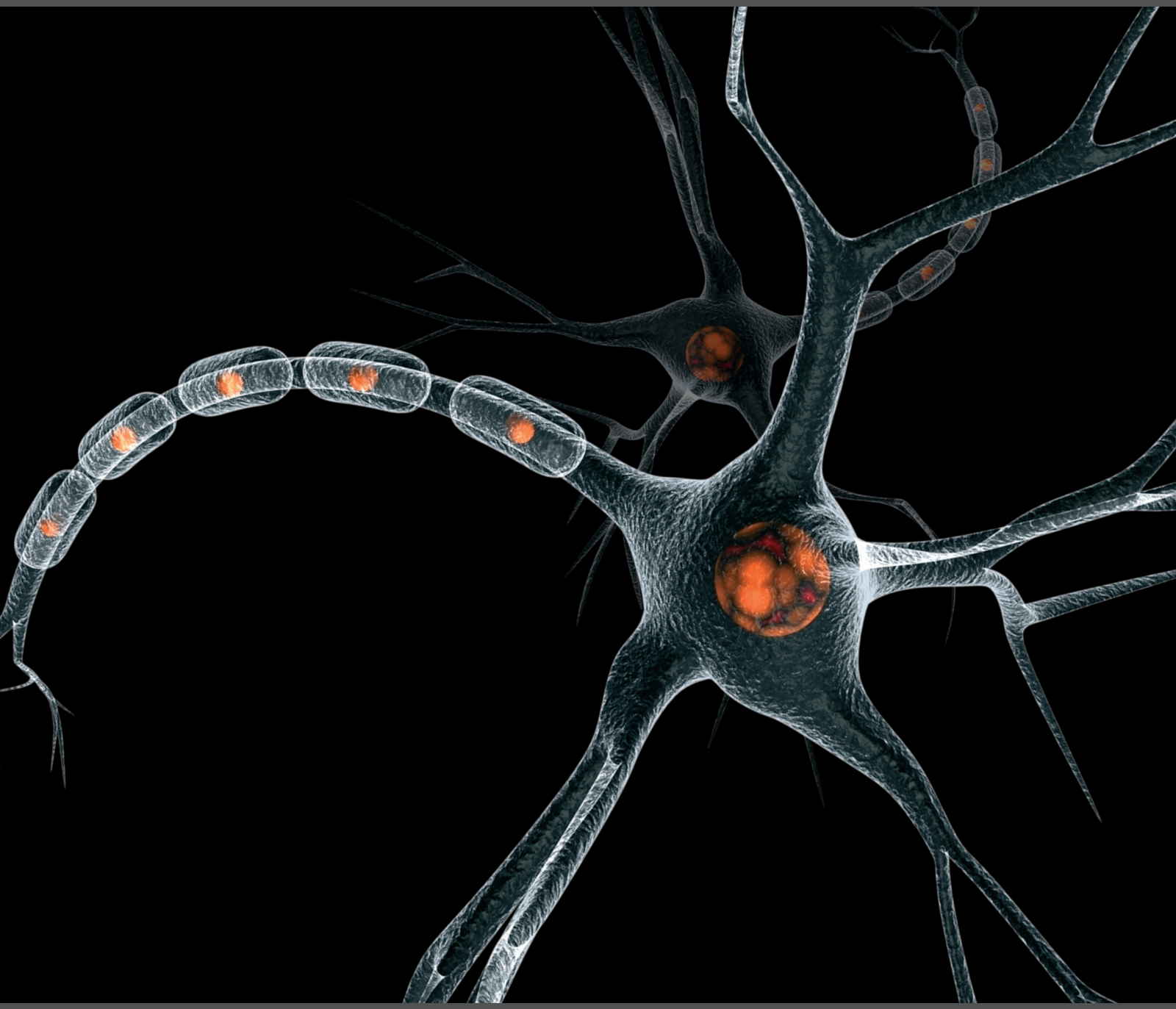
Neural Plasticity

Recent Progress in Understanding Plasticity in Neurogenetic Disorders

Guest Editors: Hansen Wang, Cara J. Westmark, Emma Frost, and Laurie C. Doering



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Editorial Board

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Editorial

Recent Progress in Understanding Plasticity in Neurogenetic Disorders

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1. Introduction

Basic research in the field of neurogenetic disorders will continue to expand our knowledge concerning the development and function of the nervous system. The greatest challenges in the field of neurogenetic disorders are defining the molecular mechanisms by which genetic mutations confer disease risk and phenotype. Neural plasticity involves a series of dynamic cellular events that orchestrate structural and functional alterations in response to experience. The study of neural plasticity provides a basic and powerful approach for unraveling the complexities of the nervous system. Investigations of plastic changes in neurogenetic diseases will shed light on the molecular and cellular mechanisms that govern function of the nervous system, in turn, leading to the discovery of potential therapeutic targets for conditions including mental impairment, neurodegeneration, epilepsy, and autism.

This special issue has targeted the most recent developments in neural plasticity as regards neurogenetic disorders. More than twenty laboratories worldwide have contributed to this special issue. The contributions have showcased the current efforts in understanding neural plastic changes and the underlying molecular and cellular mechanisms in patients and animal models of neurogenetic disorders, including a wide range of neurodevelopmental and neurodegenerative diseases. Additionally, the most promising therapeutic strategies for treating these disorders have been reviewed.

2. Plasticity in Neurogenetic Disorders

Fragile X syndrome (FXS), the most common inherited form of mental impairment and autism spectrum disorders (ASDs), is predominantly caused by a CGG repeat expansion in the 5′-UTR of the *FMR1* gene, which encodes the fragile X mental retardation protein (FMRP). With astrocytes playing a pivotal role in the development of synapses in central nervous system, C. Cheng et al. reviewed the current knowledge about the biology of astrocytes and highlighted their involvement in the developmental plasticity of FXS. Martin and Huntsman summarized studies on the mechanisms underlying plasticity deficits in FXS and emphasized that characterizing early developmental deficits in plasticity is fundamental to developing therapies for the disorder. K. Kelley et al. reviewed the studies of repeat-associated miRNAs (ramRNAs) in the transgenic zebrafish model and reported that ramRNA-induced DNA methylation of the *FMR1* 5′-UTR CGG trinucleotide repeat expansion is central to the etiology of FXS. Studies in animal models and patients have indicated that the tetracycline derivative minocycline may hold great therapeutic promise for FXS. Minocycline is thought to act via the inhibition of matrix metalloproteinases (MMPs), the zinc-dependent extracellular proteases involved in tissue remodeling and cell-cell signaling. S. S. Siller and K. Broadie summarized the recent studies on minocycline action in *Drosophila* and mouse FXS models as well as in patients, and discussed a proposed mechanism of minocycline action as an MMP inhibitor.

Rett Syndrome is a progressive neurological disorder caused by mutations in the X-linked *MECP2* gene. MeCP2 was originally known to bind methylated DNA and interact with repressor complexes to inhibit and silence its genomic targets. However, new studies have challenged this idea. R. M. Zachariah and M. Rastegar summarized the current knowledge regarding the molecular function of MeCP2 and pointed out that a collaborative effort between basic scientists and clinicians is required to address the novel and challenging concepts in MeCP2 research and to develop effective therapies for Rett Syndrome. Alterations in dendritic spines have been documented in Rett syndrome; however, C. A. Chapleau et al. reported that the lower dendritic spine density is only apparent in hippocampal CA1 pyramidal neurons of *Mecp2* mutant mice at a presymptomatic stage. This finding suggests that dendritic spine density in hippocampal neurons should not be used as a phenotypic endpoint for the evaluation of therapeutic interventions in symptomatic *Mecp2*-deficient mice and questions the role of MeCP2 in later stages of excitatory synapse and dendritic spine maintenance. The X-linked serine/threonine kinase cyclin-dependent kinase-like 5 (CDKL5) has been associated with early-onset epileptic encephalopathies characterized by intractable epilepsy, severe developmental delay, and the presence of Rett-syndrome-like features. C. Kilstrup-Nielsen et al. reviewed the current state of CDKL5 research with an emphasis on the clinical symptoms associated with mutations in *CDKL5* and the molecular mechanisms of CDKL5 function in neuronal plasticity.

Down syndrome is a neurodevelopmental disorder caused by triplication of chromosome 21 and is characterized by neurocognitive defects that range from severe intellectual disability to various patterns of neuropsychological deficits. N. Créau reviewed the main molecular and cellular findings observed in mouse models of Down syndrome and described their relationship to disease phenotypes. N. Rueda et al. also summarized studies utilizing Down syndrome mouse models but from the perspective of investigating the neurobiological substrates of mental disability in Down syndrome and testing therapies that could improve cognition. N. Cramer and Z. Galdzicki focused on hippocampal networks which are particularly impacted in Down syndrome, highlighted the neurophysiological changes that reduce the ability of trisomic neurons to undergo neural plastic adaptations, and discussed how altered plasticity may contribute to the cognitive disabilities in Down syndrome patients. Excessive GABAergic neurotransmission dampens hippocampal synaptic plasticity and contributes to cognitive impairments. Treatment with GABAA receptor antagonists results in increased plasticity and improved memory deficits in Down syndrome mice. The selective serotonin reuptake inhibitor fluoxetine can enhance plasticity in the adult rodent brain by attenuating GABAergic inhibition. Unexpectedly, M. Heinen et al. reported that adult-onset fluoxetine treatment does not improve behavioral impairments and even shows adverse seizure and mortality effects in Down syndrome mice raising the possibility of a drug/genotype interaction.

Angelman syndrome is a neurodevelopmental disorder caused by deletion or loss-of-function mutations in the maternally inherited UBE3A gene and is characterized by severe mental impairment, lack of speech, ataxia, susceptibility to seizures, and unique behavioral features. The UBE3A gene product Ube3a plays an important role in synaptic function and in regulation of activity-dependent synaptic plasticity. N. R. Jana summarized various animal models of Angelman syndrome and discussed how these models provide fundamental insight into understanding the disease biology for potential therapeutic intervention. Tuberous sclerosis complex (TSC) is caused by mutation of either the Tsc1 or Tsc2 genes, which can lead to the disinhibition of mammalian target of rapamycin (mTOR). T. Kirschstein described the animal models that have been established for tuberous sclerosis complex and discussed observed alterations in synaptic plasticity and learning in these models. Charcot-Marie-Tooth disease represents a large group of inherited peripheral neuropathies that involve both motor and sensory nerves and induce muscular atrophy and weakness. P. Juárez and F. Palau summarized the neural and molecular features of Charcot-Marie-Tooth Disease and pointed out that our understanding of the molecular pathways involved in the disease has helped to identify molecular targets for designing novel therapeutic approaches. Copy-number variations (CNVs) in the genome have been identified in several psychiatric disorders including ASDs and schizophrenia. J. Nomura and T. Takumi reviewed the creation of CNV-based animal models of psychiatric disorders and the corresponding neuroanatomical and behavioral abnormalities in these models that provide insight into human neuropsychiatric disorders.

Huntington disease is a neurodegenerative disorder caused by a tandem repeat expansion encoding a polyglutamine tract in the huntingtin protein. R. Vlamings et al. discussed the behavioral, neurophysiological, and histopathological phenotypes of transgenic Huntington disease rats that carry a truncated huntingtin cDNA fragment with 51 CAG repeats under control of the native rat *huntingtin* promoter. Different brain regions, including the hippocampus, cerebral cortex, and striatum, are affected in Huntington disease. M. I. Ransome et al. reviewed the hippocampal abnormalities, in particular the deficits of adult neurogenesis in transgenic Huntington disease mice, and discussed potential mechanisms underlying disrupted hippocampal neurogenesis and how deficits in cellular plasticity may contribute to cognitive and affective symptoms in Huntington disease.

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disorder caused by a mutation or deletion in the *survival motor neuron-1* (*SMN1*) gene. L.-K. Tsai summarized the SMN-independent therapeutic targets and strategies with demonstrated potential for the treatment of SMA. Spinal and bulbar muscular atrophy is a polyglutamine disease characterized by progressive muscle weakness and atrophy of the bulbar, facial, and limb muscles pathologically associated with motor neuron loss in the spinal cord and brainstem. Polyglutamine expansion within the androgen receptor is a disease-causing protein that results in spinal and bulbar muscular atrophy. F. Tanaka et al. reviewed current therapeutic strategies for spinal and bulbar muscular atrophy, including those based on the native functions

of the androgen receptor. Amyotrophic lateral sclerosis is a neurodegenerative disease principally affecting motor neurons. Besides motor symptoms, patients may develop cognitive disturbances or even frontotemporal dementia, indicating that amyotrophic lateral sclerosis may also involve brain regions outside of the motor regions. F. Trojsi et al. reviewed the current knowledge concerning the neuropsychological and neuropathological sequelae of amyotrophic lateral sclerosis, with a special focus on the neuroimaging findings associated with cognitive change.

Human diseases can now be modeled with relevant cell populations derived from induced pluripotent stem cells (iPSCs) that are generated with techniques that reprogram the somatic cells of patients. H. Wang and L. C. Doering reviewed recent studies using iPSCs to model various neurogenetic disorders and summarized the therapeutic implications of iPSCs, including drug screening and cell therapy for neurogenetic disorders. They highlighted the key issues associated with reprogramming that must be addressed before iPSC technology can translate to the clinic. Brain-derived neurotrophic factor (BDNF) plays essential roles in neuronal development, plasticity, and survival. Investigating the trafficking and release of BDNF is essential for understanding and potentially treating neurological disorders. D. Hartmann et al. summarized multiple techniques to investigate the transport and activity-dependent release of BDNF and their application in neurogenetic disorders. mTOR is a protein kinase involved in many neuronal functions, including dendritogenesis, plasticity, and protein synthesis. The recent literature on the neurological conditions associated with dysregulation of mTOR was covered by T. T. Gipson and M. V. Johnston. In addition, clinical trials for neurogenetic disorders with abnormalities in synaptic plasticity, and mTOR signaling were discussed.

3. Conclusion

Over the past decade, we have witnessed a dynamic expansion in the study of neurogenetic disorders. This special issue is by no means exhaustive of the research in this exciting field. We hope that the papers published herein will give our readers a broad sense of the recent progress in the field of plasticity in neurogenetic disorders as well as inspire future work that will provide a better understanding of the disease mechanisms and eventually lead to more effective treatments.

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Research Article

Hippocampal CA1 Pyramidal Neurons of *Mecp2* Mutant Mice Show a Dendritic Spine Phenotype Only in the Presymptomatic Stage

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Alterations in dendritic spines have been documented in numerous neurodevelopmental disorders, including Rett Syndrome (RTT). RTT, an X chromosome-linked disorder associated with mutations in *MECP2*, is the leading cause of intellectual disabilities in women. Neurons in *Mecp2*-deficient mice show lower dendritic spine density in several brain regions. To better understand the role of MeCP2 on excitatory spine synapses, we analyzed dendritic spines of CA1 pyramidal neurons in the hippocampus of *Mecp2*$^{tm1.1Jae}$ male mutant mice by either confocal microscopy or electron microscopy (EM). At postnatal-day 7 (P7), well before the onset of RTT-like symptoms, CA1 pyramidal neurons from mutant mice showed lower dendritic spine density than those from wildtype littermates. On the other hand, at P15 or later showing characteristic RTT-like symptoms, dendritic spine density did not differ between mutant and wildtype neurons. Consistently, stereological analyses at the EM level revealed similar densities of asymmetric spine synapses in CA1 *stratum radiatum* of symptomatic mutant and wildtype littermates. These results raise caution regarding the use of dendritic spine density in hippocampal neurons as a phenotypic endpoint for the evaluation of therapeutic interventions in symptomatic *Mecp2*-deficient mice. However, they underscore the potential role of MeCP2 in the maintenance of excitatory spine synapses.

1. Introduction

The postsynaptic sites of excitatory glutamatergic synapses in the brain, dendritic spines, are small protrusions that extend from dendrites and are associated with structural plasticity [1]. While the role of dendritic spines in models of learning and memory and synaptic plasticity continues to gain support, alterations in dendritic spine density and morphology have been consistently documented in numerous disorders associated with intellectual disabilities [2, 3]. One such disease associated with both intellectual disability and dendritic spine pathology is Rett syndrome (RTT; MIM 312750). RTT is an X chromosome-linked disorder that affects approximately 1 : 10,000–15,000 females worldwide and is the leading cause of severe intellectual disabilities in females [4]. In addition to a reduction in the size of neuronal cell bodies, a decrease in dendritic complexity of pyramidal cells was described in several brain regions [5–7]. Previous work in our laboratory using postmortem brain tissue from female RTT individuals demonstrated

that hippocampal CA1 pyramidal neurons have lower spine density than age-matched female unaffected individuals [8]. Similar qualitative observations had previously been reported in pyramidal neurons of the motor cortex [9].

Mutations in *MECP2*, the gene encoding methyl-CpG-binding protein-2, have been identified in ~90% of RTT individuals [10–12]. Using an *in vitro* organotypic slice culture system, we demonstrated that expression of *MECP2* missense mutations commonly found in RTT individuals caused a significant reduction of dendritic spine density in hippocampal pyramidal neurons, especially of the more mature mushroom type spines [8]. Consistent with these findings, neurons generated *in vitro* from induced pluripotent stem cells (iPSCs) derived from skin fibroblasts of RTT patients showed lower dendritic spine density than control neurons [13]. While these observations shed light on the role of MeCP2 in the formation and/or maintenance of excitatory synapses on dendritic spines, further characterizing the available mouse models of RTT will allow defining phenotypic endpoints to evaluate novel pharmacological interventions.

The most studied mouse models of RTT either lack the MeCP2 protein by deletion of *Mecp2* exons 3 and 4 (Bird line) [14] or express a nonfunctional mutant protein due to deletion of *Mecp2* exon 3 (Jaenisch line) ([15]; reviewed in [16, 17]). Quantitative confocal microscopy of Lucifer yellow-labeled neurons from both of these *Mecp2*-deficient mouse strains revealed lower dendritic spine density in several brain regions, including pyramidal neurons of the CA1 region of the hippocampus [18]; it should be noted that these observations were made in postnatal-day 21 mice (before overt RTT-like symptoms), and that they are consistent with the lower density of excitatory synapses estimated from VGLUT1-PSD95 puncta observed in the hippocampal CA1 region of 2-week-old $Mecp2^{tm1.1Bird}$ null mice [19]. Symptomatic $Mecp2^{tm1.1Bird}$ null mice at 5 weeks, however, showed comparable density of VGLUT1-PSD95 puncta to that in wildtype littermates [19].

To better understand the role of *Mecp2* on dendritic spine maintenance, we analyzed dendritic spine density and morphology by confocal microscopy and excitatory synapses by electron microscopy in hippocampal CA1 pyramidal neurons of presymptomatic (P7 and P15) and symptomatic (P40–60) male $Mecp2^{tm1.1Jae}$ mutant mice and their age-matched wildtype littermates [15]. Our observations indicate that dendritic spine density is lower only at postnatal-day 7 (P7), while it does not differ at P15 or later when symptoms are well established. Consistently, stereological analyses at the EM level revealed comparable density of asymmetric spine synapses between symptomatic *Mecp2* mutant and wildtype littermates. These results raise caution regarding the use of dendritic spine density in hippocampal neurons as a phenotypic endpoint for the evaluation of therapeutic interventions in symptomatic *Mecp2*-deficient mice. In addition, these observations demonstrate that proper *Mecp2* function is required for the early development of dendritic spines in CA1 pyramidal neurons and that a secondary compensatory mechanism seems to take place in symptomatic *Mecp2* mutant mice.

2. Materials and Methods

2.1. Animals. Breeding pairs of mice lacking exon 3 of the X chromosome-linked *Mecp2* gene (B6.Cg-$Mecp2^{tm1.1Jae}$, Jaenisch strain; C57BL/6 background) [15] were purchased from the Mutant Mouse Regional Resource Center at UCDavis. A colony was established at UAB by breeding wildtype males with heterozygous $Mecp2^{tm1.1Jae}$ mutant females, following the original breeding scheme [15], which is recommended by the supplier. Genotyping was performed by PCR of sample DNA from tail clips. Hemizygous male mice of the $Mecp2^{tm1.1Jae}$ mutant strain are healthy until 5-6 weeks of age, when they begin acquiring RTT-like motor symptoms, such as hypoactivity, hind limb elevation, and reflex impairments [15]. For the present studies, the experimental subjects were homozygous $Mecp2^{tm1.1Jae}$ mutant males (called *Mecp2* mutants) and wildtype male mice that were littermates of postnatal-day 7 (P7), P15, and between P40 and P60. Animals were handled and housed according to the Committee on Laboratory Animal Resources of the National Institutes of Health. All experimental protocols were annually reviewed at The University of Alabama at Birmingham and at The Università di Torino and approved by each institution's respective Institutional Animal Care and Use Committee.

2.2. DiOlistic Labeling. Mice that were postnatal-day (P7) were euthanized by cervical dislocation, and the brain was immersed in 4% paraformaldehyde (in 0.1 M phosphate buffer); hippocampi were later dissected and kept in fixative for an additional 2 hours. P15 or P40–60 mice were deeply anesthetized with ketamine (100 mg/kg) and euthanized by transcardiac perfusion with ~200 mL of 4% paraformaldehyde (in 0.1 M phosphate buffer).

After dissection, brains and hippocampi were rinsed several times in 0.1 M phosphate buffered saline (PBS) and cut into 100 μm thick coronal sections with a McIlwain tissue chopper, which were further rinsed in 0.1 M PBS and stored at 4°C until DiI labeling. To visualize dendritic spines by laser-scanning confocal microscopy, coronal sections containing the hippocampus were stained with the lipophilic fluorescent dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; InVitrogen, Carlsbad, CA, USA) by particle-mediated labeling (DiOlistics) [20, 21]. First, DiI was diluted in dimethyl chloride (methylene chloride; Sigma, St. Louis, MO, USA). Then, 20 mg of 1.1 μm tungsten particles (Bio-Rad; Hercules, CA, USA) were placed on top of a pre-cleaned glass slide and spread out evenly with two pre-cleaned razor blades. The DiI solution was added onto the tungsten particles and allowed to completely evaporate. To prevent clumping of the DiI/tungsten mixture, razor blades were used to break apart the mixture. Additionally, a small amount of polyvinylpyrrollidone (10–20 μL PVP made in fresh in 100% ethanol; Bio-Rad) was added to the DiI/tungsten mixture to further prevent particle clumping and improve their coating to the Tefzel tubing. The DiI-coated tungsten particles were then added to a glass tube with 3 mL of water and sonicated for 1 hr. After sonication, the solution was vortexed and

Table 1: Total number of mice, length of dendrites, and individual dendritic spines counted and measured in the quantitative analyses.

Genotype	Mice	Total length of dendrites (μm)	Total number of spines
P7 wildtype	3	463.96	168
P7 *Mecp2*$^{-/y}$	3	994.81	235
P15 wildtype	3	647.92	303
P15 *Mecp2*$^{-/y}$	3	676.06	385
P40–60 wildtype	5	2,107.02	2,931
P40–60 *Mecp2*$^{-/y}$	5	2,131.24	3,478

then aspirated and coated onto Tefzel tubing for 15 mins. After 15 mins, the solution was removed and the tubing was allowed to dry for 15 mins. DiI-coated tungsten bullets were shot only once onto individual hippocampal slices with a custom-modified Helios hand-held gene gun (Bio-Rad) using 75 psi He pressure through a 40 μm pore size filter [8]. After labeling with DiI bullets, slices were rinsed and stored in PBS for 15–30 min at room temperature in the dark to allow diffusion of DiI. Then, slices were postfixed with 4% paraformaldehyde and stored at 4°C overnight. Slices were finally washed with PBS and mounted on glass slides with Vectashield (Vector Laboratories, Burlingame, CA, USA). Figure 1(a) shows tungsten bullets and the resulting DiI fluorescence in a representative section of perfusion-fixed hippocampus stained by DiOlistics. It should be noted, from examining Table 1, for mice that were younger in age, the total dendritic length examined is smaller than that obtained for the older mice. This discrepancy is presumably a result of older mice having an abundant number of cells and with longer dendrites.

2.3. Laser-Scanning Confocal Microscopy. High-resolution images of spiny apical secondary or tertiary dendrites showing adequate DiI labeling were acquired from CA1 *stratum radiatum* in a Fluoview FV-300 laser-scanning confocal microscope (Olympus, Center Valley, PA, USA) using an oil immersion 60 X 1.45 NA objective lens (PlanApo, Olympus), with additional 3x digital zoom. DiI was excited with an HeNe Green laser (543 nm), and its fluorescence detected with a cube containing a 555 nm dichroic mirror and a 585 ± 40 nm emission filter (Semrock, Lake Forest, IL, USA). Series of optical sections in the *z*-axis were acquired with 0.1 μm intervals through individual apical dendritic branches. Figure 1(b) shows representative examples of a CA1 pyramidal neuron and a segment of apical dendrite stained with DiI.

2.4. Analysis of Dendritic Spine Density. Dendritic spines were identified as small protrusions that extended less than 3 μm from the parent dendrite and counted manually in maximum-intensity projections of confocal *z*-stacks using ImageJ software (W. S. Rasband, ImageJ, US National Institutes of Health, Bethesda, Maryland, USA; http://rsb.info.nih.gov/ij/, 1997–2009); protrusions that were more than 3 μm were classified as filopodia and rarely seen except in the P7 mice. Care was taken to ensure that each spine was counted only once by following its projection course through the stack of z-sections. Spines were counted only if they appeared continuous with the parent dendrite. Spine density was calculated by quantifying the number of spines per dendritic segment and normalized to 10 μm of dendrite length. Microscope calibrations were performed using 1.07 μm fluorescent latex microspheres (Molecular Probes, Eugene, OR, USA), which yielded a lateral resolution of 0.09 μm per pixel. Counting and measuring of individual spines was conducted in a blind fashion, as the genotype was unknown to the person performing the image analysis.

2.5. Dendritic Spine Classification. The categorization of different morphological spine types was performed as described [22, 23]. Briefly, geometrical dimensions of individual spines were measured in maximum-intensity projections of the *z*-stacks using ImageJ and used to calculate the L/N and H/N ratios, where L is spine length, H is the maximum head width, and N is the maximum neck width. Then, spine types were grouped as mature-shaped spines, which included type-I (stubby) and type-II (mushroom) shaped spines, or immature-shaped thin (type-III) spines, following published criteria [24]. Table 1 shows the spine density and proportions of spine types in each genotype, as well as the total number of dendritic spines counted and measured and the total dendritic length analyzed.

2.6. Electron Microscopy and Stereological Synapse Count. Brain fixation and preparation for electron-microscopy analyses were performed as described [25]. Male *Mecp2*$^{tm1.1Jae}$ mutant mice and wildtype littermates (3 per group) were anaesthetized with an intraperitoneal injection of chloral hydrate and transcardially perfused with ice cold 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4). After perfusion, brains were left in the same fixative overnight at 4°C, washed several times in 0.1 M PB, and then postfixed with 1% osmium tetroxide (in 0.1 M cacodylate buffer) for 1 hour. For resin embedding, tissues were dehydrated in a graded series of ethanol (30–100%) and infiltrated with an Epon-Araldite mixture. Ultrathin serial sections (70 nm) were cut with an ultramicrotome (Leica Ultracut, Wetzlar, Germany) and collected on single slot copper grids coated with a pioloform solution. The grids were counterstained with uranyl acetate and lead citrate and imaged in a JEM-1010 electron microscope (Jeol, Japan) equipped with a side-mounted CCD camera with 1376 × 1032 pixels (Mega View III; Soft Imaging System GmbH, Muenster, Germany). Asymmetric synapses were identified by the presence of at least three vesicles within the profile

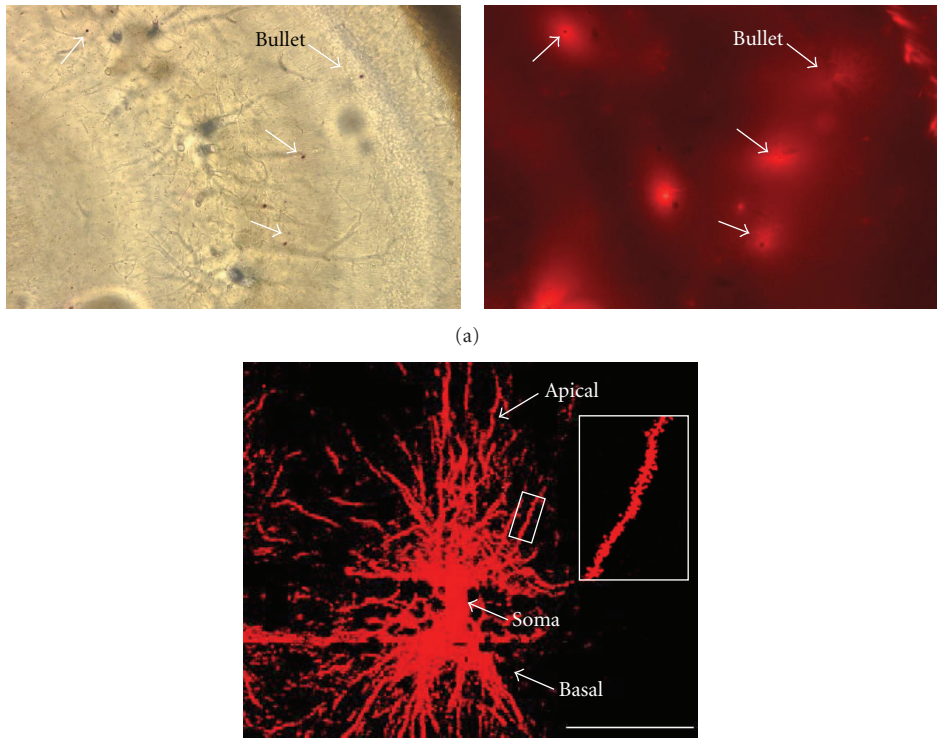

Figure 1: Dendritic spines in the CA1 region of the hippocampus visualized by DiI "DiOlistics" and confocal microscopy. (a) (left), Brightfield image of the CA1 region of a representative formalin-fixed hippocampal section (100 μm thickness) stained with DiI by particle-mediated labeling (DiOlistics) showing tungsten bullets used to deliver DiI (arrows). (right), DiI fluorescence from the same field of view. (b) Representative CA1 pyramidal neuron stained with DiI and imaged by confocal microscopy. Inset: apical dendritic segment representative of those selected for quantitative analyses of dendritic spines.

adjacent to the presynaptic membrane and the presence of a clear PSD. Unbiased stereological estimation of asymmetric spine synapses (presumptive excitatory synapses) was performed as previously described [26] by using the double dissector method [27]. In this method, images of two serial sections are acquired, the first of which is designated as the "reference section" and the second as the "look-up section." Twenty-five pairs of nonoverlapping electron micrographs were acquired for each animal in CA1 *stratum radiatum* at $\times$20,000 magnification. Within an unbiased counting frame that represented 28 μm^2, the number of synapses that were present in the reference section but absent in the look-up section were counted. The cross-section area of dendritic spines and presynaptic terminals was analyzed on the same digital images using ImageJ as previously described [26]. At least 180 synapses were analyzed per genotype.

2.7. Statistical Analyses. Averages of multiple measurements are presented as mean $\pm$ standard error of the mean (SEM). Data were statistically analyzed using unpaired Student's *t*-test using Prism software (GraphPad Software, Inc., San Diego, CA, USA). Probability values lower than 0.05 were considered statistically significant (i.e., $P < 0.05$, less than 5% probability that the observations are due to chance). When lower than this cut-off value, the actual P values are given in Section 3 (rather than just the statement "greater than" or "less than") to provide readers with more detailed information regarding the outcome of the statistical analyses. Cumulative frequencies plots were first analyzed using the normal distribution Kolmogorov-Smirnov (K-S) fitting test, and then K-S two-sample tests for subsequent paired comparisons.

3. Results

3.1. Presymptomatic Mecp2 Mutant Mice: CA1 Pyramidal Neurons Have Lower Spine Density Only in P7, While It Does Not Differ at P15. We previously showed that pyramidal neurons from rat hippocampus overexpressing human *MECP2* carrying single point missense mutations commonly found in RTT individuals have lower dendritic spine density than control neurons [8]. Consistent with lower dendritic spine density in female RTT individuals, such spine loss in mutant *MECP2*-expressing neurons was observed after 4 days of over expression *in vitro* in organotypic slice cultures. In addition, 3-week-old *Mecp2*-deficient mice have lower spine density than age-matched wildtypes [18], but older symptomatic *Mecp2* mutant mice show comparable density of VGLUT1-PSD95 puncta to that in wildtype littermates [19]. To address a potential developmental progression of this dendritic spine phenotype in *Mecp2* mutant mice ($Mecp2^{tm1.1Jae}$), we

performed quantitative analyses of dendritic spines in DiI-labeled hippocampal sections from mutant and age-matched wildtype littermates by laser-scanning confocal microscopy.

Figure 2(a) shows representative examples of maximum-intensity projections of confocal stacks from segments of secondary apical dendrites of CA1 pyramidal neurons used for quantitative analyses. Dendritic spine density in CA1 pyramidal neurons of P7 $Mecp2^{tm1.1Jae}$ mutants was lower compared to age-matched wildtype littermates (wildtype 3.65 ± 0.54 spines per 10 μm of dendritic length, n = 5 dendrite segments from 3 mice, versus $Mecp2^{tm1.1Jae}$ 2.23 ± 0.40 spines/10 μm, n = 10 segments/3 mice; P = 0.030; Figures 2(a) and 2(d)). On the other hand, dendritic spine density in P15 $Mecp2^{tm1.1Jae}$ mutant mice was not statistically different compared to age-matched wildtype littermates (wildtype 5.39 ± 1.20 spines/10 μm, n = 7 segments/3 mice versus $Mecp2^{tm1.1Jae}$ 5.87 ± 0.43 spines/10 μm, n = 9 segments/3 mice; P = 0.359; Figures 2(b) and 2(d)).

Despite differences in spine density in P7 mice, the proportions of the three main morphological types of spines did not differ between $Mecp2^{tm1.1Jae}$ and age-matched wildtype littermates (stubby: wildtype 0.49 ± 0.07 versus $Mecp2^{tm1.1Jae}$ 0.46 ± 0.08; P = 0.396, mushroom: wildtype 0.24 ± 0.02 versus $Mecp2^{tm1.1Jae}$ 0.16 ± 0.04; P = 0.110, thin: wildtype 0.27 ± 0.05 versus $Mecp2^{tm1.1Jae}$ 0.25 ± 0.06; P = 0.401). Similarly, the proportions of morphological spine types were comparable in P15 mice of both genotypes (stubby: wildtype 0.39 ± 0.01 versus $Mecp2^{tm1.1Jae}$ 0.33 ± 0.03; P = 0.087, mushroom: wildtype 0.47 ± 0.03 versus $Mecp2^{tm1.1Jae}$ 0.50 ± 0.03; P = 0.244, thin: wildtype 0.14 ± 0.03 versus $Mecp2^{tm1.1Jae}$ 0.16 ± 0.02; P = 0.269).

3.2. CA1 Pyramidal Neurons of Symptomatic Mecp2 Mutant Mice Have Comparable Dendritic Spine Density and Morphology Than Their Age-Matched Wildtype Littermates. Dendritic spine density in CA1 pyramidal neurons of P40–60 $Mecp2^{tm1.1Jae}$ mutant mice exhibiting characteristic RTT-like symptoms were not statistically different than in age-matched wildtype littermates (wildtype 14.39 ± 0.77 spines/10 μm of dendritic length, n = 35 segments/5 mice versus $Mecp2^{tm1.1Jae}$ 15.82 ± 0.86 spines/10 μm, n = 29 segments/5 mice; P = 0.110; Figures 2(c) and 2(d)). We next determined if the proportion of the three major morphological types of dendritic spines were affected in symptomatic $Mecp2^{tm1.1Jae}$ mutant mice. The proportion of stubby spines and mushroom spines, larger spines considered to be more stable, was comparable between the genotypes (stubby: wildtype 0.51 ± 0.02 versus $Mecp2^{tm1.1Jae}$ 0.53 ± 0.02; P = 0.211; mushroom: wildtype 0.33 ± 0.01 versus $Mecp2^{tm1.1Jae}$ 0.35 ± 0.01; P = 0.06). On the other hand, the proportion of the more motile and immature thin spines was lower in symptomatic $Mecp2^{tm1.1Jae}$ mutant mice (wildtype 0.16 ± 0.01 versus $Mecp2^{tm1.1Jae}$ 0.11 ± 0.01; P = 0.005).

3.3. The Density and Morphology of Asymmetric Spine Synapses in CA1 Stratum radiatum of Symptomatic Mecp2 Mutant Mice Is Comparable to That of Their Age-Matched Wildtype Littermates. Electron microscopy of asymmetric synapses on dendritic spines (presumptive excitatory) within CA1 *stratum radiatum* of symptomatic $Mecp2^{tm1.1Jae}$ mutant mice was conducted to determine if *Mecp2* alters density of excitatory synapses. Ultrastructural observations revealed a phenotype consistent with the above confocal microscopy results (Figure 3(a)). Unbiased stereological analyses revealed that the density of asymmetric spine synapses in symptomatic $Mecp2^{tm1.1Jae}$ mutant mice (2.12 ± 0.11 synapses per μm^2; n = 3 mice) was not significantly different than in wildtype littermates (2.06 ± 0.05 synapses/μm^2, n = 3 mice; P = 0.4; Figure 3(b)). Intriguingly, the cross-sectional area of individual spines (Figure 3(c)) and presynaptic terminals (Figure 3(d)) were smaller in $Mecp2^{tm1.1Jae}$ mutant mice (Kolmogorov-Smirnov test; P < 0.01), but only for the smallest asymmetric spine synapses. It should be noted that these spines have dimensions below the resolution of diffraction-limited light microscopy and thus are not detectable in our dendritic spine density measurements relying on confocal microscopy (Figure 2(b)).

4. Discussion

Quantitative confocal microscopy of DiI-labeled dendrites revealed that CA1 pyramidal neurons from P7 *Mecp2* mutant mice had lower spine density compared to age-matched wildtype littermates. These differences in spine density were not observed neither in slightly older but still presymptomatic *Mecp2* mutant mice at P15 nor in P40–60 mice that express the full spectrum of RTT-like symptoms, consistent with an analysis of the density of VGLUT1-PSD95 puncta in area CA1 [19]. Stereological analyses at the electron microscopy level revealed that the density of asymmetric spine synapses is comparable in P40–60 fully symptomatic $Mecp2^{tm1.1Jae}$ mutant mice compared to age-matched wildtype littermates. The only significant difference between genotypes during the symptomatic stage was a lower proportion of immature thin spines and a smaller cross-sectional area of individual spines and presynaptic terminals—but only for the smallest asymmetric spine synapses—in $Mecp2^{tm1.1Jae}$ mutant mice. It should be noted that the smallest asymmetric spine synapses analyzed at the EM level have dimensions below the resolution of diffraction-limited light microscopy and thus are not detectable in our measurements using confocal microscopy. Our observations during the symptomatic stage demonstrate that *Mecp2* loss-of-function causes subtle structural modifications of excitatory CA3-CA1 synapses without major changes in excitatory synapse density, as we showed in an independent EM study using random single ultrathin sections [28]. These data demonstrate that proper *Mecp2* function is required for early development of dendritic spines in CA1 pyramidal neurons and that a compensatory mechanism that normalizes spine density seems to occur later in development.

A recurrent theme in cytological studies of postmortem RTT brains is the observation of significant differences in the fine structure of dendrites. Studies of neurons from cortical regions of the brain have shown impaired dendritic branching in individuals with RTT [5]. In addition,

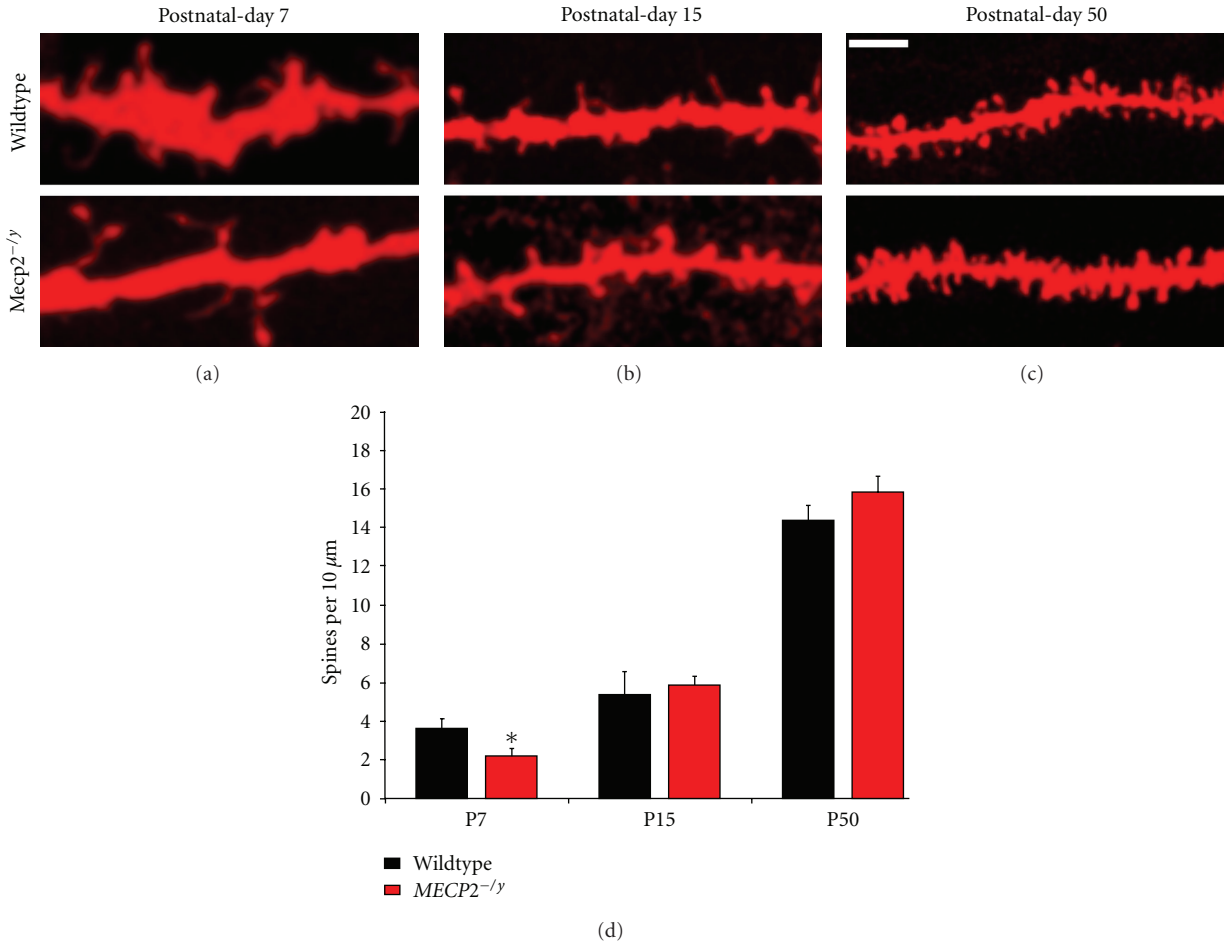

Figure 2: Quantitative analyses of dendritic spines in CA1 pyramidal neurons from *Mecp2* mutant mice and age-matched wildtype littermates. (a) Representative examples of apical dendritic segments of CA1 pyramidal neurons from P7 wildtype and $Mecp2^{tm1.1Jae}$ mice (top). Scale bar represents 2 μm. (b) Examples of apical dendritic segments of CA1 pyramidal neurons from P15 wildtype and $Mecp2^{tm1.1Jae}$ mice (top). (c) Examples of apical dendritic segments of CA1 pyramidal neurons from P40 wildtype and $Mecp2^{tm1.1Jae}$ mice (top). (d) Dendritic spine density (spines per 10 μm of dendrite). All data are expressed as mean ± SEM. *indicates $P < 0.05$.

qualitative observations of pyramidal neurons of the motor cortex of RTT individuals described segments of dendrites that were bare of spines [9]. Using quantitative confocal microscopy of DiI-labeled hippocampal sections, we recently showed that CA1 pyramidal neurons have lower dendritic spine density in female RTT individuals than in age-matched unaffected females [8]. Our results on hippocampal dendritic spine density show that male $Mecp2^{tm1.1Jae}$ mutant mice at the symptomatic stage do not recapitulate the human phenotype observed in autopsy material from female RTT individuals. While many factors could contribute to these findings (including gender differences, disease severity, seizure disorder, and X-chromosomal inactivation ratio), these results suggest that hippocampal neuron spine density in this particular mouse model is not a phenotype with sufficient face validity for RTT. However, these mice still yield relevant information on the role of *Mecp2* in the CNS. Further postmortem studies in more individuals with RTT as well as other *MECP2*-associated conditions are needed to better understand the consequences on dendritic spine density and morphology. If such studies are correlated with a detailed clinical history, they will further yield a better understanding of the contribution of other factors to the spine phenotype, such as specific *MECP2* mutations, disease severity and progression, and life-long medications.

Our previous *in vitro* studies in rat hippocampal neurons in primary culture demonstrated that either shRNA-mediated knockdown of endogenous MeCP2 protein levels or overexpression of human *MECP2* missense mutations common in RTT patients (R106W or T158M) reduced dendritic length and branching during early neuronal development [29]. Furthermore, using a postnatal rat hippocampal slice culture preparation, we observed that the knockdown of endogenous MeCP2 protein levels resulted in reduced dendritic spine density, especially of mature spines; however, overexpression of RTT-associated human *MECP2* missense mutations led to a dramatic reduction in dendritic spine density [8]. Consistently, a recent report described that neurons derived from iPSCs obtained from reprogramming of skin fibroblasts of RTT patients have reduced dendritic

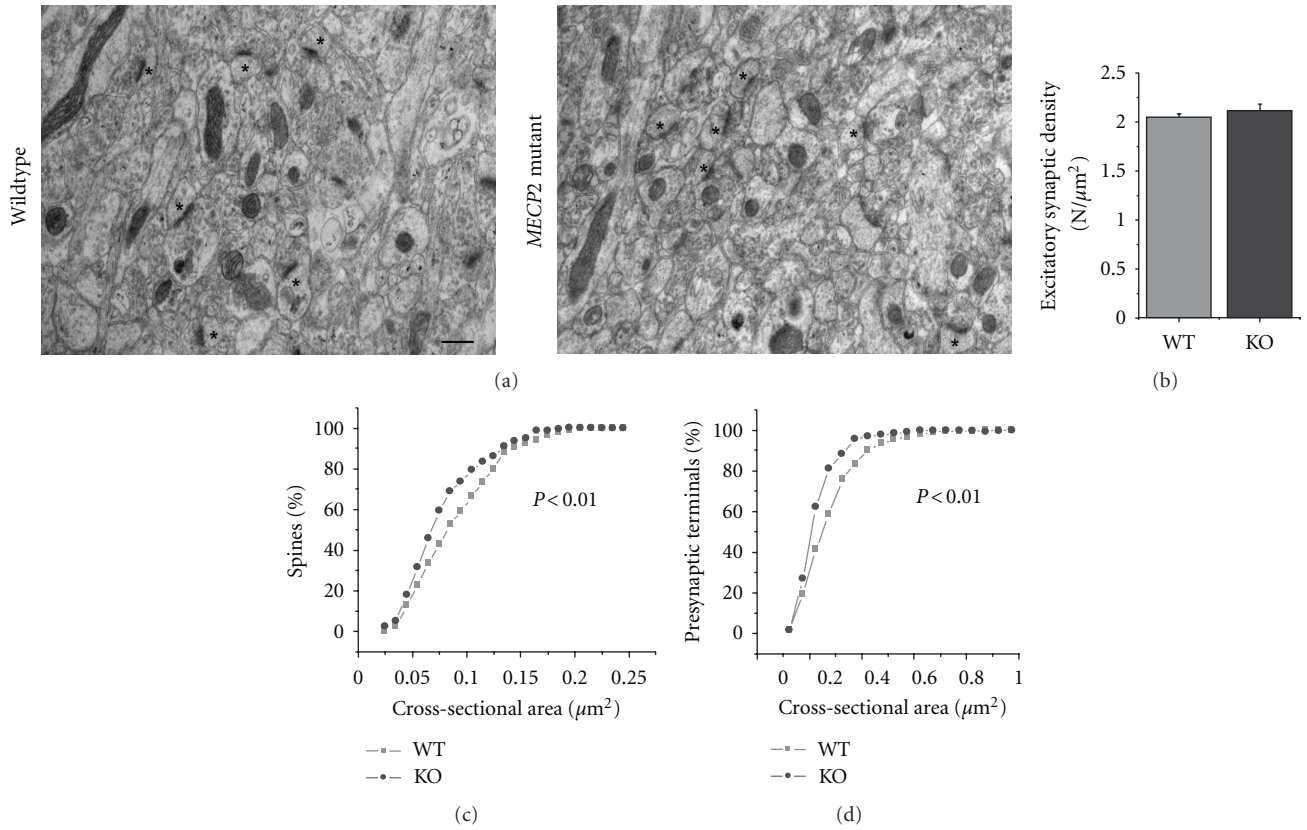

Figure 3: Quantitative electron microscopic analyses of CA3-CA1 excitatory synapses in the hippocampus of symptomatic *Mecp2*$^{tm1.1Jae}$ mutant mice and age-matched wildtype littermates. (a) Electron micrographs of *stratum radiatum* in the CA1 area of the hippocampus from wildtype and symptomatic male *Mecp2*$^{tm1.1Jae}$ mutant mice (P56). Representative excitatory synapses are indicated (asterisks). Scale bar: 400 nm. (b) Histograms show the number per unit of volume of asymmetric/excitatory CA3-CA1 synapses in CA1 *stratum radiatum*. The analysis using stereology (dissector method) revealed that *MECP2* mutation does not affect excitatory synapse number. (c) Cumulative percentage of the cross-sectional area of dendritic spines associated with asymmetric CA3-CA1 synapses. Area of the dendritic spine was determined when associated with a presynaptic terminal. (d) Cumulative percentage of the cross-sectional area of presynaptic terminals associated with asymmetric CA3-CA1 synapses. Area of the presynaptic terminals was determined when associated with a dendritic spine.

spine density [13]. While these exciting observations on patient-derived neurons provide additional evidence of the importance of dendritic spines in the neuropathology of RTT, it should be made clear that no causative biochemical underpinning has yet been established.

Transgenic mice that either lack *Mecp2* or express a mutant nonfunctional *Mecp2* peptide fragment are excellent experimental models to help determine the synaptic defects that contribute to RTT. These mice recapitulate several behavioral features of RTT and display many defects in dendritic structure and synaptic transmission and plasticity (reviewed in [16, 17]). However, they have also yielded varying results in terms of dendritic spine alterations. In contrast with the present observations, a quantitative study of two different *Mecp2* mutant lines (*Mecp2*$^{tm1.1Bird}$ and *Mecp2*$^{tm1.1Jae}$) described that pyramidal neurons from hippocampal area CA1 and layers II-III of the motor cortex have lower dendritic spine densities than their control wildtype littermates at approximately 3 weeks of age [18]. The parsimonious explanation for this apparent discrepancy is that these studies used mice of different strains and the genetic background of the different mouse lines contributed to the divergent observations on dendritic spine density. While both studies employed commercially available *Mecp2*$^{tm1.1Jae}$ mice, the genetic background is different: we used *Mecp2*$^{tm1.1Jae}$ mice on a pure C57BL/6 background inbred for more than 10 generations, while Belichenko et al. [18] used two different lines (*Mecp2*$^{tm1.1Jae}$ and *Mecp2*$^{tm1.1Jae}$) on a mixed genetic background [18]. Genetic background has been known to contribute significantly to several biological parameters. For example, neurite outgrowth is significantly different in two mouse strains where *Nogo-A* was knocked out [30]. When comparing two different *Mecp2* transgenic mouse lines maintained on different genetic backgrounds, Belichenko et al. [18] described significant differences in spine density between age-matched wildtype mice of the two different strains, strongly suggesting that genetic background contributes to the phenotype under study, for example, dendritic spine density and morphology [18].

We show here that hippocampal pyramidal neurons exhibit a dendritic spine phenotype only in neonatal (P7) mutant mice, well before excitatory synapse expansion,

while spine density in mutants recovers to wildtype levels a week later (P15) and is maintained at wildtype levels throughout the symptomatic stage (P40–60). This developmental progression of the dendritic spine phenotype is also reflected in the density of VGLUT1-PSD95 puncta, which is lower in area CA1 of 2-week-old *Mecp2* null mice, but comparable to wildtype levels at 5 weeks of age [19]. Together with our dendritic spine observations, those VGLUT1-PSD95 puncta results are consistent with the present EM analyses in symptomatic $Mecp2^{tm1.1Jae}$ mutant mice, which revealed comparable densities of asymmetric spine synapses in *stratum radiatum* of area CA1 of both genotypes [28]. Altogether, these data demonstrate that proper *Mecp2* functioning is required for dendritic spine formation during early postnatal development, and that a secondary compensatory mechanism seems to take place in symptomatic *Mecp2* mutant mice. A couple of possibilities exist as to the extent of the compensatory mechanisms necessary to bring spine density to wildtype levels. One possibility is that enhanced hippocampal network activity in *Mecp2* mutants promotes dendritic spine formation [28]. A second possibility is that deranged homeostatic plasticity promotes spinogenesis, while still affecting pyramidal neuron function [31].

Despite the lack of differences in dendritic spine density in fully symptomatic *Mecp2* mutant mice, we observed a significant, yet small, reduction in the proportion of immature thin spines in *Mecp2* mutants compared to age-matched wildtype animals. What consequence this might have on hippocampal synaptic function remains debatable; however, it is hypothesized that thin spines represent "learning spines" because of their constant changing in response to neuronal activity (e.g., LTP and LTD), while mushroom spines are considered more mature and stable "memory spines" [32]. Considering that dendritic spines are highly sensitive to the levels of neuronal activity [33], the shift in the proportion of morphological spine types could also reflect a response to the heightened neuronal activity observed in the hippocampal network of symptomatic $Mecp2^{tm1.1Jae}$ mutant mice [28]. Thus, it would be interesting to determine what role *Mecp2* has in the maintenance of thin spines and what consequences does this have on hippocampal function. One report has already demonstrated that spine motility is slowed in *Mecp2* mutant mice [34], possibly reflecting the decrease in the proportion of thin spines that we observed.

Confocal microscopy of dendritic spines in organotypic hippocampal cultures has revealed that approximately 65–70% of dendritic spines are juxtaposed to presynaptic terminals [35]. For this reason, we decided to conduct unbiased stereological analyses at the electron microscopy level to determine the density and morphology of dendritic spines that were actually connected to a presynaptic terminal, for example, asymmetric spine synapses. This approach demonstrated that the density of asymmetric spine synapses in $Mecp2^{tm1.1Jae}$ mutant mice is comparable to that of wildtype littermates, consistent with confocal microscopy of dendritic spines. Intriguingly, the areas of dendritic spines and presynaptic terminals are smaller in $Mecp2^{tm1.1Jae}$ mutant mice; however, this difference was only observed for the smallest asymmetric spine synapses. Considering that these spines have dimensions below the resolution of diffraction-limited light microscopy, they could not have been included in measurements of spine head width in confocal microscopy images (Figure 3(b)). Taken together, our observations demonstrate that while the proportion of thin spines is lower in $Mecp2^{tm1.1Jae}$ mutant mice, individual dendritic spines, and thus excitatory synapses, are smaller in volume.

In summary, our results raise caution regarding the use of dendritic spine density in hippocampal neurons as a phenotypic endpoint for the evaluation of therapeutic interventions in symptomatic *Mecp2* deficient mice. However, we present data describing the importance of *Mecp2* on spine development in neonatal mice. Future research will hopefully explain the precise molecular role of MeCP2 in the establishment of the hippocampal excitatory network and how this manifest into clinical issues.

Authors' Contribution

C. A. Chapleau and E. M. Boggio contributed equally to this work.

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Review Article

Animal Models of Psychiatric Disorders That Reflect Human Copy Number Variation

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The development of genetic technologies has led to the identification of several copy number variations (CNVs) in the human genome. Genome rearrangements affect dosage-sensitive gene expression in normal brain development. There is strong evidence associating human psychiatric disorders, especially autism spectrum disorders (ASDs) and schizophrenia to genetic risk factors and accumulated CNV risk loci. Deletions in 1q21, 3q29, 15q13, 17p12, and 22q11, as well as duplications in 16p11, 16p13, and 15q11-13 have been reported as recurrent CNVs in ASD and/or schizophrenia. Chromosome engineering can be a useful technology to reflect human diseases in animal models, especially CNV-based psychiatric disorders. This system, based on the Cre/*loxP* strategy, uses large chromosome rearrangement such as deletion, duplication, inversion, and translocation. Although it is hard to reflect human pathophysiology in animal models, some aspects of molecular pathways, brain anatomy, cognitive, and behavioral phenotypes can be addressed. Some groups have created animal models of psychiatric disorders, ASD, and schizophrenia, which are based on human CNV. These mouse models display some brain anatomical and behavioral abnormalities, providing insight into human neuropsychiatric disorders that will contribute to novel drug screening for these devastating disorders.

1. Introduction

Copy number variation (CNV) is a structural genomic variation of the human genome that may either be inherited or caused by de novo mutation. It includes translocation, inversion, duplication, triplication, and deletion. CNVs can range in size from kilobases (Kbs) to several megabases (Mbs) that have not been identified by conventional chromosomal analysis. However, recent technology of genome-wide analysis such as comparative genomic hybridization (CGH) has led to the discovery of extensive genomic structural variation [1–3]. A recent report using microarray technology revealed that as much as 12% of the human genome are variable in copy number [4]. These known CNVs are available from the interactive web-based database DECIPHER (Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources, http://decipher.sanger.ac.uk/). The DECIPHER database is a Consortium comprised of an international network of more than 100 centers and has uploaded more than 2000 cases (current statistics can be found on the DECIPHER homepage) [5].

CNVs can be de novo or familial. De novo mutations are more likely to contribute to the development of sporadic genomic disorders [6, 7]. In psychiatric disorders, ASD and schizophrenia, extension of genome-wide association studies (GWAS) have led to the discovery of both inherited and de novo sporadic CNVs. Such CNVs resulted in altering gene dosage and dosage-sensitive gene expression, which may contribute to these disorders complexities [8]. These human genetics studies have detected several CNVs (e.g., 1q21, 3q29, 10q26, 11p14, 15q11, 15q13, 16p13, 17p12, and 22q11). This discovery suggests an important role for the strict regulation of gene dosage in ASD and schizophrenia.

To understand psychiatric disorders, animal models are needed because particular experiments in human are impossible. While it is difficult to model human psychiatric

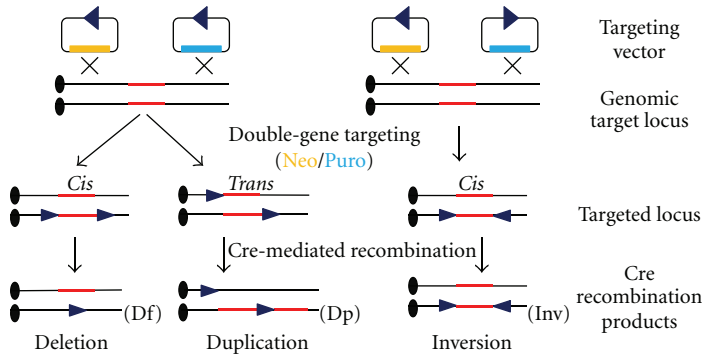


Figure 1: Chromosome engineering in mouse embryonic stem cells.

phenotypes in animals (e.g., hallucinations and delusions characteristic of schizophrenia that are human specific), animal models may contribute to the elucidation of brain anatomy, behavioral characteristics, and molecular mechanisms that reflect aspects of human phenotypes.

Although there is a strong association between genetic rearrangement and psychiatric disorders (e.g., ASD and schizophrenia), valid animal models that reflect etiology are rare. Several efforts have been made to generate mouse models of psychiatric disorders by conventional gene targeting, conditional gene targeting, and point mutation by chemical mutagens. But these techniques are not enough to reflect complex human genomic rearrangements, such as large deletions, inversions, and duplications. In this regard, the Cre/*loxP*-based chromosome engineering technique is useful to generate this kind of complex genomic rearrangements in the mouse genome. By using this chromosome engineering technique, we can accomplish CNV-based unbiased animal models of psychiatric disorders. In this paper, we focus on animal model of ASD (and schizophrenia) which was generated by chromosome engineering, principle of this technology, and discuss for future directions.

2. Chromosome Engineering in Mice

Genetic abnormalities such as point mutations, deletions, duplications, inversions, and translocations can be induced by exposure to X-ray radiation, chemical mutagens (e.g., N-ethyl-N-nitrosourea (ENU)), conventional gene-targeting, or chromosome engineering. X-ray causes DNA double-strand breaks, inducing genomic instability [16]. The chemical mutagen, ENU, induces single-base-pair substitutions in the genome causing mutations with partial functions [17, 18]. Animal models containing genes with point mutations can be used to reveal the gene's functional domain *in vivo* [19]. Both techniques are valuable but cannot predict the mutated position within the gene. Conventional gene targeting (replacement) is used to disrupt a gene (inserting markers or reporters) to determine a gene's function. Conditional gene-targeting utilizing the Cre/*loxP* and Flp/*FRT* system allows spatial and temporal control of gene expression. It has been increasingly used for gene function analysis *in vivo*.

Chromosome engineering is based on Cre/*loxP* technology, which can induce chromosome rearrangements (deletions, duplications [10, 20, 21], and inversions [22, 23]) in the mouse genome (Figure 1).

Targeting vectors can be targeted in two orientations that result in deletion, duplication, or inversion. Each targeting vector has a *loxP* site and drug selection marker, neomycin resistance (Neo), or puromycin-resistant gene (Puro). *Cis* and *Trans* indicate *loxP* sites.

Two *loxP* sites are sequentially inserted by each targeting vector into the mouse embryonic stem (ES) cell genome. Each targeting vector contains a selection marker, neomycin, or puromycin resistance gene. The vectors are manipulated by hypoxanthine phosphoribosyl transferase (HPRT) expression following Cre recombinase expression in ES cells. Transient expression of Cre recombinase induces rearrangement between *loxP* sites in the mouse genome. Clones which carry the desired chromosomal rearrangement are identified by various methods: drug selection by hypoxanthine-aminopterin-thymidine (HAT) media, genomic Southern blot analysis, fluorescent in situ hybridization (FISH), and CGH (comparative genomic hybridization) array. Although CGH array cannot identify structural chromosome aberrations such as balanced reciprocal translocations and inversions, this technique is a powerful tool to detect CNVs from genome.

To inactivate a target gene or locus by chromosome engineering, a gene target vector must be chosen or created. The Mutagenic Insertion and Chromosome Engineering Resource (MICER) (http://www.sanger.ac.uk/resources/mouse/micer/) was developed by Dr. Allan Bradley's group, the Wellcome Trust Sanger Institute and is useful as a gene-targeting vectors resource [24]. These ready to use targeting vectors can be accessed through the Ensembl mouse genome browser (http://www.ensembl.org/index.html). It is important to note that these targeting vectors use an insertion vector system rather than a replacement vector system. Given the same length of homologous sequence insertion vectors have a ninefold higher targeting efficiency than replacement vectors [25].

3. Animal Models Based on Human CNVs

In spite of a strong association between ASD (and schizophrenia) and CNV, animal models of CNV that reflect human genomic rearrangement are few. These animal

Table 1: Behavioral phenotypes of mouse models.

Human chromosomal region	Behavioral phenotypes	Reference
7q11.23 (deletion)	Increased sociability Increased acoustic startle response Cognitive deficits Growth retarded (male)	[9]
15q11-13 (duplication)	Decreased sociability Behavioral inflexibility abnormal ultrasonic vocalizations decreased spontaneous activity Increased anxiety	[10, 11]
16p11.2 (deletion)	Hyperactive difficulty adapting to change sleeping abnormalities repetitive or restricted behaviors	[12]
16p11.2 (duplication)	Hypoactive	[12]
17p11.2 (deletion)	cranio facial abnormalities Seizures Obesity	[13]
22q11.21 (deletion)	Deficits in sensorimotor gating Working memory deficit Deficit in both cued and contextual fear memory	[14, 15]

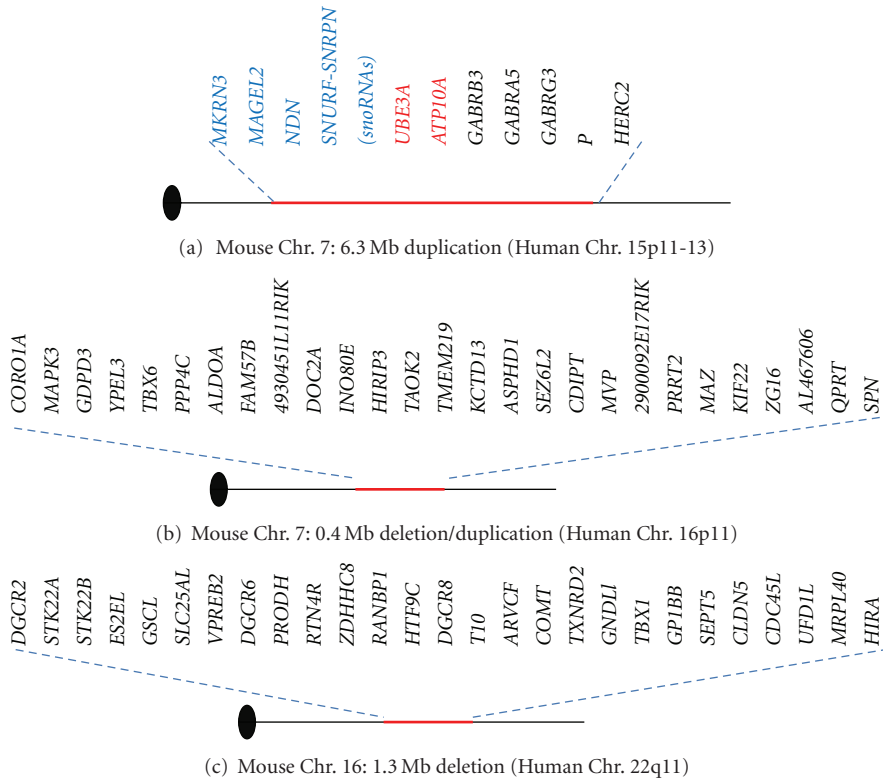
models were generated by chromosome engineering and have several psychotic phenotypes similar to those seen in patients with genomic rearrangement (Table 1). In this section we focus on 15q11-13, 16p11.2, and 22q11 locus, which are well-known copy number variant linked to ASD (or/and schizophrenia).

3.1. 15q11-13 Duplication Syndrome (ASD). Human chromosome region, 15q11-13, is a complicated region that contains *γ-aminobutyric acid receptor A (GABAA receptor)* clusters and several imprinting genes[26]. Paternally expressing genes include *MKRN3*, *MAGEL2*, *NDN*, and *SNURF-SNRPN*. Maternally expressing genes include *UBE3A* and *ATP10A*. In addition to these genes, this locus includes *noncoding small nucleolar RNAs (snoRNAs)* that are located between SNURF-*SNRPN* and *UBE3A*, which are paternally expressed and brain specific [27, 28]. Deletion or duplication of this locus causes severe neurological phenotypes. Prader-Willi syndrome (PWS) and Angelman syndrome (AS) are affected by changes in the 15q11-13 locus. Most notably, deletion of *UBE3A* has been identified to lead to AS phenotypes. Major clinical features of PWS include low birth weight, short stature, small hands and feet, severe hypotonia, feeding difficulties, obesity associated with hyperphagia starting in early childhood, mild to moderate mental retardation, and learning and behavioral problems including obsessive-compulsive disorder and autism [29, 30]. AS patients exhibit developmental delay, gait ataxia, balance disorder, frequent laughter/smiling, easily excitable personality, hyperactivity, speech impairment, microcephaly, seizures, epilepsy, and abnormal EEG (electroencephalogram) [31]. Additionally, AS patients often exhibit socialization and communication deficits, which are diagnostic criteria for ASD [32, 33].

Duplication of the 15q11-13 locus was first reported as a partial trisomy of chromosome 15 [34], and then two individuals with autistic disorder were reported [35]. This locus has been known as the most frequent cytogenetic abnormality in ASD [36, 37]. Generally patients with 15q11-13 duplication show hypotonia, delay in motor skills and language development, epilepsy, and cognitive and learning problems. Recently, Michelson et al. reported a patient with severe intractable epilepsy who has familial partial trisomy 15q11-13 inherited from a mother who has schizophrenia [38]. Autistic phenotype associated with 15q11-13 duplication, usually believed that maternal origin, *UBE3A* is involved [39–46]. Although maternal locus supposed to critical, paternally inherited patients had also developmental delay [44, 46–49]. Clinical reports have been accumulating but no mechanism has been addressed.

To address this question, Nakatani et al. generated a mouse model of human 15q11-13 duplication [10]. This mouse was generated by chromosomal engineering based on the Cre/*loxP* system, and it has a 6.3 Mb duplicated locus in mouse chromosome 7c which is highly similar to human 15q11-13 (Figure 2(a)).

Gene expression analysis revealed that paternally expressed genes, both Ndn and Snrpn, were twofold higher in paternally inherited mice (patDp/+) than wild-type (WT) mice. Similarly, maternally expressed gene Ube3a was twofold higher in maternally inherited mice (matDp/+) than WT mice. Histological analysis revealed no gross brain abnormalities. Monoamine levels in patDp/+



(a) Mouse Chr. 7: 6.3 Mb duplication (Human Chr. 15p11-13)

(b) Mouse Chr. 7: 0.4 Mb deletion/duplication (Human Chr. 16p11)

(c) Mouse Chr. 16: 1.3 Mb deletion (Human Chr. 22q11)

Figure 2: Schematic representation in studies applying chromosome engineering. Published in (a) Nakatani et al. [10], (b) Horev et al. [12], (c) Stark et al. [14]. The paternally, maternally expressed, and nonimprinting genes were labeled with blue, red, and black, respectively.

adult mice, serotonin (5-HT), and their metabolites 5-hydroxyindoleacetic acid (5-HIAA) were significantly downregulated in the midbrain and olfactory bulb. Also 5-HT content in developmental stage from postnatal 1 to 3 weeks in patDp/+ brain regions (cortex, hippocampus, cerebellum, midbrain, hypothalamus, pons, and medulla) was downregulated. This indicates 5-HT signaling during the developmental stage was significantly impaired in the brains of patDp/+ mice [10]. 5-HT influences not only mental condition (mood, social behavior, appetite, aggression and sleep) but also normal development of the central nervous system [50–52]. In addition to this, abnormal 5-HT levels have been found in ASD patient blood cells. For these reasons, 5-HT is one of the drug targets for ASD therapy. Treatment with serotonin reuptake inhibitors (SSRIs) have shown moderate success in recovering behaviors [53].

Behavioral tests revealed that patDp/+ mice display autistic behaviors such as less social interaction in the three-chamber social interaction test [54], abnormal ultrasonic vocalizations (USVs) [55] in postnatal developing pups separated from their dams, and behavioral inflexibility in the Morris Water Maze and Barnes Maze [10]. The phenotypes seen in patDp/+ mice indicate that these mice have impaired behaviors that include social interaction, communication, restricted interest, and resistance to change. These deficits correspond to human autistic phenotypes [56, 57]. Furthermore, patDp/+ mice showed anxiety-related phenotypes: decreased locomotor and exploratory activities in the open field and *Y*-maze test, and long latencies in novelty suppressed feeding test [11]. These anxiety-related phenotypes frequently accompany autistic symptoms in humans [58, 59]. Also the marble burying test, which is a useful test for the study of anxiety, obsessive-compulsive disorder (OCD), and neophobia, found that the number of buried marbles was significantly low in patDp/+ mice [11].

3.2. 16p11.2 Deletion/Duplication Syndrome (Deletion: ASD, Duplication: Schizophrenia). Deletion or duplication of the chromosome 16p11.2 locus was observed in nearly 1% of multiplex families with ASD [60]. Meta-analysis of patients with ASD and/or developmental delay estimated that 16p11.2 locus deletion is associated with a 38.7-fold increase in the odds of ASD/developmental delay. On the other hand, 16p11.2 locus duplication is associated with a 20.7-fold increase in the odds of ASD/developmental delay [60–63]. In addition to these, 16p11.2 deletion is associated with obesity [64], and duplication is associated with schizophrenia [65] as well as ASD [60, 66]. Notably, a brain anatomical abnormality (abnormal head size) has been reported to be associated with this locus. For instance, patients with the 16p11.2 deletion had statistically significant macrocephaly and those with duplication had microcephaly [67].

A mouse model of human 16p11.2 deletion (df/+) as well as duplication (dp/+) has been reported [12] (Figure 2(b)).

This mouse model was generated by Cre/*loxP*-based chromosome engineering. This locus includes 27 genes, *SPN, QPRT, c16orf54, KIF22, MAZ, PRRT2, c16orf53, MVP, CDIPT, SEZ6L2, ASPHD1, KCTD13, LOC124446, HIRIP3, CCDC95, DOC2A, FAM57B, ALDOA, PPP4C, YPEL3, GDPD3, MAPK3,* and *CORO1A*.

Young df/+ mice (before weaning) tend to be smaller than WT siblings, but as adults they are almost the same size as WT siblings and look healthy. Interestingly, 16p11.2 CNV mice, df/+ and dp/+ mice have opposite phenotypes. In a novel environmental cage, df/+ mice displayed longer distance traveled and time spent walking as compared with WT mice. In contrast, dp/+ mice traveled a shorter distance and spent less time walking as compared with WT mice. Additionally, df/+ mice were significantly active in both dark and light period. These results indicate that 16p11.2 locus affects not only physical activity but also diurnal activity and sleeping related symptoms. Also, a brain anatomical study using Magnetic resonance imaging (MRI) identified several regional changes in 16p11.2 CNV mice. For instance, df/+ mice showed increased volume of several brain regions (percentage of total brain volume): forebrain, superior colliculus, fornix, hypothalamus, mammillothalamic tract, medial septum, midbrain, and periaqueductal grey. These brain regional volumetric changes were more significant between df/+ and dp/+ than betweendf/+ and WT mice.

3.3. 22q11.2 Deletion Syndrome (22q11.2DS, DiGeorge Syndrome (DGS), Velo-Cardio-Facial Syndrome (VCFS)) (Schizophrenia). Microdeletion of chromosome 22q11 is found in 1 out of every 4000 live births, making it one of the most common interstitial deletions [68]. This 22q11.2 microdeletion causes craniofacial, cardiovascular abnormalities, immunodeficiency, hypocalcaemia, short stature, and cognitive dysfunctions [69–71]. Microdeletion of this region accounts for 1-2% of the cases of people with schizophrenia [72, 73]. Also, this locus accounts for up to 1-2% of cases of sporadic schizophrenia [74–76]. Some neuroanatomical changes have been reported in patients with 22q11.2DS as well. Volumetric reduction in total brain volume includes cortical regions (e.g., frontal, parietal, temporal, and occipital lobes), hippocampus, and cerebellum [77–86]. However, inconsistency in these neuroimaging reports may be due to the small numbers of subjects used and differences in methodology [72]. Yet these neuroanatomical reports are informative because some abnormalities are consistent with phenotypes of those who have non-22q11.2 DS-associated schizophrenia [76].

The majority of deletions in this locus are 3 Mb deletions (−90% of the cases), but 1.5 Mb deletions (<10% of the cases) contain 28 known genes which include critical genes and increased risk of mental disorders [73, 87].

The mouse chromosome 16 region is conserved with human 22q11.2. Animal models of the human 22q11.2 deletion were generated by 2 groups, and both groups used chromosome engineering [14, 88] (Figure 2(c)). These mouse models, Df(16)$A^{+/-}$ [14] and LgDel/+ [88], include 1.5 Mb critical regions, and both of them display several behavioral abnormalities, such as deficits in working memory, sensorimotor gating, and fear conditioning [14, 89–92]. Working memory deficits are becoming one of the main features of patients with schizophrenia, thus these animal models are supposed to reflect some aspects of 22q11.2 DS syndrome phenotype. In addition to behavioral abnormalities in this mouse, diminished 22q11 locus dosage disrupts cortical neurogenesis, interneuron migration [93], dendritic complexity, and formation of excitatory synapses [94]. Although, several interesting phenotypes have been reported in this mutant mouse, there are no studies published about brain structural abnormalities even though several brain abnormalities have reported in human studies. These brain anatomical changes and molecular mechanisms that underlie these phenotypes will be interesting to elucidate and will be addressed by using brain imaging techniques.

4. Future Perspectives

Application of new technologies, such as Comparative Genomic Hybridization (CGH) and next-generation sequencing, will reveal more additional genomic rearrangements related to psychiatric disorders. Thus, to analyze both phenotypes and underlying molecular mechanisms that originate from genetic rearrangements, animal models will be a powerful tool. In this context, chromosome engineering will be a valuable tool. Recently Ruf et al. [95] reported that they generated several hundred mice and embryos which have one *loxP* and LacZ site at a random genomic positions that inserted by sleeping beauty-based transposition system. These lines are mapped in Transposon and Recombinase Associated Chromosomal Engineering Resource database (TRACER, http://tracerdatabase.embl.de/fmi/iwp/res/iwp_home.html). This database is useful in creating chromosome rearrangements *in vivo*.

A logical next step is to identify responsible gene(s) in CNV. It is an orthodox approach to narrow down the region by systematically insertion of *loxP* combining the existed lines such as above TRACER. Generating Bacterial Artificial Chromosome (BAC) transgenic mice is another way to identify critical genes. BAC transgenes inserted to the genome faithfully recapitulate chromosomal endogenous gene expression, since BAC transgenic mice may appropriate animal model of gene duplication. Also transient overexpressing (or knockdown) each transcript in developmental brain is possible strategy. Recently Golzio et al. [96] identified a responsible gene *KCTD13* in 16p11.2 locus which causes brain malformation by using zebrafish. Use of these technologies in generating valid and etiology-based animal model of psychiatric disorders will contribute to the development of drugs against disorders and elucidation of molecular mechanisms that underlie these psychiatric disorders.

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Review Article

Induced Pluripotent Stem Cells to Model and Treat Neurogenetic Disorders

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Remarkable advances in cellular reprogramming have made it possible to generate pluripotent stem cells from somatic cells, such as fibroblasts obtained from human skin biopsies. As a result, human diseases can now be investigated in relevant cell populations derived from induced pluripotent stem cells (iPSCs) of patients. The rapid growth of iPSC technology has turned these cells into multipurpose basic and clinical research tools. In this paper, we highlight the roles of iPSC technology that are helping us to understand and potentially treat neurological diseases. Recent studies using iPSCs to model various neurogenetic disorders are summarized, and we discuss the therapeutic implications of iPSCs, including drug screening and cell therapy for neurogenetic disorders. Although iPSCs have been used in animal models with promising results to treat neurogenetic disorders, there are still many issues associated with reprogramming that must be addressed before iPSC technology can be fully exploited with translation to the clinic.

1. Introduction

Most of our current knowledge about the central nervous system (CNS) and neural function in patients with neurological diseases has been obtained from postmortem tissues that often represent the end stage of the disease. The inability to sample live CNS tissues impedes our progress to understand aspects of the neuropathological abnormalities that develop during the course of the disease [1–4]. Animal models can mimic genetic forms of human neurological diseases, and our understanding of the mechanisms of neurological diseases has been significantly advanced with transgenic/knockout technologies. However, these technologies are mainly limited to monogenetic disorders and thus only represent a minority of diseases. Additionally, in many cases of neurological disorders with a defined causal gene(s), modeling with animal transgenic technology is inadequate due to species differences, genetic backgrounds, or other technical challenges [3, 5, 6]. More strikingly, numerous candidate drugs with promise in animal model screening have failed when translated to human clinical trials. The failure of translation to the clinic centers on the complexity of the human brain and the difficulty to model disease specific phenotypes in nonhuman systems [5]. This situation indicates that an advancement towards more human relevant models is definitely needed to accurately study neurogenetic disorders.

The elegant and seminal work by Takahashi and Yamanaka showed that retroviral expression of a set of four genes (Oct4, Sox2, Klf4, and c-Myc) can convert somatic cells into a pluripotent state [7, 8]. Like other pluripotent stem cells, induced pluripotent stem cells (iPSCs) can be coaxed to differentiate into neurons and glial cells, as well as other terminally differentiated cell types by exposure to a combination of growth factors and cell culture conditions [9, 10]. Therefore, human iPSCs make it possible to study human CNS neuronal lineages. Neurons derived from iPSCs carry the genetic information from patients with a specific mutation or a neurological disease [3, 11, 12]. Over the past few years, the progress in cell reprogramming has accelerated the generation of iPSCs, and iPSCs have now been derived

Table 1: Genetic neurodevelopmental disorders modeled with iPSCs.

Disease	Genetic defects	iPSC derived cell types	Disease phenocopied in iPSCs or differentiated cells	Drug or functional tests
Rett syndrome	Mutation in *MECP2*	Neurons; glutamatergic neurons	Yes	Yes
	Mutation in *CDKL5*	Neurons	NA	No
Fragile X syndrome	CGG triplet repeat expansion resulting in the silencing of *FMR1*	Neurons and glia	Yes	No
Down syndrome	Trisomy 21	Cortical neurons	Yes	Yes
Angelman syndrome	Lack of *UBE3A* expression due to genomic imprinting	Neurons	Yes	Yes
Prader-Willi syndrome	Lack of expression of genes in paternal chromosome region 15q11-q13 due to genomic imprinting	Tissues of the three germ layers, including neurons	NA	No
Timothy syndrome	Mutation in the L-type calcium channel $Ca_v1.2$	Neurons	Yes	Yes

from several easily accessible human cell types, including blood cells, keratinocytes, and dermal fibroblasts [4, 5, 13–15]. The iPSC technology has opened new windows for modeling human diseases, identifying therapeutic targets, developing drug screening systems, and providing continuous autologous cell sources with potential for cell therapies [1, 5, 11, 15–20].

Here, the recent efforts and key findings when using iPSCs to model neurogenetic disorders are reviewed. The potential of iPSCs from patients as platforms for drug screening and as a source for cell therapy are presented. Additionally, some of the challenges in iPSC modeling and in iPSC based therapy of neurogenetic disorders are highlighted.

2. iPSCs for Modeling Neurogenetic Disorders

2.1. Genetic Neurodevelopmental Disorders. Genetic neurodevelopmental disorders include a wide range of diseases characterized by impairment of neuronal function during development. These conditions are monogenic or multigenic. Disease-specific iPSC lines have been generated from patients with neurodevelopmental diseases including Rett syndrome, Fragile X syndrome, Down syndrome, Angelman syndrome, Prader-Willi syndrome, and Timothy syndrome (Table 1). The iPSC based models of neurodevelopmental disorders recapitulate the early steps in neural development within genetic backgrounds that are linked to the specific disorder and may help to identify the underlying cellular and molecular mechanisms and establish novel therapeutics.

2.1.1. Rett Syndrome. Rett syndrome (RTT) is a neurodevelopmental autism spectrum disorder, caused by mutations in the methyl CpG-binding protein (*MECP2*) gene [21–25]. The iPSC derived neurons from patients with RTT reveal relevant neuronal phenotypes, such as neuronal maturation defects [26–28]. Consistent with RTT animal models and human postmortem brain tissues [29], the cell soma size of RTT neurons is decreased in comparison with nonaffected controls. Additionally, neurons derived from RTT iPSCs have fewer synapses, reduced spine densities, altered calcium signaling, and electrophysiological defects, suggesting a communication problem in RTT neuronal networks [27]. Treatment with insulin growth factor 1, a growth factor known to ameliorate the phenotype of RTT mice, improves the RTT iPSC-neuronal phenotypes, indicating that synaptic defects can be rescued in neurons derived from RTT patients [27, 30]. The iPSCs can be directed to produce glutamatergic neurons that generate action potentials and form functional excitatory synapses. A recent study found that iPSC derived neurons from heterozygous $MECP2^{308}$ mice showed defects in the generation of evoked action potentials and in glutamatergic synaptic transmission [31], as previously reported in brain slices [32–34]. These MeCP2 deficient neurons fired fewer action potentials and displayed decreased action potential amplitudes, diminished peak inward currents, and higher input resistance relative to wild-type iPSC derived neurons, suggesting that disturbed sodium channel function may contribute to the dysfunctional RTT neuronal network. These phenotypes were further confirmed in neurons derived from independent wild-type and hemizygous mutant iPSC lines, indicating that these reproducible deficits are attributable to the MeCP2 deficiency [31]. Taken together, these studies demonstrate that RTT iPSC derived neurons recapitulate deficits observed previously in primary neurons, and these identified phenotypes further indicate the requirement of MeCP2 in neuronal development and the maintenance of normal brain function.

MeCP2 is a protein involved in global DNA methylation [35–38]. The activity of L1 retrotransposons during brain development can have an impact on gene expression and

neuronal function. L1 neuronal transcription and retrotransposition in rodents are increased in the absence of MeCP2 [39–41]. Studies with neuronal progenitor cells derived from human iPSCs revealed that patients with RTT, carrying MeCP2 mutations, have increased susceptibility for L1 retrotransposition, suggesting a new potential molecular mechanism underlying RTT [42]. *MECP2* is an X-linked gene subject to random X chromosome inactivation (XCI) resulting in mosaic expression of mutant *MECP2*. The XCI status of RTT iPSCs has been inconsistent among different studies. Some reported RTT iPSCs in which the inactive X chromosome of the founder somatic cell is reactivated. Conversely, others reported that RTT iPSCs retain the inactive X chromosome of the founder somatic cell [43]. Marchetto et al. [27] found that RTT patients' iPSCs are able to undergo XCI. Cheung et al. [26] reported that iPSCs from classic female RTT patients with a functionally null mutation retained the *MECP2* mutation and an inactive X-chromosome in a non-random pattern. By taking advantage of the nonrandom pattern of XCI, they generated a pair of isogenic wild-type and mutant *MECP2* expressing RTT iPSC lines that retain the *MECP2* expression pattern upon differentiation into neurons. The mutant RTT iPSC derived neurons showed a reduced soma size compared with the isogenic control RTT iPSC derived neurons. Kim et al. [28] found that some iPSCs could maintain XCI, whereas in others the X chromosome was reactivated. They isolated iPSCs that retained a single active X chromosome expressing either mutant or wild-type MeCP2, as well as iPSCs with reactivated X chromosomes expressing both mutant and wild-type MeCP2. Consistent with RTT phenotypes, the mutant monoallelic or biallelic RTT iPSC derived neurons also showed maturation defects. Thus, the isogenic control and mutant RTT iPSC lines represent an additional promising source for investigating the pathogenesis of RTT and the role of *MECP2* in human neurons.

Classic RTT is caused by mutations in the *MECP2* gene, whereas variants could be due to mutations in *CDKL5*. Mutations in *CDKL5* have been identified both in females with the early onset seizure variant of RTT and in males with X-linked epileptic encephalopathy [44–47]. CDKL5 is a kinase protein highly expressed in neurons, but its exact function inside the cell is largely unknown [46, 48, 49]. By using iPSCs derived from fibroblasts of patients with *CDKL5* mutations, Amenduni et al. [50] demonstrated that female *CDKL5* mutated iPSCs maintain XCI and clones express either the mutant *CDKL5* allele or the wild-type allele that serve as an ideal experimental control. Furthermore, these iPSCs can be differentiated into neurons and are suitable to model the pathogenesis of *CDKL5* related disorders.

2.1.2. Fragile X Syndrome. Fragile X syndrome (FXS) is the most common inherited form mental impairment that is caused by an expanded CGG trinucleotide repeat in the 5′ untranslated region of the fragile X mental retardation (*FMR1*) gene leading to gene silencing and loss of the fragile X mental retardation protein (FMRP) [51–55]. In twenty years since the identification of the *FMR1* gene, numerous efforts have focused on understanding the consequences of loss of FMRP on neuronal development and function [51–54, 56–64]. The advent of iPSCs provides an option to study FXS in human models and allows investigations into aspects of FXS that are difficult to study in animal models. Human embryonic stem cell lines derived from embryos diagnosed with a full mutation showed that *FMR1* is unmethylated when expressed in these cells, and *FMR1* gene silencing occurs only upon differentiation of embryonic stem cells [65, 66]. In contrast, for iPSCs generated from the fibroblasts of FXS patients, *FMR1* remains methylated and transcriptionally silenced with the reprogramming process failing to reverse the methylation state of *FMR1* [66]. It seems that the current FXS iPSCs may not be suitable to model the effects of *FMR1* silencing during neuronal differentiation. A subsequent study further characterized the differentiation of FXS iPSCs into postmitotic neurons and glia [67]. In this study, the iPSC lines were generated from multiple patients with FXS. The authors found that clones from reprogrammed FXS patient fibroblast lines exhibit variation with respect to the predominant CGG repeat length in the *FMR*1 gene. In two cases, iPSC clones contained predominant CGG repeat lengths that were shorter than the corresponding input population of fibroblasts. In another case, reprogramming a mosaic patient having both normal and premutation length CGG repeats resulted in genetically matched iPSC clonal lines differing in *FMR1* promoter CpG methylation and FMRP expression. Using this panel of patient-specific FXS iPSC models, the authors demonstrated that aberrant neuronal differentiation from FXS iPSCs is correlated with the epigenetic modification of the *FMR1* gene and a loss of FMRP expression [67]. These findings provide evidence for roles of FMRP in early neurodevelopment prior to synaptogenesis and show potential for modeling FXS with iPSC technology.

2.1.3. Down Syndrome. Down syndrome (DS) is neurodevelopmental disorder caused by trisomy of chromosome 21 [68–72]. Considering that mice do not have chromosome 21, it seems unlikely to completely recapitulate the disease features in mouse DS models. However, human neural progenitor cell (NPC) lines have been generated to model DS [73], and the iPSC models provide scientists with an additional approach to study underlying mechanisms [74]. Adults with DS develop early-onset Alzheimer's disease (AD), probably due to increased expression of a gene on chromosome 21 that encodes the amyloid precursor protein (APP) [75–77]. A recent study found that cortical neurons generated from iPSCs of DS patients could develop AD pathologies over months in culture, rather than years *in vivo*. These cortical neurons processed APP resulting in secretion of the pathogenic peptide amyloid β42, which formed insoluble amyloid aggregates. The gamma-secretase inhibitor could block the production of amyloid β42. Additionally, hyperphosphorylated tau protein, a pathological marker for AD, was found to be localized to cell bodies and dendrites of AD iPSC derived cortical neurons, which mimics the phenotypes of later stages of AD [78]. The generation of iPSC lines to investigate other similar defects, such as the trisomy of other chromosomes may be rewarding.

2.1.4. Angelman Syndrome. Angelman syndrome (AS) is a neurodevelopmental disorder associated with genomic imprinting which results from a loss of function of the ubiquitin protein ligase E3A (*UBE3A*) gene [79–82]. Although iPSCs present an invaluable approach to modeling human disease, their usefulness could be limited in AS if the genomic imprinting marks are disturbed by the nuclear reprogramming of somatic cells to pluripotent stem cells. However, Chamberlain et al. [83] found that genomic imprinting was retained in AS iPSCs following nuclear reprogramming. The imprinting of *UBE3A* could be established during neuronal differentiation of AS iPSCs like normal brain tissue. In this case the paternal *UBE3A* allele was repressed in parallel to upregulation of the *UBE3A* antisense transcript. In addition, electrophysiological recordings detected AMPA-receptor-mediated spontaneous activity in mature neurons derived from AS iPSCs, indicating that functional neurons can be generated from AS iPSCs. The iPSC models will be further utilized to investigate the events related to AS, such as the developmental timing and mechanism of *UBE3A* repression in human neurons.

2.1.5. Prader-Willi Syndrome. Prader-Willi syndrome (PWS) is a neurological genomic imprinting disorder, characterized by the lack of gene expression in the paternal chromosome region 15q11-q13, while the same region in the maternal chromosome is repressed by means of DNA methylation [84–86]. Chamberlain et al. [83] found that the PWS iPSC lines show no disrupted methylation patterns in the "Prader-Willi syndrome imprinting center" (PWS-IC) in comparison to the source fibroblast cell lines. This study indicated that similar to AS, genomic imprinting of PWS can be refractory to the epigenetic erasure produced during reprogramming. Another study by Yang et al. [87] further confirmed that PWS iPSCs retain a high level of DNA methylation in the imprinting center of the maternal allele and show concomitant reduced expression of the disease-associated small nucleolar RNA HBII-85/SNORD116 [87]. Moreover, these iPSCs could readily differentiate into tissues of the three germ layers, including neurons [87]. These studies indicate that iPSCs can be used to model genomic imprinting diseases.

2.1.6. Timothy Syndrome. Timothy syndrome (TS) is a neurodevelopmental disorder caused by a missense mutation in the L-type calcium channel $Ca_v1.2$ that is associated with developmental delay and autism [88–90]. Paşca et al. [91] generated cortical neuronal precursor cells and neurons from iPSCs derived from patients with TS. The authors found that the cells from these individuals have defects in calcium Ca^{2+} signaling and activity-dependent gene expression. The cells also showed abnormalities in differentiation, including decreased expression of the genes that are expressed in lower cortical layers and in callosal projection neurons. Moreover, neurons derived from individuals with TS show abnormal expression of tyrosine hydroxylase and an increase in production of norepinephrine and dopamine. These biochemical changes were reversed by treatment with roscovitine, a cyclin-dependent kinase inhibitor and an atypical L-type-channel blocker. This study provided evidence that L-type calcium channel $Ca_v1.2$ is involved in the regulation of cortical neuronal differentiation in humans and offers new insights into the pathogenesis of autism in patients with TS.

2.2. Genetic Neurodegenerative Disorders. Genetic neurodegenerative disorders include a variety of diseases that involve the chronic and progressive loss of neuronal structure and function. To date, iPSCs have been generated from patients of many neurodegenerative disorders, including spinal muscle atrophy, familial dysautonomia, amyotrophic lateral sclerosis, Huntington's disease, Friedreich ataxia, Machado-Joseph disease, X-linked adrenoleukodystrophy, Alzheimer's disease, and Parkinson's disease (Table 2). We will comment on the current iPSC technology for these disorders in the following section and illustrate how these cell lines are helping to unravel the mechanisms of neurodegeneration.

2.2.1. Spinal Muscle Atrophy. Spinal muscular atrophy (SMA) is an autosomal recessive genetic disorder caused by mutations in the survival motor neuron 1 gene (*SMN1*) that significantly reduces SMN protein expression and leads to the selective degeneration of lower α-motor neurons [92–95]. Although patient fibroblasts have been widely used to study SMA, motor neurons provide a better model to study the anatomy and physiology in the inherent pathology. SMA was the first neurodegenerative disease to be modeled by human iPSCs. To model SMA, Ebert et al. [96] generated iPSCs from a child with a mutation in *SMN1* and from his unaffected mother. They found that iPSCs retained the capacity to generate differentiated neural tissue and motor neurons. However, the lack of *SMN1* expression and the disease phenotype of selective motor neuron death were maintained. Interestingly, two compounds, valproic acid and tobramycin, which have been known to increase SMN levels, could partially restore the reduction in the SMN protein. Recently, Chang et al. [97] reported the establishment of five iPSC lines from the fibroblasts of an SMA patient. The authors found that neuronal cultures derived from these SMA iPSC lines exhibited a reduced capacity to form motor neurons and showed abnormal neurite outgrowth in culture. Ectopic SMN expression in these iPSC lines restored normal motor neuron differentiation and rescued the phenotype of delayed neurite outgrowth. These findings indicate that the observed abnormalities are indeed caused by the SMN deficiency and not by phenotypic diversity among iPSC lines. Taken together, these studies show that human iPSCs are useful to model the specific neuronal pathology of SMA and human iPSCs represent a promising resource to screen new drug compounds and develop new therapies for SMA [98].

2.2.2. Familial Dysautonomia. Familial dysautonomia (FD) is a rare but fatal peripheral neuropathy, caused by a point mutation in the *IKBKAP* gene involved in transcriptional elongation [99–101]. FD is characterized by the depletion of autonomic and sensory neurons. The specificity to the peripheral nervous system and the mechanism of neuron loss in FD are poorly understood owing to the lack of an appropriate model system [99, 102]. Lee et al. [103] reported

Table 2: Genetic neurodegenerative disorders modeled with iPSCs.

Disease	Genetic defects	iPSC derived cell types	Disease phenocopied in iPSCs or differentiated cells	Drug or functional tests
Spinal muscular atrophy	Mutation in *SMN1*	Neuronal cultures; motor neurons	Yes	Yes
Familial dysautonomia	Mutation in *IKBKAP*	Cells of all three germ layers including peripheral neurons	Yes	Yes
Amyotrophic lateral sclerosis	Mutation in *SOD1*	Motor neurons	NA	No
	Mutation in *VAPB*	Motor neurons	Yes	No
	Mutation in *TAP-43*	Neurons and motor neurons	Yes	Yes
Huntington's disease	Excessive expansion of CAG repeat in Huntingtin gene	Neuronal precursors, striatal neurons, astrocytes	Yes	Yes
Friedreich ataxia	GAA repeat expansion in the *FXN* gene	Peripheral neurons and cardiomyocytes	Yes	No
Machado-Joseph disease	Expansion of CAG repeats in the *MJD1* (*ATXN3*) gene	Neurons, fibroblasts and glia	Yes	Yes
X-linked adrenoleuko-dystrophy	Mutation in *ABCD1*	Oligodendrocytes and neurons	Yes	Yes
Alzheimer's disease	Mutations in *PS1* and *PS2*	Neurons	Yes	Yes
	Duplication of *APP*	Neurons	Yes	Yes
Parkinson's disease	Mutation in *LRRK2*	Dopaminergic neurons	Yes	Yes
	Mutations in *PINK1*	Dopaminergic neurons	Yes	Yes
	Triplication of *SNCA*	Dopaminergic neurons	Yes	Yes
	Mutation in *PARKIN*	Dopaminergic neurons	Yes	Yes

the derivation of patient specific FD iPSCs and the directed differentiation into cells of all three germ layers including peripheral neurons. Gene expression analysis in purified FD iPSC derived lineages demonstrated tissue-specific mis-splicing of *IKBKAP in vitro*. Patient-specific neural crest precursors expressed particularly low levels of the normal *IKBKAP* transcript, suggesting a mechanism for disease specificity. FD pathogenesis has been further characterized by transcriptome analysis, and cell-based assays reveal marked defects in neuronal differentiation and migration behaviour. Furthermore, FD iPSCs were used for validating the potency of candidate drugs in reversing aberrant splicing and ameliorating neuronal differentiation and migration. This study, while limited, has laid the groundwork to use reprogramming technology for modeling FD.

2.2.3. Amyotrophic Lateral Sclerosis. Amyotrophic lateral sclerosis (ALS) is mainly characterized by muscular atrophy and weakness that accompanies a fast and progressive degeneration of motor neurons in the brain and spinal cord [104–109]. The majority of ALS cases are sporadic (sALS) and approximately 10% of cases are inherited (familial; fALS). More than 10 different genes have been implicated in ALS, including superoxide dismutase 1 (*SOD1*, *ALS1*), transactive response DNA-binding protein-43 (*TDP-43*, *ALS10*), fused sarcoma (*FUS*, *ALS6*), and vamp-associated protein B/C (*VAPB*, *ALS8*) [107–111]. Clinical trials based on ALS animal models have been disappointing, indicating a need for the exploration of new ALS models [104, 105, 112]. To date, three groups have successfully generated iPSCs from three different familial forms of ALS with previously identified mutations. Dimos et al. [113] generated iPSCs from a patient with a mutation in the *SOD1* gene. They found that the patient-specific iPSCs possess properties of embryonic stem cells and could be directed to differentiate into motor neurons. However, no assay of ALS related phenotypes was completed for the iPSCs derived motor neurons in this study. Mitne-Neto et al. [114] generated iPSC lines from patients with mutations in the *VAPB* gene as well as from noncarrier siblings (controls). They showed a significant reduction in the levels of VAPB protein in ALS8 iPSC derived motor neurons, suggesting that the reduction in VAPB could be involved in the pathogenesis of ALS8. They further demonstrated that the level of VAPB protein gradually increased during the differentiation of control iPSCs but not ALS8 iPSCs, suggesting that the ALS8 mutation causes a failure of VAPB protein upregulation during the induction of motor neurons. This regulation of VAPB is likely to happen at the posttranslational level since there is no difference in the mRNA levels during the differentiation between control and ALS8 iPSCs. These findings may be relevant to other forms of ALS, as the reduction in VAPB protein has been documented in motor neurons from sporadic ALS patients [115, 116]. The iPSCs that carry the *TDP-43* mutation could differentiate into neurons and functional motor neurons. The mutant neurons showed the cellular phenotypes of

ALS and other TDP-43 proteinopathies, including elevated soluble and detergent-resistant TDP-43 protein levels, decreased survival, and increased vulnerability to blockade of the phosphoinositide-3-kinase (PI3K) pathway [117]. Since other cells, such as astrocytes and microglia that associate with the motor neuron niche, have been shown to play a role in the pathology of ALS [118–120], it will be interesting to see if the iPSC derived astrocytes or microglia can also recapitulate the nonneuronal aspects of the disease.

2.2.4. Huntington's Disease. Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by an excessive expansion of a CAG trinucleotide repeat in the gene encoding the protein huntingtin, producing an elongated stretch of glutamines near the N-terminus of the protein [121–124]. The expanded repeat region causes a gain of function in the huntingtin protein, which then forms aggregates within the nucleus of certain neuronal cells [121, 122]. Many tissue culture models for Huntington's disease have been generated by a wide range of techniques. Modeling systems include nonneural human cell types (fibroblasts and lymphoblasts), immortalised or primary neurons from mice, and mouse and human ES cells [125–127]. These models can recapitulate many of the phenotypes seen in patients with HD. At this time, iPSCs have been generated from patients with HD [74, 128], transgenic HD monkeys, and mouse models [12, 129]. The human HD iPSCs have been used to generate neuronal precursors and striatal neurons. The HD iPSCs derived striatal neurons, and neuronal precursors contain the same CAG expansion as the mutation in the HD patient from whom the iPSC line was established. Moreover, the HD neural stem cells showed enhanced caspase activity upon growth factor deprivation, which is indicative of apoptosis [128]. Therefore, these differentiated cells are encouraging for a useful human HD cell model. The HD monkey iPSCs develop cellular features comparable to HD, including the accumulation of mutant huntingtin (htt) aggregates and the formation of intranuclear inclusions paralleling neural differentiation *in vitro* [129]. iPSCs from transgenic HD monkeys represent nonhuman primate modeling of human diseases. In the generation of iPSCs through somatic reprogramming of fibroblasts from the R6/2 transgenic HD mouse line, CAG expansion has no effect on reprogramming efficiency, cell proliferation rate, brain-derived neurotrophic factor levels, or neurogenic potential. In addition, these iPSCs do not show an increase in cell death either under self-renewal or differentiated conditions. However, genes that are involved in the alteration of the cholesterol biosynthesis pathway in HD are also affected in HD iPSC lines. Furthermore, one lysosomal gene is upregulated and the lysosome number is increased in HD iPSC lines [12]. One recent study further demonstrated that HD patient specific iPSCs were able to generate phenotypically normal, functional neurons *in vitro* and could survive and differentiate into neurons in the adult mouse brain after transplantation. However, astrocytes derived from these HD iPSCs showed a vacuolation phenotype, a phenomenon found in primary lymphocytes from HD patients [130]. These findings suggest that iPSCs from HD animals or patients can replicate some, but not all, of the phenotypes typically observed in the disease. In the future, modeling of other phenotypes, including the pathological changes only seen in the autopsy of patients and the electrophysiological changes seen in animal models may, become possible.

2.2.5. Friedreich Ataxia. Friedreich ataxia (FRDA) is an autosomal recessive disorder characterised by neurodegeneration and cardiomyopathy [131–134]. It is caused by a trinucleotide (GAA) repeat expansion in the first intron of the *FXN* gene that results in reduced synthesis of FXN mRNA and its protein product, frataxin [135, 136]. Ku et al. [137] firstly reported the derivation of iPSCs from FRDA patient fibroblasts by transcription factor reprogramming. They found that *FXN* gene repression is maintained in the iPSCs. The GAA repeats in *FXN* in iPSCs exhibit instability similar to the patient families, where they expand and/or contract with discrete changes in length between generations. The mismatch repair enzyme MSH2, which is implicated in repeat instability in other triplet repeat diseases, is highly expressed in iPSCs and occupies the *FXN* intron 1. Knockdown of MSH2 impedes repeat expansion. This study provides a possible molecular explanation for repeat expansion in FRDA. Liu et al. [138] reported the generation of iPSC lines derived from skin fibroblasts from FRDA patients. The authors found that the patient-derived iPSC lines maintain the GAA repeat expansion and the reduced FXN mRNA expression patterns that are characteristic of the patient. Interestingly, the instability of the GAA repeat length was also found within these FRDA iPSC lines. They further demonstrated that following *in vitro* differentiation, the iPSCs can produce the two cell types primarily affected in FRDA, namely peripheral neurons and cardiomyocytes. Thus, these FRDA iPSC lines have the potentials to provide powerful tools to study the cellular pathology of FRDA.

2.2.6. Machado-Joseph Disease. Machado-Joseph disease (MJD), also known as spinocerebellar ataxia type 3, is a dominantly inherited late-onset neurodegenerative disorder caused by expansion of polyglutamine (polyQ)-encoding CAG repeats in the *MJD1* (*ATXN3*) gene [139–142]. Proteolytic liberation of highly aggregation-prone polyQ fragments from the protective sequence of the *MJD1* gene product ataxin 3 (*ATXN3*) may trigger the formation of *ATXN3*-containing aggregates, the pathological hallmark of MJD [140, 141]. The levels of *ATXN3* fragments in brain tissues of MJD patients increases with disease severity, supporting a relationship between *ATXN3* processing and disease progression. The formation of early aggregation intermediates is believed to be critical for disease initiation, but the precise molecular mechanism in MJD is unknown [140, 141]. To investigate this, Koch et al. [143] generated iPSCs from MJD patients. The authors found that L-glutamate induced excitation of patient specific iPSC derived neurons initiated Ca^{2+}-dependent proteolysis of *ATXN3*, followed by the formation of sodium dodecyl sulphate (SDS)-insoluble aggregates. This phenotype could be abolished by calpain inhibition, indicating a key role of calpain in *ATXN3*

aggregation. They further demonstrated that aggregate formation depended on functional Na^+ and K^+ channels as well as ionotropic and voltage-gated Ca^{2+} channels. However, these channel effects were not observed in iPSCs, fibroblasts, or glia and hence may explain the neuron-specific phenotype of MJD. This study demonstrates the usefulness of iPSCs to investigate the aberrant protein processing associated with late-onset neurodegenerative disorders in patient-specific neurons.

2.2.7. X-Linked Adrenoleukodystrophy. X-linked adrenoleukodystrophy (X-ALD) is caused by mutations in the *ABCD1* (adenosine triphosphate (ATP)-binding-cassette transporter superfamily D member 1) gene encoding a peroxisomal ATP-binding cassette (ABC) transporter, ABCD1, which is responsible for entry of long chain fatty acids (VLCFAs; C26:0 and C24:0) into peroxisomes for degradation [144–147]. With abnormally high VLCFA levels, primary manifestations occur in the nervous system, the adrenal cortex, and the Leydig cells of the testis [144, 145]. X-ALD, with an incidence of 1 in 20,000 males, shows a wide range of phenotypic variability which does not directly correlate with *ABCD1* gene mutations [146–150]. Due to the lack of an appropriate animal model system and the inaccessibility of human oligodendrocytes *in vivo* [147, 151], iPSCs may provide a unique cellular model for studying the pathology of X-ALD. Jang et al. [152] generated iPSCs from patients with the 2 major types of X-ALD, namely, childhood cerebral ALD (CCALD) and adrenomyeloneuropathy (AMN). The authors evaluated disease relevant phenotypes by pharmacological and genetic approaches. They found that both CCALD and AMN iPSCs normally differentiated into oligodendrocytes, the cell type primarily affected in the X-ALD brain, indicating no developmental defect due to the *ABCD1* mutations. Although low in X-ALD iPSCs, long chain fatty acid (VLCFA) levels were significantly increased after oligodendrocyte differentiation. VLCFA accumulation was much higher in CCALD oligodendrocytes when compared to AMN oligodendrocytes, whereas no significant difference between CCALD and AMN neurons was reported, indicating that the severe clinical manifestations in CCALD might be associated with abnormal VLCFA accumulation in oligodendrocytes. They further showed that the abnormal accumulation of VLCFA in the X-ALD oligodendrocytes can be reduced by upregulating *ABCD2* gene expression after treatment with lovastatin or 4-phenylbutyrate. Therefore, the X-ALD iPSC model recapitulates the key events of the disease process and provides a new way to understand and diagnose X-ALD disease subtypes.

2.2.8. Alzheimer's Disease. AD is the most common age-related dementia, characterized by progressive memory loss and cognitive disturbances [153–157]. AD presents with a strong genetic predisposition [158, 159]. Mutations of presenilin 1 (*PS1*) and presenilin 2 (*PS2*) are causative factors for autosomal-dominant, early-onset familial AD (FAD) [158–161]. Yagi et al. [162] generated iPSCs from fibroblasts of FAD patients with mutations in *PS1* (A246E) and *PS2* (N141I) and characterized the differentiation of these cells into neurons. They found that FAD iPSC derived differentiated neurons have increased amyloid β42 secretion, recapitulating the molecular pathogenesis of mutant presenilins. Amyloid β42 secretion from the neurons sharply responded to γ-secretase inhibitors and modulators, indicating the potential for the identification and validation of candidate drugs. This study demonstrates that the FAD iPSC derived neurons could be an effective model of AD and provides an innovative strategy for the study of age-related neurodegenerative diseases. More recently, Israel et al. [163] reprogrammed fibroblasts from patients with FAD caused by a duplication of the *APP* gene (termed *APP* (Dp)) into iPSC lines. Compared to controls, iPSC-derived purified neurons from the *APP* (Dp) patients exhibited significantly higher levels of the pathological markers amyloid β40, phospho-tau (Thr 231) and active glycogen synthase kinase-3β (aGSK-3β), but all cells exhibited normal electrophysiological activity. Neurons from *APP* (Dp) also accumulated large RAB5-positive early endosomes compared to controls. Treatment of purified neurons with β-secretase inhibitors, but not γ-secretase inhibitors, reduced the phospho-Tau (Thr 231,) and aGSK-3β levels. These results suggest a direct role of APP proteolytic processing, but not amyloid β, in GSK-3β activation and tau phosphorylation in human neurons. More recently, Koch et al. [164] demonstrated that neurons derived from iPSCs with the *PS1* (L166P) mutation showed a partial loss of γ-secretase function, which results in the decreased production of amyloid β40 and an increased amyloid β 42/40 ratio. These neurons are also resistant to γ-secretase modulation by nonsteroidal anti-inflammatory drugs (NSAIDs). The patient-specific iPSCs thus provide a human neuronal system to study AD pathogenesis and to screen compounds for the pharmaceutical treatment of AD.

2.2.9. Parkinson's Disease. Parkinson's disease (PD) is the most common neurodegenerative movement disorder. It is due to the progressive degeneration of the dopaminergic (DA) neurons in the substantia nigra and is accompanied by the appearance of intraneuronal inclusions enriched in alpha-synuclein called Lewy bodies [165, 166]. It is becoming increasingly clear that genetic factors contribute to the complex pathogenesis of PD. Genes including *PARK2*, *SNCA*, *PARKIN*, *PINK1*, *DJ-1*, *UCHL1*, *LRRK2*, *PARK7*, *GBA*, *SNCAIP,* and *ATP13A2* have been found to be directly associated with Parkinson's disease [166–169]. The iPSCs have been successfully generated from PD patients with mutations in some of these genes [170–175].

Nguyen et al. [171] generated iPSCs that carry the mutation in the *leucine-rich repeat kinase-2* (*LRRK2*) gene and differentiated the cells into DA neurons. The high penetrance of the *LRRK2* mutation and its clinical resemblance to sporadic PD was observed in this process, suggesting that iPSCs could serve as a platform for studying PD. The authors found that the expression of key oxidative stress-response genes and the α-synuclein protein was increased in the DA neurons derived from PD iPSCs. The mutant DA neurons were also more sensitive to caspase-3 activation and cell death when exposed to stress agents. This enhanced stress sensitivity is consistent with early phenotypes of PD and

may become a potential therapeutic target for this disorder. Recently, Sánchez-Danés et al. [175] reported that DA neurons differentiated from PD iPSCs with the *LRRK2* mutation showed morphological alterations, such as reduced numbers of neurites and neurite arborization, as well as accumulation of autophagic vacuoles. These morphological alterations could be greatly exacerbated by further induction of autophagy and/or inhibition of lysosomal proteolysis, indicating autophagic compromise in DA neurons from PD iPSCs, which occurs at the level of autophagosome clearance.

Seibler et al. [172] reported the generation of iPSCs from skin fibroblasts of PD patients with nonsense or missense mutations in the *PTEN-induced putative kinase 1* (*PINK1*) gene. When differentiated into DA neurons and processed for mitochondrial depolarization, the cells showed impaired recruitment of lentivirally expressed parkin to the mitochondria, increased mitochondrial copy number, and upregulation of peroxisome proliferator-γ (PPARγ) coactivator-1α (PGC-1α), an important regulator of mitochondrial biogenesis. Importantly, these alterations were corrected by the lentiviral expression of wild-type *PINK1* in mutant iPSC derived *PINK1* neurons. This study indicates that fibroblasts from genetic PD can be reprogrammed and differentiated into DA neurons. These iPSC derived neurons exhibit distinct phenotypes that should be amenable to further studies of molecular and cellular mechanisms of PD. Triplication of *SNCA*, encoding α-synuclein, causes a fully penetrant, aggressive form of PD with dementia. The α-synuclein dysfunction is the critical pathogenic event in Parkinson's disease that leads to multiple system atrophy and dementia with Lewy bodies [167, 169]. Devine et al.[170] produced multiple iPSC lines from a SNCA triplication patient and from an unaffected first-degree relative. They found that DA neurons differentiated from the iPSCs of the patient produced double the amount of α-synuclein protein in comparison to the neurons from the unaffected relative, thus precisely recapitulating the primary cause of PD in these individuals. This model represents an exceptional experimental system to screen compounds that reduce the levels of α-synuclein and to investigate the cellular mechanisms of neurodegeneration caused by α-synuclein dysfunction. The lack of a phenotype in parkin knockout mice suggests that the human neuronal system is needed to model the disease. Jiang et al. [174] demonstrated that in DA neurons from iPSCs of PD patients with parkin mutations, the transcription of monoamine oxidases and oxidative stress are greatly increased, DA uptake is reduced, and spontaneous DA release is increased. Lentiviral expression of parkin, but not the PD related mutant, rescues these phenotypes, suggesting that parkin controls dopamine utilization in DA neurons by modulating DA neurotransmission and suppressing dopamine oxidation. This study thus provides additional targets for screening pharmaceutical therapies of PD.

One obvious limitation in the application of iPSC technology is the inability to perform experiments under genetically defined conditions. This is especially crucial to late age onset disorders in which the *in vitro* phenotypes are usually subtle and susceptible to effects of genetic background variations. To solve this problem, Soldner et al. [173] combined zinc finger nuclease (ZFN) mediated genome editing and iPSC technology. They generated sets of isogenic disease and control human pluripotent stem cells that differ exclusively at either of two susceptibility variants for PD by modifying the point mutations in the α-synuclein gene. This approach to genetically correct disease-causing point mutations in patient derived iPSCs represents significant progress in basic biomedical research and an advance for iPSC technology.

2.3. Concerns in Modeling Neurogenetic Disorder by iPSCs. Although iPSCs are promising for modeling neurogenetic disorders, there are still limitations. In most cases of neurogenetic disorders, we need to determine whether typical traits of neurogenetic disorders can be observed in the context of the iPSC models. At the molecular level, such as disorder associated protein expression or global gene expression levels, it is likely that patient iPSC derived neuronal cultures will recapitulate phenotypes of the disorders. But for late-onset neurodegenerative diseases, the patient iPSC derived neurons may not be able to show typical patient brain pathology, such as Lewy bodies in PD [5, 19, 176, 177].

On the other hand, it is also very important to reproduce or validate data derived from patient-specific iPSC lines, given the substantial phenotypic diversity of these cell lines and the genetic heterogeneity of the patients. Notably, suppression of patient iPSC culture associated phenotypes by repair of the relative genetic defects could further validate these models. Despite all these concerns, previous studies have already provided strong evidence for iPSC technology as a powerful approach to model neurogenetic disease [5, 19, 176, 177].

3. iPSC Based Therapeutic Strategies for Neurogenetic Disorders

3.1. Drug Screening and Development. iPSC technology provides a platform for the discovery of novel bioactive compounds through molecular dissection of the pathological process [5]. The inspiring examples to demonstrate the potential of iPSCs in screening drug candidates arise from the above mentioned studies in modeling neurogenetic disorders, such as RTT [27], TS [91], SMA [96, 97], FD [102, 103], MJD [143], X-ALD [152], AD [162, 163], and PD [170–172]. Currently, iPSCs have been shown to be valuable for testing small numbers of compounds for efficacy and toxicity in a specific patient or population of patients.

It is clear that iPSC technology can be a useful approach for determining which drugs or drug combinations are effective in humans or in specific patients [5, 16, 19, 102]. To make this technology more powerful, it is essential and crucial to validate the molecular and cellular phenotypes identified in iPSC derived neurons or glial cells. High-throughput drug screening and development requires uniform populations of neurons or glial cells. With the development of iPSC technology, iPSC derived neurons or glial cells will be generated in larger quantities with higher uniformity.

3.2. Cell Therapy. The generation of iPSCs from patients with neurogenetic disorders permits the production of large numbers of CNS cells with the patients' exact genotype. These cells are immune matched to the individual patient—a long desired goal of regenerative medicine. iPSCs provide autologous cell sources for cell replacement/neuroprotection strategies in patients with neurodegenerative diseases. Promising results have been reported with rodent and human iPSC derived neurons such as the improvement of the behavioural symptoms in the rat model of PD [178, 179]. In addition to the transplantation of specific neurons from iPSCs for replacement therapy, transplantation of glial cells from iPSCs can also be used for neuroprotection [180]. In patients with spinal motor neuron diseases, the problem of replacing motor neurons seems daunting, considering that these cells must extend and correctly innervate specific CNS areas. Transplanting cells as therapeutic support cells, rather than as replacement neurons, is an additional and potentially alternative mode of cell therapy for motor neuron diseases [5, 176].

The iPSC technology has largely circumvented political and ethical hurdles previously associated with human embryonic stem cell research. However, several major challenges must be overcome before cell therapy using iPSC technology can be applied clinically [5]. First, among many other safety issues, the risk of cancer must be resolved [5]. iPSC derived neurons and glial cells will not be suitable for transplantation until the oncogenic genes and retroviruses used are replaced with more controlled cell reprogramming [19, 176]. New strategies to reprogram cells in the absence of integrating viral vectors have been reported [181–183] in addition to more efficient integrative approaches [184, 185]. Second, differentiating iPSCs to the specific type(s) of required CNS cells or devising accurate methods to purify the desired cells are key priorities. Building on the progresses that have already been made using stem cells, researchers should continue to improve the understanding of directed differentiation and to develop new protocols. These protocols will bring iPSC technology one step closer to patient-matched cells or tissues for clinical transplantation. Third, it should be necessary to understand and correct any genetic defects in the patient's neurons and glial cells before they can be rationally used for cell therapy. A major concern here is that iPSC derived therapeutics may recapitulate the patient's disease process, due to their genetic propensity. In the context of single-gene disorders, such changes may theoretically be genetically repaired *in vitro* prior to transplantation [176]. For neurodegenerative disorders, such as PD, the relatively brief lifetime of the reprogrammed cells may sufficiently delay an intrinsic pathogenic program. However, cell extrinsic factors in the host patient CNS environment may promote pathogenesis in therapeutic transplanted cells [186]. If the goal is to actually repair the neural circuit, then the most significant hurdle will be the regrowth of projections to the proper target structure in a manner that respects the organization of the neural network. The mechanical barrier caused by disease or injury related gliosis may also affect the restoration of damaged neuronal networks in the adult CNS [5]. These represent some, but certainly not the only pressing issues that must be overcome before cell therapy can be translated in efficacious ways to the clinic. Despite all these potential challenges, iPSC technology, although nascent, represents a remarkable progress toward cell therapy for neurological disorders.

4. Conclusions

Given all the limitations and disadvantages discussed above, we must realize that iPSC derived modeling systems are only one tool in the array of approaches needed to understand and treat human neurogenetic disorders. In consideration of iPSC models for the advancement of therapy for these disorders, we must also understand the pathophysiology at the systems level. A full understanding will require the balancing and integration of information at multiple levels [177, 187]. The data derived from primary CNS cells, human iPSC, or other stem cell derived CNS cells, transgenic animal models, and human studies must be integrated into the broader landscape. In this landscape, each level of information will be able to inform the other to enhance our understanding as we move forward. The iPSCs fill a critical gap in our experimental approaches by providing live, functioning human CNS cells with the genetic backgrounds of patients. They provide an essential link between animal model studies and the assessment of human postmortem brain tissue and live brain functioning. Studies of human CNS cells derived from the iPSCs of patients will give us valuable insights into the mechanisms of pathogenesis and future iPSC research will facilitate drug discovery, cell therapy, and new modes of diagnosis for neurogenetic disorders [3, 19, 177].

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Review Article

Lessons Learned from the Transgenic Huntington's Disease Rats

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Huntington's disease (HD) is a fatal inherited disorder leading to selective neurodegeneration and neuropsychiatric symptoms. Currently, there is no treatment to slow down or to stop the disease. There is also no therapy to effectively reduce the symptoms. In the investigation of novel therapies, different animal models of Huntington's disease, varying from insects to nonhuman primates, have been created and used. Few years ago, the first transgenic rat model of HD, carrying a truncated huntingtin cDNA fragment with 51 CAG repeats under control of the native rat *huntingtin* promoter, was introduced. We have been using this animal model in our research and review here our experience with the behavioural, neurophysiological, and histopathological phenotype of the transgenic Huntington's disease rats with relevant literature.

1. Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder ultimately leading to death, approximately 15–20 years after the first symptoms appear. The prevalence of HD in Europe and both American continents is reported to be approximately 5–7 in 100,000 and affects both sexes with the same frequency [1]. The disease is characterized by abnormal movement (chorea and hypokinesia), cognitive impairments, and psychiatric disturbances, which are progressive. These symptoms substantially limit social and professional functioning. Treatments to prevent death, slow down progression, or delay the onset of HD are still lacking [2].

HD is caused by a trinucleotide repeat expansion, an increase in the number of CAG repeats, in the *HD* gene, (*IT15*) [3]. There is an inverse correlation between the number of repeats and the age of onset in HD [4]. The number of repeats is negatively correlated with the age of onset and positively with disease progression [5]. The *HD* gene was the first autosomal disease locus to be mapped by genetic linkage analysis in 1983 [6] and is located on the short arm of chromosome 4. The *HD* gene encodes polyglutamine repeats in the *huntingtin* (*HTT*) protein [7], which results in a stretch of polyglutamine residues, translated into a polyglutamine (polyQ) tract. The result is the formation of a mutantform of the *HTT*, which can be found in the brains of HD patients and animal models of HD.

Over the years, a number of different models for HD have been introduced varying from insects (*drosophilae melanogaster*) [11], invertebrate models as flatworms (*C. elegans*) [12], various rodent models, a transgenic ovine [13, 14], and pig model [15] to a recently developed nonhuman primate transgenic model [16]. This number of models is still limited, when compared to other neurodegenerative disorders such as Alzheimer's disease, for which currently hundreds of models exist. One explanation could be the low incidence of HD, and therefore it might not receive high priority by society and science. On the other hand, it is one of the few neurodegenerative diseases where the origin (i.e., mutation) has been so clearly described and thus where potentially a definite solution can be found.

Few years ago, the first transgenic rat model of HD (tgHD) was established by von Hörsten and coworkers [17]. The tgHD rats carry a truncated huntingtin cDNA

fragment with 51 CAG repeats under control of the native rat *huntingtin* promoter and were reported to show motor and cognitive symptoms. In addition, a progressive striatal volume loss was documented [17]. These behavioural and neuropathological phenotypes, together with the larger size of the skull and brain in comparison to mice for therapeutic interventions, motivated us to work with this animal model. We have been using this animal model in our HD research and will review here our experience with relevant literature.

2. Behavioural Phenotype

HD is well known for its motor symptoms such as chorea and bradykinesia. However, the nonmotor symptoms of HD patients are also important. Huntington (1872) already reported nonmotor symptoms, such as insanity, depression, and poor impulse control [18]. An important question is whether these nonmotor symptoms are linked to the motor symptoms of HD and whether they share a common underlying mechanism, such as a hyperdopaminergic status [10] or striatal cell damage [9]. From a clinical perspective, there is evidence that nonmotor symptoms are mainly linked to hypokinesia and not so much to hyperkinesia (i.e., chorea) [19, 20]. In this respect, it is an ongoing debate whether hypokinetic and hyperkinetic symptoms in HD have a common neuronal substrate. Different transgenic animal models have been developed to characterise the motor and nonmotor symptoms in more detail. The widely used R6/2 mouse model of disease has been found to show less anxiety-related behaviour and exhibits hypokinetic features [21], and in the tgHD rats nonmotor symptoms and hyperkinetic features were found mimicking clinical HD [17]. In the latter model, already at 3 months of age, the animals showed signs of reduced anxiety in the social interaction test, while there was no evidence of motor or cognitive dysfunction [22]. This is in comparison to human data illustrating the presence of psychiatric symptoms, such as anxiety, prior to the onset of the motor symptoms, in the early stages of the disease. Though changes in anxiety in the transgenic rats show a reduction, patients often show increased anxiety levels, not related to disease stages. This might be partly explained by the fact that increased anxiety in patients often occurs due to uncertainty about the start and/or course of the disease. This phenomenon does not exist in animals.

In one of our studies, we investigated the relationship between motor and nonmotor behaviour in the earlier stages of the disease in the tgHD rat model by using motor and nonmotor tests [23]. We found tgHD animals to be hyperkinetic (increased distance moved) in the open field test compared to their wildtype littermates at all ages tested (6, 7, 8, and 10 months of age), which was accompanied by reduced anxiety-like behaviour in the open field test and the elevated zero maze, but not in the home cage emergence test. No major changes were found in hedonia (sucrose intake test) and motivation for food (food intake test). Our data suggest that hyperkinetic features and reduced anxiety in the tgHD rats are associated behaviours and are seen in the earlier stages of the disease. These data support the hypothesis that the neuronal substrate of the hyperactivity and reduced anxiety might be similar.

In a next study, we investigated the visual object and visuospatial memory capabilities of the tgHD rats by means of the object recognition task (ORT) and the object location task (OLT), respectively [24]. Visual object and visuospatial cognition are linked to the function of the basal ganglia, the primary site of neuropathology in HD. Subsequently, memory deficits are frequently seen in HD patients and are linked to striatal, especially caudate nucleus (dorsal striatum), and hippocampal alterations [25–27].

The ORT allows the assessment of object memory and is reviewed to be specific for assessing the functionality of the ventral memory pathway including the perirhinal cortex in rats [28]. The OLT assesses the spatial component of object memory determined by the dorsal memory pathway, which gives an estimation of the functionality of the parahippocampal cortex or its rodent equivalent, the postrhinal cortex [29]. Together these brain areas reflect the medial temporal lobe memory system, which has been considered as the declarative memory system [30]. Therefore, we tested the subjects at two different time points, 10 and 16 months, corresponding to early and late stages of the HD. We found memory deficits in the tgHD rat model, for both visual object and visuospatial memory, at the early and late stages of the disease. These rodent data are comparable to data of human studies demonstrating impairment of both visual object and visuospatial memory too. Deficits in striatal cell activity, hippocampal long term potentiation (LTP) functionality, and decreased levels of Brain derived neurotrophic factor (BDNF) have been associated with memory deficits in HD patients and transgenic mouse models of HD [31–34]. Nonetheless, further studies are needed to clarify these mechanisms and their implications in the tgHD rat model. In this respect, a recent study showed the presence of prefrontostriatal processing alterations using a behavioural and electrophysiological paradigm, which could also underlie some of the cognitive symptoms seen in this model [35].

In another study, we further extended the behavioural profile of this model in the later stages of the disease. Specifically, we tested the hypothesis that these rats exhibit age- and genotype-dependent changes in cognitive performance and chorea-like symptoms (abrupt, rapid, brief, and unsustained irregular movements of the neck) [36]. Rats were evaluated in a choice reaction time task to study the cognitive performance, including reaction time and premature responding, and in an open field setting to rate the choreiform movements. We found that tgHD rats showed a clear progression of cognitive and motor impairment over time. At 15 months of age, the homozygous (+/+) tgHD rats showed only a slight impairment in the number of premature responses, whereas at 20 months of age, this impairment was significantly increased, and more cognitive deficits such as a decreased number of correct responses became apparent compared to hemizygous (+/−) and wildtype (−/−) littermates. Furthermore, the number of choreiform movements in the homozygous tgHD rats increased significantly from 15 months to 20 months of age, in comparison to hemizygous and wildtype littermates. Both cognitive and

motor impairments were significantly more pronounced in the homozygous tgHD rats as compared to the hemizygous tgHD rats, which is indicative of a gene-dose effect.

3. Neurophysiological Phenotype

The tgHD rats show a specific behavioural phenotype, consisting of hyperkinetic movements, impaired anxiety and memory parameters, and impulsivity, at different stages of the disease. One of questions arising is whether these behavioural symptoms have neurophysiological substrates.

A link between symptoms and neurophysiological substrate has been well established in another movement disorder, Parkinson's disease (PD). In PD, which is histopathologically characterized by selective, chronic, and progressive nigrostriatal degeneration, the subthalamic nucleus (STN) displays a continuous abnormal "bursting" mode of activity whereas in physiological conditions it exhibits a more or less regular pattern of discharge with intervals of burst activity [37–39]. This so-called STN hyperactivity is held responsible for at least part of the cardinal PD symptoms such as hypokinesia/bradykinesia and rigidity [40–42]. Interestingly, the discovery of STN hyperactivity has been the result of systematic scientific research. In short, in 1987 Miller and DeLong demonstrated that the STN exhibited increased neuronal activity in MPTP-treated primates, in an electrophysiological study exploring the activities of basal ganglia nuclei [43]. These observations were the basis for the pioneering work of Bergman and colleagues [44]. They showed that lesions of the STN reduced all of the major motor disturbances in monkeys rendered parkinsonian by MPTP. Benabid and coworkers introduced deep brain stimulation (DBS) as an alternative for ablative surgery in movement disorders [45] and were the first, motivated by the results of the lesion studies and by a crucial stimulation study in primates in the same year [46], to explore the effects of STN DBS in a patient suffering from advanced PD [47]. In 1995, the results of 3 patients were published showing that bilateral STN DBS resulted in marked improvement in motor symptoms [48]. This report was actually the beginning of the successful STN DBS era.

In line with the advances in PD, we aimed to investigate the metabolic and electrophysiological activities of the basal ganglia nuclei, consisting of the striatum, GP (globus pallidus, the homologue of globus pallidus externus in primates), EP (entopeduncular nucleus, rodent equivalent of the primate globus pallidus internus), substantia nigra pars compacta (SNc), and reticulata (SNr), and the STN in the tgHD rats [8]. We reasoned that mapping of neuronal and metabolic activity of the basal ganglia nuclei would help in identifying a functional substrate for symptoms and therapeutic interventions in HD. To determine the *overall* neuronal activity of all cells (supracellular) per basal ganglia nuclei, a cytochrome oxidase (COX) enzymatic staining was performed, and optical densities were measured at the level of each basal ganglia nuclei. Secondly, we performed *single-unit* electrophysiological recordings, which reflect the activity of the basal ganglia nuclei at the cellular level. Finally, to investigate the subcellular activity of the various basal ganglia nuclei we performed an immunohistochemical staining

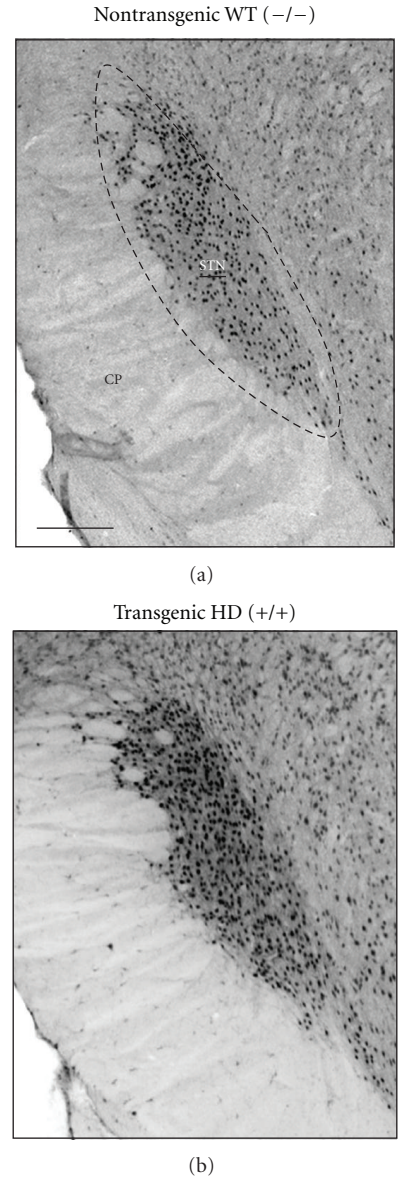

Figure 1: Representative low-power photomicrographs of frontal brain sections stained for PGC-1α showing the subthalamic nucleus (STN) of a control and a transgenic HD rat. Note the increased number of PGC-1α-containing cells STN of the tgHD rat upon close inspection in comparison with the control subject. Scale bar is approximately 250 μm. The anatomical level is approximately anteroposterior −4.16 mm from bregma according to the rat brain atlas of Paxinos and Watson of 1998. Adopted from Vlamings et al. [8].

for peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α, a transcription coactivator, and a key player in the mitochondrial energy apparatus. PGC-1α induces and coordinates gene expression that stimulates mitochondrial oxidative metabolism in various tissues. It is highly expressed in tissues with increased energy demands and large numbers of mitochondria [49]. In tgHD rats, optical density analysis showed a significantly increased cytochrome oxidase levels in the GP and STN when compared to controls. PGC-1α expression was only enhanced in the STN (Figure 1),

and electrophysiological recordings revealed increased firing frequency of the majority of the neurons in the STN and a reduced firing frequency in the GP [8].

The finding that the STN shows enhanced activity at different functional levels cannot be explained based on the classical theories on the corticobasal ganglia-thalamocortical circuits in HD [50]. Namely, the expectation would be decreased or impaired activity of the STN and an elevated GP activity due to a loss of striatal medium spiny neurons expressing D2 receptors and enkephalin. Here, we found an increase in the COX activity in the GP in the tgHD animals, which is in line with the previously mentioned theory, but we failed to find support from the PGC-1α expression and electrophysiological studies. It might well be that subtle changes in activity can be detected with COX histochemistry, which represents overall activity (sum of inhibited, excited, and unchanged cell activities). The changes in the STN were more robust and present at subcellular, cellular, and supracellular levels. We think that the explanation for this change is through the direct dopaminergic input from the SN pars compacta [51]. In the tgHD rats, we found elevated levels of tyrosine hydroxylase (TH), rate-limiting enzyme in the synthesis of dopamine, in the striatum due to increased number of dopamine-containing cells in the SN pars compacta [10]. It has been shown that higher concentrations of dopamine have a dual effect on STN neurons, increasing the firing rate and changing the pattern of firing into a more regular mode [52]. This is what we have observed here. The elevated levels of dopamine in HD seem to induce more neurobiological changes in the basal ganglia than previously expected. Another contributing factor to enhanced STN activity could be the reduced activity of the regular neurons of the GP. Altogether, our results suggested that the STN and GP play a role in the symptoms of HD and can be a potential target for therapeutic interventions.

4. Neuropathological Phenotype

The neuropathology of HD includes profound and progressive neuron death in the striatum and, to a lesser extent, in the cortex [53]. Several animal models represent some of these features of HD. For instance, injection of excitotoxins and mitochondrial toxins can mimic some aspects of the neuropathology of HD, but the resulting neuronal death is not progressive. Genetically modified mouse models have further helped to understand the pathogenesis and molecular mechanisms of the illness [54]. Major drawbacks of these models, however, are the rapid disease progression (as in the case of the most commonly used model, namely, the R6/2 mice) and the relatively small size of the mouse brain, limiting the usefulness of these mouse models for longitudinal studies and for therapeutic approaches based on surgical intervention. Except for studies reporting striatal neuron loss in the YAC128 mouse model [55], substantial striatal neuron loss has not really been observed in transgenic mouse models of HD.

In the first publication on the tgHD rats, striatal shrinkage and enlarged lateral brain ventricles in magnetic resonance images at 8 months of age were reported [17]. In a later study, the striatal neuron loss during the disease course and aggregates were documented [22]. We further characterized these changes in a histopathological study [9]. We found a reduction in striatal volume in the brains of 12-month-old tgHD rats compared with wildtype littermates (Figure 2). This age-related volume reduction was more pronounced in the medial, paraventricular part of the striatum, corresponding to the regions where the earliest neuropathological changes are seen in human HD [56].

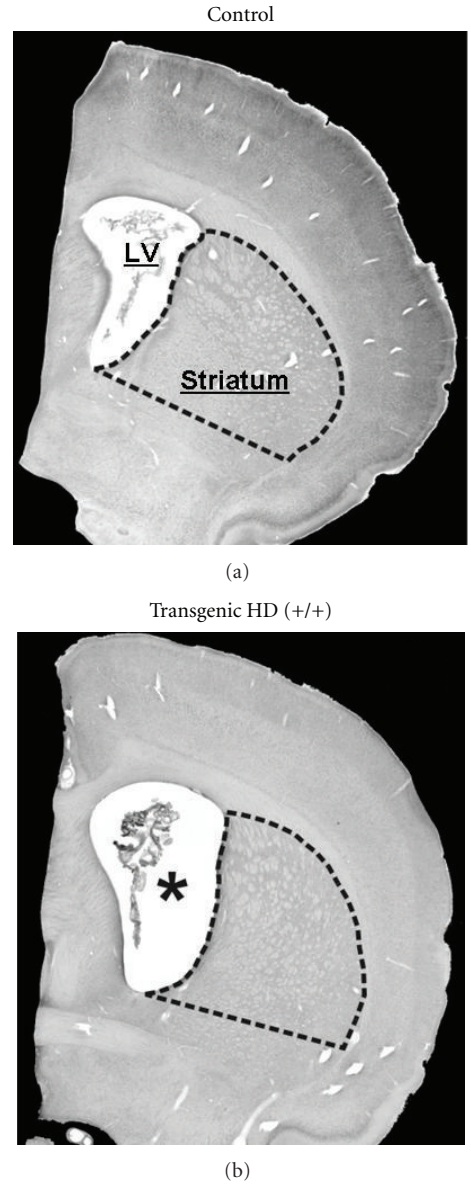

Figure 2: Representative photomicrographs of Nissl-stained frontal sections of the brains from a 12-month-old control rat and HD transgenic rat. Note the smaller striatum and the larger lateral ventricle (LV) (asterisk in b). The anatomical level is approximately anteroposterior 1.60 mm from bregma according to the rat brain atlas of Paxinos and Watson of 1998. Adopted from Kantor et al. [9].

Moreover, high-precision, design-based stereological analysis showed statistically significant neuron loss in striatum but not in frontal cortical layer V at 12 months of age. Thorough microscopical examination of Nissl stained sections revealed no neurons with abnormal morphology in the striatum of tgHD rats and no signs of astroglia activation. Nevertheless, dysregulation of neuronal firing patterns of striatal cells was found in these animals [57]. Of particular importance was the finding of dark, pycnotic pyramidal cells mostly in layer V of the frontal cortex of tgHD rats, resembling pycnotic pyramidal cells seen in the motor cortex of human HD patients [58]. Furthermore, enhanced accumulation of autofluorescent material (most probably representing lipofuscin) was specifically observed in layer V pyramidal cells of tgHD rats, resembling enhanced lipofuscin accumulation in the human disease. These findings indicate chronic neurodegenerative processes in the motor cortex of tgHD rats.

Another line of investigation is the pathoanatomical basis for HD chorea. A link with the dopaminergic system has been suggested by human postmortem studies and clinical therapy studies. Early postmortem studies showed that striatal dopamine levels, both in the dorsal and ventral striatum, were significantly higher in HD patients compared to controls [59–61]. In addition, clinical studies have shown that the chorea can be treated with dopamine antagonist or dopamine-depleting drugs [62].

The origin of elevated dopamine levels in the dorsal and ventral striatum in HD remains unknown. In another histopathological study [10], we tested the hypothesis that elevated striatal dopamine levels are caused by changes in the SNc and the ventral tegmental area (VTA), since these regions are the main source of striatal dopamine [63, 64]. We used antibodies raised against TH, the rate-limiting enzyme in the synthesis of dopamine, and by means of stereological counting methods analysed the number of TH-containing cells in the SNc and VTA, in the only experimental model of HD with chorea, the tgHD rats. We found increased expression of TH in the striatum of tgHD rats. This reflects increased striatal dopamine levels, since TH enzymatic activity highly corresponds to the cellular levels of dopamine [65]. Stereological counts of TH-containing cells revealed a substantial increase in the number of TH containing cells in both regions in tgHD rats when compared to controls (Figure 3). Our findings suggest that the origin of the increased dopamine in the striatum comes from the two main nuclei supplying the striatum with dopamine, the SNc for the nigrostriatal dopamine pathway and the VTA for the mesolimbic dopamine pathway. Since it has been demonstrated that the total number of cells in the substantia nigra in postmortem HD brains was not different from age-matched controls [66], it is possible that the increased number of TH containing cells in the SNc and VTA is the result of a change in phenotype of the non-TH-containing cells. For the SNc it is known that about 45% of the cells are non-TH containing [67]. A change in the phenotype of a TH cell, while the cell is still functional, has been documented before in the substantia nigra of mice [68] and rats [69].

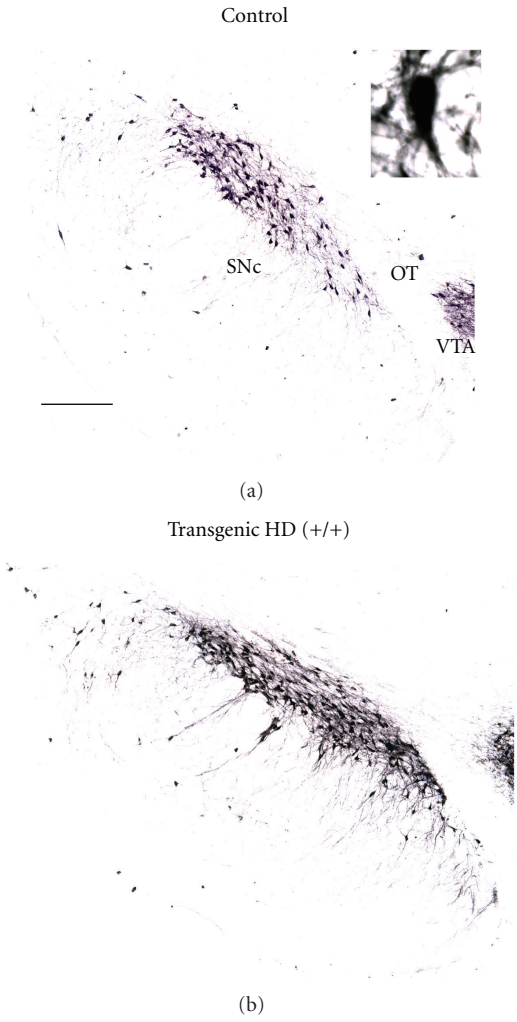

Figure 3: Representative low-power photomicrographs of frontal brain sections stained for tyrosine hydroxylase (TH) showing the substantia nigra pars compacta (SNc), optic tract (OT), and a small part of the ventral tegmental area (VTA) of a control and a transgenic HD rat. Note the increased TH-containing cell density in the SNc of the transgenic HD rats upon close inspection. The high-power photomicrograph inset in the right upper corner shows a magnification of TH-containing cells of a transgenic rat. Scale bar is approximately 250 μm. The anatomical level is approximately anteroposterior −5.2 mm from Bregma according to the Paxinos and Watson atlas of 1998. Adopted from Jahanshahi et al. [10].

5. Conclusion

We have been using the tgHD rats for several years in our research. We have experienced that these rats have a slow progressive behavioural and neuropathological phenotype. In general, we found out that the symptoms can be divided in two to three stages: early, (middle), and late. The early stage is characterised by hypermobility and reduced anxiety behaviour [23]. Although some scientists have found subtle cognitive declines [22, 35], in our hands these do not have a large impact on the animals. We have not found choreiform movements, at this early disease stage. This

stage is not accompanied with striatal cell degeneration or cortical cell damage, which occurs later in the disease [9, 22]. Nevertheless, there might already be striatal cell dysfunction [57] which continues in the middle phase (>8–10 months of age). Appearance of the first choreiform movements starts during this disease stage with an increase in the last disease stages (>15–24 months of age). This later stage is mainly characterised by the presence of more choreiform movements at the level of the head, neck, and limbs. Upon testing, the animals show impaired cognitive functioning and impulsivity-like features [24, 36]. There is a profound loss of striatal cells and signs of cortical cell damage [9]. The life expectancy of these animals is shorter than controls, probably a few months (unpublished observations).

There are some issues, which need to be considered in this animal model. The first is the effect of sex. There are differences in the behavioural phenotype between the sexes [70]. This needs to be taken into account when comparing sets of data. Therefore, in our studies, we use animals of only one sex male or female per experiment. In addition, we prefer to use merely male animals, like in most behavioural studies, to exclude any possible effects of the oestric cycle in female animals. The second is the gene-dose effect. We have been working with both homozygous (+/+) and hemizygous (+/−) transgenic animals in a few studies [10, 36]. Homozygous animals show more robust behavioural and neuropathological features [9, 36], mimicking human HD. Therefore, we decided to work with homozygous rats, only. The third issue is a potential gene-drift effect. In a recent publication, researchers could not establish robust cognitive changes in this animal model [71, 72]. One explanation could be a potential gene drift, but the existence of such mechanism still needs to be demonstrated. In our colony of animals, we have observed slight differences between homozygous rats but consistently found clear behavioural and neuropathological phenotypes.

After evaluating the different phenotypes of the tgHD rats, we consider this animal model suitable to evaluate therapeutic approaches.

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Research Article

Adult-Onset Fluoxetine Treatment Does Not Improve Behavioral Impairments and May Have Adverse Effects on the Ts65Dn Mouse Model of Down Syndrome

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Down syndrome is caused by triplication of chromosome 21 and is associated with neurocognitive phenotypes ranging from severe intellectual disability to various patterns of more selective neuropsychological deficits, including memory impairments. In the Ts65Dn mouse model of Down syndrome, excessive GABAergic neurotransmission results in local over-inhibition of hippocampal circuits, which dampens hippocampal synaptic plasticity and contributes to cognitive impairments. Treatments with several $GABA_A$ receptor antagonists result in increased plasticity and improved memory deficits in Ts65Dn mice. These $GABA_A$ receptor antagonists are, however, not suitable for clinical applications. The selective serotonin reuptake inhibitor fluoxetine, in contrast, is a widely prescribed antidepressant that can also enhance plasticity in the adult rodent brain by lowering GABAergic inhibition. For these reasons, we wondered if an adult-onset 4-week oral fluoxetine treatment restores spatial learning and memory impairments in Ts65Dn mice. Fluoxetine did not measurably improve behavioral impairments of Ts65Dn mice. On the contrary, we observed seizures and mortality in fluoxetine-treated Ts65Dn mice, raising the possibility of a drug × genotype interaction with respect to these adverse treatment outcomes. Future studies should re-address this in larger animal cohorts and determine if fluoxetine treatment is associated with adverse treatment effects in individuals with Down syndrome.

1. Introduction

Down syndrome is caused by trisomy 21 and is frequently associated with cognitive impairments. Based on the partial triplication of chromosome 16, the mouse homologue of human chromosome 21, a mouse model (Ts65Dn) has been developed that shows behavioral abnormalities, including deficient hippocampus-dependent learning and memory [1, 2]. Although most studies regarding the neurobiology of Down syndrome have been focused on neurodevelopment, recent evidence suggests that pathophysiological processes in the adult brain significantly contribute to cognitive impairments in this disorder [3–8]. In Ts65Dn mice, enhanced inhibitory synaptic transmission suppresses proper induction of hippocampal synaptic plasticity, an important cellular mechanism for learning and memory formation [6].

Strikingly, using a variety of different $GABA_A$ receptor antagonists to suppress the abnormally increased level of inhibition in adult Ts65Dn mice fully restored their learning and memory impairments without affecting wild-type controls [4]. These beneficial effects were not evident, however, with only acute administration of $GABA_A$ receptor antagonists but instead became clear only with a more prolonged (2-3 weeks) treatment [4]. This prolonged treatment was then sufficient to cause improvements in cognitive function lasting well beyond the actual treatment period [4], suggesting that treatment triggered lasting neurobiological changes. Such outcomes are reminiscent of those seen in typical antidepressant treatment of depressed individuals.

The neurobiological basis of these nonacute $GABA_A$ receptor antagonist treatment-induced behavioral modifications is currently unknown. Key insights into potential

mechanistic aspects, however, may be provided by a brief review of brain development. During postnatal brain development, inhibition plays an important role in regulating the temporal extent of critical periods (i.e., developmental time windows during which sensory input can substantially shape brain structure and function) [9, 10]. The gradually increasing levels of cortical inhibition cause the closing of these critical periods. The mature brain is therefore no longer endowed with such high levels of plasticity. Remarkably, several experimental manipulations that lower inhibitory synaptic transmission have been found to reinstate high levels of plasticity in the adult brain resembling those found in critical periods [11–13]. It is possible that high levels of plasticity, characteristic of critical periods, are actively suppressed in adults by mechanisms including increased inhibition [14]. Therefore, one strategy to enhance plasticity in the adult brain could be the removal of these constraints by lowering inhibition [14, 15].

One pharmacological manipulation particularly interesting from a translational point of view reported that ocular dominance plasticity is reinstated in the mature rat visual cortex by chronic treatment with the widely prescribed antidepressant fluoxetine, presumably also via a decrease in inhibitory synaptic transmission [11]. Vetencourt et al. used brain in vivo microdialysis to show reduced levels of extracellular GABA in the visual cortex of fluoxetine treated animals [11]. White matter LTP, a form of synaptic plasticity that is normally absent in the adult brain due to matured intracortical inhibition, was present in fluoxetine-treated animals [11]. BDNF expression was increased as a consequence of fluoxetine treatment, and intracortical BDNF administration was sufficient to cause an ocular dominance shift in response to monocular deprivation [11]. To test if reduced inhibition underlies the effects of fluoxetine on ocular dominance plasticity, the $GABA_A$ receptor agonist diazepam was administered intracortically in fluoxetine-treated mice, which fully occluded the effect of fluoxetine on ocular dominance plasticity [11].

Translation of the preclinical $GABA_A$ receptor antagonist findings in Ts65Dn mice (see above) [4, 8] to clinical populations is hampered by the fact that none of the employed $GABA_A$ receptor antagonists is currently in clinical use. $GABA_A$ receptor antagonists have narrow therapeutic windows and harsh side effect profiles and therefore have limited potential for translational applications, warranting the search for novel treatment approaches. We note that other strategies safer than $GABA_A$ receptor antagonists have been proposed for the treatment of Down's syndrome-related cognitive impairments, including $GABA_A$ receptor $\alpha 5$-selective inverse agonists and other compounds [16–19].

Here, we followed up on the finding that chronic fluoxetine treatment induced reduced levels of inhibition and enhanced plasticity [11], suggesting that chronic fluoxetine administration may have beneficial effects on cognitive dysfunction in Ts65Dn mice, similar to $GABA_A$ receptor antagonists. Treatments with selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine, influence the serotonin system and also have complex effects on GABAergic neurotransmission [20–23], including pre- and postsynaptic effects, which may include an inhibition of evoked inhibitory postsynaptic potentials due to elevated extracellular serotonin levels [23]. In the hippocampus, the serotonergic system has been proposed to be involved in shifting inhibition from dendritic to perisomal areas, which should allow for greater dendritic excitation and synaptic plasticity [22].

We tested if an oral fluoxetine treatment regime has therapeutic effects on cognitive impairments (spatial learning in the Morris water maze) in the Ts65Dn mouse model of Down syndrome. There were no beneficial effects of fluoxetine treatment on behavioral impairments in Ts65Dn mice but, instead, unexpected side effects including seizures and death due to treatment that appeared to be genotype specific.

2. Material and Methods

2.1. Mice. Experimental animals were generated by crossing C57BL/eJEiJ × C3Sn.BLiA-*Pde6b* + F1 hybrid wild-type males with B6EiC3Sn.BLiA-Ts(17^{16})65Dn/DnJ females (breeders were purchased from The Jackson Laboratories). A 1-1 mating scheme was used, and males were left in the mating cage with the female and her litter. Pups were weaned and tail biopsies for genotyping were obtained at postnatal day 21. Genotyping was performed by qPCR. Experimental animals were housed in groups of 2–4 mice per cage. We kept animals on a 12-hour light-dark cycle. Mice received water and food *ad libitum*. Experiments were carried out during the light period of the cycle. Male and female animals used for experiments were between 149 and 227 days of age at completion of the study (sex and age were approximately balanced across groups). All experiments were performed blinded to genotype and treatment. Local and federal regulations regarding animal welfare were followed.

2.2. Pharmacology. Fluoxetine hydrochloride (Sigma) was administered to the animals through the drinking water at a concentration of 0.2 mg/mL as previously described [11]. Animals were treated for 4 weeks before behavioral assessment commenced and were sacrificed after 6 weeks of treatment.

2.3. Water Maze. Initially, mice were handled for 7 days (for approximately 2 min/animal/day) to habituate the animals to investigator contact and procedural elements associated with the task. Following handling, mice were trained on the hidden version of the water maze. During water maze training, the escape platform was hidden underneath the water surface in a constant location of the pool. The pool (Med Associates) had a diameter of 1.2 m and was filled with opaque water (temperature: 22–24°C). Behavior of the animals was recorded using an automated tracking system (Ethovision XT, Noldus). During training trials, mice were placed into the pool from one of seven randomly assigned starting positions. Each mouse received four daily training trials for 5 consecutive days. Training trials were given in blocks of 2 consecutive trials. Accordingly, intertrial intervals were approximately 1 min between trials 1 and 2, as well as trials 3 and 4, and were approximately 90 min between trials 2 and 3. Training trials were completed when

mice climbed on the escape platform or when 1 min had elapsed, whichever came first. Animals were given 15 s post-trial interval on the escape platform after completion of training trials. To evaluate the accuracy with which the animals had learned the position of the escape platform, we performed a probe trial once training was completed. During the probe trial, we removed the escape platform from the pool, and animals were released into the pool from the starting position within the opposite quadrant (OQ). We determined the time that mice spent searching in the target quadrant (which previously contained the escape platform) or the other quadrants during the probe trial. Additionally, we analyzed the number of crossings of the exact target location (i.e., where the platform was during training) and compared it to crossings of analogous positions in the other quadrants. As an additional probe trial measure, we determined the average distance (proximity) to the target location and compared it to the average distance to corresponding locations in the other quadrants. To assess if the probe trial measures differed across genotypes and/or treatment groups, we performed a three-way ANOVA with genotype and treatment as between-subjects factors and quadrant as a within-subject factor. Additionally, to assess for spatial selectivity of searching during the probe trial, we performed t-tests to compare target quadrant measures to the corresponding average values of the other quadrants. Escape latencies during training were analyzed by two-way ANOVA with genotype and treatment as between-subjects factors. Swim speed during training and probe trial was analyzed by two-way ANOVA with genotype and treatment as between-subjects factors. Also, distance travelled during the probe trial was analyzed by two-way ANOVA with genotype and treatment as between-subjects factors.

2.4. Open Field. Mice were placed for 10 min in a square open field made of acrylic (footprint 27.5 cm × 27.5 cm); activity was recorded and analyzed using an automated system (Ethovision XT, Noldus). Light levels were set to 100 lux in the center of the open field.

2.5. Tissue Preparation. Mice were anesthetized and perfused transcardially with 0.9% saline and 4% paraformaldehyde in cold 0.1 M phosphate buffer (pH 7.4). Brains were extracted, postfixed in 4% paraformaldehyde over night, and subsequently transferred into 30% sucrose. Forty micrometer coronal section series were then created using a sliding microtome (Leica). Sections were stored in cryoprotectant solution (25% ethylene glycol, 25% glycerine, and 0.05 M phosphate buffer) at −20°C.

2.6. Immunohistochemistry. Every sixth section of the coronal section series mentioned above (i.e., sections 240 μm apart) was used for free-floating immunohistochemistry. Sections were rinsed in TBS, incubated in a solution containing 0.6% H_2O_2 in TBS to inhibit residual endogenous peroxidase, and blocked for 30 min in TBS with 3% donkey serum and 0.1% Triton X-100. Sections were then incubated for 48 h at 4°C in a primary antibody solution (rabbit anti-choline acetyltransferase (anti-ChAT), Millipore, 1 : 100) containing TBS, 3% donkey serum, and 0.1% Triton X-100. Subsequently, sections were rinsed in TBS, blocked in TBS with 3% donkey serum and 0.1% Triton X-100, and incubated for 1 h at room temperature in secondary antibody (biotinylated donkey anti-rabbit IgG, Dianova, 1 : 500). Next, sections were washed with TBS and subjected to 1 h incubation in avitin-biotin peroxidase complex in TBS (ABC Elite, Vector). Finally, sections were developed with diaminobenzidine (DAB, Roche). Sections were mounted, air-dried, dehydrated with a graded series of ethanol, and mounted with Permount.

2.7. Stereology and Image Analysis. Stereological analyses of cell number and cell size of choline acetyltransferase-immunostained cells were performed on section series stained against choline acetyltransferase with sections 240 μm apart, covering the entire fronto-occipital extension of the hemisphere. Analyses were carried out using a Nikon Eclipse 90i microscope equipped with Stereo Investigator (MicroBrightField). Quantitative analysis of the total number of ChAT-positive neurons in the basal forebrain including ventral diagonal band nuclei (VDB) and medial septum nuclei (MSN) was performed in an unbiased fashion using the optical fractionator method as described previously [24, 25]. This method allows for a systematic random sampling of the region of interest (ROI). The landmarks outlining the ROI (MSN and VDB) were taken from the Mouse Brain Atlas [26]. ROIs were manually marked, and pictures from every ROI were taken using the Stereo Investigator software. With a sampling grid of 90 μm × 90 μm systematically moving through the outlined ROI, we counted ChAT-positive neurons that were either within the dissector counting frame (60 μm × 60 μm) or that were touching its right/upper edge. Cell size of ChAT-immunoreactive neurons was determined on the same section series using the nucleator probe within the Stereo Investigator software. Six rays extending from the nucleus were used to mark the boundaries of the cells, allowing an estimation of cell surface area.

3. Results

To test if adult-onset fluoxetine treatment has a beneficial effect on behavioral features associated with the Ts65Dn mouse model of Down syndrome, we initiated fluoxetine or vehicle control treatment in Ts65Dn mice and 2N (diploid) littermate controls (2N/vehicle: $n = 9$ mice; 2N/fluoxetine: $n = 11$ mice; Ts65Dn/vehicle: $n = 7$ mice; Ts65Dn/fluoxetine: $n = 9$ mice).

Body weight measurements were taken after 1 month of treatment with fluoxetine or vehicle control (Figure 1; 2N, vehicle: $n = 8$ mice; 2N, fluoxetine: $n = 11$ mice; Ts65Dn, vehicle: $n = 7$ mice; Ts65Dn, fluoxetine: $n = 7$ mice). Two-way ANOVA with genotype and treatment as between-subjects factors revealed significant main effects of genotype (ANOVA genotype $F(1, 29) = 7.37$, $P = 0.01$) and treatment (ANOVA treatment $F(1, 29) = 5.42$, $P = 0.03$), while there was no significant interaction between the factors ($F(1, 29) = 0.85$, $P = 0.77$). Fluoxetine treatment reduced body weights in Ts65Dn mice relative to Ts65Dn vehicle controls (Fisher's

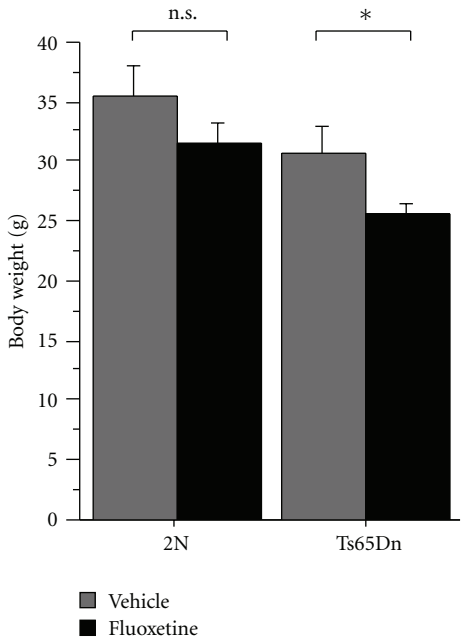

Figure 1: Body weights of Ts65Dn mice and wild-type littermate controls treated with vehicle or fluoxetine (2N, vehicle: $n = 8$ mice; 2N, fluoxetine: $n = 11$ mice; Ts65Dn, vehicle: $n = 7$ mice; Ts65Dn, fluoxetine: $n = 7$ mice). Two-way ANOVA with genotype and treatment as between-subjects factors showed significant main effects of genotype (ANOVA genotype $F(1, 29) = 7.37$, $P = 0.01$) and treatment (ANOVA treatment $F(1, 29) = 5.42$, $P = 0.03$). Posthoc analyses showed significantly lower body weights in fluoxetine-treated Ts65Dn mice compared to vehicle-treated Ts65Dn mice (Fisher's PLSD, $P = 0.04$). Body weights in fluoxetine-treated 2N controls were not significantly different from 2N vehicle controls (Fisher's PLSD, $P = 0.19$). Graph shows mean ± SEM. *denotes comparisons where $P < 0.05$. n.s. denotes comparisons where $P > 0.05$.

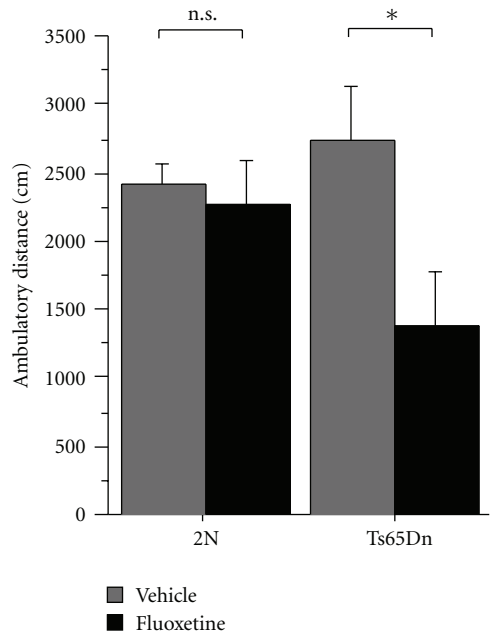

Figure 2: The figure shows the total ambulatory distance in the open field of Ts65Dn mice and wild-type controls treated with either fluoxetine or vehicle (2N, vehicle: $n = 8$ mice; 2N, fluoxetine: $n = 11$ mice; Ts65Dn, vehicle: $n = 7$ mice; Ts65Dn, fluoxetine: $n = 7$ mice). Two-way ANOVA with genotype and treatment as between-subjects factors revealed a significant effect of drug treatment on ambulatory distance ($F(1, 29) = 5.24$, $P = 0.03$) and suggested a possible genotype × treatment interaction ($F(1, 29) = 3.35$, $P = 0.08$). Posthoc comparison showed significantly reduced ambulatory distance in fluoxetine-treated Ts65Dn mice relative to vehicle-treated Ts65Dn animals (Fisher's PLSD, $P = 0.04$). In contrast, fluoxetine had no significant effect on ambulatory distance in 2N controls (Fisher's PLSD 2N/fluoxetine versus 2N/vehicle, $P = 0.5$). The graph shows means ± SEM. *denotes comparisons where $P < 0.05$. n.s. denotes comparisons where $P > 0.05$.

Table 1: Number of deaths during treatment with fluoxetine or vehicle in Ts65Dn mice and wild-type controls.

	2N	Ts65Dn
Vehicle	1 (12.5%)	0 (0%)
Fluoxetine	0 (0%)	4 (44.4%)

PLSD Ts65Dn/fluoxetine versus Ts65Dn/vehicle, $P = 0.04$). Fluoxetine had no significant effect on body weight within 2N littermate controls (Fisher's PLSD 2N/vehicle versus 2N/fluoxetine, $P = 0.19$).

Unexpectedly, during the approximate 6-week treatment period, 4 out of 9 Ts65Dn mice treated with fluoxetine died (Table 1). There were no deaths among the vehicle-treated Ts65Dn mice (0 out of 7 mice) and the fluoxetine-treated wild-type animals (0 out of 11 mice). In the vehicle-treated wild-type group, 1 out of 8 mice was found dead during the treatment period (Fisher's exact test, Ts65Dn/fluoxetine group versus collapsed data from the other groups, $P = 0.01$).

We started our behavioral assessment of fluoxetine-treated and vehicle-treated Ts65Dn mice and their respective wild-type control groups by testing general exploratory activity in an open field assay (Figure 2; 2N, vehicle: $n = 8$ mice; 2N, fluoxetine: $n = 11$ mice; Ts65Dn, vehicle: $n = 7$ mice; Ts65Dn, fluoxetine: $n = 7$ mice). Animals were placed into a novel environment, and activity levels were recorded. There was no effect of genotype on total distance travelled in the open field (two-way ANOVA with genotype and treatment as between-subjects factors: $F(1, 29) = 0.73$, $P = 0.4$). Statistical analysis showed a significant effect of drug treatment on activity levels (two-way ANOVA with genotype and treatment as between-subjects factors: $F(1, 29) = 5.24$, $P = 0.03$) and suggested a possible genotype × treatment interaction (two-way ANOVA with genotype and treatment as between-subjects factors: $F(1, 29) = 3.35$, $P = 0.08$). Activity levels were significantly reduced in fluoxetine-treated Ts65Dn mice relative to vehicle-treated trisomic mice (Fisher's PLSD, $P = 0.04$). Corresponding post hoc analysis did not show a significant effect of treatment in wild-type animals (Fisher's PLSD, $P = 0.5$).

To determine if fluoxetine treatment restores spatial learning and memory deficits in Ts65Dn mice, we tested

Ts65Dn mice and wild-type littermate controls, either treated with fluoxetine or vehicle, in the hidden platform version of the Morris water maze (Figure 3; 2N, vehicle: $n = 8$ mice; 2N, fluoxetine: $n = 11$ mice; Ts65Dn, vehicle: $n = 7$ mice; Ts65Dn, fluoxetine: $n = 7$ mice). Animals were given 4 daily training trials for 5 consecutive days. Escape latencies decreased during training in all groups (Figure 3(a)), although to a more limited extent in Ts65Dn mice (both vehicle and fluoxetine treated). Statistical analysis revealed a significant main effect of genotype with higher escape latencies in Ts65Dn mice compared to 2N controls (two-way ANOVA with genotype and treatment as between-subjects factors: $F(1,29) = 20.11$, $P = 0.0001$). There was no significant main effect of treatment (two-way ANOVA: $F(1,29) = 0.07$, $P = 0.79$) and no genotype $\times$ treatment interaction (two-way-ANOVA: $F(1,29) = 2.07$, $P = 0.16$). We analyzed swim speed during all training sessions and the probe trial using repeated-measures two-way ANOVA with genotype and treatment as between-subjects factors (Figure 3(b)). Swim speed was significantly decreased in Ts65Dn mice relative to 2N controls (repeated-measures two-way ANOVA: $F(1,29) = 9.15$, $P = 0.005$). There was no significant effect of treatment on swim speed (repeated-measures two-way ANOVA: $F(1,29) = 2.258$, $P = 0.144$) and no significant genotype $\times$ treatment interaction (repeated-measures two-way ANOVA: $F(1,29) = 0.529$, $P = 0.473$).

To evaluate how accurately the mice had learned the escape platform position during training, we performed a single-probe trial (no platform in the water tank) after completion of training (Figures 3(c), 3(d), and 3(e)). We analyzed several probe trial measures that report spatial selectivity of searching and, hence, indicate the extent of spatial learning that occurred.

First, we measured the time that the animals spent in the target quadrant (which previously contained the escape platform) and the other quadrants during the probe trial (Figure 3(c)). Statistical analysis of the quadrant occupancy data showed significant genotype $\times$ quadrant and treatment $\times$ quadrant interactions (three-way ANOVA with genotype and treatment as between-subjects factors and quadrant as within-subjects factor: genotype $\times$ quadrant interaction, $F(3,116) = 5.24$; $P = 0.002$, treatment $\times$ quadrant interaction: $F(3,116) = 5.48$, $P = 0.002$), indicating that genotype and treatment had significant effects on quadrant occupancy. Vehicle-treated 2N controls spent significantly more time in the target quadrant than the other quadrants (t-test, target quadrant occupancy versus average occupancy of the other quadrants: $P = 0.001$), indicating memory for the platform location. In contrast, in the other groups, target quadrant occupancy was not significantly different from average occupancy of the other quadrants (t-test, target quadrant occupancy versus average occupancy of the other quadrants: 2N/fluoxetine, $P = 0.292$; Ts65Dn/vehicle, $P = 0.267$; Ts65Dn/fluoxetine, $P = 0.241$).

We also recorded the number of target crossings (i.e., crossings of the exact target location, where the platform was located during training) during the probe trial, which was compared to the number of crossings over corresponding locations in the other quadrants (Figure 3(d)). With respect to target crossings, ANOVA analyses yielded a significant effect of genotype (three-way ANOVA with genotype and treatment as between-subjects factors and quadrant as within-subjects factor: $F(1,116) = 17.3$, $P < 0.0001$), reflecting an overall reduced number of crossings in Ts65Dn mice and, additionally, a possible genotype $\times$ quadrant interaction (three-way ANOVA with genotype and treatment as between-subjects factors and quadrant as within-subjects factor: $F(3,116) = 2.25$, $P = 0.09$). To further probe for spatial selectivity of searching, we asked whether animals showed significantly more crossings over the target location than over corresponding locations in the other quadrants. Fluoxetine-treated 2N mice showed significantly more crossings of the target location than the other locations (t-test, target crossings versus average crossings of corresponding locations in the other quadrants: $P = 0.010$), while this was not the case for the other groups (t-test, target crossings versus average crossings of corresponding locations in the other quadrants: 2N/vehicle, $P = 0.100$; Ts65Dn/vehicle, $P = 0.362$; Ts65Dn/fluoxetine, $P = 0.784$).

As a third measure for spatial selectivity of searching, we looked at proximity to the target (i.e., average distance to the target location, where the platform was located during training) and compared it to the average distance to corresponding locations in the other quadrants (Figure 3(e)). Three-way ANOVA with genotype and treatment as between-subjects factors and quadrant as within-subjects factor showed a significant main effect of genotype ($F(1,116) = 7.10$, $P = 0.009$), which was reflective of the overall larger distances that Ts65Dn mice had to the target position and corresponding positions in the other quadrants. This ANOVA also revealed a significant genotype $\times$ quadrant interaction ($F(3,116) = 4.10$, $P = 0.008$) and a significant treatment $\times$ quadrant interaction ($F(3,116) = 5.25$, $P = 0.002$), showing that the search pattern was influenced by genotype and treatment, such that target-preferential searching was less pronounced or absent in Ts65Dn mice/fluoxetine-treated mice. Vehicle-treated 2N mice showed significantly lower proximity values to the target than average distance to the other positions (t-test, proximity to target versus average proximity to corresponding locations in the other quadrants: $P = 0.005$), again indicating preferential searching in the relative vicinity of the target in this group. This comparison yielded nonsignificant results for the other groups (t-test, target crossings versus average crossings of corresponding locations in the other quadrants: 2N/fluoxetine, $P = 0.550$; Ts65Dn/vehicle, $P = 0.442$; Ts65Dn/fluoxetine, $P = 0.077$). As expected based on the slower swim speed in Ts65Dn mice (see above), total distance travelled during the probe trial was significantly reduced in Ts65Dn mice (Figure 3(f); two-way ANOVA with genotype and treatment as between-subjects factors: genotype effect, $F(1,29) = 10.98$, $P = 0.003$; treatment effect, $F(1,29) = 3.21$, $P = 0.084$, genotype $\times$ treatment interaction, $F(1,29) = 0.14$, $P = 0.706$).

Taken together, the probe trial revealed indications of spatially selective searching and, hence, successful spatial learning and memory in vehicle-treated (see quadrant occupancy and proximity to target) and fluoxetine-treated (see target crossings) 2N mice, but not in Ts65Dn mice,

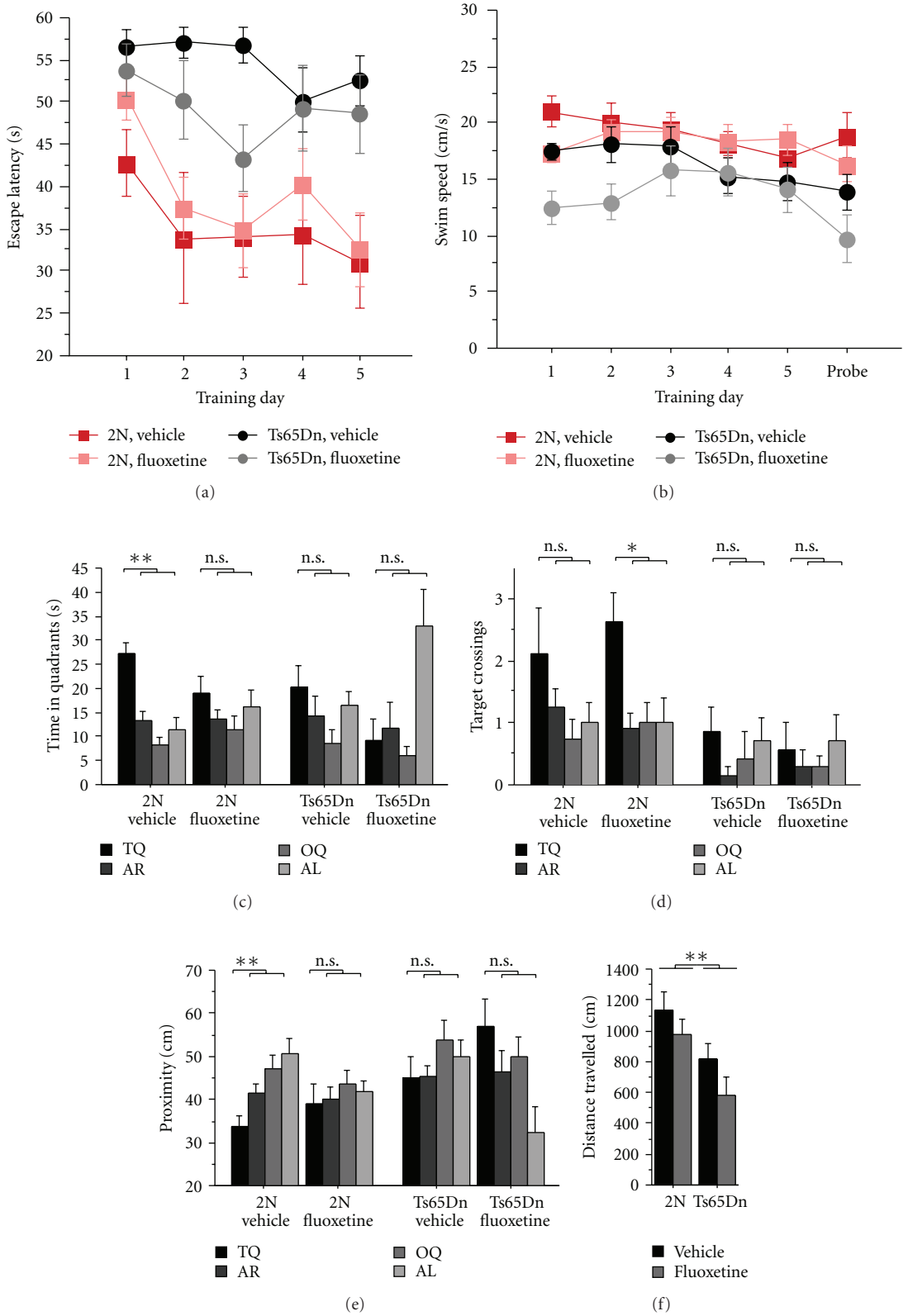

Figure 3: Results from the assessment of Ts65Dn mice and 2N controls in the hidden version of the Morris water maze (2N, vehicle: $n = 8$ mice; 2N, fluoxetine: $n = 11$ mice; Ts65Dn, vehicle: $n = 7$ mice; Ts65Dn, fluoxetine: $n = 7$ mice). (a) Escape latencies. (b) Swim speed during training trials and the probe trial. (c)–(e) Probe trial measures: (c) quadrant occupancy, (d) target crossings, (e) proximity to target, (f) total distance travelled. Pool quadrants: target quadrant (TQ), adjacent right (AR), opposite quadrant (OQ) and adjacent left (AL). Graph shows means ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$, n.s. $P > 0.05$. For details regarding statistical analyses, please see main text.

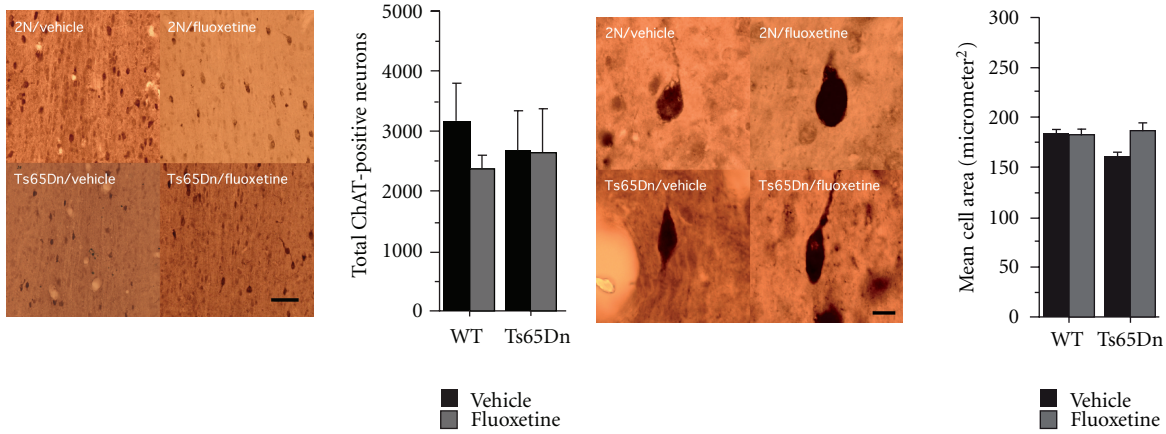

FIGURE 4: Results from stereological assessment of choline acetyltransferase-(ChAT-) positive cells in basal forebrain cholinergic nuclei in Ts65Dn mice and wild-type controls treated either with fluoxetine or vehicle control (2N, vehicle: $n = 8$ mice; 2N, fluoxetine: $n = 11$ mice; Ts65Dn, vehicle: $n = 7$ mice; Ts65Dn, fluoxetine: $n = 5$ mice). (a) Stereological counting of choline acetyltransferase-(ChAT-) positive cells in basal forebrain cholinergic nuclei. Shown are representative brain sections from 2N controls and Ts65Dn mice under treatment with vehicle or fluoxetine. Scale bar = 50 μm. The quantification is shown in the bar graph. No differences with regards to cholinergic cell number were detectable between Ts65Dn mice and 2N controls (two-way ANOVA with genotype and treatment as between-subjects factors: genotype effect, $F(1,27) = 0.04$, $P = 0.840$; treatment effect, $F(1,27) = 0.61$, $P = 0.441$; genotype $\times$ treatment interaction, $F(1,27) = 0.49$, $P = 0.491$). Graph shows means $\pm$ SEM. (b) Cell size of choline acetyltransferase-(ChAT-) positive cells in basal forebrain cholinergic nuclei from Ts65Dn mice and 2N controls. Shown are representative ChAT-stained neurons from euploid mice treated with vehicle or fluoxetine and Ts65Dn mice treated with vehicle or fluoxetine. The bar graph is depicting cell size distributions of ChAT-immunoreactive cells. Cholinergic neurons of euploid mice appeared to be larger than in Ts65Dn mice although the statistical comparison was not significant (two-way ANOVA with genotype and treatment as between-subjects factors: genotype effect, $F(1,27) = 2.2$, $P = 0.15$). Fluoxetine treatment had no significant effect on cell size, although there was a trend towards increasing cell size in Ts65Dn mice (two-way ANOVA with genotype and treatment as between-subjects factors: treatment effect, $F(1,27) = 3.08$, $P = 0.091$; genotype $\times$ treatment interaction, $F(1,27) = 3.14$, $P = 0.08$). The graph shows means $\pm$ SEM.

TABLE 2: Number of animals displaying seizures stratified by genotype and treatment group.

	2N	Ts65Dn
Vehicle	0 (0%)	0 (0%)
Fluoxetine	0 (0%)	3 (33.3%)

irrespective of treatment group. These data show clear behavioral abnormalities of Ts65Dn mice in the water maze, which are consistent with published data [2, 8, 27] and reflect motor impairments (see swim speed) and spatial learning and memory difficulties (see probe trial measures) in Ts65Dn mice. Neither motor impairments nor spatial learning deficits in Ts65Dn mice were improved by fluoxetine treatment.

During behavioral experimentation, we incidentally witnessed tonic-clonic seizures triggered by handling in a few animals (a total of 3 mice) (Table 2). All seizure episodes observed occurred in Ts65Dn mice on fluoxetine treatment. Seizures were not observed in the other groups (Fisher's exact test, Ts65Dn/fluoxetine group versus collapsed data from the other groups, $P = 0.01$).

One of the neuropathological hallmarks of both Down syndrome and Alzheimer's disease is the age-dependent loss of basal forebrain cholinergic neurons (BFCNs) [24, 28]. Previous studies pointed to a correlation between BFCN degeneration and abnormalities in memory and learning in aging Ts65Dn mice [24]. To probe whether fluoxetine is able to ameliorate or prevent early BFCN degeneration in Ts65Dn mice, we performed unbiased stereological cell counts of choline acetyltransferase (ChAT) antibody-stained sections in fluoxetine-treated animals and vehicle controls. We determined the cholinergic cell number and size (Figure 4) in the medial septal nuclei (MSN) and ventral diagonal band (VDB) of each group (2N, vehicle: $n = 8$ mice; 2N, fluoxetine: $n = 11$ mice; Ts65Dn, vehicle: $n = 7$ mice; Ts65Dn, fluoxetine: $n = 5$ mice).

No clear differences with regards to cholinergic cell number were evident between trisomic and euploid mice (two-way ANOVA with genotype and treatment as between-subjects factors: $F(1,27) = 0.04$, $P = 0.84$). Treatment also had no obvious effects on the number of ChAT-positive cells in basal forebrain cholinergic nuclei (two-way ANOVA with genotype and treatment as between-subjects factors: $F(1,27) = 0.61$, $P = 0.44$). Since neuronal atrophy precedes cell loss [28], we analyzed cell size of ChAT-positive cells in BFCN of fluoxetine-/vehicle-treated Ts65Dn mice/wild-type controls. Cholinergic neurons of euploid mice were insignificantly larger than those in trisomic mice (two-way ANOVA with genotype and treatment as between-subjects factors: $F(1,27) = 2.2$, $P = 0.15$). Fluoxetine treatment had no significant

effect on cell size, although there was a trend towards increasing cell size in Ts65Dn mice (two-way ANOVA with genotype and treatment as between-subjects factors: treatment effect, $F(1,27) = 3.08$, $P = 0.091$; genotype $\times$ treatment interaction, $F(1,27) = 3.14$, $P = 0.08$).

4. Discussion

In the present study, we set out to test whether a 4-week, adult-onset fluoxetine treatment is effective against behavioral alterations in the Ts65Dn mouse model of Down syndrome. We found no discernible benefits of this fluoxetine treatment regimen in Ts65Dn mice. In particular, we did not observe a treatment effect on the clear Ts65Dn behavioral phenotype in the water maze, which included slower swim speed and effects on probe trial measures indicative of spatial learning and memory impairments.

Adverse side effects due to fluoxetine treatment of Ts65Dn mice, however, appeared to be profound in our cohort. Four out of 9 Ts65Dn mice died while on fluoxetine treatment during which time there were no deaths in the other groups (with the exception of 1 dead wild-type/vehicle animal). These results suggest that fluoxetine may have a genotype-specific adverse effect and warrant further experimentation in larger animal cohorts that also assess the cause of death in fluoxetine-treated Ts65Dn mice.

We observed handling-induced seizures in a few mice, all of which were fluoxetine-treated Ts65Dn mice, suggesting interactive effects of Ts65Dn genotype and fluoxetine treatment on seizure susceptibility. Seizures represent a relatively rare side effect of therapeutic fluoxetine regimens in general clinical populations but are a more common feature of fluoxetine intoxications [29–31]. Several epilepsy paradigms in animal models also illustrate a proconvulsive effect of fluoxetine; for instance, fluoxetine pretreatment potentiates the convulsive effects of pentylenetetrazol (PTZ) and electrically evoked seizures [32, 33]. Collectively, these reports show that treatments with selective serotonin reuptake inhibitors, including fluoxetine, can lower seizure thresholds in humans and animal models.

Epilepsy is not uncommon in Down syndrome and may affect 1–13% of all individuals with trisomy 21 [34, 35]. Although spontaneous seizures are not a feature of the Ts65Dn model, Ts65Dn mice are more prone to audiogenic seizures than controls [36], suggesting reduced seizure thresholds in this model. In sum, the data raise the possibility that behavioral convulsions in Ts65Dn mice, described above, may result from an interaction between fluoxetine and the lowered seizure thresholds in Ts65Dn mice. Future studies should examine these effects in larger animal cohorts and include electrophysiological assessments of seizure activity.

For the present study, we adopted a fluoxetine treatment regime used previously [11], that is, administration of 0.2 mg/mL fluoxetine via the drinking water. This treatment regime was found to lower intracortical inhibition and to reinstate ocular dominance plasticity in the adult visual cortex [11]. The fluoxetine dose is higher than rodent equivalents of clinically used doses (i.e., clinically employed doses converted into equivalent surface area doses for mice/rats) but is within the range of fluoxetine dosing schemes generally used in mice and rats (e.g., [20, 37, 38]). In future work, it will be important to establish a dose-response function with respect to the side effects in Ts65Dn mice. Based on our present data, we cannot rule out the possibility that lower fluoxetine doses exert beneficial effects in the Ts65Dn model of Down syndrome. It will also be important to determine if drug $\times$ genotype interactions are caused by genotype-dependent differences in CNS effects of fluoxetine or, possibly, different pharmacokinetic profiles in Ts65Dn mice and wild-type controls (e.g., metabolism of fluoxetine, tissue distribution, etc.).

The animals used in the present study were partly in the age range in which neurodegenerative processes may slowly begin in Ts65Dn mice (i.e., >6 months of age). We assessed neurodegeneration via stereological quantification of basal forebrain cholinergic neurons and did not observe significant differences between trisomic and euploid animals (Figure 4), showing that neurodegenerative changes were not a prominent feature in our Ts65Dn cohort (consistent with other reports showing that first signs of degenerative changes affecting the cholinergic system in Ts65Dn mice emerge between 6 and 12 months; [28, 39]). Nevertheless, we cannot rule out the possibility that subtle degenerative changes were already present, yet not measureable in our cohort. Therefore, it remains possible that adult-onset fluoxetine treatment has beneficial effects in 3–6 months old Ts65Dn mice (i.e., in the narrow time window during adulthood, wherein Ts65Dn mice are thought to be free of degenerative changes).

A prior study had assessed the effect of fluoxetine treatment on hippocampal neurogenesis in 2–5 months old Ts65Dn mice [40]. Fluoxetine was administered i.p. and treatment was shorter (15 days) and involved lower dosing (5 mg/kg) than treatment in the present study. Fluoxetine treatment increased neurogenesis in Ts65Dn mice and wild-type controls [40], which is in agreement with earlier reports regarding the effects of chronic fluoxetine treatment on adult hippocampal neurogenesis [41]. Behavioral results were not reported and, hence, it remains unclear whether there was a behavioral effect associated with fluoxetine treatment of Ts65Dn mice in this study.

Another more recent study reported beneficial effects of fluoxetine, administered early postnatally, in the Ts65Dn mouse model [42]. In this study, fluoxetine was administered from P3 to P15 (s.c. injections of 5 mg/kg from P3 to P7 and 10 mg/kg from P8 to P15) followed by a BrdU injection at P15 to label proliferating cells. Fluoxetine increased cell proliferation and generation of new neurons in the dentate gyrus and subventricular zone of Ts65Dn mice and wild-type controls. Fluoxetine treatment increased the number of dentate gyrus granule cells at P45 in Ts65Dn mice to wild-type control levels, while granule cell numbers were reduced in vehicle Ts65Dn mice. Limited behavioral assessment was performed in the context of this study in juveniles (at P43). Context fear-conditioning impairments in juvenile Ts65Dn mice were improved by early postnatal fluoxetine treatment, while fluoxetine had no effects on wild-type controls. In the present study we did not find beneficial effects of fluoxetine treatment on behavioral impairments of adult

Ts65Dn mice in the Morris water maze. It is possible that early postnatal and adult-onset fluoxetine treatments have different effects on behavioral impairments in Ts65Dn mice. The protocols in our study, however, differ also in a number of other respects from the published paper (e.g., fluoxetine dose, route of administration, and duration of treatment; behavioral paradigm: here, we assessed learning in the water maze; Bianchi et al. assessed fear conditioning), which may also account for the different findings. In future studies, it will therefore be important to further explore this parameter space, that is, to establish dose-response relationships for fluoxetine treatment in Ts65Dn mice at different ages and to conduct comprehensive behavioral assessments that cover impairments of Ts65Dn mice in a range of behavioral tasks.

5. Conclusion

Here, we determined the impact of an adult-onset, chronic treatment with the antidepressant fluoxetine on behavioral alterations in the Ts65Dn mouse model of Down syndrome. We did not find a beneficial effect of fluoxetine treatment on Ts65Dn behavioral phenotypes, but instead our findings suggest the presence of genotype-dependent fluoxetine side effects; we observed seizures and mortality in treated Ts65Dn mice, but not wild-type controls. Future studies should reevaluate these findings in larger animal cohorts, determine what the nature of the possible drug $\times$ genotype interaction is (e.g., genotype-dependent differences in drug metabolism, tissue distribution, or truly differential effects of equivalent drug tissue concentrations on cellular/tissue functions), and establish dose-response relationships for these possible side effects.

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Review Article

Synaptic Plasticity and Learning in Animal Models of Tuberous Sclerosis Complex

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Tuberous sclerosis complex (TSC) is caused by a mutation of either the *Tsc1* or *Tsc2* gene. As these genes work in concert to negatively regulate the mammalian target of rapamycin (mTOR) kinase which is involved in protein translation, mutations of these genes lead to a disinhibited mTOR activity. Both the clinical appearance of this condition including tumors, cognitive decline, and epileptic seizures and the molecular understanding of the mTOR signaling pathway, not only involved in cell growth, but also in neuronal functioning, have inspired numerous studies on learning behavior as well as on synaptic plasticity which is the key molecular mechanism of information storage in the brain. A couple of interesting animal models have been established, and the data obtained in these animals will be discussed. A special focus will be laid on differences among these models, which may be in part due to different background strains, but also may indicate pathophysiological variation in different mutations.

1. Introduction

Tuberous sclerosis complex (TSC) is an inherited disease caused by a heterozygous germ line mutation of either the *Tsc1* or *Tsc2* gene that is manifested in early childhood. The pathological hallmark of this disorder is the development of hamartomas (benign tumors) arising in a number of organs including the central nervous system [1, 2]. In the brain, TSC lesions typically comprise of cortical tubers, subependymal nodules, and giant cell astrocytomas [3, 4].Hence, common symptoms related to brain lesions are epileptic seizures, mental retardation, multiple neuropsychological impairments, and even autism [5–9]. Consequently, the significant neuropsychiatric morbidity caused by this condition has inspired a number of groups worldwide to study the underlying pathomechanisms aiming to improve our functional understanding of both gene products, named hamartin (*Tsc1*) and tuberin (*Tsc2*). These proteins act in concert as a guanosine triphosphate-activating protein (GAP) towards the small G protein Rheb, which is the key regulator of the mammalian target of rapamycin (mTOR) signaling [10, 11]. Since hamartin and tuberin negatively regulate mTOR activity, which in turn phosphorylates and thereby activates important translation factors such as p70 S6 kinase 1 (S6K1) and eukaryote initiation factor 4E-binding protein (eIF4E-BP), a major role of the TSC-mTOR signaling pathway has been suggested for tumorigenesis, and both genes were initially recognized as tumor suppressors [12]. However, increasing evidence has been provided that this pathway is also considerably involved in neuronal functioning including synaptic plasticity [13–16].

Since its discovery in the early 1970s [17], a large body of evidence has been emerged supporting the key role of synaptic plasticity in memory formation at the molecular level [18, 19]. In general, synaptic plasticity is an activity-dependent change of synaptic strength which appears bidirectional. A rise in synaptic strength or long-term potentiation (LTP) is typically induced by high-frequency stimulation (>50 Hz) paradigms that are capable to activate postsynaptic NMDA receptors [19]. In contrast, long-term depression (LTD) may be a consequence of stimulation paradigms delivered at lower frequencies (1–3 Hz; [20]) or involving the activation of metabotropic glutamate receptors (mGluRs; [21–25]).

2. Synaptic Plasticity in TSC Animal Models

Several animal models of TSC are available and have already been employed to study synaptic plasticity in vitro. One

unique animal model is the Eker rat, which carries a spontaneous germ line mutation of the *Tsc2* gene and was initially described as a model for renal cancer [37].Only mild brain pathologies were observed in $Tsc2^{+/-}$ rats, albeit may be more pronounced in aged animals, and—in addition—epileptic seizures were not detected at all [26, 38–40], (Table 1). However, a "second hit" insult such as irradiation was effective in this model both in lowering seizure threshold and in producing morphological brain lesions [41]. In particular, these included cytomegalic neurons and giant astrocyte-like cells in the cortex, but also in other brain regions such as the hippocampus. With respect to learning disabilities, it is conceivable that cognitive dysfunction may be related to both cortical and hippocampal lesions. Hence, it should be mentioned that the hippocampus of irradiated $Tsc2^{+/-}$ rats exhibited multiple nodular hamartoma-like lesions with eosinophilic large cells. Since these cells were immunoreactive for glial fibrillary acidic protein (GFAP), but also for MAP2 and nestin, they seem to share both immature neuronal and glial/astrocytic features [41]. The appearance of such cells within the brain of adult irradiated as well as aged $Tsc2^{+/-}$ rats [40, 41] may reflect abnormal processes of cell migration and differentiation during corticogenesis. Synaptic plasticity, unfortunately, has not been tested in these animals. The first attempt to assess synaptic function in a TSC model was made in young naïve $Tsc2^{+/-}$ rats [27]. Notably, despite the absence of a clear neuropathology and seizures, synaptic plasticity in the hippocampus was impaired in young naïve $Tsc2^{+/-}$ rats [27]. In this early report, a rather physiological theta-burst stimulation paradigm (10 trains of 5 stimuli at 100 Hz, 200 ms apart) was applied to Schaffer collateral-CA1 synapses, which was sufficient to induce robust LTP in control animals. In mutated rats, however, this paradigm was no longer able to produce significant LTP at these synapses. Importantly, the same results were obtained when a $GABA_A$ receptor blocker was present in the bathing solution, indicating that the LTP deficit in the Eker rat model was due to *Tsc2* deficiency in CA1 pyramidal neurons rather than in GABAergic interneurons in this region. Moreover, long-term depression induced by two different low-frequency stimulation protocols (900 stimuli at 1 Hz, 2×600 stimuli at 1 Hz) was also significantly diminished in $Tsc2^{+/-}$ tissue. These findings suggested that mental retardation and cognitive decline in TSC may be due to impaired synaptic plasticity rather than resulting from brain lesions or epileptic seizures.

However, the assumption that cognitive malfunction in TSC may be due to an alteration of activity-dependent synaptic plasticity, in particular at hippocampal synapses, is plausible, but still controversial. The gene products of *Tsc1/2* act in concert in order to downregulate mTOR function [10, 11], therefore TSC is generally associated with a disinhibited activity of this important regulatory enzyme leading to increased protein translation [42, 43]. Just recently, Stoica et al. [44] have demonstrated that mTOR is indeed required for normal synaptic plasticity and long-term memory. They created heterozygous $mTOR^{+/-}$ mice, which themselves did not show altered LTP or a memory deficit phenotype. However, when hippocampal slices taken from these animals were pretreated with low concentrations of rapamycin in order to inhibit the remaining activity of mTOR, LTP was significantly reduced [44]. Moreover, the in vivo administration of low-dose rapamycin to $mTOR^{+/-}$ mice impaired contextual long-term fear memory. Importantly, the low concentration of rapamycin was subthreshold to affect LTP or long-term memory in wild-type mice, indicating that regular mTOR activity is instrumental for hippocampus-dependent forms of long-term memory.

On the neuropathological level, mTOR disinhibition in radial glia led to cortical and hippocampal dyslamination with hippocampal heterotopias and dysplastic neurons [45]. This is an extremely interesting animal model because—like in the human condition—all cells were heterozygous for the *Tsc2* deletion, and radial glia additionally lacked the second copy of the *Tsc2* gene. Unfortunately, this animal model has not yet been tested electrophysiologically. Nonetheless, it is an intriguing question whether *Tsc2* deletion and thereby disinhibited mTOR function affects learning and memory. In particular, it has been demonstrated that mTOR-dependent protein synthesis is required for late phases of LTP [46]. To assess the role of TSC-associated mTOR disinhibition in this form of LTP, heterozygous $Tsc2^{+/-}$ mice were used to record long-term potentiation in the CA1 subfield lasting for several hours [29]. Using in vitro brain slices, repetitive trains of tetanic stimulations are typically required to induce such a late phase LTP [47, 48]. However, a single train of tetanic stimulation (100 stimuli at 100 Hz), which failed to induce late phase LTP in wildtype mice as expected, elicited abnormally high levels of potentiation in $Tsc2^{+/-}$ mice-up to four hours following tetanization [29]. In contrast to the study of von der Brelie et al. [27], early LTP (i.e., 60 minutes of followup) was not different between both genotypes. Unfortunately, different stimulation paradigms (theta-burst versus tetanization) and different animal models (Eker rat versus *Tsc2* knockout mouse) impede a conclusive comparison of both studies. Nonetheless they suggest that constitutively disinhibited mTOR activity and thereby increased protein synthesis in hippocampal neurons significantly enhanced the propensity of late phase LTP, while early phase LTP, which depends on posttranslational modifications such as AMPA receptor phosphorylation via Ca^{2+}/calmodulin-dependent kinase II and protein kinase A [49, 50] rather than protein synthesis, is impaired in TSC2 animal models.

With respect to *Tsc1* gene mutations, one interesting animal model is the $Tsc1^{GFAP}$ conditional knockout mouse, in which the *Tsc1* gene was specifically inactivated in glia [51, 52]. Consequently, the gene product hamartin was absent in brain glia and the mice showed an increased astrocyte proliferation with abnormal neuronal organization, as well as frequent and severe seizures [51]. The detailed pathological examination of adult $Tsc1^{GFAP}$ mice revealed that cortical and hippocampal astrocytes expressed vimentin and brain lipid binding protein (BLBP) [52], which are marker proteins for radial glia and immature astrocytes [53, 54]. This alteration was observed throughout the hippocampus including all Cornu Ammonis subfields as well as the dentate gyrus. Hence, the authors concluded

Table 1: Overview of available data on synaptic plasticity and memory.

Animal model	Synaptic plasticity	Learning and memory	References
Tsc2$^{+/-}$ rat (Eker rat)	Reduced theta-burst stimulation-induced early phase LTP at Schaffer collateral-CA1 synapses. Reduced low-frequency stimulation-induced LTD at Schaffer collateral-CA1 synapses.	Enhanced episodic-like memory (water maze, radial maze). Mildly reduced exploration behavior (open field, novel object recognition, social exploration). No difference in anxiety-related behavior (light/dark box). No difference in hippocampus-dependent learning behavior (fear conditioning, water maze).	[26–28]
Tsc2$^{+/-}$ mouse	Enhanced tetanus-induced late phase LTP at Schaffer collateral-CA1 synapses. No difference in tetanus-induced early phase LTP at Schaffer collateral-CA1 synapses. Reduced mGluR-dependent LTD at Schaffer collateral-CA1 synapses.	Reduced hippocampus-dependent learning behavior (water maze, radial maze, context fear conditioning).	[29, 30]
Dominant negative *Tsc2* mutant mouse (ΔRG mouse)	Reduced mGluR-dependent LTD at Schaffer collateral-CA1 synapses.	Increased anxiety-related behavior (elevated plus maze). Mildly impaired hippocampus-dependent learning behavior (water maze, context fear conditioning). Impaired social interaction.	[31–33]
Tsc1^{GFAP} CKO mouse	Reduced tetanus-induced early phase LTP at Schaffer collateral-CA1 synapses.	Reduced hippocampus-dependent learning behavior (water maze, context fear conditioning).	[34]
Virus-injected mouse (*Tsc1*-deletion in CA1 neurons)	Reduced mGluR-dependent LTD at Schaffer collateral-CA1 synapses.		[35]
Tsc1$^{+/-}$ mouse		Reduced hippocampus-dependent learning behavior (water maze, context fear conditioning).	[36]

that *Tsc1* inactivation in glial cells may impair hippocampal maturation [52]. Interestingly, *Tsc1* ablation in neurons achieved by engineering mice that have a specific *Tsc1* deletion in synapsin-expressing neurons (*Tsc1*^{Synapsin} mice) [55] primarily showed ectopic enlarged and/or dysplastic neurons in the cortex and the hippocampus. Zeng et al. [34] assessed synaptic plasticity in *Tsc1*^{GFAP} animals and—again, LTP at the Schaffer collateral—CA1 synapses was tested. Despite a quite strong induction protocol consisting of four trains of tetanic stimulations (100 stimuli at 100 Hz), the potentiation after 30 minutes was negligible in the mutant mice. However, the authors also found significantly elevated glutamate levels in *Tsc1*^{GFAP} mutants, and importantly, LTP could be rescued by a low concentration of an NMDA receptor antagonist. The authors concluded that excessive glutamate release caused both excitotoxicity in the hippocampus and impaired synaptic plasticity. Since the functional role of mTOR activity might be quite diverse concerning glia and neurons, it appears difficult to reconcile the findings obtained in the *Tsc1*^{GFAP} conditional knockout mouse with those from animals carrying neuronal *Tsc2* mutations.

In summary, a uniform picture on electrically induced synaptic plasticity in various animal models of TSC might not be drawn from the available studies. On the one hand, this is certainly due to different stimulation paradigms that may evoke distinct LTP mechanisms. Alternatively, discrepancies among studies might also be attributed to previously unknown downstream effects of *Tsc1/2* gene inactivation. For instance, a recent report using a dominant negative *Tsc2* mutant (ΔRG mouse model) observed an elevated activity of extracellular signal-regulated kinase (ERK) [31]. The ERK pathway, however, has not been addressed in the investigations discussed above [27, 29, 34].

A mechanistically distinct form of long-term depression has been discovered in the CA1 stratum radiatum that depends on activation of group I metabotropic glutamate receptors (mGluR-LTD; [21–25]). A large body of evidence suggests that mGluR-LTD is mediated by protein translation-dependent AMPA receptor endocytosis [23–25, 56, 57], involving the mTOR pathway [58, 59] as well as ERK [60]. This notion has inspired three recent investigations on mGluR-LTD: (i) in *Tsc1*-deleted CA1 neurons [35], (ii) in *Tsc2*$^{+/-}$ mice [30], and (iii) in the ΔRG mouse model of TSC2 [31]. In the first paper, the stereotactic injection of a viral vector encoding a Cre recombinase-EGFP construct under the control of the synapsin promoter into the CA1 area of *Tsc1*$^{flox/flox}$ mice [61] provided an extremely elegant method to delete the *Tsc1* gene specifically in CA1 pyramidal neurons [35]. Pharmacological activation of group I mGluRs using the specific agonist (RS)-3,5-dihydroxyphenylglycine (DHPG, 100 μM, 5 min) caused an immediate as well as a long-lasting depression (i.e., mGluR-LTD) of excitatory postsynaptic currents (EPSCs) in CA1 neurons. Both types of depression were also achieved by an electrical stimulation paradigm consisting of 900 pairs of stimuli at 1 Hz (paired-pulse low-frequency stimulation, PP-LFS) in the presence of an NMDA receptor antagonist which has also been referred to as an mGluR-dependent LTD induction protocol [24]. In contrast, synaptic transmission

onto neurons that were infected with the viral construct and thus lacked the *Tsc1* gene was immediately depressed after DHPG or PP-LFS, but the induction of LTD was prevented. These findings unequivocally demonstrated that intact *Tsc1* functioning is required for mGluR-LTD in the CA1 area. However, these findings do not explain how disinhibition of mTOR activity and thus enhanced protein synthesis might compromise mGluR-LTD which was initially assumed to be more pronounced in tissue after loss of *Tsc1/2* [62].

A severe reduction of mGluR-LTD was also found in $Tsc2^{+/-}$ mice [30]. In this paper, the authors tested both standard induction protocols, that is, brief application of DHPG and PP-LFS, and thus confirmed that heterozygous deletion of the *Tsc2* gene impaired mGluR-LTD. Moreover, protein synthesis inhibition with cycloheximide mimicked this reduction, and had no effect in $Tsc2^{+/-}$ mice. On the other hand, the mGluR-LTD was restored by pretreatment with rapamycin, indicating that the impairment was due to excess mTOR activity [30]. The third recent paper again confirmed the loss of mGluR-LTD in dominant negative *Tsc2* mutant mice (ΔRG mice) and found an elevated activity of extracellular signal-regulated kinase (ERK) in these animals [31]. The ΔRG transgenic mouse expresses a dominant negative *Tsc2* gene product which binds to and recruits hamartin, but disables the TSC1/2 protein complex in terms of their Rheb-directed GAP activity [63, 64]. Surprisingly, phosphorylation of both S6K1 and eIF4E-BP remained unaltered indicating that the mTOR signaling pathway was intact. However, the ribosomal S6 protein was detected to be phosphorylated at serine residues specific for ERK1/2 activation. Thus, *Tsc2* inactivation by the ΔRG mutation caused an impairment of mGluR-LTD, which was due to overactivation of ERK1/2, but not of mTOR [31]. The authors also tested transgenic mice in which the *Tsc1* or *Tsc2* genes were specifically deleted in α-Ca^{2+}/calmodulin-kinase II-expressing neurons. In line with the other two models described above (*Tsc1*-deleted CA1 neurons using viral transfection, and the ΔRG mouse model), these strains again showed the same phenotype that is, impaired DHPG-induced LTD. Hence, from the available literature on mGluR-dependent LTD in the CA1 area, it can be concluded that genetic ablation of either the *Tsc1* or *Tsc2* is consistently associated with the loss of this form of synaptic plasticity.

3. Learning and Memory in TSC Animal Models

The manifestation of cognitive decline in TSC patients is ranging from moderate to severe, and even autistic behaviors have been observed [5–9]. To address this issue experimentally, a number of different behavioral test batteries assessing distinct brain functions in rodents have been used to characterize the phenotypes of currently available TSC animal models. The first attempt to study learning behavior and memory in TSC was again made with Eker rats [26]. Although the authors tried hard to obtain a memory-impaired phenotype in young Eker rats that were almost devoid of cerebral hamartomas [26, 38–40], the only significant difference was discovered in a delayed matching-to-place task assessing episodic-like memory [65]. First, the authors tested this specific type of memory task in a water maze, where the hidden platform was kept constant during four trials within the same experimental day, but was randomly repositioned at consecutive days. In the control condition, a trial-to-trial interval of 15 seconds was chosen resulting in no significant difference in the learning behavior between $Tsc2^{+/-}$ rats and control animals. In contrast, when the interval of the first to the second trial was prolonged to two hours (i.e., the delayed matching-to-place task), $Tsc2^{+/-}$ rats surprisingly showed significantly better performance than control littermates which was confirmed in a radial maze with similar paradigm using a randomized baiting scheme. All other behavioral tests assessing exploration and anxiety (light/dark box) as well as learning and memory (Pavlovian conditioned taste aversion test, Morris water maze hidden platform task including reversal learning, and probe trials) did not detect any behavioral constraints in $Tsc2^{+/-}$ rats [26].

Recently, the same group has published a reexamination of the Eker rat model behavioral phenotype—now comparing the naïve animal with an epileptic condition following systemic administration of kainic acid [28]. Again, they did not observe significantly different performance in learning abilities (fear conditioning, fear extinction, and Morris water maze). Thus, they were able to reproduce their previous report, but a somewhat unexpected finding was that kainic acid-injected rats also turned out to behave like controls in these learning tasks. In contrast, rearing in the open field, novel object recognition, and social exploration was in fact significantly reduced in naïve $Tsc2^{+/-}$ rats, and even more so in epileptic ones. The authors concluded that both *Tsc2* haploinsufficiency and epileptic seizures might compromise social interaction and moreover, to some extent in an additive manner. Albeit the quantitative differences obtained in these tests occasionally appeared to be less impressive, this is a potentially important issue in the clinical management of the disease.

Mice carrying a heterozygous inactivation of the *Tsc2* gene ($Tsc2^{+/-}$ mice) have already been mentioned in Section 2. In the same paper introduced above [29], the authors also looked at hippocampus-dependent learning behavior. First, the animals were allowed to learn the position of a hidden platform in the Morris water maze, then the platform was removed (probe trial), and two measures were used to assess whether the animals have learned the target position. Both the time spent in the quadrant of the prior platform position and the number of target crossings was analyzed and the performance of $Tsc2^{+/-}$ mice was found to be significantly poorer than that observed in controls. The second behavioral test (eight-arm radial maze) revealed that significantly more across-phase errors have been committed by the mutant mice. In the last test, the mice were trained in context fear conditioning using an aversive stimulus. 24 hours later, the animals were transferred either back into the training context or into a novel context, and the freezing responses were measured. With this paradigm, $Tsc2^{+/-}$ mice showed poor context specificity, that is, freezing in the novel context was not different from freezing in the training context [29].

The dominant negative *Tsc2* transgenic mouse mutant has been also been described in detail in Section 2 [31]. In two studies [32, 33], these mice have been investigated in various behavioral tasks assessing anxiety-related behavior as well as learning tasks. Anxiety-related behavior was tested in the elevated plus maze [32].This is a four-arm maze with two open arms and two arms having walls on the sides. It was mounted 1 m above the floor, and the time spent in the open arms was measured. Generally, increased levels of anxiety are associated with less time spent in these open arms. Indeed, dominant negative *Tsc2* mutants showed increased anxiety in this test. In the open field, *Tsc2* mutant mice showed a trend towards less exploration of the inner sector which may also be attributed to increased anxiety. Hippocampus-dependent learning as assessed by the hidden platform task in the Morris water maze and context discrimination after fear conditioning was only mildly affected. More recently, however, another group [33] failed to reproduce these results, and in fact observed normal Morris water maze behavior as well as normal contextual fear. However, social interaction was impaired in these mice. This is a clinically important issue which may relate to autistic behavior observed in TSC patients [5]. In conclusion, dominant negative *Tsc2* mutant mice showed markedly less impaired learning deficits compared with $Tsc2^{+/-}$ mice [29].

Cognitive performance may also be affected by *Tsc1* mutations, and two different models have been evaluated so far. In $Tsc1^{+/-}$mice [36], no preference for the target quadrant in the Morris water maze probe trial was found, and context-dependent freezing after fear conditioning was significantly less compared to wildtype littermates. Since both tests depend on intact hippocampal functioning, this study indicates a clear deficit in hippocampus-dependent learning. In addition, it is important to note that these findings were obtained despite the absence of seizures and cerebral lesions resembling the observations made with $Tsc2^{+/-}$ (Eker) rats [26, 28]. The findings in $Tsc1^{+/-}$ mice were largely confirmed in $Tsc1^{GFAP}$ conditional knockout mice [34]. Using this model, LTP was found to be impaired-as discussed above [34], and performance in the Morris water maze as well as in context fear conditioning was also affected. Thus, unlike *Tsc2* mouse models [29, 32, 33], *Tsc1* mutations were consistently associated with impaired performance in hippocampus-dependent learning paradigms [34, 36]. Social behavior has been evaluated in $Tsc1^{+/-}$ mice [36], and both social interaction as well as nest building behavior was markedly reduced in these animals indicating severe social deficits. While learning disabilities have been found to vary in *Tsc1* and *Tsc2* mutations, social behavior was consistently impaired in two studies which employed two different TSC models [33, 36].

4. Conclusions and Future Perspectives

Tuberous sclerosis complex (TSC) is a tumorous disease associated with epileptic seizures and mental retardation, sometimes even leading to autism [1–9]. The cognitive decline in these patients may be related to cortical tumors and/or to the epileptic condition. Albeit the latter has not yet been tested experimentally, there is a large body of both clinical and experimental evidence that hippocampal seizures are a leading cause of cognitive dysfunction [66–69]. Alternatively, there are no ultrastructural studies on synaptic morphology in TSC animal models with respect to the hippocampus. Thus, one can speculate whether altered synaptogenesis is causing deficits in hippocampus-dependent LTP and/or learning behavior. At least in $Tsc1^{GFAP}$ mice, it was observed that glial glutamate transport was impaired due to reduced expression of GLT-1 and GLAST protein [70]. However, the idea that cognitive deficits may arise independently from hamartomas and epileptic discharges is an attractive and yet testable hypothesis. Support for this hypothesis is at least derived from available data on animal models that are devoid of epileptic seizures and cerebral lesions presenting with deficits in synaptic plasticity and/or learning capabilities [26–28, 36]. A second line of evidence for this idea is now emerging: a series of recent papers tried to unravel the role of mTOR in long-term memory. These studies did not primarily focus on TSC pathophysiology, but showed that (i) inhibition of mTOR activity blocked both long-term synaptic plasticity and hippocampus-dependent memory [44], (ii) significantly higher mTOR activation was detected in memory-unimpaired aged rats as opposed to memory-impaired ones [71], and (iii) mTOR was required for long-term memory in mutant mice expressing autophosphorylation-deficient α-Ca^{2+}/calmodulin-kinase II [72]. Importantly, the latter study also employed electron microscopy and confirmed that behavioral long-term memory formation was accompanied with an increased synaptogenesis [72]. Thus, disinhibited mTOR activation appears to be sufficient for synaptic plasticity, synapse formation, and memory consolidation. Of course, disrupted synaptogenesis within hamartomatous lesions as well as epileptic hyperexcitability cannot be excluded as reasons to explain cognitive decline. Rather, these conditions will have an additional impact on higher cerebral functions, and further data are required to assess their relative contributions.

It is widely accepted that understanding the pathophysiology of TSC on a molecular level will be instrumental in improving the clinical management of this disease. However, although various animal models are available and have already been employed in a number of studies, occasionally considerable differences exist concerning at least synaptic physiology and in vivo behavior. Therefore, unraveling the underlying pathomechanisms of *Tsc1* or *Tsc2* mutations (e.g., with respect to cellular or regional differences) will be required to allow a more predictive correlation between cognitive dysfunction in distinct behavioral paradigms and the causative genotype.

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Review Article

From Abnormal Hippocampal Synaptic Plasticity in Down Syndrome Mouse Models to Cognitive Disability in Down Syndrome

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Down syndrome (DS) is caused by the overexpression of genes on triplicated regions of human chromosome 21 (Hsa21). While the resulting physiological and behavioral phenotypes vary in their penetrance and severity, all individuals with DS have variable but significant levels of cognitive disability. At the core of cognitive processes is the phenomenon of synaptic plasticity, a functional change in the strength at points of communication between neurons. A wide variety of evidence from studies on DS individuals and mouse models of DS indicates that synaptic plasticity is adversely affected in human trisomy 21 and mouse segmental trisomy 16, respectively, an outcome that almost certainly extensively contributes to the cognitive impairments associated with DS. In this review, we will highlight some of the neurophysiological changes that we believe reduce the ability of trisomic neurons to undergo neuroplasticity-related adaptations. We will focus primarily on hippocampal networks which appear to be particularly impacted in DS and where consequently the majority of cellular and neuronal network research has been performed using DS animal models, in particular the Ts65Dn mouse. Finally, we will postulate on how altered plasticity may contribute to the DS cognitive disability.

1. Introduction

Down syndrome (DS) results from the triplication of genes on human chromosome 21 (Hsa21) and is associated with a range of phenotypes including craniofacial changes [1, 2], cardiac defects [3], susceptibility to leukemia but with reduced occurrence of solid cancers [4, 5], and intellectual disability [6, 7]. While the presence and severity of these individual phenotypes vary among DS individuals, every individual with DS has some degree of cognitive impairment. These impairments limit the independence of DS subjects and adversely impact their quality of life. Consequently, understanding the genetic causes of cognitive dysfunction in DS has been the focus of much research in this field.

The phenomenon of synaptic plasticity has been strongly linked to cognitive processes, such as learning and memory [8, 9]. Synaptic plasticity refers to the dynamic nature of synapses, sites of communication between neurons, in which the structure, composition, or function of the synapse changes in response to network activity. Depending on the timing and strength of pre- and postsynaptic activity, synapses can either be strengthened or weakened providing a potential mechanism for memory formation and storage [10]. Structurally, synaptic connections on excitatory neurons are typically formed on the heads of dendritic spines [11]. The morphology of the spines enables compartmentalization of signaling cascades and facilitates manipulation of the structure and composition of the cell membrane by second messenger systems [12, 13]. Thus, not only is the number of spines important, as individual locations for excitatory synaptic transmission, but the shape of the individual spines also has a critical functional role.

The link between synaptic plasticity and cognitive processes such as learning and memory is frequently studied within the hippocampus, a structure involved in diverse cognitive processes such as those related to acquisition, coding, storing, and recalling information in physical or perceived spatial environments [14–16]. Multiple lines of

evidence indicate that long-lasting up- or downregulation of functional synaptic strengths, referred to as long-term potentiation (LTP) and long-term depression (LTD), respectively, are fundamental synaptic mechanisms underlying hippocampal contributions to these processes. Thus, dendritic and synaptic abnormalities in the hippocampus, either morphological or functional, would be expected to significantly impact spatial cognition. Indeed, neuropsychological investigations requiring the use of spatial information in problem solving indicate that deficits in hippocampal-mediated learning and memory processes are hallmarks of DS [17, 18]. In this paper, we will provide an overview of the morphological and behavioral evidence for altered synaptic plasticity in DS with a focus on the hippocampus and discuss the insights provided by mouse models of this neurodevelopmental disorder into the potential molecular mechanisms contributing to these deficits.

2. Evidence for Altered Synaptic Plasticity in DS: A Neurodevelopmental Impact

The basis for altered synaptic plasticity in DS can be found in changes in the physical structure of the dendrites. Alterations in the shape and densities of dendrites would be expected to adversely affect the information storage capacity of neural networks by reducing the number of potential sites for plasticity to occur. Consistent with this idea and the observed deficits in cognition associated with DS, examination of postmortem brain tissue from DS individuals reveals profound alterations in dendritic and neuronal densities and morphology across many regions of the brain beginning *in utero* and persisting throughout life. The neocortical development of DS fetuses appears normal up to at least gestational week 22 [19–21]. By 40 weeks gestation, less discrete lamination is observed in the neocortex of DS fetuses with lower and higher cell densities observed in the visual cortex and superior temporal neocortex, respectively [19, 20]. In the hippocampus, deficits begin to appear slightly earlier as DS fetuses (17 to 21 weeks of gestation) show altered morphology, reduced neuron numbers, enhanced apoptosis, and reduced cell proliferation [22–24]. These changes may result, in part, from reductions in serotonin, dopamine, and GABA levels in the fetal DS cortex [25] since, during development, neurotransmitters such as these can act as neurotrophic factors assisting with neuronal migration, axon guidance, and neurite development [26].

During the early postnatal period, significant deficits in brain weight and gross morphology as well as myelination and neuronal densities and morphology appear [27]. Initially, dendritic expansion is enhanced in DS infants, but, by the first to second year of life, this trend reverses to become a deficit [19, 28] which persists into adulthood [19, 29]. Dendritic spine numbers are reduced, and morphology altered in DS [30, 31]. Consistent with adverse changes in dendrite morphology, synaptogenesis is also aberrant in DS fetuses [19, 32, 33] and remains deficient in adulthood [34]. MRI studies reveal that DS children and young adults have smaller overall brain volumes [35, 36] with particular deficits noted in the hippocampus [36, 37]. Hippocampal volume, that continues to decrease with age in DS individuals [38], was found to be inversely correlated with the degree of cognitive impairment [36]. Cognitive tests such as the Cambridge Neuropsychological Testing Automated Battery (CANTAB) and the Arizona Cognitive Test Battery (ACTB), the latter specifically tailored to address DS deficits, indicate that hippocampal function is particularly impacted by the DS genetic condition [17, 39].

These morphological and cognitive deficits are consistent with aberrant synaptic plasticity, and, indeed, while difficult to measure directly in human subjects, evidence suggests that plasticity is reduced at least in the motor cortex of DS individuals [40]. Additionally, functional MRI (fMRI) during cognitive processing tasks reveals abnormal neural activation patterns in DS children and young adults [41, 42]. Examination of resting glucose metabolism in the cerebral cortex of DS individuals found enhanced uptake in brain regions associated with cognition suggesting cellular hyperactivity in those areas [43]. To better understand the functional consequences resulting from altered network morphologies as well as investigate potential alterations in intracellular signaling cascades contributing to aberrant plasticity, it was necessary to develop and then examine animal models of DS.

3. Modeling DS Cognitive Impairment

Over the past few decades, several mouse models of Down syndrome have been developed to further our understanding of the link between enhanced gene dosage and DS phenotypes such as altered plasticity and cognition. The Tc1 mouse model carries an almost complete, freely segregating copy of Hsa21, but the chromosome is present in only approximately 50% of cells making this a mosaic model of DS [46]. Interestingly, some genes have been deleted from the "inserted" Hsa21 [47]. It is important to note that, in spite of the mosaicism and gene deletions, many DS phenotypes have been replicated in this model [46, 48, 49]. Other mouse models have taken advantage of the homology between regions of Hsa21 and mouse chromosomes 10, 16, and 17 (Mmu10, 16, 17) making models in which these genes are triplicated highly useful in understanding the genetic basis of DS phenotypes [50, 51]. A mouse model trisomic for all Hsa21 homologous segments was recently developed and holds great promise for furthering our understanding of DS [52]. As this is a relatively new model, however, most research has been conducted using the Ts65Dn segmental trisomic mouse [53–55] which is trisomic for more than 50% genes of Hsa21 homologs [56, 57] and has well-documented DS-like deficits in behavioral tasks such as those relying upon declarative memory (novel object recognition and spontaneous alternation tasks) and the proper encoding and recollection of spatial information (radial arm and Morris water mazes) [58–63]. While the Ts65Dn mouse is the only mouse model of DS to have a freely segregating supernumerary chromosome, they are also trisomic for 60 genes that do not have Hsa21 homologs [64], and the impact

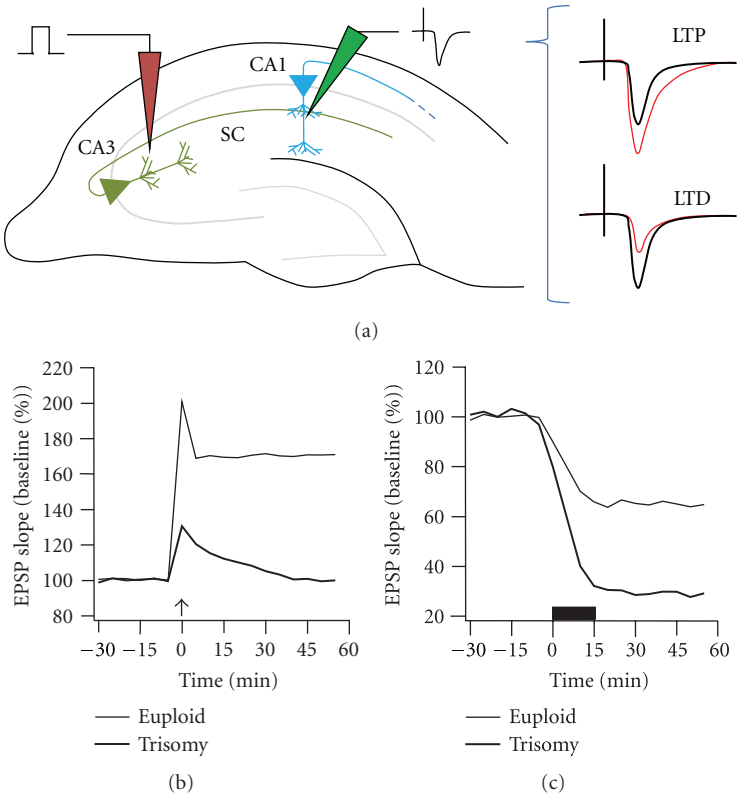


Figure 1: Depiction of altered CA1 hippocampal plasticity in Ts65Dn mice. (a) Diagram indicating electrode placement for stimulating Schaffer collaterals arising from CA3 and recording the evoked field excitatory postsynaptic potential (EPSP) in CA1. Traces to the right indicate the typical change in evoked responses (red) following LTP and LTD. (b) Simulated data depicting suppressed LTP in Ts65Dn mice. After high-frequency stimulation of SC (at arrow head), the field EPSP increases and remains enhanced in euploid mice but fails to remain elevated in Ts65Dn mice. (c) Simulated LTD data depicting exaggerated depression of evoked EPSPs following low-frequency stimulation of SC in Ts65Dn mice. (Traces in B and C based on data from [44, 45].)

of overexpression of these genes on Ts65Dn phenotypes remains to be determined.

Similar to the Ts65Dn mouse but with smaller triplicated Mmu16 segments are the Ts1Cje and Ts1Rhr mouse models. These mice display phenotypes similar to Ts65Dn mice including hippocampal dysfunction; however, the severity of the deficits is reduced [65–69]. The reduced severity of DS-like deficits in mice with fewer trisomic genes highlights one of the powerful aspects of mouse models: the ability to control expression of certain HSA21 homologs to assess their contribution to specific DS phenotypes. Those deficits associated with the hippocampus, whose function is notably altered in DS individuals [17, 39], will be the focus of the remainder of this paper.

3.1. Morphological Changes. Mouse models of DS, including the Ts65Dn strain, show similar detrimental changes in neuronal and dendritic morphologies observed in humans. The neocortex of Ts65Dn mice contains fewer excitatory neurons but an increased number of a subset of inhibitory neurons relative to euploid controls, a phenotype that was reversed by normalizing the expression levels of *Olig1/2* [70]. Additionally, regions both in the neocortex and hippocampus have decreased spine densities with larger spine volumes [71]. In the dentate granule cells of the hippocampus, there is a shift of inhibitory synaptic connections away from the dendritic shafts and onto the necks [71]. Such a change would be expected to increase the efficacy of inhibitory synaptic transmission given the significantly reduced volume of the spine neck compared to the shaft. At a finer resolution, symmetric (presumed inhibitory synapses) have greater opposition lengths in Ts65Dn while asymmetric synapses were unaltered [72], again supporting a shift towards excess inhibition in these mice. Similar but less severe changes are observed in Ts1Cje mice [67]. Beyond suppressing excitatory synaptic activity, the altered spine morphology and shift towards excess inhibition in trisomy would be expected to suppress plasticity-related signaling cascades that frequently rely on depolarization-mediated calcium influx into the postsynaptic structural domains.

3.2. Functional Changes. Synaptic plasticity in the hippocampus is often investigated in the context of long-term potentiation (LTP) in which high-frequency activation of specific inputs in the hippocampus results in a long-lasting potentiation of synaptic responses along the excited afferent pathway. First described in the anesthetized rabbit [73], this phenomenon is believed to be a fundamental mechanism underlying memory formation [8, 74] and is suppressed in Ts65Dn (depicted in Figure 1 for the CA1 region of the

hippocampus) [44, 75, 76] and Ts1Cje [66, 67] but not in Ts1Rhr mice [69] (however, see [68]) as well as mice trisomic for Hsa21 syntenic regions of Mmu16 and Mmu17 [77] or those carrying an almost complete copy of Hsa21 [46, 48].

As outlined above, structural changes suggest that inhibition is exaggerated in the trisomic hippocampus. Consistent with this idea is the observation that LTP in the dentate gyrus and CA1 regions of Ts65Dn hippocampal slices, induced by high-frequency stimulation and theta burst protocols, respectively, can be rescued by the $GABA_A$ antagonists picrotoxin [75, 76]. Blockade of $GABA_A$ receptors in hippocampal slices from Ts1Cje and Ts1Rhr mice rescues LTP deficits in the dentate gyrus in these DS mouse models as well [67, 68]. A similar treatment in Ts65Dn mice leads to an enhancement in cognitive performance [60].

In addition to suppressed LTP, hippocampi from Ts65Dn mice show enhanced long-term depression (LTD) in response to sustained activation of excitatory synapses [45, 78]. This latter effect can be reversed with the uncompetitive NMDA receptor antagonist memantine [78] and also improves the cognitive performance of Ts65Dn mice [79–81]. These results draw a clear link between altered synaptic plasticity in the hippocampus and cognitive performance in the Ts65Dn mouse model of Down syndrome.

3.3. Synaptic-Plasticity-Related Signaling Cascades. Changes in intracellular calcium concentrations are important triggers for many intracellular signaling cascades including those underlying LTP and LTD [82]. For example, the presence of a calcium chelator that buffers intracellular calcium levels in postsynaptic neurons prevents the induction of LTP [83] consistent with the hypothesis that a postsynaptic rise in intracellular calcium levels is necessary for LTP [8]. When strongly depolarized, the magnesium block of NMDA channels is lifted providing the main (but not exclusive) mechanism for calcium entry into the postsynaptic cell. Elevated intracellular calcium levels trigger a cascade of intracellular messengers that ultimately lead to the induction and maintenance of synaptic plasticity (both LTP and LTD depending on the kinetics). An excellent overview of this process can be found in several reviews [82, 84], and only key components known to be affected by trisomy (Figure 2) will be discussed here.

3.3.1. CaMKII. Activation of postsynaptic NMDA receptors (NMDARs) concomitant with the depolarization of the postsynaptic membrane is sufficient to relieve the magnesium block of NMDA channels leading to an influx of calcium into the intracellular postsynaptic space. In the case of LTP, the rise of intracellular calcium leads to the activation of calcium calmodulin-dependent protein kinase II (CaMKII), a necessary step for initiating NMDAR-dependent LTP [82]. Blocking CaMKII prevents induction of LTP, [82, 85, 86], while constitutively active forms can induce LTP [87]. During all phases of LTP (induction, early, and late), levels of phosphorylated CaMKII are increased in the hippocampus [88]. CaMKII phosphorylated at threonine 286 (Thr286) can become constitutively active providing a potential switch for initiating and then maintaining potentiation [89]. Alternatively, phosphorylation of Thr305/306 can inhibit the expression of LTP by interfering with the binding of calcium/calmodulin [90, 91]. Indeed, cognitive deficits associated with Angelman syndrome were reversed in a mouse model of the disorder by reducing the levels of CaMKII phosphorylated at Thr305/306 [92]. Thus, depending on the site of phosphorylation, CaMKII can facilitate or suppress initiation and maintenance of LTP. In Ts65Dn mice, we found that baseline levels of CaMKII phosphorylated at Thr286 are elevated in the hippocampus [93]. Excessive basal phosphorylation of the CaMKII site leading to constitutive activation could leave the DS modeling trisomic network in a saturated state unable to shift to more potentiated levels.

One of the substrates targeted by CaMKII during the initial expression of LTP is the serine 831 residue on GluR1 subunits of AMPA receptors [94, 95]. This phosphorylation leads to an increase in conductance of the AMPA channel [96] providing a rapid mechanism for enhancing glutamatergic synaptic strength. In Ts65Dn mice, we find that baseline levels of phosphorylated serine 831 in synaptically located GluR1 receptors are elevated [93]. This apparent increase in AMPA channel conductance appears not to have any significant effect on baseline excitatory synaptic transmission which is normal in the Ts65Dn hippocampus [45, 75, 76, 93]. However, it could also partially occlude the initiation of LTP in these mice by leaving Ts65Dn hippocampal excitatory synapses with fewer AMPA channels available for potentiation. This finding would be consistent with our observation of increased CaMKII in Ts65Dn hippocampus noted above [93] and the suggestion that some components of the LTP network are in an apparent saturated state in these mice.

3.4. PKA, RCAN1, Calcineurin. Protein kinase A (PKA) also plays a critical role in establishing LTP. In particular, evidence suggests that it is involved in initiating the protein synthesis required for the late phase of LTP [97, 98]. Blocking PKA activity suppresses the late phase of LTP (lasting beyond 3 hours) while leaving the early phase of LTP (less than 3 hours) unaffected [99, 100]. Transgenic mice in which PKA activity is reduced have significantly decreased late-phase LTP in CA1 but normal early LTP and perform poorly on tasks requiring long- but not short-term memory formation [101].

PKA plays a role in LTD where its substrates, such as GluR1, show increased dephosphorylation following induction [102, 103]. Dephosphorylation of GluR1 subunits should reduce the conductance levels of affected AMPA receptors [96] resulting in a reduction of synaptic strength. PKA also enhances the activity of RCAN1 [104], an inhibitor of calcineurin which contributes to AMPA receptor internalization [105] and reductions in NMDA receptor mean open time [106]. In Ts65Dn mice, we found that PKA activity is reduced in the hippocampus [93], which should adversely affect LTP by reducing protein expression required for the late phase. With respect to LTD, reduced PKA activity would result in more AMPA receptors remaining in a high

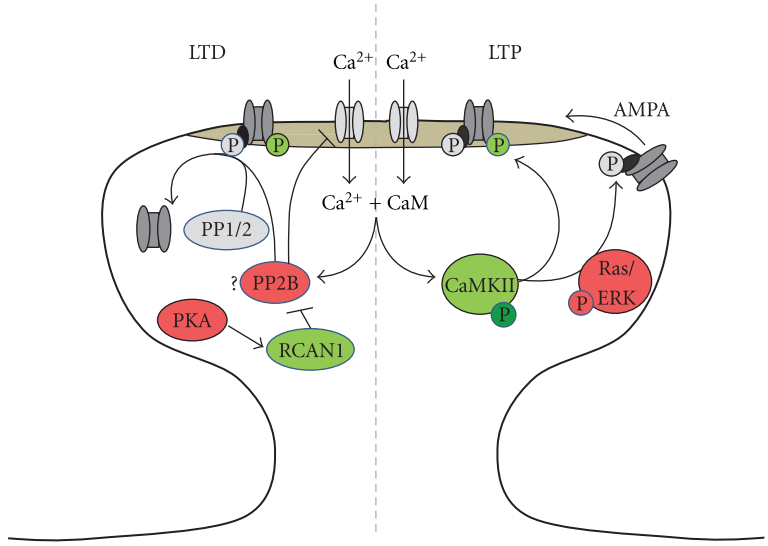


Figure 2: Alterations in intracellular signaling cascades affecting postsynaptic AMPAR response in Ts65Dn hippocampus. Green indicates elevated levels/activity at baseline, while red indicated diminished activity. During LTP (right), enhanced CaMKII and GluR1 subunit phosphorylation in Ts65Dn synapses may result in a saturated condition incapable of additional potentiation. Reduced ERK activity may reduce migration of new AMPARs into the PSD. In LTD, overexpression of RCAN1 should reduce the activity of PP2B (calcineurin) resulting in reduced internalization of AMPA receptors and potential reduction of NMDAR mean open times. Rescue of LTD in Ts65Dn mice by NMDAR antagonists suggests enhanced NMDAR activity contributes to altered LTD through yet unidentified mechanisms.

conductance state and less facilitation of RCAN1 activity. This latter effect is offset, however, by the overexpression of the gene encoding RCAN1 in DS and Ts65Dn mice [4]. How these factors contribute to the enhancement of LTD in Ts65Dn hippocampi [45, 78] remains to be determined.

3.5. Extracellular Receptor Kinase (ERK). In addition to GluR1 subunits, both CaMKII and PKA converge on another common effector, the mitogen-activated protein kinase (MAPK/ERK), that is associated with a host of synaptic-plasticity-related cellular processes [107]. In the case of hippocampal LTP, there is a rapid increase in the amount of phosphorylated ERK following induction [108] and blocking ERK activation prevents expression of LTP [109]. Cultured hippocampal neurons undergo phosphorylated ERK-dependent spine generation following LTP conditioning stimuli implicating this pathway in spine formation [110]. Additionally, it is believed that lateral diffusion of extrasynaptic AMPA receptors containing GluR1 subunits into the postsynaptic density (PSD) is a major contributor to LTP expression [111]. This process is assisted by Ras/Erk phosphorylation of stargazin on extrasynaptic AMPA receptors enabling them to be structurally secured at the synapse to PSD95 [112]. In the Ts65Dn hippocampus, ERK phosphorylation is decreased [93] suggesting decreased activity. This would be expected to adversely affect the insertion of new AMPA receptors into the PSD as well as morphological restructuring of synaptic spines observed after LTP in normal mice [113, 114].

3.6. BDNF Pathway. Brain-derived neurotrophic factor (BDNF) contributes to LTP by stimulating protein synthesis. In activating postsynaptic TrkB receptors, BDNF stimulates the PI3K pathway [115] which can initiate translation through mammalian target of rapamycin (mTOR) thereby enhancing synthesis of proteins such as CaMKIIα, GluR1, and NMDA receptor subunit 1 [116]. In Ts65Dn mice, we found that PI3K phosphorylation failed to increase following an LTP induction protocol suggesting this pathway is perturbed by trisomy [93]. Consistent with this notion, in DS individuals, BDNF blood plasma levels are approximately 5 times higher than in age-matched controls [117]. As BDNF readily crosses the blood-brain barrier [118], these levels likely reflect those present in the CNS as well.

Examination of BDNF levels in DS mouse models presents a complex picture. In Ts65Dn mice, levels of BDNF in the frontal cortex are diminished [119]. In the hippocampus, both no difference [81] and a reduction [120] compared to control have been reported. In the latter case, the reduction in BDNF levels was associated with decreased neurogenesis and was reversible through treatment with fluoxetine [120]. In the Ts1Cje mouse model of DS, BDNF is overexpressed in the hippocampus, particularly in the dentate gyrus and CA1 regions and in the dendrites of dissociated hippocampal neurons grown in culture [121]. Increased BDNF levels in Ts1Cje mice hippocampi were associated with greater levels of phosphorylated Akt-mTOR and expression of GluR1 protein which could not be further enhanced with exogenous supplemental BDNF suggesting this pathway related to synaptic plasticity is saturated in these mice preventing further contributions to LTP [121].

The discrepancies between observations in Ts65Dn and Ts1Cje BDNF levels may reflect how BDNF expression is distributed in these structures, elevated in some subregions

or subcellular compartments while diminished in others, resulting in an increased functional effect despite reduced global levels. Conversely, the differences in observed BDNF levels could be related to the different numbers of genes overexpressed in these two mouse lines [53, 65, 122] or, as mice of differing age groups were used in the studies, may reflect differences in expression levels as a function of age. Further investigation is necessary to fully align these observations. However, the observation that rapamycin has a restorative effect on phosphorylated Akt-mTOR levels in Ts1Cje suggests a potential therapeutic mechanism for improving cognition in DS individuals [121] possibly by normalizing a pathway involved in synaptic plasticity.

3.7. $GABA_B$-GIRK2 Attenuation of Synaptic Plasticity. As mentioned above, postsynaptic calcium influx is critical for LTP and LTD in the hippocampus. This initiating step relies heavily upon depolarization of the postsynaptic membrane to relieve the voltage-dependent magnesium block of NMDA channels. Any phenomenon that reduces the ability of the postsynaptic membrane to depolarize would thus be expected to adversely affect plasticity. Through its coupling to $GABA_B$ receptors, the type 2 G-protein-activated inward rectifying potassium (GIRK2) channel may act to dampen the expression of LTP in Ts65Dn hippocampus through a shunting mechanism.

GIRK2 is encoded by the gene *Kcnj6* which is located on the chromosomal segment triplicated in DS and Ts65Dn mice, and, consequently, elevated expression levels have been found in the Ts65Dn hippocampus [50, 123]. At a cellular level, overexpression of GIRK2 leads to a more hyperpolarized resting potential in cultured hippocampal neurons [124] and CA1 pyramidal neurons *in vitro* [125]. Selectively reducing the expression level of GIRK2 by crossing euploid and Ts65Dn mice with mice heterozygous for GIRK2 ($GIRK2^{+/-}$) resulted in a gene dosage-dependent change in the resting membrane potential and facilitation of LTP in GIRK2 knockout mice [50]. Selective overexpression of GIRK2 alone in mice results in cognitive deficits, reduced depotentiation (a functional reversal of potentiation at a synapse), and enhanced LTD [126].

These effects on LTP and LTD could be mediated through $GABA_B$ receptors which, in pyramidal neurons, are in closest proximity to GIRK2-contaning potassium channels near glutamatergic synapses on dendritic spines [127]. $GABA_B$ receptors are functionally linked to GIRK channels, and, indeed, whole-cell $GABA_B$-mediated potassium currents are exaggerated in Ts65Dn hippocampal neurons [50, 124, 128]. In CA1, these exaggerated currents have a greater functional impact on the distal dendrites of pyramidal neurons as opposed to those located more proximally [128]. A similar enhancement of $GABA_B$-mediated currents is also found in the dentate gyrus where the presynaptic release probability of GABA is increased [129]. Thus, GIRK channels, activated by $GABA_B$ and other G-protein coupled receptors, appear to act as a break on synaptic plasticity in the Ts65Dn hippocampus.

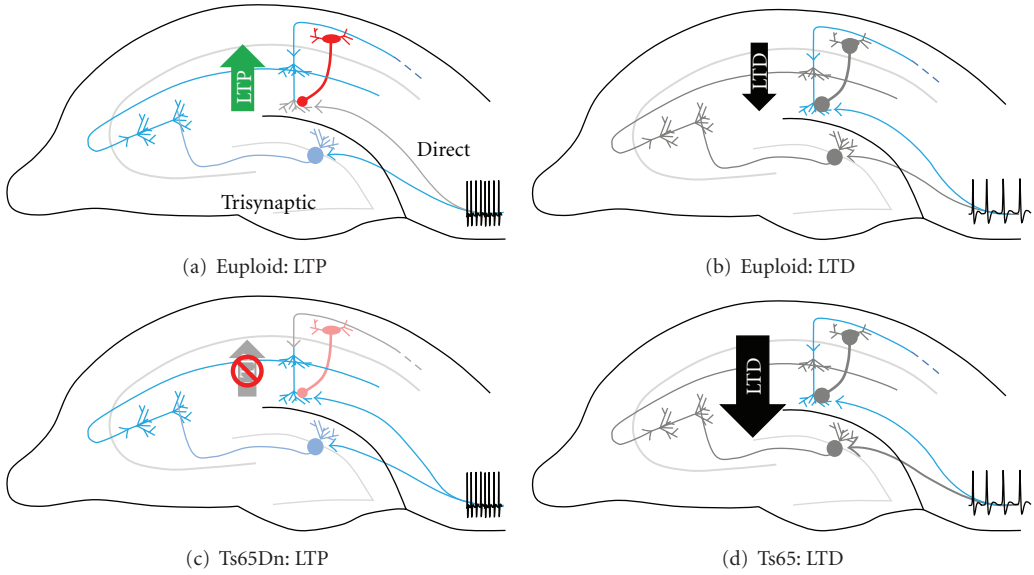
4. Potential Impact of Altered Plasticity on Hippocampal Processing

The hippocampus receives major inputs from the entorhinal cortex (EC) which converge on CA1 pyramidal through two main pathways: the perforant pathway (PP) and the temporoammonic (TA) pathway [130]. The PP pathway passes through the dentate gyrus to pyramidal neurons in CA3 before impinging upon the relatively proximal dendrites of CA1 pyramidal neurons in stratum radiatum (SR). Conversely, inputs to CA1 from TA target the distal dendrites located in stratum lacunosum molecular (SLM). In the normal hippocampus, frequency-based synaptic plasticity at the CA3-CA1 synapse, coupled with a feed-forward inhibition loop from stratum oriens alveus interneurons that suppress inputs to distal CA1 dendrites, enables segregation of information flow through these two pathways [131]. During high-frequency synaptic activity, the CA3-CA1 synapse would be expected to undergo LTP, increasing the excitatory drive to CA1 pyramidal neurons and, consequently, enhanced suppression of inputs to distal CA1 dendrites by the feed-forward inhibition loop (Figure 3(a)). Thus, TA inputs that target distal CA1 dendrites would be suppressed, and information flows through the CA3-CA1 pathway enhanced during during high-frequency events. Conversely, during low-frequency synaptic activity, the CA3-CA1 synapse would be expected to undergo LTD and become less effective. Inhibition of distal CA1 synapses would then be decreased and information flow through the TA pathway would likely be enhanced (Figure 3(b)). Diminished LTP resulting from trisomy would then interfere with this frequency-based segregation of information flow through the hippocampus. Without LTP, feed-forward inhibition would cause suppression of information flow through the TA pathway causing inputs from the two pathways to become superimposed upon and interfere with each other (Figure 3(c)). In contrast, the flow of information during low frequency signaling would likely remain intact, or potentially facilitated, since enhanced LTD at CA3-CA1 would prevent interference from this pathway (Figure 3(d)).

Electroencephalogram (EEG) recordings from DS individuals suggest that such a preferential suppression of high-frequency information flow may result from overexpression of Hsa21 genes. Compared to controls, DS individuals have increased power at low EEG frequencies and a corresponding reduction in power at higher frequencies [132]. Similar observations have been reported in Ts65Dn mice [133]. While it is not clear that hippocampal activity is accurately reflected in EEG recordings, abnormal EEGs findings are consistent with aberrant processing of high-frequency information in DS individuals and Ts65Dn mice.

5. Cognitive Therapies Targeting Plasticity

A number of studies using the Ts65Dn mouse model of DS have examined the possibility of pharmacologically reversing cognitive deficits (reviewed in [122]). Of particular note with respect to hippocampal plasticity are those targeting the



(a) Euploid: LTP (b) Euploid: LTD

(c) Ts65Dn: LTP (d) Ts65: LTD

Figure 3: Potential impact of altered synaptic plasticity on hippocampal processing in Ts65Dn mouse model of Down syndrome. Schematic of two main pathways through hippocampus arriving from the entorhinal cortex: temporoammonic (TA)—direct to CA1 distal dendrites; trisynaptic pathway from DG through CA3 to proximal CA1 dendrites. LTP and LTD are proposed to minimize interference between the two pathways [50, 131]. (a) In euploid hippocampi, high-frequency inputs induce LTP in CA1 resulting in enhanced suppression of inputs from TA by feed-forward inhibition arising from interneurons in stratum oriens. (b) Low-frequency inputs depress the trisynaptic pathway releasing distal CA1 dendrites from feed-forward inhibition and allowing information to flow through the TA pathway. (c) In Ts65Dn hippocampi, aberrant LTP in CA1 results in diminished feed-forward inhibition during high-frequency activity allowing TA inputs to become superimposed on those flowing through the trisynaptic pathway. (d) Enhanced LTD would be expected to facilitate flow of low-frequency information through the direct TA pathway in Ts65Dn mice.

excess GABAergic inhibitory tone or NMDA receptors whose activation, as outlined above, is a critical step in initiating LTP and LTD.

Application of the $GABA_A$ receptor antagonist, picrotoxin to hippocampal slices from Ts65Dn mice rescues LTP in the dentate gyrus [75] and CA1 region [76]. Chronic administration of low doses of picrotoxin or other $GABA_A$ receptor antagonists (pentylenetetrazole or bilobalide) improves cognition in Ts65Dn mice suggesting that the efficacy of this class of pharmacological agents could be tested for reversing impaired cognition in DS [60]. However, as overinhibition of $GABA_A$ receptors can induce seizures, translating these findings to humans requires great caution. Careful screening of similar drugs or design of pharmacological compounds with similar blocking capabilities but reduced propensities for inducing seizures may prove to be effective treatments. Currently, a small molecule targeting $GABA_A$ receptors developed by F. Hoffmann-La Roche Ltd (Pharmaceutical pipeline molecule RG1662 http://www.roche.com/roche_pharma_pipeline.htm) is in clinical trials with the goal of safely improving cognition in DS individuals.

Braudeau et al. [134, 135] are currently investigating a similar promising inhibitor that targets the alpha-5 subunit of $GABA_A$ receptors.

Another pharmacological avenue targets aberrant NMDA receptor-mediated signaling apparently present in Ts65Dn mice. The uncompetitive NMDA receptor antagonist memantine improves the cognitive performance of Ts65Dn mice [79] and normalizes hippocampal LTD [78]. Memantine is an FDA approved and fairly well-tolerated drug already in use for treating dementia in Alzheimer disease. Clinical trials assessing the safety, tolerability, and efficacy in alleviating DS cognitive phenotypes are currently underway [57].

In addition to pharmacological approaches, behavioral therapies have been shown to improve cognition in Ts65Dn mice. When housed in enriched environments (larger cage with novel objects such as toys and running wheels), trisomic mice performed as well as euploid littermates in the Morris water maze and had normalized hippocampal LTP [136, 137]. Interestingly, environmental enrichment was effective for trisomic females but not trisomic males potentially due to social and physical factors associated with the new environments [138].

The benefits of environmental enrichment appear to be linked in part to regulation of excess inhibition in the neocortex and hippocampus. Release of GABA from synaptosomes isolated from the hippocampus and neocortex is elevated in Ts65Dn mice, an effect that is reversed by environmental enrichment [137]. In adult rats with amblyopia (via monocular deprivation during critical period), environmental enrichment reversed visual deficits reduced GABA levels in the visual cortex contralateral to the deprived eye while increasing plasticity [137]. It thus appears possible to regulate aberrant levels of inhibition in trisomic mice behaviorally without pharmacological intervention and achieve similar behavioral outcomes without the concerns

associated with nonspecific actions or adverse side-effects of drugs.

Deficits in neurogenesis in the dentate gyrus and forebrain subventricular zone in Ts65Dn mice are also reversed following environmental enrichment [139] and may add an additional therapeutic layer to the beneficial effect of a decrease in inhibitory tone. A structural benefit of environmental enrichment appears lacking; however, as, unlike euploid mice, this treatment has failed to significantly increase dendritic branching and spine density in Ts65Dn mice [140].

Early behavioral intervention techniques designed to improve development in DS children show great promise [141, 142] suggesting that this comparatively easily translatable therapeutic approach, either used alone or in combination with pharmacological agents, could potentially increase cognitive capacities in DS individuals.

6. Conclusion

Synaptic plasticity is believed to be the process central to learning and memory. This belief is bolstered by experiments where drugs that normalize aberrant plasticity in hippocampal slices isolated from mouse models of DS also confer improvements in cognition in in intact adult mice. The initiation and maintenance of plastic changes involve structural and compositional modifications of synapses that depend on intracellular signaling cascades. The reduced excitatory neuronal densities and deficits in dendritic morphologies present in individuals with DS diminish the capacity of their neural networks in general to undergo neuroplastic adaptations. Combined with the deficits in signaling pathways reported in Ts65Dn mice, evidence strongly suggests that synaptic plasticity is severely impaired in DS neural networks. By understanding how plasticity is perturbed, we can design therapies to reverse these phenotypes and ultimately improve cognition in DS individuals.

Acknowledgments

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Review Article

Molecular and Cellular Alterations in Down Syndrome: Toward the Identification of Targets for Therapeutics

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Down syndrome is a complex disease that has challenged molecular and cellular research for more than 50 years. Understanding the molecular bases of morphological, cellular, and functional alterations resulting from the presence of an additional complete chromosome 21 would aid in targeting specific genes and pathways for rescuing some phenotypes. Recently, progress has been made by characterization of brain alterations in mouse models of Down syndrome. This review will highlight the main molecular and cellular findings recently described for these models, particularly with respect to their relationship to Down syndrome phenotypes.

1. Introduction

Down syndrome (DS) is the most frequent human aneuploidy (1/800 births). DS is characterized, in part, by cognitive impairment, which is present to some degree of severity in all affected individuals [1], and by neuropathological alterations similar to those observed in the brains of Alzheimer's disease patients (over 40 years in DS) [2–4]. Specific deficits of the nervous system in DS individuals affect learning, memory, language, and movement [5–8]. These deficits are associated to alterations in volume, in grey matter density and altered neuronal circuits of different regions of the brain [9–13]. DS typically results from the presence of three complete copies of human chromosome 21 (trisomy 21, T21) [14]. Due to the presence of this extra copy of chromosome 21 (HSA21), DS phenotypes are expected to be associated with a gene dosage effect: genes on HSA21 are present in three copies rather than two, leading to 50% overexpression (or 1.5-fold expression levels). Transcriptome and proteome studies have shown that, indeed, a global gene dosage effect is present; however, interestingly, expression of a number of trisomic genes varies: some are compensated (near 1), while others are underexpressed (less than 1) or highly overexpressed (more than 1.5). These changes may vary depending on the cellular component and likely result from gene or protein interactions in pathways or in protein complexes (e.g., complex subunits). These variations have been observed in T21 as well as in different organs of mouse models of DS and as a result of aging [15–21]. Thus, defining which HSA21 genes (or murine orthologs) are particularly responsible for disease phenotypes is crucial: identifying the molecular and cellular variations in conjunction with overexpression will help determine their associations with the phenotype and aid in testing potential molecules for phenotypic rescue.

2. Mouse Models of DS

Mouse models have been critical to our understanding of the molecular genetics of DS. Several models have been constructed: some have an additional copy of a chromosome segment orthologous to HSA21 [22, 23], others have additional copies of individual genes from HSA21 or their mouse orthologs [24, 25]. Though more recent models have increased our understanding of the consequences of adding one copy of a specific gene or a segment containing multiple genes, the most extensively studied models are the Ts65Dn [22] and Ts1Cje [23] mice, which carry large segmental trisomies for mouse chromosome 16 (MMU16) (Figure 1). These models recapitulate several phenotypes of

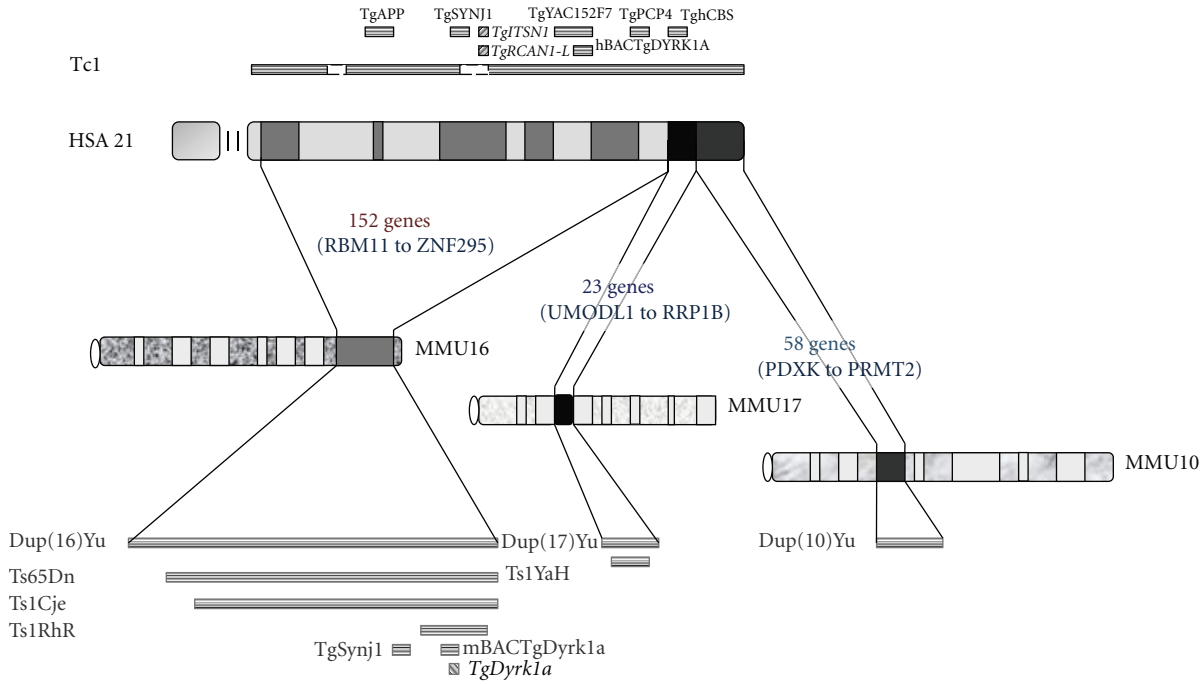

Figure 1: HSA21 (with main cytogenetic bands) and its ortholog segments in the mouse genome (MMU16, MMU17 and MMU10) are indicated. Main mouse models and those reported in this review are indicated in black for human genes, and in gray for mouse genes. Models with cDNA constructs are indicated in italics. Representation of their localisation is not to scale. Tc1 [32]; TghAPP [33]; TgSYNJ1 [54]; *TgITSN1* [55]; *TgRCAN1-L* [56]; TgYAC152F7 [34]; hBACTgDYRK1A [24]; TgPCP4 [38]; TghCBS60.4 [57]; Ts65Dn [22]; Ts1Cje [23]; Ts1RhR [58]; TgSynj1 [54]; *TgDyrk1a* [59]; mBACTgDyrk1a [25]; Dup(16)Yu, Dup(17)Yu and Dup(10)Yu [29, 31]; Ts1Yah [30].

DS, including reduced brain volume, significant learning and memory impairment, and altered synaptic plasticity measured in hippocampal long-term potentiation (LTP). However, recent investigations into the exact gene composition of these models have shown that, in addition to the duplicated segment of MMU16, Ts65Dn, which results from a translocation onto MMU17, contains a duplication for proximal genes of MMU17 and Ts1Cje contains a deletion of a 7-gene span of MMU12 [26–28]. More recent trisomic models [29–31], constructed using the Cre/loxP-mediated chromosome engineering strategy, have integrated only segments of the mouse chromosomes orthologous to HSA21—MMU16, MMU17, and MMU10 (Figure 1)—eliminating any potential confounding effects from additional genetic aberrations. Another model, Tc1, is a transchromosomic model transmitting a copy of a portion of HSA21 spanning over 75% of the original chromosome [32]. The human genes present on this chromosome are, indeed, expressed in the mouse, confirming that specific models may bear either a human or mouse gene, as previously demonstrated with YACs containing human genes [33, 34]. Interestingly, the Tc1 model may also be useful for evaluation of effects of T21 mosaicism because the transchromosome appears to be retained in only 50–60% of Tc1 adult brain cells [35]. In fact, Papavassiliou et al. [36], in studying the rate of T21+ cells in the buccal mucosa and lymphocytes of individuals with T21 mosaicism, found a positive correlation between patient IQ range and percentage of T21+ cells in their tissues. Thus, the presence of trisomy in at least 50% of brain cells may have strong implications for cognitive development.

Transgenic models introducing a specific HSA21 gene or murine ortholog, and for which molecular and cellular studies have been performed, are presented along with trisomic models in Figure 1 and Tables 1 and 2. Tables 1 and 2 summarize the main studies identifying molecular and cellular changes in these models.

3. DS Transcriptome

Gene expression studies have provided much-needed insight into global expression changes occurring in DS. In particular, microarrays have been employed to determine the transcriptome of cells and even brain structures. Ts1Cje and Ts65Dn transcriptomes were analyzed at various developmental timepoints (see Table 1). Changes in transcript levels were observed for genes in three copies, mirroring copy number (i.e., near 1.5-fold). However, more specific analyses of expression changes, as in the cerebellum of Ts65Dn [44], suggest that the genetic backgrounds of trisomic mice may impart individual variations onto expression changes. Further, such inter-individual variations are observed at the protein level [52]. Interestingly, in Tc1 mice genes from HSA21 are expressed at embryonic day (E) 14.5, indicating that these genes are transcribed during mouse embryonic development [32]. Recall that this model leads to a mosaic composition of adult organs with cells containing or not

Table 1: Significant quantitative transcript variations observed in the DS mouse models (trisomics and transgenics, see Figure 1). Results are classified from top to bottom with increasing age of the mice studied: age in embryonic days (E), postnatal days (d) and month (m). Names in bold for genes present in 2 copies. Transcriptome methods used: C (cDNA arrays); N (Northern); M (microarrays); Q (quantitative-RT-PCR); R (RT-PCR). Gene names are indicated according to gene nomenclature (Gene Cards: http://www.genecards.org/).

Brain structures	Models	Age	Up	Down	Method	Additional comment	Target	Rescue	References
E11–E13 telencephalon, mesencephalon + diencephalon	TgYAC152F7	E11.5, E12.5, E13.5	Dyrk1a		Q, M	Dysregulation of the Rest pathway			[37]
Embryonic, brain hemispheres, cerebellum	Ts1Cje; TgPCP4	E11.5, E14.5, 4 m	Pcp4		Q				[38]
Embryonic total	Tc1	E14.5	—		R	Expression human genes			[32]
Embryonic	mBACTgDyrk1a	E14.5	Dyrk1A		Q				[25]
Total brain	Ts1Cje	birth	mean: 1.435		M				[39]
Cerebellum	Ts1Cje	birth	**Ptch**, **Shh**		M				[26]
Cortex, brain	Ts65Dn	8 d	**Vip**, **Vipr1**		Q				[40]
Total brain	Ts65Dn	1 m	62% of 3-copy genes		Q				[18]
Hippocampus, frontal cortex, substantia nigra	Ts65Dn	78–92 d	Kcnj6		Q				[41]
Hippocampus	TgYAC152F7	3 m	Dyrk1a	**Bdnf**, **Trkb**	Q		Dyrk1a	Bdnf, Trkb	[42]
Hippocampus	TS65Dn	3 m	Gart, Ifnar2, Kcnj6, Itsn1, Hcls, Sod1		M		Gabra5	Bdnf	[43]
Cerebellum	Ts65Dn	3-4 m	range (0.84–2.93); mean 1.45		M				[44]
Cortex, midbrain, cerebellum	Ts65Dn	4 m	mean: 1.63, 1.3, 1.37		C, M				[17]
Forebrain	Ts65Dn	4 m	App, Sod1, **ApoE**		N				[45]
Hippocampus (rescue), prefrontal cortex	Ts65Dn	5-6 m	mir155, mir802, **Mef2c**	**Creb1**, **Mecp2**	Q		mir-155, mir-802	Mecp2, Mef2c, Creb1	[46]
Brain hemispheres	Ts65Dn	4 to 12 m	App, Sod1, Dyrk1a		Q	increase with age			[21]
Brain	Ts65Dn	6-7 m		**Gfap**	Q				[47]
Hippocampus, cortex, raphe nuclei	Ts65Dn	9.5 m	**Vip**, **Vipr1**		Q				[48]
Hippocampus	Ts65Dn	10 m		**Bdnf**	Q		Nmdar	Bdnf	[49]
Total brain	Ts65Dn	11 m	47% of 3-copy genes		Q				[18]

Table 1: Continued.

Brain structures	Models	Age	Up	Down	Method	Additional comment	Target	Rescue	References
Hippocampal CA1	Ts65Dn (m + f)	12–24 m	**Htr2c**	**Cdk5**, **Ntf3**	Q				[50]
Medial septum, hippocampus	Ts65Dn	18 m	App		Q				[51]

HSA21, detecting the molecular consequences in adults at the transcriptional level may be more difficult. Interestingly, Tc1 mice have impaired short-term memory but normal long-term memory [35]; both features are affected in Ts65Dn mice [53]. These contrasted phenotypes in adult mice suggest that part of the functional alterations in DS results from strong modifications in proliferation and/or differentiation steps of neural components of various brain structures—processes that are established during embryogenesis. Notably, however, the absence of several genes on the human chromosome transmitted in Tc1 may also influence the functionality of the adult brain (Figure 1).

More recent evidence indicates that alternative splicing may play a role in differentiating the brain transcriptome in DS mouse models as well as in DS. Proteins involved in the splicing machinery are modulated and alternative exons of key synaptic transcripts (neuroligins, TrkB, AChE, Mapt) can be expressed, suggesting a different control of the transcriptome in the disease state. Modulated splicing factors (ASF, Srp55, Srp75, Srp30, SC35) were identified at the global protein level or at the phosphorylation level depending on the brain regions explored as well as a result of aging. Notably, at least one HSA21 gene appears to be responsible for dysregulation via splicing factor phosphorylation: *Dyrk1A*. This *proline-directed serine/threoninekinase* colocalizes with some of these splicing factors and, further, regulates biogenesis of the splicing speckle compartment [60–62]. In adults with DS, *Dyrk1a* overexpression appears related to overexpression of the 3R isoform transcript of *microtubule-associated protein tau* (*Mapt*), which is predominant in neurofibrillary tangles, suggesting a new role for *Dyrk1a* in neuronal degeneration [61, 63–65].

In addition to protein-coding RNAs, several functional RNAs do not lead to the translation into a protein (noncoding RNA). MicroRNAs (miRNA) belong to the small noncoding RNAs class and have been shown acting on the regulation of translation of gene transcripts either by degradation or repression, thus influencing the content of the proteome. Mounting evidence suggests that miRNAs affect brain development and function [66]. Five miRNAs are transcribed from HSA21, three of which are clustered [46]. HSA21 miRNAs (miR-99a, let-7c, miR-125b-2, miR-155, and miR-802) are overexpressed in the DS brain from fetal to adult stages [46, 67, 68]. In the Ts65Dn mouse model, only miR-155 and miR-802 (both in 3 copies) have been found to be overexpressed in brain [69]. The authors found also that the transcription of the *methyl-CpG-binding-protein* (*Mecp2*) gene, which is mutated in Rett syndrome, is decreased. Intracerebroventricular injection of Ts65Dn with antisense RNA for these two miRNAs (antagomirs) normalizes the expression of *Mecp2* and *Creb* (*cyclic AMP responsive element binding protein*)as well as the Mecp2-regulated gene *Mef2c* (increased in Ts65Dn) [69]. Other possible involvement of miRNAs in brain alterations of DS and mouse models require further investigation [70].

4. DS Proteome

Alterations in the transcriptome in DS is expected to have direct implications on the proteome. The brain proteome has been studied using different quantification methods, but its modulations are more difficult to approach on a large scale. Quantitative immunohistochemistry is complementary to these approaches, since it can reveal which cells may be more affected by protein expression changes. Indeed, it is necessary to determine whether any fluctuations in protein expression result from changes at the cellular level or changes in the proportion of cells expressing the protein(s). Current research targeting potential pathways have led to an increase in studies identifying the proteome changes within specific brain structures in DS models.

Table 2 recapitulates significant protein changes (up or down) observed in the trisomic and transgenic mice in function of age and brain structures. Interestingly, these data show that the proteins level even in the same mouse model may increase with age (App and Sod1—which are in 3 copies), may depend on the brain structures (Synaptophysin (Syp) up in cortex versus down in hippocampus; Gaba-b receptor 2 (Gabbr2) up in hippocampus versus down in thalamus) or may be increased from early stages to adult (Map2, Ntf3), though all developmental stages are not yet studied.

5. Morphological and Cellular Changes in Brain Structures

The universal presence of cognitive impairment in DS has made understanding the structural and cellular changes in the DS brain the focus of much research effort. Reduction in cerebellum volume is a feature of Down syndrome and is recapitulated in Ts65Dn and to a lesser extent in Ts1Cje models. Interestingly, changes in volume or cellular density appear to differ between regions of the brain, suggesting that gene dosage differentially affects brain structure development [9, 10, 89, 90]. Similarly, enlargement of the lateral ventricle, another alteration in brain morphology, has been observed in both DS and mouse models of DS, specifically Ts1Cje, Ts2Cje [91], Ts65Dn, and mBACTg*Dyrk1a* [25].

Table 2: Molecular changes observed in DS mouse models: Proteome.

Brain structures	Models	Age	Up	Down	Method	Additional comment	Treatment	Target	Rescue	References
Embryo E11; E14 SNC	Ts1Cje; TgPCP4	E11; E14	Pcp4, **Tubb3**, **Map2c**; **Calb2**		W, I					[38]
Neonatal brain	Ts65Dn	P0	**Map2**, **Ntf3**	**Syp**	W, I					[71]
Cortex	mBACTgDyrk1a	P0	Dyrk1a, **Gap43**		W					[25]
Hippocampus	Ts65Dn	P2	**Ptch1**		I					[72]
Hippocampus	Ts65Dn	P25	Kcnj6	**Gabbr2**	W					[73]
Medial septum	Ts65Dn	P2–20 m		**p75Ngfr** (6 m)	I					[45]
Thalamus, medulla oblongata	Ts65Dn	1 m	**Gabbr2**		W					[74]
Hippocampus	Ts65Dn	15–45 d		neuron, glia	I		fluoxetine		neurogenesis	[75]
Brain	Ts65Dn	49–66 d		**KIF17**	W	involv. NR2B transport				[76]
Hippocampus	Ts65Dn	2–4 m	**P(CaMKIIa, AKT)**, **Glur1**, **p(Ser831)-Glur1**	**pERK**	W					[77]
Hippocampus, frontal cortex	Ts65Dn	80 d	Kcnj6, **Kcnj3**		W					[41]
Brain	Ts65Dn	3–5 m	App, Synj1		W					[54]
Brain	TgSYNJ1	3–5 m	Synj1		W					[54]
Cortex	mBACTgDyrk1a	3 m	Dyrk1A, **Ccnd1**, **Syp**, **Map2**		W					[25]
Hippocampus	Ts65Dn	1 m, 4 m, 12 m	App (12 m)		W		RS86 (agonist)	Chrm1	App increase (12 m) in Ts and 2N	[78]
Basal forebrain, hippocampus, paraventricular nucleus	Ts65Dn	3 m		**Nos1** in MSN, DB, PVN	I					[79]
Brain	Ts65Dn, TgSYNJ1	3–5 m	**PtdInsP**	**Ptdlns(4,5)P2**	E		Synj1 gene copy		yes	[54]
Hippocampus	Ts65Dn	4 m	**Ntf3**, **Cdk5**	**Syp**	W					[71]
Brain, hippocampus, cortex, striatum	Ts65Dn	4–12 m	App, Sod1, Dyrk1A, sAPP-alpha and -beta (12 m)		W					[21]
Brain hemispheres, cerebellum	Ts1Cje; TgPCP4	4 m	Pcp4		W, I					[38]
Somatosensory cortex	Ts65Dn	4-5 m	**Syp**, **Gad67**, **Calb**, **Calb2**, **Parv**		I					[80]
Brain hemispheres	Ts65Dn	5–12 m	increase with age: App, Sod1		W					[21]

Table 2: Continued.

Brain structures	Models	Age	Up	Down	Method	Additional comment	Treatment	Target	Rescue	References
Hippocampus (rescue), prefrontal cortex	Ts65Dn	5-6 m	**Mef2c**	**Creb1**, **Mecp2**	W					[46]
BFCN, hippocampus	Ts65Dn, Ts1Cje	6 m, 12 m	App, **Vchat hipp. termini**	**Ngf transport**	W, I	App copy numb. dep.				[81]
Hippocampus	Ts65Dn	7-8 m	Tiam1, Dyrk1a		W		MK801 ip	Nmdar	no	[52]
Cortex	Ts65Dn	7-8 m	Tiam1, Itsn1, Dyrk1A, **p(AKT, ERK, GSK3b)**		W		MK801 ip	Nmdar	Dyrk1a, **pERK1, 2**	[52]
Hippocampus	Ts1Cje	7-8 m	Dyrk1a; **pERK1,2**		W		MK801 ip	Nmdar	Dyrk1a; **pERK1,2**	[52]
Cortex	Ts1Cje	7-8 m	Itsn1, Dyrk1a, **p(AKT, ERK, GSK3b**)		W		MK801 ip	Nmdar	Dyrk1a	[52]
Medial septum, hippocampus	Ts65Dn (m + f)	7–18 m	**microglia (CD45+)**	**Calb1 (hippo)**	I		minocycline (7–10 m)	inflammation	CD45, Calb1	[82]
Hippocampus, medial septum, locus coerelus	Ts65Dn	10 m		**Calb1 (H)**, **Chat (MS)**, **Th (LC)**	I		memantine (4–10 m)	Nmdar	no	[49]
Hippocampus, olfactory bulb, frontal cortex, cerebellum	Ts65Dn	10–19 m	**Chat (10m, all ages in cerebellum)**, **Glul (19m)**	**Chat in medial septum**, **AChe**	A					[83]
Medial septum BFCN	Ts65Dn	12 m		**p75Ngfr**	I					[84]
Hippocampus	Ts65Dn	12 m	**Chat**		A					[78]
Medial septum	Ts65Dn	12 m		**Chat**	I					[78]
Cerebellum	Ts65Dn	10–12 m	**Gfap**, **Sap/Jnk activation**		I, W	axonal damage				[85, 86]
Hippocampus, cingulate cortex	Ts65Dn/Ts1Cje	12–15 m	**Map2/ no**		I					[87]
Medial septum	Ts65Dn	18 m		**Chat**	I		Ngf infusion	Ngf transport	number and size	[84]
Fronto-parietal cortex, hippocampus	Ts65Dn	19 m	**Gfap**		W					[83]
Hippocampus, frontal cortex	TghAPP	24 m	Abeta 42, **UPS**	**alpha-**, **beta-secretase**, **Ngf**	E		RS86 (agonist)	Chrm1	Ngf, Abeta 42 increase (no)	[88]

Cell proliferation is also altered in DS and in mouse models, suggesting a relationship between alterations in volume and altered cell numbers in brain structures. In cortex, hippocampus, and cerebellum, region volume and neuronal populations are affected [58, 92–96]. These defects in proliferation alter the neuron as well as the astrocyte number and percentage. Recently, proliferation impairment in neural cell precursors of Ts65Dn was shown to involve inhibition of the hedgehog pathway [72]. This finding extends those of Roper et al. [97], who linked hedgehog to decreased granular cell progenitor (GCP) production in the cerebellum of Ts65Dn. Sonic hedgehog (*Shh*), produced by the cerebellar Purkinje cell, typically activates GCP proliferation during cerebellar development, but this pathway is defective in DS models. Similarly, a defect in *Shh* mitotic response is present in neural crest progenitors of these mice [98]. Inhibition of the hedgehog pathway can occur through overexpression of a fragment of amyloid precursor protein (*App*, in 3 copies in the Ts65Dn), AICD (App intracellular domain). Through increased binding of AICD to the *Ptch1* (*Patched*, SHH *receptor*) promoter and histone hyperacetylation, *Ptch1* is overexpressed [72]. However, silencing of *Ptch1* restores proliferation of neural cell precursors. Indeed, AICD has been shown to act as a transcriptional regulator for its own gene (*App*) as well as other genes [99]. Reduced cerebellar volume also occurs in Ts1Cje mice (2 copies of *App*), but to a lesser extent than in Ts65Dn (3 copies of *App*), suggesting that other 3-copy genes contribute to the proliferation defect through the Shh receptor *Ptch1* [26] or other molecules.

Notably, these proliferation defects may be associated with the surprising lack of medulloblastoma and neuroblastoma tumors observed in Down syndrome [100–102]. In DS models, several genes involved in the regulation of the cell cycle, namely, cell-cycle-dependent kinases *p21Cip1* [103] and *p27Kip1* [104, 105], are differently affected and induce a dysregulation of the cell cycle. These proteins as well as *Ptch1*, the receptor for Shh [72], have been shown to be important players in medulloblastoma induction [106]. Thus, the alteration in neural proliferation, while likely contributing to cognitive impairment in DS, may protect against these type of tumors. Additionally, increased *Dyrk1a* [107] and *Pcp4* [38, 108, 109] expression are associated with premature neuronal differentiation at early embryonic stages, which may also guard against these tumors by driving neurons to a more mature state.

Interestingly, increased dosage of murine *Dyrk1a* leads to an increase in neurons and glial cells in the thalamus VPL-VPN while other structures, like the somatosensory cortex, though increased in volume, do not show any change in the numbers of these cellular components [25]. Thus, proliferation may be differentially affected in particular regions and cell types during development, as has been visualized in the DS brain [10, 110].

Adult neurogenesis occurs at two major sites in the brain: the subventricular zone of the lateral ventricule and the subgranular zone of the dentate gyrus of the hippocampus (human and mouse). Though the physiological relevance of adult neurogenesis is still under debate, it may have strong implication in new acquisition of memory. Adult neurogenesis is impaired in Ts65Dn hippocampus [111] and can be reversed by treatment with fluoxetine, an inhibitor of serotonin (5-HT) reuptake [112]. Recent experiments using the same molecule rescued neurogenesis in Ts65Dn not only in hippocampus but also other structures (striatum, neocortex) and involved the rescue of expression of the neurotrophic factor BDNF [75], which is crucial for neuron survival. Indeed, BDNF levels (RNA and protein) depict a complex situation in DS that may result partly from a newly identified mechanism acting in brains of DS models: regulation of local translation [113]. BDNF RNA levels are decreased in DS and mouse models, but circulating levels of BDNF are higher in DS [42, 114, 115]. In Ts1Cje, increased BDNF release in the hippocampus occurs through different regulators of synaptic local translation, suggesting a more fine-tuned regulation of this neurotrophic factor. Further, the new hypothesis proposed by Troca-Marín et al. [113] of a positive-feedback loop involving BDNF and the Akt-mTOR pathway suggests new avenues for treatment. This type of regulation may involve other molecules important for brain function, as has already been shown for *Dscam* [116]—which occurs in 3 copies in the mouse models—and still needs to be explored.

Other molecules and pathways contributing to DS neuropathology have been extensively studied. For example, Map2, a microtubule-associated protein present in the soma and dendrites of mature neurons, is increased in hippocampus and cingulate cortex of Ts65Dn, independent of age [71, 87]. Map2 immunolabeling reveals thicker, shorter, and less-tapered dendrites in aged Ts65Dn adult neurons. Further, during embryonic cell differentiation in culture, abnormal neurite branching was observed in neurons of fetal T21 [117] and Tc1 [118], combined with an increase in secondary to primary dendrites. Abnormal dendrites have been previously observed during early development in DS cortex; the overdevelopment of dendritic trees in the visual cortex of DS patients at birth, despite dendritic atrophy later during infancy [119, 120], suggests that temporally different mechanisms may contribute to abnormal maturation of neurons in DS. Though different 3-copy genes might contribute to these changing phenotypes [38, 59, 121], the mechanisms of altered cytoskeletal dynamics remain unexplained.

Another neuronal phenotype in DS is the excitation-inhibition imbalance shown to play a central role in brain malfunction; reducing overinhibition represents a current goal for ameliorating cognitive dysfunction [122, 123]. Overinhibition may result from an increase in inhibitory neurons [80, 95], an increase in inhibitory synapses [124, 125], an increase in efficiency of inhibitory synapses [126], an increase in stimulation of GABAergic ouput neurons [127], or a decrease in these excitatory components [128]. Moreover, in relation to Girk2 overexpression (*Kcnj6* in 3 copies) which regulates the GABA-B receptor at dendrites, the balance between GABA-B and GABA-A inhibition is altered in Ts65Dn hippocampus [41, 73, 129]. In Ts65Dn cortex, excitatory neurons exist in the same proportions in control and Ts65Dn brains throughout development; interneurons, however, are increased in Ts65Dn brains. Further, these interneurons show an increased excitability in basal

conditions [95]. Reducing copy numbers of *Olig1* and *Olig2* transcription factors required for oligodendrocyte specification and differentiation [130], rescues the number of cortical interneurons of Ts65Dn [95]. Finally, additional circuitries of neurotransmitter release as well as neuropeptide signaling are impaired ([54, 79, 131, 132]; Table 2).

Though the global composition of Ts65Dn synapses does not differ from controls reduced CaMKIIalpha and increased peptide phosphorylation, potentially important for synaptic function, have been found; synaptojanin 1 (Synj1), which is important for synaptic vesicle recovery and is triplicated in Ts65Dn, is also increased [133]. Additionally, spine morphology and spine density differ [134, 135], but the global level of synaptophysin, a marker of presynaptic vesicles, appears reduced [71]. Decreased spine density has been observed in Ts65Dn hippocampus and temporal cortex [135–137]. Further, synapse enlargement is present in hippocampus, with an associated decreased length of spine neck [135]. Similarities are evident in spine morphology between Ts1RhR [58], Ts1Cje, and Ts65Dn, but with increased severity of phenotype with increased number of genes in 3 copies [134, 138]. Moreover, the trisomy in Ts1RhR is sufficient to induce a decreased average in spine density in the fascia dentata [138]. Finally, endocytosis may be altered by increased levels of Itsn1 [55, 139], *Dyrk1A* [140], Synj1 [141], and interaction with other genes in 3 copies [142]. Together, these anomalies may lead to altered synaptic plasticity, as visualized at the level of hippocampal LTP, and likely regulate learning processes.

Glial cells are another structurally and functionally important component of the brain, serving as support and as regulators of synapse connectivity; they are also present at the blood-brain barrier. Glial fibrillar acidic protein (GFAP) is commonly used to identify these cells. During early development in DS hippocampus and frontal lobe, an increase in GFAP-positive cells is observed [143, 144], together with a more mature morphology [144]. This may result from a preference for glial cell production over neuron production, as seen during the differentiation of neural precursor cells [117, 145–148]. An increase in glial cells has been identified in the Ts65Dn hippocampus during early postnatal development [149]. However, in adult Ts65Dn brain, a decrease in GFAP transcript was observed [47]. Moreover, dysfunction of Ts65Dn astrocytes [40] coupled with an increase in beta-catenin in the microvessels of Ts65Dn brain [150, 151], two important components of the brain-blood-barrier, suggest that its function might be altered.

Interestingly, in aged DS brains, a reduced glial cell number has been observed in the cortex [152], and alterations in the morphology of astroglial cells develops with age [153]. Further, increased GFAP in the frontoparietal cortex and hippocampus of aged Ts65Dn mice revealed gliosis [83]. Thus, altered glia may play a role in the modified functionality of brains of DS mouse models. Notably, alterations in Purkinje axons in the cerebella of Ts65Dn have been observed from 10 months of age, while astrogliosis appears later [85, 86]. These results suggest that the Ts65Dn cerebellum is not protected against neuronal degeneration, which may be detected earlier by specific modifications of neuronal properties.

Finally, identification of aging processes related to Alzheimer's disease pathology are under investigation in DS models. APP has been suspected as a major player in this pathology and increased copy number of *APP* in human is associated with Alzheimer's disease [154]. Other genes on HSA21 may either protect against or enhance the effects of the increase in APP [21, 64, 65]. Aged Tc1 mice (18 months) have an increase in tau phosphorylation and neurofibrillary tangles, features not present in young animals. Further, a correlation with the level of Dyrk1A was found, but only in aged mice [65]. In this model, human proteins like APP, SYN1, ITSN1, and RCAN1 may be absent, suggesting they do not play a role in that process [35]. Transgenic mice with a copy of the entire *APP* [33] or *SYNJ1* [54] gene have been already constructed, but mice transgenic for *ITSN1* and *RCAN1* were constructed with heterologous promoters. Thus, although elevated phospho-tau was observed in transgenic *TgRCAN1-L* [56] mice, confirmation in a model with the entire gene is needed to further understand the role of these genes in Alzheimer's disease pathology.

6. Genes and Pathways Targeting

Thanks to these rapid advances in understanding the specific brain alterations in DS, therapeutic approaches are being developed. The first therapeutic assay targeted the specific loss of basal brain cholinergic neurons (BFCN) observed after 6 months in Ts65Dn. This specific loss, due to altered transport of nerve growth factor (NGF), was rescued by infusion of NGF [84], demonstrating the potential for phenotype reversal. As excitation-inhibition imbalance has emerged as a strong target, recent approaches have targeted the potential pathways at the roots of the observed over-inhibition. Fernandez et al. [123], by using an inhibitor of the GABA-A receptor (pentylenetetrazole, PTZ), reversed the phenotype of Ts65Dn, confirming that GABA, the major inhibitory neurotransmitter of the central nervous system, is involved. Though multiple approaches are currently being tested (see Table 1), only two recent approaches have tried to identify—on a large scale—correlations between molecular changes and behavioral changes induced by a therapeutic molecule, in adults of DS models.

Braudeau et al. [43, 155] analyzed the transcriptome of mice submitted to memory processing using the Morris water maze paradigm following treatment with an inhibitor of the GABA-alpha5 receptor, the GABA-alpha5 promnesiant inverse agonist (alpha5IA). The GABA-alpha5 receptor (*Gabra5*) is specifically expressed in the hippocampus and, thus, its modulation directly involves hippocampal function. In combination with the expression of early genes, specific 3-copy genes were modulated significantly: 6 transcripts were upregulated (*Kcnj6*, *Sod1*, *Itsn1*, *Hcls*, *Gart*, *Ifnar2*) and 3 were downregulated (*App*, *Kcnj6*, *Sod1*) in Ts65Dn following treatment. Moreover, a set of 5 3-copy genes (including *Pcp4*, *Hmgn1*, *Cbr1*, and *Gabpa*), as well as BDNF, showed an interaction between genotype and treatment, suggesting a close relationship with this pathway.

Rescue of BDNF expression can also be obtained using green tea polyphenols (PGT) [42] and memantine [49] (see

Tables 1 and 2). BNDF level rescue is associated with rescue of learning impairments, and thus plays a critical role in our understanding of DS and its potential therapies.

Regulation of the glutamate receptor, NMDAR, may be altered by several genes of HSA21, namely, through the calcineurin pathway. MK-801, a noncompetitive antagonist of NMDAR, may rescue memory retention, in particular, during aging. Locomotor activity of Ts65Dn and TS1Cje was evaluated in relation to different doses of MK-801 which block this receptor with a high affinity [52]. It was given at a dose leading to the same level of induced locomotion in the two strains. Proteins fractions (nuclear, cytosolic and membranous) of hippocampus and cortex were analyzed for their level in phosphorylation for proteins belonging to the Mapk pathway and for Tiam1, Itsn1, and Dyrk1a. Overexpression of these proteins was observed in Ts65Dn and Ts1Cje. Interestingly, a partial decrease in *Dyrk1a* and modified phosphorylation of MAPK proteins was observed in a genotype-specific pattern, suggesting that the genes responsible are at different locations on the trisomic segments [52, Table 2]. Interestingly MK-801 and memantine restore the phospho-mTOR level in Ts1Cje hippocampal dendrites [113]. But it is still to be proved that such treatment will benefit to the Ts65Dn memory impairment [111].

As an noninvasive approach, "environmental enrichment" that combines sensorimotor to social stimulations, may impact at the behavioral and molecular levels [156, 157]. Standardized methods (starting age, type of stimulation) may be needed to compare the changes observed and help understand why it benefits preferentially to Ts65Dn females.

Finally, molecular and cellular analyses in DS mouse models and DS brains show a clear correlation, though brain regions may vary in their specific features, confirming the utility of mouse models of DS for testing therapeutic treatments [158]. The number of therapeutic approaches in DS mouse models is rapidly increasing, with accompanying tests for behavioral rescue. However, little is known about the molecular and cellular consequences of these treatments; assessing these consequences will be crucial for future research and for any potential translation into the clinic.

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Review Article

Astrocytes and Developmental Plasticity in Fragile X

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A growing body of research indicates a pivotal role for astrocytes at the developing synapse. In particular, astrocytes are dynamically involved in governing synapse structure, function, and plasticity. In the postnatal brain, their appearance at synapses coincides with periods of developmental plasticity when neural circuits are refined and established. Alterations in the partnership between astrocytes and neurons have now emerged as important mechanisms that underlie neuropathology. With overall synaptic function standing as a prominent link to the expression of the disease phenotype in a number of neurodevelopmental disorders and knowing that astrocytes influence synapse development and function, this paper highlights the current knowledge of astrocyte biology with a focus on their involvement in fragile X syndrome.

1. Introduction

In recent years, it has been revealed that astrocytes perform a significantly wider range of functions than previously appreciated. Interest in astrocyte function has increased dramatically because of their newly discovered roles in synapse formation, maturation, efficacy, and plasticity. Today, astrocytes are recognized as multifunctional cells with well-defined essential neuron supporting functions. Mounting evidence suggests that these versatile cells participate in a multitude of diverse processes in the central nervous system (CNS). These roles include regulating blood flow, providing much needed energy to neurons, and supplying the building blocks of neurotransmitters that fuel synapse activity [1]. However, the roles of astrocytes are not restricted to supporting neuronal function [2]. The addition of their role in synaptic function to the known repertoire of astrocyte activities over the past decade has enhanced our conception of their seminal importance in normal functioning of the adult brain. More comprehensive reviews highlighting astrocyte function include Jacobs et al. [3], Wang and Bordey [4], and Kimelberg [5].

In the developing nervous system, the assembly of synaptic circuits is a complex and dynamic process, requiring the coordinated exchange of signals between pre- and postsynaptic neurons and neighbouring glia [6]. The formation, maintenance, and modulation of synaptic connections are required for normal CNS function and ongoing plasticity. In the diseased nervous system, however, the structural and functional integrity of synaptic connections is often modified or lost, resulting in profound cognitive and behavioral deficits. Yet until recently, no exact roles had been identified for astrocytes in the pathogenesis of specific CNS diseases. While some aspects of the mechanisms underlying the formation, maintenance, and plasticity of CNS synapses in the developing and diseased nervous system have been elucidated, many more remain enigmatic.

As our knowledge about astrocytic function in normal physiology has expanded, exploration into their likely role in disease pathology has followed. In the case of fragile X syndrome (FXS), a compelling case can be made for the abnormal dysfunction of astrocytes. FXS is the most common form of inherited mental impairment, and it typically results from the transcriptional silencing of the *FMR1* (*fragile X mental retardation 1*) gene and loss of the encoded protein, FMRP (fragile X mental retardation protein) [7]. FXS symptoms include neurodevelopmental delay, anxiety, hyperactivity, and autistic-like behavior. FMRP was once thought to be expressed solely in neurons; however, it was later shown to

have specific roles in astrocytes. In fact, it appears that the expression of FMRP is developmentally regulated. Pacey and Doering [8] found that FMRP is expressed in early development in cells of the glial lineage both *in vitro* and *in vivo*.

Although few studies focus specifically on the role of astrocytes, recent work provides important examples of how a better understanding of astrocyte biology during development can enhance our knowledge about human disease. In this paper, we discuss the landmark findings and recent advances in our understanding of astrocytes and their featured roles in regulating synapse formation, maturation, and synaptic transmission. Further, we assess how astrocytes contribute to the extensive plasticity that occurs during development, highlighting the dynamic morphology of astrocyte processes and their involvement in synaptic development. Lastly, we explore the means by which perturbations in astrocyte function may contribute to neurological diseases, such as FXS, in the context of synaptic defects. We propose here that, by investigating the precise roles of astrocytes during neurological disease, we are likely to achieve a broader understanding of how the brain works, in addition to new insights into disease prognosis, diagnosis, and treatment.

2. Astrocyte Diversity

Astrocytes, or astroglia, are named with the Greek root word "astro," which means star. They were so named due to their "stars in the night sky" appearance obtained from Golgi stained samples [9]. In the late nineteenth century and the early twentieth century, Camillo Golgi and Santiago Ramón y Cajal noticed that, although different astrocytes share a stellate feature, their morphology is extremely diverse, perhaps as diverse as neurons. Since Cajal's time, modern scientists have confirmed the morphological diversity of astrocytes *in vitro* and *in vivo* [10, 11].

Astrocytes are divided into two main classes distinguished on the basis of their morphology and primary location [12, 13]. Protoplasmic astrocytes are classically found in the grey matter of the brain. Their processes, which are long, thick, and highly ramified, are closely associated with synapses as well as blood vessels [11]. In the hippocampus, protoplasmic astrocytes ensheath more than half of the synapses, most of which are excitatory [14]. The other subtype is composed of fibrous astrocytes found mainly in the white matter of the brain, where their processes pass between nerve fibers. In contrast to protoplasmic astrocytes, fibrous astrocyte processes are long, cylindrical, smooth, and branch infrequently.

Astrocytes are also far more morphologically complex than initially appreciated [15]. The morphology of a mature mammalian astrocyte is spectacular. From the cell soma radiate primary branches that gradually divide into finer and finer processes to generate a dense network of delicate terminal processes, which associate very closely with synapses. A number of immunological markers have been used over the years to characterize astrocyte morphology. Until recently, our understanding has been predominantly based on classical immunostaining with the widely used astrocyte marker GFAP (glial fibrillary acidic protein, an intermediate filament protein), which grossly underestimates the complexity of astrocytes and their interactions with neurons and other cells [16]. GFAP only reveals the structure of primary branches, which represent a meager ~15% of the total volume of the astrocyte. Other markers include ALDH1Ll (aldehyde dehydrogenase 1 family, member L1) [17], Glt-1 (glial glutamate transporter 1), and GLAST (glutamate-aspartate transporter) [18]. To date, no marker has been identified that is expressed exclusively in mature astrocytes. Moreover, no pan-astrocytic marker has been identified with which to determine the fraction of astrocytes that are GFAP+, although recent studies on ALDH1L1 seem promising [17].

Recent physiological and gene expression profiling studies indicate that astrocytes, like neurons, are a diverse cell population with distinct properties in different brain regions and at different periods of development [19]. For instance, astrocytes are crucial at every step of neural development. They function as neural stem cells and guide axon projections; they promote synapse formation and maintain neuronal survival [20, 21]. Astrocytes also differ in their proliferation potential. Subsets of astrocytes, or astrocyte-like cells, in the adult subventricular zone (SVZ) and in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus act as neural stem cells, whereas most astrocytes in other parts of the adult brain do not normally proliferate [22]. Heterogeneity of astrocytes, however, is not exclusive across brain regions, as it can also exist within the same areas of the brain [23]. The number and size of astrocytes in the brain also vary between species relative to species brain size and cognitive ability. For example, the human brain contains several more populations of astrocytes than the rodent brain, and human astrocytes are threefold larger than their rodent counterparts [24]. Therefore, these classifications may not be adequate to appreciate the full extent of astrocyte diversity.

Astrocytes have unique cytoarchitectural and phenotypic features that ideally position them to sense their surroundings and respond in dynamic ways to changes in their microenvironment [25]. Astrocytes are, therefore, well suited to share synaptic function with neurons as they extend numerous processes, forming highly organized anatomical domains with little overlap between adjacent cells. They are also interconnected into functional networks via gap junctions. The territory of a single astrocyte is estimated to contact between 300 to 600 dendrites and upwards of 10^5 synapses [16, 26]. This extensive synaptic interaction not only ensures that astrocytes are able to fulfill their metabolic support roles but also positions astrocytes to directly influence the structure and function of the synapse [27]. While some astrocyte processes (which express a wide range of receptors and ion channels) closely ensheath synapses, others are in close contact with intraparenchymal blood vessels via specialized processes called endfeet. In line with this, astrocytes have been shown to play an important role in neurovascular and neurometabolic coupling [23].

3. Astrocytes Influence Synapse Formation and Development

In the past decade, astrocytes have emerged as essential regulators of synaptic connectivity. The formation of synaptic contacts is paramount for the proper development and function of the CNS. Although most neurons are produced during embryonic stages, the major waves of synaptogenesis follow and depend on astrocyte production. Given their proximity to synapses, astrocytes can directly promote and regulate these processes through both secreted and contact-mediated signals.

3.1. Secreted Signals. The traditional assumption that neurons are intrinsically able to form synapses led early studies on synaptic development to focus on neuronal signals and surface molecules. Remarkably, neurons cultured with media conditioned by astrocytes control the number and effectiveness of synapses [28–30], indicating that soluble factors secreted from astrocytes play an important role in synapse formation. Some of the factors released by astrocytes that mediate these effects have been identified. These include matricellular proteins [31], such as thrombospondins (TSPs-1–4), SPARC, SPARC-like 1 (Hevin), and tenascin C, which are all expressed by astrocytes in the CNS of rodents.

A possible role for glial involvement in CNS synaptogenesis was first elucidated by a series of studies on rat retinal ganglion cells (RGCs). Cholesterol complexed to apolipoprotein E (ApoE) released by astrocytes increases the number of glutamatergic synapses in RGC cultures. When cholesterol is applied directly to cultured RGCs, the frequency of spontaneous synaptic events increases. The researchers further demonstrated that cholesterol acts to increase the quantal content of synaptic vesicles and the overall efficacy of vesicle release [32]. This is in concert with other findings that cholesterol is an essential component of synaptic vesicle production whose presence serves as a limiting factor in vesicle formation [33].

The RGC culture technique has also been used to identify other key synaptogenic-secreted factors including Thrombospondins 1 and 2 (TSP-1 and TSP-2), members of oligomeric extracellular proteins. Christopherson and colleagues identified TSPs as the signals coming from astrocytes that can induce an increase in synapse number [34]. When directly applied to RGC cultures, TSP-1/2 increased the number of immunochemically identified synapses nearly 3-fold. Immunodepletion of TSPs from astrocyte-conditioned medium (ACM) decreased its synaptogenic effect down to control levels indicating that TSPs are the key synaptogenic component of ACM.

The expression of TSP-1/2, which is elevated in the developing brain when the majority of synapses are formed (during postnatal week 1), ceases in the mature adult brain (by postnatal week 3). This suggests that astrocytes downregulate pathways that strongly promote synapse formation when the synaptogenic period of neurons is reduced, and other TSP genes may be functioning to stabilize synaptic structures. Besides TSPs 1 and 2, other TSPs (TSPs-3–5) are detected in mammals [35]. Astrocytes have been found to express mRNAs for TSP-3 and -4. In contrast to other TSPs, TSP-4 expression is only detected in mature astrocytes after P17 [17]. This suggests that TSP-4 could represent the adult isoform of TSP in the CNS and is important for the control of synaptogenesis and enhanced plasticity in the adult brain. Recently, gabapentin receptor $\alpha 2\delta$-1 has been identified as the TSP receptor responsible for mediating excitatory CNS synaptogenesis [36]. Despite the critical role of TSPs in promoting synaptogenesis, additional signals are likely required for synapse maturation, as TSP-induced synapses are ultrastructurally normal, but postsynaptically silent, underscoring the complexity of astrocyte contribution to synapse formation. A more recent study has identified two closely homologous glypicans, glypican-4 and glypican-6, as astrocyte-secreted proteins that are sufficient to increase AMPA glutamate receptor levels on synapses, thus inducing postsynaptic function [37].

Additionally, tenascin-C (TN-C), another extracellular matrix glycoprotein, seems to play a role in synaptogenesis and synaptic function [38, 39]. TN-C is highly expressed by astrocytes during early stages of development, while its expression ceases in the adult CNS [40], with the exception of specific cell populations, particularly those in close proximity to areas of active neurogenesis, such as the hippocampus, subventricular zone borders, and the rostral migratory stream. Following stimulation of synaptic activity, TN-C was found upregulated in the hippocampus within a few hours [41]. In TN-C knockout mice, stimulation of Schaffer collaterals resulted in a reduction of long-term potentiation (LTP) at CA1 synapses, whereas CA1 long-term depression (LTD) was completely abolished [42, 43]. These expression patterns reveal important roles for TN-C in the remodeling of the CNS, both during development and in adulthood.

Notably, astrocyte-secreted factors do not act exclusively to promote excitatory synaptogenesis. In fact, recent studies reveal astrocyte contributions to inhibitory synapse formation and function in cultured hippocampal neurons. While astrocyte-expressed extracellular matrix protein Hevin has been found to induce the formation of synapses in cultured RGCs [44, 45], its homolog, SPARC, which is also secreted by astrocytes, antagonizes the synaptogenic function of Hevin, thereby acting as a negative regulator of synapse formation [44]. SPARC expression is typically high in early development, where it then becomes downregulated in certain parts of the brain by the time of synaptogenesis. Alternatively, Hevin expression increases with development in agreement with synapse formation and is also present in adulthood, most likely functioning in the maintenance of existing synapses. Unlike TSP-1 and TSP-2, the expression of which is decreased during maturation, Hevin and SPARC mRNA levels remain high even in the adult. Taken together, the secretion of both positive and negative regulators of synapse formation allows astrocytes to regulate the timing and location of synapse formation with greater precision.

Moreover, a recent study has provided evidence that astrocytes play a role in the elimination of redundant synapses during development. In the developing postnatal brain and retina, immature astrocytes seem to be a source

of a signal that triggers the expression of complement component C1q in developing neurons [46]. C1q's best-known role in the innate immune system is to opsonize or "tag" unwanted cells or debris for elimination. C1q localizes to synapses that are thus tagged for elimination through the activation of the complement cascade and deposition of C3b, an opsonin derived from the proteolytic activation of the complement component C3. Mice deficient in C1q or the downstream complement cascade protein C3 exhibit large sustained defects in CNS synapse elimination, as shown by the failure of anatomical refinement of retinogeniculate connections and the retention of excess retinal innervation by lateral geniculate neurons. Also, C1q-deficient mice show enhanced neocortical excitatory synaptic connectivity and epileptiform activity [47]. Together, these findings implicate a role for astrocytes during the critical period when neural circuits are formed.

3.2. Contact-Mediated Effects. While astrocyte-secreted factors induce the formation and function of synapses, other evidence proposes further regulatory roles for astrocytes through contact-mediated mechanisms. An elegant study by Hama et al. [48] provided evidence that astrocytes upregulate synapse formation by the process of adhesion. Local contact with astrocytes via integrin receptors facilitated excitatory synaptogenesis through the activation of protein kinase C (PKC) in individual dissociated hippocampal neurons. The researchers observed that PKC activation, while initially focal, subsequently spread throughout the entire neuron. Thus, propagation of PKC signaling could signify an underlying mechanism for global neuronal maturation following local astrocyte adhesion.

Astrocyte processes, which are highly mobile, contribute to the stabilization of new synapses during synaptogenesis. Astrocytes may induce local structural and functional modifications of dendritic segments or individual synapses through a contact-mediated mechanism involving bidirectional ephrin/EphA signaling [49–51]. Membrane-bound ligands on astrocytes, such as ephrin-A3, have been shown to upregulate spine morphology in the hippocampus, suggesting local activation of EphA receptors on spines by astrocytic ephrin-A3. Dendritic spines are small protrusions visible on dendrites of neurons that serve as postsynaptic sites for excitatory input [52–54]. Live imaging of organotypical hippocampal slice preparations showed that astrocytes rapidly extend and retract fine processes to engage and disengage from postsynaptic dendritic spines [55]. Studies with two-photon microscopy that tracks the dynamics of astrocyte processes and the fate of dendritic protrusions also revealed contributions of astrocyte contact [56]. Dendritic protrusions with astrocyte contacts had a longer lifetime and were morphologically more mature. Thus, dendritic protrusive activity and transient contacts with astrocytes act to stabilize newborn synapses and promote subsequent spine maturation. Spine dynamics are largely controlled through changes in cytoskeletal proteins [57]. Expressing a dominant negative mutant Rac1, a GTPase that mediates actin motility, reduces astrocyte process motility and provides evidence that cytoskeletal rearrangements underlie motility, similar to mechanisms of spine extension and retraction [56, 57].

The development of inhibitory synapses can also be modulated by astrocyte contact. Liu et al. [58] showed that local contact between neurons and astrocytes significantly increased the amplitude and density of $GABA_A$ currents in developing hippocampal neurons. This contact-dependent increase in GABAergic synaptic activity relied on Ca^{2+} signaling in astrocytes. In addition, astrocytes were shown to regulate Cl^- gradient in cultured spinal cord neurons and convert GABAergic synapses from excitatory to inhibitory [59]. This finding is particularly exciting given the importance of local GABAergic inhibitory circuits in both activity-dependent wiring of developing neural circuits and the consolidation of critical period plasticity [60, 61].

Overall, these studies reveal that contact-mediated signaling between astrocytes and neurons is important for the structure and maintenance of synaptic connections and suggests a model in which physical and molecular interactions between neurons and astrocytes provide instructive cues that control synapse formation, morphology, and plasticity.

4. Astrocytes Modulate Synaptic Transmission

As our understanding of the extent of their influence at the synapse unfolds, it is much more apparent that astrocytes are well poised to modulate multiple aspects of synaptic plasticity than was previously imagined. A turning point in our understanding of astrocytes was elicited by the recognition of their active communicative properties [62–64]. Networks of astrocytes can act in concert to influence transmission among neighbouring synapses. Astrocytes, which are bidirectional, can communicate and exchange information with both pre- and postsynaptic elements. Communication is primarily controlled by the change in Ca^{2+} concentrations, causing excitability within the astrocyte [64–66].

Astrocytes use their ability to respond to neurotransmitters and secrete neuromodulators to actively regulate a number of processes involving synaptic plasticity [67–69]. In addition to secreting factors that influence and modulate synapse formation, astrocytes are known to release factors that can directly affect synaptic transmission. Briefly, of the gliotransmitters released by astrocytes [70], the most well characterized and extensively reviewed are glutamate [71, 72], adenosine triphosphate (ATP) [73], and D-serine [74, 75]. Glutamate serves as the principal excitatory neurotransmitter in most regions of the CNS, and its release from astrocytes has been shown to modulate synaptic transmission [76]. Glutamate released from neurons activates metabotropic glutamate receptors on astrocytes, leading to an increase in astrocyte Ca^{2+} concentrations and a subsequent astrocytic release of glutamate. D-serine, perhaps the most interesting, is an important neurotransmitter that serves as a coagonist with glutamate, promoting NMDA (N-methyl-D-aspartate) receptor activity at synapses in the hypothalamus [75]. Moreover, astrocytes release ATP to communicate with each other and other glia by activating

purine receptors localized on neighbouring cells [73]. These findings have led to the establishment of a new concept in synaptic physiology, the tripartite synapse, in which astrocytes exchange information with neuronal synaptic elements [6, 67, 77]. Consequently, astrocytes are an integral part of the synapse, being involved not only in passive homeostatic control of adequate conditions for synaptic function, but also actively in synaptic function [78].

5. Astrocytes and Pathology: Contributions to Neurological Disorders

With an evident role of astrocytes in normal neural function at all cellular and molecular levels, it is not surprising that astrocytes contribute in some capacity to almost all pathological conditions of the nervous system [79–84]. For most disorders, it remains unclear whether astroglial changes are causative of the disease or if they merely represent an accompanying phenomenon. Accordingly, astrocyte-dysregulated function has been fundamentally linked with the progressive pathology of ischemic stroke, epilepsy, and to a number of neurodegenerative disorders including, but not limited to, amyotrophic lateral sclerosis, Huntington's disease, and Parkinson's disease. Further involvement of astrocytes has also been implicated in the development of neurodevelopmental disorders such as Rett syndrome (RTT), Down syndrome (DS), Fragile X (FXS), and autism. Among these conditions, FXS has emerged as the prototypical disorder in which to study how altered signaling may lead to synaptic defects and dysfunctional neural circuitry underlying pathology [85]. Both dysregulated astrocyte signaling and abnormal synaptic function stand as prominent contributing factors to the learning disability phenotype expressed in FXS.

6. Fragile X Neurobiology

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability [7]. It affects approximately 1 in 4,000 males and 1 in 6,000 females and is characterized by cognitive impairments, attention deficits, and autistic-like behaviors [86]. FXS is caused by an expanded CGG trinucleotide repeat in the 5′ untranslated region of the *FMR1* gene leading to gene silencing and the consequent loss of FMRP expression [87, 88].

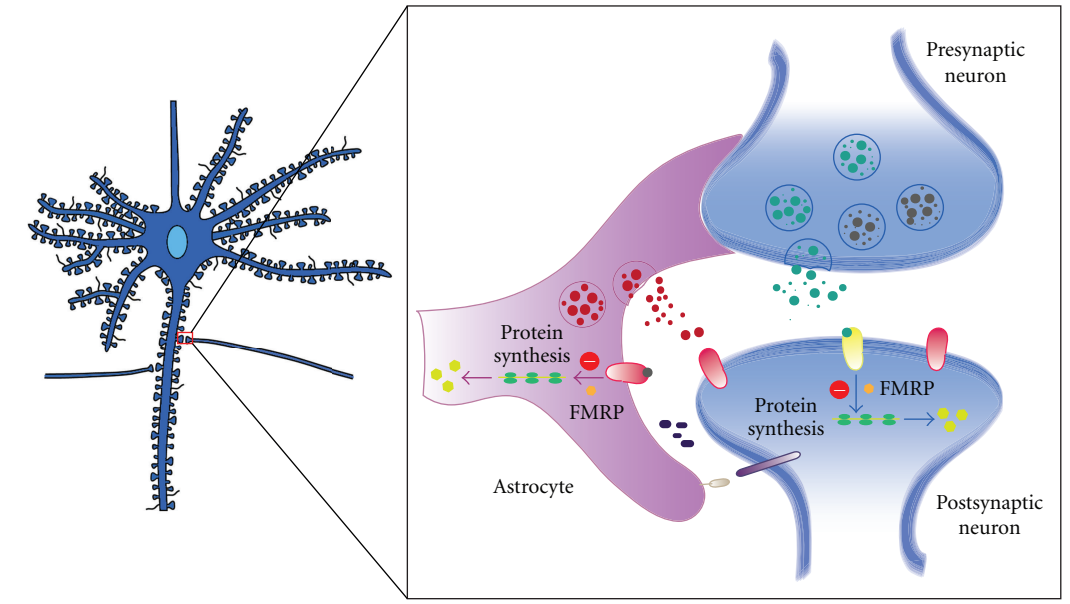
To understand the etiology of the synaptic phenotypes that accompany FXS, it is first important to discuss the purported function of FMRP. FMRP acts as a regulator for the transport and local translation of specific synaptic mRNAs in response to neural stimulation [89]. FMRP is found in growth cones, immature axons, and mature dendrites, as well as dendritic spines [90]. Accumulating evidence suggests roles for FMRP in synapse development, elimination, and plasticity. The loss of FMRP results in the aberrant expression of its mRNA targets, which in turn leads to functional deficits that characterize FXS. The reason that FMRP has been implicated in synaptic plasticity is on the basis of dendritic spine abnormalities and exaggerated long-term depression (LTD) displayed by *FMR1* mutant mice. This finding led to the "mGluR" theory of FMRP, whereby synaptic signaling of metabotropic glutamate receptor 5 (mGluR5) leads to the localized translation of *FMR1* mRNA [91]. As such, the newly synthesized FMRP acts as a translational repressor of specific target mRNAs, resulting in the downregulation of mGluR5 activity through a negative feedback loop [92] (Figure 1). Several exceptional reviews on the genetic and clinical features of FXS or molecular functions of FMRP include Bear [93], Huber et al. [92], Garber et al. [94], and Bassell and Warren [89].

6.1. FXS Animal Models. Current knowledge surrounding the pathophysiology of FXS has been greatly advanced by the development of animal models [95]. These transgenic animals do not carry the trinucleotide expansion but do have functional deletions of FMRP. The first model developed was the *FMR1* knockout (KO) mouse [96], which recapitulates behavioural and cognitive deficits reminiscent of the human condition. Drosophila and zebrafish models also exist and have contributed to our understanding of the conserved roles of FMRP in neural development [97–99]. Although they are not perfect models of the human disease, they have helped to reveal the cellular and molecular mechanisms underlying FXS, and they have immensely enhanced glial-neuronal research.

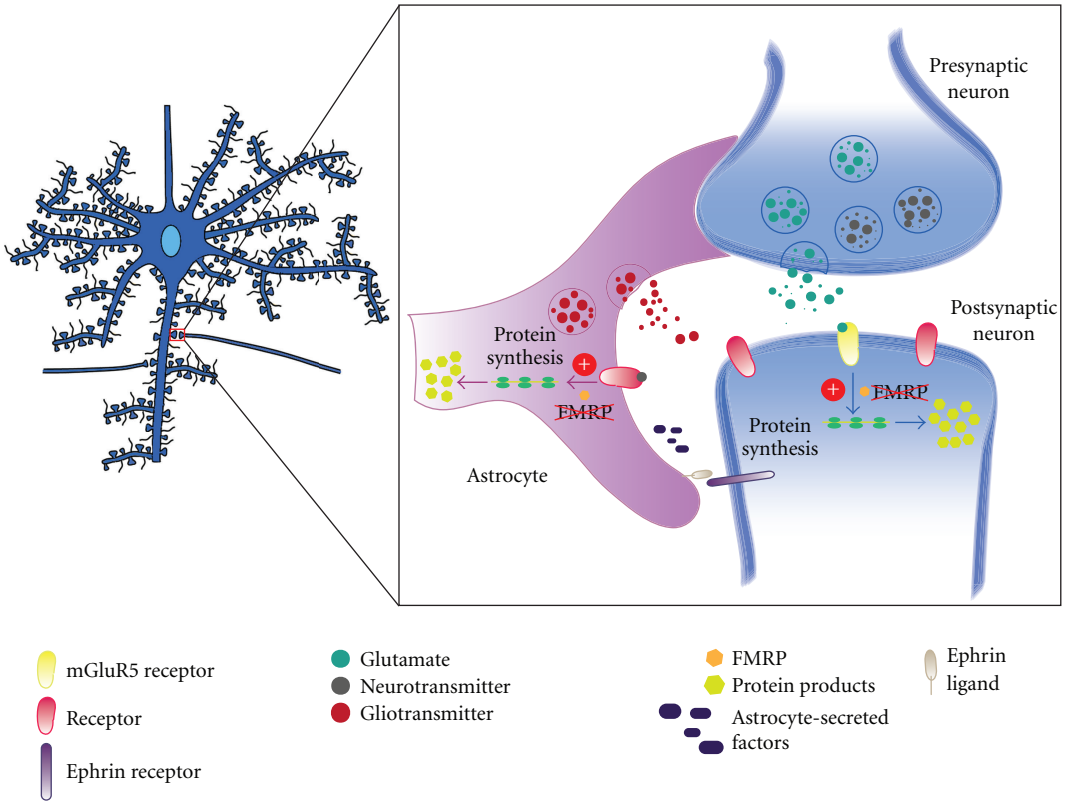
6.2. FXS Spine Dysgenesis. During development, the first postnatal weeks of the mammalian brain are characterized by extensive plasticity. Selective elimination or pruning of inappropriate synaptic connections occurs for the proper formation and establishment of neural circuitry. Current models regarding the neurobiological changes that underlie Fragile X have largely focused around the synapse. This is based in part on the structural synaptic changes and alterations in synaptic function, which are observed in human patients and FXS animal models.

Filopodial spine morphology has long been a common hallmark of disease. Spines develop around the time of synaptogenesis and are dynamic structures that continue to undergo remodeling over time. Developmental changes in the shape of dendritic protrusions reflect the progressive replacement of thin, elongated, and highly motile filopodia, characteristic of immature neurons, with more stable spines that acquire a mature morphology [100]. Spine morphogenesis is fundamental to the development of neuronal networks and the regulation of synaptic plasticity.

Some of the first neuroanatomical findings associated with mental impairment were alterations in dendritic spine structure [101]. The first such evidence of altered synapse structure in FXS came from analysis of postmortem cortical tissue, which exhibited an increased number of dendritic spines relative to control individuals [102]. This data revealed that excitatory synapse number was increased in FXS patients and further provided a potential mechanism for the increased rates of epilepsy in FXS. It was additionally noted that a large proportion of the spines of FXS patients appeared abnormally long, thin, and tortuous, a phenotype reminiscent of the immature spine precursors (filopodia),



(a) Under normal conditions, astrocytes can promote synaptogenesis via direct and/or indirect contact with neurons through the release of soluble factors. Astrocytes also release a variety of neuroactive substances (gliotransmitters) to modulate synaptic transmission and plasticity. Astrocyte FMRP plays an important role in shaping the neuron morphology and synaptic protein profiles. FMRP has been shown to inhibit translation of specific mRNAs.



(b) In the FXS disease state, nonfunctional FMRP in neurons leads to the dysregulation of synaptic protein synthesis and abnormal dendritic morphologies. FMRP may play a similar role in astrocytes as in neurons, functioning as a negative regulator of protein translation. In FMRP-deficient mice, the inability to repress translation is lost. mGluR5 stimulation, associated with dysregulated FMRP protein levels, results in increased levels of FMRP targeting mRNAs. Basal protein levels encoded by these target mRNAs become significantly elevated and thus improperly regulated. Aberrant spine and dendritic morphology is apparent through increased branching and an abundance of immature spines (filopodial projections).

Figure 1: The role of astrocytes in FXS. It is becoming increasingly apparent that, in addition to presynaptic terminals and postsynaptic dendritic spines, synapses contain a third element: the fine processes of the astrocyte, which intimately enwrap the first two elements.

and indicative of alterations in synapse development and/or function. At this point, it was not clear if the excess filopodia-like spines in FXS represented functional synapses or immature synapse precursors.

Much of the evidence for a role for FMRP in synaptic and neurite pruning is derived from the *Drosophila melanogaster* model of FXS (dFXR). During development, FMRP has been shown to control the pruning of immature dendrites in developing neurons. In support of a pruning function for dFXR, most neurons of dFXR null flies exhibit an overgrowth and elaboration of axons and dendrites into the peripheral and CNS [98, 103–106].

Parallel to human studies, work with the *FMR1* KO mouse has largely confirmed the spine phenotype observed in FXS patients. Numerous studies agree that *FMR1* mutant brains display an increase in long, thin, immature dendritic spines [102, 107] mirroring human neuroanatomical abnormalities [102]. It is important to note that many of these defects in spines and in synaptic/circuit plasticity occur during critical periods of development in the first postnatal weeks, coinciding with the maximal expression of FMRP. However, the existence and/or magnitude of the spine alterations in the *FMR1* KO mouse varies according to brain region, developmental age, and genetic background, indicating the complex and multifactorial regulation of spines.

In a study by Cruz-Martin et al. [108], spines of L2/3 layered pyramidal neurons were imaged at various developmental stages, and it was revealed that *FMR1* KO mice demonstrated a delay in the stabilization of dendritic spines, due to high turnover during the second postnatal week [108]. This happens to correspond to the time when FMRP protein expression is highest in the cortex [109]. In the absence of FMRP, hippocampal neurons have fewer spines that colocalize with synaptic markers, which suggests a loss of functional spines [90]. This provides compelling evidence that FXS might be caused by a failure in the transition from filopodia (earliest dendritic protrusions) to mature spines, consequently resulting in an increase of immature synapses. The failure of spines to stabilize during the critical period in the barrel cortex strongly suggests that *FMR1* KO mice could have problems in maintaining the proper balance between stable and dynamic connections that is necessary to establish mature synapses. Since dendritic spines are the primary sites of excitatory synapses and information exchange in the CNS, perturbations in their structure and function can result in synaptic and circuit alterations leading to disrupted brain function and pathology.

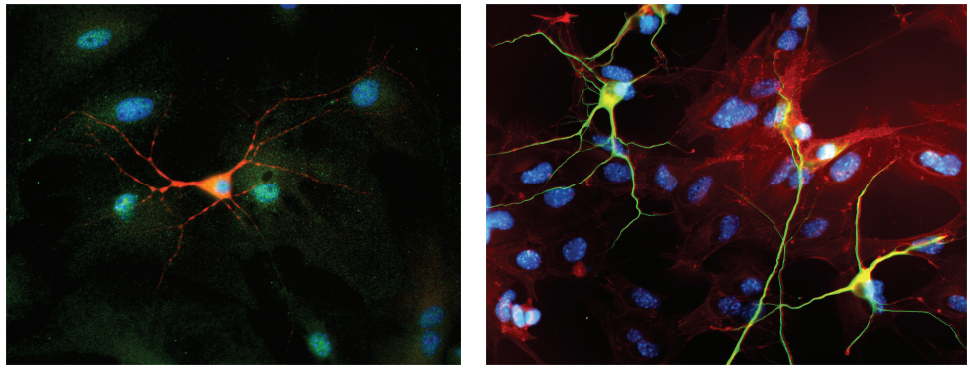
7. Astrocyte Involvement in Fragile X

While it has been recognized that astrocytes play multiple critical roles in the regulation of normal CNS function, the possibility that astrocyte-specific dysfunction might cause diseases that manifest as pathologies of neurons is a relatively recent idea. Previously, it was thought that FMRP expression in the brain was exclusively confined to neurons. FMRP had been reported in oligodendrocyte precursor cells, but not mature oligodendrocytes [110]. Our laboratory initially identified FMRP in the astrocyte lineage in the FXS mouse [111]. When studying stem and progenitor cells from the brains of wild-type (WT) and knockout (KO) FXS mice, approximately half of the cells in culture coexpressed FMRP and GFAP. Parallel immunocytochemical studies *in vivo* also showed the coexpression of FMRP and GFAP in the embryonic and adult developing hippocampus.

With the identification of FMRP in astrocytes and knowledge of their role in synaptogenesis, our laboratory was prompted with further experiments to explore neuronal development and synapse formation in FXS [112]. Utilizing a coculture design adapted by Jacobs and Doering [113], hippocampal neurons (E17) and cortical astrocytes (P0-1) were independently isolated to explore four different combinations of neuronal-astrocyte cultures (WT neurons + WT astrocytes, WT neurons + *FMR1* KO astrocytes, *FMR1* KO neurons + WT astrocytes, *FMR1* KO neurons + *FMR1* KO astrocytes). The cells were grown for 7, 14, or 21 days *in vitro* and then processed for immunocytochemistry to analyze morphological and synaptic profiles. Examples of the cocultures are shown in Figure 2. These experiments are novel and exciting as they are the first to establish a potential role for astrocytes in the altered neurobiology of FXS.

The first group of experiments focused on neurons in each of the four combinations to elucidate the effects of FMRP on dendritic morphology and excitatory synapse expression. The neurons were studied with antibodies directed against the neuronal (dendritic) marker, MAP-2, the presynaptic protein synaptophysin, and excitatory postsynaptic protein, PSD-95, respectively. Through Sholl analyses, morphological assessments were performed on neurons under parameters of dendritic branching and the area of the cell body. Synaptic protein distribution was determined by the quantification of synaptic puncta (spots of intense staining). WT neurons grown on *FMR1* KO astrocytes exhibited significantly altered dendritic arbor morphologies, with a shift toward a more compact and highly branched dendritic tree. Specifically, WT neurons grown on *FMR1* KO astrocytes resulted in a decrease in the length of the longest primary dendrite and area covered by dendritic arbor, and an overall increase in branch number and density in comparison to their WT counterparts. These neurons also displayed a significant reduction in the number of pre- and postsynaptic protein aggregates. However, when the *FMR1* KO neurons were cultured with WT astrocytes, the alterations in dendritic morphology and synaptic protein expression were remarkably prevented. In fact, their morphological characteristics and synaptic protein expression approached the appearance of normal neurons grown with WT astrocytes. These experiments were the first to suggest that astrocytes contribute to the abnormal dendritic morphology and the dysregulated synapse development seen in FXS.

In the next phase of this research, we wanted to determine if these altered characteristics represented a developmental delay imparted by the *FMR1* KO astrocytes [114]. Focusing on WT neurons grown in the presence of WT or *FMR1* KO astrocytes, we evaluated the dendritic arbor morphology and synaptic protein expression at 7, 14, and 21 days in culture. Our results revealed that WT neurons grown with *FMR1*



(a) (b)

Figure 2: Examples of Fragile X astrocyte-neuron cocultures. (a) *FMR1* WT astrocytes + WT neurons double labeled with MAP-2 (neuron in red) and gephyrin (astrocytes in green); (b) *FMR1* KO astrocytes + WT neurons identified with MAP-2 (neurons in green) and GFAP (astrocytes in red); DAPI—nuclei (blue).

KO astrocytes displayed significantly altered morphological and synaptic protein profiles at 7 days (when compared to the WT condition). Strikingly, by 21 days in culture, these differences were no longer significantly different from normal. In light of these findings, it appears that astrocytes in the FXS mouse may contribute to the altered characteristics of neurons seen in FXS in a developmentally regulated manner. Thus, these results suggest that timing is crucial in brain development. Despite these outcomes, it is noteworthy that conclusions about synapse maturity cannot be drawn. It is possible that the increase in synapses observed in the neurons grown on *FMR1* KO astrocytes reflects an increased number of immature synapses. Given that the dendritic spine is the site for the majority of excitatory synapses, this finding would be in agreement with numerous studies that identified neurons in FXS with an abnormally high number of immature dendritic spines. As a note, the methods used in the current study did not permit the assessment of alterations in dendritic spine morphology.

7.1. Outstanding Questions and Future Approaches. Understanding the role of astrocytes in human neurological diseases requires a comprehensive picture of how astrocytes develop and what roles they play in development. Given these findings, it is highly plausible that FXS astrocytes lack functional FMRP, specifically at a time during development when astrocyte support of neuron growth and synapse formation is vital, and that this lack of FMRP could contribute to the abnormal neuron phenotype seen in FXS. However, it is uncertain whether the alterations in astrocytes are due to a lack of FMRP or if they are abnormal because they develop and function in a diseased microenvironment. Also, if the absence of FMRP in astrocytes is the primary source of dysfunction, how are these effects translated to neurons? For instance, is astrocyte-neuron signaling disrupted due to a lack of astrocyte FMRP? How, where, and when do these signals act? Is the abnormal astrocyte-neuron communication mitigated by a membrane associated or a soluble factor? Could it be a combination of both direct and indirect contact? These questions, among many others, about the FXS astrocyte are now important targets for FXS research. The answers will allow us to gain a full understanding of the underlying neurobiology that contributes to the morphological phenotype seen in FXS and in the potential of a future treatment for individuals with FXS.

Recent evidence indicates that the interface between astrocytes and neurons is necessary for normal synapse development, including synaptic pruning. Dendritic spines, which are highly dynamic during development, become more stable in the adult brain; thus, a correlation exists between age-dependent spine dynamics and the plasticity of the brain. This decrease in spine motility in the mature brain could be attributed to the close association of astrocytes with synapses, with astrocytes providing both physical constraints that inhibit spine movement as well as molecular interactions that stabilize spines. Importantly, EphA4R (expressed on dendritic spines) interacts downstream with members of the Rho/Ras pathways, suggesting that EphAR/ephrin-A interactions may underlie aspects of actin-driven astrocyte motility observed during synapse formation [27, 115]. Interferences in these interactions may result in the destabilization of newly formed spines [49]. Therefore, this raises the possibility that *in vivo* defects in dendritic spine development are at least partly related to neuron-glia interactions during development.

Astrocyte involvement has also been fundamentally implicated in neurodevelopmental disorders such as RTT and DS. A common finding in many of these studies is that astrocyte dysfunction has profound non-cell-autonomous effects on surrounding neurons. In fact, synaptic function and structure may be a converging point of malfunction. RTT, which is an X-linked neurodevelopmental disorder, is caused by the loss of the transcriptional repressor methyl-CpG-binding protein (MeCP2). A study by Ballas et al. [116] showed that wild-type hippocampal neurons cocultured with cortical astrocytes or conditioned medium from *Mecp2*-deficient mice had abnormally stunted dendrites, suggesting that *Mecp2*-deficient astrocytes may dominantly affect normal neuronal development. Furthermore, in DS

patients, cognitive deficits have been associated with structural changes in the architecture and alterations in dendritic spine number. Garcia et al. [117] found that DS astrocytes are directly involved in the development of spine malformations and reduced synaptic density. These researchers also indicated that the astrocyte-secreted protein TSP-1 possesses a potent modulatory effect on spine number and morphology. Taken together, these studies serve to identify astrocyte dysfunction as a significant factor of spine and synaptic pathology.

Future experiments will focus on the assessment of dendritic spines in FXS and the role of direct/indirect neuronal-astrocyte cell contact in the altered developmental sequences that we observed in our tissue culture paradigm. It is highly conceivable that the absence of astrocyte FMRP would directly affect spine morphology or dynamics via dysregulated protein synthesis, and a defect in the maturation of dendritic spines could explain deficits in the intellectual ability seen in individuals with FXS.

8. Closing Remarks

Armed with novel experimental techniques, powerful imaging tools, and a better understanding of astroglia, neuroscientists are uncovering a new view of the synapse. Neuroscientists are now in a better position to explore and uncover the long-standing mysteries of astrocytes and gain new insights into the cellular and molecular underpinning of the nervous system. The recent findings discussed in this paper place astrocytes in an important position to actively exchange signals with neurons and other glial cells to coordinate synaptic networks. Astrocytes secrete soluble factors that enhance synaptogenesis and release neuroactive molecules that mediate plasticity. Both astrocyte contact and secreted factors are important in regulating synapse formation and function. While studies help to distinguish the effects of astrocyte contact from secreted factors on neuronal form/function, it is unlikely that they are separate *in vivo*. Also, given the central role of the synapse in neuronal communication and plasticity, it comes as no surprise that dysregulation of the synapse accounts for many, if not most, of pathological and developmental disorders in the brain. Thus, the involvement of astrocytes and how they interface with neuronal circuitry should be taken into consideration when interpreting future studies in the pathophysiology of FXS and/or other related neurological diseases.

A unifying theme from these recent findings is that astrocytes can promote the development and plasticity of synaptic circuits. Much of the current literature surrounding FXS focuses on synaptic control of protein synthesis because it appears to be proximal to the biology of FMRP and the pathogenesis of the disease in multiple animal models. In addition to targeting synaptic protein synthesis, other therapeutic approaches show promise, for example, in changing the balance of excitation to inhibition by enhancing GABA signaling [118]. Whether different approaches will converge on the same pathophysiological processes or whether they will target distinct aspects of the disease remains to be determined. As we continue to expand our understanding, insights into how these mechanisms may be perturbed in FXS and other diseases states may pave the way for promising future therapeutic interventions and treatments. Potential modes of pharmacological therapy should indeed concentrate on the astrocyte as a "gatekeeper" of neuronal health and function.

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Review Article

Understanding the Pathogenesis of Angelman Syndrome through Animal Models

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Angelman syndrome (AS) is a neurodevelopmental disorder characterized by severe mental retardation, lack of speech, ataxia, susceptibility to seizures, and unique behavioral features such as easily provoked smiling and laughter and autistic features. The disease is primarily caused by deletion or loss-of-function mutations of the maternally inherited *UBE3A* gene located within chromosome 15q11-q13. The *UBE3A* gene encodes a 100 kDa protein that functions as ubiquitin ligase and transcriptional coactivator. Emerging evidence now indicates that UBE3A plays a very important role in synaptic function and in regulation of activity-dependent synaptic plasticity. A number of animal models for AS have been generated to understand the disease pathogenesis. The most widely used model is the *UBE3A*-maternal-deficient mouse that recapitulates most of the essential features of AS including cognitive and motor abnormalities. This paper mainly discusses various animal models of AS and how these models provide fundamental insight into understanding the disease biology for potential therapeutic intervention.

1. Introduction

In 1965, Dr. Harry Angelman first described that 3 of his child patients showed severe mental retardation, jerky movements, excessive laughter, and abnormal physical development. He called them "puppet children" because they resembled puppets with their flat heads. All three showed typically common behavioural features that led him to suggest the possibility of a distinct syndrome. Later the disease was named as Angelman syndrome (AS). Children with AS show developmental delay, lack of speech, ataxia, learning disability, flat occiput, seizures, tongue protrusion, and uncontrollable laughter. Individuals suffering from this disorder show hyperactivity and restless behaviour, wide gait, hypotonia, microcephaly, widely spaced teeth, abnormal EEG patterns, hypopigmentation with blond hair and light eyes, love for water, and dysmorphic features like prominent chin and deep set eyes [1, 2]. Intellectual disability has been described as a feature of AS in almost all studies including the first report by Dr. Angelman [2, 3]. The severity of intellectual disability varies amongst the individuals. Many cases of AS seem to associate with autism [4, 5], which is characterized by reduced social interaction, lack of communication, and stereotypic behavior [6].

2. Genetics of AS

The cause behind AS remained unknown until the late eighties. High resolution chromosome banding technique revealed that one of the AS patient had a deletion of chromosome 15q11-12 [7]. This was confirmed when a group of children with severe mental retardation, ataxia, and seizures were shown to have a deletion in the proximal long arm of chromosome 15 [7, 8]. Although this deletion had already been reported in Prader-Willi Syndrome (PWS) earlier [9, 10], these children showed features suggestive of AS rather than PWS. The difference in the manifestation of the two syndromes proposed that the genes responsible for both syndromes might be closely associated but definitely distinct. Later AS was mapped within the 15q11-13 region of the chromosome [11]. Another major breakthrough came when it was found through RFLP (restriction fragment length polymorphisms) that deletion in the maternal copy of the chromosome led to AS in contrast to the paternal

inheritance of PWS [12, 13]. While 60–70% of the AS cases showed large (3-4 Mb) de novo deletions in chromosome 15 [14], less than 5% of cases showed uniparental paternal disomy (UPD) [15, 16], and 2-3% cases occurred due to imprinting defects [1, 2]. The remaining 25% of the cases had unknown origin but few of them were observed to be familial [17]. A recent clinical study with 160 AS patients suggested that characteristic EEG patterns could be an important biomarker in AS and might predict the underlying genetic cause [18].

In 1994, two candidate genes were mapped to the AS critical region, E6-AP (E6 associated protein encoded by the *UBE3A* gene) and PAR-2 for Prader-Willi/Angelman region-gene-2. Soon mutations in the *UBE3A* gene were found in around 5–10% cases of AS [19, 20]. Discovery of point mutations in *UBE3A* gene strongly implicated *UBE3A* as the gene responsible for AS [20, 21]. Although we cannot dismiss the involvement of other genes in AS, *UBE3A* is the only gene to date whose dysfunction is sufficient to manifest the AS phenotype in number of animal models. It is also important to mention that along with various other chromosomal aberrations identified in autism, maternal deletions and duplication in the proximal region of 15q (region deleted in most cases of AS) are a common cause of autism [22, 23]. *UBE3A* gene was suggested as a strong candidate for autism because of its imprinted nature and maternal dominance [22, 24]. A whole genome wide screening for copy number variation revealed *UBE3A* as one of the affected genomic loci in autism [25]. A map of the maternal and paternal human chromosome region 15q11-13 containing multiple genes is shown in Figure 1.

3. UBE3A/E6-AP Protein

UBE3A gene is located within the q11-q13 region on chromosome 15 in humans while it is found on the proximal region of chromosome 7 in mice [26]. It encodes a 100 kDa protein known earlier as E6-AP (E6 associated protein) [27, 28]. *UBE3A* gene encodes five mRNA subtypes generated by alternate splicing that give rise three protein isoforms [29]. The functional significance of different isoforms is still unclear. The murine homolog is slightly longer with 885 amino acids. There is about 99% similarity in human and murine E6-AP/UBE3A protein [27]. E6-AP/UBE3A belongs to the HECT (homologous to E6-AP C-terminus) domain family of E3 ubiquitin ligases in the ubiquitin proteasome system (UPS). These proteins exit with large diversity and promote degradation of short lived or abnormal proteins by transferring multiubiquitin molecules to them as a degradation signal [30]. The members of the HECT family share a ~350-residue conserved C-terminal region called the HECT domain [31, 32]. UBE3A is the founding member of the family, discovered based on its interaction with viral E6 oncoprotein to target p53 for proteasomal degradation in cells infected with human papilloma virus (HPV) [28].

UBE3A is also demonstrated to act as a transcriptional coactivator of steroid hormone receptors [45–47]. UBE3A is shown to interact with number of cellular proteins that indicate its involvement in multiple cellular function including cell cycle regulation [48–52], synaptic function and plasticity [53–58], and cellular protein quality control [59–61]. A list of identified substrates and possible cellular function of UBE3A is shown in Table 1.

4. Mouse Models of AS

The first attempt to model AS was made in 1992 [62]. This group successfully made a model for PWS with maternal duplication in the central region of chromosome 7 but failed to make the same for AS with paternal duplication. While the imprinting was expected in the central region on the mouse chromosome 7, (which was considered homologous to the human region 15q11-13 deleted in PWS/AS) the actual imprinting seen in the partial UPD mice was more proximal on the chromosome. Hence this was not considered an appropriate model for AS. A few years later, based on detailed investigation by the same group, this mouse model was strongly put forward as a model for AS [40]. Detailed study in this model suggested that the imprinted proximal region earlier identified in fact should be included in the putative PWS/AS segment. The mouse model showed various features like gait ataxia, abnormal limb clasping, startle response, and hyperactivity. The cerebral hemispheres did not show any gross abnormality or cell loss but cortical thinning was noticed. Reduction in the size of the cerebellum was also shown. Abnormal EEG, a typical feature of AS [63, 64], is also recorded in these mice. Soon after the discovery of *UBE3A* mutations in AS individuals [65, 66], this model was further characterized for the expression of *UBE3A,* and found that the expression of this gene was absent in the hippocampus, cerebellar Purkinje cells, and olfactory bulb (mitral-cell layer) of the mice [67]. This shows that majority of the expression observed in these areas is from the maternal allele. Using RNA *in situ* hybridization, it was shown that the cortex showed reduced levels of the *UBE3A* transcript, while there was no change in the anterior commissure and optic chiasm. This suggests that the *UBE3A* gene has varied expression in different region of the brain. Areas like the cerebral cortex, which show reduced expression, have slight predominance of maternal expression, while optic chiasm and anterior commissure have equal expression from both the maternal and paternal alleles. Imprinting in the AS brain was reported around the same time [24, 68], but Albrecht et al. [67] failed to detect imprinting in the whole mouse brain. Therefore, they looked into different parts of the brain and concluded that *UBE3A* is imprinted only in certain areas of the brain. The absence of *UBE3A* had no effect on the number of Purkinje cells or the overall cytoarchitecture of the brain in UPD mice.

The most widely used model of AS is the *UBE3A* knockout mice. This mouse was generated by a deletion mutation in exon 2 of *UBE3A* gene thereby inhibiting the formation of a functionally active protein [33]. Mice generated were termed wild-type $UBE3A^{m+/p+}$, heterozygous $UBE3A^{m-/p+}$- or $UBE3A^{m+/p-}$ (depending upon the parental inheritance), and homozygous $UBE3A^{m-/p-}$ (null) for the mutation. The maternal deficient heterozygous mice $UBE3A^{m-/p+}$ exhibited reduced brain weight, ataxia,

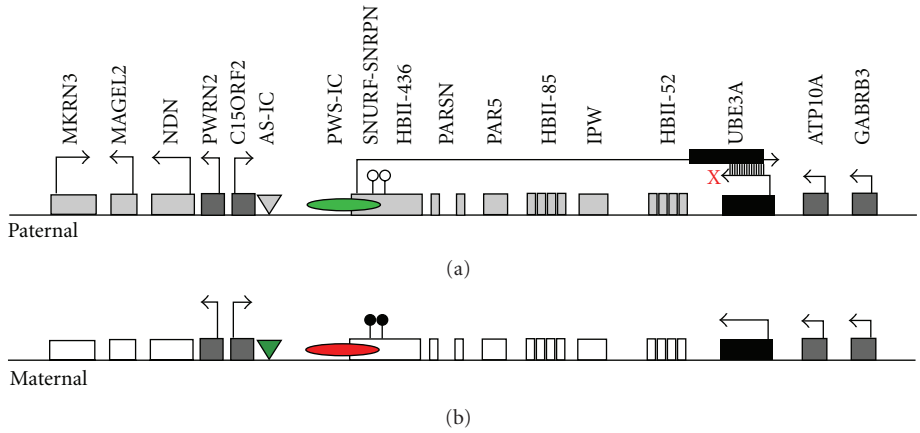

Figure 1: Imprinting map of the human chromosome 15q11-13 region around AS imprinting centre (AS-IC). Paternal and maternal chromosome 15q11-13 regions around AS-IC and PWS-IC are represented in (a) and (b), respectively. Paternally expressed genes (gray boxes), maternally expressed genes (black boxes), maternally repressed genes (white boxes), and biallelically expressed genes (dark gray boxes) are represented with arrows marking transcription start sites. Right arrow indicates gene transcription on "+" strand, whereas left arrow indicates gene transcription on "−" strand. AS-IC (triangle) and PWS-IC (ellipse) are shaded depending on histone modification in the area. AS-IC is dormant (gray triangle) on paternal chromosome, whereas on the maternal chromosome it is acetylated and methylated at H3-lys4 (green triangle), thus active. PWS-IC is active on paternal chromosome (green ellipse) since it is also acetylated and methylated at H3-lys4. However, PWS-IC at the maternal chromosome is methylated at H3-lys9 and repressed (red ellipse). Differentially CpG methylated region (DMR1) in SNRPN exon 1 overlaps with PWS-IC partially. Note that DMR1 on maternal but not paternal chromosome is methylated (black pin). UBE3A-ATS (antisense transcript) originating upstream of SNRPN can either be a degradable complex with *UBE3A* transcript or prevent the extension of *UBE3A* transcript (collision or upstream histone modifications represented by "X").

Table 1: Mouse models of AS and their phenotypes.

Animal models	Associated phenotypes
UBE3A$^{m-/p+}$ mice. Deletion of maternal Exon 2 of *UBE3A* [33–37].	Cognitive and motor deficits and inducible seizures. Loss of *UBE3A* expression in neurons, reduced dendritic spine density and defect in hippocampal LTP.
UBE3A$^{m-/p+}$ mice. Deletion of maternal Exons 15 and 16 of *UBE3A* [38].	Cognitive and motor problems, decreased REM sleep, and abnormal EEG, seizures. Loss of *UBE3A* expression in neurons.
DelUBE3A-Gabrb3$^{m-/p+}$ mice. 1.6 Mb maternal deletion disrupting *UBE3A*, *Atp10a*, and *Gabrb3* loci [39].	Increased ultrasonic vocalization, spontaneous seizures, abnormal EEG, impaired learning and memory. Loss of *UBE3A* expression in neurons.
Mice generated with paternal duplication of central region of chromosome 7 (homologous to the human region 15q11-13) [40].	Abnormal EEG, Gait ataxia, abnormal limb clasping, and startle response, hyperactivity. Loss of expression of *UBE3A* in Purkinje cells, hippocampus and olfactory bulb.
Mice created with maternal deletion of central part of chromosome 7 through inheritable transgene insertion [41].	Behavioural abnormalities are not reported. Mice show imprinted expression of *UBE3A* in cerebellum.
Mice created with paternal duplication of chromosome 7 (corresponding to the region of human chromosome 15q11-13) [42].	Abnormal ultrasonic vocalization, poor social interaction, and anxiety. Reduced *UBE3A* expression in brain.
Mice with imprinting defect mutation (corresponding to human AS-IC) [43].	Behavioural phenotypes are not reported. Reduced *UBE3A* expression in brain.
Mice with large radiation-induced deletion of p30PUb [44].	Behavioural phenotypes are not reported.

Number in the brackets indicates references.

motor impairment, and abnormal EEG pattern. Around 20–30% of maternal-deficient and null mice exhibited audiogenic seizures. The maternal deficient mice also showed context-dependent learning and memory impairment and deficits in hippocampal long-term potentiation. UBE3A expression was imprinted in hippocampus and cerebellar Purkinje cells, and p53 level was increased in the Purkinje cells of *UBE3A*$^{m-/p+}$ mice [33]. This genetic model successfully captured many of the classical features associated with AS and provides a tool to discover molecules and

pathways affected by the absence of UBE3A, mainly the ones responsible for cognitive and motor function.

Detailed immunohistochemical and immunoblot analysis later revealed that *UBE3A* in these mice is imprinted throughout the brain. Various areas of the brain like cortex, striatum, midbrain, and hypothalamus in addition to hippocampus, cerebellum, and olfactory bulbs showed predominant expression from the maternal copy of the chromosome [34, 35, 69]. It was reported that along with the neurons, parvalbumin and calretinin positive GABAergic interneurons also expressed *UBE3A* solely from the maternal allele. Peripheral tissue like liver, heart, and lungs in AS mice showed more than 50% reduction in the levels of *UBE3A* expression, showing that maternal expression was predominant even in the other tissues [69].

Further behavioural characterization in this model showed that $UBE3A^{m-/p+}$ mice have motor deficits suggestive of a dysfunctional cerebellum [70]. A novel finding was that these mice had a different licking behaviour than the wild-type mice, with more number of licks at greater intervals. It is possible that the difference in the lick behaviour is due to the loss of synchrony between breathing and swallowing and correlates with the feeding and swallowing difficulties seen in AS children [19, 71]. Although the motor deficits observed in $UBE3A^{m-/p+}$ mice are thought to be due to dysfunction of cerebellar Purkinje cells, a recent report indicated probable abnormalities in nigrostriatal pathway [33, 72, 73]. The $UBE3A^{m-/p+}$ mice showed reduced number of dopaminergic neurons in the substantia nigra accompanied by poor performance in behavioural paradigms sensitive to nigrostriatal dysfunction [74]. This is further supported by the fact that two patients with AS have been shown to manifest typical features of Parkinson's disease like tremors, cogwheel rigidity, and bradykinesia and were responded to levodopa, which is widely used for the symptomatic treatment of Parkinson disease [75]. However, similar disabling tremor in AS patients also has been treated differently [76, 77].

Lately, there have been major advancements in understanding the molecular basis of the cognitive deficits associated with AS. The level of the inhibitory phosphorylation at Thr305 of the calcium/calmodulin-dependent protein kinase II (CaMKII) in the hippocampus of the $UBE3A^{m-/p+}$ mice was increased leading to reduction in the activity of the protein [36]. The role of CaMKII in the induction of LTP is well established. All the behavioural and learning deficits observed were reversed when a mutation was introduced to block the inhibitory phosphorylation of CaMKII [78]. A very important advancement came with the study of Yashiro et al. [35]. $UBE3A^{m-/p+}$ mice were shown to have impaired experience-dependent synaptic plasticity in the visual cortex. Brief monocular deprivation revealed that $UBE3A^{m-/p+}$ mice do not show ocular dominance plasticity. This impairment is reversible, and late postnatal deprivation of sensory inputs again restores plasticity of the synapses. These observations suggest that absence of *UBE3A* leads to the inability to modify or rearrange synapses as per the requirement in activity-dependent synaptic plasticity. It is hypothesized that this could occur either due to decreased number of excitatory synapses or due to decreased efficiency of neurotransmitter release. The second probability is in turn dependent on the calcium levels and receptor trafficking which very well correlates to the CaMKII levels. It was observed that the visual cortical circuitry and the retinotopic map are formed normally, but the basal dendrites show reduced spines in $UBE3A^{m-/p+}$ mice [37]. This was consistent with the earlier studies [34]. Absence of *UBE3A* plays a crucial role in the postnatal experience driven period [35, 37]. This correlates with the AS patient history of normal birth but delayed developmental milestones. Cognitive development and development of speech are events that depend on the external sensory experience [79]. Failure of these important processes in AS patients could mean that *UBE3A* is indeed required for remodeling of the circuitry. The work so far emphasizes that *UBE3A* is not directly involved in circuit formation but is crucial in experience-dependent synaptic remodeling.

Recently, the exact role of *UBE3A* in experience-driven synaptic plasticity was elucidated at the molecular level [54]. UBE3A mRNA and protein levels are regulated by synaptic activity. UBE3A levels are increased after treatments with kainic acid, KCl, NMDA (N-methyl-D-aspartic acid), glutamate, and bicuculline in primary neuronal cultured cells, while novel environment increases the levels of *UBE3A* in mice brain compared to standard laboratory caged mice. The promoter of *UBE3A* gene is under the control of activity-dependent transcription factor MEF2. The increase in levels of UBE3A with glutamate stimulation and decrease with inhibitors of glutamate receptors clearly puts forth the role of UBE3A in synapse development. Many substrates of UBE3A have been discovered but none were directly implicated in the loss of synaptic plasticity. HA-ubiquitin transgenic mice were crossed with $UBE3A^{m-/p+}$ mice, and the proteins that showed reduced ubiquitination were studied. Sacsin was one of the substrates of UBE3A as it showed reduced ubiquitination in knockout mice as compared to wild type. Sacsin is mutated in Charlevoix-Saguenay spastic ataxia, a disorder similar to AS [80]. It is mainly expressed in the neurites of the neurons [81]. The exact role of sacsin in modulation of synapses remains unknown. But sacsin could be one of the causes of the motor deficits seen in AS patients, considering its involvement in disorders with ataxia. Arc was another substrate discovered, which is responsible at least in part for the rigidity seen at the UBE3A deficient synapses. Arc regulates surface expression of AMPARs (alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionate receptors). Increased Arc expression leads to decreased surface AMPARs while decrease in Arc levels leads to increase in the AMPARs at the surface. Arc promotes the endocytosis of GluA1 type of AMPARs. Lack of *UBE3A* leads to accumulation of Arc, which subsequently results in increased internalization of the AMPARs. UBE3A regulates the surface expression of AMPARs through ubiquitination and proteasomal degradation of Arc. This effect is reduced in presence of catalytically inactive mutants of UBE3A. The decrease in the expression of AMPARs affects the synaptic transmission. There is a reduction seen in the AMPA/NMDA current ratio, which is due to the loss of AMPARs as there

was no change in NMDARs. The RhoGEF ephexin5 was also discovered as an UBE3A interacting protein. It has a role in restricting the neuron to form only the required number of synapses [54, 56].

Mice expressing UBE3A-YFP fusion protein exclusively from the maternal copy is a very promising tool to carefully study the microscopic abnormalities in AS [34]. Study focusing on the cellular localization of UBE3A helped to elucidate the probable functions of this protein. UBE3A-YFP fusion protein localized mainly in the nucleus with detectable expressions in the cell soma and dendrites. The UBE3A protein was found in the pre and postsynaptic compartments and was localized in the growth cones of hippocampal neurons in primary culture [34, 69]. This mouse model showed biallelic expression of *UBE3A* in GFAP-positive astrocytes lining the ventricular area. In other brain regions GFAP-positive astrocytes seems to exhibit imprinted expression [34]. Although the absence of UBE3A did not affect dendritic branching in any of the imprinted neurons, a detailed microscopic study showed that the dendritic spines had abnormal structures. In the absence of any gross cellular or structural changes in the brain, it is hypothesized that absence of UBE3A is necessary either for the formation or maintenance of the dendritic spines. This is probable since the activity of phospho CaMKII is reduced in maternal deficient animals, and CaMKII is known to help in activity-dependent spine formation. This correlates very well with the observations made in a pathological study in AS brain as well [82]. Further investigation in this mouse model can give major insights into the role of UBE3A during synaptogenesis even at a single synapse level.

UBE3A is shown to interact with and coactivate nuclear steroid hormone receptors [45, 46, 83]. Absence of UBE3A renders both male and female mice less fertile compared to the wild-type controls [47]. *UBE3A* null male mice show reduced testis size, lesser sperm count, decreased sperm ability to penetrate ova and reduced prostate size. In *UBE3A* knockout female mice, there is reduced oocyte production and smaller ovary size. All these findings indicate that coactivator role of UBE3A is important in reproductive function. But whether the loss of coactivator function of UBE3A is associated with any abnormalities in brain function leading to AS are not very clear. Recently, we have shown that the defective glucocorticoid hormone receptor signaling in $UBE3A^{m-/p+}$ mice brain could lead to increased stress and anxiety in these mice. These mice also exhibited decrease in the number of parvalbumin-positive GABAergic interneurons in their hippocampus [84].

Yet another mouse model of AS was generated by inactivating the exons corresponding to the human exons 15 and 16 from the *UBE3A* gene [38]. A LacZ reporter was introduced after the deletion site to detect the expressing protein albeit truncated. The expressed UBE3A does not show ligase activity, and the β-galactosidase activity is seen in the brain wherever maternal copy expresses the truncated protein. This mouse model showed motor deficits, learning and memory impairments, and an abnormal EEG characteristics of AS, but seizures were absent in this model. UBE3A was imprinted in the hippocampus, basket cells in the cerebellum, as well as in the frontal cortex. Cells in the ventricular ependyma showed LacZ expression both in maternal and paternal *UBE3A* deficient mice, which is consistent with the observation that the ventricular GFAP positive cells express biallelic *UBE3A* [34]. This model confirmed the finding that imprinting is specific to neurons and not astrocytes. Interestingly, it was observed that the progenitor cells do not show imprinted expression, but imprinting is acquired by embryonic day 10 in mouse. Neurons specifically expressed the maternal sense *UBE3A,* while the antisense *UBE3A* was expressed only from the paternal copy [85]. Surprisingly, there was no imprinted expression in the cerebellar Purkinje cells which is a deviation from the other studies [33, 34, 69]. As the protein is truncated only in the C-terminal HECT domain, the transcriptional coactivator function is still might be active in the animals. Absence of imprinted expression in Purkinje cells is a major drawback of the model and could be a reason for unaltered p53 levels. Interestingly, this mouse model showed disrupted sleep wake cycle seen in most of the AS children [2, 86]. Using this mouse model, another group [87] has shown that the deficiency of *UBE3A* leads to impaired neurogenesis and changes in the hippocampal plasticity. The immediate early genes *c-fos* and *arc*, associated with neuronal long-term plasticity and memory formation, showed reduced expression in the maternal deficient mice brain.

A knockout mouse model of the $GABA_A$ (γ-amino butyric acid) receptor $\beta 3$ subunit (GABRB3) showed most of the behavioural features like epilepsy, abnormal EEG pattern, learning deficits, and poor motor coordination [88]. Absence of $\beta 3$ subunit leads to neonatal deaths and cleft palates in the animals. The deletions in *GABRB3* are heritable, but since this gene is not imprinted in the brain, *GABRB3* only adds to the phenotypic characteristics and is not a direct cause of AS [89]. Mutation in *UBE3A* is sufficient to show the cardinal features of AS, although deletion of *GABRB3* might contribute to a more severe phenotype [88, 90]. A new mouse model of AS, has been reported recently that tries to replicate the most prevalent form of the syndrome [39]. A 1.6 Mb region spanning from *UBE3A* to *Gabrb3* was deleted to generate this mouse model [39]. Homozygous mutations showed phenotype similar to the *Gabrb3* null mutant. These homozygous null mice showed cleft palate and lethality around the time of birth. The maternal deficient mice of this region, on the other hand, showed no developmental abnormality. They showed spontaneous seizure activity and abnormal EEG. Like the earlier $UBE3A^{m-/p+}$ mice, these mice also showed impairment in motor activity and learning and memory. The anxiety related behavior was assessed in these mice and found that maternal deficient mice spent more time in dark areas as compared to the wild-type or paternal deficient mice. Maternal deficient mice with deletion of this region exhibited contextual fear and spatial learning deficits. These mice also showed abnormal pattern of ultrasonic vocalizations [39]. These may correlate with the lack of speech and impaired communication seen in AS patients.

Another mouse model was generated with an inheritable transgene insertion (Epstein-Barr virus Latent Membrane

Protein 2A, *LMP2A*) into the central part of chromosome 7 of mouse [41]. The deletion created by transgene insertion led to formation of either PWS or AS model in a parent-of-origin manner. Inheritance of the deletion from the paternal allele led to formation of PWS, while maternal transmission led to an AS model. *UBE3A* was imprinted in the cerebellum in these mice. Behavioural studies were not reported in this model. Around 70% of the cases in humans are due to deletions in the 15q11-13 region. This model, therefore, represents the widely prevalent condition of AS and, therefore, should be characterized for better understanding of disease pathogenesis and developing therapeutics. Several other mouse model have been generated based on AS imprinting defect mutation [43, 91], radiation-induced mutation removing multiple genes including *UBE3A* [44], and duplication of the AS-PWS locus [42]. Although all of these mouse models reported reduced expression of *UBE3A*, their neurobehavioral phenotype are not well characterized. A list of AS mouse models are shown in Table 2. Interestingly, mice over expressing triple the dose of *UBE3A* showed autism traits like impaired communication, defective social interaction, and increased repetitive stereotypic behavior [92]. These findings along with others [54] clearly indicate that UBE3A plays a very important role in synaptic function, and its altered function could be linked with both AS and autism. In addition to these mouse models, human induced pluripotent stem cell model of AS or mouse differentiated embryonic stem cell model of AS were also developed [93, 94]. These models will be useful to understand the developmental timing and mechanism of *UBE3A* silencing in neurons as well as disease biology.

5. Fly Models of AS

Drosophila models have also been generated in order to understand the pathogenesis of AS. d*UBE3A*, the homologue of human *UBE3A*, is deleted imprecisely such that the corresponding protein is not formed [95]. Lack of d*UBE3A* is not lethal and the flies born show no morphological abnormality. However, they do show motor abnormalities when tested on motor specific tasks. They have impaired long-term memory formation and abnormal circadian rhythms. Missense mutations analogous to the ones found in AS patients were also used to study their effect. These catalytically inactive mutants show the same behavioral deficits like the d*UBE3A* null flies. Very importantly, this report studies the effect of over expression of d*UBE3A*. The gain-of-function model in this case is particularly informative since the deletion of d*UBE3A* does not lead to any morphological abnormality. Over activity of d*UBE3A* in general is lethal to the flies. Promoter specific expression in the eyes and wings leads to aberrant morphology of the organs.

Another fly model corroborated the findings of mouse models of the disease [96]. The group studied RNAi d*UBE3A* flies in addition to the deletion mutants. In an interesting approach, they also studied flies by mosaic analysis with a repressible cell marker (MARCM) in which a single neuron is injected with GFP labeled genetic mutation while the surrounding neurons continue to have a wild genotype. Using these advanced techniques, they found that d*UBE3A* is necessary for dendritic arborization in a cell autonomous manner. Absence of d*UBE3A* leads to reduced formation of terminal dendritic branching. Surprisingly, over expression of d*UBE3A* also causes reduction in dendritic branching in the fly, suggesting that the levels of *UBE3A* are critical in formation of the dendrites. The fly, model would be useful in identifying and characterizing the substrates of UBE3A and understanding the disease pathogenesis.

Table 2: Cellular functions regulated by UBE3A.

Identified substrates	Cellular functions
HHR 23A, Src family tyrosine kinase Blk, P53, P27, PML tumor suppressor [48–52].	Cell growth and differentiation
Steroid hormone receptors like androgen receptor, glucocorticoid receptor, mineralocorticoid receptor [45, 46, 84].	Coactivator of steroid hormone receptors
Arc, RhoA-GEF ephexin5, Rho-GEF Pbl/ECT2 [54–58].	Synaptic function and plasticity
Polyglutamine proteins, α-synuclein, misfolded proteins [59–61].	Cellular protein quality control

Number in the brackets indicates references.

6. Conclusions and Future Perspectives

It is evident from the existing literature that the loss of expression of maternal-inherited *UBE3A* is primarily responsible for AS, although we cannot completely rule out the possibility of other disease-modifying gene like *GABRB3*. Dysfunction of UBE3A is sufficient to produce phenotypes resembling to AS in different animal models. Most extensively used *UBE3A*-maternal deficient mice replicate many essential features of AS including cognitive and motor deficits. This mouse model provided enormous insight in understanding the disease pathogenic mechanism. Clinical features of AS like cognitive and motor deficits, sleep disturbance, feeding difficulties, and altered synaptic plasticity have a molecular or electrophysiological correlate due to the studies performed in animal models. A recent clinical study reported that specific EEG pattern could be an important biomarker of AS and might indicate the underlying genetic cause [18]. This can be further tested in various mouse models to validate the results. Most interestingly, UBE3A-maternal deficient mice show significant impairment in activity-dependent synaptic plasticity indicating the role of UBE3A in regulation of synaptic function and plasticity [54]. The experience-dependent synaptic plasticity is shown to be modulated by number of ways [97]. Therefore, this novel role of UBE3A can be exploited further for possible therapeutic intervention of AS. In fact one report demonstrated neuregulin-ErbB4 signaling is associated with abnormal synaptic plasticity in $UBE3A^{m-/p+}$ mice, and inhibitors of ErbB reverse the contextual fear memory deficits [53]. The cognitive deficits observed in $UBE3A^{m-/p+}$ mice were also rescued upon adeno-associated virus vector-mediated expression of *UBE3A* into the brain [98].

Since the paternal copy of *UBE3A* is epigenetically silenced in neurons, it is possible that the reactivation of paternal expression could be an exciting therapeutic strategy. Clinical trials were conducted in AS children using methylation-promoting dietary supplements (creatine, folic acid vitamin B12, metafolin, and betaine) in order to up-regulate the *UBE3A* expression (by suppressing the expression of UBE3A antisense transcript). Unfortunately, there were no significant improvements of intellectual disabilities or abnormal EEG patterns in AS children [99, 100]. Interestingly, a very recent report has demonstrated that topoisomerase inhibitors activate the dormant expression of UBE3A in neurons [101]. This is an exciting development. However, treatment of such drugs could also alter the expression of other genes and, therefore, lead to other complications. Further studies are required to investigate possible role of these topoisomerase inhibitors in the recovery of behavioral abnormalities in animal models. Most preferable strategy could be targeted knockdown of the antisense transcript. Enriched environment or neuronal activity (that can trigger experience-dependent synaptic development) also has been demonstrated to increase the expression of *UBE3A* [54]. Therefore, various cognitive training paradigms in early developmental stage could potentially improve cognitive and motor deficits in AS children by increasing the expression of UBE3A. All together, the field is now passing through an exciting phase, and we all are hoping for a major breakthrough in therapeutic intervention of AS.

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Review Article

Pathological Plasticity in Fragile X Syndrome

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Deficits in neuronal plasticity are common hallmarks of many neurodevelopmental disorders. In the case of fragile-X syndrome (FXS), disruption in the function of a single gene, *FMR1*, results in a variety of neurological consequences directly related to problems with the development, maintenance, and capacity of plastic neuronal networks. In this paper, we discuss current research illustrating the mechanisms underlying plasticity deficits in FXS. These processes include synaptic, cell intrinsic, and homeostatic mechanisms both dependent on and independent of abnormal metabotropic glutamate receptor transmission. We place particular emphasis on how identified deficits may play a role in developmental critical periods to produce neuronal networks with permanently decreased capacity to dynamically respond to changes in activity central to learning, memory, and cognition in patients with FXS. Characterizing early developmental deficits in plasticity is fundamental to develop therapies that not only treat symptoms but also minimize the developmental pathology of the disease.

1. Introduction

The capacity for appropriate, dynamic, and effective neuronal plasticity is essential for the normal development and function of mature neuronal networks. Neuronal plasticity can be defined as the ability of a neuron or network to functionally alter in response to changes in input or activity. These alterations can occur at the synaptic, neuromodulatory, cell intrinsic, or circuit level and underlie many of the diverse functions within the central nervous system (CNS) such as the development and refinement of connections, learning and memory, regulation of behavior, and cognition. Problems arise in these functions when plasticity mechanisms operate abnormally and neuronal networks improperly develop in response to activity-dependent experience. Accordingly, abnormal neuronal plasticity is a hallmark in many developmental and cognitive disorders including fragile-X syndrome (FXS), the most prevalent inherited cause of intellectual disability and autism spectrum disorder (ASD) [1–3].

FXS is an X-linked, single gene disorder caused by dysfunction in the transcription of the *FMR1* gene that codes for fragile X mental retardation protein (FMRP) [4, 5]. The syndrome results from an irregular expansion of CGG repeats in the 5′ untranslated region of the *FMR1* gene. Greater than ~200 repeats of this trinucleotide sequence promote hypermethylation and chromatin condensation upstream of the coding region causing transcriptional silencing of *FMR1* and a subsequent lack of expression of its protein product FMRP [5]. FMRP is expressed in a variety of mammalian tissues but is highly concentrated in the brain and testes [6–10]. In the brain FMRP is located both pre- and postsynaptically and functions mainly as a translational regulator, especially at the synapse [9, 11–15]. It is known to associate with a myriad of neuronal mRNA molecules and an estimated 8% of synaptically targeted mRNA [16–18]. Studies also show that FMRP can function in the nucleus as an mRNA chaperone, binding specific mRNA as part of a ribonucleoprotein (RNP) complex to transport it from the nucleus to the appropriate cytosolic location for protein translation [19]. In humans with FXS, the loss of FMRP results in a variety of neurological symptoms widely associated with imbalances in excitation/inhibition and dysfunctional plasticity in critical brain regions such as the cortex, hippocampus, and amygdala. These symptoms include mild-to-severe intellectual disability, social anxiety and autistic

behaviors, increased incidence of epilepsy, attention-deficit hyperactivity disorder (ADHD), and sensory hypersensitivity [2, 20–24]. Shortly after the pathological CGG expansion in the *FMR1* gene and the absence of its protein product were identified as the source of the disorder [4, 5], an *Fmr1* KO mouse model of the disease was generated to study the consequences of the loss of FMRP in FXS neuronal networks [25]. These mice display phenotypes consistent with the symptoms of FXS in humans including problems with learning and memory, social interaction, hyperactivity, hypersensitivity, and susceptibility to seizures [19, 25–27]. In addition, early studies of FMRP indicated that the protein was highly expressed in the dendritic shafts and spines of neurons. These observations along with the observation of a higher density and higher proportion of elongated dendritic spines [28–30] in neurons from both humans with FXS and the *Fmr1* KO mouse led researchers to hypothesize that FXS might primarily be a synaptic plasticity disorder. Subsequently, researchers in Bear's group published the first evidence of pathological plasticity in excitatory hippocampal synapses of the *Fmr1* KO mouse in the form of exaggerated protein translation- and group-I-metabotropic-glutamate-receptor- (GpI mGluR-) dependent long-term depression (LTD). This evidence led to the proposal of the "mGluR theory of FXS" [15, 31] that identifies FMRP as a key downstream regulator of GpI mGluR activation (specifically mGluR5). The theory has been strengthened in recent years by evidence that mGluR5 antagonists or genetic reduction of mGluR5 expression can at least partially rescue both synaptic and behavioral phenotypes in *Fmr1* KO mice [26, 32–40]. However, more detailed examinations into the role of FMRP in controlling activity-dependent protein translation reveal a complex role of the protein in the regulation of activity dependent synaptic, cell intrinsic, and homeostatic plasticity.

The purpose of this paper is to summarize studies that explore the role of FMRP in the regulation of these types of plasticity and their deficits in FXS. We review evidence for the extensive role of GpI mGluRs as well as highlight recently discovered mGluR-independent roles of FMRP. Finally, we discuss how these aberrant processes affect development of neuronal networks in FXS. Our discussion will focus on how pathological plasticity in the disorder effectively reduces the range and stability of responses FXS networks can have in response to changes in activity and/or experience. We emphasize promising areas of study that may advance therapies to alter the course of the pathology and partially restore an effective dynamic range for plasticity in diseased networks. These advances may ultimately reduce the severity of the syndrome and improve responses to current and future therapies for this disease and related autism spectrum disorders.

2. The mGluR Theory and Synaptic Plasticity Mechanisms in FXS

Synaptic plasticity is commonly associated with functional changes of pre- and postsynaptic neuronal elements following patterned activity that discretely strengthen (potentiation) or weaken (depression) synapses. FMRP was first connected to synaptic plasticity when researchers identified the protein as upregulated in response to the GpI mGluR agonist 3,5-dihydroxyphenylglycine (DHPG) [41]. This compound induces GpI mGluR-dependent and translation-dependent LTD in the CA1 region of the hippocampus. In this form of LTD, ionotropic glutamate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPARs), undergo internalization decreasing synaptic strength in response to low-frequency stimulation or DHPG [42]. Soon after the discovery, Huber and colleagues showed the first evidence of pathological plasticity in FXS in the form of enhanced GpI mGluR-dependent LTD in CA1 slices from *Fmr1* KO mice [15]. Because FMRP functions as a negative regulator of translation [12, 14] and is upregulated in response to mGluR activation [41], "the mGluR theory of FXS" was proposed. According to the theory, AMPAR receptor internalization and synaptic destabilizing protein-dependent processes go unchecked in mice lacking functional FMRP. Therefore, protein synthesis related to mGluR activation overall is dysregulated [15, 31, 43].

Since the initial proposal of the mGluR theory, mGluR-dependent and -independent synaptic plasticity mechanisms have been thoroughly evaluated in the *Fmr1* KO mouse. The results of these studies reveal highly region- and modality-specific dysfunction in postsynaptic plasticity mechanisms. In the cerebellum of *Fmr1* KO mice, for instance, mGluR1-dependent LTD is enhanced similar to hippocampal area CA1 [44]. However, N-Methyl-D-aspartic acid (NMDA) receptor-mediated non-mGluR-dependent long-term potentiation (LTP) is not affected in hippocampal circuits in these mice [15, 45–47] revealing the specificity of FMRP for regulating mGluR-dependent plasticity. In other regions such as deep somatosensory cortical layers in which non-mGluR-dependent and mGluR-dependent LTP mechanisms coexist, mGluR-dependent LTP is not enhanced but absent [48]. Furthermore the mGluR5 selective antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) cannot rescue this phenotype in *Fmr1* KO mice [48]. Similar deficits in mGluR-dependent LTP were revealed in the basolateral amygdala of these mice [49]. Although seemingly contradictory to the "overactivation of mGluR mediated protein synthesis" hypothesis put forth by the mGluR theory, these results might be explained by an upregulation of mGluR-dependent processes during development that may have washed out and/or eliminated this type of plasticity from the particular synapse. Network alterations as a result of the loss of FMRP or enhancement of mGluR signaling during development could also explain the attenuation. In the basolateral amygdala deficits in mGluR-dependent LTP were accompanied by decreases in basal synaptic transmission [49]. In accordance with these possible network alterations, similar attenuations in non-mGluR-dependent LTP exist in areas like the anterior cingulate cortex (ACC) and lateral amygdala in *Fmr1* KO mice [50]. The role of FMRP and mGluRs in development is further discussed in the section below. Alternatively, LTP attenuation could result from the upregulation of other proteins normally regulated by FMRP that affect synaptic plasticity. One example is the dendritically located voltage-gated potassium channel Kv4.2,

which regulates the induction of NMDA receptor-dependent LTP by theta burst stimulation. This channel is overexpressed in the dendrites of CA1 pyramidal cells in young *Fmr1* KO mice, and these mice show deficits in this type of LTP. Blocking Kv4.2 with heteropodatoxin HpTx2 restores LTP in *Fmr1* KO synapses [51].

Recent studies also characterize deficits in presynaptic plasticity in FXS related to the loss of FMRP in presynaptic terminals. Using isolated sensory-to-motor neuron cocultures derived from *Aplysia*, Till and colleagues (2011) knocked down the *Aplysia* homolog of FMRP (ApFMRP) in either the presynaptic or postsynaptic neuron and evoked LTD with pulses of FRMF amide. They identified enhanced LTD consistent with mGluR-dependent hippocampal LTD if the FMRP knockdown was applied to the postsynaptic cell *or* the presynaptic cell indicating a crucial role of presynaptic protein regulation to regulate LTD [52]. Another study examined presynaptically regulated short-term depression (STD) in *Fmr1* KO hippocampal excitatory synapses. Neurons from *Fmr1* KO mice exhibited enlarged vesicle pools and increased vesicle turnover that correlated with reduced STD when compared to wild-type mice. Consequently, these synapses showed increased responses to replicated high-frequency place field stimuli. These data indicate a strong presynaptic requirement for regulation by FMRP in this type of processing [53].

3. Neuromodulatory Endocannabinoid Plasticity in FXS

Region-selective and mechanism-dependent alterations in plasticity in FXS are not exclusive to disruptions in excitatory neurotransmission. Exaggerated signaling through mGluR5 receptors can alter the strength and duration of inhibitory neurotransmission in a form of chemical plasticity of excitatory circuits. GABA release is modulated by both membrane depolarization and through presynaptic receptors that act to reduce the amount of neurotransmitter in the synapse [54–56]. One mechanism involves the synthesis and release (or mobilization) of endocannabinoids—endogenous neuromodulatory lipids that target type 1 cannabinoid receptors (CB1Rs) on the presynaptic terminals of inhibitory interneurons [55]. Activation of Gp1 mGluRs enables the mobilization of endocannabinoids in the postsynaptic neuron and retrogradely modulates GABA release through a mechanism known as depolarization-induced suppression of inhibition (DSI) [57]. The binding to CB1Rs on the presynaptic terminal of the inhibitory interneuron leads to a transient suppression of voltage-gated calcium channel activity thus inhibiting GABA release. These mechanisms require heightened neuronal activity—an environment that exists in brain circuitry of *Fmr1* KO mice [7, 58]. In the CA1 region of the hippocampus, enhanced mGluR signaling leads to excessive endocannabinoid mobilization in *Fmr1 KO* mice and enhanced suppression of inhibitory transmission [59]. This increase in the suppression of inhibition is proposed as a potential contributor to the hyperexcitable phenotype in the *Fmr1* KO hippocampus [59]. In hippocampal circuitry, endocannabinoid modulation of DSI likely involves specific inhibitory circuits relegated to perisomatic targeting interneurons [60]. Therefore, with respect to endocannabinoid mobilization in the FXS brain, the loss of FMRP may selectively affect specific inhibitory circuits and leave other circuits intact.

In the cerebral cortex endocannabinoid mobilization can retrogradely modulate the release of presynaptic GABA [61] or act to hyperpolarize a specialized type of inhibitory interneuron known as the low threshold spiking (LTS) cell through endogenous autocrine release [54]. In this mechanism, sustained action potential activity activates voltage-gated calcium channels for the influx of calcium in LTS interneurons that triggers the synthesis of endocannabinoids. The binding of endocannabinoids to CB1Rs expressed within the same neuron function to activate G-protein-coupled inward-rectifying potassium (GIRK) currents, resulting in a prominent hyperpolarization that can last for several minutes [54]. This mechanism is known as slow self-inhibition (SSI) and is specific to cortical LTS interneurons [54, 62]. While there is no known abnormality in FXS for this type of interneuron, it is likely affected in FXS. Both Group I and Group II mGluRs selectively activate cortical LTS interneurons causing sustained action potential firing during drug application [63, 64]. Our studies show that DHPG-induced mGluR activation of LTS interneurons is abnormal in *Fmr1* KO mice [65]. mGluR activation of LTS interneurons in the developing and mature brain is critical for the proper synchronization of cortical excitatory neurons at behaviorally relevant frequencies [63, 64, 66, 67]. Therefore, alteration of mGluR signaling in this specific type of interneuron likely has wide-reaching ramifications in developing and mature cortical networks.

4. Intrinsic and Homeostatic Plasticity in FXS

The extent of pathological plasticity related to excessive GpI mGluR signaling is not restricted to the synapse. Upregulation of mGluR-regulated processes can fundamentally alter the excitability of the neuron and subsequently modify network dynamics. In 1998, Wong and colleagues demonstrated that GpI mGluR stimulation with the agonist DHPG increased epileptiform burst frequency and duration in hippocampal area CA3 pyramidal cells [68, 69]. These bursts are related to ictal discharges during seizures [70]. They showed that the increase in burst duration was protein synthesis-dependent because the prolongation of bursts persisted after agonist washout and did not occur in the presence of the protein synthesis inhibitors anisomycin or cycloheximide [69]. Subsequent investigations have further characterized the GpI mGluR-mediated prolonged discharges to occur in area CA3 without blockade of GABA receptors [71, 72], to resist generation by repeated glutamatergic synaptic activation alone (without exogenous agonist, i.e., DHPG)

[73], and to require GpI mGluR-dependent mRNA translation by way of the tyrosine kinase-extracellular signal-regulated kinase (ERK) 1/2 signaling pathway [72, 74]. Studies implicate a key voltage-dependent cation current, $I_{mGluR(V)}$, as a mechanism underlying GpI mGluR-dependent epileptogenesis [74, 75]. $I_{mGluR(V)}$ upregulation requires phospholipase C β1, outlasts acute mGluR activation, is protein synthesis-dependent, specifically tyrosine kinase-ERK signaling pathway-dependent, and blockade of the current suppresses DHPG-induced epileptogenesis [74]. $I_{mGluR(V)}$ is a persistent current with a threshold of around -65 mV (near resting potential) and reversal potential at approximately -10 mV [74, 76, 77]. Its activation induces long bursts of action potentials (up to 12 seconds) and creates a bistable resting membrane potential in CA3 pyramidal cells. Together these properties along with the recurrent synapses of the CA3 network produce epileptiform discharges when $I_{mGluR(V)}$ is sufficiently activated [74, 75].

Since FMRP is a central regulator of the ERK pathway [72, 74, 78], its presence is crucial to the control of $I_{mGluR(V)}$ activation. Although synaptic activation of GpI mGluRs alone is insufficient to produce $I_{mGluR(V)}$-dependent synchronized bursting in wild-type CA3 pyramidal cells [74, 79], these discharges can be induced in *Fmr1* KO hippocampal slices by upregulating glutamatergic transmission alone (via GABAa receptor blockade) without the addition of a GpI mGluR agonist like DHPG [74, 80, 81]. In effect, the *Fmr1* KO CA3 pyramidal cell is predisposed to a persistent activation of $I_{mGluR(V)}$ that thereby renders the CA3 network susceptible to plastic adjustments in favor of the generation of prolonged epileptiform discharges [74, 80, 81]. Moreover, this maladaptive plasticity may be accentuated further by the GABAergic deficits known to exist in the mature *Fmr1* KO hippocampus and elsewhere [7, 82, 83]. Taken together, this combination of deficits produces a network that is likely more susceptible to hyperexcitability and epileptogenesis when faced with relatively normal increases in neuronal activity. Evidence from audiogenic seizure behavioral assays suggests that this susceptibility underlies decreased seizure threshold in *Fmr1* KO mice and seizures in humans with FXS [27, 36].

The lack of FMRP in the above case likely perturbs the homeostatic balance that translational repression would have on the local increase in expression of $I_{mGluR(V)}$ allowing excitation to spread unchecked from the synapse to the whole cell and network. FMRP has recently been implicated in another form of homeostatic plasticity, *mGluR-independent*, retinoic-acid- (RA-) dependent synaptic scaling. Synaptic scaling is an increase in synaptic strength in response to a prolonged reduction in activity. Observation of the phenomenon is usually achieved *in vitro* by blocking synaptic activity and NMDA receptors with tetrodotoxin (TTX) and aminophosphonovalerate (APV), respectively. This type of plasticity is fundamental for perfecting neuronal connectivity, stabilizing the network, and setting the operational range for coding by the network [84, 85].

RA synthesis increases in response to reductions in activity and crucially regulates synaptic scaling by inactivating the translational repressor retinoic acid receptor α (RARα) [86]. The release of this repressor allows synthesis of key proteins required to strengthen the synapse [87]. These proteins include the AMPAR components GluR1 and GluR2 which serve to strengthen the synapse in response to activity-dependent RA signaling [87, 88]. Using hippocampal cultures Soden and Chen (2010) determined that FMRP is required for the RA-dependent increases in AMPAR insertion and synaptic strength. Cultures from *Fmr1* KO mice did not show synaptic scaling but viral introduction of FMRP into *Fmr1* KO cells restored synaptic scaling in response to RA. The researchers subsequently used modified FMRP constructs to show that FMRP binding to mRNA is required to reduce elevated protein synthesis and induce scaling by upregulation of AMPARs. In addition they showed that homeostatic scaling requires FMRP-directed interaction with actively translating ribosomes to upregulate AMPAR insertion. Therefore both FMRP binding to mRNA and FMRP-directed interaction with active ribosomes are necessary to upregulate AMPARs in synaptic scaling, while mRNA binding alone is sufficient to downregulate increased protein synthesis (presumingly resulting from GpI mGluR overactivation) [88].

These data therefore support a dual role for FMRP, first, in translational suppression at the synapse regulating mGluR-dependent Hebbian plasticity and, second, in homeostatic translation induction in response to decreases in activity. Although these roles seem contradictory, they are performed by different mechanisms and therefore may act in concert to dynamically regulate networks. Since homeostatic plasticity sets the dynamic coding range of the network and stabilizes and balances activity levels [84, 85], deficits in this plasticity could further weaken already compromised Hebbian plasticity at the synapse in FXS by failing to maintain the strength of established connections [88]. Deficits in plasticity are regionally diverse in FXS (see above); therefore interactions between synaptic and homeostatic mechanisms in different regions likely result in varied alterations in the operational range and coding capacity of the network. In the hippocampus, for instance, enhanced mGluR-dependent LTD lowers coding capacity, and faulty homeostatic mechanisms may exacerbate the problem by shrinking the range of activity to which the network would respond. These issues loom larger when we consider an understudied question in the field: how does aberrant plasticity effect the establishment of compromised FXS networks during development? The answers to this question may provide the necessary insight to develop therapies to lessen the severity of the disease through earlier therapeutic intervention and improve lifelong response to treatments.

5. Pathological Plasticity in the Development of FXS Networks

The same kinds of plasticity that govern learning, memory, and cognition in the mature network refine the developing network especially during developmental critical periods. Critical periods are discrete time windows during which

the connectivity of a developing network can be adjusted and refined [84, 89, 90]. After the critical period closes, opportunities for extensive network alterations drop tremendously, and experience no longer modifies networks to the same extent [84, 89, 90]. We can empirically recognize these decreases in plasticity capacity with age when we try to learn new skills like playing a musical instrument after childhood and young adulthood. Critical periods have been especially studied in the cortex and present in a hierarchical fashion [90]. That is, primary cortical sensory areas tend to have earlier critical periods than integrative cortical centers. Disruptions during the critical period of network development can drastically and *permanently* alter the ability of the network to accurately respond to normal activity resulting in irregular sensory processing. Hubel and Wiesel's pioneering studies in the primary visual cortex of the cat notably indicated the permanent loss of visual acuity in adulthood of an eye deprived of experience in the visual critical period [91]. In humans, congenitally deaf children receiving cochlear implants develop hearing and speech most successfully if they receive the implant before ~7-8 years old [92]. Similarly, monaural deprivation in rats only results in interaural imbalance and tonotopic cortical map reorganization if deprivation occurs in young animals versus adult rats [93]. Critical periods for cortical map development have been characterized in the somatosensory system of rodents [94, 95] and the human [96] and animal visual system as well (reviewed in Berardi et al. [97]).

Patients with ASD of various etiologies generally experience deficits in sensory processing consistent with disrupted critical periods leading some to postulate that autism is a "critical period disorder" [89]. FXS is no exception. For example, tactile defensiveness, or hypersensitivity to a normally mild stimulus, is common in FXS [98, 99], and ocular dominance plasticity in response to monocular deprivation is disrupted in *Fmr1* KO mice [26]. Although not yet extensively studied, several lines of evidence indicate that the pathological plasticity mechanisms and associated deficits discussed in this paper are prime candidates to affect critical periods of FXS network development.

Firstly, FMRP and GpI mGluRs are expressed early in development and participate in activity-dependent processes. FMRP is expressed embryonically in the human and mouse [6, 10, 25, 100–102], and its expression in sensory cortex is regulated by neuronal activity, that is, whisker movements [103, 104]. As detailed above, FMRP has dual pre- and postsynaptic roles at the synapse to regulate activity-dependent plasticity. GpI mGluRs are also developmentally expressed in rodents and humans [105, 106]. Besides activity-dependent synaptic plasticity, these mGluRs regulate many early developmental processes including cell proliferation and survival of neural progenitors [105, 107, 108] and laminar organization of developing cortex through expression in cortical plate Cajal-Retzius cells [109].

Secondly, synaptic balance in FXS networks is faulty. The range and dynamics of plastic mechanisms are severely compromised in the ability to code experience/activity-dependent changes (Hebbian) and maintain those changes (homeostatic) as described above. In addition, either subsequently to or concurrently with excitatory transmission problems, GABA network maturation is crucially disrupted in a region-specific manner (reviewed in Paluszkiewicz et al. [110]). Key regions such as the hippocampus, cortex, striatum, and amygdala display up- or downregulations of GABA receptors, glutamic acid decarboxylase (GAD65/67), GABA transporters, and GABA synthesis and release depending on the particular network [7, 83, 111–114]. Importantly studies show that critical period plasticity is defined by excitation/inhibition balance in developing networks [115, 116]. Specifically, GABAergic transmission is important with regard to initiation, prolongation, and termination of the critical period [116, 117]. Defective temporal and spatial interactions between maturing excitatory and inhibitory networks then could easily modify the time course of critical periods.

Thirdly, recent evidence reveals critical period problems in *Fmr1* KO mice that could be related to dysfunctional plasticity resulting from the loss of FMRP and/or dysregulated mGluR mechanisms. For instance, *Fmr1* KO mice have abnormal ocular dominance (OD) plasticity. When challenged with brief monocular deprivation (3 days) starting at postnatal day 28 (P28), wild-type mice display depression in the visual responses from the deprived eye followed 4 days later by potentiation of responses from the nondeprived eye. *Fmr1* KO mice instead show immediate potentiation of nondeprived eye responses and insignificant deprived eye depression [26, 118].

In rodent layer IV somatosensory (barrel) cortex, the critical period for thalamocortical plasticity normally occurs in the first postnatal week (through P7), with NMDA-dependent LTP from thalamocortical afferents peaking at around postnatal day 4 (P4). In *Fmr1* KO mice, this critical period is delayed past P7 with LTP levels remaining elevated into the second postnatal week before quickly dropping to wild-type levels by adulthood [103]. The barrel cortex normally develops a stereotypical somatotopic map of thalamic inputs that receive afferents from and respond to individual rodent vibrissae during the first postnatal week [119]. This process appears to proceed as planned in layer IV for map arrangement except for a delay in the reorganization of cells. Normally cell bodies concentrate in the barrel septa (the border between barrels) versus the barrel hollow at P7 but this process is deficient at this time period in *Fmr1* KOs, and septa cell density is low [120]. Perhaps significantly, the normal close of the critical period (P7) coincides with an elevation in FMRP expression in wild-type mice that is obviously absent in the *Fmr1* KO [120]. Synaptic proteins downstream of GpI mGluRs and NMDARs such as PLC-β1 and SynGAP, respectively, are downregulated at this time point in *Fmr1* KO cortex as well [120]. Both of these proteins' mRNAs are targets of FMRP [18].

Subsequently in layer IV, dendritic localization at the end of the second postnatal week in *Fmr1* KO mice is delayed with more dendrites remaining in the barrel septa at around P14 instead of concentrating in the barrel hollow. Furthermore morphology of those dendrites skews preferentially toward immature filopodia versus the mature mushroom head phenotype as has been reported in mature cortex and

hippocampus in *Fmr1* KOs [120–122]. Also in layer IV at P14, there exists a decreased excitatory drive of local fast-spiking (FS), parvalbumin-positive interneurons that persists into adulthood [58].

Following these layer IV perturbations, a succeeding critical period in the refinement of layer IV to layer II/III connections is affected in *Fmr1* KO mice [123]. Layer IV to layer III ascending connections are weakened at P14 with scattered axonal arbors similar to diffuse dendritic arbors in layer IV at this time point [120]. In addition normal layer IV to layer III synaptic depression in response to activity deprivation does not exist [123].

Critical period plasticity in the cortex is temporally progressive not only from primary areas to integrative areas but also from input layers (layer IV) to integrative (layer II/III) and output layers (layer V/VI) within the same cortical area. Based on this concept and the limited evidence of critical period alterations in the somatosensory cortex in *Fmr1* KO mice, we might predict that as network development progresses, abnormalities caused by plasticity deficits can either compound, normalize by way of compensatory mechanisms, or suspend network development altogether. In fact we probably observe a mixture of these phenomena in FXS. Studies indicate that many of the developmental phenotypes just described normalize by adulthood and thus may simply represent developmental delays. For example delayed increases in LTP in layer IV barrel cortex return to wild-type levels by adulthood (P21) [103]. Layer IV to layer III connections eventually normalize in the mutant mouse [123]. Even some behavioral phenotypes diminish or disappear with maturity including increased seizure susceptibility in *Fmr1* KO mice [124] and epilepsy in humans with FXS [3, 20]. However some phenotypes observed during critical period maturation remain in adulthood, notably deficient excitatory drive of inhibition [58], dendritic morphological immaturity [120–122], and learning and memory deficits [25, 26].

Based on the dynamics of plasticity in *Fmr1* KO mice discussed above, we can propose a general temporal model of effective plasticity in which critical periods (at least those of primary cortical areas) are delayed and restricted in FXS (Figure 1). In the FXS brain, even within critical periods though, plasticity is inefficient, compromised by persistent deficits. Then, as the network approaches maturity persistent deficits acting on compromised networks result in decreased capacity for effective plasticity in FXS. Mechanisms responsible for deficits in developmental and postdevelopmental time periods likely have similarities and differences. Then in order to fully understand how the lack of FMRP affects network development, we must discern what plasticity mechanisms are employed during development, the nature of those mechanisms, and the time point at which those mechanisms are crucial for proper network maturity.

We know that both FMRP and Gp I mGluRs are developmentally expressed and regulated [103, 105, 106, 123], yet little to no studies focus on the role of mGluR-dependent processes during early developmental time points in FXS. Ample evidence shows that pharmacological reduction of GpI mGluR function or genetic reduction of mGluR

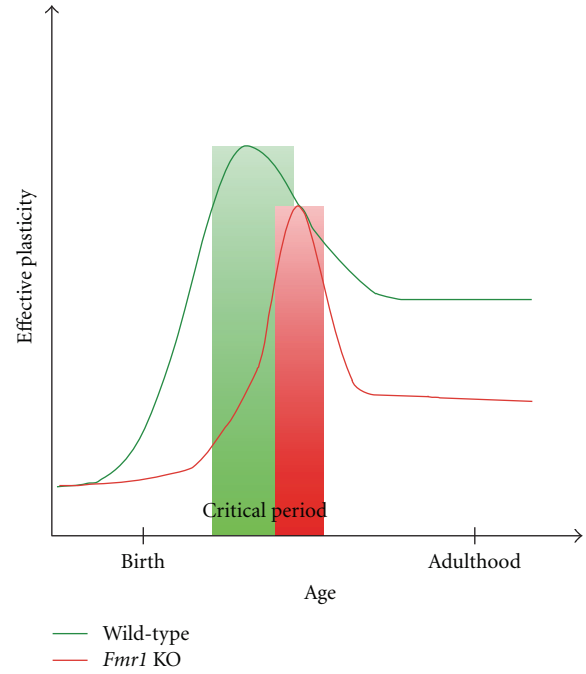

Figure 1: General model of effective plasticity in wild-type versus *Fmr1* KO mice. In primary somatosensory cortex of wild-type mice the capacity for effective plasticity increases rapidly from birth, peaking during the critical period of network development and then normalizing into adulthood. *Fmr1* KO mice display a delay in the increased expression of plasticity mechanisms [103] that normalizes at approximately the same developmental time point as wild-type mice [103, 123]. However, persistent deficiencies in plasticity such as dentritic spine dynamics [122] compromise effective plasticity throughout network development in *Fmr1* KO mice.

expression can rescue FXS phenotypes [26, 40, 125] (see http://www.clinicaltrials.gov/). In the case of OD plasticity, *Fmr1* KO mice heterozygous for a knockout of mGluR5 (*Grm5* +/−), and therefore expressing a 50% reduction in mGluR5 protein, showed the same response as wild-type mice to monocular deprivation [26]. Similar rescues of deficits in spine morphology, increased basal protein synthesis, fear extinction, audiogenic seizures, and learning and memory deficits have been successful [2, 32–34, 36, 37, 39]. However, these experiments, including OD plasticity, focus almost exclusively on alterations in adult animals. Genetic reduction of mGluR5 shows similar effects as pharmacological reduction, but the contribution of developmental versus acute alterations in function cannot be determined by analysis at a single time point.

The role of mGluRs is likely important and unique at early developmental periods in FXS. In hippocampal area CA1 at least, GpI mGluR-mediated LTD undergoes a developmental switch from presynaptically mediated, protein synthesis-*independent* plasticity to postsynaptically mediated protein synthesis-*dependent* plasticity involving internalization of AMPARs. This switch occurs between P8 and P21 which corresponds to the time period of major critical periods in cortical development including primary

somatosensory, auditory, and visual [97]. Although GpI mGluRs and FMRP are expressed in these regions during these early periods, no studies to date have elucidated the mechanism of interaction or investigated possible changes in FXS. Given the success of GpI mGluR inhibition to rescue phenotypes and improve symptoms in FXS [26, 40, 125], one might expect that inhibition of this transmitter system may show similar results at early developmental time points. However, Cruz-Martín and colleagues (2010) demonstrate that this is not necessarily the case. In cortex of *Fmr1* KO mice at 2 weeks of age, they observed a delay in spine maturation and increase in dendritic spine turnover. Application of an mGluR5 antagonist did not rescue this phenotype but instead *increased* spine length and motility, an effect directly opposite of that observed in older animals [26, 121]. Whether or not the differences are related to a mechanistic switch of mGluR function downstream of the receptor is unknown, but the evidence indicates that seemingly similar dysfunctional phenotypes (i.e., dendritic morphology) can have different or additional etiologies depending on the developmental time point investigated. Furthermore, reduction of mGluR activity early in development may be deleterious rather than helpful in patients with FXS.

Elucidating the early role of FMRP in developmental plasticity mechanisms in FXS is therefore essential to understanding how the loss of the protein modifies networks and how to improve those negative modifications through targeted treatments. These investigations should not be limited to mGluR mechanisms. Other mechanisms discussed in this paper may also play a role in early development. Endocannabinoid-mediated enhancement of inhibition is developmentally regulated [126] and could play a role in decreased or delayed cortical connectivity in *Fmr1* KO. Furthermore mGluR regulation of LTS interneurons may be disrupted as described above which could affect the fine tuning of cortical circuits in development [65, 67]. These abnormalities result in faulty synchronization of synaptic inhibition and in turn disrupt DHPG-induced action potential synchronization in cortical pyramidal neurons [65]. FMRP-regulated homeostatic mechanisms may participate early in development as well to hone network connectivity [87, 127]. Although not discussed in detail in this paper (see Paluszkiewicz et al. 2011 [110]), GABAergic regulation of development likely plays a crucial role in regulating early developmental plasticity. GABAergic inhibition and the balance of excitation and inhibition define critical periods [90, 116, 117]. In particular, the developmental maturation of parvalbumin (PV) positive FS interneurons and their connectivity may regulate critical periods, at least in visual cortex [128, 129]. Since this connectivity develops defectively in *Fmr1* KO mice as indicated by a decreased excitatory drive of these PV positive cells at a relatively early developmental time point (P14) [58], determining the integration of inhibitory and excitatory plasticity represents an important target for future research. Relatively new genetic tools in the form of *Fmr1* conditional KO and conditional "ON" mice that utilize the Cre-lox system to express *Fmr1* only in excitatory or inhibitory neurons [130, 131] will enable researchers to separate the roles of FMRP in regulating development of excitatory versus inhibitory circuits.

6. Conclusions

Neuronal plasticity establishes and maintains connectivity and defines the operational range and coding capacity of neuronal networks. In FXS, the absence of a single protein, FMRP, perturbs the balance in a diverse array of plasticity mechanisms from Hebbian to homeostatic, which alters the establishment and maintenance of this operational range and coding capacity of FXS networks in a developmentally and regionally specific way. Most of these mechanisms likely involve dysregulation of processes downstream of GpI mGluR signaling as a result of the absence of a key transcriptional regulator in FMRP. However, as researchers begin to investigate early developmental processes in FXS, a more diverse role of FMRP in plasticity mechanisms begins to emerge that may provide new avenues for treatment that alter pathological plasticity underlying the disease progression. As Krueger and Bear describe in a recent review (2011), the key to treating a developmental disorder like FXS relies on treatment at the critical developmental time point where developmental progression diverges from the norm [125]. Unfortunately circuit alterations precede behavioral signs of pathological disturbances. By investigating dysfunctional plasticity related to the loss of FMRP earlier in development, we can better identify that point of divergence and design treatments that not only correct abnormal plasticity and thereby their behavioral correlates but also minimize the establishment of the plasticity-deficient networks in the first place.

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Review Article

Hippocampal Neurogenesis, Cognitive Deficits and Affective Disorder in Huntington's Disease

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Huntington's disease (HD) is a neurodegenerative disorder caused by a tandem repeat expansion encoding a polyglutamine tract in the huntingtin protein. HD involves progressive psychiatric, cognitive, and motor symptoms, the selective pathogenesis of which remains to be mechanistically elucidated. There are a range of different brain regions, including the cerebral cortex and striatum, known to be affected in HD, with evidence for hippocampal dysfunction accumulating in recent years. In this review we will focus on hippocampal abnormalities, in particular, deficits of adult neurogenesis. We will discuss potential molecular mechanisms mediating disrupted hippocampal neurogenesis, and how this deficit of cellular plasticity may in turn contribute to specific cognitive and affective symptoms that are prominent in HD. The generation of transgenic animal models of HD has greatly facilitated our understanding of disease mechanisms at molecular, cellular, and systems levels. Transgenic HD mice have been found to show progressive behavioral changes, including affective, cognitive, and motor abnormalities. The discovery, in multiple transgenic lines of HD mice, that adult hippocampal neurogenesis and synaptic plasticity is disrupted, may help explain specific aspects of cognitive and affective dysfunction. Furthermore, these mouse models have provided insight into potential molecular mediators of adult neurogenesis deficits, such as disrupted serotonergic and neurotrophin signaling. Finally, a number of environmental and pharmacological interventions which are known to enhance adult hippocampal neurogenesis have been found to have beneficial affective and cognitive effects in mouse models, suggesting common molecular targets which may have therapeutic utility for HD and related diseases.

1. Introduction

Toward the close of the nineteenth century, the Neuron Doctrine emerged as a fundamental theory of the nervous system [1]. A basic tenet of the Neuron Doctrine was that specialized cells—neurons—formed the elementary structural and functional units of the nervous system. Embedded within the formulation and refinement of the Neuron Doctrine was born the concept of neuronal plasticity or structural modifiability of the brain [2]. Arguably, and perhaps unwittingly, the developing theory of neural plasticity was linked with functional roles in learning and memory. However, the ability of the brain to modify its connections was tempered by the overriding edict that the adult neuronal population was stable. In other words, production of new neurons—neurogenesis—was complete prior to brain maturation. Thus another basic tenet of the Neuron Doctrine was that the adult mammalian brain was devoid of ongoing neurogenesis. This view of adult neurogenesis pervaded neuroscience for a substantial portion of the twentieth century, holding sway in spite of early contradictory evidence [3, 4]. With technical advancements and overwhelming evidence, adult neurogenesis in the mammalian brain became a widely accepted concept by the close of the twentieth century [5]. Addition of new neurons to established circuitry of the adult brain affords a novel form of neuronal plasticity beyond the structural and synaptic plasticity of existing neurons. Adult neurogenesis is considered to play an etiological role

in, and provide a therapeutic target for, many neurodegenerative conditions [6]. The potential of manipulating adult neurogenesis to treat the cognitive deficits associated with Huntington's disease has been recently covered in detail [7]. This review will focus on an emerging association between adult neurogenesis and hippocampal dysfunction and its relationship to affective and cognitive symptoms in Huntington's disease.

2. Hippocampal Neurogenesis and Huntington's Disease

The hippocampus is a key structural element of the limbic system and has been intensively investigated as a mediator of learning and memory [8]. Beyond cognition, however, impaired hippocampal neuronal plasticity is thought to be a significant neural substrate underlying the development of depressive behavior [9, 10]. Major depressive disorder encompasses cognitive impairment and both impaired cognition and depression feature in the spectrum of symptoms experienced by HD patients [11]. A significant step toward the general acceptance of adult neurogenesis was the generation of evidence for neuron production in the adult human hippocampus [12]. Since this study, there has been, and continues to be, considerable debate regarding the functional significance of hippocampal neurogenesis in human health and disease [13]. Hypotheses have been put forward that posit (1) impaired neurogenesis elicits the development of depression and (2) that efficacy of antidepressants relies on intact hippocampal neurogenesis [14–16]. Refinement (or refutation) of the "neurogenesis hypothesis" of depression will have an impact not only on our understanding of pathogenic mechanisms but also on the development of new treatments. Recent studies using transgenic HD mice may further inform the neurogenesis hypothesis of depression and provide preclinical direction for more effective HD treatments.

Adult hippocampal neurogenesis (AHN) is a highly regulated multistep process involving proliferation of neuronal stem/precursor cells, neuronal differentiation and migration, and finally neuronal maturation and integration into the existing hippocampal circuitry [17]. A diversity of factors targets these regulatory steps to affect the net rate of AHN. A significant foundation for the neurogenesis hypothesis of depression arises from data that shows neurotransmitter systems involved in the development and treatment of depression also regulate levels of AHN [14]. Various transgenic rodent models of HD have been found to exhibit affective and cognitive abnormalities reflecting clinical data in HD patients. For example, R6/1 and R6/2 transgenic lines of HD mice have behavioral deficits that include impaired hippocampal-dependent spatial cognition [18–20]. However, depressive-like behavior also manifests in R6/1 HD mice prior to cognitive and motor symptoms [21, 22].

Serotonin is a key neurotransmitter system involved in the etiology and treatment of depressive disorders [23, 24]. Selective serotonin uptake inhibitors (SSRIs), such as fluoxetine (Prozac) and sertraline continue to be a core pharmacological intervention for major depressive disorder [23, 24]. A significant mechanism of action of the antidepressant effects of SSRIs involves potentiation of serotonergic neurotransmission through inhibition of synaptic clearance. Reflecting the incidence of HD patient depression, depressive-like behavior manifests in premotor symptomatic R6/1 HD mice [21, 25]. Early analysis of R6/1 HD mice showed a reduction in hippocampal stem/precursor cell proliferation leading to a net reduction in AHN [26]. Further studies in R6/1 [25, 27, 28] and R6/2 [29–31] lines of mice have extended our understanding of the AHN deficit. Could the impaired AHN in R6/1 HD mice cause the development of the depressive-like phenotype? Administration of fluoxetine to R6/1 HD mice rescued net AHN and ameliorated the depressive-like behavior and a hippocampal-dependent cognitive deficit [25]. This data was supported by subsequent studies in which both acute and chronic sertraline ameliorated a more extensive battery of depressive-like behaviors in female R6/1 HD mice [22, 32]. These data accord with the basic propositions of the neurogenesis hypothesis of depression, where impaired basal AHN levels coincide with depressive-like behavior and the efficacy of pharmacological antidepressants coincides with restoring AHN. Despite neuron production being central to the neurogenesis hypothesis of depression, whether this involves specific deficits at key regulatory points or simply a net AHN reduction remains unclear. Our findings using the R6/1 model have recently questioned whether the neurogenesis hypothesis of depression applies in the context of HD [32]. Activity of specific serotonin receptor subtypes differentially affects net AHN by targeting proliferation, differentiation and survival [33]. Thus tonic serotonin receptor activity may play a role in the maintenance of basal AHN rates. This notion holds particular importance in light of our recent studies showing an imbalance of the serotonergic system in R6/1 HD mice [21, 22, 34]. Impaired serotonergic function in the hippocampus of R6/1 HD mice could play a causative role in reduced AHN in R6/1 HD mice. However, provision of serotonin appears capable of ameliorating depressive-like behavior in R6/1 HD mice without rescuing net AHN levels. Such an observation does not support the neurogenesis hypothesis of depression.

The yeast artificial chromosome (YAC) mouse model of HD carries a full-length mutant human huntingtin gene with varying CAG repeats lengths [35]. Reflecting the R6 transgenic lines, YAC128 mice (128 CAG repeats) also develop depressive-like behavioral symptoms and hippocampal-dependent cognition deficits [36, 37]. In contrast to the R6/1 study, fluoxetine provided no significant efficacy in redressing depressive-like symptoms in YAC128 mice [38]. Hitherto studies on YAC128 mice have shown that the early onset of depressive-like behavior does not progress in severity with advancing age [37]. Further studies have shown that adult hippocampal neurogenesis deficits in YAC128 mice are progressive and appear to be relatively intact at an age coincident with the manifestation of depressive-like behavior [39]. The progressive deficits in adult hippocampal neurogenesis appear to more closely correlate with the onset of spatial memory and learning impairments in YAC128 mice

[36, 37]. Moreover, evidence derived from YAC128 mice does not support the notion that deficits in adult hippocampal neurogenesis precipitate depressive-like behavior, however, these neurogenesis deficits do retain an association with impaired hippocampal-dependent cognition. Finally, a new study has revealed sexually dimorphic affective dysfunction and adult neurogenesis (neuronal maturation but not cell proliferation) deficits in the Hdh^{Q111} knock-in mouse model of HD [40]. Thus, for all of the HD mouse models investigated AHN is abnormal, suggesting that this is a robust effect of the HD gene mutation expressed in different contexts.

Physical activity is advocated to maintain mental health including the prevention and amelioration of depression and anxiety [38]. Physical activity was one of the first physiological factors found to both increase AHN and improve hippocampal function in rodents [41]. The pertinent question arises: does AHN mediate the anti-depressant effects of exercise? It appears that increases in the proliferative steps of AHN are associated with the antidepressant effects of exercise [42]. However, while exercise alleviates depressive-like behavior in R6/1 HD mice, it does not concomitantly elevate neural stem/precursor cell proliferation in the hippocampus [32]. Furthermore, environmental enrichment, which delays onset of affective, cognitive and motor deficits in R6/1 HD mice [19, 21, 43], has only subtle effects on AHN in these mice [27]. Thus, redressing AHN impairment is not necessary for antidepressant effects in R6/1 HD mice. However, part of the neurogenesis hypothesis of depression posits that reduced basal rates of AHN precipitates the development of depression. The early observed lower rates of proliferation of neural stem/precursor cells in R6/1 HD mice [26] are not reflected in a recent postmortem analysis in HD patients [44]. One caveat of this human postmortem finding, using immunohistochemical analysis of endogenous cell-cycle markers, is that the patients who had eventually died from HD (after one or more decades of disease progression) are at a much later stage of progression than was examined in HD mice, and may also have received treatments, such as SSRIs, that are known to affect AHN. Furthermore, AHN is known to decline with age and in this postmortem study [44], where the average age of controls and HD subjects at death was close to 60 years, the baseline cell proliferation detected in the dentate gyrus appeared to be low. Thus, a "floor effect" could mean that any early HD-induced AHN deficit would decrease over time due to age-dependent declining control levels of AHN.

Further consideration relevant to the apparent discrepancies between mouse models and the human postmortem study resides in the observation that the transgenic and knock-in lines have CAG repeat expansions that are in excess of the pathological repeats observed in most patients (although juvenile-onset HD patients do generally have >70 CAG repeats). Nevertheless, the R6/1 and YAC mouse lines show strong construct and face validity, with adult-onset symptoms and molecular abnormalities that do model clinical HD. Furthermore, there is evidence of neuronal cell loss in the hippocampus of HD patients at postmortem [45] as well as hippocampal volumetric evidence from MRI studies [46]. The issue of whether AHN deficits occur in clinical HD will only be definitely answered with new brain imaging approaches which are sensitive to *in vivo* changes in neurogenesis and can be performed longitudinally on gene-positive subjects.

Another molecular correlate of pathogenesis in the hippocampus of R6/1 HD mice, which may contribute to AHN deficits as well as affective and cognitive abnormalities, is decreased brain-derived neurotrophic factor (BDNF) [20, 47, 48]. BDNF is a potent stimulator of adult neurogenesis [49, 50]. In particular, BDNF has been associated with hippocampal synaptic plasticity and neurogenesis and implicated in enhanced hippocampal-dependent cognition. Further evidence for a pathogenic role of BDNF dysregulation in HD has been provided by other animal and cell models of HD, as well as other neurodegenerative diseases (reviewed by [51]). Thus, along with the serotonergic dysregulation discussed above, BDNF and its associated signaling pathways (including the TrkB receptor) are likely candidates to help explain the AHN deficits in HD mice. It is possible that BDNF dysregulation could contribute to affective and cognitive deficits independently of effects on AHN, although this has yet to be experimentally tested. Enhanced voluntary exercise and environmental enrichment were found to differentially affect BDNF expression in the hippocampus of R6/1 HD mice and wild-type controls, which may provide insight into molecular mechanisms mediating the beneficial effects of such environmental manipulations [20, 47, 48]. Other evidence linking BDNF to the beneficial effects of environmental enrichment and exercise on AHN and cognition, particularly with respect to "pattern separation" during memory formation, has been recently discussed [52]. These and other findings have led to BDNF being considered a therapeutic target for various psychiatric and neurological disorders [53].

Our present data in R6/1 HD mice suggest that despite reduced net rates of AHN there is no change in proliferation at an age coincident with the presence of depressive-like behavior (Ransome and Hannan, submitted). Collectively, these data are consistent with the possibility that specific deficits in hippocampal proliferation contribute to a subset of cognitive deficits and/or depressive-like behaviors, which can be alleviated through restoring proliferation levels. In the case of R6/1 HD mice, basal proliferation is normal and, therefore, the development of concomitant depressive-like endophenotypes is presumably elicited from other pathologies. This notion is strengthened by the observation that exercise in R6/1 HD mice restores 5-HT1A receptor function thus explaining the amelioration of depressive-like behavior in the absence of AHN effects [32] (Ransome and Hannan, submitted).

3. Clinical Significance

What is the relevance for HD patients? The collective data using environmental and pharmacological interventions suggest basal serotonergic dysfunction in the hippocampus plays a causative role in depressive-like behavior in R6/1

HD mice. Efforts to ameliorate depression should focus on this system, and perhaps others such as catecholamine and neurotrophin signaling, rather than AHN per se. Another feature to emerge from these recent studies is the evidence for sexual-dimorphism in the development of major depressive disorder. Clinical depression has a higher incidence in females compared to males [54, 55], which intuitively suggests sex-hormone involvement. Much interest continues to surround differential regulation of AHN dependent on sex [56]. Recent studies have highlighted sex-specific AHN increases in response to fluoxetine treatment [57]. Could the neurogenesis hypothesis of depression manifest differently in males and females? Our endeavors to elucidate the role of AHN in the etiology of psychiatric illness have demonstrated that rates of proliferation, differentiation, and survival of adult hippocampal neurons are similar between male and female mice [58]. Furthermore, our current work on R6/1 HD mice shows no change between male and female hippocampal stem/precursors cell proliferation (Ransome and Hannan, submitted). This is reflected in YAC128 mice, in which impaired adult hippocampal neurogenesis manifests similarly in both males and females [39]. The decrease in hippocampal serotonin levels in R6/1 HD mice is more severe in females compared to males, while males respond less to sertraline treatment [22]. Moreover, these sexually dimorphic observations in R6/1 HD mice are consistent with the hypothesis that serotonergic dysfunction (and possibly changes in other molecular systems such as catecholamine and neurotrophin signaling) rather than AHN deficits are central to the manifestation of depressive-like behavior. Again, YAC128 HD mice show a contrasting result, whereby the depressive phenotype manifests equally in males and females [37].

With respect to the link between AHN and cognition in rodents, there are clinical studies in which similar memory deficits have been identified in HD patients, for example, a recent study involving a human analog of the Morris water maze [59]. The clinical incidence of cognitive dysfunction in HD appears to largely manifest equally in male and female patients, although most studies do not assess for potential sex effects. In contrast, it would be expected, based on depression studies in the broader population, that depression would be more common in females than males in those with the HD gene mutation. Surprisingly, this question has not been systematically addressed and is worthy of investigation.

Late-onset hypogonadism is posited to be a readily treatable predisposing factor to depression in elderly men [60]. Clinical analysis shows that male HD patients have lower testosterone levels [61]. This study determined that testosterone levels inversely correlated with the severity of the manifest cognitive impairment but not depressive-like symptoms in these male HD patients. We recently explored the potential of testosterone therapy for cognitive impairment in HD using R6/1 HD mice. Chronic testosterone at supraphysiological doses provided efficacy in restoring testosterone levels, androgen receptor expression, and testicular function in R6/1 HD male mice [62]. However, testosterone therapy did not induce AHN nor rescue cognitive impairment. Androgens can exert anxiolytic effects in mice. Given that our study showed AHN deficits were refractory to the stimulating effects of testosterone in R6/1 HD mice, this model may provide a valuable tool in elucidating the mechanism of testosterone's antidepressant effects including an obligatory role of AHN. This would provide additional evidence toward refining (or refuting) the neurogenesis hypothesis of depression, should testosterone therapy prove to reverse the depressive-like symptoms.

4. Concluding Remarks

The discovery and characterisation of adult neurogenesis in the mammalian hippocampus has elicited several hypotheses regarding its physiological roles, including contributions to neural systems mediating specific cognitive and affective functions. Accumulating evidence suggests that AHN contributes to specific cognitive processes such as spatial pattern separation [17, 63]. The discovery that pharmacological antidepressants stimulate hippocampal neurogenesis, which in turn appeared to be necessary for therapeutic efficacy of such drugs, provided a significant impetus for the generation of the neurogenesis hypothesis of depression [64, 65]. Accumulating evidence has seen refinements of the hypothesis, where depression manifests at least partly from hippocampal dysfunction precipitated by neurogenesis deficits [66]. Nevertheless, the hypothesis remains controversial and requires further testing in validated animal models. On balance, our data derived from R6/1 HD mice suggest that depression symptoms associated with HD are more consistent with the serotonergic vulnerability hypothesis of depressive disorders [67], although other molecular abnormalities, such as disrupted neurotrophin and catecholamine signaling, may also be involved.

The potential role of AHN deficits in the pathogenesis of cognitive and affective symptoms in HD has also not yet been fully tested in animal models. Transgenic animal models, such as HD mice, provide a unique system in which the progressive development of affective, cognitive, and motor deficits can be delineated over time, and the preceding molecular and cellular changes can be closely correlated with onset of specific endophenotypes. Similarly, environmental and pharmacological inventions which induce affective and cognitive benefits in HD mouse models can be used to explore molecular and cellular mechanisms of pathogenesis. The psychiatric and cognitive symptoms of HD are amongst the earliest and most devastating in this currently incurable disease. While adult neurogenesis deficits may contribute, other cellular processes, such as synaptic function and plasticity, are known to be disrupted in HD mouse models (e.g., [18, 68–70]) and are likely to be involved in the generation of specific psychiatric and cognitive endophenotypes. By tackling this unique disease with validated animal models that integrate pathogenic processes at molecular, cellular and systems levels, we hope to attain more sophisticated understanding of the complex etiologies involved and thus develop effective therapeutic approaches for HD and related disorders.

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Review Article

Mechanism of Repeat-Associated MicroRNAs in Fragile X Syndrome

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The majority of the human genome is comprised of non-coding DNA, which frequently contains redundant microsatellite-like trinucleotide repeats. Many of these trinucleotide repeats are involved in triplet repeat expansion diseases (TREDs) such as fragile X syndrome (FXS). After transcription, the trinucleotide repeats can fold into RNA hairpins and are further processed by *Dicer* endoribonuclases to form microRNA (miRNA)-like molecules that are capable of triggering targeted gene-silencing effects in the TREDs. However, the function of these repeat-associated miRNAs (ramRNAs) is unclear. To solve this question, we identified the first native ramRNA in FXS and successfully developed a transgenic zebrafish model for studying its function. Our studies showed that ramRNA-induced DNA methylation of the *FMR1* 5′-UTR CGG trinucleotide repeat expansion is responsible for both pathological and neurocognitive characteristics linked to the transcriptional *FMR1* gene inactivation and the deficiency of its protein product FMRP. FMRP deficiency often causes synapse deformity in the neurons essential for cognition and memory activities, while *FMR1* inactivation augments metabotropic glutamate receptor (mGluR)-activated long-term depression (LTD), leading to abnormal neuronal responses in FXS. Using this novel animal model, we may further dissect the etiological mechanisms of TREDs, with the hope of providing insights into new means for therapeutic intervention.

1. Introduction

More than 97% of a human genome consists of noncoding DNA, the function of which was unknown until recent years. Variations between individuals' noncoding DNA can sometimes manifest into biological and clinical dysfunction. MicroRNA (miRNA) is a subclass of noncoding RNA that is involved in a wide variety of physiological and developmental events, including developmental timing, embryonic patterning, cell fate determination, cell lineage differentiation, cell proliferation, apoptosis, organogenesis, growth control, and metabolism [1, 2].

MiRNAs are single-stranded molecules consisting of about 18 to 27 ribonucleotides in length and regulate the expression of other protein-coding genes through an intracellular gene silencing mechanism named RNA interference (RNAi). MiRNAs can be located within the noncoding DNA or protein-coding region of DNA [2, 3]. After transcription, instead of being translated the primary miRNA transcript (pri-miRNA) is processed by *Drosha*-like endoribonucleases to a hairpin-like stem-loop precursor, termed "pre-miRNA." Further processing of the precursor by *Dicer*-like endoribonucleases results in a single-stranded mature miRNA which subsequently forms an RNA-induced silencing complex (RISC) with argonaute proteins and binds complementarily to matched sequences of one or more messenger RNAs (mRNAs) for executing targeted gene silencing through either direct mRNA degradation or translational suppression.

Many introns and untranslated regions (UTRs) of mRNAs also contain tri- or tetranucleotide repeat expansions, capable of being transcribed and processed into repeat-associated microRNAs (ramRNAs) [4–7]. Intronic miRNA is a subset of miRNA that is derived from the noncoding DNA regions of a gene, such as the intron or 5′- and 3′-UTR. In vertebrates, the biogenesis of intronic miRNAs involves five steps [8, 9]. First, miRNA is transcribed—as a long primary precursor microRNA (pri-miRNA)—by type II

RNA polymerases (Pol-II) from the intron or UTR of a primary gene transcript [3]. Second, after intron splicing, the long pri-miRNA is excised by spliceosomal components and may be further processed by other *Drosha*-like RNaseIII endonucleases/microprocessors to form precursor microRNA (pre-miRNA) [8–11]. However, intronic miRNA precursors may also bypass *Drosha* processing [12]. During the third step, the pre-miRNA is exported out of the cell nucleus into the cytoplasm, by Ran-GTP and exportin receptors [13, 14]. Fourth, once in the cytoplasm, a *Dicer*-like endoribonuclease cleaves the pre-miRNA to form mature miRNA [9, 10]. Finally, the mature miRNA is assembled into a ribonuclear particle (RNP) to form an RNA-induced silencing complex (RISC) or RNA-induced transcriptional silencing (RITS) complex for executing RNAi-related gene silencing mechanisms [9, 10, 15, 16].

Although the biogenic pathways of small interfering RNA (siRNA)/small hairpin RNA (shRNA) and miRNA are thought to be relatively comparable, many characteristics of the mechanistic components are distinctly different from each other [17, 18]. In zebrafish, we have observed that the stem-loop structure of intronic pre-miRNA is involved in strand selection for mature miRNA during miRNA-associated RISC (miRISC) assembly [10]. Furthermore, unlike the siRNA/shRNA pathway, excessive RNA accumulation can be prevented by the intracellular nonsense-mediated decay (NMD) mechanism, a specific RNA degradation system for unstructured spliceosomal introns [9]. These findings indicate that the siRNA/shRNA pathway is likely lacking some advanced properties required for the regulation of intronic miRNA generation and function.

Given that natural evolution leads to more complex and variable introns in higher animals and plants for the coordination of gene expression volumes and interactions, an intronic repeat expansion or deletion may cause dysregulation of some miRNA biogenesis or miRNA-targeted interactions and thus lead to triplet repeat expansion diseases (TREDs). As shown in Table 1, currently identified TREDs include dentatorubral-pallidoluysian atrophy (DRPLA), fragile X mental retardation syndrome (FXS), Friedreich ataxia (FRDA), Huntington's disease (HD), myotonic dystrophy (DM), spinobulbar muscular atrophy (SBMA), and a number of spinocerebellar ataxias (SCAs). One commonality between these TREDs is that they all express mutant genes with elevated expansion of either CGG/CCG (FXS/FXTAS) or CAT/CTG (others). However, the correlation between the intron-encoded repeat-associated microRNA (ramRNA) and its related TRED remains to be determined. In order to understand the role of a specific ramRNA in the pathogenic mechanism, we must first identify the structure and function of the RNA molecular associated with a TRED. For years the existence of ramRNA has been speculated [5, 7, 19]. Several groups have suggested a correlation between RNA toxicity and TREDs [20–26]; however, there has been no evidence linking a specific ramRNA to a TRED. In this paper, we will describe the process of discovering the first ramRNA identity and how it was used as a tool to establish a transgenic animal model for studying its function *in vivo*.

2. Discovery of ramRNAs in FXS

In 2006, our group successfully found and isolated the first native ramRNA identity, miR-*fmr1*, which is involved in the pathogenetic development of fragile X syndrome (FXS) in a zebrafish model [4, 6]. There are two isoforms of the primary miR-*fmr1* ramRNAs, miR-*fmr1-27* and miR-*fmr1-42*, both of which are derived from the *fmr1* 5′-UTR CGG repeat region approximately 65-nucleotide upstream of the translational start codon (accession number NM_152963) (Figure 1(b)) [4]. These two isoforms contain the same seed and core sequence to interact with the zebrafish *fmr1* gene and/or its gene transcripts. Northern blotting of the two miR-*fmr1* isoforms isolated from either the cytoplasm or nucleus of the pallium neurons further demonstrated that miR-*fmr1-42* is the only ramRNA accumulated in the nucleus of the FXS neurons [4]. Accompanying nuclear miR-*fmr1-42* accumulation, a significant increase of genomic DNA methylation in the *fmr1* 5′-promoter upstream region was also identified using bisulfite sequencing assays [4]. FXS-related DNA methylation occurs mostly in the CpG-rich binding sites of several *fmr1*-associated transcriptional cofactors, such as NRF1 (GCGCGC), SP1 (GC box), and USF1/USF2 (E box), resulting in transcriptional silencing of the *fmr1* gene. The tissue-specific expression pattern of both miR-*fmr1* ramRNAs in the zebrafish brain has also been identified using fluorescent *in-situ* hybridization (FISH) with a locked nucleic acid (LNA) probe directed against the miR-*fmr1* seed and core sequence [4, 6]. As shown in Figures 1(a) and 1(c), the normal expression pattern of miR-*fmr1* is limited in the neuronal bodies and nuclei but not the dendrites of the hippocampal-cortical junction, hippocampal stratum radiatum, and cerebellum neurons. In FXS brains, the presence of miR-*fmr1* is however further extended into the dendrites of these neurons and hence causes synaptic deformity. Such broader miR-*fmr1* distribution throughout the dendrites may serve as a marker for FXS diagnosis.

It should be noted that miR-*fmr1-42* has a unique pre-miRNA structure consisting of (a) multiple loops and short matched stems in a relatively long hairpin precursor, (b) a nuclear import signal (NIS) motif (probably to allow the reentry of the mature ramRNA into the cell nucleus), and (c) a C/G-rich gene binding motif to recruit DNA methylation machinery (Figure 1(b)) [4]. Deletion of the NIS motif from the miR-*fmr1-42* precursor has been shown to significantly increase miR-*fmr1* accumulation in the cytoplasm, but not the nucleus of the neurons, suggesting that NIS is responsible for the nuclear entry of miR-*fmr1-42* [4]. These characteristics support a novel disease model in which mature ramRNAs originating from the trinucleotide repeat expansion of a gene can reversely bind back to the corresponding triplet repeat regions of the gene. Individuals with more trinucleotide repeats in the gene generate more mature ramRNAs. As more ramRNAs binding back to the targeted gene, DNA methylation of the triplet repeat regions of the gene occurs, consequently leading to targeted gene inactivation. Due to our discovery of ramRNA and its function in DNA methylation, this ramRNA-induced DNA

Table 1: Triplet repeat expansion diseases (TREDs) that have been identified in humans.

TRED disorders	Site of pathogen	Expansion	Repeat no.
Dentatorubral-pallidoluysian atrophy (DRPLA)	*Atrophin-1*, exons	CAG	49–88
Fragile X syndrome (FXS)	*FMR1*, 5′-UTR	CGG	>200
Fragile X-associated tremor ataxia syndrome (FXTAS)	*FMR1*, 5′-UTR	CGG	55–200
Fragile X syndrome E (FRAXE)	*FMR2*, 5′-UTR	CCG	200–900
Friedreich ataxia (FRDA)	*Frataxin*, intron	GAA	200–1,700
Myotonic dystrophy type 1 (DM1)	*DMPK*, 3′-UTR	CTG	50–1,000
Myotonic dystrophy type 2 (DM2)	ZNF9, *intron 1*	CCTG	75–11,000
Huntington's disease (HD)	*Huntingtin*, exon 1	CAG	40–121
Huntington's disease-like 2 (HDL2)	*JPH3*, intron, exon, or 3′-UTR	CTG	66–78
Spinobulbar muscular atrophy (SBMA)	*Androgen receptor*, intron	CAG	38–62
Spinal cerebellar ataxia (SCA) types 1–3, 7	*Ataxin 1–3, 7*, exons	CAG	37–300
SCA type 8 (SCA8)	(ncRNA)*UD	CTG	>74
SCA type 17 (SCA17)	*TBP*, exon	CAG	47–63

* UD: undefined.

methylation model may provide further important insights into the mechanism underlying specific gene inactivation in TREDs.

3. Correlation between ramRNA and FXS

FXS is one of the most common neuropsychiatric and mental retardation disorders in humans, affecting approximately one in 2000 males and one in 4000 females [27]. In boys, characteristic features of FXS include a long face, prominent ears, large testes, delayed speech, hyperactivity, tactile defensiveness, gross motor delays, and autistic behaviors. Much less is known about girls with FXS. The disease is caused by a dynamic mutation (expansion of microsatellite-like trinucleotide—(cytosine-guanine-guanine)—repeats or termed r(CGG)) at an inherited fragile site on the long arm of the X chromosome, located at the *FMR1* gene. Due to the dynamic nature of this mutation, trinucleotide repeats can increase in length—and hence in severity—from generation to generation, from person to person, and even within a given person. Patients with FXS have an increased number of r(CGG) > 200 copies in the 5′-UTR of the *FMR1* gene [20, 28, 29]. The CpG-rich r(CGG) expansion region is often heavily methylated, with a methyl group replacing the hydrogen atom of cytosine (C) and thus the cytosine is conversed to 5-methylcytosine in the *FMR1* 5′-UTR. Such r(CGG) expansion and methylation leads to physical, neurocognitive, and emotional characteristics linked to the *FMR1* inactivation and the deficiency of its protein product FMRP.

FMR1 encodes an RNA-binding protein, FMRP, which is associated with polyribosome assembly in an RNP-dependent manner and is capable of suppressing translation through an RNAi-like pathway that is important for neuronal development and plasticity. FMRP also contains a nuclear localization signal (NLS) and a nuclear export signal (NES) for shuttling specific mRNAs between nucleus and cytoplasm [30, 31]. Hence, excessive expression of r(CGG)-derived ramRNAs during embryonic brain development may cause early *FMR1* gene inactivation, leading to the pathogenesis of FXS. Two theories have been proposed to explain this *FMR1* inactivation mechanism in FXS. First, Handa et al. [5] found that noncoding RNA transcripts transcribed from the *FMR1* r(CGG) expansion can fold into RNA hairpins and are further processed by RNaseIII *Dicer* to suppress the *FMR1* expression. Second, Jin et al. [19] proposed that miRNA-mediated gene methylation may occur in the CpG regions of the *FMR1* r(CGG) expansion, which are targeted by hairpin RNAs derived from the 3′-end of the *FMR1* expanded allele transcript. Conceivably, the *Dicer*-processed hairpin RNAs may trigger the formation of RITS assembly on the homologous r(CGG) sequences and result in transcriptional repression of the *FMR1* chromatin locus; yet, the real mechanism was unclear at that time.

4. Vector-Based ramRNA Expression System

Ongoing neuroscience research on FXS using animal models (such as the *FMR1*-deleted mouse and fly) has provided a wealth of information in subcellular, cellular, and intercellular networks to delineate the neurobiology of this disorder. Still none of these models demonstrate the pathogenic role of noncoding RNAs in FXS etiology. To overcome this barrier, we have developed and established the first ramRNA-mediated loss-of-*FMR1*-function zebrafish strain as a viable animal model for studying the aforementioned r(CGG)-derived miRNA-induced FXS theory [4, 6, 32, 33]. This novel *in vivo* model may also be used to develop and test drugs or therapies for the treatment of FXS. Our previous studies have shown that effective mature miRNAs can be generated from an artificial intron inserted in a zebrafish vertebrate gene [3, 34]. As demonstrated in Figure 2, the intron containing pre-miRNA structures is cotranscribed with its encoding gene by a type-II RNA polymerase (Pol-II) and further excised by spliceosomal components to form mature miRNAs. Because this intronic miRNA biogenesis pathway is

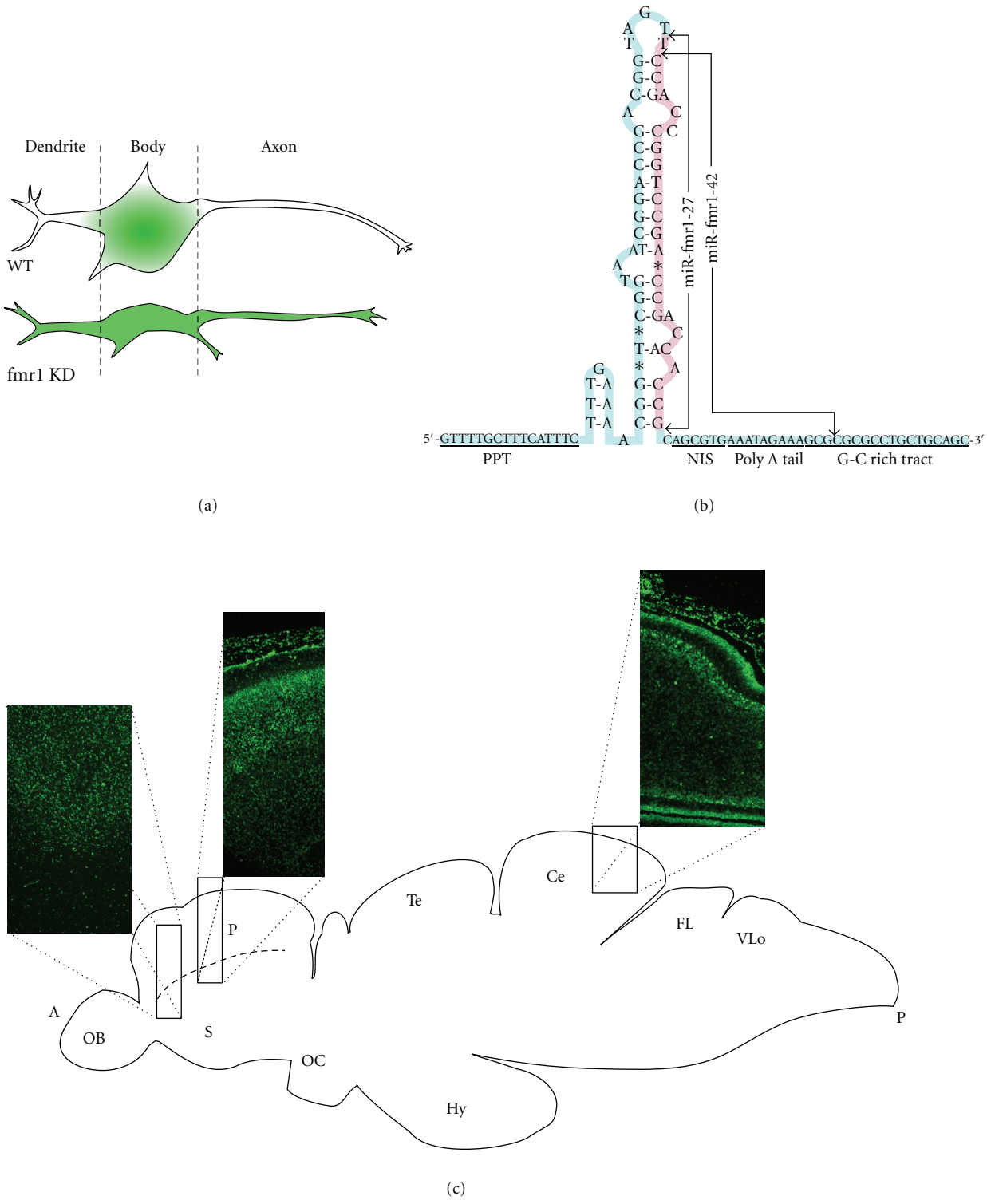

Figure 1: (a) Depiction of the distribution of miR-*fmr1* in wild-type and FXS zebrafish neurons. (b) Sequence diagram of the miR-*fmr1* precursor with both isoforms labeled. Polypyrimidine tract: PPT, nuclear import signal: NIS. (c) Map of wildtype zebrafish brain showing *in situ* hybridization expression patterns of miR-*fmr1* ramRNAs from three sections: (1) cross-section of the lateral pallium, (2) longitudinal section of the pallium-neocortical junction, and (3) longitudinal section of the cerebellum [4].

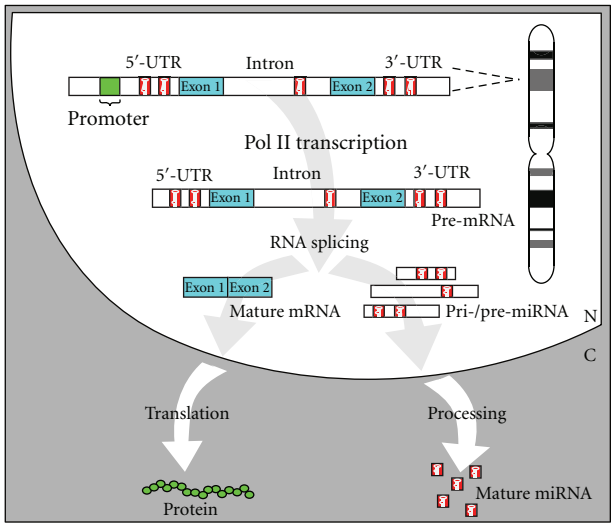

Figure 2: Schematic representation of the mechanism of intronic miRNA expression. After transcription, the miRNA-containing intron is sliced out of the transcript; after further processing by the enzyme *Dicer*, mature miRNA is released and exported out of the nucleus. Meanwhile, the exons are linked together to form mature mRNA which is also transported out of the nucleus for translation.

coordinately regulated by intracellular Pol-II transcription, RNA splicing, and NMD mechanisms, the resulting miRNA effector is safe, effective, and powerful as a new genetic tool for regulating targeted gene function [8, 9, 33]. Using this Pol-II-mediated intronic miRNA expression system, we observed target-specific RNAi effects of various man-made miRNAs in mouse and human cell lines *in vitro* [3, 33, 35] as well as mouse skin, chicken embryo, and zebrafish *in vivo* [4, 6, 9, 32]. Based on similar expression designs, Zhou et al. [36] and Chung et al. [37] have also observed that both native intergenic and intronic miRNAs possess the same RNAi effectiveness, while the use of intronic miRNA allows coexpression of a protein marker with the miRNA at a defined expression ratio. Given that there are currently over 1000 native miRNA species found in vertebrates and many more new miRNA homologs continue to be identified, we are able to utilize this intronic miRNA expression system as a transgenic tool for generating target-specific loss-of-gene-function animal strains or cell lines for evaluating the gene function of interest.

Previously, several kinds of vector-based RNAi systems have been developed based on a directly exonic shRNA expression mechanism, using type-III RNA polymerases (Pol-III) [38–40]. Although some of these studies have succeeded in maintaining constant gene silencing effects *in vivo* [41, 42], they failed to provide tissue-specific RNAi efficacy in a cell population due to the ubiquitous existence of Pol-III activities. Moreover, because Pol-III machinery often reads through a short DNA template in the absence of proper termination, large double-stranded RNA (dsRNA) products (e.g., >30 base pairs) may be synthesized and cause unexpected interferon cytotoxicity, particularly in the vertebrates [43, 44]. Such a problem may also result from the competitive conflict between the Pol-III promoter and another vector promoter (i.e., LTR and CMV promoters). Sledz et al. [45] and Lin and Ying [35] have reported that high concentrates of siRNA/shRNA (e.g., >250 nM in human T cells) can result in strong cytotoxicity similar to that caused by long dsRNAs. Notably, Grimm et al. [46] further demonstrated that the Pol-III-directed RNAi systems often generate high concentrated siRNA/shRNA which can oversaturate the cellular miRNA pathway, resulting in global miRNA inhibition and cell death. In view of these problems, a Pol-II-mediated intronic miRNA expression system has the advantage of its autoregulation by the cellular RNA splicing, and NMD mechanisms [9, 33, 47], both of which degrade excessive RNA accumulation to prevent possible cytotoxicity.

The Pol-II-mediated intronic miRNA expression system is designed around a recombinant gene construct containing one or more splicing-competent RNA introns, namely, SpRNAi [3, 8, 9]. Structurally, the SpRNAi consists of several consensus nucleotide elements such as 5′-splice site, branch-point motif, polypyrimidine tract, and 3′-splice site. A pre-miRNA or pre-miRNA cluster insert is placed within the SpRNAi intron sequence between the 5′-splice site and the branch-point motif. This portion of an intron would normally form a lariat structure during RNA splicing and processing. The spliceosomal U2 and U6 snRNPs, both helicases, may be involved in the unwinding and excision of the lariat RNA fragment into pre-miRNA; however, the detailed processing mechanism remains to be elucidated. Moreover, the SpRNAi contains a multiple translational stop codon motif (Ts codons) in its 3′-proximal region, which, if presented in a premature mRNA, will signal diversion of the premature mRNA processing to the nonsense-mediated decay (NMD) pathway and thus eliminates excess RNA accumulation in the cell. This feature guarantees the safety of the intronic miRNA biogenesis pathway.

Using this intronic miRNA expression construct, we have tested various hairpin-like miRNA precursors (pre-miRNAs), many of which resulted in mature miRNAs with full capacity for triggering RNAi-associated gene silencing effects in mouse, rat, and human cell lines *in vitro* [3, 33, 35] and in mouse, chicken, and zebrafish *in vivo* [4, 6, 10, 32]. Further advances in the intronic miRNA expression system have also been reported in mice; Chung et al. [37] successfully performed ectopic expression of a cluster of polycistronic miRNAs, which were processed into multiple miRNAs via the cellular miRNA pathway. This kind of Pol-II-driven miRNA expression has several advantages over the conventional Pol-III-directed siRNA/shRNA expression systems. First, Pol-II expression can be tissue specific, whereas Pol-III expression cannot. Second, Pol-II expression is compatible with the native miRNA pathway, while Grimm et al. [46] have reported some incompatibility in the Pol-III-directed siRNA/shRNA expression systems. Third, excessive RNA accumulation and cytotoxicity can be prevented by the NMD mechanism of a cellular intronic expression system, but not a direct expression system [46, 48]. Finally, one Pol-II is able to express a large cluster (>10 kb) of polycistronic shRNAs/miRNAs, which can be further excised into multiple shRNAs/miRNAs via the native miRNA pathway, so as to

prevent the promoter conflict that often occurs in a vector system containing multiple promoters.

5. Transgenic Animal Model of FXS

Animal models mimicking the human developmental events and diseases are essential tools for the advancement of biomedical research. Zebrafish (*Danio rerio*), a fresh water tropical fish, has set an impressive record as an *in vivo* viable model for studies of mechanisms involving in embryogenesis, organogenesis, physiology, and behavior; developmental neuroscience has also benefited from research using the zebrafish model. Advantages of using zebrafish include low cost, easy maintenance, rapid life cycle, small size, and embryonic transparency. Also, zebrafish exhibit fast development (i.e., nervous system precursors presented by 6-7 hour postfertilization (hpf); first neuron formed by 18–24 hpf), large generation number (i.e., clutch sizes from a single mating pair range between 100 to 200 embryos), and the phenotypes can be easily assessed in many high-throughput assays [49–51]. Screening genetic suppressors in zebrafish will advance the understanding of loss-of-gene-function phenotypes that are related to certain diseases and help identify logical drug target candidates. In addition, screening for morphological or behavioral mutants is often more time- and cost-effective than the equivalent assays in mouse. These advantages have provided great advances in understanding the detailed pathological mechanisms underlying brain disorders that may lead to functional and behavioral defects. For example, zebrafish possess three *FMRP*-related genes, *fmr1, fxr1,* and *fxr2* that are orthologous to the human *FMR1, FXR1,* and *FXR2* genes, respectively [52]. The expression patterns of these genes in zebrafish are also consistent with those in mouse and human [52, 53], suggesting that zebrafish is one of the best models for studying human *FMRP*-related disorders.

To investigate the molecular mechanism of r(CGG)-derived ramRNA-mediated *FMR1* inactivation, we developed a transgenic FXS model in zebrafish, in which fish *fmr1* is silenced by overexpression of an isolated r(CGG) expansion from the *fmr1* 5′-UTR [4, 6]. Our previous reports have demonstrated the use of a pantropic retroviral vector, *pLNCX2-rT*, to deliver a recombinant SpRNAi-containing red fluorescent protein (*SpRNAi-RGFP*) transgene that is able to express desired miRNA precursors (pre-miRNA) in a ubiquitous *actin* promoter-driven green EGFP-expressing Tg(UAS:gfp) strain zebrafish, Tg(*actin*-GAL4:UAS-gfp) [10, 32]. In this FXS model, an isolated *fmr1* 5′-UTR r(CGG) expansion (accession number NW_001511047 from the 124001st to 124121st nucleotide) was incorporated into the pre-miRNA insertion site of the SpRNAi intron. The original weak *fmr1* promoter (100–1000 copies of mRNA per cell) was further replaced by a fish gamma-aminobutyric acid receptor 2 (*GABA R2*) promoter (5000–15,000 copies of mRNA per cell) to boost the expression of the isolated *fmr1* 5′-UTR r(CGG) expansion. The *pLNCX2-rT* vector was previously modified from a pseudotyped Moloney Murine Leukemia virus, *pLNCX2* (Clontech, Palo Alto, CA), by replacing the original *CMV* promoter with an isolated fish *GABA R2* promoter and then inserting the *SpRNAi-RGFP* transgene into the gene cloning site of the *pLNCX2* construct, so as to form a transgenic *pGABAR2-rT-SpRNAi-RGFP* retroviral vector. Given that *GABA R2* and *FMR1* genes are closely coexpressed in many major brain areas in particular, cortex, hippocampus and cerebellum [54, 55], the infection of *pGABAR2-rT-SpRNAi-RGFP* retroviral vector generated a novel transgenic zebrafish strain displaying a full spectrum of FXS disorders.

The *pGABAR2-rT-SpRNAi-RGFP* vector so obtained was injected directly into one-cell-stage fertilized eggs or used to prepare high-titer retroviruses for infecting the 1–10 hpf-stage zebrafish embryos [4, 6, 10, 32]. Transgenic F0 zebrafish obtained from this process were selectively separated into four groups based on their different *fmr1* knockdown levels, as determined by Western blot analysis, including <50%, 50%–75%, 75%–90%, and >90% knockdown of *fmr1* expression. The zebrafish showing above 90% *fmr1* knockdown were too unstable to be raised into a transgenic line. We succeeded in raising zebrafish with 75%–90% *fmr1* knockdown to sexual maturity. These fish were then crossed with one another to generate the F1 founder line with a stable 75%–85% *fmr1* knockdown rate. After genotyping and transgene sequencing analyses, the F1 and F2 transgenic lines exhibited two copies of the transgene in a consistent genomic insertion site located in the chromosome 18 close to the 3′-side of the LOC565390 locus region—a region that encodes no gene. We have also showed that fish with >90% *fmr1* knockdown possess on average 3–5 copies of the transgene located in two to three genomic insertion sites. Concomitant insertion is known to frequently occur in high-titer retroviral infection.

The principle of this loss-of-*fmr1*-function zebrafish model and human FXS is based on the same molecular interaction between the r(CGG)-derived ramRNA and the *FMR1* 5′-UTR r(CGG) expansion. Both mechanisms result in similar pathological defects triggered by ramRNA-mediated *FMR1* inactivation. We found that increasing the expression of *fmr1* 5′-UTR r(CGG) expansion results in a corresponding elevation of miR-*fmr1* concentration over 6-fold in the transgenic zebrafish with 75%–85% *fmr1* knockdown [4, 6]. Because we only isolated 30% of the whole *fmr1* 5′-UTR r(CGG) expansion region, each transgene—after *GABA R2* promoter-driven transcription—would approximately create a total 2–4-fold increase in miR-*fmr1* production. As a result, the zebrafish with 75%–85% *fmr1* knockdown express 6-fold more miR-*fmr1* than the wild-type zebrafish, similar to the difference between human FXS (>200 copies) and normal (<50 copies) r(CGG) expansion expression. During native embryonic development, excessive expression of r(CGG)-derived ramRNAs (over 4 fold) is sufficient to inactivate *FMR1* gene transcription [4, 6]. We also found that both human and fish FXS models present similar pathological abnormalities in synaptic connectivity and neuronal plasticity. Fish with 50%–75% *fmr1* knockdown may be related to fragile X tremor/ataxia syndrome (FXTAS), which expresses a moderate increase of r(CGG) expansion (~120 copies) and often displays elevated *fmr1* mRNA but not FMRP protein levels, accordingly.

6. Same Abnormalities Observed in Human and Zebrafish FXS

Despite some notable differences in the size, the overall organization of major brain components in zebrafish is highly similar to the human brain [56, 57]. As in other vertebrates, zebrafish possess all of the classical sense modalities such as vision, hearing, olfaction, taste, tactile, balance, and sensory pathways. We have compared the phenotypes of human and zebrafish FXS in detail to provide an informative groundwork for the use of this novel r(CGG)-derived ramRNA-mediated animal model for FXS-related research and drug development. Our previous studies using fluorescent three-dimensional (3D) micrograph have shown abnormal neuron morphology and connectivity in the embryonic brains of the FXS fish, reminiscent of those found in human FXS [4, 6]. In the normal fish lateral pallium (similar to human hippocampal-neocortical junction), wild-type neurons present normal dendrite outlines and are well connected to each other, whereas the *fmr1*-knockdown FXS transgenics exhibit thin, strip-shape neurons, similar to the abnormal dendritic spine neurons in human FXS. Synaptic deformity frequently occurs in the *fmr1*-knockdown neurons, indicating that the functional role of *FMR1* is to maintain neuronal plasticity. Similar alterations in synaptic plasticity have been observed to be a major physiological damage in human FXS, particularly in the hippocampal stratum radiatum, layer IV/V cortex and sometimes cerebellum of severe cases [58–61].

FMR1 mRNA is present in dendritic spines and translated in response to activation of the type 1 metabotropic glutamate receptors (mGluR-1) in synaptoneurosomes [62, 63]. The activation of mGluR1 stimulates a phosphorylation cascade, triggering rapid association of some mRNAs with translation machinery near synapses and leading to protein synthesis of the mRNAs [62]. FMRP protein is, however, a translational inhibitor that binds with the mRNA species involved in regulation of microtubule-dependent synapse growth and function, including its own mRNA [20, 64, 65]. Such translational suppression in dendritic spines is though to be crucial for eliminating immature synapses and enhancing synaptic strength during brain development. Changes in spine shape are often coupled to the absence of FMRP function in FXS patients [58]. Thus, an increased density of long, immature dendritic spines found in the *fmr1*-knockdown FXS neurons may provide new insights into the role of FMRP in synaptic maturation and pruning. Based on the present evidence, not only FMRP protein but also miR-*fmr1* ramRNA can modulate the expression of certain neural genes involved in synaptic development and maturation.

In three-month-old male FXS zebrafish, excitatory synapses in slices of the pallium-neocortical junction were found to exhibit diminished long-term potentiation (LTP), as compared with wild-type controls [4]. LTP in hippocampus is a learning-related form of synaptic plasticity and is highly involved in changes found in abnormally shaped dendritic spines [66]. This result observed in *fmr1*-knockdown FXS neurons indicates that deficits in hippocampal-cortical LTP mechanisms likely contribute to cognitive impairments in FXS disorders. On the other hand, postsynaptic stimulation of mGluR increases neural protein synthesis and subsequently triggers internalization of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors. This process is crucial for the expression of long-term depression (LTD), which refers to a long-lasting decrease in synaptic strength to below the normal baseline level. Given that FMRP is a downstream gene stimulated by mGluR to reversely quench this LTD process, deficiency of FMRP in FXS neurons hence interrupts this feedback regulation mechanism and leads to LTD overamplification. In particular, the pallium neuron LTD is augmented in the absence of *fmr1* [4], suggesting that exaggerated LTD may be also responsible for aspects of abnormal neuronal responses in FXS, such as autism. This exaggerated LTD, however, can be inhibited by treating the brain slices of the FXS fish with mGluR-specific agonists, such as 3,5-dihydroxyphenyglycine (DHPG). These findings in FXS zebrafish raise a possibility in FXS-associated autism, which is also supported by other evidence that induction of mGluR1-dependent LTD is enhanced in pyramidal cells of the hippocampus in *FMR1*-deleted mice [60]. Thus, altered LTP and LTD in FXS hippocampal neurons may explain how and why such *FMR1* inactivation hinders the normal learning and cognition process in the brain, which is important for the development of human intelligence quotient (IQ).

7. Conclusions

In sum, our studies have established a novel animal model and possible etiological mechanism for FXS (Figure 3), in which excessive expression of ramRNAs derived from the *FMR1* 5′-UTR r(CGG) expansion results in nuclear ramRNA accumulation and hence inactivated the *FMR1* gene transcription through promoter DNA methylation. Similar to miRNA biogenesis, *Dicer1* endoribonuclease is required for ramRNA processing. Rad54l and MeCP2 also play a crucial role in the RITS assembly of the ramRNAs responsible for the *FMR1* promoter methylation. The pathological outcomes of this ramRNA-mediated *FMR1* gene silencing were corresponded to the neurodegenerative and cognitive impairments in FXS disorders, like neuronal deformity, immature synapse formation, long dendritic spine shaping, LTP diminishment, and mGluR-LTD augment. In current studies, we overexpressed one-third of the wild-type *fmr1* 5′-UTR r(CGG) expansion region and found one effective ramRNA, miR-*fmr1-42*; it is estimated that the full *FMR1* r(CGG) expansion in FXS may generate more than 12 kinds of ramRNAs. These findings signify a high similarity between the real human FXS and our ramRNA-induced FXS animal model, which may shed light on new therapeutic interventions.

The list of developmental and degenerative diseases that are caused by expansion of microsatellite-like genomic repeats continues to grow. Many of the trinucleotide repeats are predicted to encode miRNAs; nevertheless, no repeat-associated miRNA (ramRNA) has ever been identified before

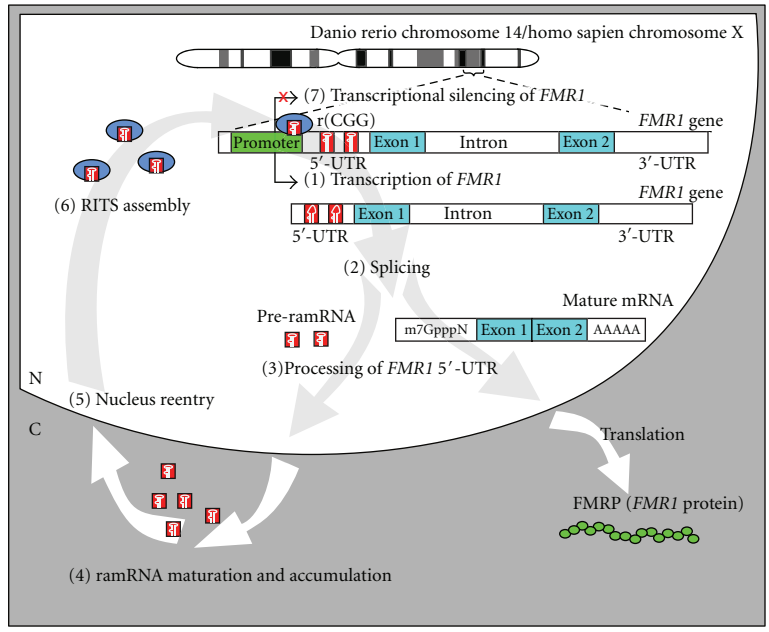

Figure 3: Proposed mechanism of ramRNA-mediated *FMR1* inactivation in FXS. Fragile X mental retardation 1 (*FMR1*) contains a trinucleotide CGG repeat region (r(CGG)) located in the 5′-UTR of the gene. Expansion of this repeat region of *FMR1* to over 200 copies results in loss of FMRP expression. Based on current data, pathological silencing of FMRP occurs in seven steps. (1) *FMR1*, including the r(CGG) region, is transcribed at an early embryonic stage (day 10 in humans and 12 hours postfertilization in zebrafish). (2) Splicing of the gene transcript to form mature mRNA. During this process r(CGG) molecules are released. (3) The r(CGG) molecules are further processed into repeat-associated miRNA precursors (pre-ramRNA) and exported out of the nucleus. (4) Pre-ramRNA is further processed by the enzyme *Dicer* or a *Dicer*-like endoribonuclease. Mature miR-*FMR1*s accumulate in the cytoplasm near the nucleus. (5) Some miR-*FMR1*s containing a nuclear import signal (NIS) reenter the nucleus by an unknown mechanism. (6) As nuclear miR-*FMR1*s concentrations rise within the nucleus, they may begin to form complexes for RNA-induced transcriptional silencing (RITS). (7) RITS complexes accumulate near *FMR1* promoter and interact with Rad541 and MeCP2, leading to transcriptional silencing of *FMR1* through a CpG methylation mechanism. Consequently, ramRNA-mediated transcriptional silencing of *FMR1* results in loss of FMRP expression, which is observed in ~99% of patients with FXS.

our studies. We established three important breakthroughs in the understanding of r(CGG)-derived ramRNA function in FXS. First, mature ramRNAs, namely, miR-*fmr1*, can be generated from the 5′-UTR r(CGG) expansion of the *fmr1* gene in zebrafish, matching the previously predicted epigenetic disease model of human FXS. Second, the ramRNA-induced FXS zebrafish can be raised and maintained to show the same neural defects found in human FXS. Finally, the normal expression pattern of miR-*fmr1* in wild-type zebrafish is limited within the cytoplasm of neuronal bodies, whereas the presence of r(CGG)-associated ramRNAs in FXS neurons can extend into the compartments of nuclei and dendrites, consequently leading to transcriptional *fmr1* inactivation. These findings confirm the feasibility of using this novel FXS animal model for studying ramRNA-mediated pathogenesis and neuropathology, which may be common in human FXS patients but difficult to identify in other *FMR1*-deleted animal models. In addition, our novel ramRNA overexpression approach may provide further insights into the molecular mechanism of brain-specific trinucleotide repeats for understanding how a ramRNA affects human IQ. Given that there are still many more microsatellite-like nucleotide repeats in the human genome, which may code for a variety of ramRNA species, as might be expected, learning how to use the newly established intronic miRNA expression system for exploiting the functional roles of these ramRNAs *in vivo* will be a forthcoming challenge.

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Review Article

What We Know and Would Like to Know about CDKL5 and Its Involvement in Epileptic Encephalopathy

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In the last few years, the X-linked serine/threonine kinase cyclin-dependent kinase-like 5 (CDKL5) has been associated with early-onset epileptic encephalopathies characterized by the manifestation of intractable epilepsy within the first weeks of life, severe developmental delay, profound hypotonia, and often the presence of some Rett-syndrome-like features. The association of CDKL5 with neurodevelopmental disorders and its high expression levels in the maturing brain underscore the importance of this kinase for proper brain development. However, our present knowledge of CDKL5 functions is still rather limited. The picture that emerges from the molecular and cellular studies suggests that CDKL5 functions are important for regulating both neuronal morphology through cytoplasmic signaling pathways and activity-dependent gene expression in the nuclear compartment. This paper surveys the current state of CDKL5 research with emphasis on the clinical symptoms associated with mutations in *CDKL5*, the different mechanisms regulating its functions, and the connected molecular pathways. Finally, based on the available data we speculate that CDKL5 might play a role in neuronal plasticity and we adduce and discuss some possible arguments supporting this hypothesis.

1. Introduction

The kinase CDKL5 (Cyclin-Dependent Kinase-Like 5) was initially identified through a positional cloning study aimed at isolating disease genes mapping to Xp22. Sequence analysis revealed homologies to several serine-threonine kinase genes and identified one protein signature specific for this subgroup of kinases, therefore, leading the authors to name the gene *STK9* (Serine Threonine Kinase 9) [1]. Within the next five years, mutations in this gene were found in epileptic patients; in 2003, Vera Kalscheuer suggested STK9 to be a chromosomal locus associated with X-linked infantile spasms (ISSX) [2]. In particular, by sequence comparison, the authors discussed the resemblance of the kinase domain of STK9 to the MAP kinase family and hypothesized a role in the MAP kinase cascade, a hypothesis still to be tested. Given the strong similarity to some cell division protein kinases [2], the *STK9* gene subsequently appeared as *CDKL5/STK9* and, eventually, got renamed *CDKL5*.

A study of the evolutionary conservation of the human CDKL5 protein sequence (GeneBank accession n°. CAI42485) identified several orthologs in vertebrates with large blocks of homology diffused through the whole protein. Fish orthologs diverge the most from the human protein [3], while no orthologs have been found in invertebrates or in plants (unpublished data).

Despite the clear involvement of CDKL5 for proper brain development, as evidenced from the available clinical and molecular data, CDKL5 functions are still far from being understood. This paper examines the current status of CDKL5 research, highlighting the clinical symptoms involving CDKL5, the diverse isoforms so far described and their regulation of expression, the molecular pathways associated with it, and the most urgent studies required to better understand its functions and its possible involvement in neuronal plasticity.

2. Clinical Aspects Associated with *CDKL5* Mutations

Even though the first patients mutated in *CDKL5* were two girls affected by X-linked infantile spasms [2], subsequent cases were reported in female patients with a clinical phenotype mimicking Rett syndrome (RTT), which is in most cases caused by mutations in *MECP2* [4, 5]. Because of that, the first genetic screenings for *CDKL5* mutations occurred mainly in cohorts of patients with RTT syndrome, or a variant of it, with no *MECP2* mutations. By comparing the clinical phenotypes of patients with *CDKL5* mutations already described in literature, it became clear that almost all of them presented early epilepsy, starting from 10 days to 3 months after birth. Therefore, in the last years, *CDKL5* screenings have been extended to cohorts of both genders in which patients were characterized by epileptic encephalopathy. Interestingly, Intusoma et al. [6] suggested in a recent paper that screening among patients having intractable seizures with an onset before 6 months of age gives a higher score than screening among *MECP2*-negative RTT patients; this score is even increased when RTT-like features are shown as well. In detail, the authors estimated that clinical sensitivity among females with intractable seizures starting before the ages of 12, 6, and 3 months were 4.7, 11.6, and 14.3%, respectively. Stunningly, this value rises to 31.3% when some typical RTT symptoms are included in the phenotype [6]. Importantly, these results are in good concordance with other publications.

Albeit few milder cases have been reported, the clinical phenotype of girls mutated in *CDKL5* is in general comparable between several reports [7–9]. In particular, early drug-resistant epilepsy, usually starting in the first months of life, tends to be the most common feature. Seizures are in general highly polymorphic and many different seizure types can also occur in the same patient, changing with time. Complex partial seizures, infantile spasms, myoclonic, generalized tonic-clonic, and tonic seizures have all been reported. Very often, patients treated with antiepileptic drugs face a seizure-free honey-moon period, which, unfortunately, is followed by relapses. The clinical and EEG data, available so far, do not permit to recognize any exact pattern; conversely, no specific abnormalities have been clearly detected by MRI [2, 10–12]. In addition, it has been proposed that stereotypic hand movements, severe hypotonia, and impaired psychomotor development are usually associated with *CDKL5* mutations and common to the general clinical manifestation of RTT patients [7–9, 12]. Whereas most of the RTT features presented by *CDKL5* patients become evident when they get older [12], these patients lack the apparent normal development followed by regression that is usually considered a diagnostic criteria for Rett syndrome [13]. Furthermore, differently from RTT, *CDKL5* patients are generally characterized by residual hand use, poor eye fixation with avoidance of eye contact, visual impairment, and feeding difficulties. Indeed, cortical visual loss has been described by several reports [12, 14–16], and Moseley and colleagues have recently reported that many young CDKL5-positive children exhibit significant dysphagia, requiring PEG/PEJ placement [16]. Eventually, autonomic features such as breathing irregularities, cold extremities, and gastrointestinal disturbances might occasionally be found in *CDKL5* patients, whereas they are consistently found in Rett syndrome.

An interesting diversity between patients mutated in *MECP2* or *CDKL5* emerges from the clinical severity shown by male patients with *CDKL5* mutations with respect to those of the same gender carrying *MECP2* mutations. Indeed, it is generally accepted that *MECP2* mutations causing classic Rett syndrome in females lead to encephalopathy and death in males with a normal chromosome complement within the first years of life. The same mutations, instead, lead to Rett syndrome in XXY backgrounds or in males with somatic mosaicisms. Eventually, mutations with minor clinical relevance in females can lead to RTT in males [17]. On the contrary, the most recent reports suggest that clinical severity does not differ between males and females with *CDKL5* mutations [10, 18, 19]. Furthermore, whereas *MECP2* mutations are rarely found in males, Liang et al. estimated that, in a cohort of patients affected by epileptic encephalopathy, *CDKL5* mutations have a frequency of 5% in males and 14% in females [10]. While these studies suggest that *CDKL5* testing should be considered independently of the gender, they also lead to an important question: why do mutations in an X-linked gene lead to a similar outcome in females and males? Even though no answers are yet available, we believe that, in the future, it will be important to address whether CDKL5-depleted neurons affect the phenotype of wild-type neurons. Anyhow, these results suggest that whereas a major source of the phenotypic variability associated with *MECP2* mutations is given by the pattern of X chromosome inactivation, this does not seem to influence the clinical outcome determined by CDKL5. Accordingly, a study describing a male with Klinefelter syndrome (47, XXY) and a large CDKL5 COOH-truncation presenting a phenotype comparable to those of other CDKL5-positive boys suggests that the presence of a wild-type *CDKL5* allele and a balanced pattern of X-inactivation does not reduce the severity of the disease [20].

3. The *CDKL5* Gene and Protein Isoforms

The human *CDKL5* gene occupies approximately 240 kb of the Xp22 region and is composed of 24 exons of which the first three (exons 1, 1a, 1b) are untranslated, whereas the coding sequences are contained within exons 2-21 (Figure 1). Two splice variants with distinct 5′UTRs have been found: isoform I, containing exon 1, is transcribed in a wide range of tissues, whereas the expression of isoform II, including exons 1a and 1b, is limited to testis and fetal brain [2, 3]. Alternative splicing events lead to at least three distinct human protein isoforms. The original *CDKL5* transcript generates a protein of 1030 amino acids ($CDKL5_{115}$; 115 kDa). While $CDKL5_{115}$ is expressed mainly in testis, two recently identified transcripts are likely to be relevant for CDKL5 brain functions [3, 21]. As depicted in Figure 1, these two isoforms are characterized by an altered C-terminal region. Importantly, one of these ($CDKL5_{107}$ in Figure 1) is

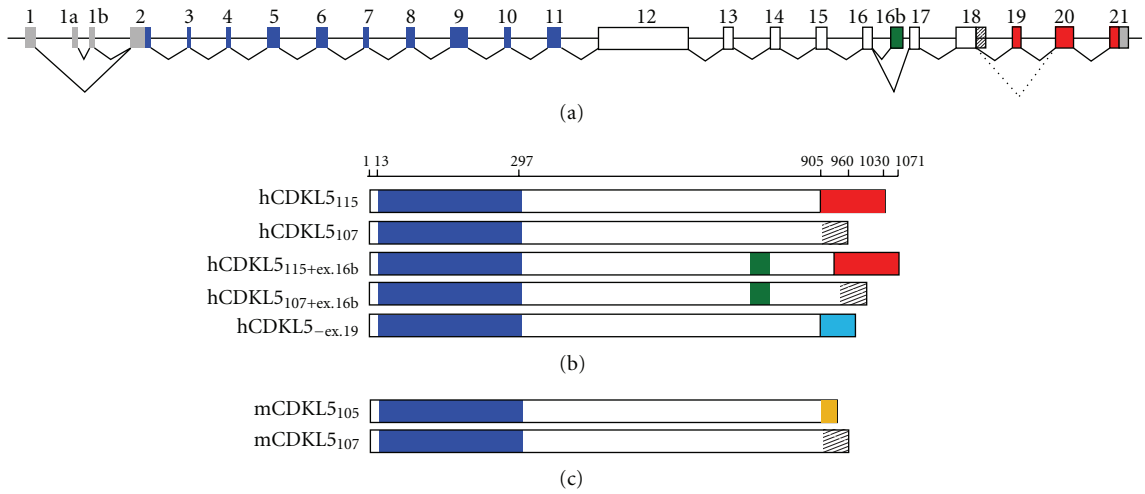

Figure 1: The genomic structure of *CDKL5* and its splice variants. (a) The human *CDKL5* gene with nontranslated sequences in grey and exons encoding the catalytic domain in blue. Exons encoding the common C-terminal region appear in white, whereas isoform-specific sequences are shown in red, green, and as hatched. (b) hCDKL5 protein isoforms differing in the C-terminal region. $CDKL5_{115}$ [22] contains the primate-specific exons 19–21. In $CDKL5_{107}$ [3], intron 18 is retained. The inclusion of exon 16b would generate $CDKL5_{115+ex.16b}$ and/or $CDKL5_{107+ex.16b}$ [21, 23]. $hCDKL5_{-ex19}$ is a hypothetical splice variant in which exon 19 is excluded generating an alternative C-terminus (light blue; personal communication Limprasert.) (c) The murine CDKL5 isoforms. $mCDKL5_{105}$ harbors a distinct C-terminal region encoded by a mouse-specific exon 19 (orange). As in humans, the retention of intron 18 generates the common $CDKL5_{107}$ isoform [3].

common in a number of species, including mouse, which renders *Cdkl5* mouse models of significant relevance for studying CDKL5 functions [3]. Interestingly, an alternative splice variant, containing yet another distinct C-terminus, has been predicted through a bioinformatics simulation (ECgene analysis; personal communication Dr. Limprasert).

Interestingly, two isoforms selectively expressed in rat neurons and glial cells have recently been described [24]. By western blotting of mouse CDKL5 in a pure glial culture, a weak signal sensitive to siRNA of CDKL5 and migrating below the major band in a brain extract can be detected (our unpublished results). This suggests that a glial-specific CDKL5 isoform is expressed also in mouse.

4. *CDKL5* Mutations and Their Influence on the Phenotypic Outcome

The CDKL5 protein belongs to the CMGC family of serine/threonine kinases (including cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK), and CDK-like kinases) and is characterized by an N-terminal catalytic domain (amino acids 13–297), homologous to that of the other CDKL-family members. CDKL5 is unique in this family of kinases as it displays an unusual long tail of more than 600 amino acids without obvious similarity to other protein domains but with a high degree of conservation between different CDKL5 orthologs that differ only in the most extreme C-terminus. Besides the ATP-binding region and the serine/threonine protein kinase active site (amino acids 13–43 and 131–143, resp.), CDKL5 is characterized by a Thr-Xaa-Tyr motif (TEY) at amino acids 169–171, whose dual phosphorylation is normally involved in activating, among others, kinases of the MAP kinase family. Moreover, putative signals for nuclear import (NLS) and export (NES) have been found in the C-terminus of the protein, as shown in Figure 2.

The limited number of CDKL5 targets has not yet allowed drawing a consensus sequence for this kinase; however, the presence of a critical arginine-residue in the kinase subdomain VIII suggests that CDKL5 might be a proline-directed kinase. Moreover, as some of the other CMGC protein kinases, CDKL5 appears capable of autophosphorylating its TEY motif [39].

Almost 90 different *CDKL5* patients, harboring a wide range of pathogenic mutations, have been described so far, including missense and nonsense mutations, small and large deletions, and frameshifts and aberrant splicing (Figure 2). Even though the small number of cases does not permit to draw any conclusive data, hot-spots have been suggested so far only for few mutations (indicated with an asterisk in Figure 2). By analyzing the distribution of missense mutations, it appears evident that they localize mainly in the catalytic domain, thus confirming the relevance of the kinase activity of CDKL5 for proper brain function and/or development. On the contrary, truncating mutations can be located anywhere in the gene, leading to CDKL5 derivatives of various lengths. Again, the relevance of the rather uncharacterized C-terminal part of CDKL5 is suggested by the fact that many pathogenic alterations involve the C-terminus. Importantly, in a recent publication, Bahi-Buisson and colleagues [12] analyzed patients' explants for their expression levels of CDKL5 transcripts. Some nonsense and frame shift mutations caused a significant reduction or the complete absence of the mutated transcripts, whereas other mutations, probably not subjected to the process of nonsense-mediated decay, were expressed similarly to the

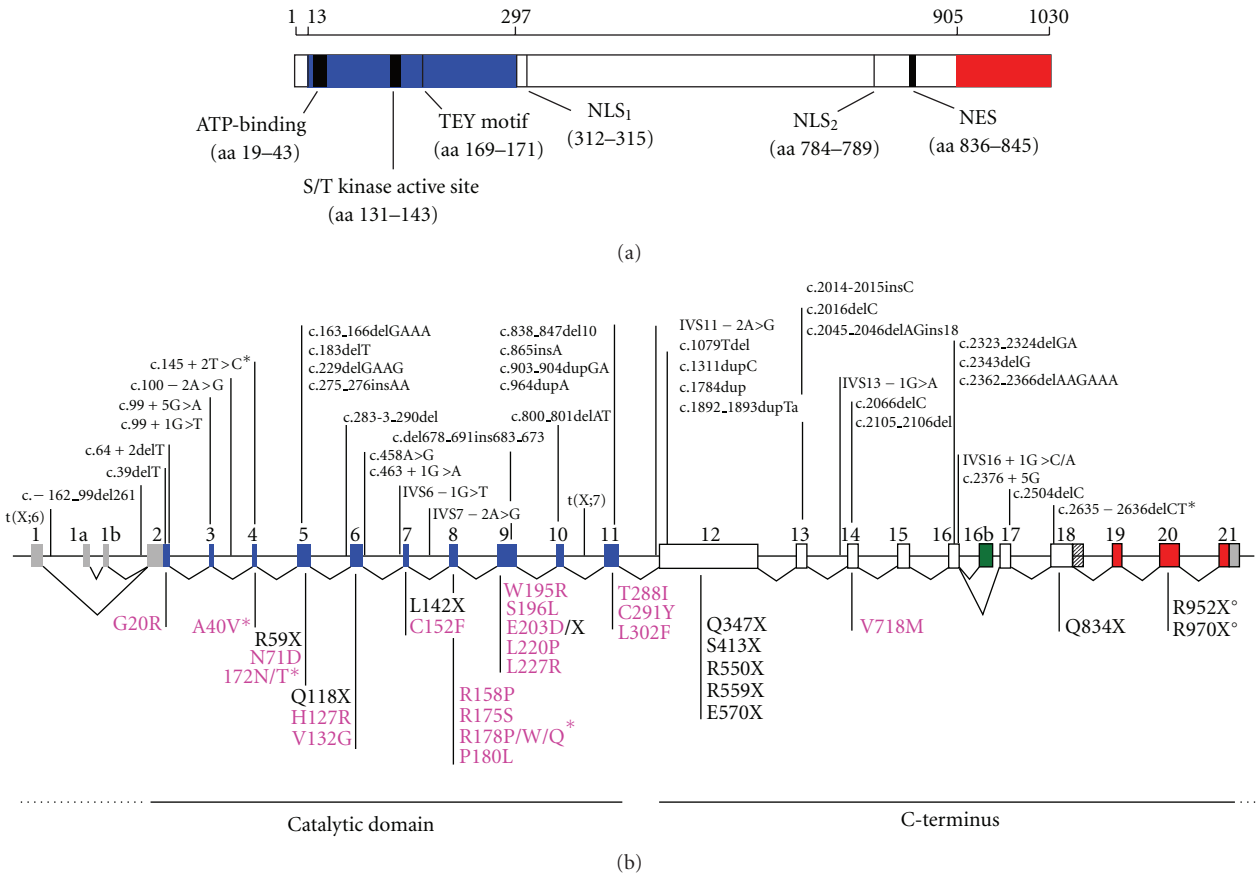

Figure 2: Pathogenic *CDKL5* mutations. (a) Schematic representation of $CDKL5_{115}$ with the functional domains and signatures indicated. *NLS:* nuclear localization signal; *NES:* nuclear export signal. (b) All mutations in *CDKL5* reported to date are indicated corresponding to their location within the gene. Mutations shown above the *CDKL5* gene are deletion and frame shift mutations as well as splice variants indicated with cDNA nomenclature. Missense and nonsense mutations (fuchsia and black, resp.) are represented with amino acid nomenclature below the *CDKL5* gene. *: recurrent mutations; °: uncertain pathogenicity. The indicated mutations have been referred by: t(X;6) [2]; c.-162_99del261 [25]; c.39delT [10]; c.64+2delT [12]; c.99+1G>T [12, 26]; c.99+5G>A [8, 27]; c.100-2° >G [9]; c.145+2T>C [12, 25, 28]; c.229delGAAG [12]; c.183delT [5]; c.163_del166delGAAA [18, 29]; c.275_276insAA [8]; c.283-3_290del [8]; c.del458A>G [9]; c.463+1G>A [9]; IVS6-1G>T [30]; IVS7-2A>G [31]; c.del678_691ins683_673 [30]; c.800_801delAT [12, 26]; t(X;7) [2]; c 838_847del10 [22]; c.865insA [12]; c.903_904dupGA [25]; c.964dupA [18]; IVS11-2° >G [30]; c.1079Tdel [10]; c.1311dupC [12, 26]; c.1784dup [8]; c.1892_1893dupTA [12, 26]; c.2014-2015insC [10]; c.2016delC [12]; c.2045_2046delAGins18 [12, 26]; IVS13-1G>A [5]; c.2066delC [18]; c.2105_2106del [8]; c.2323_2324delGA [12, 26]; c2343delG [22]; c.2362-2366delAAGAAA [30]; IVS16+1G>C/A [30, 31]; c.2376+5G [25]; c.2635_2636delCT [12, 26, 29]; G20R [32]; A40V [9, 12, 26, 33]; R59X [30, 34]; N71D [7]; I72N [31]; I72T [25]; Q118X [12, 26]; H127R [25]; V132G [7]; L142X [12]; C152F [4]; R158 [35]; R175S [4]; R178P [9, 19]; R178W [7, 9]; R178Q [10]; P180L [30]; S196L [32]; E203D [7]; E203X [36]; L220P [12, 26, 33]; L227R [9]; T288I [19]; C291Y [19]; L302F [10]; Q347X [7, 10]; S413X [10]; R550X [25, 28]; R559X [10, 20]; E570X [36]; V718M [12]; Q834X [12, 26, 37]; R952X [6]; R970X [38].

wild-type *CDKL5* mRNA. Even though, so far, no reports have demonstrated the existence of the truncated CDKL5 proteins in human cells, the molecular effects of some pathogenic CDKL5 mutations (missense and truncating derivatives in the background of the $CDKL5_{115}$ isoform) have been addressed through the overexpression of mutated derivatives in nonneuronal cell lines [33, 39–41]. To summarize such data (including our unpublished results), we can state that, as expected, missense mutations generally impair the kinase activity of CDKL5 and can therefore in most cases be considered loss-of-function mutations. Further studies are needed to evaluate whether, as hypothesized, a significant reduction of the catalytic activity of CDKL5 influences its subcellular distribution. A regulatory role for the tail of CDKL5 has emerged from the characterization of few C-terminal truncating derivatives. In fact, it seems to act as a negative regulator of the catalytic activity of CDKL5 and to modulate the subcellular localization [39, 41]. In particular, since both the pathogenic derivatives L879X and R781X confine CDKL5 to the cell nucleus, we can possibly state that the very last portion of CDKL5 acts localizing the protein to the cytoplasm. Moreover, since the leucine-rich NES-like motif, shown in Figure 2, is preserved in the L879X derivative, such motif seems insufficient for driving CDKL5 to the cytoplasm. Whether truncated CDKL5 mutants act as loss- or gain-of-function proteins

still remains to be understood. Indeed, if expressed, they would be mislocalized hyperfunctional derivatives; however, as described below, since CDKL5 seems to exert its functions both in the nucleus and the cytoplasm, it remains possible that the absence of CDKL5 from the cytoplasm might also contribute to the pathogenic phenotype. To conclude and by integrating the described results with a number of studies reporting pathogenic duplications of X chromosome regions including CDKL5 [9, 42–45], it remains possible that a tight regulation of CDKL5 levels and/or activity is essential for the proper function of the central nervous system, therefore making the search for pathogenic *CDKL5* gene duplications relevant.

So far, no clear genotype-phenotype correlation of CDKL5 mutations has been established. Some reports suggested that mutations in the COOH-terminus originate milder clinical pictures than those caused by mutations in the catalytic domain, but others state that the nature of the mutation does not correlate with the clinical heterogeneity. Accordingly, Weaving et al. [5] reported of two genetically identical CDKL5-mutated twin girls with a significant discordant phenotype. Indeed, one proband showed a clinical phenotype overlapping RTT, whereas her twin sister showed autistic disorder and mild-to-moderate intellectual disability. Since both girls were characterized by random X-inactivation, we believe that their phenotypic differences can be attributed to modifier genes that have been differentially influenced by environmental and/or epigenetic factors. Moreover, a recent report [6] identified an R952X mutation with incomplete penetrance and uncertain pathogenicity. Since this novel mutation occurs in exon 20 that, as described, might not be highly expressed in brain, we speculate that modifier genes affecting CDKL5 splicing might be responsible for the observed penetrance.

Future studies, focused on the identification of direct and indirect partners of CDKL5, will help defining its functions and might lead to the identification of modifier genes representing relevant targets for therapeutic approaches.

5. Modulation of CDKL5 Abundance and Localization

Expression studies in human and mouse tissues have shown that *CDKL5/Cdkl5* mRNA is present in a wide range of tissues besides the brain, where the transcripts levels are the highest [1, 3]. Indeed, whereas *CDKL5* mRNAs can easily be detected in tissues such as testis, lung, spleen, prostate, uterus, and placenta, they are barely present, or under detection levels, in heart, kidney, liver, and skeletal muscle. Even if the data regarding CDKL5 protein expression in different tissues are somewhat conflicting, the transcription data have been partially confirmed by western blotting using adult rat extracts [24, 40]. To draw a conclusion, what is clear and commonly accepted is that, in the brain, CDKL5 levels reach the highest in conjunction with the development and differentiation of this organ. CDKL5 in fact is only weakly present during embryogenesis to get strongly induced in the early postnatal stages until P14, where after it declines [41].

A detailed analysis of *Cdkl5* expression in adult mouse brain (Figure 3) shows that its mRNA levels are particularly high in the adult forebrain. Interestingly, higher expression levels are detected in the most superficial cortical layers, particularly involved in the connection of the two hemispheres through the corpus callosum; a slightly higher abundance of mRNA in the frontal cortical areas might suggest a role for CDKL5 in the physiology of such brain districts. Notably, fairly strong staining is detected in the motor cortex and the cingulate cortex, an area of high interest for the origin of a wide plethora of mental diseases. Of interest, high levels of expression are detected in the pyriform cortex and, possibly, in the entorhinal cortex. The hippocampus, a brain area that partly shares the same developmental origin as the cortex, shows very high levels of *Cdkl5* mRNA in all the CA fields, but in the dentate gyrus, possibly in accordance with the establishment of *Cdkl5* transcription in fully mature neuronal phenotypes, given that the DG neuronal population undergoes adulthood neurogenesis. Considering the fair expression levels of *Cdkl5* in the striatum, we assume that the glutamatergic and the gabaergic neurons are by far the two cellular types expressing most of the brain *Cdkl5*. Accordingly, very low, if any, expression was detected in dopaminergic areas such as the substantia nigra or the ventral tegmental area or in noradrenergic areas such as the locus coeruleus. Of interest, however, very high levels of *Cdkl5* transcripts are detected in several thalamic nuclei, including the geniculate nuclei. In the cerebellum, *Cdkl5* mRNA is expressed in all the lobules and, possibly, in the Purkinje cells; its levels, however, appear significantly lower when compared to the other brain areas. Specific experiments aimed at elucidating *Cdkl5* expression in all the cerebellar cell types will shed light on its transcriptional pattern.

Carouge and colleagues have initiated the characterization of *Cdkl5* transcriptional regulation [46]. Interestingly, in the rat gene, they found a CpG-rich sequence of 0.8 kb (from −346 to +490), well conserved in the mouse and human counterparts. The authors demonstrated that DNA methylation involving this area inhibits *Cdkl5* expression and that the kinase gene is a target of the repression mediated by MeCP2. Even though these data contrast earlier reports where *CDKL5/Cdkl5* mRNA levels were found unaltered in RTT patient lymphocytes or brains of *Mecp2*-null mice [5, 22], we would like to mention that our unpublished results appear to confirm a role of MeCP2 in inhibiting CDKL5 expression. In the future, it might be interesting to discern whether these contradictory results stem from the capability of MeCP2 to act on different genes according to the specific cellular type. Eventually, CDKL5 transcription appears regulated upon different stimuli and depending on the specific brain district. Indeed, a significant reduction of *Cdkl5* mRNA has been observed in the striatum of rats treated with cocaine, whereas this reduction was not revealed in frontal cortexes of the same animals. As complementary results, the levels of the *Cdkl5* transcript appear reduced incubating PC12 cells with serotonin [46].

Concerning the protein expression, the available data suggest that the levels of the kinase more or less coincide

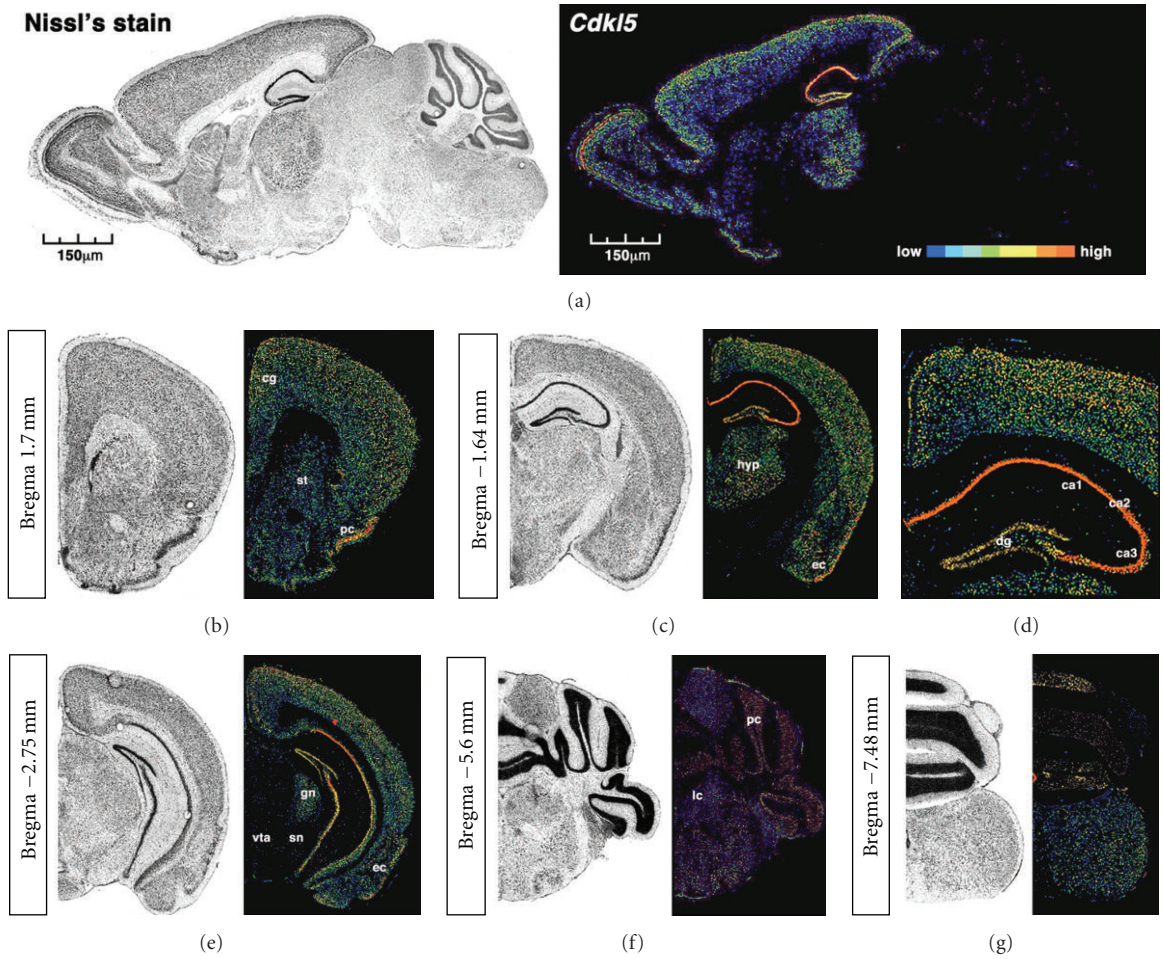

Figure 3: *Cdkl5* expression patterning in the adult male mouse brain (C57BL/6J, postnatal day 56). Images are collected from the Allen Brain Atlas website (http://www.brain-map.org/; probe name RP_051219_02_C01; experiment ID 74000486 for sagittal samples, experiment ID 75042239 for coronal samples). Each Nissl-stained section is coupled by a quantitative *in situ* hybridization screening at different sectioning levels. The color bar in (a) might be used to follow transcription level intensity. (b), (c), (d), (e), (f), and (g) are representative of different brain levels, therefore, different brain areas; in particular: cg: cingulated cortex, st: striatum, pc: piriform cortex, hyp: hypothalamus, ec: entorhinal cortex, dg: dentate gyrus, ca1,2,3: hippocampal CA fields, vta: ventral tegmental area, gn: geniculate nuclei, sn: substantia nigra, lc: locus ceruleus, pc: Purkinje cells.

with those of mRNA in the adult brain. At the cellular level, CDKL5 is easily detectable in virtually all NeuN-positive neurons while it is expressed at very low levels in the glia [24].

Several mechanisms seem to regulate CDKL5 functions and levels. The presence of different CDKL5 splice variants, of which some, as mentioned, appear to be enriched in brain, indicates that alternative splicing is involved in regulating CDKL5 functions. At the functional level, we still need to understand whether the different CDKL5 isoforms have distinct functions. The only difference that has been observed so far is that $CDKL5_{107}$ appears to be more stable than the longer human $CDKL5_{115}$ isoform [3], whereas the subcellular localization of exogenous CDKL5 derivatives ($CDKL5_{115}$, $CDKL5_{107}$, and $CDKL5_{115+16b}$) is grossly identical [3, 21].

CDKL5 functions seem to be regulated both through its subcellular localization and through its synthesis and degradation. In brain, CDKL5 is initially predominantly cytoplasmic and progressively accumulates in the nucleus, starting from roughly P14 when approximately 40% of total CDKL5 can be detected in this compartment. However, CDKL5 gets significantly translocated to the nucleus only in certain brain areas: in the cerebellum, for example, more than 80% of CDKL5 remains cytoplasmic while in the cortex it is almost equally distributed between cytoplasm and nucleus [41]. Exogenous CDKL5 shuttles constitutively between the two main compartments in cultured non-neuronal cells through an active nuclear export-dependent mechanism involving the C-terminus of the protein and the CRM1 nuclear export receptor. Interestingly, however, in resting hippocampal neurons the protein is not dynamically shuttling and its nuclear exit appears to be regulated by specific stimuli. In fact, glutamate treatment induces nuclear export of CDKL5 leading to its accumulation in the cytoplasm [47]. In the future, it will be interesting to understand whether (a) posttranslational modifications or interactions with other proteins are involved in regulating the

nuclear export/import of CDKL5, (b) the enzymatic activity of the protein is modulated by its localization and interaction with other factors, and eventually (c) which other stimuli influence the localization and activity of CDKL5.

As mentioned, the degradation of CDKL5 seems also to be involved in regulating its functions. The long $CDKL5_{115}$ isoform is, in fact, rapidly degraded in a proteasome-dependent manner in transfected cells. Conversely, the human and mouse $CDKL5_{107}$ isoforms are more stable and their quantity is not significantly increased by the proteasome inhibitor MG132, thus indicating that the very C-terminal region, from amino acid 905, contains signals responsible for this degradation [3]. In neurons, however, CDKL5 levels are strongly reduced upon sustained glutamate treatment or other cell death signals [47]. Why neurons degrade CDKL5 upon cell death induction and how this degradation is regulated still remains to be elucidated.

6. Is CDKL5 Involved in Synaptic Function, Structure, and Plasticity?

Modulation of cellular activity is the force that regulates dendritic growth and morphology during neuronal development, affecting locally the synapses and regulating transcription at the nuclear level. Similarly, activity-induced calcium influx alters synaptic function in mature neurons, modifying synaptic strength and gene expression [48]. Therefore, extracellular cues and neuronal adaptive responses equally cooperate through signaling mechanisms that flow from the synapses through the cytoplasm to, eventually, the nucleus. Mutations in components of these signaling pathways might thus result in impaired information processing in the brain, therefore leading to neurodevelopmental, neuropsychiatric, and neurodegenerative disorders. In particular, Rett syndrome, whose connection with CDKL5 has already been discussed, is characterized by a number of synaptic deficits [49]. Is CDKL5 actually involved in neuronal plasticity? Even though the lack of mouse models for *Cdkl5* functions does not permit to draw a definitive picture, there are several pieces of evidence supporting this hypothesis.

6.1. Cdkl5 Expression Correlates with Neuronal Maturation and Maintenance. In recent years, it has become clear that dendrites and spines are dynamic structures that during early postnatal development undergo a significant remodeling necessary for synapse function and plasticity. As development proceeds up to the adulthood, spines continue to be remodeled in response to diverse stimuli such as LTP and LTD; these changes are considered of high relevance for learning and memory.

As already mentioned, CDKL5 expression correlates, both *in vitro* and *in vivo,* with neuronal maturation, reaching the highest levels of expression when neurons acquire a mature phenotype and suggesting an involvement of the kinase in neuronal differentiation and arborization [24, 41, 47]. Interestingly, even if CDKL5 levels seem to slightly decrease in adult brain, they are significantly higher when compared to nonneuronal tissues. Therefore, it is possible to speculate that CDKL5 might play a role in maintaining neuronal functions in addition to maturation. Furthermore, since, as already stated, CDKL5 intracellular distribution changes upon neuronal maturation and its nuclear fraction peaks in adult brain, such fraction may be involved in adult synaptic plasticity.

6.2. Cdkl5 Affects Neuronal Morphogenesis through Cytoskeleton Rearrangements. The morphology of neuronal extensions and their spines influences the electrophysiology and behavioral output of the cell. Indeed, arborization defects have already been reported in brain disorders, such as RTT and Fragile X, in which experience-dependent neuronal maturation and plasticity are disrupted [50]. Interestingly, by RNAi and overexpression of CDKL5 in cultured rat cortical neurons, Chen and colleagues demonstrated that CDKL5 is a critical regulator of neuronal morphogenesis and dendritic arborization. *In vivo*, the kinase shows overlapping functions and affects neuronal migration [24]. Even if future studies should investigate whether a malfunction of CDKL5 also impairs synaptic spine morphology, it is important to mention that CDKL5 colocalizes with F-actin in the growth cone and interacts with Rac1 [24]. Rac1 belongs to the Rho GTPase family of proteins that promotes the formation and/or maturation of spines by remodeling the actin cytoskeleton of neuronal spines [51]. Functional experiments suggested that CDKL5 influences neuronal morphogenesis by acting upstream of Rac1.

6.3. CDKL5 Functions Respond to Neuronal Stimuli. A comprehensive knowledge of the stimuli affecting CDKL5 expression and activities will help to understand how its deficiency impacts brain functions. However, so far, only limited information is available. In particular, it has been demonstrated that *BDNF*, an activity-regulated gene encoding a neurotrophin already involved in several neurological and psychiatric disorders including RTT, induces a transient phosphorylation of CDKL5. The kinase is required for the capability of BDNF to activate Rac1 [24]. Furthermore, we demonstrated that CDKL5 is transported outside the nucleus into the cytoplasm in response to activation of extrasynaptic NMDA receptors (NMDA-R). Recent publications suggest that extrasynaptic NMDA-Rs have a role in LTD and dephosphorylation of CREB; alterations in the cross-talk between synaptic and extrasynaptic receptor activities might play an important role in seizures [52–54]. Of interest, we discovered that CDKL5 is degraded by the proteasome in response to extended glutamate bath stimulation or other death stimuli [47]. These results, linking CDKL5 to programmed cell death pathways, appear particularly intriguing considering that (1) local cell death and pruning enable proper brain development and (2) proteolysis by the ubiquitin-proteasome pathway is emerging as a new mechanism controlling synaptic plasticity. As an example, the ubiquitin ligase Ube3A is associated with human cognitive defects, including Angelman syndrome, and it has been hypothesized that defective degradation of different Ube3A substrates, such as Arc, might contribute to the Angelman clinical manifestations [55].

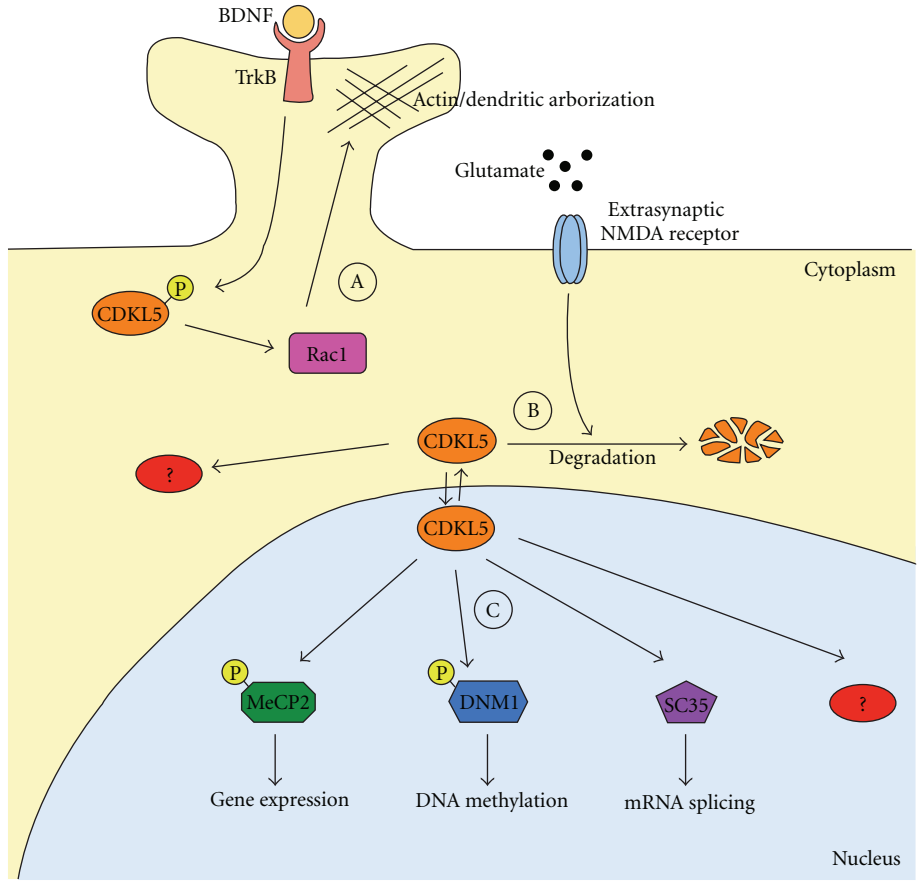

Figure 4: A model depicting the different functions of CDKL5 in the cytoplasmic and nuclear compartments. As described in the text, CDKL5 functions are occurring both in the cytoplasm and in the nucleus. A. In the cytoplasm, CDKL5 is involved in the regulation of actin cytoskeleton and dendritic arborization. This function is mediated by the interaction of CDKL5 with Rac1. Importantly, a link between BDNF, CDKL5 phosphorylation, and Rac1 activation has been suggested by Chen et al. [24]. B. In the cytoplasmic compartment, the levels of CDKL5 are regulated by degradation. Furthermore, we assume that several phosphorylation targets of CDKL5 remain to be identified. C. In the nucleus, CDKL5 has been proposed capable of interacting with and phosphorylating MeCP2 and DNMT1, thereby influencing gene expression and DNA methylation. Furthermore, the protein has been shown to colocalize with RNA speckles involved in RNA splicing. As for the cytosol, we assume that several other targets of CDKL5 remain to be discovered. In this model, we have neglected the capability of the nuclear CDKL5 to influence dendritic arborization [24] and the transcriptional regulation of *CDKL5*.

6.4. CDKL5 Might Regulate the Functions of Epigenetic Factors and Transcriptional Regulators. As already mentioned, bidirectional signaling pathways link synapses to the nucleus, and, moreover, sensory experiences influence gene expression. Regarding this topic, little evidence suggests that CDKL5 has a role in regulating gene expression. Chen and colleagues [24] demonstrated that the ability of CDKL5 to influence dendritic growth occurs both in the cytoplasm and in the nucleus and requires its catalytic activity. Accordingly, we, and others, have demonstrated that, in cultured cell lines, exogenously expressed CDKL5 interacts with the transcriptional repressor MeCP2 [22, 40] and seems capable of phosphorylating the methyl binding protein *in vitro*. Indeed, the two human CDKL5 isoforms ($CDKL5_{107}$ and $CDKL5_{115}$) immunopurified from cultured cell lines induce the phosphorylation of a recombinant MeCP2, purified from *E. coli* [3, 22, 39]. Identical results are routinely obtained in our lab using the murine CDKL5 (unpublished results). To this regard, two previous publications were unable to obtain a CDKL5-mediated phosphorylation of MeCP2 [40, 56]. It is, however, important to mention that the experimental conditions were quite different; indeed, one paper was using a truncated CDKL5 derivative that lacks the identified MeCP2-interacting domain [56], the second one uses a lower abundant, immunopurified MeCP2 [40]. It is still unclear whether CDKL5 influences the phosphorylation state of MeCP2 *in vivo*. Anyhow, it must be recalled that several papers suggest that, in neurons, MeCP2 functions as a dynamic epigenetic factor, regulating gene transcription during learning and memory through activity-dependent phosphorylation of specific serine residues [57–60]. The neuronal activity-induced phosphorylation of MeCP2 is required for proper dendritic/synaptic development and behavioral responses to experience [61, 62]. Intriguingly, recent evidence suggests that postmitotic neurons exploit epigenetic mechanisms, including DNA methylation, to consolidate and stabilize cognitive-behavioral memories. Indeed, inhibition of DNA methyltransferases (DNMT) in

the hippocampus affects contextual fear memories and LTP [63]. The connection of CDKL5 to epigenetics and gene expression seems also reinforced by its possible interaction with DNMT1 [56] and by the already mentioned induction of *Cdkl5* expression in response to MeCP2 ablation, DNMT inhibition, and histone deacetylase inhibition [46].

To conclude, we believe that CDKL5-signaling cascades are involved in synaptic plasticity and learning, affecting spines, dendritic branching, and actin cytoskeleton in the cytoplasm and activity-dependent gene expression in the nucleus (Figure 4). Clinical, genetic, and biological data suggest a functional relationship between CDKL5 and MeCP2, leading to hypothesize that common biological network(s) are disrupted when either gene is mutated. We speculate a possible mechanism in which CDKL5 works upstream of MeCP2, directly or indirectly influencing its phosphorylation state and activity. According to this model, while mutations in *MECP2* mainly lead to classic RTT, altered CDKL5 activity would determine, among others, certain phosphorylation-dependent MeCP2 dysfunctions, bringing to a subset of RTT symptoms. Other uncharacterized CDKL5 targets could also be deregulated leading to phenotype specifically associated with mutations in CDKL5. However, as already proposed [46], a transcriptional control of MeCP2 over *CDKL5* might also exist, therefore, reinforcing the link between the two proteins. The two models should not be considered mutually or temporally exclusive. Considering all above, the identification of the cytoplasmic and nuclear phosphorylation targets of CDKL5, together with possible transcription events directly or indirectly affected by the kinase, represents an important future milestone towards understanding its neuronal functions.

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Review Article

Neural and Molecular Features on Charcot-Marie-Tooth Disease Plasticity and Therapy

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In the peripheral nervous system disorders plasticity is related to changes on the axon and Schwann cell biology, and the synaptic formations and connections, which could be also a focus for therapeutic research. Charcot-Marie-Tooth disease (CMT) represents a large group of inherited peripheral neuropathies that involve mainly both motor and sensory nerves and induce muscular atrophy and weakness. Genetic analysis has identified several pathways and molecular mechanisms involving myelin structure and proper nerve myelination, transcriptional regulation, protein turnover, vesicle trafficking, axonal transport and mitochondrial dynamics. These pathogenic mechanisms affect the continuous signaling and dialogue between the Schwann cell and the axon, having as final result the loss of myelin and nerve maintenance; however, some late onset axonal CMT neuropathies are a consequence of Schwann cell specific changes not affecting myelin. Comprehension of molecular pathways involved in Schwann cell-axonal interactions is likely not only to increase the understanding of nerve biology but also to identify the molecular targets and cell pathways to design novel therapeutic approaches for inherited neuropathies but also for most common peripheral neuropathies. These approaches should improve the plasticity of the synaptic connections at the neuromuscular junction and regenerate cell viability based on improving myelin and axon interaction.

1. Introduction

Charcot-Marie-Tooth disease (CMT) is a clinical and genetic heterogeneous group of inherited motor and sensory peripheral neuropathies (HMSN) that affect 17–40 per 100,000 inhabitants [1, 2]. Mendelian segregation in families may follow either autosomal dominant, autosomal recessive, or X-linked patterns. Autosomal recessive forms are described more frequently in specific populations and geographical areas such as the Mediterranean basin. Molecular genetic studies and positional cloning, and more recently exome sequencing approaches, have unraveled a wide number of genes involved in the etiology of CMT disease [3–5]. Molecular genetic studies have been very successful for defining the gene nosology and classification of inherited peripheral neuropathies; more than 40 genes have so far been identified to be associated with CMT and related neuropathies, including rare clinical variants (http://www.molgen.ua.ac.be/CMTmutations/). As an immediate consequence, genetic testing has become an important tool in clinical practice of CMT, and patients and families have been beneficiated of a more specific genetic counseling. CMT is caused by mutations in genes that encode proteins with different locations, including compact and noncompact myelin, Schwann cells, and axons, and that are involved in very different functions, which include compaction and maintenance of myelin, transport through myelin, transcription regulation associated with myelination, cell signaling, cytoskeleton formation, axonal transport, mitochondrial dynamics and metabolism, vesicle and endosomal trafficking, and chaperones. Whatever the metabolic or structural defect that primarily affects the myelin or the

axon, the final common pathway in peripheral neuropathies is represented by an axonal degenerative process that, in most cases, mainly involves the largest and longest fibers [6–8]. Signals from axons determine whether or not a Schwann cell will alter its phenotype and make myelin. Alternatively, Schwann cell abnormalities may induce axonal degeneration with or without demyelination. Progress has been made toward understanding how particular mutations cause disease, but pathogenic mechanisms remain largely unknown.

The PNS is a complex network of myelinated and nonmyelinated nerves of varying diameters. A myelinated nerve fiber consists of a single continuous neuronal process, the axon, surrounded along the outside by serially arranged Schwann cells, which enwrap the associated axon with their cell membrane in a multilayered specialized structure, the myelin sheath. During development, the acquisition of a myelinating phenotype by the Schwann cell appears to be in response to as yet not understood cues from the axons [9]. When the Schwann cell establishes a one-to-one association with an axon at the promyelinating stage of its development, the program of myelination is started and becomes a myelinating Schwann cell. In contrast, Schwann cells that do not establish this relationship with an axon do not activate the program of myelin gene expression and become nonmyelinating Schwann cells [10]. Interestingly, this decision process is directed by the axons, as maintenance of myelin depends on axon and axon integrity. Other example of the influence of the axons on Schwann cells is the establishment of an electrically insulate node of Ranvier. Numerous molecules mediate specific aspects of the interactions between peripheral axons and Schwann cells [11] including MAG, p75, IGF1, integrins, and TGF-β. Neuregulin 1 (Nrg1) and its receptors, the ErbB receptors tyrosine kinases, have emerged as key regulators of axon-Schwann cell interactions at every stage. Spinal cord motoneurons, dorsal root ganglia (DRG) sensory neurons, and autonomic neurons express Nrg1 [12], and Schwann cell express both ErbB2 and ErbB3 receptors [13]. In addition to its previously known roles in proliferation and myelination, Nrg1 type III controls Schwann cell migration. Talbot's group have recently demonstrated that Nrg1 type III is an essential signal that controls Schwann cell migration to ensure that they are present in the correct numbers and positions in developing nerves [14]. Inherited demyelinating neuropathies provide examples of how the axons are also dependent on Schwann cells. The molecular studies on the progressive axonal degeneration seen in demyelinating CMT rodent models have demonstrated that they are likely to be the result of abnormalities in Schwann cell-axonal interactions [15–17].

2. CMT: Inheritance and Phenotypes of Motor and Sensory Neuropathies

CMT disease refers to peripheral neuropathies that affect both motor and sensory nerves. They are classically subdivided into "primary demyelinating" forms (CMT disease type 1 or CMT1), which are defined by a characteristic reduction of nerve conduction velocity (NCV) and segmental demyelination and remyelination, and "primary axonal" forms (CMT2) that show preservation or mild reduction of NCVs and loss of axons, namely, those of large diameter ($\geq 8\,\mu$m). As information about CMT2 is increasing, it has become evident that the distinction between CMT1 and CMT2 is less clear-cut than what was originally believed [18]. Diagnosis of CMT2 can be difficult; however, as many authors have noted, this type of CMT appears to have greater variability in its clinical presentation than CMT1. The CMT clinical phenotype is the consequence of a progressive axonal loss and degeneration affecting preferentially the longest axons, whatever the underlying primary pathogenic mechanism, either a myelinopathy and/or axonopathy [6, 7, 19]. CMT is predominantly a large-fibre neuropathy, but as sural nerve biopsies sometimes show, small fibres may be involved as well. In spite of the surprising variability of genes involved in the pathogenesis of CMT, common molecular pathways have been identified within Schwann cells and axons that cause these genetic neuropathies [5]. A review of some of most frequent forms is useful to define relevant clues to the pathogenesis of CMT and to sum up therapeutic interventions oriented to modulate the plasticity of these neuropathies (Figure 1).

2.1. Autosomal Dominant Demyelinating Neuropathies: CMT1. Approximately 60% of CMT patients show a predominantly demyelinating peripheral neuropathy and are classified as CMT1. The main subtype is **CMT1A** [20, 21], accounting for 40–50% of all CMT cases, which is associated with an autosomal dominant 1.4 Mb duplication on chromosome 17p11.2 that includes the peripheral myelin protein 22 gene (*PMP22*) [22, 23], a dosage-sensitive gene, expressed predominantly in the compact myelin of Schwann cells of the PNS. Less commonly, point mutations in *PMP22* have been also associated to CMT1. The mirror mutation, that is, the 1.4 Mb deletion [24, 25], and more rarely nonsense or frameshift *PMP22* mutations [26, 27] cause the myelinopathy called hereditary neuropathy with liability to pressure palsies (HNPPs) [28, 29]. Thus, duplicated or deleted of *PMP22* gives rise to demyelinating neuropathies and secondary axonal loss or abnormalities by a mechanism of gene dosage. When overexpressed in cultured cells or in transgenic mice overexpressing the human gene, PMP22 reaches late endosomes and forms protein aggregates that are ubiquitinated [30]. Removal of preexisting aggresomes formed by endogenous PMP22 is aided by autophagy [31]. A second cellular mechanism that can influence protein aggregation is the heat shock response. In proteasome-inhibited cells, overexpressed wild-type and mutant PMP22, as well as the spontaneous aggregates in neuropathic mouse nerves, recruits heat shock proteins [31]. The formation of aggresomes is a protective response of the cell that concentrates misfolded proteins in a central location to activate an autophagic response [32]. Fortun et al. propose a protective role for chaperones in preventing the accumulation of misfolded proteins. Elevated

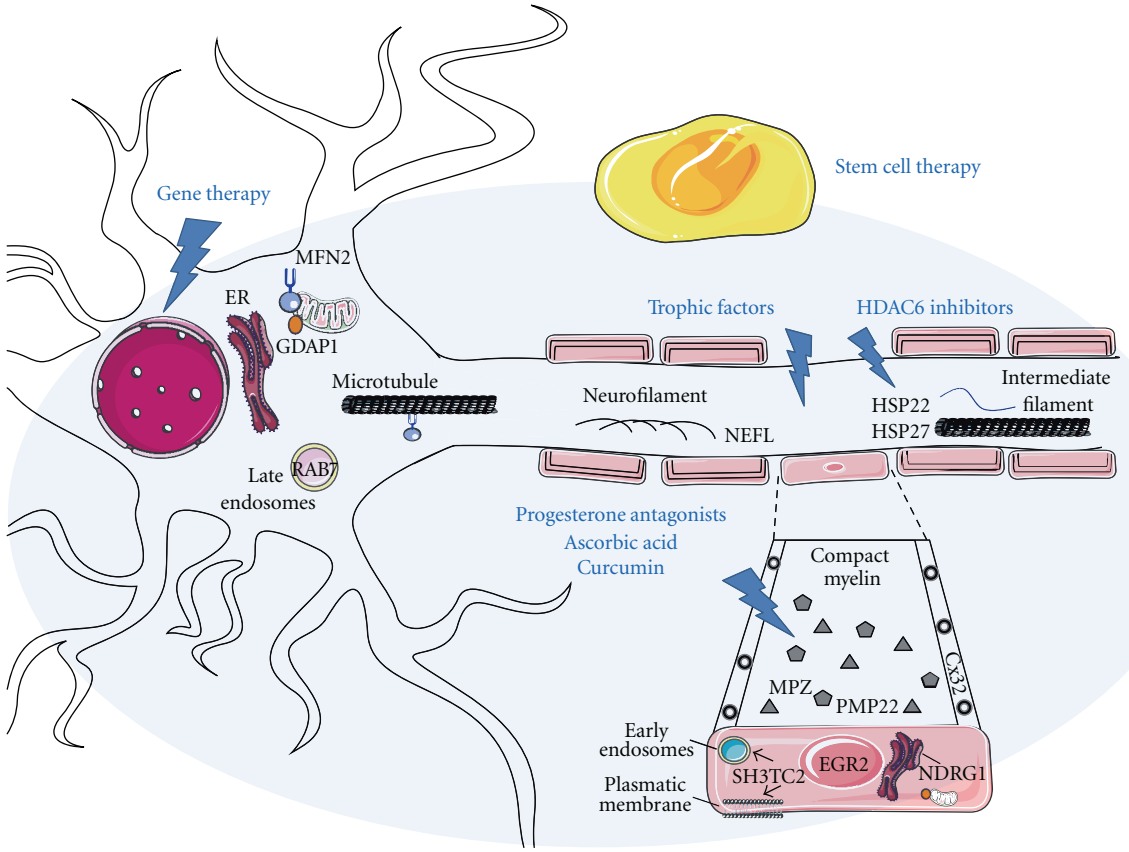

Figure 1: Peripheral nerve structure and cell localization of some CMT proteins at either the Schwann cell and myelin or the neuronal axon. (a) PMP22 and P0 are structural proteins located at the compact myelin and Cx32 at the noncompact myelin in the paranode (and also at the Schmidt-Lanterman incisures). Some other demyelinating CMT-associated molecules are SHT3TC2 at the plasmatic membrane and related to early endosomes and endosome recycling, the transcription factor ERG2 working in early promyelination programme, and NDRG1 that is ubiquitously expressed and has been proposed to play a role in growth arrest and cell differentiation, possibly as a signaling protein shuttling between the cytoplasm and the nucleus. Proteins mainly related to axonal CMT are associated with neurofilaments (NEFL), late endosomes (RAB7), mitochondria, endoplasmic reticulum and microtubules (MFN2 and GDAP1), or intermediate filaments (HSP22 and HSP27). (b) A ray sign indicates the main location where drugs or advanced therapies are acting. Stem cell therapy is represented as an open shadow grasping the neuron soma and axon and the Schwann cell. Trophic factors may be delivered as a drug but also by means of gene vectors or as a part of the local function of therapeutic stem cells. This figure was produced using Servier Medical Art (http://www.servier.com/servier-medical-art/powerpoint-image-bank).

PMP22 expression might perturb Schwann cell function by interfering with the intracellular sorting of PMP22 and other proteins, leading to overloading of the protein degradation machinery. Although demyelination is the pathological and physiological hallmark of CMT1A, the clinical signs and symptoms of this disease, progressive weakness, and sensory loss are produced by axonal degeneration [6]. The characteristic features of the PMP22 mutant Trembler and Trembler-J mice [33], particularly the minimal or abnormal myelination and reduced axonal diameter, have made them very attractive models to define the mechanisms by which defective peripheral myelination can modify axonal properties.

CMT1B is caused by mutations in the major myelin protein zero gene (*MPZ*), which comprises approximately 50% of myelin protein, and is necessary for both normal myelin structure and function [34, 35]. To date there are more than 150 different mutations in *MPZ* known to cause CMT1B in patients, which include missense, nonsense, small insertion/deletion, and splice site mutations. Based on clinical studies, Shy and coworkers' [36] patients fall into two distinct phenotype groups: one causing delayed motor development and marked slow nerve conduction and a second one usually associated with late-onset neuropathy, which allows developmental myelination, but eventually leads to axonal degeneration with minimal evidence of demyelination. It is difficult to make genotype-phenotype correlations because mutations in *MPZ* impair the adhesive function of myelin protein zero (P0), its subcellular trafficking, or both. Either abnormal gain-of-function effects (toxicity of misfolded protein) or reduced amounts of P0 (haploinsufficiency) could, therefore, underlie the clinical phenotype [37]. Pennuto and collaborators demonstrated that the unfolded protein response (UPR) activated by overload of misfolded proteins in the ER was responsible for demyelination in a CMT1B mouse model [38]. A recent

work has identified that an increased gene dosage of *MPZ* is directly involved in the pathogenesis of human peripheral nerves [39].

2.2. Autosomal Dominant Axonal Neuropathies: CMT2. CMT2 has a highly heterogeneous genotype. Mutations in the mitofusin 2 gene (*MFN2*) cause CMT2A and account for about 20% of CMT2 cases. Other less frequently mutated genes are *MPZ* (CMT2J), which also causes CMT1B, and the neurofilament light chain gene (*NEFL*) [40, 41].

CMT2A MFN2 participates in the fusion pathway of the mitochondrial dynamics [42, 43] and is also involved in the relationship of mitochondria with endoplasmic reticulum (ER); furthermore, depletion of MFN2 causes a disruption of mitochondrial dynamics and abnormalities in Ca^{2+} homeostasis [44]. Mechanisms that have been proposed to explain the pathophysiology of CMT2A associated with MFN2 dysfunctions include a defect in mitochondrial fusion, leading to a loss of mtDNA, and impairment in oxidative phosphorylation and cell bioenergetics [45]. Current models propose that a mitochondrial transport defect could be the cause of CMT2A. Zhao et al. were the first to link axonal cargo transport dysfunction to CMT2A [46]. Based on several studies [47, 48], it is tempting to speculate that MFN2 could be part of a motor complex involved in anterograde movement of mitochondria. So far, two transgenic mouse models expressing pathogenic mutations have been generating, $Mfn2^{T105M}$ [49] and $Mfn2^{R94Q}$ [50]. Loss of MFN2 profoundly and selectively disrupts axonal mitochondrial transport [51], which indicates its integral role in the regulation of mitochondrial transport, and the important implications for understanding the pathophysiology of CMT2A.

To date, up to 18 neurofilament light (*NEFL*) mutations have been associated with axonal **CMT2E** [52, 53]. A conditional mouse model, carrying the P22S mutation, mimics many aspects of the human CMT2E disease, including motor disability, abnormal muscle morphology, and denervation events [54]. These results highlight the importance of the integrity of the neurofilament network for neuronal function and suggest that the disease symptoms caused by the $NEFL^{P22S}$ mutation might result from axonal transport defects rather than deleterious effects of large neurofilament aggregates.

2.3. X-Linked CMT. With a frequency of about 10%, **CMTX1** [55] is the second most common inherited neuropathy. CMTX1 is genetically defined by mutations in the gene *GJB1,* which encodes the gap junction protein connexin-32 (Cx32) on the Xq13 chromosome [56]. Cx32 is localized in the noncompacted myelin sheath of large diameter fibers, forming the functional channels that allow for the rapid transport of ions and small nutrients between coupled cells [37]. Although Cx32 expression is not limited to the peripheral nervous system (it is also expressed by white matter oligodendrocytes), Cx32 mutations are associated only with CMT [57]. So far, more than 270 mutations that alter the structure of Cx32 have been reported, and most of these probably cause a partial or complete loss of function. *Cx32*-deficient mice have prominent adaxonal changes at the ultrastructural level, and a similar pathomechanism is observed in humans [58].

2.4. Autosomal Recessive CMT: Demyelinating CMT4 and Axonal AR-CMT2 Variants. In 2002, Baxter et al. [59] and Cuesta et al. [60] demonstrated that mutations in the ganglioside-induced differentiation-associated protein-1 (*GDAP1*) gene cause autosomal recessive CMT neuropathy. This finding was fascinating, as the two reports differed markedly with respect to the phenotypes of their families. Cuesta et al. described families who had an axonal phenotype (**AR-CMT2K**), whereas Baxter's families showed a demyelinating phenotype (**CMT4A**). Both slow and normal NCVs have been reported in patients, and many of the cases show a severe phenotype and have their onset in childhood. However, mild forms segregating as an autosomal dominant phenotype have also been reported [61]. Mutations have been described in every exon and include missense, nonsense, splicing site, short deletions, and insertion mutations. GDAP1 belongs to a glutathione S-transferase enzyme subfamily [62] that is mainly expressed in neurons [63, 64] but also in Schwann cells [65]. GDAP1 is a mitochondrial protein [63] located in the mitochondrial outer membrane (MOM) acting as a regulator of mitochondrial dynamics [65, 66]. The effect of *GDAP1* mutations in mitodynamics seems to depend on the inheritance pattern [67]. Overexpression of GDAP1 in COS7 or HeLa cells causes mitochondrial fragmentation or fission and a substantial accumulation of mitochondria around the nucleus. Rescue experiments in *Saccharomyces cerevisiae* defective mutans for fission and fusion genes have shown that *GDAP1* rescues the phenotype of the fission-associated gene *Fis1*. In particular, the recovery of G2/M delay suggests that both Fis1p and GDAP1 may affect the interaction of mitochondria with microtubules [68], an aspect that may relate GDAP1 to mitochondrial transport or movement in axons.

CMT4C neuropathy, which is caused by mutations in the *SH3TC2* gene, is the most common cause of the autosomal recessive form of demyelinating CMT. *SH3TC2* is specifically expressed in Schwann cells and is necessary for proper myelination of peripheral axons. Analysis of the murine model of CMT4C revealed that the capacity of SH3TC2-deficient Schwann cells to properly myelinate underlying axons is affected at the early stages of myelination, which is in line with the early onset of the neuropathy reported in CMT4C patients [69–71]. However, its exact role in myelin biology remains to be determined. Recent data demonstrated that SH3TC2 localizes at the plasma membrane and in endocytic vesicles [72–74] and that it interacts with the small GTPase Rab11, which is known to regulate the recycling of internalized membranes and receptors back to the plasma membrane. Further protein binding studies and transferrin receptor trafficking assays revealed that SH3TC2 together with Rab11 indeed affect the dynamics of endocytic recycling [75].

3. Cellular and Molecular Bases of Nerve Regeneration and Plasticity in CMT Neuropathies

The discovery of many genes involved in CMT disease has provided a unique opportunity to understand the critical molecular pathways involved in peripheral axon stability and length-dependent peripheral nerve disease [17, 18]. An important concept in peripheral neuropathy is that many types are characteristically length dependent; that is, the longest axons in the body are affected first and most profoundly. The length-dependent distribution supports the concept that the major site of pathology is in the axon itself, rather than the cell body. Furthermore, it suggests that shorter axons are either less susceptible or better able to compensate for certain insults that are longer axons, leading to the degeneration of the distal regions of the longest axons first [76]. Peripheral neuropathies presenting a distal nonterminal axonopathy represent the most common nerve diseases. Their long-term outcome depends on the balance of two processes: the degree or rate of axonal degeneration and the ability of the nascent axon tips to regenerate efficiently. One strategy to alter these processes would be to improve the efficiency of regeneration by using trophic factors such as neurotrophins [77], moving them from bench-to bedside. Prolonged denervation could lead to decreased regeneration capacity because of reduction in the expression of regeneration-associated Schwann cell molecules, such as neurotrophic factors and receptors. Therefore, Schwann cells might remain in a growth-supportive mode for prolonged periods or they have to be transformed into a competent premyelinating state to initiate and complete myelination. The functional significance of regeneration is to allow reinnervation of target organs and restitution of their corresponding functions. The materials for axonal growth are mainly provided by the cell body via axonal transport [78, 79], but more recently the contribution of local axonal synthesis and degradation of proteins has been identified [80, 81]. Increased energy demands on the neuron to propagate action potentials, and decreased trophic factor support from denervated Schwann cells or muscle are other potential mechanisms that may also contribute to axonal degeneration in demyelinating neuropathies (reviewed in [16]). An unanswered question with respect to all CMT1 forms is why mutant Schwann cells fail to support axonal function and survival [37]. An important direction is the development of therapeutic strategies that enhance axonal regeneration and promote selective target reinnervation; in addition, modulation of the central nervous system reorganization to improve functional recovery but also diminishing undesirable consequences has been proposed as well [82].

4. CMT Pharmacological and Biological Therapies

CMT disease course and severity vary according to CMT type, causative gene, and mutation change, but considerable phenotypic variability may occur also within the same CMT type. Understanding the molecular pathogenesis of inherited neuropathies is essential for the development of rational therapies (Figure 1). While much remains to be learned, it is clear that most are caused by the expression of a mutant allele(s) in myelinating Schwann cells or neurons. For recessive neuropathies, in principle it is possible to "replace" the defective gene by introducing a normal version. For dominant neuropathies, the situation is even more complex, as these are likely to be caused by a toxic gain of function that is not necessarily related to the normal function of the gene product. Nevertheless, for dominant demyelinating neuropathies caused by altered gene dosage, reestablishing the normal level of gene expression might be of benefit. The pathogenic mechanism in CMT1A duplication is attributed to an excess gene copy number of *PMP22*, leading to protein overexpression [28], and factors that modify the expression levels of *PMP22* might potentially be effective for treatment. A proof of concept that demyelination can be reversed by normalizing expression of *PMP22* was provided through a transgenic mouse model [83]. Research is focused on developing new treatment strategies to target the regulation of *PMP22* gene dosage. Two compounds that have been shown to alter *PMP22* mRNA levels in rodents are ascorbic acid and progesterone and progesterone antagonists.

Ascorbic acid reduced the severity of neuropathy in transgenic mice overexpressing *PMP22*, an animal model of human CMT1A, compared with untreated mice [84]. Ascorbic acid promotes myelination *in vitro* and decreases *PMP22* mRNA levels through a cAMP-mediated pathway [84, 85]. Evidence of efficacy of ascorbic acid in the animal model prompted initiation of randomized controlled trials to test the efficacy of ascorbic acid in patients with CMT1A. Results from a phase 3, multicentre, placebo-controlled, double-blind randomized trial to assess the efficacy and tolerability of chronic ascorbic acid treatment in patients with CMT1A in Italy and the UK have been just published [86]. Unfortunately, ascorbic acid supplementation had no significant effect on neuropathy compared to placebo after 2 years followup, suggesting that no evidence is available to support treatment with ascorbic acid in adults with CMT1A.

It is known that progesterone and derivates are able to increase *MPZ* and *PMP22* gene expression *in vitro* [87]. In order to test if progesterone can modulate the progressive neuropathy caused by moderate overexpression of *PMP22,* Sereda and collaborators [88] administrated daily subcutaneous injections of progesterone and the **progesterone antagonist**, onapristone, to a transgenic rat model of CMT1A during 7 weeks and showed that onapristone reduced *PMP22* mRNA by 15%, resulting in clinical and neuropathological improvement. A long-term study reaffirmed these results and shed light to the axonal degeneration process seen in CMT1A patients, by reducing progressive muscle atrophy and preventing axonal loss without altering myelin sheath thickness [89]. Unfortunately, onapristone and currently available progesterone antagonists are too toxic to be safely administered to CMT1A patients, so further research is ongoing to develop suitable compounds for future clinical trials. To accomplish this goal, the Charcot-Marie-Tooth Association (CMTA) has established

the Strategy to Accelerate Research (STAR) to specifically fund CMT-related research (http://www.cmtausa.org/). High-throughput screens are trying to identify compounds that are already FDA approved, so it could accelerate the drug development process and start phase III clinical trials in 3–5 years.

Another interesting and promising molecule is **curcumin**, which plays a role stimulating the translocation of misfolded proteins from the endoplasmic reticulum to the plasma membrane, thereby reducing cytotoxicity of the mutant proteins. This mechanism might be helpful for selected CMT1A and CMT1B forms, in which various *PMP22* and *MPZ* mutations are known to cause intracellular accumulation of mutant proteins. Oral curcumin mitigates the clinical and neuropathologic phenotype of *Trembler-J* mouse model of CMT1A, inhibiting Schwann cell apoptosis and increasing axonal caliber and myelin thickness. Furthermore, this positive clinical response to curcumin occurs in a dose-dependent manner and is reversed after withdrawal of treatment without side effects. Recent cell-based studies showed that mutant P0 could accumulate in the ER and induce apoptosis. This aggregation-induced apoptosis was abrogated by pretreatment with curcumin [90]. These findings suggest a potential therapeutic role of curcumin in selected forms of inherited peripheral neuropathies. There is also evidence that *MPZ* mutations with ER-retention of the mutated protein cause UPR activation rather than apoptosis [38] so this mechanism could also be relevant as a therapeutic target.

Regarding axonal CMT, in a transgenic mouse model for mutant *HSBP1*-induced CMT2 and distal HMN type 2B [91], mutant HSBP1, also known as HSP27, leads to severe axonal transport defects induced by a decrease in acetylated tubulin abundance in peripheral nerves. The phenotype was partially restored and the axonal transport defects were rescued when mice were treated with **HDAC6 inhibitors**. Histone deacetylase 6 (HDAC6) is the major enzyme with α-tubulin deacetylating activity. For this study the authors used a nonspecific inhibitor, trichostatin A (TSA), and two highly selective inhibitors: tubacin and tubastatin A, which resulted to be more effective compared to TSA when axonal transport and CMT phenotype were assessed. As some HDAC6 inhibitors have entered into clinical trials for cancer treatment and other neurological disorders such as Friedreich's ataxia [92], it is rationale to think that the same drug approach could be tested in CMT patients.

Schwann cell pathology damages the delicate myelin-axon interaction and can lead to axonal degeneration [6], but pronounced axonal pathology has also been observed even in genetic models in which axons are associated with normal appearing myelin sheaths. Thus, a therapeutic approach focusing on preventing this intimate connection could be providing **trophic factor support** to degenerating axons, which may be useful for a number of CMT neuropathies, either primary myelinopathies or primary axonopathies. The main conceptual problem with this approach is the diversity of trophic factors and PNS neurons, especially because different kinds of neurons respond to different trophic factors [16]. Only neurons with the proper receptors typically respond to a given growth factor. Receptor-mediated, retrograde axonal transport delivers the trophic factor to neuronal cell bodies, where they act. Axonal elongation requires an adequate substrate of trophins and tropic factors, provided by reactive Schwann cells and the extracellular matrix within the degenerated nerve [93]. Another potentially approach involves the manipulation of Schwann cell-axonal signal transduction pathways. Neuregulin-1 enhances axonal regeneration [94] by acting on Schwann cells as PNS neurons do not express neuregulin receptors. Axons express neuregulin-1 type III (Nrg1-III) on their surface, which binds to ErbB receptors on Schwann cells as part of a process that initiates myelination. Therefore, Nrg1-III acts as a juxtacrine signal. Nrg1-III binds to ErbB3 and promotes ErbB2 phosphorylation of tyrosine residues in the cytoplasmic domain of both ErbB2 and ErbB3 receptors [95]. Activation of the ErbB receptors leads to signaling through multiple signaling cascades including PI3K/Akt, Erk1/2, JNK, and FAK [96]. Three families of trophic factors are particularly important for PNS neurons: the neurotrophin family, the glial-derived neurotrophic factor (GDNF), and the ciliary neurotrophic factor (CNTF) family of cytokines. Sahenk and collaborators studied the ability of mutant Schwann cells to respond to exogenous neurotrophin-3 (NT-3) in two CMT1A animal models [97]. NT-3-treated animals presented improved nerve regeneration and the associated myelination process. Furthermore, at the early stages of regeneration-associated myelination, NT-3 stabilizes the axonal cytoskeleton locally by inducing neurofilament phosphorylation when axon sprouts become enwrapped by Schwann cells [77]. In contrast, BDNF, which belongs to the neurotrophin family but is not part of the Schwann cell survival loop [98], showed no effect upon axonal growth or cytoskeletal neurofilament pathology. Unfortunately, despite the promising results in animal studies, no studies have been successful in humans, probably due to a poor delivery and short half-lives of the trophic and growth factors. Targeting the correct combination of trophic factors to neurons or Schwann cells at the optimal time may be necessary to achieve meaningful results. A combination of trophic factors or engineered "pan-neurotrophic factors" [99] might be more beneficial than a single factor.

The short half-life of most neurotrophic factors would require either multiple administrations or a continuous infusion of the therapeutic molecules in order to achieve an adequate and effective local concentration. Knowing the molecular basis of inherited diseases prompts immediate consideration of **gene therapy**. Gene therapy can be defined as a strategy to transfer biologically relevant genetic material (usually mutant genes or genes delivering trophic and growth factors) into affected cells in the body to treat disease. For instance, delivering neurotrophic factors to the healing nerve ends, favoring survival and regeneration of both sensory and motor axons, could, ultimately, allow the recovery of nervous functions [100]. Viral vectors and plasmid DNA have been widely used for treating human disease models and patients. Expression of the gene might be modulated by the introduction of regulatory elements for the controlled or

tissue-specific expression of the desired molecule. Until now, three major classes of viral vectors, based on adenovirus, adeno-associated virus (AAV), and herpes simplex virus (HSV), have been exploited to target the PNS. Although not originally neurotropic, adenoviral and AAV vectors are able to transduce spinal sensory and motor neurons after either intramuscular or intraneural injections. When injected at the site of a nerve injury, these vectors are retrogradely transported to motor neuron cell bodies and can thus be exploited to deliver therapeutic genes along the route of the nerve. However, the strong absorption of both adenoviral and AAV vectors to skeletal muscle fibers might represent a limitation for efficient neuronal transduction and retrograde transport [101]. A recent study in mice has characterized the tropism and transduction efficiency of different AAV pseudotypes after sciatic nerve injection. Among the pseudotypes tested, AAV2/1 transduced both Schwann cells and neurons, AAV2/2 infected only sensory neurons, and AAV2/8 preferentially transduced Schwann cells, proving the utility of AAVs as gene therapy vectors [102]. A few approaches have used plasmids as vehicles to deliver therapeutic genes to peripheral nerves. In these cases, the skeletal muscle has been the preferred site for delivery and expression of the transgene. For instance, intramuscular delivery of a plasmid encoding for vascular endothelial growth factor (VEGF) showed a protective role against myelin wasting and axonal loss [103]. Viral vectors have been modified so that they are unable to cause disease. Unfortunately, they have caused immunologic reactions, which currently limit their use. In contrast, plasmid DNA is nonimmunogenic, but it is characterized by poor delivery efficiency, and proteins made from it have only been produced in target organs for a short time. Progress in this area demands more sophisticated delivery systems and more knowledge of the molecular pathogenesis of neuropathies.

Schwann cells, and their basal lamina, represent the key component of nerve regeneration, as they serve as scaffolds for the regenerating axons, which grow through the empty basal lamina tubes. Schwann cells, however, have limited clinical applications since the culture of an adequate quantity of cells to achieve optimal conditions for transplantation in nerve conduits is time consuming and requires particular care for *in vitro* expansion and a constant input of growth factors. Moreover, Schwann cells are not easily accessible without nerve biopsy and bear the need to sacrifice an autologous nerve, with the related complications. Due to all these difficulties, the field of **stem cell therapy** for peripheral neuropathies has been explored. Adult stem cells show ability to differentiate into neuroprogenitor-type cells [104, 105]. Stem cells could be differentiating into neurons, which will generate new axons to contact their targets or Schwann cells enwrapping demyelinating axons and secreting trophic factors. Bone-marrow-derived mesenchymal stem cells (MSCs) can be induced to differentiate into Schwann cells [106], improving myelin formation and nerve regeneration *in vivo* after their transplantation into different models of peripheral nerve injury [107, 108]. Adipose tissue has been also indicated as a novel and promising source of multipotent cells (adipose-derived stem cells, ASCs), which can be differentiated into a neuronal phenotype [109, 110], and in terms of clinical use, they may be harvested by conventional liposuction procedure under local anaesthesia. The frequency of stem cells in adipose tissue is 100- to 1000-fold higher than that in bone marrow, which is a considerable advantage as it reduces the period of expansion of the stem cells prior to differentiation. Terenghi's group showed how ASCs could be differentiated towards a Schwann cell-like phenotype, expressing markers like S-100, glial fibrillary acidic protein (GFAP) and P75 neurotrophin receptor and enhancing neurite outgrowth in an *in vitro* co-culture model [111]. More recently, expression of myelin proteins P0 and PMP22 after differentiation of both ASC and MSC [112] and the neurotrophic potential shown *in vitro* from differentiated adipose-derived stem cells (dASCs) with a brief term *in vivo* study have been described [113].

5. Conclusions

Charcot-Marie-Tooth disease is a generalized disorder of motor and sensory peripheral nerves. Three major points deserve attention: (1) disease pathophysiology of both myelinopathies and axonopathies forms are the consequence of altered Schwann cell-axon communication; abnormal intercellular contact and signaling induce neurodegeneration and axonal loss, which ultimate produce muscular atrophy and weakness, (2) the primary cause is the genetic mutation in any of the more than 40 genes causing CMT or related neuropathies, and (3) a major therapeutic target is the regeneration of cell viability based on improving myelin and axon interaction. Therapies promoting plasticity changes in axons and Schwann cells require not only new therapeutic drug, gene, or cell approaches but also proper delivery systems targeted to the pathological cellular structures.

Conflict of Interests

Both P. Juárez and F. Palau have no conflict of interest.

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Review Article

Widespread Structural and Functional Connectivity Changes in Amyotrophic Lateral Sclerosis: Insights from Advanced Neuroimaging Research

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Amyotrophic lateral sclerosis (ALS) is a severe neurodegenerative disease principally affecting motor neurons. Besides motor symptoms, a subset of patients develop cognitive disturbances or even frontotemporal dementia (FTD), indicating that ALS may also involve extramotor brain regions. Both neuropathological and neuroimaging findings have provided further insight on the widespread effect of the neurodegeneration on brain connectivity and the underlying neurobiology of motor neurons degeneration. However, associated effects on motor and extramotor brain networks are largely unknown. Particularly, neuropathological findings suggest that ALS not only affects the frontotemporal network but rather is part of a wide clinicopathological spectrum of brain disorders known as TAR-DNA binding protein 43 (TDP-43) proteinopathies. This paper reviews the current state of knowledge concerning the neuropsychological and neuropathological sequelae of TDP-43 proteinopathies, with special focus on the neuroimaging findings associated with cognitive change in ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS), also known as motor neuron disease, is a progressive disorder causing degeneration of the motor system at all levels, from the cortex to the anterior horn of the spinal cord. Approximately 5% of cases are familial, whereas the bulk of patients diagnosed with the disease are classified as sporadic as they appear to occur randomly throughout the population. A large hexanucleotide repeat expansion in the first intron of the C9ORF72 gene is resulted, the most common genetic cause of familial ALS (FALS). It was detected in more than one-third of FALS cases of European ancestry and in nearly one-half of Finnish FALS cases [1].

Despite the early view of ALS as a neurodegenerative disease that exclusively affects the motor system, growing evidence supports the new concept of ALS as a multisystem disease also affecting executive functions, behavior, language, and other cognitive domains, functionally associated, in general, with temporal and frontal lobes [2, 3]. A detectable, although variable in magnitude, degree of cognitive involvement has been found in many patients with ALS. Indeed, 5–15% of ALS patients meet criteria for frontotemporal dementia (FTD), while a substantial percentage of patients without dementia may show mild to moderate executive (approximately from 22 to 35%) and behavioral (up to 63%) dysfunctions [3–5]. In support of these clinical evidences, immunohistochemical findings suggest that ALS may affect the frontotemporal network and, furthermore, is considered part of a broader clinicopathological spectrum now known as TAR-DNA binding protein 43 (TDP-43) proteinopathies which also include FTD [6, 7].

Structural and functional magnetic resonance imaging (MRI), positron emission tomography (PET), and single photon emission-computed tomography (SPECT) studies have corroborated the theory of frontotemporal impairment in ALS with approximately half of the patients displaying at least mild abnormalities [8–25]. In particular, the diffusion tensor imaging (DTI) findings of reduced white matter (WM) integrity in the frontal, temporal, and parietal lobes and in the corpus callosum suggest that a widespread WM involvement may underlie both cognitive and functional changes in ALS [17–25].

As the brain is a complex system of interacting structures, relevant contributions have been derived from resting-state functional magnetic resonance imaging (RS-fMRI), a novel technique that evaluates the spontaneous fluctuations in the Blood Oxygen Level-Dependent (BOLD) signals with subjects being completely at rest [26, 27], which proved to be particularly suitable to explore functional interactions between cerebral networks in ALS. Indeed, local degeneration of motor neurons was found to be accompanied by a widespread effect on brain networks [21, 22, 28, 29]. A whole body of evidence, including the aforementioned ones, leads to the novel conception of ALS as a multisystem disease that affects not only primary motor connections but also the connectivity between primary motor regions and supplemental motor and extra-motor regions.

Given that neuropsychological and neuropathological findings may be interpreted as the cognitive and histopathological correlates of disease-related loss of the structural brain integrity in ALS, with a consequent reorganization of cortical networks, we will review current neuropsychological, neuropathological, and neuroimaging knowledge within a framework of cognitive and connectivity changes in ALS, along with some recent hypotheses about pathogenesis.

2. Cognitive and Behavioral Changes in ALS

It is now recognized that the ALS-dementia (ALS-D) syndrome is not a random association. It occurs in at least 5% of patients with ALS and includes a set of different subtypes [4, 5]. Clinically, the most common syndrome appears to be very similar to FTD, characterized by personality change, breakdown in social conduct, and impairment of abstraction, planning, set shifting, and organizational skills [2]. Compared to ALS without symptoms of FTD, the prognosis of ALS with comorbid FTD (ALS-FTD) is more unfavorable, and the median survival of patients with ALS-FTD is shorter than that of ALS patients by approximately 1 year [30].

One subgroup of ALS-D patients presents at onset with a predominantly aphasic syndrome, characterized by changes in speech (slowing of speech or dysarthria, anomia, neologisms, echolalia, and semantic paraphasias) [31, 32], although bulbar involvement in the tongue and throat may frequently obscure these language-specific symptoms.

Therefore, patients with ALS can have features of progressive nonfluent aphasia (PNFA), semantic dementia (often atypical), or both. Verbal fluency has been the most frequently investigated executive task in ALS research and has been found to be impaired in the majority of cognitive studies in ALS (e.g., [2, 8, 13]). Furthermore, a number of neuropsychological studies have found deficits among nondemented ALS patients in tasks of confrontation naming, conceptual semantic processing, and syntactic comprehension [33], in conjunction with paraphasias, decreased phrase length, and deficits in phrase construction [5, 34].

In comparison to the findings of impaired executive functioning, memory abilities are less consistently disrupted in ALS [34, 35], with poor performance on memory tasks considered indicative of a failure of encoding information, again implicating a frontal lobe impairment [30].

Behavioral changes are now recognized as another feature of ALS [4]. The term behaviorally impaired (ALSbi) has been recently introduced to describe ALS patients who display frontal behavioral signs but do not meet the full criteria for FTD. Thus, according to current consensus criteria [36], diagnosis of ALSbi requires that the patient meets at least two nonoverlapping supportive diagnostic features from Hodges' criteria [37] for FTD. Although cognitively normal patients with ALS can have profound behavioral abnormalities, cognitive and behavioral impairments can coexist in 25% or more of ALS patients [3].

Disinhibition, irritability, emotional blunting, lack of empathy, and especially apathy have been reported in several cohorts of ALS patients [38–41]. Although the most commonly applied instrument to compare the behavioral changes in several neurodegenerative diseases is the Frontal Systems Behavior (FrSBe) Scale, which is able to assess apathy, disinhibition, and executive dysfunction [42], additional investigations are needed to rule out the potential confounding effects of motor impairment, depression, and recall bias on the evaluation of behavioral modifications by using this in conjunction with measures of mood or other similar scales that account for mood and motor weakness [41].

Furthermore, instrumental markers of cognitive and behavioral impairment in ALS might be useful tools for disease management, promoting the quality of life of both patients and caregivers. In this regard, structural imaging techniques (DTI and voxel-based morphometry, VBM) have allowed to investigate the neuroanatomical correlates of some frontal symptoms, like apathy, the most prominent behavioral feature in ALS [43, 44]. Therefore, in the future, it will be assessed by longitudinal analyses whether DTI and VBM measures may have a predictive value as biomarkers of behavioral impairment in ALS.

3. The Neuropathological Basis of Cognitive Impairment in ALS

Together with the advancements of research in neuropsychology, neurobiological studies have inferred a relationship between the density and distribution of pathological abnormalities and cognitive changes in both ALS and FTD. Indeed, by comparing the neuropathologic features of ALS and sporadic FTD, a common and characteristic pathologic finding has emerged in ALS, the ubiquitin-only inclusion body (UBI), at the level of spinal anterior horns, hippocampus,

frontotemporal and parietal neocortices [45, 46], and basal ganglia [47, 48], showing higher density and more widespread distribution of inclusions in cognitively impaired ALS patients than in cognitively normal ALS patients [45, 48].

On the basis of these new insights the hypothesis that a common undiscovered proteinopathy underlays both sporadic ALS and FTD was formulated. After TDP was identified as the main disease protein in the majority of FTD cases [49], the ubiquitinated compact and skeinlike inclusions characteristic for ALS were also found to be composed of TDP-43 [49, 50], thereby providing strong evidence that ALS and FTD are part of a clinicopathological continuum of multisystem diseases, the so-called TDP-43 proteinopathies [7]. In fact, immunohistochemical whole-brain analyses of autopsied ALS/FTD cases revealed TDP-43 deposits in multiple brain areas within and also beyond the pyramidal motor system, including the nigrostriatal system, neocortical and allocortical areas, and, to a variable extent, the cerebellum, although there were regional differences in the pathological burden between the various clinical phenotypes [6, 7]. Moreover, the presence or absence of cognitive behavioral dysfunction has been associated with the topographic distribution of cortical TDP-43 inclusions (i.e., predominant involvement of the frontal gyrus in patients with behavioral and/or dysexecutive symptoms, and of the temporal cortex and the angular gyrus in case of language dysfunction) [6, 7, 51].

Furthermore, cortical involvement with pathological TDP-43 aggregations was usually accompanied by subcortical TDP-43 pathology, in particular in areas directly adjacent to the affected cortex [7]. This finding points toward an involvement of subcortical U-fibers, implicated in connecting multiple cortical areas, suggesting that such subcortical involvement may underlie both cognitive and functional changes in ALS.

Within the ALS/MND-FTD spectrum disorders, multiple genes appear to drive a similar phenotype characterized by neuroglial inclusions immunoreactive to phosphorylated TDP-43. Specifically, mutations in transactivation response DNA-binding protein (TARDBP) gene and in other genes associated with neuronal (NCIs) or glial (GCIs) cytoplasmic inclusions (i.e., fused in sarcoma/translocation in liposarcoma or FUS/TLS, C9ORF72, and progranulin or PGRN) have been identified in several familial or sporadic ALS and ALS/FTD cases [1, 52], and in subsets of FTD [52, 53]. Remarkably, the availability of well-characterized human pathological material in brain banks has yielded the potential to study this disease pathway by creating transgenic animal models, based on different genes but resulting in a common pathology [54–56].

Neuropathological findings from human autopsy studies [51, 57] and experimental models [54–56] of ALS/MND have also suggested that neuronal loss is noncell autonomous and glial cells contribute significantly to neurodegeneration within motor and extra-motor areas. Interestingly, recent evidence suggests that in ALS/MND cytoplasmic protein aggregate inclusions occur also in GCIs in multiple areas. This is especially true for oligodendroglial cells, in some cases showing a significant correlation between the topographic distribution of GCIs and the different clinical subsets of ALS-D [56, 58]. However, taking the neuropathological findings together, it is clear that the idea of a specific frontotemporal dysfunction underlying cognitive impairment in ALS is only partially valid. Instead, this model should be refined in favor of a broader network involving dysfunction in multiple areas, mainly focused on frontotemporal regions that have major connections with posterior areas as well as subcortical and limbic structures.

4. Functional Imaging Studies

The whole-brain analysis of functional brain activity has undoubtedly played a crucial role towards a better understanding of the in vivo pathology of ALS over the last two decades.

The earliest single photon emission-computed tomography (SPECT) with 99mTc-hexamethylpropylene, that indirectly evaluated functional brain activity by measuring the regional cerebral uptake of glucose, identified reduced tracer uptake in the frontal lobes of some patients with ALS-D [59, 60]. A number of subsequent SPECT studies have also reported widespread frontotemporal lobe involvement in ALS patients with or without cognitive impairment [10, 11]. Moreover, to explore the relationship between activation and cognitive functions, reduction of regional Cerebral Blood Flow (rCBF) in frontal and temporal areas (anterior and medial orbitofrontal cortex, anterior and medial frontal cortex, and anterior temporal lobes) was correlated to neuropsychological performance, revealing a more marked and widespread pattern of perfusion impairment in patients with ALS-FTD (reduction of rCBF also in the posterior frontal, parietal, and occipital lobes bilaterally) [60, 61] and a significant correlation between memory impairment (abnormal retrieval processes) and frontal hypoperfusion in patients with classical ALS [33]. However, not all previous SPECT studies reported significant correlations between measures of rCBF and neuropsychological data [62], probably because of the different clinical characteristics of the enrolled patients (variability of onset and clinical course of the disease, and of the degree of functional and cognitive impairment) and the different methodologies used.

(18F)2-Fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET) studies, assessing regional cerebral metabolic rates for glucose (rCMRGlc), also found significantly decreased rCMRGlc in the frontal cortex and superior occipital cortex in classical ALS patients compared to controls, revealing a significant correlation between mild frontal dysfunction and reduced glucose metabolism in the frontal cortex and thalamus [63].

More recently, Flumazenil PET studies assessed the regional flumazenil binding to the benzodiazepine subunit of the Gamma-aminobutyric acid A (GABAA) receptor, as a potential marker for cortical neuronal loss or dysfunction [64, 65]. Reduced [11C]-flumazenil binding in ALS, associated with poorer offline performance on written verbal fluency tasks and Graded Naming Test [66], was reported in the inferior and middle frontal gyri, the superior temporal

gyrus, and anterior insula [67], in agreement with earlier neuropathological findings in ALS-aphasia [31, 32]. Further evidence of extra-motor involvement in ALS, using [11C]-flumazenil PET, was also provided by Lloyd et al. [68] who found significant bilateral reductions in the prefrontal cortex, Broca's area, right temporal cortex, the parietal cortex, and right visual association cortex.

In the last two decades, functional activation studies have proven invaluable in exploring disease-related effects in ALS patients on the physiologic activity of different neural systems. The development of new acquisition protocols has allowed the study of the brain, both structurally and functionally, also with the hope of discovering sensitive and specific biomarkers for monitoring the progressive extent of the multisystem degeneration in ALS [69]. However, given that some conditions like hypoxia and hypercapnia might influence brain cognitive and functional modifications [70, 71], their confounding effects should be avoided in the assessment of MRI research projects. Furthermore, it is to take into account that respiratory processes (i.e., natural fluctuations in the depth and rate of breathing and changes in levels of carbon dioxide) can contribute substantially to the measured BOLD signal time series, and removing their effects is an important consideration for fMRI studies of neural function [72, 73].

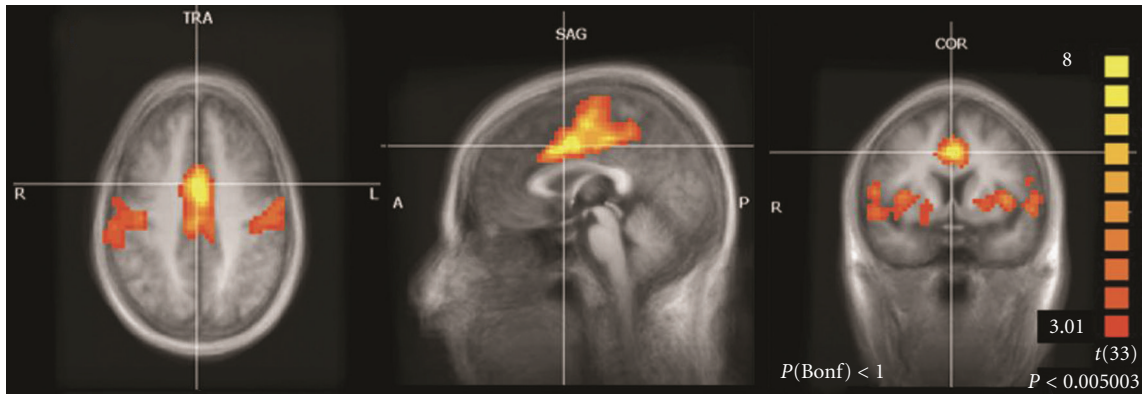
Remarkably, a widespread frontotemporal lobe involvement has been shown consistently in PET and fMRI studies using both cognitive and motor tasks. For instance, cognitive impairment was examined in a series of studies by Abrahams et al. [12, 13], who compared rCBF during a task of executive function (verbal fluency/word generation) in patients with impaired offline verbal fluency scores (ALSi) and unimpaired offline fluency scores (ALSu). ALSi patients showed reduced activation in the dorsolateral prefrontal cortex (DLPFC), premotor cortex, insular cortex, and thalamus, confirming previous findings [8]. Moreover, Abrahams et al. [74] have also used fMRI to further assess whether word retrieval deficits and underlying cerebral abnormalities are executive in nature, or whether they represent a language dysfunction. They compared ALS patients to matched healthy controls during performance of two tasks: verbal fluency and confrontation naming. The ALS group demonstrated impaired activation in the DLPFC, the anterior cingulate gyrus, and the inferior frontal gyrus (implicated in letter fluency), in the supramarginal gyrus and the temporal lobe auditory association areas (implicated in the phonological store component of working memory and phonological and lexical processing, resp.), and in the occipitotemporal pathway (involved in confrontation naming).

PET and fMRI studies associated with motor tasks have been consistently applied to investigate cortical reorganization of the motor system. In fact, Kew et al. [9], for the first time, demonstrated that ALS patients performing a stereotyped and self-generated PET motor task showed marked activation abnormalities in sensorimotor, parietal association, and anterior cingulate cortices. More recent fMRI studies confirmed these findings about the cortical plasticity in ALS [75, 76]. Moreover, to assess the effects of motor neuron degeneration on both cortical and subcortical areas, Tessitore et al. [77] conducted an fMRI study while a group of ALS patients performed a simple visually paced motor task. In comparison to controls, patients with ALS exhibited reduced activity in the right parietal association cortex, involved in the execution of visually guided movements and strongly connected to the cortical motor area, and heightened activity in the left anterior putamen, also implicated in motor execution. When comparing patients with greater UMN involvement to patients with greater LMN involvement, there were significant differences in the anterior cingulate cortex and right caudate nucleus, with more robust activation of these areas in the group with greater UMN involvement. These results provided further evidence for altered functional responses in brain regions subserving motor behavior in patients with sporadic ALS, reflecting previous morphometric and PET results that had revealed significant impairment of extra-motor regions such as the prefrontal and parietal cortices [46, 67, 68].

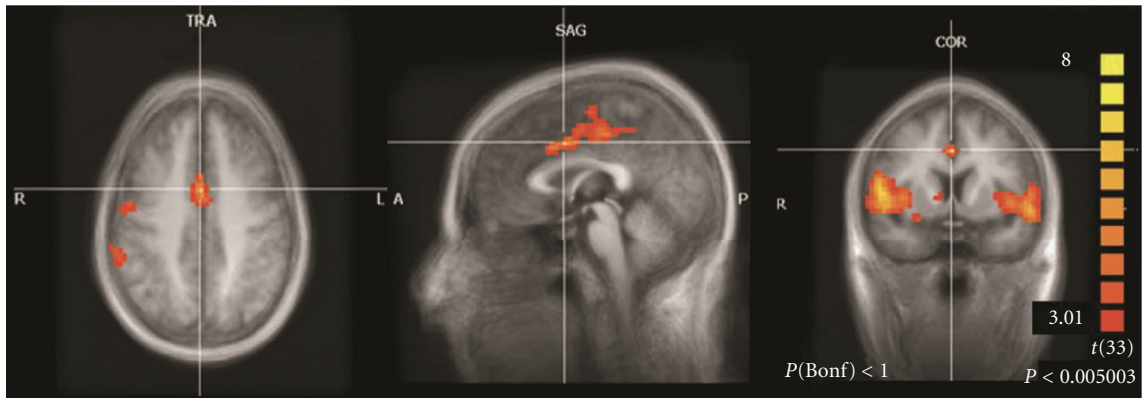
The increased striatal activation in patients with ALS was interpreted as a compensatory response to increasing functional demands in the context of affected cortical motor areas, even during the execution of a simple motor task. Therefore, the striatal pattern of activation may indicate the need of the ALS patient group to recruit the basal ganglia system (normally recruited in adaptive control of more complex motor behaviors) as a compensatory circuitry to perform simple motor tasks as well as controls. This finding was consistent with previous PET results concerning an abnormal recruitment of nonprimary motor areas in ALS [9], also interpreted as a pattern of functional adaptation to the corticospinal tract (CST) dysfunction. Thus, it has been suggested that such increased activation during motor tasks may reflect cortical plasticity, as new synapses and pathways are developed to compensate for the selective loss of pyramidal cells in the motor cortex [76] with consequent proliferation of synaptic processes in less affected brain areas. Significantly, patterns of functional adaptation were also detected in ALS by neurophysiological findings, derived from electroencephalography (EEG) and magnetoencephalography (MEG) studies [78, 79], and in case of motor recovery after ischemic stroke [80], in other neurodegenerative disorders [81, 82], and in the aging brain [83].

Further evidence of cortical adaptive changes in the affected brain of ALS patients is derived from fMRI studies that investigated random hand movements against rest [84]. Once again during such a motor task, patients with ALS showed increased cortical activation bilaterally, extending from the sensorimotor cortex posteriorly into the inferior parietal lobule and inferiorly to the superior temporal gyrus. In addition, ALS patients showed reduced activation in the DLPFC extending to anterior and medial frontal cortex [84].

More recently, to assess fMRI longitudinal data on activation changes in different clinical stages of the disease, Mohammadi et al. [85] investigated motor activations in three groups of ALS patients with different degrees of weakness, and a subset of those patients was scanned on multiple occasions. Two distinct stages of neuroplastic changes were identified: first, an increase of the activated area in contralateral sensorimotor cortex, and second, a reduction



(a) Healthy



(b) Patients

Figure 1: Sensorimotor network (SMN) in the healthy controls (a) and ALS patients (b) groups that Tedeschi et al. [28] examined by RS-fMRI (independent component analysis, ICA). The amount of coherent RS-fMRI fluctuations within this network appeared strongly reduced in the ALS population.

of signal change and beta weights with increasing weakness. The increase of the activated area was interpreted as a result of decreased intracortical inhibition, which seems to play a determinant role in regulating plasticity in both neurodevelopmental and neurodegenerative disorders [64, 65], and the reduction of movement-related signal change and beta weights as a consequence of loss of upper motor neurons.

A novel focus of neuroimaging research concerns the analysis of functional connectivity of spatially remote brain regions. To this purpose, the whole-brain analysis of functional connectivity by RS-fMRI appears important in developing a better understanding of specific motor or cognitive functions by exploring highly reproducible networks at rest, the so-called resting-state networks (RSNs) [27, 86]. Theoretically, during rest there exist spontaneous coherent fluctuations of the BOLD signal in different brain areas that are functionally connected.

Mohammadi et al. [87] examined, for the first time, the RSNs activity in ALS and demonstrated significant changes in the sensorimotor network (SMN), mainly the premotor area (Brodmann area-BA 6). In addition, in comparison to healthy controls, ALS patients demonstrated a significantly weaker connectivity of the default mode network (DMN) in the ventral anterior cingulate cortex, posterior cingulate cortex, and the left and right inferior parietal cortex, regions that have been linked to higher level executive functions. Indeed, this finding would support previous neuropsychological evidences of a dysexecutive syndrome in ALS patients [2–4, 8, 10, 63].

Later, Tedeschi et al. [28] designed an RS-fMRI study in ALS not only to assess functional RSNs but also to examine the possible interaction between neurodegeneration and aging, which has been reported to induce physiological age-related modulation effects on fMRI signal fluctuations especially in the DMN [88, 89]. The amount of coherent RS-fMRI fluctuations within the SMN network appeared strongly and significantly reduced in the ALS population especially in primary motor cortex (PMC) regions (Figure 1), in agreement with those reported by Mohammadi et al. [87]. Furthermore, the frontoparietal network (FPN), which includes the main RSNs in the cognitive domain, presented a selective suppression of signal fluctuations in ALS patients in two clusters of the right FPN (the superior frontal gyrus and the supramarginal gyrus). These effects in a cognitive executive network like the right FPN are consistent with the

frontal executive dysfunction that has been largely described in ALS [2–4].

Remarkably, in the ALS patient group there was a statistically significant interaction between neurodegeneration and aging (disease-by-age effect) in the DMN, specifically, in the posterior cingulate cortex (PCC). In fact, this effect resulted capable of inverting the trend of negative correlation between functional connectivity and age observed in the sex- and age-matched control group. This finding is in line with recent evidence that neurodegenerative dementias may be associated with increased functional connectivity within unaffected (or affected at later stages) networks with less evident functional decline [90, 91]. Particularly, posterior cortical functions have been shown to survive or even thrive in patients with FTD [92, 93] in contrast to Alzheimer's disease that, like normal aging, damages the posterior part of the DMN [94].

Interestingly, the positive modulation on the spontaneous functional connectivity of the posterior part of DMN described by Tedeschi et al. [28] in an ALS population appears to be similar to the RS-fMRI trend observed in the behavioral variant of FTD [94] and may be interpreted as the functional expression of a possible compensatory mechanism of the default system to the combined effect of degeneration and aging. However, a recent study using animal and cellular models of ALS pathophysiology [95] has linked neurodegeneration and aging to specific strategies of neuroprotection by which the cell damage is contrasted with adaptive mechanisms against the physiological stress implied by aging.

5. Structural Neuroimaging

Morphometric studies by volumetric MRI were originally used in ALS for the in vivo investigation of region-specific volume reductions and have enabled the detection of subtle yet significant cortical and subcortical changes in the frontal and temporal lobes [96, 97].

In recent years, the development of advanced automated imaging analysis, based upon construction of statistical parametric maps, allowed detailed anatomic studies of brain morphometry. Particularly, voxel-based morphometry (VBM) allows a fully automated whole-brain measurement of regional brain atrophy by voxelwise comparison of gray matter (GM) and white matter (WM) volumes between groups of subjects [98]. The most consistent finding of VBM studies in ALS involves GM atrophy in several regions of the frontal (i.e., the anterior cingulate, middle and inferior frontal gyrus, BA 8, 9, and 10) and temporal lobes (i.e., temporal poles, superior temporal gyrus, temporal isthmus) [14–17, 28, 99].

Among the authors who investigated GM volumetric changes in ALS, Mezzapesa et al. [15] and Grossman et al. [16] reported significant correlations between measures of cognitive function and cortical atrophy in classical ALS patients.

Mezzapesa et al. [15] detected a gray matter volume decrease in several frontal and temporal areas bilaterally in patients with ALS, whose performances on Symbol Digit Modalities Test were significantly worse compared with controls. Therefore, the presence of mild whole-brain volume loss and regional frontotemporal atrophy seemed to be related to the cognitive impairment in patients with ALS.

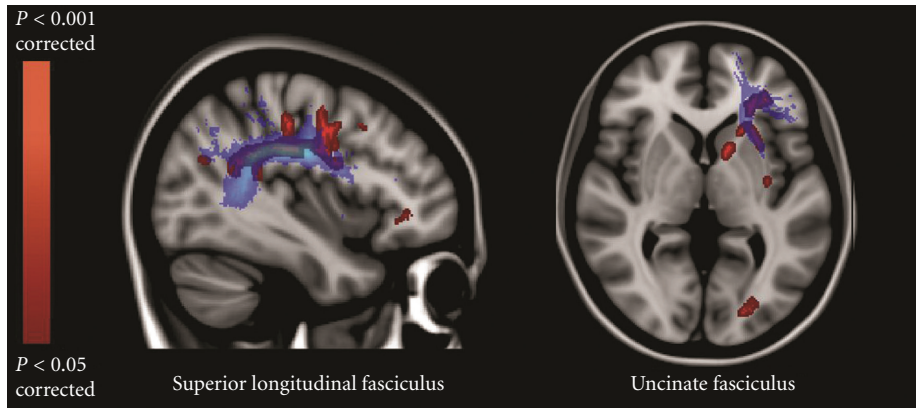
Grossman et al. [16] showed atrophy in several regions including the frontal, temporal, limbic, and occipital lobes. From a neuropsychological point of view, patients showed significant difficulty on measures requiring action knowledge compared to object knowledge, and performances to this kind of tasks were highly correlated with cortical atrophy in motor regions. Interestingly, scores on tests of both action and object knowledge were correlated with decreased GM volume in inferior frontal cortex and DLPFC, known to be involved in components of semantic memory. Therefore, deficiency in semantic access in patients with ALS partially reflects the degeneration of motor system mediation of tasks requiring knowledge of action features, while also reflecting degeneration of prefrontal regions responsible for both action and object knowledge.

To identify a marker of upper motor neuron degeneration, a surface-based cortical morphology technique has also been applied in ALS measuring cortical thickness, surface, and volume. Cortical morphology analyses revealed specific thinning in the precentral gyrus (preCG) [21, 100, 101] correlated with CST damage evaluated by DTI in combined analyses [21, 100]. A significant direct association was not found between measures of cortical thickness and cognitive impairment, although relative thinning in temporal regions was associated with a rapidly progressive disease course [101].

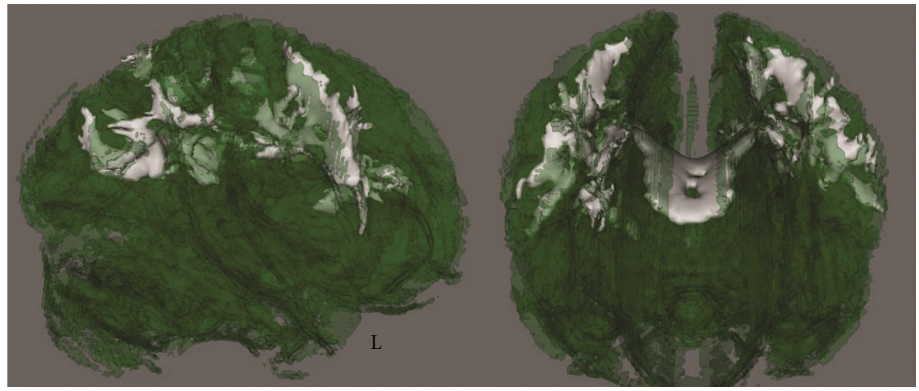
DTI studies of ALS have developed along two main directions: (i) a voxel-by-voxel evaluation of whole-brain WM and (ii) measurements of specific tracts by positioning regions of interest (ROIs). The first type of analysis involves the coregistration of each person's scan to a common template and can be performed without an a priori hypothesis. With this method, anisotropy maps are coregistered into a standard space, allowing comparisons of anisotropy value between groups. Moreover, this approach based on whole-brain DTI analysis may result in higher accuracy in detecting widespread microstructural disease-related WM changes rather than by using an ROI-based method.

Recent whole-brain DTI analyses reported regions of WM damage in ALS via voxel-based [17–19, 23, 102, 103], tract-based spatial statistics (TBSS) [18, 20, 22, 24, 104], and High Angular Resolution Diffusion Imaging (HARDI) [25] approaches. Most of these studies found changes of fractional anisotropy (FA) and mean diffusivity (MD) not only in the CSTs but also in the corpus callosum [18, 20, 23, 24, 102] and the frontal and temporal lobes [17, 18, 23, 29].

Recently, new insights in the assessment of corticomotor connectivity changes in ALS were obtained by acquiring HARDI scans along with high-resolution structural images (sMRI) [25]. A significant reduction in mean FA within a number of intra- and interhemispheric WM connections associated with the preCG and postcentral (postCG) gyri was found in ALS participants compared to controls, in



(a)



(b)

Figure 2: Regional FA reductions in ALS patients compared with healthy controls in frontal (associative) tracts (TBSS DTI analysis performed by Cirillo et al. [24]). In (a), blue shows the superior longitudinal and the uncinate fasciculi (derived from the Johns Hopkins University White-Matter Tractography atlas [106, 107]), whilst red shows significant FA decrease in ALS patients ($P < 0.05$, corrected). (b) illustrates 3D renderings of the FA skeleton (green), where white shows regional FA reductions in patients. Remarkably, these diffusivity changes resemble those which have been described in patients with the behavioral variant of frontotemporal dementia.

agreement with other DTI analyses (i.e., FA decrease in anterior cingulate, superior longitudinal, inferior longitudinal, inferior occipitofrontal, and uncinate fasciculi) (Figure 2) [24, 25]. Once again this DTI pattern of predominantly frontal WM injury clearly reflects the frontal executive dysfunction that has been extensively described in several cohorts of patients with ALS [2, 3] and is consistent with similar diffusivity changes described in patients with the behavioral variant of FTD [105].

By combining DTI and graph analytical network approaches (examination of the organization of widespread functional brain networks or connectome), Verstraete et al. [29] found a significantly impaired structural network overlapping bilateral primary motor regions (precentral gyrus and paracentral lobule, BA 4), bilateral supplementary motor regions (caudal middle frontal gyrus, BA 6), parts of the left basal ganglia (pallidum), and right posterior cingulate and precuneus in ALS. Therefore, the neurodegeneration process seems to affect not only the primary motor connections but also the connectivity between primary motor regions and supplemental motor areas. The authors hypothesize that the disease starts in the precentral gyrus and progresses along the structural connections of the primary motor regions towards secondary motor regions, as suggested by both DTI [20, 24] and graph analytical network evidence. Alternatively, brain plasticity might be potentially attributed to the reduced motor connectivity. This takes into account that the connectivity changes reported in DMN in ALS patients [28, 87] were in agreement with the findings of impaired structural connectivity of the motor network to the precuneus and PCC, key regions of the DMN.

To investigate the functional correlates of the structural changes, combined MRI studies have been recently performed in ALS. A multiparametric analysis by Verstraete et al. [21], based on a network perspective, by combining cortical thickness, DTI and RS-fMRI techniques, demonstrated a decline of structural integrity (i.e., significant reduction of cortical thickness in the preCG and in microstructural organization of rostral CST) with preserved functional organization of the motor network in ALS (Figure 3). Moreover, the local connectedness was found to be related with disease progression. Accordingly with these results,

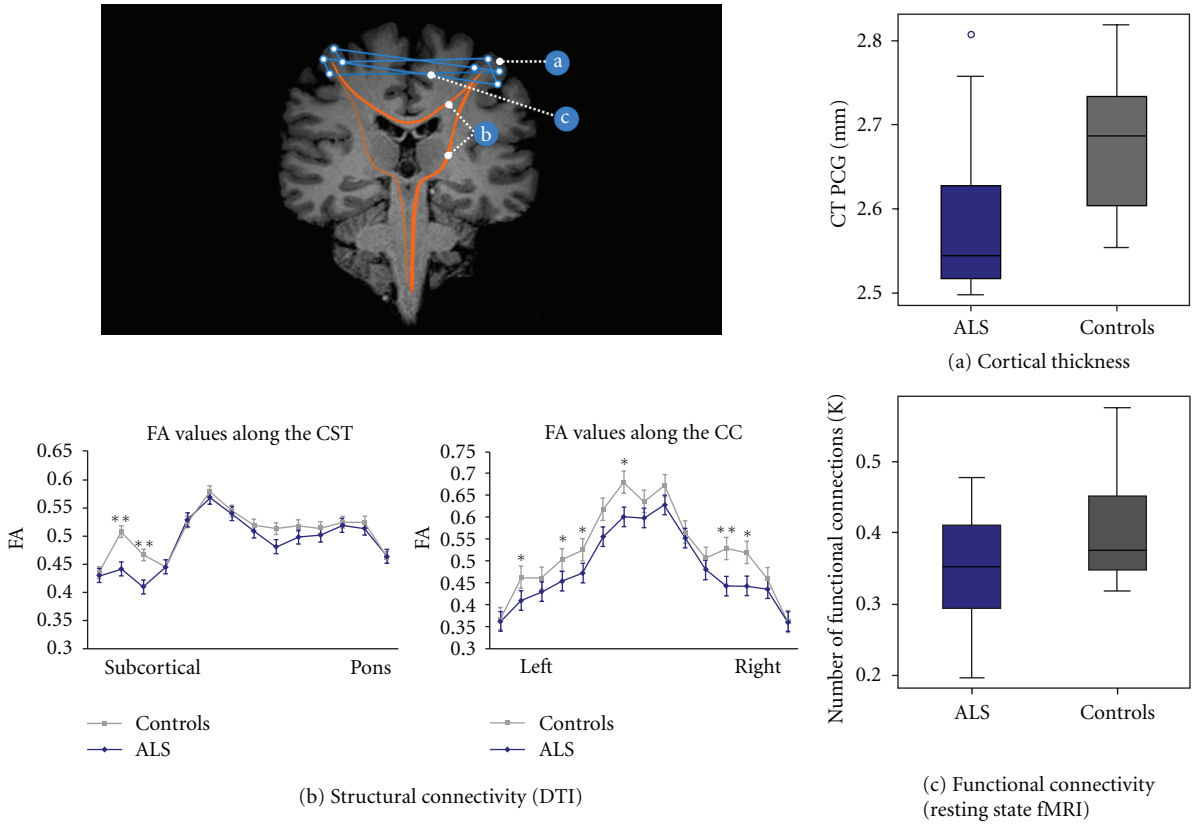


Figure 3: (a) Cortical thickness (CT) in patients with ALS versus controls in the preCG in mm ($P = 0.04$), corrected for age and whole brain CT. (b) Fractional anisotropy (FA) values along the CST and the corpus callosum, evaluated by DTI analysis, in patients with ALS and controls ($^{**}P < 0.01$; $^{*}P < 0.05$). (c) Number of functional connections in patients with ALS versus controls, corrected for age (threshold 0.40) ($P = 0.14$): this result was indicative of a relative sparing of functional connectivity in patients (derived from Verstraete et al. [21]).

another MRI study of connectivity in ALS by Douaud et al. [22] demonstrated an increased functional connectivity directly associated with an impaired ALS-specific grey matter network (predefined by the consistent regions of WM damage), spanning sensorimotor, premotor, prefrontal, and thalamic regions (Figure 4). Patients with a slower rate of disease progression (not only longer disease duration) presented connectivity values more comparable to those of healthy controls. Therefore, these findings prompted speculation as to whether connectivity changes might have a more active role in pathogenesis. In fact, one hypothesis is that increased functional connectivity arises as a result of loss of central nervous system interneurons influence, reflected in the hitherto unexplained variable compartmentalization of pathology within upper and lower motor neuron populations [108]. This interneuronopathy may cause a generalized hyperexcitability in the motor cortex, as also shown by several electrophysiological findings (i.e., derived from transcranial magnetic stimulation or TMS, and event-related potentials or ERP studies) [109–111], and appears also corroborated by histopathological [41] and flumazenil PET [67, 68] evidence.

Studies of lower motoneurons in the animal model of the disease have given important clues to the downstream mechanisms of cell death in the spinal cord, where the earliest damage appears to occur in the interneurons in lamina VII known as Renshaw cells [112]. It was then hypothesized that dysfunction or loss of Renshaw cells may have important consequences for spinal connectivity and motor control. Speculatively therefore, an excitotoxic pathway common to upper and lower motor neurons populations might result from an unopposed glutamatergic activity [108]. However, abnormal presymptomatic development of lower motor neuron connectivity has not been seemed a prerequisite for subsequent neuromuscular pathology in a mouse model of severe spinal muscular atrophy (SMA) [113].

Finally, an era of multimodal MRI studies, combining several advanced techniques, along with neuropsychological, genetic, and histopathological information, might lead to a comprehensive assessment of neurodegeneration in ALS, including disease mechanisms and monitoring of disease progression and therapeutics. Remarkably, with the model of Alzheimer's Disease Neuroimaging Initiative (ADNI) [114] in mind, Oxford University (UK) hosted international scientists at the first Neuroimaging Symposium in ALS (NISALS; November 2010), which led to the development of consensus guidelines on image acquisition and analysis, with the aim of retrospective data sharing to further explore the feasibility of MRI as a surrogate marker in future therapeutic trials for ALS [69].

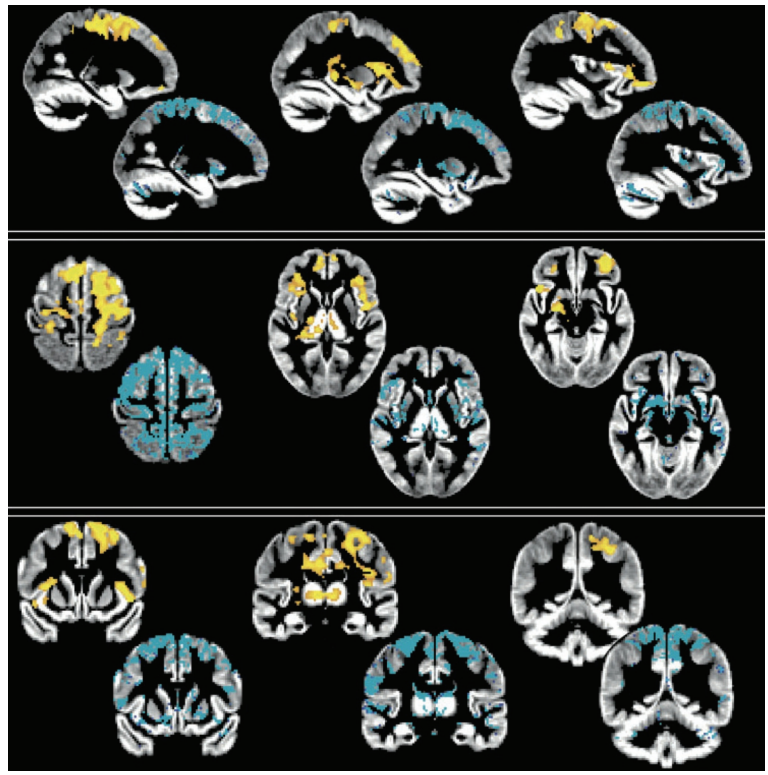


Figure 4: Increase of functional connectivity and lower structural connectivity in a population of ALS patients studied by Douaud et al. [22]. The spatial distribution of the significant increase of functional connectivity in patients (red-yellow scale: $P < 0.05$, corrected) corresponded to the areas where the patients had lower structural connectivity, evaluated by using tract-based spatial statistics and probabilistic tractography, in comparison to healthy controls (in blue, thresholded at 10 streamlines of difference on average) (derived from Douaud et al. [22]). By permission of Oxford University Press.

6. Concluding Remarks

The involvement of frontotemporal areas in ALS and the existence of overlap syndromes with dementia types have been recognized for decades. Functional imaging studies have confirmed that functional changes beyond the primary motor network are a common feature in ALS patients and may reflect an attempt of the ALS brain to compensate for the effect of motor neurodegeneration by neural plasticity within unaffected or less affected structures subserving cognitive domains. Alternatively, the abnormal functional connectivity may arise as a result of loss of interneurons inhibitory influence, with widespread and variable effects on upper and lower motor neuron populations.

We believe that future studies based on neuropsychology, advanced imaging, molecular pathology, and genetics will further enhance our understanding of the relationship between motor system dysfunction and cognition and provide valuable information on the physiopathological mechanisms underlying the complex interaction between the multiple affected systems in ALS.

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Review Article

Current Status of Treatment of Spinal and Bulbar Muscular Atrophy

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Spinal and bulbar muscular atrophy (SBMA) is the first member identified among polyglutamine diseases characterized by slowly progressive muscle weakness and atrophy of the bulbar, facial, and limb muscles pathologically associated with motor neuron loss in the spinal cord and brainstem. Androgen receptor (AR), a disease-causing protein of SBMA, is a well-characterized ligand-activated transcription factor, and androgen binding induces nuclear translocation, conformational change and recruitment of coregulators for transactivation of AR target genes. Some therapeutic strategies for SBMA are based on these native functions of AR. Since ligand-induced nuclear translocation of mutant AR has been shown to be a critical step in motor neuron degeneration in SBMA, androgen deprivation therapies using leuprorelin and dutasteride have been developed and translated into clinical trials. Although the results of these trials are inconclusive, renewed clinical trials with more sophisticated design might prove the effectiveness of hormonal intervention in the near future. Furthermore, based on the normal function of AR, therapies targeted for conformational changes of AR including amino-terminal (N) and carboxy-terminal (C) (N/C) interaction and transcriptional coregulators might be promising. Other treatments targeted for mitochondrial function, ubiquitin-proteasome system (UPS), and autophagy could be applicable for all types of polyglutamine diseases.

1. Introduction

Spinal and bulbar muscular atrophy (SBMA) was first described in 1897 by a Japanese neurologist, Kawahara [1], and has been known worldwide as Kennedy's disease since 1968 when reported by Kennedy [2]. It is characterized by the degeneration and loss of lower motor neurons in the brainstem and spinal cord, and patients present with weakness and wasting of the facial, bulbar, and limb muscles, along with sensory disturbances and endocrinological abnormalities [3, 4]. SBMA is an X-linked trinucleotide polyglutamine disease, caused by an abnormal expansion of tandem CAG repeat in exon 1 of the androgen receptor (AR) gene on chromosome Xq11-12 [5]. In normal individuals, the CAG repeat ranges in size between 9 and 36, and expansion over 38 and up to 62 is pathogenic [5, 6]. Polyglutamine-expanded mutant AR accumulates in nuclei, undergoes fragmentation, and initiates degeneration and loss of motor neurons [7, 8].

So far, nine polyglutamine diseases are known including SBMA, Huntington's disease, dentatorubral-pallidoluysian atrophy, and six forms of spinocerebellar ataxia (SCA), known as SCA1, SCA2, SCA3, SCA6, SCA7, and SCA17 [9, 10]. These diseases share several features such as late-onset, progressive neurodegeneration, anticipation, somatic mosaicism, and accumulation of misfolded mutant proteins in the nuclei or cytoplasm of neurons [8–13]. Expanded polyglutamine tracts form antiparallel beta-strands held together by hydrogen bonds formed between the main chain of one strand and the side chain of the adjacent strand. This leads the polyglutamine protein to acquire a nonnative beta-sheet conformation, which results in the accumulation of misfolded protein into microaggregates/oligomers and inclusions [3, 14]. Accumulation of polyglutamine-expanded protein into inclusions is considered to be protective [15–17], while diffuse nuclear microaggregates/oligomers might be toxic [18]. These aggregates and inclusions contain

components of the ubiquitin proteasome system (UPS) and molecular chaperons, which attempt to degrade or refold the polyglutamine-expanded proteins [19]. Thus, these common features of aggregates and inclusions observed in polyglutamine diseases suggest that the expanded polyglutamine tract itself seems to be deeply involved in the pathogenesis.

However, the observation that the same genetic mutation in nine different proteins results in nine different diseases highlights both the significance of a specific protein context other than the polyglutamine tract and the role of normal protein function in the pathogenesis of polyglutamine diseases [20]. Direct evidence that native protein functions and interactions may mediate toxicity comes from an animal model in which overexpression of wildtype AR harboring nonexpanded polyglutamine tract results in pathology resembling SBMA [21]. In the majority of polyglutamine diseases, neither the primary function nor the native interactors of the disease proteins are well known. SBMA represents an exception because AR protein structure and function as a ligand-dependent transcription factor are well characterized. AR belongs to the family of steroid hormone receptors and is composed of an amino-terminal domain, a DNA-binding domain, and a ligand-binding domain [22]. In the inactive state, AR is confined in the cytoplasm in association with heat shock proteins (HSPS). Testosterone binding to AR leads to the dissociation of AR from Hsps and causes nuclear translocation (Figure 1) [3, 23]. Also, ligand binding induces conformational changes of AR such as intra- or inter-molecular amino/carboxy-terminal (N/C) interactions (Figure 1) [3, 24]. Nuclear translocation of AR is followed by DNA binding to androgen-responsive elements, which in turn leads to recruitment of coregulators and expression regulation of androgen-responsive genes (Figure 1). These native functions and sequential processing of AR have important roles for the pathogenesis and therapy development of SBMA.

In SBMA, expanded polyglutamine tracts are associated to lower levels of transcription of androgen-responsive genes [25, 26], which in turn lead to mild androgen insensitivity symptoms such as gynecomastia, feminized skin changes, testicular atrophy, and oligospermia/azoospermia causing reduced fertility [27]. However, dysregulation of androgen-responsive genes does not likely contribute to the neurological symptoms of SBMA, because complete androgen insensitivity syndrome associated with total loss of AR function has no signs of neurodegeneration [28], and AR knock out mice are also normal in motor neuron functions [29].

So far, therapeutic interventions have been developed to target a number of events occurring through native AR functions upon ligand binding. Although no treatments have been established in SBMA, this review illustrates several therapeutic strategies based on the native function of AR and the common mechanisms shared by polyglutamine diseases.

2. Therapeutic Interventions to Inhibit Nuclear Transport of Mutant Androgen Receptor (AR)

Due to the X-linked transmission, SBMA exclusively affects males and is transmitted by clinically unaffected or mildly manifesting female carriers. A unique gender-specific feature of SBMA is well recapitulated in both vertebrate and invertebrate animal models of the disease [30, 31]. In transgenic mice expressing polyglutamine-expanded mutant AR, the disease fully manifests only in males due to higher levels of circulating androgens [30, 32, 33]. Importantly, decrease of androgen levels by castration of transgenic male mice prevents neurodegeneration, while treatment of transgenic female mice with testosterone induces disease manifestations [30]. In a fly model of SBMA, neurodegeneration occurs only if the flies are reared in a hormone-containing food [31], further supporting the ligand-dependent neurotoxicity of pathogenic AR. The prerequisite for SBMA pathogenesis is both the existence of ligand and nuclear translocation of mutant AR. This is shown by the observation that cytoplasmic retention of mutant AR by deletion of the nuclear localization signal suppresses polyglutamine-AR toxicity in SBMA mouse model [34].

Leuprorelin is a potent luteinizing hormone-releasing hormone analog that decreases the production of testosterone and its more potent derivative, dihydrotestosterone (DHT), and has been used for the treatment of a variety of sex hormone-dependent diseases including prostate cancer, endometriosis, and central precocity [22]. Treatment of SBMA mice with leuprorelin reduced both polyglutamine-AR nuclear aggregation and inclusion formation in spinal cord as well as skeletal muscle and reversed the behavioral and histopathological phenotypes (Figure 1) [35]. These dramatic therapeutic effects of leuprorelin observed in a mouse model of SBMA were translated into a phase II clinical trial, and the patients treated with leuprorelin for 144 weeks exhibited significantly greater functional scores and better swallowing parameters than those who received a placebo [36]. Leuprorelin significantly diminished the serum level of creatine kinase and decreased mutant AR accumulation in scrotal skin of treated patients [36]. Of note, leuprorelin inhibited the nuclear accumulation and/or stabilization of mutant AR in the motor neurons of the spinal cord and brainstem of an autopsied patient who received it for 2 years [36]. More recently, a larger randomized placebo-controlled multicentric clinical trial of this drug showed no definite effect on motor functions, although swallowing function improved in a subgroup of patients whose disease duration was less than 10 years [37].

Another potent drug for hormonal intervention is the 5-α-reductase inhibitor, dutasteride. The observation that motor neurons degenerating in SBMA express high levels of 5-α-reductase suggests that the conversion of testosterone to DHT represents a potential therapeutic target (Figure 1) [3]. However, the effectiveness of dutasteride was not proven in a 2-year double-blind placebo-controlled trial evaluated by the primary outcome measure of quantitative muscle assessment (QMA) [38].

Although the results of these clinical trials are inconclusive, their findings do not exclude the possibility that ligand-targeted hormonal therapies slow the progression of SBMA. As an example of the effectiveness of longer administration of leuprorelin, a 75-year-old male SBMA patient who received leuprorelin for 5 years due to coexisting prostate cancer was

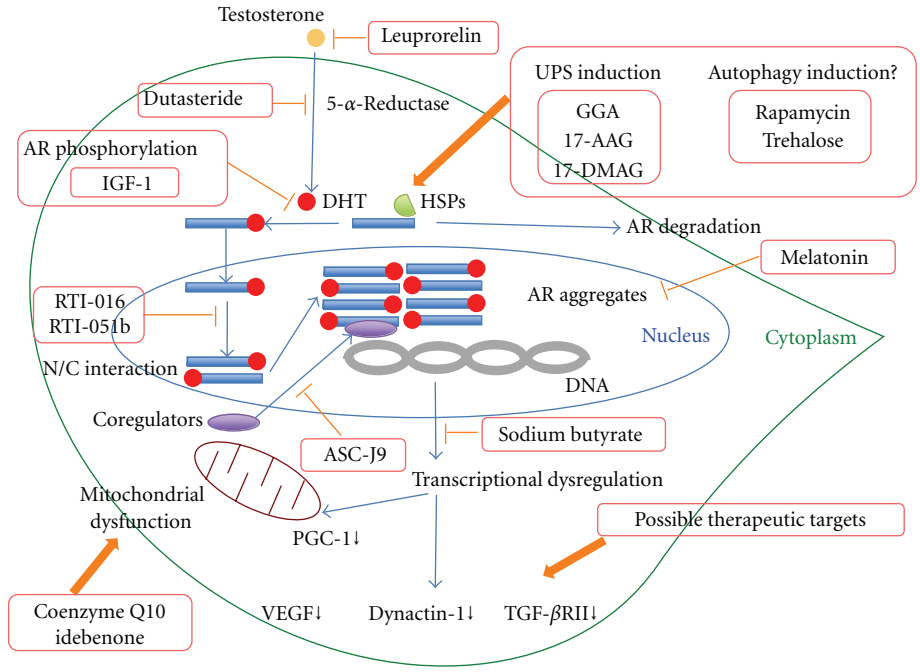

Figure 1: Potential disease-modifying therapies for spinal and bulbar muscular atrophy (SBMA). Ligand-induced nuclear translocation of mutant androgen receptor (AR) is a critical step of motor neuron degeneration in SBMA. In order to block this step, androgen deprivation therapies using leuprorelin and dutasteride have been developed. AR phosphorylation is another potential treatment strategy through attenuation of ligand binding. Insulin-like growth factor-1 (IGF-1) reduces mutant AR toxicity through phosphorylation of AR at the Akt consensus sites. Amino-terminal (N) and carboxy-terminal (C) (N/C) interaction of mutant AR is critical for toxicity, and this interaction is blocked by selective AR modulators such as RTI-016 and RTI-051b. The SBMA modifier melatonin blocks toxic fibrillar and induces nontoxic annular aggregates. As a transcription factor, the binding of AR to DNA in the nucleus is followed by the recruitment of a variety of transcriptional coregulators. 5-Hydroxy-1,7-bis(3,4-dimethoxyphenyl)-1,4,6-heptatrien-3-one (ASC-J9) disrupts the interaction between AR and its coregulators and yields a therapeutic effect. In SBMA, histone acetylation is impaired, resulting in transcriptional dysregulation. Sodium butyrate, histone deacetylase (HDAC) inhibitor is effective at this step. Furthermore, transcriptionally attenuated genes such as vascular endothelial growth factor (VEGF), dynactin-1, and transforming growth factor β receptor type II (TGF-βRII) are also possible therapeutic targets. Decreased expression of peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1) is one of the causes of mitochondrial dysfunction, and treatments with the antioxidants coenzyme Q10 and idebenone have been developed targeting mitochondria. Mutant AR is degraded through induction of the ubiquitin-proteasome system (UPS) by acyclic isoprenoid geranylgeranylacetone (GGA) and heat shock protein 90 (Hsp90) inhibitors such as 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) and 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG). Autophagy induction using rapamycin and trehalose is also effective for AR degradation in fly and cell models of SBMA. However, the opposite results concerning autophagy augmentation therapy were recently reported in SBMA knock-in mice.

reported to show long-term stabilization of motor function even when the treatment was started in the advanced stage of the disease [39].

Besides the hormonal interventions, attenuation of ligand binding might be another therapeutic strategy for inhibition of nuclear transport of mutant AR. Ligand binding is at least partly mediated by phosphorylation of the mutant AR. Interestingly, substitution of the AR at two Akt consensus sites, S215 and S792, with aspartate, which mimics phosphorylation, reduces ligand binding, ligand-dependent nuclear translocation, transcriptional activation, and toxicity of expanded polyglutamine AR [40]. Furthermore, in motor neuron-derived MN-1 cells toxicity associated with polyglutamine-expanded, AR is rescued by coexpression with Akt [40]. Insulin-like growth factor-1 (IGF-1) reduces mutant AR toxicity in cultured cells through phosphorylation of AR at the Akt consensus sites [41]. Interestingly, augmentation of IGF-1/Akt signaling by overexpressing a muscle-specific isoform of IGF-1 selectively in skeletal muscle ameliorated the neurological phenotypes, extended the life span, and rescued not only muscle but also spinal cord pathology of SBMA transgenic mice [41]. This finding also indicates skeletal muscle as a viable target tissue for therapeutic intervention in SBMA. These results highlight that AR phosphorylation is another potential target for therapeutic intervention through inhibition of nuclear transport of mutant AR in SBMA (Figure 1).

3. Therapy Targeted for Conformational Changes of Androgen Receptor (AR) and Transcriptional Regulation

Ligand-mediated nuclear localization of the mutant AR is necessary but not sufficient for SBMA pathogenesis. Upon

ligand binding, the AR undergoes several conformational changes including the interdomain interaction between the 23FQNLF27 motif near the amino terminus and the activation function-2 (AF2) domain near the ligand-bound carboxyl terminus (N/C interaction) [42, 43]. This N/C interaction is critical for toxicity through stabilizing the AR and enhancing hormone binding [44, 45]. Selective androgen receptor modulators such as RTI-016 and RTI-051b prevent the N/C interaction and ameliorated AR aggregation and toxicity while retaining AR transcriptional function, highlighting a novel therapeutic strategy for SBMA (Figure 1) [45].

Another strategy for reducing toxicity of mutant AR is based on alteration of the morphology of the oligomers. Recently, Jochum and colleagues demonstrated that the pathogenic AR mutants formed oligomeric fibrils up to 300–600 nm in length, whereas annular oligomers 120–180 nm in diameter were formed by the nonpathogenic receptors [46]. They showed that melatonin ameliorated the pathological phenotype of the SBMA fly model through the conformational change of the polyglutamine-expanded oligomers from the toxic fibrillar forms to nontoxic annular forms (Figure 1) [46].

As a ligand-dependent transcription factor, the binding of AR to DNA is followed by the recruitment of a variety of transcriptional coregulators, both coactivators and corepressors of transcription [47, 48]. In most steroid receptors, AF-2 domain plays a major role in receptor transactivation by serving as the interaction surface for transcriptional coregulators [3]. K720A and E897K mutations to the AF-2 attenuated polyglutamine-expanded AR toxicity in a *Drosophila* model of SBMA [20], suggesting that this toxicity requires DNA binding followed by association with coregulators through the AF-2 domain. In motor neurons, one of the key AR coregulators is the cAMP response element-binding (CREB)-binding protein (CBP), a transcriptional coactivator for neuronal survival factors [49]. In SBMA, through the sequestration of CBP by expanded polyglutamine aggregates [49, 50], many different transcription factors compete for functionally limiting levels of CBP, resulting in transcriptional disturbance. These observations raise the possibility of a therapeutic approach using compounds that disrupt the interaction of AR with transcriptional coregulators. One such compound is curcumin-related 5-hydroxy-1,7-bis(3,4-dimethoxyphenyl)-1,4,6-heptatrien-3-one (ASC-J9). ASC-J9 disrupts the interaction between AR and its coregulators including ARA70 and CBP (Figure 1) and markedly ameliorates phenotypes of SBMA transgenic mice by decreasing mutant AR aggregation [51]. ASC-J9 did not change the serum testosterone level in contrast to hormonal therapies associated with reduction of testosterone causing side effects on sexual functions.

CBP exerts its transcriptional coactivating function through histone acetyltransferase (HAT) activity. Overexpression of CBP rescued histone acetylation and neurodegeneration in cell and animal models of SBMA [50, 52] in association with subsequent restoration of gene transcription, whereas histone deacetylase (HDAC) inhibitor also acetylates histone, suggesting that it may be of therapeutic value. Oral administration of sodium butyrate, an HDAC inhibitor, ameliorated neurological phenotypes as well as increased acetylation of nuclear histone in neural tissues of a mouse model of SBMA (Figure 1) [53]. Beneficial effects of this compound, however, were seen within a narrow therapeutic window of dosage.

Downstream targets associated with decreased expression through transcriptional dysregulation in SBMA include vascular endothelial growth factor (VEGF), dynactin-1, and transforming growth factor β (TGF-β) receptor type II [54–57]. The importance of VEGF on maintenance of motor neuron is highlighted by motor neuron loss in mice with a homozygous deletion in the hypoxia-response element site in the VEGF promoter region [58]. Moreover, mutant AR-induced death of motor neuron-like cells (MN-1 cells) could be rescued by VEGF supplementation [54]. Dynactin-1 is a critical component of dynein/dynactin complex, a microtubule motor protein essential for retrograde axonal transport [59, 60], and its mutation was identified as the cause of a slowly progressive, autosomal dominant form of lower motor neuron disease [61]. In the mouse model of SBMA, pathogenic AR impairs retrograde axonal transport via transcriptional dysregulation of dynactin-1 [56]. TGF-β signaling was demonstrated to play a crucial role in the survival and function of adult neurons [62]. Transcriptional inhibition of TGF-β receptor type II suppressed nuclear translocation of phosphorylated Smad2/3, a key step in TGF-β signaling in the spinal motor neurons of SBMA mice and patients [57]. Targeting these molecules and as-yet-unknown transcriptionally dysregulated molecules might be another effective therapeutic approach (Figure 1).

4. Therapy Targeted for Ubiquitin-Proteasome System (UPS) and Autophagy System

The two major intracellular mechanisms for the degradation of misfolded proteins are the ubiquitin-proteasome system (UPS) and lysosome-mediated autophagy [23]. The degradation and removal of mutant AR may be obtained through overexpressing different Hsps, such as Hsp40 and Hsp70 through UPS pathway [63–65]. Moreover, the C-terminus of heat shock protein 70 interacting protein (CHIP) interacts with hsp90 or hsp70 and ubiquitylates misfolded proteins trapped by molecular chaperones and degrades them [19, 66]. Similar effects can also be induced by oral administration of the acyclic isoprenoid geranylgeranylacetone (GGA) [67]. GGA increased the levels of expression of Hsp70, Hsp90, and Hsp105, leading to inhibition of cell death, and amelioration of the neuromuscular phenotype of SBMA mice via activation of heat shock factor-1 and reduction of nuclear accumulation of polyglutamine-expanded AR (Figure 1) [67].

In addition, Hsp90 inhibitors are able to promote the clearance of Hsp90 client proteins including misfolded mutant AR through the UPS. Treatment with potent Hsp90 inhibitors, such as 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) or its derivative 17-(dimethylaminoethyl-amino)-17-demethoxygeldanamycin (17-DMAG), enhanced proteasomal degradation of the monomeric and

aggregated mutant AR, reduced motor neuron degeneration, and increased survival in SBMA mice (Figure 1) [68–70]. It is to be noted that 17-AAG also disrupts the interaction between hsp90 and other client proteins, including steroid receptors, such as glucocorticoid receptor (GR), estrogen receptor-α (ERα), and retinoid X receptor-α (RXRα) [71], rendering them more susceptible to degradation and leading to unwanted side effects. Interestingly, a recent report shows that in motor neuron-derived cells 17-AAG removes misfolded AR species and aggregates by activating the autophagy system rather than UPS [72].

This report indicates the importance of link and equilibrium between these two degradative systems [23]. In particular, HDAC6 plays an important role in the functional relationship between UPS and autophagy [73]. Compensatory autophagy is induced in response to UPS impairment in the SBMA fly model in an HDAC6-dependent manner [73]. Furthermore, HDAC6 overexpression rescued degeneration associated with UPS dysfunction accelerating the rate of mutant AR clearance through autophagy [73].

Other therapeutic approaches to augment autophagy induction are the use of rapamycin, an mTOR inhibitor [73], and trehalose [34, 74]. However, a recent report using knock-in SBMA mouse suggests that autophagy activators are unlikely to be effective therapeutics for the subset of protein aggregation disorders such as SBMA where nuclear localization of the mutant protein is required for toxicity [75]. This suggests the need for due care in the use of autophagy augmentation therapies.

5. Modulation of Mitochondrial Function

Mitochondrial dysfunction has been implicated in various neurodegenerative diseases, including Huntington's disease, amyotrophic lateral sclerosis and Friedreich's ataxia [76, 77]. Expression of the mutant AR in cell cultures results in depolarization of the mitochondrial membrane and an elevated level of reactive oxygen species, which is blocked by treatments with the antioxidants coenzyme Q10 and idebenone (Figure 1) [78]. Mitochondrial dysfunction in SBMA is caused either by the indirect effects on the transcriptional repression of nuclear-encoded mitochondrial genes such as the peroxisome proliferator-activated receptor-γ-coactivator 1 (PGC-1) (Figure 1) and the mitochondrial specific antioxidant superoxide dismutase 2 or through direct effects of the mutant protein on mitochondria or both [78]. Therapeutic interventions targeted for mitochondrial function, UPS, or autophagy system might be promising treatments for all types of polyglutamine diseases.

6. Conclusions

SBMA is the first identified member of nine polyglutamine diseases [5], and due to its advantages through the well-investigated disease-causing protein structure and function, it has a leading place among polyglutamine diseases, especially from the standpoint of development of disease-modifying treatment as represented by hormonal therapies. However, despite dramatic efficacy in animal studies, hormonal therapies are not successfully translated into the clinical field at present [37, 38]. In consideration of the limited availability of participants, together with the slow progression of symptoms, clinical trials of SBMA should be carefully designed in terms of endpoints, sample size, duration, and inclusion and exclusion criteria [79, 80]. Other than hormonal therapies, therapeutic strategies have been developed to target many steps of the exertion of mutant AR toxicity as described in this article. It is possible that together with hormonal therapy preventing nuclear translocation of mutant AR, additional therapies targeted for the molecular events occurring after this translocation will strengthen the therapeutic effect for SBMA.

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Review Article

Multiple Approaches to Investigate the Transport and Activity-Dependent Release of BDNF and Their Application in Neurogenetic Disorders

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Studies utilizing genetic and pharmacological manipulations in rodent models and neuronal cultures have revealed myriad roles of brain-derived neurotrophic factor (BDNF). Currently, this knowledge of BDNF function is being translated into improvement strategies for several debilitating neurological disorders in which BDNF abnormalities play a prominent role. Common among the BDNF-related disorders are irregular trafficking and release of mature BDNF (mBDNF) and/or its prodomain predecessor, proBDNF. Thus, investigating the conditions required for proper trafficking and release of BDNF is an essential step toward understanding and potentially improving these neurological disorders. This paper will provide examples of disorders related to BDNF release and serve as a review of the techniques being used to study the trafficking and release of BDNF.

1. Introduction

Molecular, electrical, and structural properties of neurons are all regulated in part by the most widely expressed neurotrophin in the mammalian central nervous system (CNS), brain-derived neurotrophic factor (BDNF). Mature BDNF (mBDNF) binds with high affinity to the tyrosine kinase receptor, TrkB, and most of the effects of BDNF are due to the intracellular cascades resulting from BDNF-TrkB signaling. The prodomain predecessor of BDNF, proBDNF, is also secreted, but preferentially binds to the pan-neurotrophin receptor p75NTR, triggering intracellular cascades with very different effects than those elicited by BDNF-TrkB signaling [1, 2].

BDNF and proBDNF play essential roles in neuronal development, plasticity, differentiation, and survival (see [3, 4] for reviews). With such widespread function, there is a substantial possibility for BDNF malfunction. In Alzheimer's disease patients, BDNF mRNA was reduced 50% in nuclei of the basal forebrain, the origin of the cholinergic neurons that innervate the cortex and hippocampus, and a region associated with the cognitive deterioration seen in Alzheimer's [5]. Neuronal survival is compromised in Huntington's disease as a result of an attenuated TrkB-Shc signaling pathway [6]. Thus, many neurological disorders have been associated with BDNF, with abnormalities occurring anywhere from the transcription of the *BDNF* gene to the receptor-mediated signaling cascades.

Between the transcription and signaling of BDNF are the equally important trafficking and release of it. BDNF in its pro- and/or mature form is packaged in the trans-Golgi network (TGN) and will see one of two possible fates: storage in dense-core granules for release via activity-regulated secretory pathways, or smaller BDNF vesicles can bud off from the TGN for release through a constitutive pathway (reviewed in [7]). Mice with a genetic knockout (KO) of carboxypeptidase E, a transmembrane receptor in the TGN that directs proBDNF to the secretory pathway, exhibited a wide range of problems in dendritic spine morphology, accompanied by behavioral complications related to learning and memory [8]. A recent study found very different patterns of intracellular cascades, yielding large observable

Table 1: Summary of advantages and disadvantages of using BDNF ELISA, BDNF-eGFP, BDNF-pHluorin.

Method	Primary purpose	Advantages	Disadvantages	References
BDNF ELISA	Quantify levels of BDNF in homogenized tissue, or levels of released BDNF if using ELISA *in situ*	Genetic manipulations are unnecessary. Endogenous BDNF levels can be quantitatively measured with pg sensitivity. proBDNF-specific ELISA kits are available. If using ELISA *in situ,* the amount of released BDNF can be accurately quantified, and tissue need not be destroyed.	Cannot identify sites of BDNF release, and cannot observe trafficking of BDNF vesicles. ELISA is performed over the course of hours, and changes in BDNF cannot be observed in real time. ELISA *in situ* inhibits BDNF-TrkB signaling.	[13, 19, 20, 22, 24, 51, 57, 59, 65]
BDNF-eGFP	Visualize BDNF trafficking and release in real time	BDNF vesicle dynamics can be observed throughout the cell. Sustained vesicle release can be observed by a relative decline in fluorescence. Can be used in conjunction with western blot to determine relative levels of mBDNF and proBDNF. Downstream signaling of BDNF-eGFP release is similar to that of BDNF.	cDNA plasmids for BDNF-eGFP must be introduced by transfection or viral transduction, which can be harsh on cells, and/or can lead to artificial overexpression of BDNF. Cannot quantify absolute levels of released BDNF.	[11, 12, 28–31, 33, 35, 36]
BDNF-pHluorin	Visualize BDNF vesicle release kinetics in real time	The pH sensitivity of pHluorin enables discrimination between sustained and transient vesicular fusion, which is indicative of how much BDNF is diffusing out of each vesicle. Downstream signaling of BDNF-pHluorin release is similar to that of BDNF.	Because fluorescence is quenched at low pH, BDNF-pHluorin is difficult to track while inside of acidified vesicles, making this tool unsuitable for BDNF trafficking studies. The same disadvantages as BDNF-eGFP also apply.	[10, 85]

differences in spine morphology, resulting from acute or gradual exogenous BDNF application, even when final BDNF concentration was held constant [9]. These results demonstrate that not only are concentrations of BDNF important for neuronal function, but so are the ways in which BDNF is released—either constitutively (analogous to gradual and chronic application) or in an activity-dependent manner (analogous to rapid and acute application).

Within recent years, knowledge of BDNF trafficking and release, and the techniques used to study them, has improved dramatically [10–13]. With these advancements, research is now able to shift from investigation of normal BDNF dynamics to the study of abnormal BDNF dynamics as they pertain to particular disorders. However, only some such investigations have been conducted [14–16]. In this paper, three tools currently being used to study BDNF trafficking and/or release are reviewed: BDNF ELISA, BDNF tagged with enhanced green fluorescent protein from the jellyfish *Aequorea victoria* (BDNF-eGFP), and BDNF-pHluorin, which is tagged with the superecliptic variant of pHluorin, a pH-sensitive mutant of eGFP (see Table 1). Discussion will then shift towards the value of these methods in studying the role of BDNF in the following neurological disorders: (i) Val66met, a subclinical condition defined by a common single nucleotide polymorphism in (SNP) in the pro region of BDNF. It is associated with memory complications and decreased hippocampal and cortical volume [17]; (ii) neurodevelopmental disorders such as Rett syndrome and Fragile X syndrome; (iii) Huntington's disease, an inherited neurodegenerative disorder.

2. BDNF ELISA

Enzyme-linked immunosorbent assay (ELISA) involve linking a protein-specific antibody to an enzyme (e.g., horseradish peroxidase), which then processes common conjugated substrates to yield an absorbance reading that is proportional to the amount of antibody bound to the antigen being studied. Standard curves using known concentrations of antigen are made so that the absorbance can be used to ascertain the unknown concentration of the antigen. The sandwich variation of ELISA is most commonly used to study BDNF levels. Recently, a product has been designed to specifically detect proBDNF via ELISA (Adipobioscience), but these do not appear to have been widely used as

of yet; most studies have utilized standard BDNF ELISA, which is unable to discriminate between mBDNF and proBDNF.

Prior to BDNF ELISA, BDNF mRNA levels served as the most common measure of cellular BDNF content. It was clear using RT-PCR or *in situ* hybridization techniques that BDNF transcripts increase in an activity-dependent manner [18], but the amount of BDNF protein being produced could not be quantified because of very small endogenous concentrations [19]. The first experiment to use BDNF ELISA definitively showed discrepancies between BDNF mRNA and protein levels; there were numerous temporal and spatial differences in the dynamics of the mRNA and protein, suggesting involvement of posttranslational mechanisms that rendered BDNF mRNA an incomplete measure of the presence of BDNF [19]. There are now commerciallyavailable kits for BDNF ELISA (Promega's BDNF Emax ImmunoAssay System) that have become a staple in studies comparing BDNF levels across different brain regions and experimental groups [20]. Additionally, a variant named BDNF ELISA *in situ* has been shown to be much more accurate in detecting release of endogenous BDNF in primary neuronal cultures, making it possible to detect the minute changes in extracellular BDNF that occur upon electrical stimulation [13].

With BDNF ELISA, temporal and spatial information cannot be attained—only the amount of BDNF present or released in a sample throughout a protracted time span can be determined [21]. The major advantages in using ELISA are genetic manipulation is not necessary; blood serum levels of BDNF are reflective of brain BDNF levels [22] and can be readily measured in human subjects using BDNF-ELISA [23]; endogenous BDNF release and/or levels can be detected at a sensitivity of 1–3 pg/mL [19, 24]; and BDNF can be measured without destroying the tissue being studied—perfused liquids can be measured for their BDNF content [13, 25]. These last two points are particularly important in that they offer BDNF ELISA an advantage over Western immunoblotting. However, Western immunoblots can be more specific than ELISA in distinguishing BDNF and proBDNF [26], and in elucidating the cascades involved in BDNF signaling effects [9].

3. BDNF-eGFP

Several types of mammalian proteins have been tagged with wildtype green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* and, given that the subcellular localization of the fusion proteins are intact, these tagged proteins allow the study of their trafficking mechanisms (reviewed in [27]). The first BDNF-eGFP fusion protein was generated by inserting the rat preproBDNF gene into a CMV promoter-driven vector expressing eGFP using DNA recombination [28]. The primary localization of BDNF-eGFP to somatodendritic compartments matched previous immunocytochemical findings, showing that BDNF-eGFP was trafficked identically to the endogenous BDNF protein [28]. Importantly, the synthesis and posttranslational processing of BDNF-GFP were also reported to be largely the same as endogenous BDNF [11, 28]. However, a recent study showed that the ratio of proBDNF-eGFP/mBDNF-eGFP is higher than the endogenous proBDNF/mBDNF ratio [29].

BDNF-eGFP offered the ability to image dynamic activity of BDNF, enabling the discovery that BDNF travels transsynaptically in an activity-dependent manner, providing great support that BDNF is involved in synaptic plasticity [11]. Anterograde transport and release from axon terminals [11], autocrine and paracrine dendritic BDNF release [12], and the need for back-propagating action potentials in dendritic release [30] are among other important findings that were only possible with time-lapse BDNF-eGFP imaging in live neurons. However, higher than endogenous levels of BDNF expression have been necessary thus far to aid in the visualization of BDNF-eGFP [28, 31].

A unique advantage offered by fluorescent tagging is the ability to observe BDNF trafficking, which involves packaging, transport, and localization of BDNF. With trafficking abnormalities implicated in Alzheimer's disease [32], Huntington's disease [33], and others (reviewed in [34]), the ability to image BDNF with temporal and spatial resolution is critical. Issues with BDNF-eGFP include overexpression of BDNF, inability to distinguish mBDNF and proBDNF, transfection is often inefficient and/or harsh, and there is a requirement for *in vitro* preparation.

Figure 1 shows an example of a hippocampal neuron in primary culture after transfection with BDNF-eGFP, with subsequent immunolabeling with SGA2, a marker of secretory granules. This image shows that BDNF-eGFP is trafficked into SGA2-positive secretory granules, evidence that it is processed similarly to endogenous BDNF. We are currently using this approach for time-lapse imaging of BDNF trafficking in neurons obtained from *Mecp2*-deficient mice, a model of Rett syndrome (see below).

Several methods for introduction of the BDNF-eGFP construct are available, including liposome-based reagents (e.g., Lipofectamine) and viral transductions. Viral transduction may offer a gentler method of inducing the expression of BDNF-eGFP in cells. The landmark study by Egan et al. [35] transduced BDNF-eGFP plasmids into hippocampal cultures using a Sindbis virus in their discovery that val-BDNF is trafficked differently than met-BDNF (discussed below, [35]). Several studies have since used Sindbis virus-mediated transduction to investigate trafficking and release of variants of BDNF [36]. One mouse model has been developed in which the human BDNF (hBDNF) gene with a C-terminal eGFP gene was integrated into the mouse genome [37]. The hBDNF-eGFP gene, particularly its exons I and IV, is upregulated in response to neuronal activity, recapitulating the behavior of wildtype mouse BDNF [38]. However, hBDNF-eGFP in this mouse line is expressed in addition to the endogenous untagged BDNF, which leads to BDNF overexpression with unknown consequences [38]. A knock-in mouse model in which the endogenous BDNF is replaced by eGFP-tagged BDNF would be a way to circumvent the issue of BDNF overexpression that is prevalent in BDNF-eGFP research. Because of the incredible complexity of the

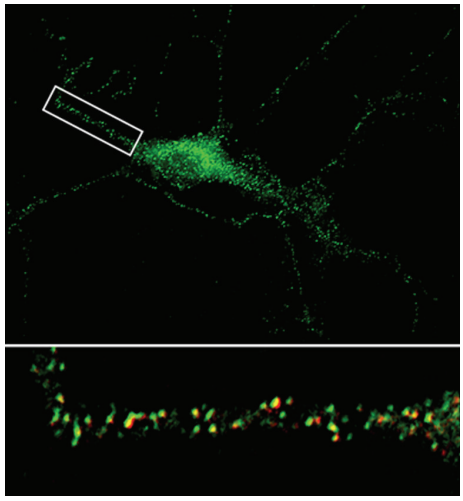

Figure 1: A pyramidal neuron expressing BDNF-eGFP. Insert shows BDNF-eGFP puncta in dendrites, which colocalize with the secretory granule marker SG2 (red).

rodent BDNF gene, a BDNF-eGFP knock-in mouse has been difficult to generate. However, knock-in mice have been generated that replace wildtype BDNF with the val66met BDNF polymorphism [39]. Thus, a knock-in BDNF-eGFP mouse is plausible and would be an important advancement in BDNF research, as it could even open the possibility for live *in vivo* imaging of BDNF at least in the cerebral and cerebellar cortices, which are accessible to multiphoton excitation microscopy through thinned skulls or cranial windows.

4. BDNF-pHluorin

Because of a vesicular proton ATPase, the lumen of secretory granules (including neurotransmitter-containing vesicles) is typically at pH ~ 5.5, meaning that a protein with pH sensitivity in this range can be used as an optical indicator of vesicular release. Genetic mutagenesis of key amino acids that surround the chromophore in wildtype eGFP-generated pHluorin, a mutant with its fluorescence intensity highly variable in the 5.5–7.4 pH range [83], taking advantage of natural variations in fluorescence that occur in the four different protonation states of the GFP chromophore [84]. Synaptobrevin, a membrane protein involved in vesicular release, was tagged on its luminal portion with pHluorin [83]. Because pHluorin fluorescence increases upon exposure to the extracellular environment, it was clear when and where synaptobrevin-containing vesicles fused [83], illustrating how tagging proteins with pHluorin can provide information that standard eGFP tagging cannot. However, when pHluorin-containing vesicles are quenched, they hardly fluoresce, meaning that pHluorin cannot be used in monitoring vesicle trafficking. An additional concern is that, if pHluorin-tagged proteins diffuse out of a vesicle (i.e., cargoes like BDNF), there is a net fluorescence decrease because the local concentration of pHluorin molecules decreases sharply when vesicle fusion is sustained [10, 85]. Similar to BDNF-eGFP, experimental concerns for BDNF-pHluorin include overexpression of BDNF and potentially harsh transfection methods; so far, viral transduction has not been used for BDNF-pHluorin.

BDNF-pHluorin was first used in the discovery that synaptotagmin-4 (syt4) regulates exocytosis of BDNF secretory vesicles [85]. This chimeric protein was also used to characterize BDNF release in response to different types of stimulation; theta burst stimulation (TBS) and other LTP-inducing stimulations showed the highest amounts of BDNF release [10]. These two papers comprise the entire body of published BDNF-pHluorin research. Both studies show that BDNF-pHluorin fluorescence increases transiently following neuronal depolarization but only in axonal puncta, meaning that BDNF vesicle fusion proceeds though a process of several repeated fusions, followed by re-acidification and requenching of BDNF-pHluorin, that is, kiss-and-run (Figure 2). On the other hand, the intensity of BDNF-pHluorin puncta in dendrites always showed a net decrease following depolarizing stimulation, indicating full vesicular fusion and BDNF discharge (Figure 2). However, fluorescence increases in the initial fusion events could be detected in both axonal and dendritic puncta using total internal reflectance fluorescence (TIRF) microscopy and faster image acquisition rates [10], enabling detection of many types of single vesicle fusion events. Thus, these studies illustrate how BDNF-pHluorin can be very useful in determining vesicular release kinetics of BDNF. It would be of great interest to investigate if BDNF vesicular release kinetics is altered in BDNF-related disorders, but none such studies have been conducted to date.

Figures 3 and 4 show unpublished work from our laboratory using a BDNF-pHluorin plasmid from the Poo laboratory [10]; same results were obtained with a BDNF-pHluorin plasmid from the Chapman laboratory [85]. BDNF-pHluorin in cultured hippocampal neurons is sensitive to pH, as demonstrated by its quenching when switching from artificial cerebrospinal fluid (ACSF) containing 50 mM ammonium chloride (NH_4Cl) to control ACSF (Figure 3). To confirm the viability of neurons transfected with BDNF-pHluorin and their responsiveness to extracellular field stimulation, Ca^{2+} imaging was performed in neurons labeled with the fluorescent dye fura-2AM. Upon stimulation with a 1 sec-20 Hz train at 20 V delivered by Pt wires, Ca^{2+} transiently increased in several dendrites (Figure 4). Using the same stimulation intensity but for longer duration, dendritic BDNF-pHluorin puncta showed activity-dependent destaining consistent with full fusion and BDNF discharge from secretory granules (Figure 5). Current studies in our laboratory use BDNF-pHluorin to study activity-dependent BDNF release in neurons from a mouse model of Rett syndrome.

So far, all studies of BDNF release agree upon the requirement for intracellular Ca^{2+} elevations, and the varying effects of different stimulation frequencies on BDNF release, with TBS and other LTP-inducing protocols eliciting the greatest amounts of BDNF secretion [10, 12, 13, 21]. None of the methods described in this review can provide measures of the effects of BDNF-receptor signaling. Standard Western

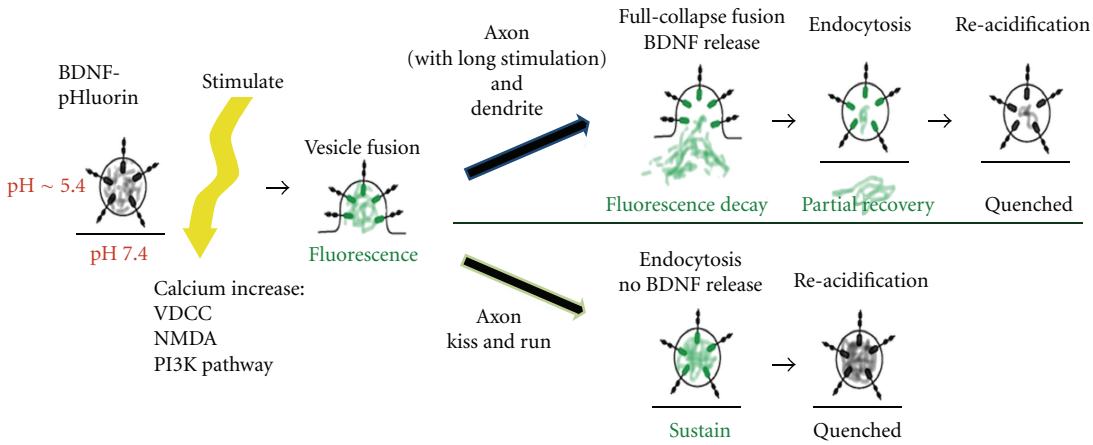

Figure 2: Schematic illustration of how BDNF-pHluorin fluoresces, both inside the vesicle and upon axonal and dendritic vesicular fusion. Before stimulation, BDNF-pHluorin protein shows little fluorescence. If intracellular calcium increase occurs upon electrical stimulation, then BDNF-pHluorin vesicle may fuse, opening up to the pH 7.4 extracellular space, causing a transient spike in fluorescence that can be detected by TIRF microscopy. Different styles of fusion between axon and dendrite are shown, as explained in [10] and in the text. After sustained vesicular fusion as occurs in dendrites, fluorescence will decrease as a result of BDNF-pHluorin diffusion out of vesicles. Kiss-and-run fusion as occurs in stimulated axons will rather show an increase in fluorescence because minimal pHluorin diffuses out of the vesicle. The sticks represent synaptotagmin-4; see [85] for details. Modified from Figure 4(e) in [85].

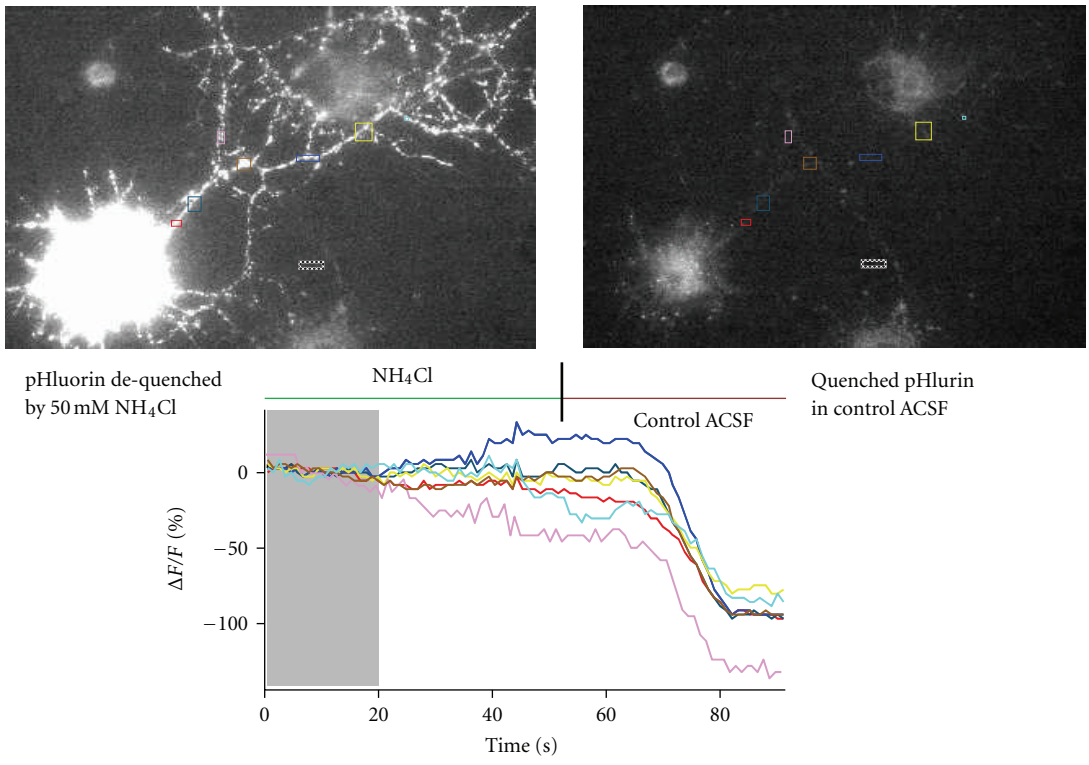

Figure 3: Left panel shows a BDNF-pH expressing hippocampal neuron in the presence of 50 mM NH_4Cl. Right panel shows the same cell 80 sec after standard ACSF solution was applied. The graph shows the time course of the fractional change of BDNF-pH intensity (background-subtracted delta F/F_0) from pixels within the color-coded regions of interest (ROIs) shown in the panels above. The change from NH_4Cl-containing ACSF to control buffer quenches BDNF-pH within acidic secretory granules. Time-lapse was performed in an inverted microscope with a Hg-lamp and a cooled CCD camera. Neurons were imaged with a 60x 1.45 NA oil-immersion objective, exposure times ranged from 50–100 ms, and images were taken at 1 frame per second (fps).

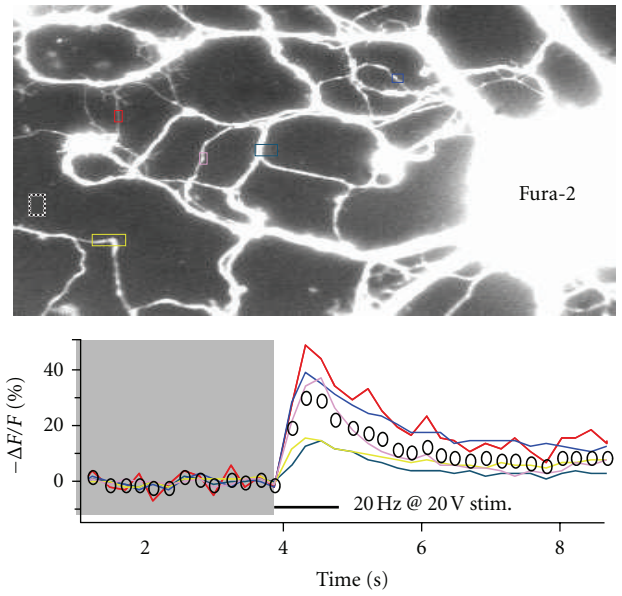

Figure 4: To confirm the viability of neurons transfected with BDNF-pH and their responsiveness to extracellular field stimulation, Ca^{2+} imaging was performed in neurons labeled with fura-2 AM. Upon electrical stimulation through field Pt wires, Ca^{2+} transiently increased in several dendrites. The graph shows the time course of background-subtracted delta F/F_0 of 380 nm-excited fura-2 fluorescence intensity (510 nm emission) from the colored ROIs shown in the image. Images taken at 4 fps.

immunoblotting, and newly developed immunofluorescence assays that do not require tissue homogenization [27] can specifically detect qualitative and quantitative molecular responses to BDNF, complementing the methods reviewed here.

5. Application to Neurogenetic Disorders

5.1. Val66Met BDNF Polymorphism. An estimated 30–50% of people worldwide are homozygous or heterozygous for a single nucleotide polymorphism (SNP) that has only been observed in the human *BDNF* gene [40]. With this SNP, valine is changed to methionine in the 66th residue of the pro region of proBDNF. This mutation has not been shown to affect expression or signaling of mature BDNF, but improper sorting of met-BDNF-GFP from the trans-Golgi network into secretory vesicles dramatically reduced the amount of vesicles in dendritic compartments, leaving much of the met-BDNF-GFP in the perinuclear region rather than in synaptic regions [35, 41]. Using BDNF ELISA with chronic KCl depolarization, it was very evident that the activity-dependent release of met-BDNF was substantially less, but constitutive release of val-BDNF and met-BDNF was nearly identical [35]. Coimmunoprecipitation showed that the protein sortilin binds to the pro region of BDNF and directs it from the trans-Golgi network into the regulated secretory pathway; the association with sortilin was stronger in val-BDNF than it was in met-BDNF, explaining the trafficking defects observed with met-BDNF [14].

Behavioral and fMRI studies of the consequences of the Val66Met BDNF polymorphism were the first to link human behavior and brain morphology with BDNF action [40]. Subjects heterozygous for met-BDNF scored significantly lower on episodic memory tasks, but not a factual recall task [35]. These results emphasize the importance of regulated BDNF release in memory, particularly in the hippocampus, where BDNF mRNA expression is highest [42], and where it promotes the survival and differentiation of newborn neurons [43]. Additional influences of the BDNF polymorphism were seen in fMRI studies, which revealed that hippocampal activation patterns were abnormal during a memory task [35], and hippocampal and dorsolateral prefrontal cortex volumes were ~10% less in met-BDNF carriers [17].

The results from these behavioral and imaging studies illustrate a range of macroscopic effects that can be predicted by studies of molecular activity: less overall BDNF is being released [35] and several lines of evidence suggest that cleavage of met-proBDNF into mature BDNF is impaired compared to val-proBDNF, meaning that LTP and dendritic arborization will be further impaired because of reduced TrkB activation. In colocalization studies using BDNF-YFP (yellow fluorescent protein), it was recently shown that an association between sortilin and proBDNF at the TGN facilitates cleavage of proBDNF by the protease furin [44]. Because met-proBDNF binds less strongly to sortilin than val-proBDNF, it is highly plausible that cleavage of met-proBDNF is less efficient than cleavage of val-proBDNF. Additionally, proBDNF-GFP was shown to be copackaged into dense-core granules of the activity-dependent secretory pathway with tissue plasminogen activator (tPA) and plasminogen—two proteins that interact to make plasmin [45, 46]. This means that met-proBDNF is no longer colocalized to secretory granules with its primary extracellular protease, so that proBDNF may predominate over mBDNF in the extracellular space. Recent evidence corroborates this idea—the extracellular ratio of mBDNF:proBDNF dramatically increased under depolarizing conditions [29]; without proper activity-dependent release, this ratio maybe reduced, and less mBDNF-TrkB signaling may help to explain the deficits in memory and hippocampal volume seen in Met-BDNF carriers. This latter study illustrates how BDNF-eGFP can be useful in methods besides fluorescence microscopy: anti-GFP antibodies were used to pull down BDNF-eGFP and proBDNF-eGFP in cellular media, and western blotting then quantified relative amounts of BDNF-eGFP and proBDNF-eGFP to show that mBDNF is the dominant species released following high-frequency stimulation [29]. The extracellular mBDNF:proBDNF ratio has yet to be directly studied in Val66Met. This would be an important aspect of the disorder to study, given that extracellular cleavage of proBDNF is essential for LTP, and proBDNF preferentially binds the p75 receptor, which has pro-apoptotic cascades [45, 47].

5.2. Neurodevelopmental Disorders Associated with Intellectual Disabilities. There is a well-studied link between the impairment of activity-dependent refinement of synapses and the

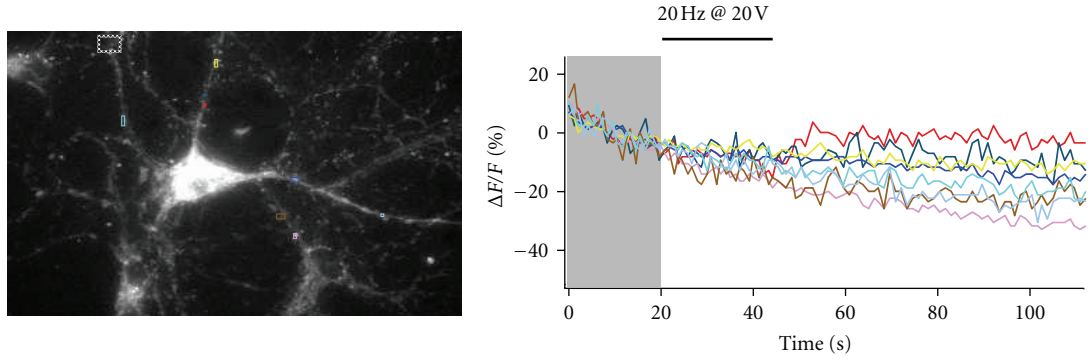

Figure 5: Neuron expressing BDNF-pHluorin and loaded with the Ca^{2+} indicator fura-2 AM (image is of 488 nm-excited BDNF-pHluorin). This cell was sensitive to pH (as in Figure 4) and responded to electrical stimulation with Ca^{2+} transients (using 380 nm excitation, as in Figure 5). Consistent with activity-dependent BDNF release, dendritic BDNF-pHluorin puncta show decreases in intensity (~20% deltaF/F) as a result of BDNF-pHluorin discharge from vesicles following full fusion.

neurodevelopmental disorder Rett syndrome (RTT), a debilitating disorder that affects ~1 : 15,000 females worldwide (reviewed in [34]). Rett syndrome first manifests itself 6–18 months after birth, with common symptoms being difficulty breathing and deterioration of acquired motor, language, and social skills [48]. Origins of the disorder have been confirmed to be mutations that yield loss of function in *MECP2*, a gene on the X chromosome that encodes for the transcriptional regulator protein MeCP2. MeCP2 binds to A/T-rich sites near methylated CpG islands and can recruit transcriptional repressor and/or activator proteins that can then modify chromatin (reviewed in [49]). Interestingly, one of the many genes regulated by MeCP2 is the *Bdnf* gene. *BDNF* mRNA levels are lower in autopsy brain samples from RTT individuals [50] and BDNF protein levels are decreased in *Mecp2*-deficient mouse models, with more severe decreases in BDNF are associated to more severe RTT-like symptoms [51]. The mechanisms underlying MeCP2 control of BDNF have long been debated, but recent evidence shows that MeCP2 acts to repress transcription of multiple microRNAs that bind to the 3′UTR region of BDNF mRNA and subsequently reduce BDNF protein levels, as measured by ELISA [52]. This finding may explain how loss of MeCP2 function leads to impaired BDNF expression.

The influence of BDNF on RTT is widespread. Autopsies showed that, in hippocampus [53] and neocortex [54] of RTT patients, there was a pronounced decrease in dendritic growth and spine density (but see [55]). This is possibly explained by the reduced expression and phosphorylation of microtubule-associated protein 2 [54], processes which are both modulated by BDNF [56]. Increased expression of BDNF, measured by Western blots [57], BDNF-GFP [31], or ELISA [58], caused improvements in synaptic function, dendrite length, and respiratory function in Rett syndrome mouse models. RTT patients with the Val66Met BDNF polymorphism showed more severe symptoms and an increase in the risk of seizure onset, suggesting that BDNF trafficking and secretion are altered in RTT, not just BDNF expression levels [59]. This idea gains credence with ELISA *in situ* evidence that the total amount of activity-dependent BDNF release is equal to wildtype levels in the *Mecp2*-/y mouse model, even when BDNF expression was less than half wildtype levels and constitutive secretion was less than wildtype levels [16]. Thus, a larger readily releasable pool (RRP) of BDNF in RTT was suggested, but BDNF ELISA cannot confirm this; BDNF-eGFP should be used to follow up this investigation because it can reveal the relative quantities of BDNF vesicles that localize to neurites and therefore can help to uncover an altered RRP of BDNF vesicles in RTT.

Dendritic spine morphology is regulated by activity-dependent BDNF release and synthesis [60]. Abnormal spine morphologies have long been associated with genetic neurodevelopmental disorders, particularly RTT and Fragile X syndrome (FXS) [61, 62]. Postmortem studies of RTT patients showed reduced dendritic spine density in the hippocampus, with a greater ratio of thin/mature spines than in wildtype hippocampus [63]. To aid in future investigations, induced pluripotent stem cells (iPSCs) were recently generated from RTT patient fibroblasts and were differentiated into neurons that recapitulated many characteristics of neurons in RTT patients, namely, reduced spine density [63]. Mature neurons and proliferative neural precursor cells were generated in the process, opening the exciting possibility of sustaining cell lines that recapitulate the RTT phenotype [63]. iPSCs derived from patients afflicted with a neurogenetic disorder open an exciting new avenue in neuroscience research, which thus far has been seldom utilized in studying the involvement of BDNF in neuropathologies.

In Fragile X syndrome, an inherited autism-spectrum disorder associated with intellectual disability that affects 1 : 4000 males and half as many females, mutations in the *FMR1* gene cause a functional absence of Fragile X mental retardation protein (FMRP), a protein that reduces translation by recruiting 4E-BP proteins to the 5′ end of mRNAs [64, 65]. Importantly, FMRP can also work to increase translation by transporting mRNA, such as Rab3a and BDNF mRNA to neurites [65, 66]. Indeed, levels of Rab3a protein, which is important in activity-dependent dense core vesicle docking and fusion at the

pre-synaptic terminal, were substantially reduced in tissues from *Fmr1* knockout mice, causing dysfunctional release of neuropeptide vesicles [65]. This finding might provide an explanation as to why hippocampal neurons from *Fmr1* knockout mice show impaired LTP that can be restored by application of recombinant BDNF, even though levels of proBDNF and mBDNF were not significantly different between wildtype and *Fmr1* knockout mice [67], and BDNF was in fact increased in the hippocampus of *Fmr1* knockout mice in another study, as measured by BDNF ELISA [66]. Considering that the levels of BDNF are not negatively affected by loss of FMRP function, but dense core vesicle docking is affected, it is very plausible that BDNF vesicle docking and fusion is affected in FXS, contributing to the observed deficits in LTP and learning [68]. Additionally, one study has linked BDNF trafficking with FXS symptoms, showing an increased propensity for epilepsy in FXS-afflicted men who have the improperly sorted met-BDNF allele [69]. Future studies utilizing BDNF-eGFP or BDNF-pHluorin in neurons from FXS models could elucidate the ways in which BDNF trafficking and vesicle fusion are affected in FXS, providing clues as to why LTP and dendritic spine maturation are impaired in FXS.

There is also evidence that BDNF is upstream of FMRP activity: BDNF, but not neurotrophin-3 application, and overexpression of TrkB was shown to slightly reduce the expression of the *Fmr1* gene, implicating BDNF-TrkB signaling in the negative regulation of FMRP levels [70]; BDNF also caused dissociation of the FMRP translational repression complex, allowing translation to occur specifically in dendritic compartments [64]. The impact of abnormal communication between BDNF and FMRP that is expected to occur in FXS has not been extensively studied (see [66]), and deserves further investigation.

5.3. Huntington's Disease. In contrast to the neuropathologies discussed thus far, Huntington's disease (HD) is a neurodegenerative disorder with symptoms usually appearing in middle age [71]. Its most characteristic symptoms are related to loss of motor function as a result of basal ganglia degeneration, but often before motor symptoms become manifest there are cognitive deficits in verbal learning and working memory, tasks associated with hippocampal and prefrontal areas [72]. HD is a rare inherited disorder that affects 5–10 in 100,000 Caucasians, with fewer reported cases in Japanese populations [73]. HD is associated with 36 or greater CAG repeats in the *huntingtin* gene, producing an abnormal huntingtin protein (htt) with an extended tract of glutamine that is encoded by the CAG repeats. The severity of the disease is proportional to the number of CAG repeats, as the polyglutamine tail causes self-association of htt mutants that can lead to htt aggregates [73]. Unlike those seen in Alzheimer's disease, these mutant htt aggregates do not have intrinsic toxicity, but vesicle trafficking and endocytic recycling are severely impaired in mutant htt, leading to dendritic spine degeneration and eventual cell death ([74, 75], but see [76]).

BDNF-eGFP vesicles colocalized with wildtype and mutant htt, but only wildtype htt was shown to increase velocity and efficiency of BDNF transport to synapses. This effect appeared specific for BDNF vesicles, as mitochondrial transport was unaffected [33]. htt-mediated axonal transport of BDNF requires Huntington-associated protein 1 (HAP1); HAP1 associates with the pro domain of BDNF-eGFP, and this association is reduced with mutant htt and with met-BDNF [15], likely because of impaired complexation with sortilin [44]. This finding suggests impaired activity-dependent release and axonal transport, but normal constitutive release, in HD as a result of improper proBDNF-HAP1-htt interactions. The importance of this finding is apparent when considering that the striatum, the region defined by neurodegeneration in HD, contains very low levels of BDNF mRNA [77] but still requires BDNF protein for survival of striatal inhibitory neurons in mouse HD models [20, 78]. This means that the striatum requires anterograde corticostriatal transport of BDNF in order to avoid striatal degeneration [77–79]. Thus, as revealed by BDNF-GFP, the abnormal anterograde trafficking of BDNF by htt and HAP1 is a major component of striatal neuron degeneration in HD.

Transcription of the *Bdnf* gene is also affected in HD. When wildtype htt was overexpressed, BDNF ELISA showed higher levels of BDNF, and the opposite effect was seen with mutant htt. This is a result of wildtype htt releasing the transcriptional repressor REST/NRSF from a 23 bp DNA sequence called RE1/NRSE that was discovered in one particular *Bdnf* exon. Mutant htt was unable to derepress transcription of the *Bdnf* gene, explaining the reduced BDNF levels seen in HD brains (reviewed in [80]). Other studies corroborate these data, finding that cortical levels of BDNF mRNA negatively correlate with the progression of HD in a mouse model [81], and that overexpression of forebrain BDNF results in increased levels of striatal BDNF, and significant reduction in HD phenotype mouse model [26]. With so many facets of HD mediated by BDNF, there is a clear potential for pharmacological applications: upregulation of BDNF by activity-inducing ampakines restored synaptic plasticity and memory in hippocampus of an HD mouse model by stabilizing actin polymerization in dendritic spines, a process severely impaired in HD [82]. BDNF-GFP trafficking studies, and BDNF ELISA studies of protein levels have proven invaluable thus far in elucidating the role of BDNF in HD. However, the quantal release of BDNF in HD has yet to be visualized, an application well suited for BDNF-pHluorin.

6. Conclusion

BDNF is a neuropeptide with a diverse array of functions that modulate neuronal function during early brain development and throughout life. The dynamics of its intracellular transport and activity-dependent regulated release are complex and require sophisticated approaches that are currently in development. To start, methods to study BDNF transport and release must be dynamic in order to properly follow its

behavior in live cells. Thanks to the development of BDNF-GFP and BDNF-pHluorin, we are beginning to understand the ways in which BDNF contributes to neuronal function in health and disease. Therapies are currently being developed that will aid in restoring BDNF abnormalities to normal, thereby slowing the progression of diseases like Huntington's and Rett syndrome. Future studies can use the tools discussed in this review to test the efficacy of experimental compounds on BDNF transport and release, using the restoration of proper BDNF dynamics as a preclinical endpoint. Hopefully, these studies will yield novel therapies for the wide range of neurogenetic disorders associated with BDNF dysfunction.

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Review Article

Therapy Development for Spinal Muscular Atrophy in SMN Independent Targets

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Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disorder, leading to progressive muscle weakness, atrophy, and sometimes premature death. SMA is caused by mutation or deletion of the *survival motor neuron*-1 (*SMN1*) gene. An effective treatment does not presently exist. Since the severity of the SMA phenotype is inversely correlated with expression levels of SMN, the *SMN*-encoded protein, SMN is the most important therapeutic target for development of an effective treatment for SMA. In recent years, numerous SMN independent targets and therapeutic strategies have been demonstrated to have potential roles in SMA treatment. For example, some neurotrophic, antiapoptotic, and myotrophic factors are able to promote survival of motor neurons or improve muscle strength shown in SMA mouse models or clinical trials. Plastin-3, cpg15, and a Rho-kinase inhibitor regulate axonal dynamics and might reduce the influences of SMN depletion in disarrangement of neuromuscular junction. Stem cell transplantation in SMA model mice resulted in improvement of motor behaviors and extension of survival, likely from trophic support. Although most therapies are still under investigation, these nonclassical treatments might provide an adjunctive method for future SMA therapy.

1. Introduction

Spinal muscular atrophy (SMA) is characterized by motor neuron degeneration with muscular atrophy, paralysis, and an attenuated lifespan [1]. The disease is the leading genetic cause of infantile mortality [2]. SMA exhibits an autosomal recessive pattern of inheritance with an incidence of 1 in 6,000–10,000 newborns and a carrier frequency of about 1 : 35 [2, 3]. Based on age of onset and achievement of motor milestones, SMA has been subdivided into four clinical types: severe (type I; Werdnig-Hoffmann disease), intermediate (type II), mild (type III; Kugelberg-Welander disease), and adult forms [4]. Most SMA patients harbor deletions, mutations, or conversions of the telomeric copy of the *survival motor neuron* gene (*SMN1*) [5, 6]. The centromeric *SMN* gene (*SMN2*) is present in all SMA patients, but is unable to compensate for the *SMN1* gene defect as the primary transcript of *SMN2* gene is defectively spliced [5, 6]. Currently, there are no curative therapies for SMA. Since there is an inverted correlation between the amount of SMN protein and disease severity [7, 8], SMN has been the most important therapeutic target for development of SMA treatment [9, 10]. However, some SMN independent targets and therapeutic strategies have been demonstrated to have the potential to benefit SMA [11–20]. Although most are still under investigation, these nonclassical therapies might provide an adjunctive method for future SMA therapy.

2. Disease Mechanisms

Although pathogenesis of SMA has been investigated extensively, some of the detailed disease mechanisms are still not fully understood. Figure 1 showed the genetics in SMA. The SMN is a 38-kDa protein expressed in both the cytoplasm and nucleus of all cells [21]. SMN serves as a chaperone in the assembly of spliceosome precursors by combining small nuclear RNA (snRNA) molecules with Sm proteins

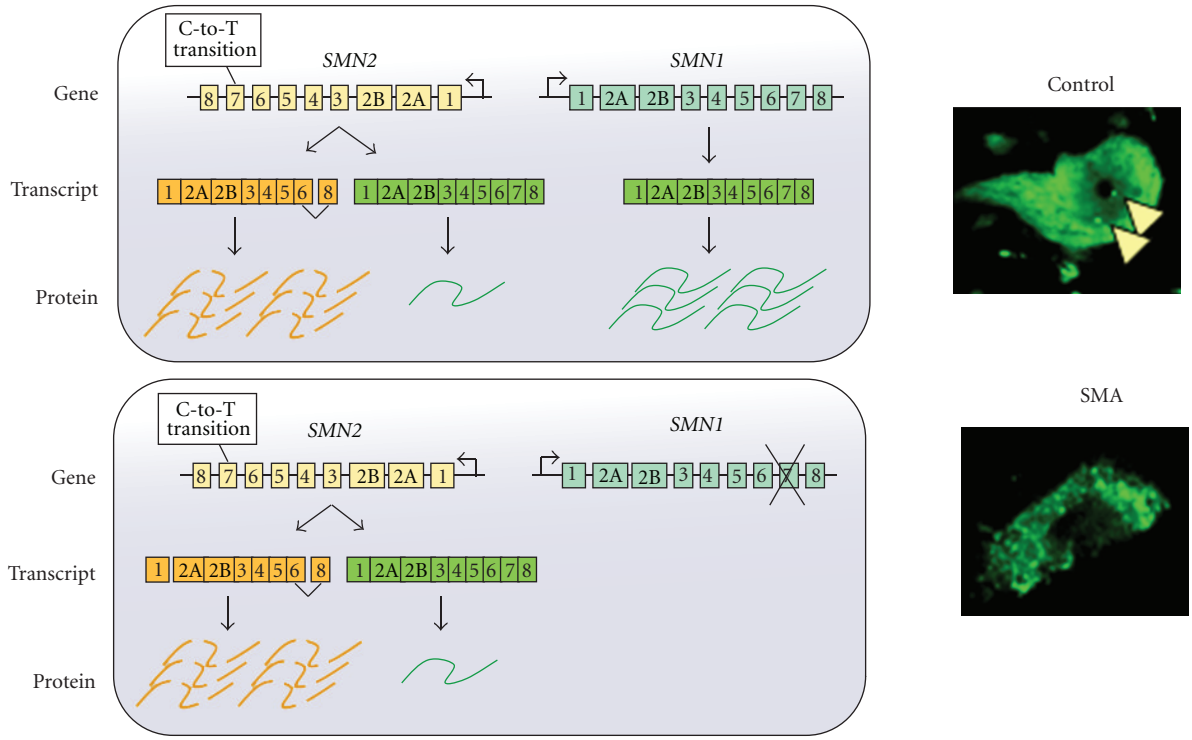

Figure 1: Schematic diagram of the *SMN1* and *SMN2* genes. Humans are the only species that carry both *SMN1* and *SMN2* genes, located in the human 5q11.2–13.3 region [5, 168]. The *SMN1* and *SMN2* genes differ by five nucleotide exchanges [6]. Among them, a translationally silent cytosine to thymidine exchange at position 6 of exon 7 is responsible for the skipping of exon 7 during splicing of the *SMN2* gene [6]. The C-to-T transition abolishes an exonic splice enhancer site and generates a new exonic splicing silencer domain for the last coding exon [169, 170]. Subsequently, through alternative splicing, most of the translating SMN protein from the *SMN2* gene lacks the C-terminal residue and becomes less stable and relatively inactive [171]. In normal situation, abundant SMN protein is produced mainly from *SMN1* gene with a little amount from *SMN2* gene. The spinal motor neuron from a wild-type mouse thus expresses a high level of SMN in both cytoplasm and nucleus with several gems (arrow head) as compared to that in an SMA mouse. With homozygous mutation of the *SMN1* genes, all SMA patients still have at least one *SMN2* gene copy [6]. While complete loss of SMN expression is embryonically lethal [172], the small amount of full-length SMN protein produced by the *SMN2* gene (about 20%) prevents lethality in SMA patients, but has insufficient SMN levels to assist in recovery from spinal motor neuron death [28].

to generate small nuclear ribonucleoproteins (snRNPs) [22, 23]. The snRNP assembly activity is dramatically reduced in spinal cord from SMA model mice and the degree of snRNP assembly impairment correlates with disease severity [24]. Therefore, SMN plays a critical role in pre-mRNA splicing. Evidence shows that SMN is also involved in the stabilization and maturation of the neuromuscular junction and the transportation of axonal mRNAs in motor neurons [25–27]. SMN-deficient motor neurons exhibit severe defects in clustering voltage-gated calcium channels in axonal growth cones [26]. An alteration of calcium channel distribution might influence neurotransmitter release, causing dysfunction and immaturation of neuromuscular junction [25, 28]. In addition, the SMN protein can form granules that are transported and associated with β-actin mRNA in neuronal processes [29]. The close relationship of SMN and β-actin has further demonstrated that motor neurons derived from SMA model mice have shortened axons and small growth cones, which are also deficient in β-actin mRNA and protein [30]. Therefore, SMN has a function in maintaining proper neuronal machinery via assistance in splicing process and establishing adequate communication between the muscles and nerves at the motor end plate through stabilization of the neuromuscular junction. The loss of maintenance and communication might thus trigger the cascade of events that probably results in motor neuron death.

SMA mouse models have been generated through mouse *Smn* knockout and human *SMN2* transgenic methods [8, 31]. These mice reveal spinal motor neuron degeneration, muscle atrophy, and impaired motor performances similar to SMA patients. The disease severity of these SMA mice is also inversely correlated to the copy number of the *SMN2* transgenes [8, 31]. These findings confirm that SMA is directly caused by SMN deficiency. Denervation of neuromuscular junction precedes spinal motor neuron loss in SMA mice [25]. Neuromuscular junction can form and function normally prior to the postnatal onset of disease [32]. Afterward, abnormal neurofilament accumulation and functional disruption at the neuromuscular junction become evident [25]. Alongside these morphological and functional changes at the neuromuscular junction, studies on the spinal cord of SMA mice showed an apparent failure of expression of genes that cluster in postnatal developmental pathways [33]. Subsequently, through still unknown mechanisms,

motor neurons degenerate in spinal anterior horn regions probably through cell apoptosis [16, 34], and muscle atrophy and motor dysfunction become apparent.

Recently, congenital heart defects have been recognized as additional important phenotypes especially in type I SMA patients, including atrial septal defects, dilated right ventricle, and ventricular septal defects [35]. The histological studies in SMA model mice also showed that cardiac remodeling starts at the embryonic stage in the severe SMA mice while motor neurons are not yet visibly affected at this stage. After birth, there is progressive cardiac fibrosis, which may result from oxidative stress [36]. SMA mice also suffer from severe bradyarrhythmia characterized by progressive heart block and impaired ventricular depolarization, which may be related to defective sympathetic innervation [37]. Notably, systemic restoration of SMN expression is able to diminish the cardiac defects accompanied with prolonged lifespan, implying that cardiac abnormalities are playing a critical role on SMA pathogenesis [38, 39].

3. SMN Dependent Therapy

Since SMN levels generally correlate with disease severity in SMA patients and mouse models [7, 8, 31, 40], SMN is the best therapeutic target for development of SMA treatment. Various strategies to increase the SMN levels have been tested in SMA mouse models and some of them have even showed promising beneficial effects [9, 10]. Until now, none of them have been demonstrated to be consistently robust or produce continual benefits in SMA patients. These therapeutic strategies are divided into small molecules, antisense oligonucleotides (ASO), and viral vector-mediated gene therapy.

All SMA patients have at least one copy of the *SMN2* gene, providing an opportunity for manipulation of the *SMN2* gene expression [6]. The mode-of-action for a potential SMA therapy using small molecules mainly includes restoration of the *SMN2* splicing pattern, activating the *SMN2* promoter, and extending the half-life of SMN mRNA or protein [10]. The potential drugs include histone deacetylase (HDAC) inhibitors such as sodium butyrate [41], phenylbutyrate [42], valproic acid (VPA) [43], trichostatin A [44], SAHA [45], and LBH589 [46], as well as hydroxyuria [47], sodium vanadate [48], aclarubicin [49], indoprofen [50], bortezomib [51], and aminoglycosides, such as tobramycin, amikacin [52], TC007 [53], and G418 [54]. Since there are still no drugs that have shown consistent benefits in clinical trials [55, 56], finding an effective treatment with distinct therapeutic mechanisms, such as SMN independent targets, is necessary for future SMA therapy.

Among these small molecules, VPA is the drug being studied most extensively and has been used in patients with epilepsy and bipolar disorders for decades [57]. VPA treatment increased levels of SMN transcripts and protein in fibroblasts derived from SMA patients through upregulation of serine/arginine-rich (SR) proteins, which are involved in regulating *SMN2* exon 7 recruitment [58, 59]. Autophagy, the degradation of cytosolic components in lysosomes, maintains neuronal homeostasis; its dysfunction has been linked to various neurodegenerative diseases, possibly including SMA [60]. VPA is also an autophagy enhancer, which activated autophagic pathways and attenuated rotenone-induced toxicity in SH-SY5Y cells [61]. In addition, VPA upregulates some antiapoptotic factors such as Bcl-2 and Bcl-xl, perhaps via activation of ERK44/42 [43, 62, 63]. Probably through multiple therapeutic effects, VPA reduced motor neuron degeneration, muscle atrophy, and motor dysfunction in SMA mice [43, 64], and a small group of SMA patients showed obvious improvement in muscle strength after daily VPA treatment [65, 66]. Despite these encouraging results, large clinical trials did not confirm the beneficial effects of VPA in SMA patients [67–69]. These disappointing outcomes may contribute to different pharmacokinetics and bioavailability between rodents and humans as well as dose-limiting intolerance and drug adverse effects [9]. In addition, the responses of VPA treatment showed intrapatient and interpatient variability in the study using fibroblasts and lymphoblasts from SMA patients [70], probably indicating that tissue and individual factors may affect the VPA effects with unknown reasons.

Using ASO to inhibit the splicing silencer for *SMN2* exon 7 leads to restoration of the normal *SMN2* splicing pattern [71]. The effects of ASO were further improved through the incorporation of a binding platform with ASO for recruitment of SR protein to the *SMN2* exon 7 region [71]. These bifunctional ASOs were able to achieve nearly 100% exon 7 inclusion and enhance SMN expression up to 2- to 3-fold in cell-based assays [72]. Injection of ASO into cerebroventricles elicited a robust induction of SMN protein in the brain and throughout the spinal cord and extended the lifespan of SMA mice [73]. A recent study demonstrated that systemic delivery of ASO resulted in dramatic prolongation of lifespan in SMA mice and the effects were much better than those with intracerebroventricular delivery of ASO (median survival, 108 versus 16 days) [39]. These findings suggest that ASO therapy has great potential in this field and extra-CNS targeting is required to rescue the SMA phenotype. However, another similar study showed different results that early intracerebroventricular delivery of ASO had a better outcome than intravenous ASO delivery [74], which suggests that therapeutic methods for ASO treatment still need further investigation and optimization.

Direct injection of adeno-associated viral vector serotype 8 (AAV8) carrying *SMN* into both cerebroventricles and upper lumbar spinal cord of SMA mice showed a robust increase in lifespan by 880% with less motor neuron degeneration and abnormal architectures of neuromuscular junction [75]. However, augmented SMN is expressed in thoracolumbar regions, but sparse in the cervical cord, which may suggest poor diffusion of AAV in subarachnoid space. In contrast, intravenous AAV serotype 9 (AAV9) injection has shown success in affecting widespread gene delivery in entire spinal cord [76]. Intravenous injection of AAV9 carrying human codon-optimized *SMN1* at postnatal day 1 recovered most motor function, neuromuscular physiology, and lifespan in SMA mice [77]. Notably, postnatal day

1 treatment resulted in the maximal transduction of the motor neurons, while postnatal day 10 treatment led to glia-predominant transduction [77]. This shift in cell type specificity was probably because of the closure of the blood brain barrier that occurs within the first week of life in neonatal mice [78]. When the blood brain barrier is mature and patent, virions are probably not able to penetrate out of vessels smoothly to access motor neurons, but only encounter the endothelial wrappings of astrocyte end feet. Since blood brain barrier likely matures in as early as human neonatal period [79], the AAV9 transduction efficacy should further be tested in nonhuman primates of different ages to identify the optimal temporal window for future therapy.

4. SMN Independent Targets and Treatment

4.1. Neuroprotection, Antiapoptosis, and Myotrophic Effects

4.1.1. Insulin-Like Growth Factor-1. Insulin-like growth factor-1 (IGF-1) is a trophic factor mainly secreted by the liver and circulates at high levels in the bloodstream. IGF-1 is a key molecule involved in normal brain growth and function [80] and may have a neuroprotective effect by inhibiting neuronal death in Huntington's disease and spinocerebellar ataxia [81, 82]. IGF-1 also enhances axonal outgrowth of corticospinal motor neurons [83]. *Igf1*-null mice show some phenotypic similarity to SMA mice, such as small size and generalized muscle dystrophy, with most of them dying at birth [84]. Notably, serum IGF-1 level was decreased in SMA mice, and systemic increase of SMN expression using the ASO strategy in SMA mice was accompanied with restoration of serum IGF-1 to normal levels [39]. Interestingly, mRNA levels of IGF-binding protein, acid labile subunit (IGFALS), but not IGF-1, was reduced in SMA mice. IGFALS binds to IGF-1 and IGF-binding protein 3 to form a stable ternary complex, extending the half-life of IGF-1 from 10 minutes to 12 hours [85]. Therefore, the low serum IGF-1 level in SMA mice is likely related to downregulation of IGFALS, and IGF-1 may be one of the factors that contribute to the pathogenesis of SMA [39].

IGF-1 treatment has been shown to improve disease phenotypes in rodent models of motor neuron diseases such as amyotrophic lateral sclerosis (ALS) [86] and spinal and bulbar muscular atrophy (SBMA) [87]. For SMA, transgenic expression of IGF-1 in skeletal muscle of SMA mice resulted in an increase in myofiber size and a modest improvement in median survival [11]. Delivery of a plasmid DNA vector encoding IGF-1 by intracerebroventricular injection into newborn SMA mice also increased body mass and provided a modest improvement in median survival [12]. However, intracerebellar viral delivery of IGF-1 reduced motor neuron degeneration, but did not improve motor function in the mildly affected SMA mice [88]. Therefore, the effects of IGF-1 and IGFALS-related therapy using different treatment strategies in SMA still require further investigation.

4.1.2. Ciliary Neurotrophic Factor. Schwann cells close to neuromuscular endplates play a major role in triggering terminal sprouting [89]. These cells express ciliary neurotrophic factor (CNTF), and lack of CNTF expression strongly reduces terminal sprouting and motor unit size [13]. In a mouse model of ALS, the depletion of synaptic vesicles precedes the loss of synapses; CNTF could prevent the depletion of synaptic vesicles and thus maintain function of neuromuscular junctions [90]. CNTF treatment using CNTF-secreting stem cells or by local CNTF injection into skeletal muscle led to better maintenance of peripheral motor axons in a mouse mutant, progressive motor neuronopathy (*pmn*) [91, 92].

In a severe type of SMA mice, the sprouting and enlargement of motor units do not normally occur. In contrast, the architecture and function of neuromuscular junctions in heterozygous *Smn* (+/−) mice are relatively preserved, despite some loss of spinal motor neurons [13]. However, completed knockout of CNTF in heterozygous *Smn* (+/−) mice reduces the sprouting responses of the nerve terminals accompanied with reduced muscle strength [13]. These results imply that CNTF may be able to compensate loss of motor neurons by sprouting from remaining motor axon terminals so that neuromuscular endplates remain innervated; CNTF may thus guide the way for new therapies for SMA. Although systemic CNTF treatment elicited severe adverse effects including fever and cachexia in ALS patients [93], muscle or CNS targeting CNTF therapy might offer a chance to reduce these side effects and show benefits in SMA.

4.1.3. Cardiotrophin-1. CNTF and Cardiotrophin-1 (CT-1) are both members of the IL-6 family, which bind a common receptor complex requiring leukemia inhibitory factor receptor (LIFR) and gp130 [94]. CT-1, an important cardioprotective cytokine, also has beneficial effects in neuromuscular systems [95]. CT-1 is essential in normal motor neuron development and is also able to support long-term survival of motor neurons as demonstrated in culture cells and rats with axotomy [96]. In addition, overexpression of CT-1 in *pmn* and ALS mice both significantly delayed disease onset, reduced degeneration of motor neurons and axons, and preserved the terminal innervation of skeletal muscles [97, 98]. For SMA mice, intramuscular injection of adenoviral vector expressing CT-1, even at very low doses, prolonged survival, delayed motor defects, and diminished motor axonal degeneration and aberrant synaptic terminals [14]. Although most of studies regarding CT-1 are focused on diseases in the cardiovascular system, CT-1 might still be a valuable therapeutic agent for motor neuron diseases through neurotrophic effects.

4.1.4. Bcl-xL and Bax. Degeneration of spinal motor neurons in SMA is mediated in part through apoptosis [16, 34]. In the Bcl-2 family, Bcl-xL and Bax are important regulators of cell death in the nervous system when cells have matured. Bcl-xL is an antiapoptotic member of the Bcl-2 family and acts by inhibiting proapoptotic members of the Bcl-2 family through heterodimerization [99]. Bcl-xL was downregulated in SMA patients and model mice [17, 100]. Bcl-xL overexpression can protect against motor neuron death in cultured primary

motor neurons [101] and embryonic motor neurons with *SMN* knockdown [102]. Interestingly, Bcl-xL overexpression in SMA mice reduced motor neuron degeneration, preserved motor function, and prolonged lifespan without changes in SMN expression levels [17]. In addition, Bax protein is a major proapoptotic member of the Bcl-2 family. *Bax* knockout SMA mice had milder disease severity and longer lifespan with less spinal neuronal degeneration than SMA littermates with wild-type *Bax* genes [16]. Therefore, effects of Bcl-xL and Bax may not be simply through apoptotic pathways, but through unknown mechanisms to salvage neural function in SMA. The ratio of Bcl-xL/Bax is thus another attractive target, where the potential to increase Bcl-xL and decrease Bax expression may be of benefit to SMA patients.

4.1.5. Riluzole. Riluzole, a 2-aminobenzothiazole, is the only disease-modifying therapy available for ALS [103]. Although riluzole is known to modulate excitatory neurotransmission mainly through inhibition of glutamate release, the precise neuroprotective mechanisms remain largely speculative [104]. In SMA mice, riluzole improved median survival and reduced aberrant cytoskeletal organization of motor synaptic terminals [105]. However, a small phase I clinical trial, enrolling 7 riluzole-treated and 3 placebo-treated type I SMA infants, demonstrated no significant differences in survival and the change in motor abilities after riluzole treatment [106]. Nevertheless, further analysis showed that 3 patients in the riluzole group presented an unusual disease course and were still alive at the age of 30 to 64 months. The pharmacokinetics of riluzole in SMA patients has recently been investigated [107], and the long-term benefits of riluzole still warrant large clinical trials for SMA patients.

4.1.6. Gabapentin. Gabapentin is a GABA analogue and has been used clinically for patients with seizures and neuropathic pain for more than 10 years [108]. Gabapentin could also have a neuroprotective action in part by reducing the pool of releasable glutamate in neurons, thereby diminishing the excitotoxicity potential [109, 110]. Although gabapentin treatment showed marginal reduction in disease progression in a phase II clinical trial for ALS patients [111], the following phase III clinical trial did not reveal significant benefits after gabapentin treatment for 9 months [112]. For SMA, the first clinical trial of gabapentin enrolled 84 type II and III SMA patients. There was no difference between the gabapentin and placebo groups in any outcome measure including changes in muscle strength, pulmonary function, or motor functional rating scale after 12 months of treatment [113]. However, another clinical trial which enrolled 120 type II and III SMA patients showed a significant improvement in muscle strength of legs at both 6 and 12 months after gabapentin treatment [114]. Meta-analysis of these two trials did not successfully demonstrate the beneficial effects of gabapentin in SMA [56].

4.1.7. β-Adrenergic Agonist. β2-Adrenergic agonist, such as salbutamol (albuterol in the United States), enhanced muscle strength in aged rats [115], human healthy volunteers [116], and some pathological conditions [117, 118]. In a pilot clinical trial, thirteen type II or III SMA patients receiving salbutamol for 6 months showed significant increase in myometry, forced vital capacity, and lean body mass [119]. A further larger trial enrolling 23 type II SMA patients consistently got similar results that functional scores were better after daily salbutamol treatment for 6 or 12 months [120]. Notably, the drug did not produce any major side effects [119, 120]. The mechanism of action of β2-adrenergic agonists on human skeletal muscles to enhance muscle strength is not completely understood. Interestingly, salbutamol also promoted exon 7 inclusion in *SMN2* transcripts and thus increased levels of full-length transcripts of *SMN2* in SMA fibroblasts [121]. In SMA patients, daily salbutamol significantly and consistently increased *SMN2* full-length transcript levels in peripheral leukocytes, and the response was directly proportional to *SMN2* gene copy number [122]. Considering bifunctional therapeutic effects and safety of salbutamol, large randomized double-blinded placebo-controlled clinical trials are mandatory.

4.1.8. Follistatin. Myostatin is a member of the TGF-β family and functions as a potent negative regulator of muscle growth [123]. Inhibition of myostatin increases muscle mass and strength in wild-type rodents and improves the pathophysiology of a mouse model for muscular dystrophy [124, 125]. Follistatin is a cystine-rich glycoprotein, which binds to and inhibits several TGF-β family members, including myostatin [126]. Follistatin delivered by intramuscular injection of recombinant viral vectors increased muscle mass in mouse models of both ALS and Duchenne muscular dystrophy [127, 128]. Since SMA also features diffuse muscle atrophy, inhibition of myostatin may also be a therapeutic strategy. Intraperitoneal injection of recombinant follistatin in SMA model mice increased muscle mass, improved motor function, and prolonged lifespan by 30% without changes in SMN protein levels in spinal cord and muscles [15]. However, other studies detected no phenotypic alteration in transgenic overexpression of follistatin or ablation of myostatin in SMA mice [129, 130]. The reason for this discrepancy is unclear and the effects of follistatin for SMA treatment still need further validation.

4.2. Axonal Dynamics

4.2.1. Plastin-3. Although SMA-affected siblings usually develop similar disease severity in terms of their age at onset and the progression of disease [131], a small proportion of individuals with homozygous *SMN1* mutation are fully asymptomatic despite carrying an identical number of *SMN2* copies as their affected siblings, suggesting the influence of modifier genes [132, 133]. The first potential SMN-independent disease modifier, plastin-3, was recently identified from six SMA-discordant families with eight fully asymptomatic females who had inherited the same *SMN1* and *SMN2* alleles as their affected siblings [18]. Increased

levels of plastin-3 were also found to correlate with a mild SMA phenotype in female patients, independently of SMN protein levels [18, 134].

Plastin-3, an actin binding protein, is a regulator of actin filament organization and is expressed in almost all solid tissues, including the human brain, spinal cord, and muscles [18]. Plastin-3 colocalizes with SMN in granules throughout motor neuron axons, and plastin-3 protein levels are reduced in brain and spinal cord of an SMA mouse model [18, 135]. In SMN-depleted neuronal PC12 cells and primary mouse motor neuron cultures derived from SMA mice, plastin-3 overexpression was able to recover from axon outgrowth defects [18]. Notably, overexpression of plastin-3 or its orthologues also led to diminishment of axon defects and disease severity in SMN depleted zebrafish embryos, *Drosophila*, and *C. elegans* [18, 136]. SMN has been shown to moderate and restrict the negative function of profilin IIa on actin polymerization [137]. Profilin IIa is another actin binding protein, and knockdown of profilin IIa results in stimulation of neurite outgrowth, while overexpression of profilin IIa reduces neurite number and size [138]. Knockout of profilin IIa in SMA model mice was able to restore abnormal low plastin-3 levels. However, the phenotype of these SMA mice was not ameliorated despite the depletion of profilin IIa and restoration of plastin-3 levels, which suggests that other components of actin dynamics are also critically affected in SMA [135]. Although some questions need to be answered, such as the mechanisms behind plastin-3 in SMA and effects of plastin-3 upregulation in SMA mouse models, plastin-3 may become an important SMN-independent therapeutic target for SMA in the future.

4.2.2. Cpg15. The candidate plasticity-related gene 15 (cpg15) is highly expressed in the developing ventral spinal cord and can promote motor axon branching and neuromuscular synapse formation [139, 140]. Cpg15 mRNA colocalizes with SMN protein in axons and is locally translated in growth cones [141]. HuD is a neuron-specific RNA-binding protein and also an interacting partner of SMN [141–143]. Cpg15 may be an mRNA target for the SMN-HuD complex and SMN deficiency reduced *cpg15* mRNA levels in neurons [141]. Most importantly, cpg15 overexpression partially recovered from motor axonal deficits in zebrafish with SMN deficiency [141]. Therefore, cpg15 appears to be a crucial downstream effecter of SMN in neurons and may serve as a modifier of SMA disease by regulating axon extension and axon terminal differentiation.

4.2.3. Rho-Kinase Inhibitor. Rho-kinase signaling is a major regulatory pathway of actin dynamics, and Rho-kinase activation is associated with dendritic simplification, and reduced spine length and density [144]. Rho-kinase activity is upregulated in SMN-depleted PC12 cells and SMA model mice [145, 146]. The migratory capacity of the U87MG astroglioma cells was attenuated by knockdown of SMN through abnormal activation of Rho-kinase pathway [147]. Normally, SMN binds to profilin IIa to form complexes, and Rho-kinase may phosphorylate profilin IIa [148]. Through competition between SMN and Rho-kinase for binding to profilin IIa, SMN deficiency results in a decrease in SMN-profilin IIa complexes and stronger interaction of profilin IIa with Rho-kinase [148]. Subsequently, hyperphosphorylation of profilin IIa in SMA leads to inhibition of neurite outgrowth. Therefore, Rho-kinase inhibition might be able to correct the effect of SMN reduction in SMA to achieve an adequate ratio of de-/phosphorylated profilin IIa.

Notably, treatment of SMA model mice with Rho-kinase inhibitor Y-27632 or Fasudil led to a significant prolongation in survival, improvement in integrity of neuromuscular junction, and increase in muscle fiber size without altered SMN expression or increase in the number of spinal motor neurons [146, 149]. Since Fasudil has been successfully applied in many clinical trials for other neurological and vascular diseases based on its neuroprotection, vasodilatation, and immune modulation effects [150], the results of Fasudil therapeutic studies for SMA patients are anticipated.

4.3. Stem Cells

4.3.1. Neural Stem Cells. A diagnosis of SMA is usually made following a patient's initial presentation of muscle weakness, at which there would be substantial spinal motor neuron loss [64]. Both SMN dependent and independent treatments described above could only prevent disease progression, but not regain lost motor neurons, while stem cell therapy might provide a possibility for cell replacement. Fetal-derived neural stem cells (NSCs) are able to self-renew and are multipotent with the capacity of producing neurons (including motor neurons), astrocytes, and oligodendrocytes [151]. NSCs can be isolated from mouse embryonic spinal cords and differentiated toward a motor neuron cell fate by priming with retinoic acid and sonic hedgehog. Intrathecal injection of these primed NSCs in *nmd* mice, another model of motor neuron disease, resulted in improvement of abnormal phenotypes and extension of survival [152]. In addition, NSCs derived from human fetal spinal cord delayed disease onset and prolonged lifespan after being transplanted directly into spinal cord of ALS mice [153, 154].

In a severe type of SMA mouse model, intrathecal injection at postnatal day 1 with primed NSCs derived from mouse embryonic spinal cord also promoted motor neuron survival, improved motor function, and prolonged lifespan [19]. Although some grafted cells expressed motor neuron markers, there was no direct evidence suggesting that the beneficial effects resulting from the formation of functional motor units by the transplanted cells. Transplantation of undifferentiating NSCs also showed a significant increase in survival of SMA mice, although not as efficient as the effects of NSCs primed into a motor neuron fate [19]. Therefore, the observed benefits of NSCs in SMA model mice were likely related to trophic support.

4.3.2. Embryonic Stem Cells. Although fetal-derived NSC transplantation in SMA mice showed promising effects, their derivation from a spinal cord source impedes further clinical implementation because of ethical and technical

issues [155]. On the other hand, embryonic stem cells might be easier to obtain and are also able to differentiate *in vitro* and *in vivo* into NSCs and a motor neuron fate [156]. Intraspinal grafting of embryonic stem cell-derived motor neurons resulted in a significant improvement in motor behaviors in the ALS rat [157]. For SMA, embryonic stem cell-derived NSCs transplanted intrathecally in SMA model mice migrated to spinal anterior horn and improved motor function and lifespan [20]. Although the grafted stem cells integrated appropriately into the parenchyma, and expressed both neuron- and motor neuron-specific markers, there was again no evidence of newly generated motor neuron outgrowth to the muscles. In one previous study, a boy with ataxia telangiectasia received intracerebellar and intrathecal injection of human fetal NSCs. Four years later, he was diagnosed with a donor-derived multifocal brain glioneuronal neoplasm [158]. To increase the differentiation rate of embryonic stem cells into NSCs before transplantation, the above SMA study used drug-selectable embryonic stem cell lines that ganciclovir and G418 have been applied for selection against undifferentiated embryonic stem cells and for neuroepithelial cells, respectively. Usage of these drug-selectable stem cells not only promoted transplantation safety, but also produced superior treatment results as compared to using wild-type embryonic stem cells [20].

4.3.3. Induced Pluripotent Stem Cells. Since the first report on reprogramming of mouse fibroblasts into so-called induced pluripotent stem (iPS) cells by the expression of oct3/4, Sox2, c-Myc, and Klf4 in 2006 [159], reprogramming of human somatic cells to a pluripotent state was achieved using similar approaches [160, 161]. The iPS cells can be differentiated into cells of endodermal, mesodermal, or ectodermal origin, and further lineage restriction can obtain specific neural subtypes or astrocytes. Recently, iPS cells have been successfully generated from fibroblasts of SMA patients [162, 163]. The SMA-specific iPS cells exhibited a reduced capacity to form motor neurons and an abnormality in neurite outgrowth that ectopic SMN expression rescued these abnormal phenotypes [163]. These iPS cells provide a novel opportunity in disease modeling for investigating SMA pathogenesis and can be used in screening novel compounds for SMA treatment.

The use of fetal-derived cells or embryonic stem cells for transplantation is hurdled by problems of availability, the possibility of immune rejection, and ethics. In contrast, the source of iPS cells is unlimited, and iPS cells can be transplanted autologously. Transplantation of normal neurons derived from iPS cells reduced abnormal phenotypes in a murine model of Parkinson's disease [164]. Notably, when iPS cell-derived neural precursor cells from a patient with Parkinson's disease were transplanted into the striatum of a Parkinson's disease rat model, the donor cells differentiated into dopaminergic neurons, survived in the rodent brain for several months, and reduced the abnormal motor asymmetry [165]. For autologous iPS cell transplantation in SMA, iPS-derived neural precursor cells or motor neurons should be pretreated to express a high level of SMN before transplantation. Until now, there is still no cell transplantation report using iPS cells in SMA.

5. Conclusions

In various neurological disorders, many diseases, such as Parkinson's disease, epilepsy, and multiple sclerosis, are treated clinically with multiple drugs in combination to enhance the therapeutic effects. Motor neurons may also require additional support to optimally respond to SMN-based treatment. In the past two decades, there has been tremendous progress in SMA regarding genetics, pathophysiology, and therapeutics. Some useful strategies to enhance SMN expression have been developed, and some novel SMN-independent therapeutic targets have been discovered. While SMN acts to modulate and correct the neuromuscular junction for functional improvement, SMN-independent targets could play a role of extension in the survival of motor neurons and reduce the influence of SMN depletion in axonal dynamics.

The two currently available stem cell transplantation studies for SMA have only demonstrated benefits likely with trophic support without evidence of functional cell replacement [19, 20]. To generate functional motor units, the grafted stem cells should be able to differentiate into motor neurons, appropriately project the axons a long distance toward corresponding muscles, and form functional synapses within neuromuscular junctions. In a virus-induced rat model of motor neuron degeneration, mouse embryonic stem cell-derived motor neurons transplanted into spinal cord could survive, extend axons, form functional motor units, and promote recovery from paralysis [166, 167]. The successful development of motor units in the above studies may result from a combination approach, which includes administration of dibutyryl-cAMP, rolipram, cyclosporine, and glial cell line-derived neurotrophic factors to promote motor neuron survival, circumvent myelin repulsion, prevent immune rejection, and enhance axonal outgrowth, respectively. Therefore, cell replacement therapy using stem cells for SMA is not totally impossible; however, there is still much to be accomplished in cell therapy before being applied clinically to treat motor neuron diseases.

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Review Article

Mouse Models of Down Syndrome as a Tool to Unravel the Causes of Mental Disabilities

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Down syndrome (DS) is the most common genetic cause of mental disability. Based on the homology of Hsa21 and the murine chromosomes Mmu16, Mmu17 and Mmu10, several mouse models of DS have been developed. The most commonly used model, the Ts65Dn mouse, has been widely used to investigate the neural mechanisms underlying the mental disabilities seen in DS individuals. A wide array of neuromorphological alterations appears to compromise cognitive performance in trisomic mice. Enhanced inhibition due to alterations in $GABA_A$-mediated transmission and disturbances in the glutamatergic, noradrenergic and cholinergic systems, among others, has also been demonstrated. DS cognitive dysfunction caused by neurodevelopmental alterations is worsened in later life stages by neurodegenerative processes. A number of pharmacological therapies have been shown to partially restore morphological anomalies concomitantly with cognition in these mice. In conclusion, the use of mouse models is enormously effective in the study of the neurobiological substrates of mental disabilities in DS and in the testing of therapies that rescue these alterations. These studies provide the basis for developing clinical trials in DS individuals and sustain the hope that some of these drugs will be useful in rescuing mental disabilities in DS individuals.

1. Introduction

Trisomy 21, or Down syndrome (DS), is the most common genetic cause of intellectual disability. It affects 1 in 850–1000 infants [1] and is characterized by a number of phenotypes, including cardiovascular, skeletal, and motor alterations. However, the most prominent feature of DS is an intellectual disability that affects 100% of the individuals with this condition. DS individuals typically display an average Intelligence Quotient (IQ) of 50 (ranging from 30 to 70) [2] and show an array of altered cognitive and behavioral phenotypes, including the incomplete and delayed acquisition of motor [3], linguistic [3, 4] and visual-spatial abilities [3], impairments in learning and memory [3–6], and neurobehavioral disorders [4] and have a higher risk of developing Alzheimer-like dementia by the age of 40 [7, 8]. Great inter-individual variability, however, is present in both the nature and the intensity of all of these conditions.

In recent years, the question of how trisomy of Hsa21 leads to this set of phenotypes has been a matter of debate. Two hypotheses have been proposed to account for this phenomenon: the "amplified developmental instability" hypothesis [9] and the "gene-dosage effect" hypothesis [10–12]. The first hypothesis proposes that trisomy of Hsa21 causes a general alteration in developmental homeostasis that leads to the DS phenotypes; the "gene-dosage effect" proposal maintains that these alterations result from the overexpression of a subset of genes and their encoded proteins.

The analysis of DS cases resulting from partial trisomies of Hsa21 and the development of a number of mouse models of this condition have provided insight on the causative role of dosage-sensitive genes on DS phenotypes. These studies have yielded evidence that support both theories; while the role of single dosage-sensitive genes on different phenotypes has been confirmed, research has also demonstrated that many of these DS features are due to the complex effects of multiple Hsa21 genes (see [13]) and their interactions with genes of other chromosomes.

To identify the biological mechanisms underlying different pathologies and to evaluate the efficacy of novel therapies, thousands of animal models of human disorders have been developed. For an animal model of a human disorder to

be considered valid, it has to satisfy three criteria: *construct*, *face*, and *predictive* validity [14, 15]. *Construct* validity relates to the similarity between the etiology of the human and the animal disorder (e.g., in the case of mouse models of DS, the triplication of Hsa21 genes). *Face* validity refers to how well the model mimics the molecular, cellular, physiological, and behavioral phenotypes observed in humans. *Predictive* validity requires that the new knowledge obtained in the animal model makes accurate predictions of what will be found in the human condition. This validity is particularly important for unraveling the neurobiological causes of the cognitive deficits found in DS that cannot be assessed in humans for ethical or practical reasons and for developing and testing new therapies.

In the following sections, we will summarize (1) the similarities between the genetic overdose of various mouse models of DS and human trisomy 21; (2) the concordance between the behavioral, neuromorphological, and neurochemical phenotypes of DS mouse models and the human condition; (3) the knowledge obtained in these animals about the neurobiology of DS that have yielded the development and analysis of several therapeutic strategies that could potentially be used to attenuate cognitive impairments in DS individuals.

2. Mouse Models of Down Syndrome

The long arm of Hsa21 contains approximately 552 genes, 166 of which are orthologous to genes localized in syntenic regions of three mouse chromosomes: Mmu16 (110 orthologous genes), Mmu17 (19 orthologous genes), and Mmu10 (37 orthologous genes) [16]. Based on these homologies, several mouse models that are trisomic for different sets of Hsa21 genes have been developed (Figure 1). The first attempt to create a mouse model of DS was to develop a mouse, named Ts16, which was trisomic for the entire Mmu16 [17]. However, this model does not resemble the DS aneuploidy because Mmu16 presents syntenies with regions of Hsa3, Hsa8, Hsa16, and Hsa21; thus, it has triplicated many genes that are not in trisomy in DS and, consequently, does not exhibit good construct validity. Furthermore, Ts16 embryos die in utero, making it impossible to test phenotypes in young and adult mice, thus restricting the face and predictive validities of this model.

The next approach adopted was the generation of mouse models with partial trisomies of sets of Mmu16 genes orthologous to those found in Hsa21. In 1993, Davisson et al. [18] created the Ts65Dn mouse, which is now the most commonly used and best characterized model of DS. This mouse bears a partial trisomy of a segment of Mmu16, extending from the Mrp139 to the Znf295 genes, and contains approximately 92 genes orthologous to Hsa21 genes [16]. Additionally, Ts65Dn mice also carry a trisomy of ~10 Mb of Mmu17 containing 60 genes nonhomologous to Hsa21 [19]. Thus, this model does not have perfect construct validity because many of the orthologous genes found in Hsa21 are not triplicated in this mouse and because a set of genes not triplicated in DS are in trisomy in this model. However, as detailed below, the Ts65Dn mouse is currently the model that displays the best face validity. Additionally, in some cases, DS results from a partial trisomy of different regions of Hsa21, and there is strong evidence that some regions of this chromosome contribute more to the DS phenotype [12, 20]. Moreover, according to the "gene-dosage effect" hypothesis, different DS phenotypes are determined by the increased dosage of only a subset of genes. A comparison of the phenotypes in Ts65Dn mice with those of other partial trisomic models (see below) suggests that the set of genes triplicated in this model contribute to several DS phenotypes, including cognitive and neuroanatomical impairments (Tables 1 and 2).

The Ts2Cje model carries the same segment of Mmu16 triplicated in the Ts65Dn mouse but is translocated to chromosome 12 [21]. Although this model also shows some of the DS-relevant phenotypes found in the Ts65Dn mouse, it has not been fully characterized.

Several other segmental trisomic models of different segments of Mmu16, 17, and 10 have been created. In the late 90s, Sago and coworkers generated two mouse models with the triplication of two different regions of Mmu16: the Ts1Cje mouse, which presents a trisomy of 81 genes localized in the region of Mmu16 that extend from Sod1 to Znf295 [22]; the Ms1Ts65 mouse, which has a partial trisomy of 33 genes mapped in the region of Hsa21 that extend from App to Sod1 [23]. In addition, to evaluate the influence of the so-called Down syndrome critical region (DSCR), Olson et al. [24] developed the Ts1Rhr mouse, a model that is trisomic for the Cbr1-Orf9 region of Mmu16, which contains 33 genes. Finally, Li et al. [25] generated a mouse trisomic for the complete Hsa21 syntenic region on Mmu16 (between Lipi and Zfp295) containing 110 orthologous genes, the Dp(16)1Yey/+ mouse [16].

To model the trisomy of Hsa21 orthologous genes located in Mmu17, two mouse models have been created: the Ts1Yah mouse, trisomic for 12 genes in the Mmu17 region, syntenic to the subtelomeric region of Hsa21 [26] and the Dep(17)1Yey/+ mouse which is trisomic for the entire Hsa21 syntenic region on Mmu17 that contains 19 orthologous genes [16, 27, 28]. Additionally, Vacík et al. [29] created the Ts43H model, a mouse that is trisomic for 30 Mb of Mmu17 containing over 300 genes but only ~20 of them are orthologs of Hsa21 genes. Therefore, this is not a valid DS mouse model.

The last segmental trisomic mouse generated is a mouse that models the trisomy of Hsa21 orthologous genes located in Mmu10. The Dp(10)1Yey mouse is trisomic for the region of Mmu10 syntenic to the distal part of Hsa21 containing 37 orthologous genes [16, 27].

After the tree partial trisomic models for all the Hsa21 syntenic regions on Mmu10 (Dp(10)1Yey/+), Mmu16 (Dp(16)1Yey/+) and Mmu17 (Dep(17)1Yey/+) were established, Yu et al. [27] cross-breeded them to generate a mouse that is trisomic for the entire Hsa21 syntenic regions on Mmu10, Mmu16, and Mmu17 chromosomes: the Dp(10)1Yey/+Dp(16)1Yey/+; Dep(17)1Yey/+ mouse. This is a promising new model with excellent construct and face validities, as it shows several DS phenotypes [27].

The Tc1 model is a mouse in which the entire human Hsa21 has been triplicated [30]. This mouse shows different

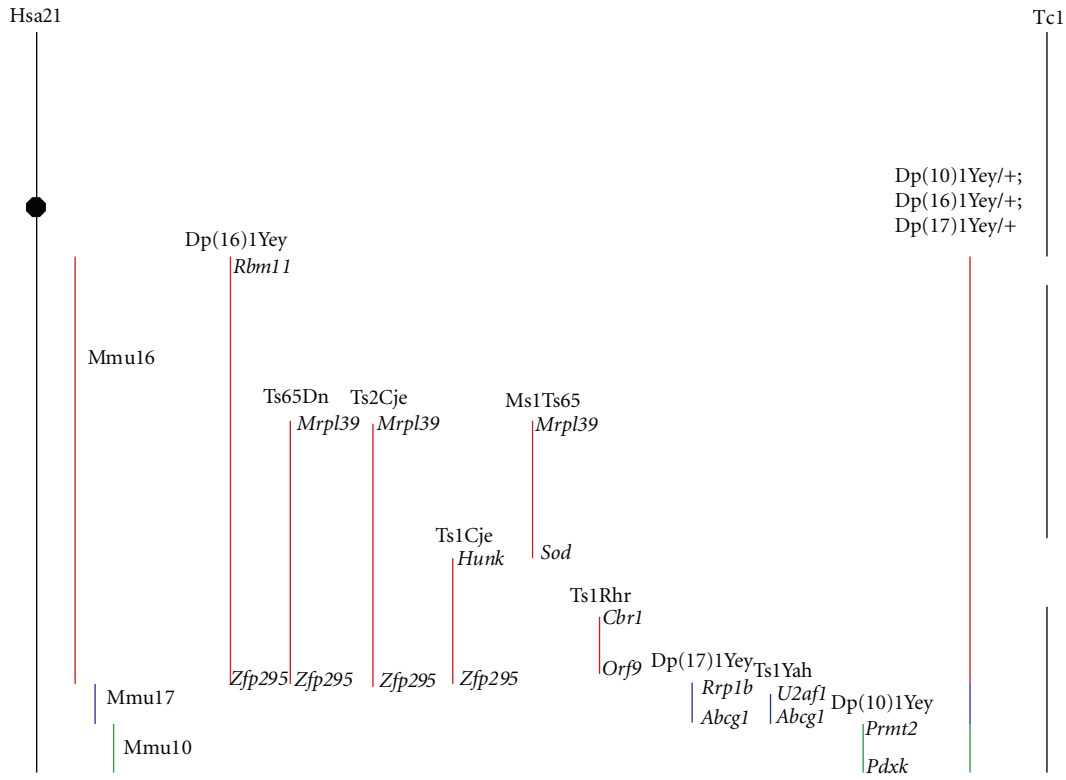

Figure 1: Schematic representation of Hsa21 and syntenic regions of Mmu16, Mmu17, and Mmu10 and the different mouse models trisomic for different sets of genes orthologous to those of Hsa21. The flanking genes found at the boundaries of the triplicated region in each model are written in italics. Modified from [3, 137].

DS-relevant phenotypes [30–32], although its characterization is not as complete as those performed on the different segmental trisomic models. However, the Tc1 mouse presents variable levels of mosaicism of the extra chromosome in different tissues, confounding the analysis of phenotypic consequences. In addition, although the starting material was an intact Hsa21, delections occurred and this mouse has triplicated ~83% of the genes on Hsa21.

Finally, to study the role of particular genes in the DS phenotypes, a number of mouse models with the triplication of single genes and trisomic mice in which the expression of only one of the genes triplicated in DS have been normalized have been created (see [13]).

3. Cognitive and Behavioral Deficits in Mouse Models of DS

This section describes the similarities between the cognitive and behavioral disturbances found in various mouse models of DS compared to the human condition (Table 1).

Motor dysfunction is a hallmark of DS. Hypotonia, hyporeflexia, reduced muscular strength, disturbances in striate muscle control, and delays in the acquisition of fine and gross motor skills are found in DS individuals from early childhood [33–35].

Ts65Dn mice are not impaired in sensorimotor abilities such as forelimb strength, postural skills, equilibrium, and climbing [36, 37]. However, these mice show poorer balance and motor coordination [38]. Ts65Dn mice are hyperactive in the dark [36, 39, 40] and in other settings that provoke caution and lack of movement in normal animals, such as in open-field and plus-maze tests [36, 41–43]. This hyperactivity has been proposed to be due to a failure to inhibit activity or as a deficit in the ability to attend to relevant stimuli [44–46]. Attention deficits in Ts65Dn mice have been confirmed by Driscoll et al. [47]. Unlike Ts65Dn, the other models that are partially trisomic for different segments of Mmu16 are not hyperactive. Ts1Cje and Ms1Ts65 mice do not exhibit altered spontaneous activity [23], and Ts1Rhr mice display normal performances in the open-field test [48]. However, Tc1 mice present higher spontaneous locomotor activity, reduced ability to habituate to new environments, and several deficits of motor coordination and balance in the rotarod and static-rod tests [32].

Similar to DS [6], Ts65Dn mice are impaired in performing hippocampal-dependent tasks, such as spontaneous alterations in the T-maze, contextual fear conditioning, novel object recognition [49, 50], and spatial memory in the radial arm maze [51–54] and the Morris water maze tests [23, 40, 42, 45, 55]. Ts65Dn mice also show deficits in learning an operant conditioning paradigm [56].

Table 1: Behavioural and cognitive alterations in DS and in the different DS mouse models.

Trisomy	Hsa21	Segment of Mmu16						Segment of Mmu17		Segment of Mmu10	Segment of Mmu16, Mmu17, and Mmu10	Hsa21
	DS	Dp(16)1Yey/+	Ts65Dn	Ts2Cje	Ts1Cje	Ms1Ts65	Ts1Rhr	Dep(17)1Yey/+	Ts1Yah	Dp(10)1Yey/+	Dp(10)1Yey/+; Dp(16)1Yey/+; Dep(17)1Yey/+ Yu et al., 2010	Tc1
Motor skills	Delayed acquisition		Delayed acquisition									
Motor coordination	Impaired		Impaired									Impaired
Activity and attention	Reduced attention		Hyperactivity and reduced attention		Normal activity	Normal activity	Normal activity					Increased spontaneous activity
Context discrimination		Impaired	Impaired					Unchanged		Unchanged	Impaired	
Spatial learning and memory	Impaired	Impaired	Impaired		Impaired	Impaired		Unchanged	Enhanced	Unchanged	Impaired	Impaired
Working and reference memory	Impaired		Impaired									Impaired
Novel object recognition			Impaired				Impaired		Impaired			
Operant conditioning			Impaired									

Ts1Cje and Ms1Ts65 mice display poor performance in hippocampal-dependent tasks such as the T-maze [52] and the Morris water maze [22, 23]. Ts1Rhr mice are impaired in spontaneous alterations in the T-maze task [48] and show altered long-term memory in the novel object recognition test [48] but not in the Morris water maze [57]. Dp(16)1Yey/+ mice showed impaired performance in the Morris water maze and in the contextual fear conditioning test [27].

Regarding the two mouse models trisomic for segments of Mmu17, Ts1Yah mice are impaired in the novel object recognition and Y-maze test, but their performance in the Morris water maze is enhanced [26]; however, Dp(17)1Yey+ do not show alterations in performance in the later task or in the contextual fear conditioning test [28].

The Dp(10)1Yey/+Dp(16)1Yey/+; Dep(17)1Yey/+ mouse is impaired in the Morris water maze and in the contextual fear conditioning test [27]. Finally, Tc1 mice show altered performance in the novel object recognition test but not in the T-maze [30].

In summary, most of the above-mentioned mouse models show characteristic DS cognitive and behavioral phenotypes, although they differ in the degree of impairment.

4. Neuromorphological Alterations in DS and DS Mouse Models

Several mechanisms have been proposed to be the neurobiological correlates of intellectual disability in DS, including altered neurogenesis, hypocellularity, altered synaptic development, increased inhibition, and neurodegeneration. Table 2 summarizes the main neuromorphological and electrophysiological alterations found in DS individuals and in the different mouse models of DS, and Table 3 describes the outcome of several studies that tested the ability of different therapeutical approaches to rescue different phenotypic alterations in the Ts65Dn mouse and in DS individuals.

4.1. Reduced Volume and Hypocellularity. In DS individuals, the volume of the brain is reduced, beginning at early developmental stages [57–62]; in adults, the reduction in size reaches approximately 20% [63, 64], and, during aging, neurodegeneration further deteriorates this scenario [65].

The brain volume of Ts65Dn, Ts1Cje, and Ts2Cje mice is also reduced during the embryonic period, but not after birth or during adulthood [39, 52, 66–68]. Ms1Rhr mice also show reduced brain volume [66], as does the Ts1Rhr mouse, at 4 months of age [69] but not at later stages [48]. Thus, most DS mouse models do not show changes in total brain volume during adulthood.

A number of studies demonstrated that brain areas are dissimilarly affected. Reduced volumes of the hippocampus, entorhinal, frontal, prefrontal, and temporal cortices, amygdala, cerebellum, brain stem nuclei, and mammillary bodies of the hypothalamus have been reported in children and adults with DS [63, 64, 70–78].

Consistently, size and anatomical alterations have been found in some brain regions of trisomic mice. The hippocampus and the cerebellum appear to be the most affected structures. Spatial learning is known to depend on the functional integrity of the hippocampus, a structure that plays a key role in information encoding and retrieving in the CNS [79, 80]. In Ts65Dn mice, the hippocampal granule cell layer and hilus show reduced volume [54, 81–83]. The hippocampal area of Ts1Cje mouse is not reduced [52], and the Ts1Rhr mice show greater volume of the posterior hippocampus [48].

In DS individuals, cell density is not compromised in early gestation [60, 62, 84], but neuron number is reduced in late gestation (after weeks 19–23). Indeed, the number of neurons in the hippocampus, parahippocampal gyrus, cerebellum and neocortex of fetuses [60, 61, 85], and newborn DS individuals [60, 62, 84] is reduced. Hypocellularity persists in different areas of the brain [86] and cerebellum [37] of children and adults with DS.

Ts65Dn mice show reduced cell density during prenatal (E 18.5) and early postnatal stages in the neocortex (P8) [67]. At 1 month of age, Ts65Dn mice display a normal number of neurons in the hippocampal CA1–CA3 areas [81]. However, CA1 neuron density is lower in older (17-18 month) Ts65Dn mice [87, 88]. The dentate gyrus (DG) of Ts65Dn mice has fewer granule cells at all examined ages [54, 81, 87, 89, 90]. However, in 18-month-old Ts1Cje mice, the thickness of the granule cell layer and molecular layer of the DG is not affected [52].

The cerebellum has been traditionally implicated in motor coordination, but there is increasing evidence for the role of this structure in higher cognitive processes, including attention, cognitive flexibility, and memory [91]. Consistent with what is found in DS, the volume of the cerebellum is significantly reduced in Ts65Dn, Ts1Cje, and Ts1Rhr mice [37, 48, 66, 92]. However, Ms1Ts65 mice do not show changes in cerebellar volume [92].

As expected from the reduced cerebellar volume of DS and trisomic mice, the cerebellum of Ts65Dn mice has a lower number of granule and Purkinje cells from early postnatal stages to adulthood [37, 93, 94]. Ts1Cje, Ms1Rs65, and Tc1 mice also show a decrease in cerebellar granule cell density [30, 92].

One of the anatomical substrates of learning and attention is the septohippocampal cholinergic system [95, 96]. In the aging DS brain, there is a loss of cholinergic neurons [97, 98]. Several studies have demonstrated an age-dependent decline in cholinergic markers in Ts65Dn mice. Starting at 6 months of age, Ts65Dn mice basal forebrain cholinergic neurons show a loss of the cholinergic phenotype. These neurons show a decrease in choline acetyltransferase (ChAT) and $p75^{NGFR}$, a neurotrophin receptor localized in basal forebrain cholinergic neurons, immunoreactivity. Furthermore, there is a decrease in the size of these cholinergic neurons cell bodies [82, 99–102].

In conclusion, persistent hypocellularity is one cause of intellectual disability in the trisomic condition. The following sections will describe the evidence showing that these neuroanatomical alterations could be caused by impaired cell proliferation, increased apoptosis and/or neurodegeneration.

Table 2: Neuromorphological and electrophysiological alterations DS and DS mouse models.

Trisomy	Hsa21	Segment of Mmu16						Segment of Mmu17		Segment of Mmu10	Segment of Mmu16, Mmu17, and Mmu10	Hsa21
	DS	Dp(16)1Yey/+	Ts65Dn	Ts2Cje	Ts1Cje	Ms1Ts65	Ts1Rhr	Dep(17)1Yey/+	Ts1Yah	Dp(10)1Yey/+	Dp(10)1Yey/+; Dp(16)1Yey/+; Dep(17)1Yey/+ Yu et al., 2010	Tc1
Brain volume	Reduced		Reduced	Reduced during the embryonic period	Reduced during the embryonic period	Reduced	Reduced at 4 months of age					
Neuronal density	Reduced		Reduced	Reduced	Not affected							
Cerebellar volume	Reduced		Reduced		Reduced	Not affected	Reduced					Reduced
Cerebellar neuronal density			Reduced		Reduced	Reduced						Reduced
Neurogenesis	(i) Impaired neural precursor proliferation (ii) Slowing of the cell cycle (iii) Impaired neurodifferen-tiation (iv) Impaired cerebellar neurogenesis		(i) Impaired neural precursor proliferation (ii) Slowing of the cell cycle (iii) Impaired neurodiffer-entiation (iv) Impaired cerebellar neurogenesis	(i) Impaired neural precursor proliferation	(i) Impaired neural precursor proliferation (ii) Impaired neurodiffer-entiation (iii) Impaired cerebellar neurogenesis							
Dendrites and dendritic spines	(i) Impaired morphology (ii) Reduced density		(i) Impaired morphology (ii) Reduced density	(i) Impaired morphology (ii) Reduced density	(i) Impaired morphology (ii) Reduced density		(i) Impaired morphology (ii) Reduced density					

Table 2: Continued.

Trisomy	Hsa21	Segment of Mmu16						Segment of Mmu17		Segment of Mmu10	Segment of Mmu16, Mmu17, and Mmu10	Hsa21
	DS	Dp(16)1Yey/+	Ts65Dn	Ts2Cje	Ts1Cje	Ms1Ts65	Ts1Rhr	Dep(17)1Yey/+	Ts1Yah	Dp(10)1Yey/+	Dp(10)1Yey/+; Dp(16)1Yey/+; Dep(17)1Yey/+ Yu et al., 2010	Tc1
Synaptic density			(i) Reduced (ii) Size of presynaptic boutons and average length of synaptic clefts are increased		(i) Size of presynaptic boutons and average length of synaptic clefts are increased							
Inhibition			(i) Increased number of inhibitory synapses (ii) Decreased number of excitatory synapses (iii) Redistribution of inhibitory synapses (iv) Increased number of GABAergic interneurons		(i) Redistribution of inhibitory synapses							
Electrophysiology	(i) EEG abnormalities (ii) EEG coherence differences (iii) Alterations in event-related potentials	(i) Impaired hippocampal LTP	(i) Impaired hippocampal LTP		(i) Impaired hippocampal LTP		(i) Impaired hippocampal LTP	(i) Enhanced hippocampal LTP		(i) Unchanged LTP		(i) Impaired hippocampal LTP

Table 3: Therapeutical approaches tested in DS and in the Ts65Dn mouse.

Therapies	DS	Ts65Dn
Targeting neurogenesis		
(i) Fluoxetine	(i) Not tested	(i) Restores BDNF levels, neurogenesis, dendritic maturation and branching and cognition
(ii) Lithium	(ii) Not tested	(ii) Restores neurogenesis
(iii) SAG 1.1	(iii) Not tested	(iii) Restores neurogenesis and cognition
(iv) Environmental enrichment	(iv) Improves cognition	(iv) Restores neurogenesis, improves cognition, no effect on dendritic arborization in TS mice
Targeting inhibition		
(i) Picrotoxin	(i) Not tested	(i) Rescues LTP and cognition
(ii) Bilobalide	(ii) Not tested	(ii) Rescues LTP and cognition
(iii) PTZ	(iii) Not tested	(iii) Rescues LTP and cognition
(iv) α5IA	(iv) Not tested	(iv) Rescues cognition
Targeting NMDA receptor functioning		
(i) Memantine	(i) No effect	(i) Improves cognition, reduces APP levels
Targeting NA functioning		
(i) L-DOPS	(i) Not tested	(i) Rescues cognition
(ii) Xamoterol	(ii) Not tested	(ii) Rescues cognition
Targeting neurotrophins		
(i) NGF	(i) Not tested	(i) Rescues BFCNs altered size and number
(ii) Peptide 6 (CNFT)	(ii) Not tested	(ii) Improves learning and memory, enhanced neurogenesis
(iii) EGCG	(iii) Not tested	(iii) Rescued BDNF levels, brain size, and LTP in the Dyr1A Tg mouse
(iv) Neurotrophin	(iv) Not tested	(iv) Prevents decline in BDNF expression, improves cognition
Targeting inflammatory activity		
(i) Minocycline	(i) Not tested	(i) Inhibits microglia activation, prevents neuron loss, improves working, and reference memory
Neuropeptides		
(i) NAP and SAL	(i) Not tested	(i) Rescues acquisition of neurodevelopmental milestones, increases ADNP levels and rescued ADNP levels
Targeting oxidative stress: antioxidants		
(i) SGS111	(i) Not tested	(i) No effect on cognition
(ii) Vitamin E	(ii) No effect	(ii) Reduced oxidative stress, improved cognitive performance, reduced cholinergic neuron pathology, and increased cell density in the DG
(iii) Combined antioxidant supplementation	(iii) No effect	
(iv) Folinic acid/ folinic acid + antioxidants	(iv) Beneficial effect on developmental age/no effect	
Estrogens	(i) Not tested	(i) In females enhanced cognition, increased the size and number of cholinergic neurons and NGF levels
Targeting AD neuropathology		
(i) DAPT	(i) Not tested	(i) Reduced beta-amyloid levels, rescued cognition
(ii) Donepezil	(ii) No effect/small effect	(ii) No effect

4.2. Neurogenesis

4.2.1. Neurogenesis in Trisomic States. Neurogenesis is severely compromised in DS from early developmental stages. Impaired neuronal precursor proliferation, slowing of the cell cycle, and altered differentiation are thought to account for altered neurogenesis.

In DS fetuses, a reduced number of dividing cells is found in the dentate gyrus (DG) and lateral ventricle [60, 89]. Reduced proliferation of neural precursor cells is also found in mouse models of DS. In Ts65Dn mice, reduced neural precursor proliferation is found in the neocortical ventricular zone (VZ) during embryonic stages [67]. However, in these mice, a larger progenitor population of inhibitory neurons has been found in the embryonic medial ganglionic eminence [103].

Reductions in neural progenitor cells and neuroblasts, leading to altered neurogenesis, in the embryonic neocortex

and subventricular zone (SVZ) of Ts1Cje and Ts2Cje mice have also been reported [68, 104, 105].

It has been proposed that the extra copy of Hsa21 in DS delays the mitotic cell cycle of neuronal precursors, thereby affecting cell proliferation. Accordingly, a slowing of the cell cycle in different trisomic conditions has been demonstrated. In Ts65Dn mice, the cell cycle is extended during embryonic stages in CA3 [67] and in early postnatal life in the DG [89]. The expression of two regulators of the G2/M and G1/S transitions, Ccnb1 and Skp2, is decreased in newbornTs65Dn cerebellar granule cell precursors [82]. Hewitt et al. [104] also observed dysregulated expression of genes involved in cell cycle control in Ts1Cje mice.

Impaired differentiation also appears to contribute to the smaller number of neurons in DS brains [106, 107]. Ts1Cje neural progenitors have a reduced capacity to differentiate into neurons [104, 105].

Adult hippocampal neurogenesis has been demonstrated in many species, including rodents [108–111]. During the entire life span, cell proliferation takes place in the SVZ and in the subgranular zone (SGZ) of the DG, where a pool of multipotent progenitor cells is located. In the SGZ, newborn neurons migrate into the granular cell layer (GCL) and establish functional connections in the dentate molecular region, where they receive excitatory synaptic input from perforant path afferents [111]. Increasing evidence indicates that adult hippocampal neurogenesis is implicated in the establishment of long-term potentiation (LTP) and has a role in hippocampal-dependent learning and memory [112–114]. Interestingly, we have showed a negative correlation between performance in the Morris water maze and the number of proliferating cells in the DG of Ts65Dn mice and euploid littermates (Figure 2).

In Ts65Dn mice, cell proliferation in the SVZ is reduced from birth to adulthood [54, 114, 115]. In the DG, proliferation impairments have also been reported in newborn [81, 89], young [54, 116], middle-aged [117], and aged [83] Ts65Dn mice. Adult (3-month-old) Ts1Cje and Ts2Cje mice also present severe neurogenesis reduction in the SGZ [118].

Cerebellar neurogenesis is also affected by trisomy. DS fetuses show reduced neurogenesis in the external granular layer (EGL) of the cerebellum and in the VZ [61]. Newborn (P0, P2, and P6) Ts65Dn mice also show reduced proliferation of cerebellar granule cell precursors in the EGL [93, 119]; their cell cycle is also dramatically slowed, and the G1 and G2 phases are the most affected [119]. One-month-old Ts65Dn mice show reduced proliferation of the granule neurons of the internal granular layer [94]. In Ts1Cje mice, proliferation of cerebellar granule cells is reduced at birth but normal at postnatal days 3 and 7 [120]. Differentiation is also altered in the cerebellum of Ts65Dn mice because a smaller percentage of cells acquire a neuronal phenotype [119]. Reductions in cerebellar neurogenesis in Ts65Dn mice seem to be due to the decreased response of granule cell precursors to the mitogenic factor Sonic hedgehog (Shh) [93].

It can be concluded that neurogenesis impairment, due to reductions in neural precursors, cell cycle timing and differentiation, is a hallmark of trisomic conditions from prenatal to adult stages. This altered proliferation is likely to be one of the mechanisms responsible for the widespread hypocellularity leading to altered synaptogenesis, connectivity, synaptic plasticity, and cognitive disabilities.

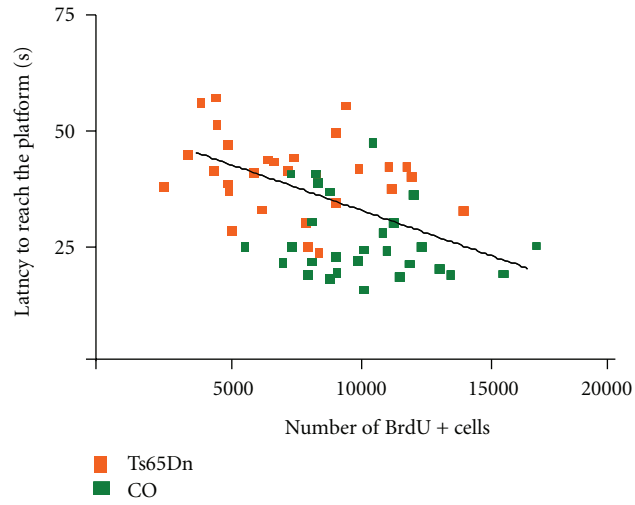

Figure 2: Correlation between performance in the Morris water maze (mean latency to reach the platform) and the number of BrdU+ cells in the DG of Ts65Dn and euploid littermates (Rueda et al., unpublished results; Pearson's R: -0.4647; $P < 0.001$).

4.2.2. Trisomic Genes and Neurogenesis Impairment. A number of trisomic genes in DS have been proposed to play a role in the proliferation impairment found in this condition. One of the genes overexpressed in the DS brain is DYRK1A (dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A), an orthologous gene to the Drosophila gene minibrain [121]. DYRK1A codes for a serine-threonine protein kinase [122], which has important transcription factors as substrates and, consequently, appears to be implicated in multiple biological pathways. DYRK1A is essential for normal postembryonic neurogenesis [123, 124]. This gene plays a role in neuronal progenitor proliferation, neurogenesis, and neurodifferentiation, and regulates neuronal development, brain volume and cellular density in different brain areas [124–127]. The DYRK1A protein also modulates CREB (cAMP response element-binding protein) activity, which participates in synaptic plasticity signal transduction pathways [126]. Overexpression of DYRK1A inhibits proliferation, induces premature differentiation of neural progenitor cells in the developing mouse cerebral cortex, and impairs G1/G0-S phase transition in rat hippocampal progenitor cells [128, 129]. Recent studies have suggested that the DYRK1A gene could be a potential therapeutic target in DS because the inhibition of DYRK1A expression rescued several DS relevant phenotypes. Ortiz-Abalia et al. [130] demonstrated that the normalization of the Dyrk1A expression in the striatum of TgDyrk1A mice, through the injection of an adeno-associated virus type 2-mediated Dyrk1A RNA inhibitor (AAVshDyrk1A), rescued motor alterations in these animals.

The Olig1 and Olig2 genes are also overexpressed in DS individuals. These genes encode transcription factors that have been implicated in neurogenesis and oligodendrogenesis [131–133]. Chakrabarti et al. [103] have demonstrated the implication of these genes in the regulation of the number of inhibitory neurons during embryonic development. The normalization of Olig1 and Olig2 expression in Ts65Dn mice rescued the abnormal interneuron production and the balance between excitatory and inhibitory transmission [103].

The APP gene is triplicated in DS and in most DS mouse models, and it is thought to play a role in different DS phenotypes, such as the development of AD pathology. It has been proposed that the APP gene may also be involved in the altered neurogenesis characteristic of the trisomic condition. Trazzi et al. [115] have recently related increased levels of the APP fragment AICD to the overexpression of the negative regulator of the Shh pathway, Ptch1, in Ts65Dn mice neural precursors and to their proliferation impairment. APP overexpression may also alter the differentiation of newly born cells by acting upon the Notch pathway, which is implicated in the acquisition of a glial phenotype [134]. Notch is upregulated in the cortex of DS and AD patients and in DS fibroblasts [135]; therefore, it could shift the balance towards a glial phenotype rather than a neuronal phenotype in newly born cells.

4.2.3. Therapies Targeting Neurogenesis. The implication of adult neurogenesis defects in DS-related cognitive impairments suggests that therapies targeted to rescue neurogenesis may be of value in treating intellectual disability in DS individuals.

The selective serotonin reuptake inhibitor fluoxetine is an antidepressant that has been shown to increase neurogenesis in the mouse DG and SVZ [113, 136]. Chronic treatment with fluoxetine restored neurogenesis in adult Ts65Dn mice [116]. Bianchi et al. [54] have recently shown that Ts65Dn mice treated with fluoxetine during the first two weeks of postnatal life showed rescued proliferation in the DG and SVZ, differentiation, and survival. Furthermore, this treatment restored brain derived neurotrophic factor (BDNF) expression, total granule cell number, and cognitive performance in a contextual fear conditioning task.

Another drug that markedly increases neurogenesis in the DG of adult normal mice is lithium, a drug prescribed for the treatment of bipolar depression [113]. Lithium treatment for 1 month rescued neurogenesis in the SVZ of 12-month-old Ts65Dn mice [114].

As mentioned above, the Shh pathway plays a key role in granule precursor cell (GPC) proliferation. Drugs targeting this pathway rescue neurogenesis alterations. Treatment of Ts65Dn mice with an activator of the Shh pathway, SAG 1.1, increased mitosis, restored cerebellar granule cell precursor populations [93], and rescued the cell proliferation of neural progenitors from the SVZ and DG [115]. Furthermore, a single injection of SAG 1.1 to newborn Ts65Dn mice restored cognition in these mice when they became adults [137].

Active care programs are one of the most successful therapeutic interventions used in DS individuals. In rodents, environmental enrichment has been associated with morphological, physiological, and cognitive improvements. These changes include increases in cortical weight and thickness, hippocampal neurogenesis, dendritic branching, length, number of dendritic spines and size of dendritic spines [138–140], facilitation of long-term potentiation [141, 142], and more efficient learning during different tasks [143–145].

Exposure of Ts65Dn mice to environmental enrichment for 7 weeks modulated spatial memory in a sex-dependent manner [55]. Environmental enrichment improved the performance of Ts65Dn females in the Morris water maze but lowered the performance of Ts65Dn male mice. In a subsequent study [42], it was shown that the deterioration found after environmental enrichment in Ts65Dn males was likely due to the stress induced by increased intermale aggression when the animals were housed in large groups. Enriching TS males in large groups (8–10) produced a large deterioration of performance in the Morris water maze and an increase in corticosterone plasma levels, effects that were not found when TS mice were housed in standard laboratory conditions or enriched in groups of 2-3.

Chakrabarti et al. [146] have recently shown that environmentally enriching groups of 2-3 Ts65Dn mice per cage increased cell proliferation and neurogenesis in the DG and SVZ of both male and female mice. It was proposed that this cellular response could underlie the cognitive improvements seen after special care programs in DS individuals.

Voluntary exercise is beneficial for cognition in both normal rodents and mouse models of altered cognition [110, 140, 147–149]. It has been suggested that these beneficial effects could be mediated, at least in part, by enhanced hippocampal neurogenesis [148, 150]. We have demonstrated that voluntary physical exercise improved the performance of TS mice in the Morris water maze but did not restore the neuromorphological phenotype (neurogenesis and hypocellularity in the DG), which suggests that the cognitive improvements produced by exercise were not mediated by neurogenesis-dependent mechanisms [83].

4.3. Apoptosis. Apoptosis or programmed cell death is physiologically involved in nervous system development and aging. It has been proposed that the hypocellularity found in DS brains could also be due to increased cell death. However, thus far, studies on the apoptotic processes in the trisomic condition have led to contradictory results. Some groups have reported increases in the number of apoptotic cells in DS brains [60, 151] and in Ts65Dn [89] and Ts1Cje [105] mice. In addition, changes in apoptotic regulatory proteins in different structures of DS brains have been found [152–156]. However, other studies have failed to find differences or have shown a reduced rate of apoptotic cell death in human and mouse trisomies [89, 157]. We have recently shown a downregulation of the antiapoptotic Bcl-Xl protein in the hippocampus of adult Ts65Dn mice, without changes in other pro- or antiapoptotic proteins in the cortex or the hippocampus [158]. Furthermore, we did not find any evidence of changes in molecular or cellular markers of apoptosis, suggesting that programmed cell death is not likely to play a role in the hypocellularity found in these mice brains.

4.4. Dendritic Hypotrophy. Altered synaptic plasticity is an additional mechanism that may underlie intellectual disability in DS individuals. Synaptic plasticity includes alterations in the number and the functional characteristics of synapses, which are mostly localized to dendrites and dendritic spines [159, 160].

Numerous studies have demonstrated impaired dendritic morphology in trisomic conditions. Although normal or even increased dendritic branching has been reported in DS fetuses and neonates [161–163], in DS children, neurons of the motor [164], visual [163, 165], and parietal cortex [166] show dendritic hypotrophy. These anomalies continue throughout the lifespan; in DS adults, the visual cortex, CA1, and CA3 are characterized by the presence of dendrites of shorter length and dendritic trees with reduced branching that progressively degenerate [161, 165, 167, 168]. Dendritic spines also show altered morphologies in the DS brain. Starting from infancy, spines are less numerous and smaller [168, 169], and their density is reduced to a greater extent in older DS individuals with AD [167, 170].

Mouse models also resemble the dendritic pathology of DS. In adult Ts65Dn mice, neocortical pyramidal neurons show a reduction in the length and arborization of dendrites and in the density of spines [171]. Spine density is also reduced in the granule cells of the DG in Ts65Dn, Ts1Cje, Ts2Cje, and Ts1Rhr mice [21, 48, 52, 172, 173]. In addition, in Ts65Dn, Ts1Cje, and Ts2Cje mice, these DG spines are characterized by several morphological anomalies, including an increase in the size of the heads and a decrease in the length of the necks [21, 52, 172]. Ts1Rhr mice also display enlarged spine heads, but no changes have been found in the morphology of spine necks of neurons in the cortex or the DG [174].

4.4.1. Therapies Targeting Dendrites and Dendritic Spines. These anomalies in dendritic tree arborization, spine density, and morphology lead to reduced density of synapses and compromised synaptic function in DS individuals. Several groups have tested the value of various therapeutic strategies for rescuing dendritic pathologies.

Based on the observation that fluoxetine favors dendritic development in normal animals [175], Bianchi et al. [54] reported that the early administration of fluoxetine (P3–P15) restored dendritic maturation and dendritic branching of DG neurons in Ts65Dn mice. As mentioned above, this drug also rescued neurogenesis and cognitive deficits in this mouse model.

The enhancement of dendritic arborization and spine density has been firmly established as one of the positive effects of environmental enrichment [138, 139, 176]. Because environmental enrichment has been shown to improve cognition in female but not male Ts65Dn mice [55], Dierssen et al. [171] tested the effect of this experimental protocol on dendritic morphology. The authors found that the enriched environment increased dendritic length and spine density in the basal dendritic trees of the neocortical pyramidal cells of euploid animals but had no effect on Ts65Dn mice [171]. Thus, enhancements in dendritic arborization and spine density do not appear to be the mechanism by which enrichment improves cognition in Ts65Dn females.

4.5. Synaptic Pathology. As predicted by the reduced length and number of branches of dendrites and density of spines, the trisomic condition is characterized by a reduced number of synaptic contacts and alterations in synaptic plasticity. Ts65Dn mice show a reduction in synaptic density in the neocortex and CA1 at P9 [67] and in the hippocampal DG, CA1, and CA3 regions in adulthood [88]. However, the size of presynaptic boutons and the average length of synaptic clefts are increased in the cortex and hippocampus of Ts65Dn and Ts1Cje mice [52, 69, 172].

Not only the number and characteristics of synapses but also the relative distribution of different types of synapses is altered in trisomic mice, leaving the balance between excitatory and inhibitory synapses shifted toward increased inhibition in the trisomic brain. Ts65Dn mice have less excitatory (asymmetric) synapses in the temporal cortex, DG, CA1, and CA3 [88, 177], and glutamatergic synapses are reduced in the hippocampus of Ts65Dn mice [90]. An increased number of inhibitory synapse markers [172] have been reported in the DG of these mice, but no changes in the number of symmetric or asymmetric synapses were found in the fascia dentata of Ts65Dn mice [69]. Ts65Dn and Ts1Cje brains also show a redistribution of inhibitory synapses, with a relative decrease in inputs to the dendritic shafts and an increase in inputs on spine necks [52, 172]. An increased number of GABAergic interneurons in the somatosensory cortex of Ts65Dn mice have been reported [178], which implies an enhancement of inhibitory synapses. Finally, Chakrabarti et al. [103] have found enhanced neurogenesis of inhibitory neurons in the forebrain of Ts65Dn mice, which led to an increased inhibitory drive.

Overall, these morphological and functional disturbances compromise the physiological properties of synapses, possibly leading to cognitive impairments in DS and trisomic mice.

5. Electrophysiological Alterations in DS and Mouse Models of DS

DS individuals present small electroencephalographic (EEG) abnormalities. EEG alpha activity is relatively preserved in young individuals with DS, but older patients with dementia present abnormal activity [179]. EEG coherence differences [180] and alterations in event-related brain potentials (ERPs) have also been reported in DS individuals [181].

In mouse models of DS, altered synaptic plasticity in the hippocampus has been consistently reported. Hippocampal long-term potentiation (LTP) is considered to be the electrophysiological substrate of learning. Ts65Dn mice display reduced LTP in the hippocampal CA1 and DG regions [52, 182–186]. Similarly, Ts1Cje, Ts1Rhr, Dp(16)1Yey/+, and Tc1 mice show reduced hippocampal LTP [28, 30, 31, 48, 52]. However, Dep(17)1Yey/+ mice showed enhanced LTP [28].

It has been proposed that altered synaptic plasticity in the hippocampus of DS mouse models results from increased inhibition due to unbalanced excitatory and inhibitory

neurotransmission [172, 186, 187]. Reduced activation of NMDA receptors is thought to hinder LTP induction in trisomic mice [30, 48, 52, 186]. Enhanced hippocampal long-term depression (LTD) has also been reported in the Ts65Dn mouse [183]. Scott-McKean and Costa [188] have demonstrated that increased cerebellar LTD, mediated by exaggerated NMDAR-dependent mechanisms, could be rescued by the administration of the NMDA receptor antagonist memantine.

In addition, overinhibition in the hippocampus of Ts65Dn mice has also been shown to be dependent on $GABA_A$ receptors [172] because the $GABA_A$ antagonist picrotoxin rescued the reduction in LTP induced by theta-burst stimulation (TBS) in these mice [184]. Furthermore, Kleschevnikov et al. [189] have shown that both $GABA_A$ and $GABA_B$ receptor-mediated components of evoked inhibitory postsynaptic currents (IPSCs) were significantly higher in Ts65Dn mice, suggesting an increase in presynaptic release of GABA. Thus, both $GABA_A$ and $GABA_B$ receptors are implicated in the reduced synaptic efficiency found in the DG of Ts65Dn mice.

The G-protein-activated inwardly rectifying potassium channel 2 (Girk2) gene is overexpressed in DS individuals. Girk channels generate a $GABA_B$ receptor-dependent slow inhibitory postsynaptic potential in hippocampal neurons [190]. It has been proposed that an increase in Girk2 gene expression may produce overinhibition in hippocampal neurons and contribute to LTP failure in the trisomic condition [191].

5.1. Therapies Targeting Overinhibition. Because overinhibition in the trisomic brain appears to underlie LTP impairments and, therefore, alter learning and memory processes, a number of studies have tested drugs that reduce GABA-mediated inhibition in an attempt to rescue the electrophysiological substrates of cognition.

It is well established that the $GABA_A$ receptor system plays an important role in cognition. Nonselective positive modulators of the $GABA_A$ receptor disrupt learning and memory processes [192–194], while nonselective negative modulators improve cognitive processes [195–197]. Reducing inhibition in the Ts65Dn brain by administering the $GABA_A$ antagonists picrotoxin (PTX), bilobalide (BB), or pentylenetetrazole (PTZ) restored LTP and cognition in the object recognition test in these mice [185]. Rueda et al. [198] confirmed that chronic PTZ treatment also rescued Ts65Dn mice performance in the Morris water maze.

However, non-selective $GABA_A$ negative modulators cannot be safely used to improve cognition due to their anxiogenic and proconvulsant effects [199]. Among the different $GABA_A$ receptor subtypes, $GABA_A$ $\alpha 5$ subunit-containing receptors are known to facilitate cognition in hippocampal-dependent tasks [200, 201]. Moreover, selective $GABA_A$ $\alpha 5$ negative allosteric modulators, also called inverse agonists, have cognition-enhancing effects without anxiogenic or proconvulsant side effects [202–204]. A functionally selective $GABA_A$ $\alpha 5$ inverse agonist, $\alpha 5$IA, has been shown to rescue learning and memory deficits in TS mice without inducing anxiogenic and convulsant side effects [205].

Further support for the efficacy of reducing GABA-mediated overinhibition to improve cognition in trisomic mice comes from a recent report that demonstrated that environmental enrichment reduced the release of GABA in the hippocampus and visual cortex of Ts65Dn mice while rescuing spatial learning and hippocampal LTP [206].

6. Altered Neurotransmission and Receptors

Alterations in several neurotransmitters and changes in the expression and function of their receptors, in both DS individuals and mouse models of this condition have been demonstrated. These impairments may be responsible for other phenotypes found in trisomic conditions, such as defects in neurogenesis, synaptic transmission, and cognition. Dopamine, taurine, and histamine levels have been shown to be altered in the brains of DS fetuses and adults [97, 98, 207–209]. The main neurotransmitter and receptor alterations in DS and in the Ts65Dn mouse model are summarized in Table 4.

6.1. GABA. GABA is reduced in DS fetuses [207]. However, as predicted from the enhanced inhibition of the trisomic brain, an increase in the number of inhibitory neurons has been found in Ts65Dn mice due to the overexpression of the Olig1 and Olig2 genes (see above) [103, 178]. Furthermore, it has been suggested that enhanced presynaptic GABA release may be responsible for the increased hippocampal inhibitory postsynaptic potentials (IPSPs) observed in these mice [189].

A number of alterations have been reported in the expression of various GABA receptor subunits. In neurospheres from fetuses with DS, upregulation of the $\alpha 2$ and downregulation of the $\alpha 5$ and $\beta 3$ subunits of the $GABA_A$ receptor have been reported [210]. In the hippocampus of Ts65Dn mice, reductions in the number of $\beta 2$ and $\beta 3$ subunits of the $GABA_A$ receptor were found [69]. Brain synaptosomes of Ts65Dn mice show a reduction in $GABA_A$ $\alpha 1$ receptor expression [211]. Changes in the R1 subunit of the $GABA_B$ receptor have also been reported in Ts65Dn mice [69]. However, Kleschevnikov et al. [189] did not find changes in the levels of $GABA_A$ or $GABA_B$ receptor subunits by western blot analysis.

$GABA_A$ activity is known to regulate neuronal proliferation, migration, differentiation, and integration of newly generated neurons [212–214]. The enhanced $GABA_A$-mediated inhibition shown by Ts65Dn mice could, therefore, be implicated in the alterations in neuronal proliferation and survival found in these mice.

6.2. Excitatory Transmitters. Increased inhibition in the trisomic condition is also caused by alterations in excitatory transmission. Although similar levels of glutamate [215] are found in fetuses with and without DS, decreased levels of aspartate and glutamate have been found in several areas of the adult DS brain [97, 98, 208].

As detailed above, altered hippocampal LTP in trisomic mice suggests disturbances in NMDA receptor signaling. In Ts65Dn mice, a reduction of the GluR1 subunit of the AMPA receptor [69] and of the NR2A and NR2B subunits of

Table 4: Neurotransmitter and receptor alterations in DS and in the Ts65Dn mouse model of Down syndrome.

	DS	Ts65Dn
GABA	(i) Reduced in fetuses	(i) Increased number of GABAergic interneurons
Excitatory transmitters	(i) Decreased levels of glutamate and aspartate in adults	(i) Alterations in the composition of the AMPA and NMDA receptor (ii) Alterations in the signalling mechanisms downstream the NMDA receptor
5-HT	(i) Deficits of 5-HT in the frontal cortex (ii) Reduced levels of the 5-HT_{1A} receptor	(i) Unchanged levels of 5-HT (ii) Reduced levels of the 5-HT_{1A} receptor
Ach	(i) Deficits in the cholinergic system and in ChAT activity	(i) Reduced levels of markers for Ach (ii) Increased ChAT activity
NA	(i) Reduced levels in adult brains (ii) Altered β-adrenoceptor function in aged brains	(i) Loss of locus coeruleus neurons starting at 6 months of age (ii) Altered β-adrenoceptor function
Neurotrophins		
(i) BDNF (ii) NT3 (iii) NGF	(i) Reduced levels in fetuses	(i) Reduced levels (ii) Increased levels (iii) Reduced levels

the NMDA receptor [216] has been reported. However, other studies failed to find changes in the GluR1 subunit in brain homogenates or changes in the NR2A and NR2B subunits in synaptosomes of these mice [211].

Ts65Dn and Ts1Cje mice exhibit hypersensitivity to the locomotor stimulatory effect of MK-801, an NMDA receptor channel blocker [217].

Alterations in the signaling mechanisms downstream of the NMDA receptor have also been reported; the hippocampi of Ts65Dn mice show disturbances in the calcium/calmodulin-dependent protein kinase II (CaMKII), phosphatidylinositol 3-kinase (PIP3K)/Akt, extracellular signal-regulated kinase (ERK), protein kinase A (PKA), and protein kinase C (PKC), all of which have been shown to be involved in synaptic plasticity [218].

One of the targets of the NMDA receptor is the protein phosphatase calcineurin (CaN). The DSCR1 gene encodes a protein that inhibits CaN, and this gene is overexpressed in the Ts65Dn brain [219]. The inhibition of CaN activity increases the mean open time and opening probability of the NMDA receptor [220]. Memantine, a partial agonist of the NMDA receptor, often prescribed for the treatment of AD-dementia, acts as an open-channel blocker and has been proposed to mimic the actions of CaN and restore the function of the NMDA receptor. Costa et al. [221] demonstrated that the acute administration of memantine improved contextual fear conditioning in Ts65Dn mice. Chronic treatment with memantine also improved Ts65Dn mice performance in the Morris water maze [90] and in the novel object recognition test and water radial arm maze [222]. Memantine slightly reduced brain APP levels and normalized the levels of hippocampal excitatory synapses in Ts65Dn mice [90]. However, memantine did not rescue Ts65Dn morphological alterations, as the number of hippocampal granule [90], basal forebrain cholinergic, and locus coeruleus neurons [222] remained low in memantine-treated Ts65Dn mice. Nevertheless, these mice showed increased BDNF levels in the hippocampus and the prefrontal cortex.

In spite of the rescue induced by memantine of several DS-relevant phenotypes in the Ts65Dn mouse, a recent randomized double-blind clinical trial failed to find any benefit of memantine administration for 52 weeks on cognitive impairment and dementia in DS individuals over 40 years of age [223].

6.3. Serotonin. Deficits in serotonin (5-HT) have been reported in the frontal cortex of DS fetuses [207] and in adult DS brains [97, 98, 209]. However, Ts65Dn mice show unchanged levels of 5-HT in the hippocampus [54], and no alterations were found in the histological analysis of serotonergic neurons of the dorsal and medial raphe nuclei of these mice [224]. 5-HT has a role in neurogenesis, neuronal differentiation, dendritic development, axon myelination, and synaptogenesis [225]. Thus, the reduction of this transmitter in DS fetal and adult brains may underlie a number of altered neuromorphological and cognitive phenotypes.

The 5-HT_{1A} receptor has also been implicated in the regulation of neurogenesis [113, 226, 227]. Reduced levels of the 5-HT_{1A} receptor have been reported in the DS brain at birth [228], in hippocampal neurospheres, and in the hippocampus of newborn Ts65Dn mice [54]. Thus, reduced 5-HT_{1A} receptor expression may underlie the defective neurogenesis found in Ts65Dn mice [54]. Moreover, treatment with the 5-HT_{1A} reuptake inhibitor fluoxetine rescued the expression levels of this receptor in Ts65Dn mice, suggesting that this effect may underlie the rescue of proliferation produced by this drug, as previously mentioned.

6.4. Acetylcholine. One of the anatomical substrates of learning and attention is the septohippocampal cholinergic system [95, 96]. A number of studies have demonstrated alterations of this system in the trisomic condition. Deficits in the cholinergic system have been found in DS fetuses [207], and choline acetyltransferase (ChAT) activity is reduced in the brains of adults with DS [97, 98].

Although a normal number of cholinergic neurons is found in young Ts65Dn mice, basal forebrain cholinergic neurons (BFCNs) degenerate with age in these mice [99–102]. However, ChAT activity is increased in the cortex and hippocampus of 10-month-old Ts65Dn mice, likely in an attempt to compensate for the reduced number of cholinergic neurons [82, 100, 229, 230].

6.5. Noradrenaline. The levels of noradrenaline (NA) are normal in DS fetuses [207] but are reduced in adult DS brains [97, 98], likely as a consequence of the neurodegeneration of the locus coeruleus [231, 232]. Ts65Dn mice also show a loss of locus coeruleus neurons starting at 6 months of age [53].

Ts65Dn mice show unchanged numbers of β-adrenoceptors in the cortex and hippocampus; however, their function is altered. Basal production of cyclic AMP in the hippocampus of TS mice was impaired. In addition, the responses of adenylyl cyclase to the stimulation of β-adrenoceptors with isoprenaline and of the catalytic subunit with forskolin were both severely depressed [233, 234]. Aging DS brains also show a dramatic reduction in basal and stimulated cAMP production [235].

NA has been shown to play a role in neurogenesis, as neuron proliferation is enhanced or impaired following increases or reductions in NA transmission, respectively [236]. Therefore, altered NA transmission in the trisomic condition may also play a role in the impairment of adult hippocampal neurogenesis. Furthermore, a link between noradrenergic afferents from the locus coeruleus to hippocampal neurons and contextual learning has been demonstrated [237]. This hippocampal-dependent cognitive process is impaired in individuals with DS [6] and in Ts65Dn mice [53]. A recent study by Salehi et al. [53] demonstrated that enhancing NA transmission through the administration of L-Threo-3, 4-dihydroxyphenylserine (L-DOPS), a synthetic amino acid that is metabolized by NA-containing neurons to produce NA, or xamoterol, a ß1-adrenergic receptor partial agonist, rescued contextual learning in Ts65Dn mice. These authors hypothesized that, given the finding that NA can activate or inhibit GABAergic neurons and that GABA can increase the release of NA, there could be an overlap in the mechanisms by which $GABA_A$ antagonists and NA-enhancing drugs improve learning in Ts65Dn mice.

6.6. Neurotrophins. The role of neurotrophins (NT) in neuronal survival, differentiation, migration, and synaptic plasticity is well documented [238–240]. Consequently, alterations in their expression may alter many aspects of neurodevelopment.

The reduced expression of BDNF has been observed in the hippocampus of DS fetuses [241], and the reduced expression of both BDNF and the tyrosine kinase receptor TrkB has been observed in the cerebral cortex of DS fetuses [242]. Young Ts65Dn mice also show reduced BDNF levels in the hippocampus [54, 243] and in the frontal cortex during adult stages [244]. Because BDNF has a role in neuronal survival and differentiation [213, 215], it is a natural target for several treatments to restore neurogenesis in the trisomic brain. In Ts65Dn mice, fluoxetine restored BDNF expression, survival of newborn cells, differentiation, and granule cell number.

NT-3 is increased in the hippocampus of newborn and adult Ts65Dn mice [245], potentially in an attempt to compensate for the neuronal loss found in these mice.

Nerve growth factor (NGF) is generated in the hippocampus and retrogradely transported to the soma of BFCNs [239]. NGF levels are reduced in the hippocampus of young Ts65Dn mice [50], and the retrograde transport of NGF to the basal forebrain is hindered in older Ts65Dn and Ts1Cje mice [50, 100]. NGF enhances the survival, differentiation, and maintenance of neurons, including BFCNs [239]. The administration of NGF to Ts65Dn mice rescued the altered size and number of BFCNs [100].

Peptide 6, an active region of ciliary neurotrophic factor (CNTF), modulates the CNTF pathway by inhibiting the antineurogenic activity of leukemia inhibitory factor, thereby increasing neurogenesis [246]. Administration of peptide 6 to Ts65Dn mice reduced learning and memory deficits, enhanced the pool of neural progenitor cells in the hippocampus, and increased the level of synaptic proteins crucial for synaptic plasticity [247].

Considering the role of Dyrk1A in neuronal progenitor proliferation, neurogenesis, and neurodifferentiation, it has been suggested that molecules targeting this gene could provide therapeutic benefits to DS phenotypes. Epigallocatechin gallate (EGCG), an antioxidant extracted from green tea, is an inhibitor of the protein kinase DYRK1A [248].

The chronic administration of EGCG from conception to adulthood rescued BDNF levels in the hippocampus of Dyrk1a transgenic mice [241]. Concomitant to this neurotrophic factor normalization, these mice presented an increase in brain volume and improved cognitive performance. Other studies have demonstrated that the acute administration of EGCG normalizes hippocampal LTP in Ts65Dn mice [249]. However, EGCG affects a wide array of signal transduction pathways including the MAPK, PI3K/AKT, Wnt, and Notch pathways [250], which are altered in Ts65Dn mice [135]; thus, its beneficial effects could be mediated by mechanisms different from Dyrk1A inhibition.

Finally, Fukuda et al. [243] have recently demonstrated that the chronic administration of the analgesic neurotrophin to Ts65Dn mice prevents the age-dependent decline in hippocampal BDNF expression. This treatment also enhanced the performance of these mice in the radial arm maze. It has been proposed that the analgesic action of neurotrophin is mediated by the noradrenergic and GABAergic systems [251]; therefore, the cognitive-enhancing effects could also be determined by improvements in the function of these transmitter systems.

7. Neurodegeneration

Although neurodevelopmental alterations occurring from early embryonic stages are likely to cause intellectual disability, there are a number of neurodegenerative mechanisms in DS that complicate this scenario. Atrophy of a number of structures, including the hippocampus, amygdala [71, 252],

Table 5: Neurodegenerative processes in DS and in the Ts65Dn mouse.

	DS	Ts65Dn
Neuroinflammation	(i) Activated microglia and increased levels of proinflammatory cytokines	(i) Activated microglia
Oxidative stress	(i) Increased	(i) Increased
AD neuropathology	(i) Cholinergic neuron loss, plaques, and tangles	(i) Cholinergic neuron degeneration, increased APP and β-amyloid levels

the corpus callosum, and the parietal, frontal, and occipital cortices [77, 78], has been reported in nondemented adult DS brains. Furthermore, neuroinflammation, increased oxidative stress, and the development of AD neuropathology are hallmarks of DS (Table 5).

7.1. Neuroinflammation. DS and AD brains are characterized by activated microglia, and increased levels of proinflammatory cytokines that lead to neuroinflammation are likely involved in neurodegeneration [253, 254]. The activation of microglia may play a role in the loss of basal forebrain cholinergic neurons in Ts65Dn mice.

Minocycline is a semisynthetic tetracycline that inhibits neuronal death and reduces inflammatory activity by blocking inflammatory mediators [255]. The chronic administration of minocycline to adult Ts65Dn mice inhibits microglia activation in the basal forebrain and hippocampus, prevents the loss of cholinergic neurons in the medial septal nucleus, attenuates the loss of hippocampal calbindin-positive neurons, and improves working and reference memory in these mice [102].

7.2. Neuropeptides. Vasoactive intestinal peptide (VIP) is neuroprotective, as it promotes the release of several survival factors from astrocytes and regulates neuropeptide release from glial cells, including activity-dependent neuroprotective protein (ADNP) and activity-dependent neurotrophic factor (ADNF) [256]. The active peptide fragments of ADNP and ADNF, NAPVSIPQ (NAP), and SALLRSIPA (SAL) have been shown to protect neurons from oxidative stress and limit the severity of traumatic head injuries, stroke, and the toxicity associated with the Aβ peptide [257, 258].

In cultures of DS cortical neurons, treatment with SAL or NAP increases neuronal survival [259]. In Ts65Dn mice, prenatal treatment with these two peptides rescued the acquisition of neurodevelopmental milestones [260], increased the reduced levels of ADNP, and normalized the levels of the NMDA receptor subunits NR2A, NR2B, and the $GABA_A$ receptor subunit $\beta 3$ [216]. Furthermore, subchronic treatments with D-NAP and D-SAL to adult Ts65Dn mice rescued learning and memory and ADNP and NRD2 levels [256].

7.3. Oxidative Stress. In DS individuals and in the Ts65Dn mouse, there is an overexpression of SOD1, the gene responsible for the formation of superoxide dismutase, an enzyme that modifies oxygen free radicals into hydrogen peroxide. The overproduction of hydrogen peroxide leads to the overproduction of highly reactive oxygen free radicals, which damage cell membranes, including the mitochondrial membrane, and deteriorate lipids, proteins, and mitochondrial DNA. This set of alterations is called oxidative stress. Evidence for increased mitochondrial superoxide production in DS individuals has been repeatedly demonstrated [261, 262]. Therefore, in this condition, some cells are under the permanent threat of oxidative stress with mitochondrial damage, which deteriorates cell life, facilitating aging, and death. This increase in oxidative stress occurs during pre- and postnatal development. Increased oxidative stress in the fetal stages can modify processes such as neurogenesis, differentiation, migration and net connexion, as well as survival [261, 263–265].

In an attempt to reduce oxidative stress-induced neurodegeneration, several groups have tested the efficacy of various antioxidants to reduce the altered phenotypes in Ts65Dn mice. SGS-111, an analogue of the nootropic piracetam, has been shown to increase neuronal survival and prevent the accumulation of intracellular free radicals, peroxidative damage, and the development of neurodegenerative changes in both normal and DS cultured neurons [265]. However, the chronic administration of SGS-111 to Ts65Dn mice from conception to adulthood did not rescue their cognitive alterations [266]. Conversely, the administration of another antioxidant, vitamin E, to Ts65Dn mice during adult stages [267] or from conception throughout their entire life [268] reduced markers of oxidative stress, improved cognitive performance, reduced cholinergic neuron pathology in the basal forebrain, and increased cell density in the DG.

A recent report [269] revealed a positive effect of folinic acid on developmental age in children with DS. Folinic acid has an antioxidant effect and is known to be involved in CNS development. Folate deficiency causes neurological, psychiatric, and cognitive disorders, and DS probably involves either folate deficiency or defective folate use [270]. However, in a randomized controlled trial, Ellis et al. [271] failed to find any efficacy of antioxidants and folinic acid supplementation in children with DS cognitive development. In addition, a number of studies on the effects of antioxidant supplementation in children and adults with DS did not find any benefit of this treatment on cognition. Salman [272] performed a systematic review of eleven randomized controlled trials on the effects of dietary supplements (vitamins and/or minerals) on cognitive function in subjects with DS. None of these trials reported cognitive enhancing effects in individuals with DS. Moreover, in a two-year randomized, double blind, placebo-controlled trial daily oral

antioxidant supplementation (α-tocopherol, ascorbic acid, and the mitochondrial cofactor: α-lipoic acid) did not produce any improvement in cognitive functioning nor a stabilization of cognitive decline in adults with DS [273].

7.4. Estrogens. Because estrogens maintain the function of basal forebrain cholinergic neurons, it has been proposed that the administration of estrogens may be useful in reducing the loss of these neurons in AD and DS individuals [274].

The chronic administration of estrogens to aged female Ts65Dn mice enhanced cognition, increased the size and number of cholinergic neurons, increased the levels of NGF in the medial septum [275], restored the number of cholinergic terminals in hippocampus, and restored the levels of the dendritic marker Map2 [276].

7.5. AD Neuropathology. One of the genes triplicated in the trisomic condition is APP. In DS individuals, the increased expression of this gene leads to the increased production of β-amyloid, which is thought to be responsible for the amyloid plaque pathology and degeneration of BFCNs found in 100% of DS individuals over 40 years of age. Ts65Dn mice also show age-related elevations in the levels of the APP protein [277] and the β-amyloid peptide [278] in the cortex and hippocampus. In these mice, the overexpression of APP has also been implicated in the degeneration of both the cholinergic and noradrenergic neurons that provide strong modulatory inputs to the hippocampus [279]. Thus, this age-related noradrenergic and cholinergic deafferentation is likely compromising hippocampal function.

To test the effect of β-amyloid reductions on the Ts65Dn mice altered phenotypes, Netzer et al. [278] administered the gamma secretase inhibitor DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester). This treatment reduced β-amyloid levels and rescued spatial learning in these mice. Because β-amyloid is a regulator of the glutamatergic system, the authors proposed that the cognitive enhancing effects of DAPT could be mediated by an enhancement and/or a regulation of excitatory synaptic transmission.

Given the role of the cholinergic system in cognition and the degeneration of this system in AD and DS individuals, it has been proposed that pharmacological enhancements of this system could help diminish cognitive deterioration in these conditions. Donepezil is an acetylcholinesterase inhibitor that is widely prescribed to enhance cholinergic transmission in the treatment of AD dementia. However, the chronic administration of donepezil did not improve learning and memory in Ts65Dn mice [198]. Similarly, donepezil administration to young adult individuals with DS has produced ambiguous results [280–283].

Piracetam is a drug that shows cognitive-enhancing effects in patients with a number of cognitive disorders and dementia [284] and in several animal models. Although the mechanisms underlying these effects are not known, it has been proposed that piracetam may be enhancing cholinergic and modulating glutamatergic transmission [284]. However, piracetam treatment did not improve cognitive impairments in children with DS [285] or in the Ts65Dn mouse [286].

8. Concluding Remarks

The first partial trisomic models, the Ts65Dn and Ts1Cje models, demonstrated that DS phenotypes could be recapitulated in mice. More recently, knockout and transgenic mice for individual genes and new animals that are trisomic for different regions of orthologues of Hsa21 regions are helping to identify dosage-sensitive genes involved in DS phenotypes. Although some of these triplicated genes may play a role individually, it appears that DS phenotypes arise from the complex effects of groups of Hsa21 genes.

In the last 20 years, the characterization of these animal models of DS, particularly the Ts65Dn mouse, has been enormously useful to understand of the neurobiological basis of intellectual disability. Several mechanisms have been proposed to underlie this altered cognition, including impaired neurogenesis leading to hypocellularity in the cortex, hippocampus, and cerebellum, altered dendritic morphology, altered synapses, increased inhibition and neurodegeneration. The new knowledge of the pathogenic mechanisms in DS individuals has been applied to the development of new pharmacotherapies. Several drugs have been shown to rescue neurogenesis, hypocellularity, electrophysiological deficits, and cognitive alterations in the Ts65Dn mouse. These studies provide the basis for developing clinical trials in DS individuals and sustain the hope that some of these drugs will be useful in rescuing intellectual disability in DS individuals.

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Review Article

Matrix Metalloproteinases and Minocycline: Therapeutic Avenues for Fragile X Syndrome

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Fragile X syndrome (FXS) is the most common known genetic form of intellectual disability and autism spectrum disorders. FXS patients suffer a broad range of other neurological symptoms, including hyperactivity, disrupted circadian activity cycles, obsessive-compulsive behavior, and childhood seizures. The high incidence and devastating effects of this disease state make finding effective pharmacological treatments imperative. Recently, reports in both mouse and *Drosophila* FXS disease models have indicated that the tetracycline derivative minocycline may hold great therapeutic promise for FXS patients. Both models strongly suggest that minocycline acts on the FXS disease state via inhibition of matrix metalloproteinases (MMPs), a class of zinc-dependent extracellular proteases important in tissue remodeling and cell-cell signaling. Recent FXS clinical trials indicate that minocycline may be effective in treating human patients. In this paper, we summarize the recent studies in *Drosophila* and mouse FXS disease models and human FXS patients, which indicate that minocycline may be an effective FXS therapeutic treatment, and discuss the data forming the basis for the proposed minocycline mechanism of action as an MMP inhibitor.

1. Introduction

Fragile X syndrome (FXS), the most common heritable cause of intellectual disability and autism spectrum disorders [1], has a prevalence of roughly 1 in 4000 males and 1 in 6000 females [2, 3]. While a hallmark of FXS is low IQ (~40), patients present with a wide spectrum of behavioral, physical, and neurological symptoms [4–6]. Behavioral problems include hyperactivity and hypersensitivity to sensory stimuli, anxiety and mood disorders, disrupted sleep patterns, defects in cognitive learning and memory consolidation, and impaired social skills [1, 4, 5, 7–14]. Often, a speech deficit is the first symptom leading to a FXS clinical diagnosis [4, 5]. Although there are usually not significant non-neurological medical impairments associated with the syndrome, FXS patients typically display male macroorchidism, macrocephaly with prominent ears and a long, thin face, joint hypermobility, and flat feet [4, 5, 15–17]. Elevated electroencephalogram (EEG) activity is characteristic, and epileptic seizures are present in ~20% of FXS patients, typically with remittance by adulthood [18].

FXS is caused by loss of the *fragile X mental retardation 1* (*FMR1*) gene product, FMRP [19], typically due to expansion (>200) of the CGG trinucleotide repeat in the 5′-untranslated gene region, leading to subsequent hypermethylation and gene silencing [20]. FMRP is an mRNA-binding protein known to regulate mRNA stability, trafficking, and translation [21–26], with roles in the activity-dependent regulation of synaptic development and plasticity [26–33]. Numerous studies support the "mGluR theory of FXS" that suggests enhanced group 1 metabotropic glutamate receptor 5 (mGluR5) signaling is responsible for deficits in synaptogenesis, dendritic spine morphology, long-term potentiation (LTP) and depression (LTD) in the disease state [5, 27, 28, 30–32, 34–43]. Consequently, many studies have focused on mGluR inhibitors, such as 2-methyl-6-phenylethynyl-pyridine (MPEP), as a therapeutic intervention for FXS [31, 38–40, 43–47], with considerable

success. For example, in the *Drosophila* disease model, MPEP effectively prevents cellular synaptic deficits and behavioral learning and memory impairments [31, 42, 45, 46]. While some mGluR inhibitors cannot be used in FXS patient treatment due to toxicity and bioavailability limitations (e.g., MPEP), other drugs, such as the selective mGluR5 inhibitor fenobam, are currently in human clinical trials [48, 49]. Lithium, an inhibitor of GSK3β, a downstream effector of mGluR5 signaling, is also being taken to clinical trials with promising results as a potential FXS therapeutic treatment [50].

In addition to the promise of mGluR5 pathway interventions, several recent reports suggest minocycline as another potential avenue of FXS therapeutic treatment [51–54]. Used for decades as an antibiotic and acne treatment, the second-generation, semisynthetic tetracycline derivative minocycline has a long half-life, highly lipophilic characteristics, and easily crosses the blood-brain barrier [55]. In addition to its antibiotic actions, minocycline also functions as an anti-inflammatory agent via inhibition of several molecules, including COX-2, iNOS, and p38 MAPK, and as an anti-apoptotic agent via inhibition of caspases, among many other putative modes of action [55, 56]. Central to its role in FXS, minocycline is known to inhibit matrix metalloproteinases (MMPs), a family of secreted and membrane-tethered zinc-dependent extracellular proteases with roles in tissue remodeling and intercellular signaling [57–60]. Functioning through one or more of these modes of action, minocycline has been shown to have neuroprotective effects [55] and has been previously suggested to be useful in the treatment of several neurodegenerative diseases, including multiple sclerosis [61], amyotrophic lateral sclerosis (ALS) [62, 63], Huntington's disease [64], Parkinson's disease [65], and Alzheimer's disease [66].

In the FXS disease state, several recent studies have proposed that minocycline exerts its therapeutic effects via MMP inhibition [51, 53]. In humans, a diverse array of at least 24 MMPs functions to cleave components of the extracellular matrix (ECM), including both secreted and cell membrane proteins [57–59]. In general, MMPs contain a pro domain that is cleaved to activate the extracellular protease, an enzymatic zinc-containing catalytic domain, a linker domain, and a hemopexin domain [57–59]. MMPs are part of the metzincin family of proteases, named for a conserved methionine residue and zinc in the protease catalytic sites, which includes ADAMs (a disintegrin and metalloproteinase) and ADAM proteases with thrombospondin motifs (ADAMTSs) [57–59, 67]. In the central nervous system, MMPs have been implicated in axonal guidance, synaptogenesis, neurotransmission, synaptic plasticity, and behavioral learning [57, 58, 67]. MMPs are endogenously inhibited by tissue inhibitors of MMPs (TIMPs), with 4 family members in humans [68, 69]. Along with MMPs, TIMPs are also expressed in the central nervous system, where they also regulate synaptic mechanisms and behavioral outputs [67–69].

In this paper, we review recent evidence that minocycline acts as an effective therapeutic treatment in FXS genetic animal models and human FXS patient clinical trials. We then summarize current data supporting an "MMP inhibition mechanism" by which minocycline may remediate the FXS disease state. Finally, we consider possible pathways through which the MMP/TIMP and FMRP pathways could intersect in the formation of FXS pathogenesis.

2. Minocycline in Fragile X Animal Models

Minocycline was first revealed as a possible FXS therapeutic treatment in the mouse disease model [51]. The *FMR1* knockout (KO) mouse is a well-validated model of the human disease state [30, 40, 51, 71, 72], which displays macroorchidism, hyperactivity, and some learning/memory deficits [71–73]. As with cortical postsynaptic dendritic spine morphogenesis defects in FXS patient brain autopsies [74, 75], *FMR1* KO mice exhibit an immature dendritic spine profile with more long, thin filopodia-like spines and a proportional lack of mature short, stubby, mushroom-like spines [76–79]. This synaptic maturation/overgrowth defect has been reported to vary in severity/penetrance in different brain regions and may be developmentally transient, with the defect being most prominent during neural circuit refinement stages of early postnatal development [80, 81]. The dendritic spine phenotype of *FMR1* KO mice has been linked with defects in postsynaptic group 1 mGluR signaling activity, as with defects in synaptic plasticity [82]. This postsynaptic dendritic spine defect has long been considered the FXS neuroanatomical hallmark and was the logical choice to first assay the effects of minocycline treatment.

In 2009, Bilousova and colleagues provided the first evidence for the therapeutic effectiveness of minocycline in the *FMR1* KO mouse with a thorough examination of dendritic spine profiles of hippocampal neurons both *in vitro* and *in vivo* [51]. This study showed that 20 μM minocycline promoted maturation of dendritic spines in control hippocampal neuronal cultures. In *FMR1* KO neurons, minocycline treatment in culture (20 μM for 17 hours) or fed to mice (30 mg/kg/day in drinking water) shifted the immature dendritic spine profiles towards normal dendritic spine profiles (Table 1). It is important to note that no change was reported in dendritic spine length or the total number of spines between *FMR1* KO untreated and treated conditions; rather, there was solely a proportional shift of the number of immature to mature spines upon minocycline treatment [51]. Following this neuroanatomical analysis, the same study examined the effect of minocycline treatment on *FMR1* KO mouse behavior [51]. Minocycline was again fed to newborn mice via their drinking water at levels of 30 mg/kg/day. At 3 weeks of age, treated *FMR1* KO mice were found to be less anxious in an elevated plus maze assay (Table 1). Moreover, minocycline treatment resulted in better memory in a passive Y maze, compared to untreated *FMR1* KO mice [51]. Additionally, a follow-up study was very recently conducted by this same group [70]. In *FMR1* KO adult male mice, it was found that the rate of ultrasonic vocalizations (USVs) produced during mating is reduced. No other acoustic property deficits were identified. Interestingly, minocycline treatment restored a normal rate of USVs in the *FMR1* KO

Table 1: Summary of recent minocycline treatment trials in *Drosophila* and mouse disease models, and human clinical studies. The columns display the systems, minocycline dosages, phenotypes tested, treatment outcomes and side effects.

Model	Dosage	Phenotypes tested	Treatment effects	Side effects	Study
Drosophila	20 μM in larvae; 1 mM in adult (oral feeding)	Synpatic structure of NMJ, sLN_v, and MB neurons	Prevention of all neuroanatomical defects	None	Siller and Broadie, 2011 [53]
Mouse	20 μM for 17 hrs *in vitro*; 30 mg/kg/day *in vivo*	Immature dendritic spine profiles, anxiety, memory defects, decreased rate of USVs	More mature dendritic spine profiles, less anxious, memory improvements, increased rate of USVs	More mature dendritic spines	Bilosuova et al., 2009; [51] Rotschafer et al., 2012 [70]
Human	50 mg BID (low dose) 100 mg BID (high dose)	Behavioral symptoms	Better language and social communication skills, less anxiety, more attentive; Better irritability, stereotypy, hyperactivity, inappropriate speech subscales on ABC-C	GI issues, diarrhea, loss of appetite, dizziness, headaches	Utari et al., 2010; [54] Paribello et al., 2010 [52]

mouse (Table 1). Thus, minocycline was shown to be effective at ameliorating both neuroanatomical and behavioral FXS defects in the mouse disease model.

Recently, a study employing the *Drosophila* FXS disease model corroborated and expanded these data, reinforcing the therapeutic potential of minocycline treatment [53]. Over more than a decade of research, the *Drosophila* system has been firmly established as a highly validated model of the human disease state [25, 26, 31, 42–47, 83–99]. *Drosophila* contains a single *FMR1* homolog, *dFMR1*, compared to the three Fragile X family genes present in mammals (*FMR1*, *FXR1,* and *FXR2*). Only human *FMR1* rescues the broad range of neurological phenotypes caused by *dFMR1* KO, with human *FXR1/2* having no activity, showing that *FMR1* function has been evolutionarily conserved and that human *FMR1* requirements can be effectively dissected in the *Drosophila* FXS disease model [84, 99]. Like human patients and the mouse model, *dFMR1* KO flies exhibit synaptic overgrowth and synaptic immaturity in a range of neural circuits, including motor neurons [84, 86, 99], clock neurons [84, 87, 88, 99], and learning/memory neurons [26, 42]. Likewise mimicking the human disease condition, *dFMR1* null animals display macroorchidism and deficits in spermatogenesis, hyperactivity and circadian arrhythmicity, and strong deficits in learning formation and memory consolidation [46, 83, 85, 87, 88, 92]. The breadth and FXS disease relevance of these phenotypes makes *Drosophila* an excellent system to examine minocycline's effectiveness in treating the disease state.

In 2011, Siller and Broadie provided a follow-up study examining the therapeutic effectiveness of minocycline in the *dFMR1* KO in a thorough examination of synaptic architecture in a range of disparate neural circuit types [53]. Synapse structure was analyzed in three locations: (1) the well-characterized glutamatergic neuromuscular junction (NMJ) in the peripheral musculature, (2) the pigment dispersing factor (PDF) neuropeptidergic small ventrolateral (sLN_v) clock neurons in the central brain, and (3) Kenyon cell neurons of the brain mushroom body (MB) learning and memory center (Table 1). In all three circuit types, the *dFMR1* KO displays the same characteristic synaptic overgrowth and overelaboration, including an expanded synaptic arbor domain, increased synaptic branching, and increased supernumerary synaptic boutons with prominent structurally immature synaptic sites [25, 26, 42, 47, 86, 87]. Minocycline was fed to *dFMR1* KO mutants in their food during larval development in the range of 2–20 μM and to adults at the higher dosage of 1 mM (Table 1), resulting in a dosage-dependent improvement of synaptic architecture towards the wildtype state [53]. Interestingly, the CNS synaptic deficits responded better to minocycline treatment than the NMJ defects, although the reason for this difference is currently unknown. At the NMJ, minocycline treatment completely prevented the accumulation of immature synaptic boutons and partially prevented the overabundance of mature boutons, but had no effect on the defect in synaptic branching in *dFMR1* KOs [53]. In the brain, both in clock neurons and in MB Kenyon cells, minocycline treatment both prevented the excess synaptic branching and completely rescued the overelaboration of synaptic boutons in *dFMR1* KOs (Table 1). Unlike the mouse study, the *Drosophila* study found no effect of minocycline treatment on wildtype synaptic architecture [53]. Moreover, the *Drosophila* study contained no behavioral analyses. However, at a neuroanatomical level, the *Drosophila* study corroborates the findings of the mouse study and adds further evidence for the effectiveness of minocycline as a broad-spectrum FXS therapeutic treatment in multiple classes of neural circuits [51, 53].

3. Minocycline in Fragile X Patients

Taken together, the two animal model studies strongly suggest minocycline may be an effective FXS treatment. However, the obvious question still remains: does the drug effect translate to human FXS patients? In 2010, two studies began to provide insight into this question with early clinical trials [52, 54]. Utari and colleagues studied 50 FXS patients, given minocycline for 2 weeks or longer, mainly to assess

the safety of the drug as a FXS treatment [54]. Of the 50 patients examined, 21 reported side effects, with the most common being gastrointestinal problems, including diarrhea and loss of appetite (Table 1). Most patients reported side effects as mild. One patient did experience coloring of the nails, but none reported tooth discoloration. Of the FXS patients followed throughout the course of the study, most displayed improvements in several areas, including language, attention, social communication, and anxiety [54]. A small subset of patients exhibited worsening in two areas: hyperactivity and moodiness (Table 1). Thus, this study suggests minocycline may be effective in FXS treatment with only mild side effects, and the results warrant a followup, controlled study to more closely examine minocycline effectiveness.

Paribello and colleagues performed an open-label, add-on minocycline treatment trial with 19 FXS patients aged 13–32 years followed for an 8-week treatment period [52]. One patient dropped out due to side effects, and two other patients developed asymptomatic seroconversion of antinuclear antibodies, a diagnostic in autoimmune disorders. None of the other patients reported serious side effects that were attributed to the minocycline treatment, although dizziness, headaches, sleepiness, and diarrhea were reported as mild side effects (Table 1). In this trial, significant improvements occurred in behavioral outcomes using the Aberrant Behavior Checklist-Community Edition (ABC-C) [52]. Four out of five subscale scores showed significant improvement, including the irritability subscale, which was used as the primary outcome measure, and stereotypy, hyperactivity, and inappropriate speech subscales, which were employed as secondary outcome measures (Table 1). Positive results using the clinical global improvement scale (CGI) and the visual analog scale for behavior (VAS) as measures were also reported for the majority of minocycline-treated FXS patients [52]. Interestingly, given the choice to extend minocycline treatment for 1 year, 18 of the 19 families independently decided to continue treatment based on their perceptions of behavioral improvements. Taken together with the Utari study, both sets of data strongly suggest that minocycline is a relatively safe and potentially effective treatment for FXS patients [52, 54]. However, a double-blind, placebo-controlled clinical treatment trial is still necessary to provide concrete evidence that minocycline is a positive and effective FXS therapy.

4. Mechanism of Action: MMP Inhibition

The 2009 mouse FXS model study provides good evidence that minocycline acts through inhibition of secreted matrix metalloproteinase-9 (MMP-9) [51]. In other neurological disorders, such as multiple sclerosis, minocycline has similarly been found to be effective as an MMP-9 inhibitor [55]. Bilousova and colleagues performed both Western Blot and gel zymography analyses to assess differences in MMP-9 levels and enzymatic (gelatinase) activity in the mouse hippocampus [51]. In *FMR1* KO (P7) mice, levels of active MMP-9 as well as MMP-9 gelatinase activity were both increased compared to controls (Figure 1). Importantly, minocycline treatment of the *FMR1* KO decreased both hippocampal active MMP-9 protein levels and hippocampal gelatinase activity towards the wildtype condition [51]. Interestingly, MMP-9 treatment of wildtype hippocampal cell cultures induced immature dendritic spine profiles with a greater proportion of long, thin filopodia-like dendritic spines, mimicking the *FMR1* KO state [51], a finding that has since been validated via genetic methods [100]. Together, this evidence suggests that upregulation of secreted active MMP-9 is a novel aspect of the molecular pathology of FXS and that MMP-9 inhibition is the mechanism of action of minocycline in alleviating FXS phenotypes (Figure 1).

In the follow-up *Drosophila* study, Siller and Broadie greatly extended testing of this MMP hypothesis, taking advantage of the fly's relative genetic simplicity (2 MMPs in *Drosophila* compared to 24 in mammals; 1 TIMP in *Drosophila* compared to 4 in mammals) [53, 57, 58, 60, 101]. The two *Drosophila* MMPs include secreted MMP-1 and membrane anchored MMP-2, with a good antibody probe available for MMP-1 only. MMP-1 expression levels and gelatinase activity showed no significant differences in the *dFMR1* KO compared to control, at least at the NMJ synapse with immunocytochemistry and *in situ* zymography and in whole-brain Western Blots (Figure 1) [53]. Nevertheless, to begin to test the MMP hypothesis, the endogenous TIMP inhibitor was genetically overexpressed in the *dFMR1* KO background to mimic the proposed minocycline effect. At the NMJ, TIMP overexpression was highly efficacious in suppressing the synaptic structural overelaboration characterizing the *dFMR1* KO, restoring the synaptic branching and excess mature and immature bouton formation to the wildtype condition [53]. Conversely, TIMP overexpression causes early developmental lethality and tracheal deformations prior to death [60, 102, 103], and *dFMR1* removal bidirectionally suppressed these TIMP overexpression phenotypes. Importantly, a *dFMR1*; *MMP-1* double KO mutant displayed the same reciprocal suppression of phenotypes, with prevention of *dFMR1* KO synaptic architecture defects and rescue of *MMP-1* KO tracheal defects and early lethality [53]. Together, these data provide excellent evidence for a specific genetic interaction between the TIMP/MMP-1 and FMRP pathways (Figure 1). Taken with the previous mouse FXS model study showing specific upregulation of secreted MMP-9, reduced upon minocycline treatment, the combined data set strongly suggests that minocycline is inhibiting MMPs to exert its alleviatory actions on FXS phenotypes [51, 53].

A critical question is to determine how the FMRP and TIMP/MMP pathways intersect (Figure 1). FMRP is best known as a negative translational regulator, and, therefore, it is possible that FMRP directly inhibits MMP expression, resulting in MMP upregulation in the FXS disease state. Based on known FMRP functions, this interaction could happen at the level of regulating MMP mRNA stability or translation, or FMRP could secondarily influence MMP protein function, secretion, or localization via acting on MMP-interacting proteins (Figure 1). However, numerous more indirect interactions between the two pathways are also

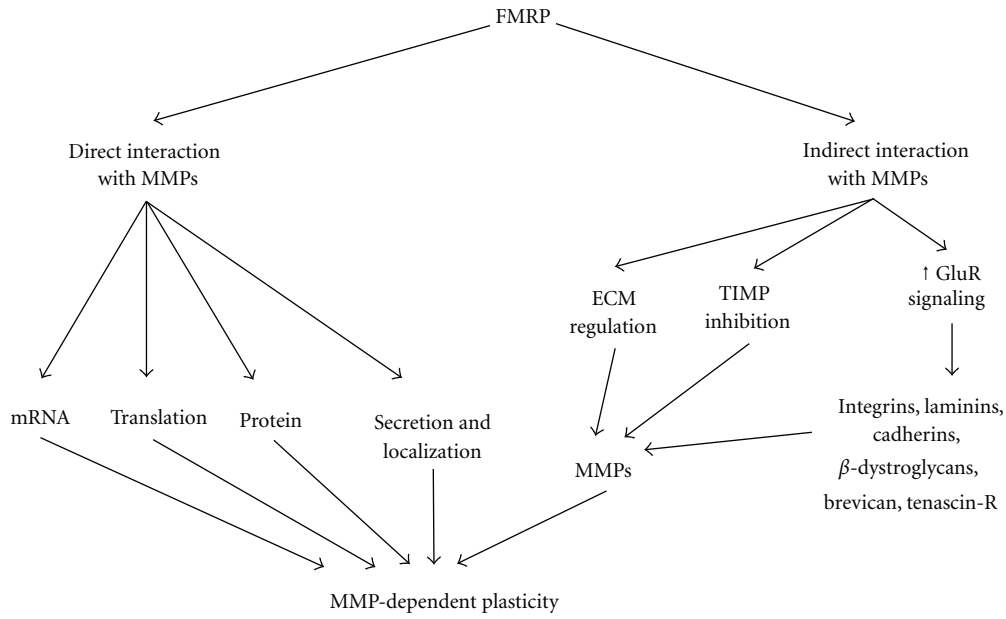

Figure 1: Schematic of potential interaction mechanisms between FMRP and MMPs. FMRP may more directly regulate MMPs at the level of transcript stability, translation, or protein function. FMRP may more indirectly convergently interact with MMPs in the regulation of extracellular matrix (ECM) components and their receptors, via the endogenous TIMP regulatory mechanism, or via glutamate receptor (GluR) signaling.

possible. FXS is a disease of enhanced glutamate receptor signaling, associated with defects in synaptic morphogenesis and plasticity (LTD/LTP) [35, 37]. Several studies have shown that NMDA glutamate receptor signaling causes local MMP-9 release, leading to MMP-mediated synaptic plasticity events [104–106], presumably via extracellular proteins, such as integrins, laminins, cadherins, β-dystroglycan, brevican, and tenascin-R, which have all been implicated in hippocampal LTP [57, 58, 67] (Figure 1). Indeed, recent work implicates integrin β1 signaling as the mechanism by which MMP-9-mediated changes in dendritic spine morphology occur [100]. Furthermore, MMP-dependent synapse remodeling can be blocked by NMDA receptor inhibitors, and NMDA receptor activity has been shown to increase MMP-9 activity, suggesting another possible link [58]. Moreover, MMPs play roles in axonal-dendritic structural remodeling [58, 67]; for example, with MMP-9 present in mammalian dendritic spines and MMP-1 present at the *Drosophila* glutamatergic NMJ [53, 107, 108]. *MMP-9* KO mice display deficiencies in hippocampal LTP, and other changes, for example, after spinal cord injury, can induce elevated gelatinasez activity, showing that careful control of MMP expression levels is critical to synaptic regulation [105, 109]. In addition, MMP inhibition may lead to TIMP signaling changes due to decreased levels of MMP-bound TIMP versus increased levels of free unbound TIMP [110]. For example, the balance between MMP-7 and TIMP-1 was recently shown to be important for pro-nerve growth factor (NGF) cleavage and neuroprotection following kainite-induced seizures [111], which could possibly provide a link with seizure manifestations in FXS patients. Thus, while it is unclear how the MMP and FMRP pathways intersect, it is clear that a number of intriguing possibilities need to be explored (Figure 1).

5. Conclusions and Future Directions

FXS is a devastating neurological disease characterized by a broad spectrum of cellular and behavioral symptoms [4, 5]. While much attention has been focused on mGluR5 inhibitors as a potential avenue of disease treatment [31, 45–47], a significant amount of new evidence from *Drosophila* [53], mouse [51], and human studies [52, 54] suggests that the common tetracycline derivative minocycline may be a new and highly effective treatment alternative (Table 1). These recent reports indicate that minocycline may be a broad-spectrum treatment with only mild side effects. The clear next step is to pursue a double-blind, placebo-controlled FXS clinical trial of minocycline effectiveness. Importantly, it may be critical to test minocycline in young children as it is probable that treatment effectiveness may be linked, in part, to developmentally transient events of neural circuit formation and/or refinement. Of course, it is to be hoped that the inherent plasticity of the nervous system will also make adult minocycline treatments effective. Thus, minocycline holds the real possibility of being an accessible and cost-effective broad treatment for the disease. Importantly, minocycline has long been FDA approved, greatly facilitating its rapid dissemination to the FXS community. This is indeed an exciting development for families afflicted by this devastating neurological disease.

Both *Drosophila* and mouse studies point toward MMP inhibition being the mechanism of minocycline action in

FXS [51, 53]. However, another possibility that must be considered arises from the fact that minocycline also functions as an antibiotic by inhibiting bacterial translation, and a similar function could be predicted to antagonize the effect of losing FMRP translational repression, causing elevated translation [21]. Although this should not be a consideration at the levels of minocycline used in the recent FXS studies (Table 1), which are the same dosages as used for treatment of acne and bacterial infections, such as neurosyphilis [112, 113], it remains a strong possibility to be investigated as potentially FXS patients may be more sensitive toward minocycline treatment than healthy individuals. Significant research into minocycline's effects on eukaryotic ribosomes, especially in FXS, has not been done and must be performed extensively to determine if the minocycline mechanism of action is through its ability to regulate translation. In addition, the available evidence does not rule out the possibility of other mechanisms of minocycline action, such as p53 MAPK regulation [55, 114, 115]. Nevertheless, the recent *Drosophila* study strongly indicates specific genetic interplay between the MMP and FMRP pathways that should be the focus of investigation in the immediate future [53]. It will be important to fully study the roles of TIMP and MMPs in the context of synaptic development/refinement and to define overlapping and distinct synaptic functions of the different MMP family members (i.e., membrane-anchored versus secreted). Moreover, a great limitation in the recent *Drosophila* study was that it only examined synaptic structure defects, and it is imperative to extend the work to the examination of minocycline/MMP involvement at the level of synaptic function/plasticity and behavioral outputs in the *Drosophila* FXS model. Besides the neuronal mechanisms, it is also important to note that the genetic interaction between MMP and FMRP occurs also in nonneuronal tissues, because *MMP-1* nonneuronal phenotypes and overall lethality are rescued by *dFMR1* removal [53]. A similar nonneuronal *FMR1* function is also revealed by the joint symptoms of FXS patients, strongly implicating an ECM component of the human disease state. Moving forward, it will be important to understand how TIMP/MMP dysfunction fits into the larger picture of FXS pathogenesis, to provide both a greater understanding of TIMP/MMP roles at the synapse, as well as to bring to light possible new therapeutic targets for FXS.

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Review Article

Plasticity and mTOR: Towards Restoration of Impaired Synaptic Plasticity in mTOR-Related Neurogenetic Disorders

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Objective. To review the recent literature on the clinical features, genetic mutations, neurobiology associated with dysregulation of mTOR (mammalian target of rapamycin), and clinical trials for tuberous sclerosis complex (TSC), neurofibromatosis-1 (NF1) and fragile X syndrome (FXS), and phosphatase and tensin homolog hamartoma syndromes (PTHS), which are neurogenetic disorders associated with abnormalities in synaptic plasticity and mTOR signaling. *Methods*. Pubmed and Clinicaltrials.gov were searched using specific search strategies. *Results/Conclusions*. Although traditionally thought of as irreversible disorders, significant scientific progress has been made in both humans and preclinical models to understand how pathologic features of these neurogenetic disorders can be reduced or reversed. This paper revealed significant similarities among the conditions. Not only do they share features of impaired synaptic plasticity and dysregulation of mTOR, but they also share clinical features—autism, intellectual disability, cutaneous lesions, and tumors. Although scientific advances towards discovery of effective treatment in some disorders have outpaced others, progress in understanding the signaling pathways that connect the entire group indicates that the lesser known disorders will become treatable as well.

1. Introduction

Brain plasticity, the developing brain's ability to change in response to either positive experiences or negative experiences, is a critical component of pediatric neurology. The major types of plasticity in the developing brain include *adaptive plasticity*—occurs in response to learning or recovering from injury or disability; *impaired plasticity*—results from brain injury due to an acquired or neurogenetic disorder; *maladaptive plasticity*—a plastic response leading to a new disorder; *plasticity as the brain's Achilles' heel*—a mechanism, such as selective vulnerability of neurons, which creates risk for injury [1]. Basic cellular mechanisms of plasticity include overproduction of neurons followed by reduction via apoptosis [2]; continued production of new cells from stem cells in the hippocampus and lateral ventricle throughout life [3]; activity-dependent synaptic plasticity through receptor trafficking; activity-dependent production of growth factors; overproduction of synapses and axodendritic connections followed by pruning, activity-dependent stabilization of dendrites and axons; regulation of DNA expression by epigenetic regulation [1]. Although there are many disorders associated with impaired plasticity, this paper will highlight the clinical features, neurobiology associated with dysregulation of mTOR, preclinical studies, and clinical trials in tuberous sclerosis complex (TSC), neurofibromatosis-1 (NF1), and fragile X syndrome (FXS), as well as phosphatase and tensin homolog hamartoma syndromes (PTHS), neurogenetic disorders linked by abnormalities in synaptic plasticity and mTOR (mammalian target of rapamycin) signaling.

Table 1: Diagnostic criteria.

(a) TSC. Definite TSC: two major or one major and two minor features; probable; TSC: one major and one minor feature; possible TSC: one major or two or more minor features

Major features	Minor features
Cortical tubers	Dental enamel pits
Subependymal nodules	Hamartomatous rectal polyps
Subependymal giant cell astrocytoma	Bone cysts
Hypomelanotic macules (3 or more)	Cerebral white matter radial migration lines
Shagreen patch	Gingival fibromas
Facial angiofibromas or forehead plaque	Nonrenal hamartoma
Multiple renal nodular hamartomas	Retinal achromatic patches
Nontraumatic ungual or periungual fibromas	"Confetti" skin lesions
Cardiac rhabdomyoma	Multiple renal cysts
Pulmonary lymphangiomyomatosis and/or renal angiomyolipomas	

(b) NF1. Presence of two or more clinical features

Family history of NF1	Neurofibromas or plexiform neurofibromas
Six or more cafe-au-lait spots	Axillary or groin freckling
Lisch nodules	Skeletal abnormalities—tibial dysplasia or shin bone thinning
Optic glioma	

(c) FXS

Full mutation >200 CGG repeats
Premutation 50–230 CGG repeats

(d) PTHS (Only Cowden syndrome has diagnostic criteria). Cowden syndrome. Operational diagnosis: mucocutaneous lesion alone if: 6 or more facial papules, 3 or more trichilemmoma; cutaneous facial papules and oral mucosal papillomatosis; oral mucosal papillomatosis and acral keratosis, or 6 or more palmoplantar keratosis; or two or major criteria, including macrocephaly or adult Lhermitte-Duclos disease; or one major or three minor criteria; or four minor criteria

Pathognomic criteria	Major criteria	Minor criteria
Adult Lhermitte-Duclos	Breast cancer	Intellectual disability
Mucocutaneous lesions	Thyroid cancer	Other thyroid lesions
	Macrocephaly	GI hamartomas
	Endometrial cancer	Fibrocystic breast disease
		Lipomas; fibromas
		Genitourinary tumors or malformations

EIF4E (No diagnostic criteria).

2. Methods

Pubmed was searched using the following search strategies: mTOR and/or neurology; mTOR and/or plasticity; mTOR and/or TSC; mTOR and/or NF1; mTOR and/or FXS; mTOR and/or PTHS; plasticity and/or neurology; plasticity and/or TSC; plasticity and/or NF1; plasticity and/or FXS; plasticity and/or PTHS. Clinicaltrials.gov was searched by disorder without language or country of origin restrictions for active studies through 11/30/11.

2.1. Tuberous Sclerosis Complex (TSC)

2.1.1. Clinical Features. Tuberous sclerosis complex (TSC) has an incidence of 1/6000 and may be defined clinically by the presence or absence of major and minor features associated with the disorder and genetically by spontaneous or inherited mutations in TSC1 or TSC2. Major neurologic features include brain lesions-subependymal nodules, subependymal giant cell astrocytomas, and cortical tubers, intractable epilepsy in 60–90% [4–6], autism in up to 61% [7, 8], intellectual disability in 45% [9], and self-injury in 10% [10]. TSC has also been associated with pulmonary, cardiac, and cutaneous lesions (Table 1).

2.1.2. Neurobiology of mTOR Dysregulation. Overexpression of the serine/threonine protein kinase mammalian target of rapamycin (mTOR) results from disruption of either TSC1 or TSC2. Typically, TSC1 and TSC2 form a complex, which inhibits Rheb (ras homologue expressed in brain), an activator of mTOR. The consequences of mTOR overexpression include abnormally rapid cell growth and hyperactivation of mRNA translation, which may lead to impaired synaptic plasticity in TSC [11] (Figure 1).

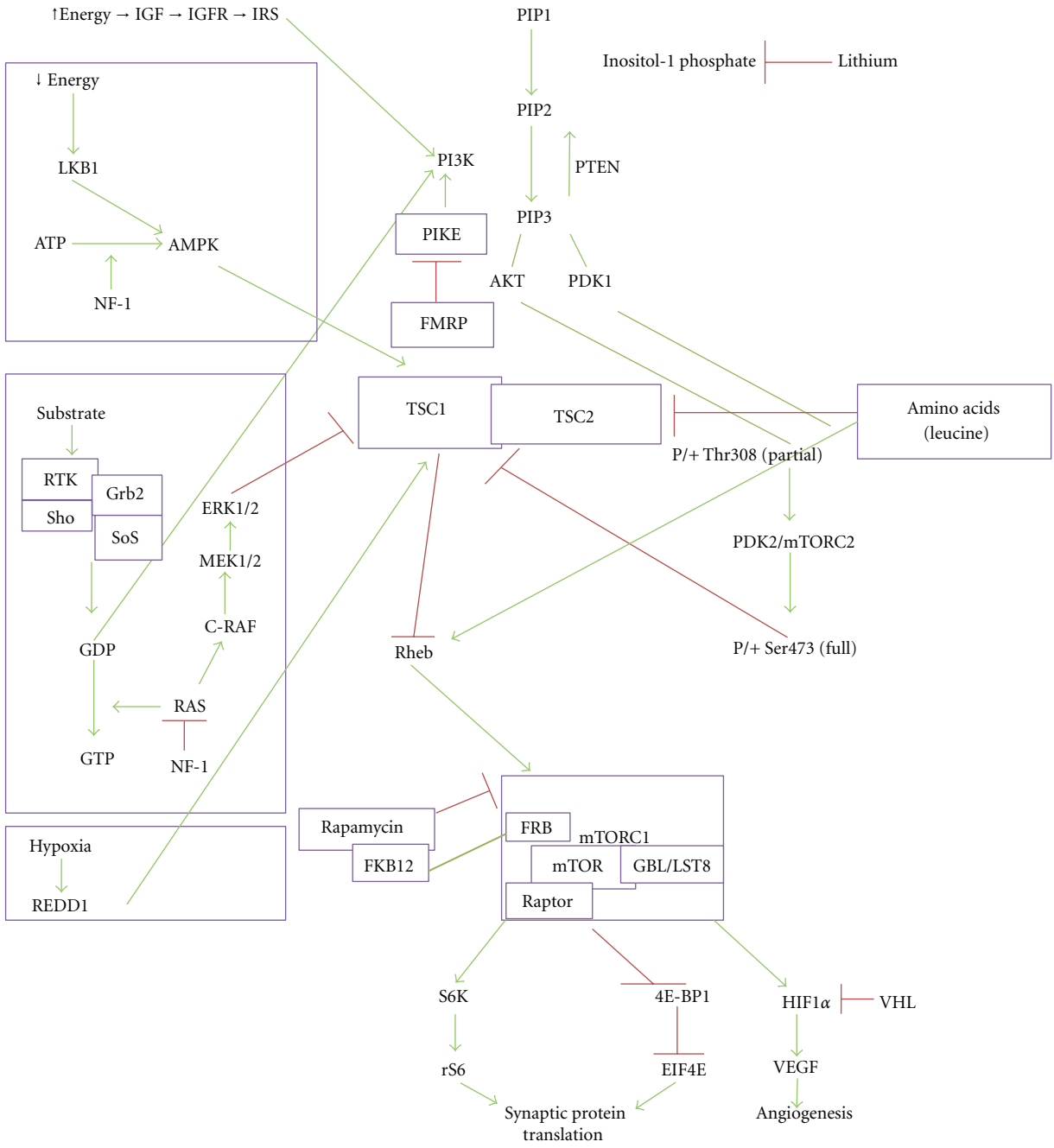

Figure 1: Pathways associated with neurogenetic conditions linked by mTOR and impaired synaptic plasticity. AKT-v—akt murine thymoma viral oncogene homolog 1; AMPK—adenosine monophosphate kinase; ATP—adenosine triphosphate; EIF4E/4EBP1—eukaryotic translation initiation factor 4E-binding protein 1; ERK—extracellular signal-related kinase; FKB12-FK506—binding protein family; FMRP—fragile X mental retardation protein; GBL/LST8—(mTOR-associated protein, LST8 homolog); GDP—guanosine diphosphate; GTP—guanosine triphosphate; GDP—guanosine diphosphate; HIF1α—hypoxia inducible factor 1, alpha subunit IGF—insulin-like growth factor; IGFR—insulin-like growth factor receptor; IRS—insulin receptor substrate; LKB1—serine threonine kinase 11; mTORC1—mammalian target of rapamycin complex 1; mTORC2—mammalian target of rapamycin complex 2; MEK—dual specificity mitogen-activated protein kinase 1; NF-1—neurofibromatosis 1; P/+ Thr308—phosphorylation of threonine, position 308; P/+ Ser473—phosphorylation of serine, position 473; PDK1—pyruvate dehydrogenase kinase, isozyme 1; PDK 2—pyruvate dehydrogenase kinase, isozyme 2; PI3K—phosphoinositide-3-kinase; PIKE—phosphoinositide 3-kinase enhancer; PIP1—p21-activated protein kinase-interacting protein 1 (PAK1 interacting protein 1); PIP2—phosphatidylinositol 4,5-biphosphate; PIP3—phosphatidylinositol (3,4,5)-triphosphate; PTEN—phosphatase and tensin homolog; RAS—Ras p21 protein activator 1 or RAS GTPase activating protein; REDD1—regulated in development and DNA damage responses; RHEB—Ras homologue expressed in brain; RTK—receptor tyrosine kinase; S6K—ribosomal protein S6 kinase; TSC—tuberous sclerosis complex; VEGF—vascular endothelial growth factor; VHL—von Hippel Lindau.

2.1.3. Preclinical Models. Impaired synaptic plasticity as a consequence of a disruption in either TSC1 or TSC2 has been supported by results from preclinical studies. Abnormalities in long-term potentiation (LTP) and long-term depression (LTD) were found in the $Tsc2^{+/-}$ Eker rat, which carries a spontaneous germline mutation [12]. Abnormal late-phase LTP induction and hippocampal-dependent learning deficits was observed in $Tsc2^{+/-}$ adult mice and improved after rapamycin treatment [13].

Metabotropic glutamate receptor-mediated long-term depression (mGluR-LTD) was impaired in $Tsc2^{+/-}$ mice and related to decreased translation of proteins required for stabilization of LTD. Potentiation of mGluR5 activity led to restoration of normal LTD [14]. mGluR-LTD was also deficient in a neuronal model of *Tsc1* [15].

Prolonged neuronal hyperexcitability, typically associated with epilepsy, has also been supported by recent studies as a possible mechanism of impaired synaptic plasticity in TSC [16]. Hyperexcitability in cortical tubers has been linked to abnormalities of glutamate receptor expression [17]. This hyperexcitability was maintained despite the absence of cortical tubers from brain sections of an individual with TSC and $Tsc1^{synapsin}$ *conditional*knockout mice, a neuronal model of TSC in an earlier study [18, 19]. An astrocyte-specific model of TSC, $Tsc1^{GFAP}$ conditional knockout mice, was also characterized by abnormally elevated glutamate [20]. Astrocytic dysfunction in the uptake of extracellular potassium may explain the hyperexcitability in this model [21].

2.1.4. Clinical Trials. Guided by preclinical observations, investigators have completed studies to reduce the burden of neurologic disease in individuals with TSC. A clinical trial of everolimus for subependymal giant cell astrocytomas (SEGAs) achieved the primary outcome of reduction in the size of SEGAs, supporting similar results from a case series [22–24]. Everolimus is now FDA-approved for reduction in the size of SEGAs that are nonsurgically resectable. Positive outcomes from these studies have led investigators to consider rapamycin for additional neurologic conditions, such as autism [25]. The ability of everolimus to improve cognition is currently under investigation (http://www.clinicaltrials.gov/; NCT01289912).

2.2. Neurofibromatosis 1 (NF1)

2.2.1. Clinical Features. Neurofibromatosis 1 (NF1), a disease caused by an inherited mutation in NF1, has an incidence of 1 in 3500 [26]. NF1 can be diagnosed by identification of the genetic mutation or the presence of two or more clinical features—family history of NF1; six or more cafe-au-lait spots; neurofibromas; plexiform neurofibromas; axillary or groin freckling; Lisch nodules (a hamartomatous nodule of melanocytes on the iris); skeletal abnormalities such as tibial dysplasia or thinning of the shin bone; or optic glioma. Associated conditions include cognitive impairments, pilocytic astrocytomas, and neuropathological abnormalities characterized by MRI hyperintensities, megalencephaly, and thalamic lesions. Cognitive impairment is the most common source of neurological impairment in children with NF1, affecting as many as 81% of children [27]. Neuropathological abnormalities associated with impaired cognition have been identified in some cases. Megalencephaly associated primarily with increased white matter volume was identified in individuals with NF1-associated ADHD [28]. Abnormalities in gray matter volume and enlargement of the corpus callosum have also been associated with cognitive impairment [29]. NF1 has also been characterized by the presence of MRI T2-hyperintensities (nonenhancing bright areas of unknown etiology), sometimes referred to as UBOs (unidentified bright objects). An early study employing sibling comparison found distribution of these lesions to be predictive of lower IQ [30]. Subsequent studies have also supported the role of these lesions in cognition [31, 32]. A longitudinal profile revealed changes in these lesions with childhood regression followed by recurrence in early adolescence [33].

2.2.2. Neurobiology of mTOR Dysregulation. Disinhibited RAS MAPK signaling underlies the molecular basis of disease, and mTOR hyperactivity has also been identified in preclinical models [34]. NF1 encodes neurofibromin, a GTPase activating protein, which normally leads to inactivation of Ras. Mutations in neurofibromin lead to overactivation of Ras activity, followed by enhanced activation of the Ras-MAPK signaling pathway as well as PI3K and ERK 1/2 which both inactivate the TSC1/TSC2 complex releasing inhibition of Rheb and allowing activation of mTOR. However, there may be pathways leading to dysregulation of mTOR in NF1 that differ from other conditions [34]. mTOR hyperactivity in *Nf1* leads to increased astrocyte proliferation, an effect not shared by preclinical models of *Pten*, *Tsc1*, *Tsc2*, or overexpression of *Rheb* [35]. Phospho-histone-H3 rather than phosphor-S6 or Ki67 correlated with response to rapamycin in *Nf1* mice [36]. Neurofibromin also interacts with caveolin-1 [37] and nucleophosmin [38].

2.2.3. Preclinical Models. Long-term potentiation was impaired by increased hippocampal inhibitory transmission in mice heterozygous for a germline mutation in *Nf1* ($Nf1^{+/-}$). However, restoration of LTP deficits and reversal of cognitive impairments was achieved with pharmacological inhibition of Ras using lovastatin, an HMG CoA reductase inhibitor [39] and BMS 191563, a farnesyltransferase inhibitor [40]. Farnesyltransferase inhibitors demonstrated inhibition of Rheb and subsequent inhibition of mTOR in $Tsc1^{-/-}$ *and* $Tsc2^{-/-}$ mouse embryonic fibroblasts [41]. Inhibition of ERK also led to restoration of early-phase and long-term LTP [42].

2.2.4. Clinical Trials. Simvastatin in children with NF1 improved object assembly, a secondary outcome in a randomized trial, but there was no difference in primary outcome [43]. Preliminary results of a subsequent of lovastatin in children with NF1 revealed improvement in verbal and nonverbal memory [44].

2.3. Fragile X Syndrome (FXS)

2.3.1. Clinical Features. Fragile X syndrome (FXS) is the leading cause of inherited intellectual disability and has a full mutation gene frequency of 1 in 2500 [45, 46]. Associated neurologic conditions include autism, anxiety, and ADHD [47, 48]. Definitive diagnosis relies on genetic confirmation and individuals may be classified as full mutation if there are greater than 200 CGG repeats within the promoter of the fragile X mental retardation-1 gene (FMR1) and premutation if there are 50 to 230 repeats [49].

2.3.2. Neurobiology of mTOR Dysregulation. These abnormal CGG repeats result in suppression of FMR1 gene transcription and subsequently reduced to absent fragile X mental retardation protein (FMRP) [50, 51]. Loss of FMRP releases inhibition of PIKE, which activates PI3K and leads to increased mTOR activity. The "mGluR theory" proposes that elevation of group I mGluRs (mGluR1 and mGluR5) glutamate receptors leading to reduced insertion of AMPA receptors into the postsynaptic membrane is one of the central mechanisms of impaired synaptic plasticity in FXS, and this has been supported in experimental models [52]. Increased mGluR5 activity and reduced insertion of AMPA receptors leads to long-term depression (LTD) due to reduced AMPA-mediated synaptic activity.

2.3.3. Preclinical Models. Using preclinical models, specific interactions among synaptic proteins and FMRP have been identified. Initially, abnormal synaptic translation of CaMKIIa, PSD-95, and GluR1/2 mRNAs was observed in the Fmr1 knockout mouse [53]. Subsequent studies revealed regulation of expression of PSD-95 by FMRP, miR125a, and mGluR.

Phosphorylation of FMRP induces the creation of an AGO2-miR125a complex, which inhibits PSD-95 mRNA. mGluR stimulation, however, causes dephosphorylation of FMRP, which leads to activation of translation of PSD-95. In *Fmr1* KO mice, miR125a is reduced in addition to the reduction in FMRP [54]. In addition to hyperactivity of group 1 mGluR and mGluR-LTD, abnormally increased signaling of mTOR in hippocampus was discovered in *Fmr1* KO mice, providing a link between mGluR elevation and abnormalities in synaptic plasticity leading to cognitive impairment. Loss of FMRP releases inhibition of PIKE, which activates PI3K and leads to increased mTOR activity as measured by four methods. Abnormally increased mTOR leads to an abnormal increase in cap-dependent translation of synaptic proteins and subsequent abnormalities in synaptic plasticity. Inhibition of PI3K activity resulted in normal levels of phosphorylated mTOR. Increased PTEN activity, mediated by dephosphorylation, was discovered in *Fmr1* KO mice and may serve as a feedback inhibition to compensate for abnormally increased PI3K since PTEN dephosphorylates PI3K, which reduces phosphorylation and activation of Akt [55].

In a Drosophila model of FXS, treatment with mGluR antagonists during development resulted in reversal of neuropathology, abnormal courtship behavior, and impaired memory. Partial reversal of impaired memory and abnormal courtship behavior without change in neuropathology was seen in treated adults. Conversely, treatment led to impairment in wild-type control flies [56].

Reduction in genetic function of mGluR5, achieved by crossing *Fmr1* mutant mice with heterozygous mGluR5 mutant mice, rescued many of the core phenotypic features in the *Fmr1* KO mouse [57]. Treatment of *Fmr1* KO mice with either an mGluR1 antagonist (JNJ) or an mGluR5 antagonist (MPEP) led to similar, but slightly different neurologic and behavioral improvements. Marble burying, a measure of repetitive behavior, was reduced without reduction in activity in *Fmr1* KO and WT mice. MPEP eliminated audiogenic-induced seizures. Motor learning also improved with MPEP in *Fmr1* KO mice. Prepulse inhibition, a measure of sensorimotor gating, known to be abnormally increased in *Fmr1* KO mice was not affected by JNJ or MPEP [58]. Abnormalities in prepulse inhibition were linked to abnormalities in presynaptic short-term plasticity in mice models of schizophrenia [59].

Prolonged UP states, a marker of cortical hyperexcitability in *Fmr1* KO mice was found to be due to a non-translation-related function of mGluR5, and treatment with MPEP reversed this phenomenon [60]. In addition to long-term postsynaptic plasticity, abnormalities in short-term presynaptic plasticity were also identified in *Fmr1* KO mice and may also contribute to cognitive impairment [61]. Another approach utilized $GABA_A$ receptor agonist in *Fmr*1 KO mice, resulting in restoration of amygdala-based deficits in neuronal excitability, reduced prepulse inhibition, and alleviation of hyperactivity [62]. The behavioral effects of genetic reduction of mGluR1 and mGluR5 by 50% were observed in *Fmr1* KO mice. Reduction in mGluR1 led to decreased activity, whereas reduction in mGluR5 led to decreased active social behavior and decreased thermal sensitivity. Neither genetic reduction resulted in changes in memory, motor responses, sensorimotor gating, audiogenic seizures, and responses related to anxiety and perseveration [58].

2.3.4. Clinical Trials. Human studies have led to the identification of the behavioral/cognitive profile of Fragile X as well an endophenotype of autism in Fragile X distinguished by social withdrawal [63–65]. Comparison of patients with FXS with and without autism supported the previously identified endophenotype of social withdrawal in FXS-associated autism by the finding of decrease in the left temporal gyrification index, an indicator of cortico-cortical connectivity and organization [66]. Recent significant scientific discoveries have culminated in human clinical trials targeting different aspects of the neurobiological impairments in FXS. AFQ056, an mGluR5 antagonist, resulted in different responses dependent upon the methylation status of FMR1. Patients with full methylation of FMR1 and no detectable FMR1 mRNA in the blood responded positively to treatment with improvement in inappropriate speech, stereotypic behavior, and hyperactivity [67]. Additional trials focused on antagonizing mGluR5 include a trial of

fenobam, which reduced anxiety, hyperarousal, improved accuracy in continuous performance tasks, and prepulse inhibition of startle [68]; acamprosate, which in three young adult patients, resulted in improvement in communication and global clinical improvement (CGI-I) [69]. Results are pending from an open label phase I study of STX107 (Seaside Therapeutics) and a phase II trial of RO4917523 (Hoffman-LaRoche) (clinicaltrials.gov). Other mechanisms that may lead to repair of the impaired plasticity associated with FXS have also been examined. Phospholipase C and glycogen synthase kinase-3, linked to Gp1 mGluR signaling, have been targeted using lithium, which resulted in improvement in cognition and adaptive skills [70]. Ampalex (CX516) is an ampakine (binds AMPA receptors) that increases hippocampal LTP by slowing receptor deactivation [71, 72]. Evaluation of ampalex in a placebo-controlled phase II trial for Fragile X-associated autism did not reveal differences in the primary outcome of memory or any of the secondary outcomes: overall functioning, attention/executive functioning, language, or behavior [73]. Minocycline, a broad spectrum antibiotic and analogue of tetracycline, has been found to have neuronal effects. In C57BL/6J mice, minocycline increased phosphorylation of GluR1 and subsequent insertion of AMPA receptors *in vivo* and *vitro* [74]. Study of minocycline in *Fmr1* KO mice revealed behavioral improvement: reduction in anxiety and improved exploration as well as neuropathological improvement—dendritic spine maturation associated with inhibition of abnormally elevated matrix metalloproteinase-9 (MMP-9) in hippocampal neurons [75]. The observations in *Fmr1* KO mice were supported in a Drosophila model of FXS where treatment with minocycline or genetic elimination of mmp1 reverses synaptic structural abnormalities [76]. Open-label treatment with minocycline in individuals with FXS led to significant improvement in irritability [77]. Cholinergic deficits in FXS, confirmed in individuals by ^{1}H magnetic resonance spectroscopy, were targeted using donepezil in an open label study with noted improvement in continuous naming, attention difficulties, and total ABC score as well as irritability and hyperactivity [78]. Reduction in glutamate using riluzole in an open-label study corrected abnormal activation of ERK; however, improvement in the primary outcome-repetitive, compulsive behavior was not achieved [79]. A single-dose, placebo-controlled trial of oxytocin for social anxiety in FXS resulted in improvement in eye gaze towards the examiner in a social challenge [80]. Aripiprazole, an atypical antipsychotic that is a partial D2 and 5-HT1A agonist as well as a 5-HT2A antagonist, improved scores on CGI-I and ABC-irritability [81]. Baclofen, a $GABA_B$ receptor agonist, inhibited seizures in *Fmr1* mice [82]. A phase II, randomized double-blind study of arbaclofen has been completed with results pending, and a phase III study of arbaclofen is now recruiting (http://www.clinicaltrials.gov/).

2.4. PTEN-Associated Conditions

2.4.1. Clinical Features. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a phosphatase which limits cell growth by apoptosis and cell cycle arrest. Conditions linked by a genetic mutation in PTEN have been collectively termed phosphatase and tensin homologue hamartoma syndromes (PTHS) and include Juvenile Polyposis, Lhermitte-Duclos disease, Bannayan-Riley-Ruvalcaba, Cowden Syndrome, Proteus-syndrome, and Proteus-like conditions. Cowden syndrome and Bannayan-Riley-Ruvalcaba have been associated with autism and intellectual disability.

Cowden syndrome has an estimated prevalence of 1/200,000 and may be diagnosed by the presence of either pathognomonic criteria or a specific combination of major and minor criteria. Severe and progressive macrocephaly (>2 S.D.) associated with autism should prompt consideration of the diagnosis and led to publication of the first reported case of Cowden syndrome-associated autism and epilepsy [83]. A similar pattern with the addition of a lipoma and thyroid adenoma led to the identification of Bannayan-Riley-Ruvalcaba Syndrome (BRRS) in a nine-year-old girl [84]. A retrospective review of 114 patients analyzed for PTEN mutations revealed mutations in 18% of those with macrocephaly in addition to either ASD or ID [85]. This contrasts with a PTEN mutation in one of eighty-eight children (1%) with macrocephaly and ASD [86]. BRRS does not have established diagnostic criteria; however, macrocephaly is usually the most striking feature. Identification of Cowden and Bannayan-Riley-Ruvalcaba syndrome in the same family raises the possibility of the two syndromes being the same syndrome with variation in phenotypic expression [87].

Functional analysis of the consequence of PTEN germline mutations from individuals with autism spectrum disorders was compared to PTEN germline mutations in individuals with PTHS in a humanized yeast-based bioassay and revealed greater preservation of PTEN PIP3 phosphatase activity in those with ASD [88].

2.4.2. Neurobiology of mTOR Dysregulation. PTEN is important in mTOR signaling since it removes a phosphate from phosphatidylinositol 3,4,5-triphosphate (PIP3). This conversion from PIP3 to PIP2 negates the activity of PI3K and results in elevation of mTOR since the processes downstream—Akt activation, Akt-mediated phosphorylation and inhibition of TSC2, release inhibition of Rheb which activates mTOR.

2.4.3. Preclinical Models. Evidence of impaired synaptic plasticity in PTEN mutations has been identified in *Pten* conditional knockout mice. Neuropathological features include enlarged neuronal nuclei and cell bodies, increased density of dendritic spines, abnormalities in axonal myelination, and weakening of excitatory synaptic transmission in hippocampal neurons between CA3 and CA1 as evidenced by impaired EPSPs, normal presynaptic function, and reduced long-term potentiation [89]. Cre-driven deletion of *Pten* in cortical and hippocampal neurons of mice was associated with hyperactivity of the mTOR pathway as well as hypersensitivity to stimuli, social interaction abnormalities, ectopic dendrites, increased axonal synapses, and macrocephaly associated with neuronal hypertrophy [90].

Table 2: Mechanisms of impaired synaptic plasticity, mTOR dysregulation, and therapeutic targets.

Condition	Gene (chromosome)	Mechanism of impaired synaptic plasticity impairment	mTOR physiology	Therapeutic targets
TSC	TSC 1 (9) or TSC2 (16)	↓mGluR-LTD	↑mTOR	mTOR antagonists mGluR 5 agonist
NF1	NF1 (17)	↓LTP↑GABA	↑mTOR	Ras antagonists ERK antagonists
FXS	FMR1 (X)	↑mGluR-LTD	↑mTOR	mGluR5 antagonists mGluR5/mGluR1 genetic reduction $GABA_A$ receptor agonist PLC/GSK3 antagonist (lithium) AMPA receptor agonist MMP 9 antagonist
PTHS	PTEN (10)	↓LTP	↑mTOR	mTOR antagonists
EIF4E-associated autism	EIF4E (4)	unknown	Downstream of mTOR	None developed

2.4.4. Clinical Trials. A pilot study is now recruiting for an open label trial of sirolimus, an mTOR inhibitor, in adult patients with Cowden syndrome, tumors, and germline PTEN mutations (http://www.clinicaltrials.gov/).

2.5. EIF4E-Associated Autism. Synaptic translation mediated by EIF4E is a common and final process of the pathways associated with PTEN, mTOR, and FMRP and serves a critical role in learning and memory [91, 92]. Linkage to chromosome 4q, the region containing EIF4E has been shown in genome-wide linkage studies [93, 94]. After identification of a translocation involving the region containing EIF4E in a young boy with autistic regression, investigators screened for mutations among families with two autistic siblings and found EIF4E mutations in two related families [95].

3. Conclusions

Review of recent literature reveals significant advances in our ability to understand the pathogenesis of several neurogenetic conditions associated with intellectual disability and autism that have been considered to be idiopathic and untreatable. In this paper, we have highlighted recent discoveries in neurogenetic conditions united primarily by dysregulation of mTOR and evidence of impaired synaptic plasticity (Table 2). In addition to autism and intellectual disability, some of these conditions also share an association with cutaneous lesions and tumor development. Based on this knowledge, it is reasonable to hope that these disorders could become treatable in the near future. Investigators have already begun the process of connected research, as exemplified by the work of Auerbach et al. who simultaneously examined models of TSC and FXS and created a model by crossing the two models to discover that the same intervention, modulating metabotropic glutamate receptor 5, demonstrates efficacy for both models in opposing directions [14]. Continuing to examine the link between these disorders is likely to lead to a greater chance of discovery for all of them. Tools needed to translate basic science research into clinical trials which yield definitive results include refined genotypic and phenotypic characterization, detailed knowledge of the natural history of the conditions, knowledge of optimal therapeutic windows, valid biomarkers, and expertise in clinical trials.

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Review Article

Linking Epigenetics to Human Disease and Rett Syndrome: The Emerging Novel and Challenging Concepts in MeCP2 Research

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Epigenetics refer to inheritable changes beyond DNA sequence that control cell identity and morphology. Epigenetics play key roles in development and cell fate commitments and highly impact the etiology of many human diseases. A well-known link between epigenetics and human disease is the X-linked *MECP2* gene, mutations in which lead to the neurological disorder, Rett Syndrome. Despite the fact that MeCP2 was discovered about 20 years ago, our current knowledge about its molecular function is not comprehensive. While MeCP2 was originally found to bind methylated DNA and interact with repressor complexes to inhibit and silence its genomic targets, recent studies have challenged this idea. Indeed, depending on its interacting protein partners and target genes, MeCP2 can act either as an activator or as a repressor. Furthermore, it is becoming evident that although Rett Syndrome is a progressive and postnatal neurological disorder, the consequences of MeCP2 deficiencies initiate much earlier and before birth. To comprehend the novel and challenging concepts in MeCP2 research and to design effective therapeutic strategies for Rett Syndrome, a targeted collaborative effort from scientists in multiple research areas to clinicians is required.

1. Introduction

The term epigenetics refers to inheritable changes in gene expression that control cellular phenotype and fate decisions without alterations in the underlying DNA sequence [1]. In eukaryotes, two main epigenetic regulations are exerted through modifications on DNA and DNA-bound histone proteins. In general, histone modifications are dynamic and include acetylation, methylation, isomerization, phosphorylation, sumoylation, and ubiquitination [1, 2]. The combination of such modifications confers enormous flexibility in terms of functional response of an individual cell towards extracellular signals and environmental stimuli. Certain modifications such as histone methylation can display additional layers of complexity regarding their methylation degree and undergo mono-, di-, or tri-methylation of lysine residues [2, 3]. Furthermore, combinations or sequential additions of different histone marks can affect the chromatin organization and subsequently alter the expression of the corresponding target genes [4]. Conventionally, DNA methylation was considered to be a stable epigenetic mark, although this notion is being challenged by recent reports of active DNA demethylation [5]. In mammals, DNA methylation strictly happens at the cytosine residues in the context of CpG dinucleotides. The methylation of DNA molecules is processed by a group of enzymes called DNA methyltransferases (DNMTs). The mammalian DNMT family consists of 5 proteins (DNMT1, 2, 3A, 3B, 3L). DNMT1 is involved in maintaining the DNA methylation pattern during replication, while DNMT3A and DNMT3B act as *de novo* methyltransferases. DNMT3L is essential for the establishment of maternal genomic imprints during oocyte development, and DNMT2 is classified as part of the DNMT family; however it has very weak catalytic activity [6].

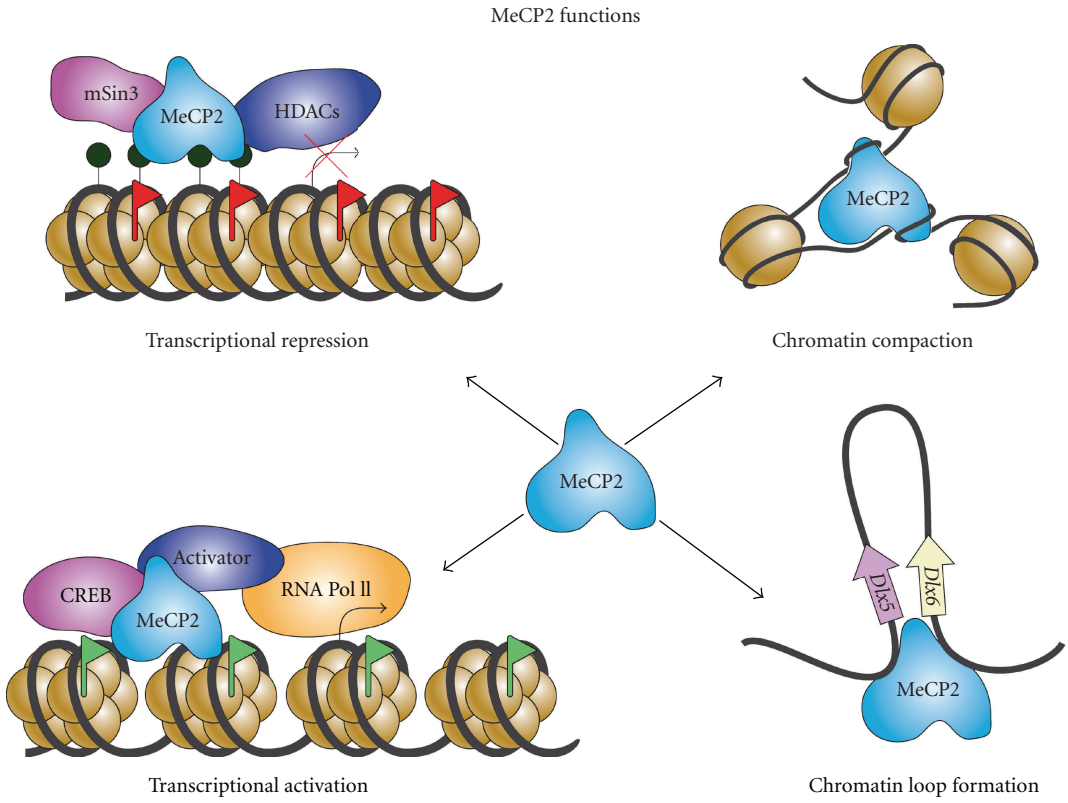

Figure 1: The diverse functions of MeCP2 in gene regulation and chromatin organization.

DNA methylation is often associated with transcriptional repression and has been linked to the tissue-specific regulation of genes [7], expression of imprinted genes [8], and X-chromosome inactivation in females [9]. In general, DNA methylation affects gene expression in two ways: (i) directly, by altering the binding sites of transcription factors, or (ii) indirectly, *via* recruitment of proteins that recognize and bind to the methylated DNA and in turn modulate gene expression. The first group of proteins that were discovered with the potential of binding to methylated DNA were the MBD (methyl-binding domain) protein family members. The mammalian MBD family consists of 5 nuclear proteins, MBD 1–4 and MeCP2 (Methyl CpG binding protein 2). With the exception of MBD3, all MBD proteins share a conserved methyl-binding domain, through which they bind to methylated DNA [10]. MBD3 lacks such ability due to a critical mutation in its MBD domain [11]. *MECP2* is an X-linked gene, which was discovered as the prototype member of the DNA methyl binding proteins (MBPs) [12]. Mutations in *MECP2* are the primary cause of Rett Syndrome (RTT), a neurological disorder predominantly affecting young females. RTT is characterized by an apparently normal development for the first 6–18 months after birth, followed by regression of acquired motor and language skills [13–15]. In addition to Rett Syndrome, mutations in *MECP2* have been observed in patients with classical autism, neonatal encephalopathy, and X-linked mental retardation [16–19].

Studies on MeCP2 have yielded surprising results in terms of the diversity of its functions (Figure 1) with enormous potential for epigenetic regulation of target gene expression. MeCP2 was initially identified as a methyl-binding protein [20]. Further investigations on MeCP2 function led to the discovery of its role as a transcriptional repressor and association with corepressor complexes such as mSin3A and HDACs [21, 22]. This was not surprising, since DNA methylation itself was considered to be a repressive mark. However, a genomewide search for MeCP2 genomic distribution in SH-SY5Y cells led to two surprising observations: (i) MeCP2 was found to be associated often with transcriptionally active genes; (ii) only 2.2% of the most methylated promoters were bound by MeCP2. The presence of MeCP2 at the active promoters was later observed in mouse hypothalamus, where MeCP2 was observed to be bound to approximately 85% of genes which were misregulated by overexpression or absence of MeCP2 [23]. These studies highlight the many facets of MeCP2 functions and emphasize the need to further study its known functions. In this review, we will discuss the role of MeCP2 in chromatin structure and nuclear architecture of neurons, its competition with the linker histone H1, the *MECP2* transcript products and diverse functional domains of MeCP2 protein, as well as MeCP2 expression and genomic targets in neurons and glia.

2. The *MECP2* Gene Structure and Its Splice Variants

The *MECP2* gene maps between *L1CAM* and the *RCP/GCP* loci in Xq28 and undergoes X-Chromosome Inactivation

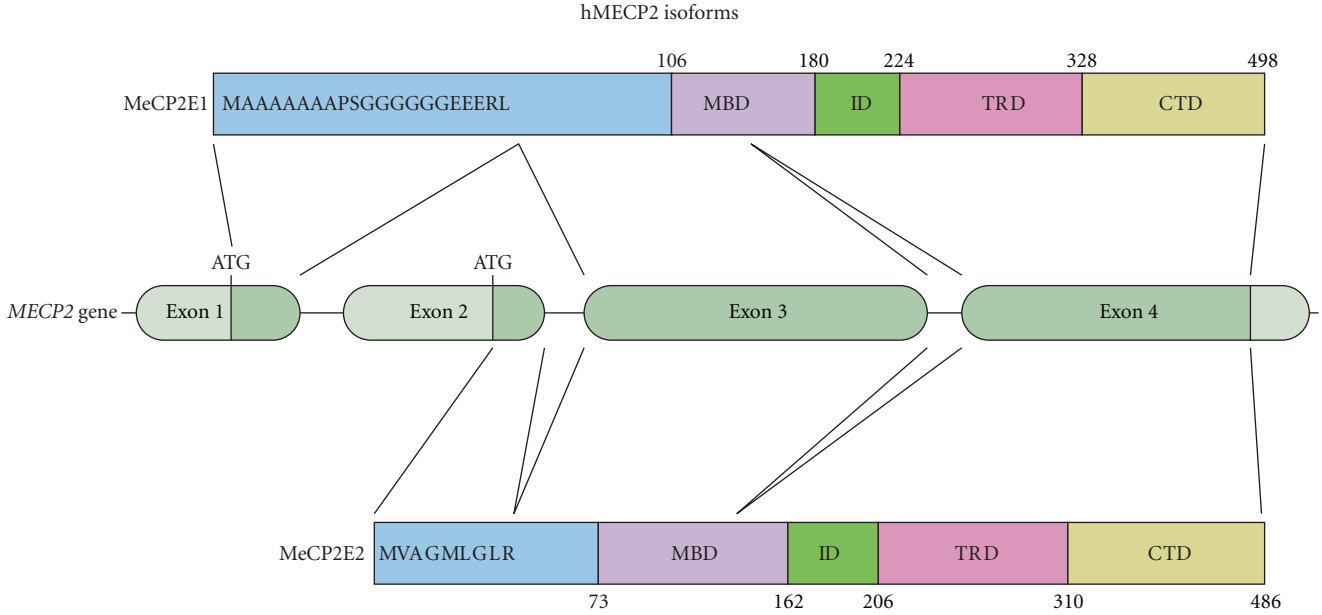

Figure 2: *MECP2* gene and protein isoforms. Schematic illustration of the gene structure of *MECP2* and the different domains of the two protein isoforms, MeCP2E1 and MeCP2E2. The primary amino acid composition of the N-terminus of MeCP2E1 and MeCP2E2 is depicted.

(XCI) in females [24, 25]. The genomic locus of *MECP2* spans approximately 76 kb and consists of four exons encoding two different isoforms (MeCP2E1 and MeCP2E2), due to alternate splicing of exon 2 (Figure 2). The more abundant E1 isoform contains 24 amino acids encoded by exon 1 and lacks the 9 amino acids encoded by exon 2, whereas the start site for the E2 lies within the exon 2 [26]. Of the two isoforms, *MECP2E1* is more efficiently translated and show 10X more expression than *MECP2E2* in brain. *Mecp2* has a large, highly conserved 3′UTR that contains multiple polyadenylation sites. Alternative 3′UTR usage leads to three distinct transcripts, short 1.8 kb and long 10 kb transcripts, with the latter including a highly conserved (8.5 kb) 3′UTR, and a third additional low abundance transcript of approximately 5–7 kb [27]. MeCP2 is a nuclear protein that is mainly colocalized with densely methylated heterochromatin in mouse cells. The differential expression of *Mecp2/MECP2* transcripts can be subjected to tissue- and developmental stage-specific regulation. In the brain, differential transcript expression patterns for the two isoforms have been detected [28]. The transcript levels are high during embryogenesis with a postnatal decrease, but increasing again towards adulthood. On the other hand, the protein levels are low during embryogenesis and increase postnatally upon neuronal maturation [29].

MeCP2E1 and E2 isoforms only differ in their N-terminal sequences, sharing the functional MBD and Transcriptional Repression Domain (TRD), and it seems likely that their functional properties overlap considerably. However, several observations point towards the possibility that the two isoforms might indeed have subtle yet etiologically relevant nonredundant functions. The MeCP2E1 isoform has a putative serine residue within its N-terminus, which is absent in MeCP2E2 isoform [26]. Furthermore, differential expression of the two isoforms at the transcript level has been demonstrated in the developing mouse brain. *Mecp2E2* mRNA was enriched in the dorsal thalamus and layer V of the cerebral cortex, while more *Mecp2E1* transcripts were detected in the hypothalamus than in the thalamus between P1 and P21 [28]. Whether this reflects a similar variation of the protein expression pattern remains to be determined. Mutation analysis in RTT patients has shown that exon 1 mutations can lead to severe RTT phenotypes. Some of these mutations do not seem to affect the transcription of MeCP2E2, suggesting that MeCP2E2 alone might not be able to compensate for the loss of MeCP2E1. Although mutations in all domains of MeCP2 have been identified in RTT patients, none have been reported to be in the MeCP2E2-specific exon 2. However, a number of point mutations have been identified that are unique to the MeCP2E1, indicating that MeCP2E1-specific mutations are sufficient to cause RTT. The possibility of functional redundancy between the two isoforms has been further investigated recently by a group studying the RTT phenotype rescue capabilities of each isoform. This study showed that MeCP2E1 alone is capable of compensating for overall MeCP2 deficiency in mice, in a dose-dependent manner. While MeCP2E2 also achieved phenotypic rescue, the degree of rescue was significantly higher with MeCP2E1, even at lower dosage levels [30]. The results of this study suggest that the two isoforms have both redundant and nonredundant functions.

3. MeCP2 Protein Structure, Interacting Protein Partners and Posttranslational Modifications

The main functional domains of MeCP2 are the MBD, the TRD, and the C-Terminal Domain (CTD). The MBD facilitates binding to methylated CpG dinucleotides and the preference for adjacent A/T-rich motifs [31]. It is also capable

of binding to nonmethylated DNA sequences such as the four-way DNA junctions [32]. However, the role of MeCP2 as a transcriptional repressor is mostly mediated through its TRD domain. The TRD interacts with corepressor complexes such as mSin3A, further recruiting HDAC1 and HDAC2, and thereby acting as a link between DNA methylation and chromatin remodelling [21]. The TRD domain further facilitates MeCP2 interaction with other partners including c-SKI [33], YY1 [34], and YB1 [35]. MeCP2 CTD is believed to have critical functions, as transgenic mice lacking MeCP2 CTD display many RTT phenotypes [36]. Recently, MeCP2 has been shown to have dual functions, also acting as a transcriptional activator *via* interaction with CREB [23], although no interacting domain has been mapped. *In vitro*, MeCP2 is known to influence chromatin compaction and nucleosome clustering [37]. In neurons, MeCP2 is also known to suppress spurious transcription of repetitive elements, thereby reducing "transcriptional noise" [38, 39].

A crucial aspect of MeCP2 that has not been fully explored is the functional effect of its Posttranslational Modifications (PTMs). Although several modifications have been detected for MeCP2, only two phosphorylation modifications have been studied in detail. Of these, phosphorylation of serine 421 (S421) is linked to neuronal activity and is known to modulate MeCP2-regulated *Bdnf* transcription [40]. Interestingly, phosphorylation of serine 80 (S80) is removed upon neuronal activity [41]. The same group detected two other phosphorylations, S399 and S424, in resting and active neurons, respectively. It would be interesting, however, to characterize any potential cross-talk between these specific PTMs in MeCP2, as seen in histones [42, 43]. Furthermore, knock-in mice models of S80 and S421/S424 showed opposing effects of the modifications on locomotor activity, implying differential function of MeCP2 based on its PTM [41]. This shows that MeCP2 PTMs enhances its capability to function dynamically within neurons, thus emphasizing the necessity of characterizing other PTMs of MeCP2.

4. The Expression Pattern of MeCP2

MeCP2 is widely expressed among various tissues, with higher expression in the brain. Expression studies in rodents, macaque, and humans have revealed a similar pattern of heterogeneous MeCP2 expression in brain [29, 44–49]. MeCP2 expression pattern within different brain regions follows the developmental maturation of the central nervous system, being initially detected in the earliest developing structures such as brainstem and thalamus [29, 49, 50]. In rodents, MeCP2 expression in the olfactory bulb precedes synaptogenesis [47, 48]. In general, MeCP2 expression is highest in neurons, with lower levels of the protein being detected in glia [51]. Within neurons, MeCP2 expression is lower in immature neurons and highest in postmitotic neurons [52]. The elevated levels of MeCP2 expression in mature neurons are maintained throughout adulthood, implying its importance in postmitotic neuronal function. To understand how MeCP2 deficiency impairs brain function, much effort has been focused on the neuronal cell-autonomous effects of MeCP2 deficiency, due to its high expression in mature neurons. Previous data indicate that MeCP2 deficiency in neurons is sufficient to cause RTT-like neurological phenotypes in mouse [53]. Recent studies investigating the effects of *Mecp2* deletion in specific neuronal population have observed differential phenotypic outcomes [54, 55]. These observations imply that various RTT phenotypes might be generated as a consequence of MeCP2 deficiency in specific neuronal populations. To date, a possible contribution of astrocyte dysfunction to RTT has not been fully examined, mainly due to the previous assumption that MeCP2 is not expressed in astrocytes.

In 2009, MeCP2 expression in glial cells was shown by independent groups [51, 56, 57], with significantly lower detection of MeCP2 in glia compared to neurons. MeCP2 deficient astrocytes showed functional abnormalities and were unable to support proper neuronal growth. Furthermore, MeCP2 deficient neurons were capable of exerting a nonautonomous effect on MeCP2 wild type astrocytes, and negatively regulating them. Another study on MeCP2 expression in microglia showed that MeCP2 deficiency in microglia leads to elevated secretion of glutamate and contributes to neuronal abnormalities in RTT. Perhaps the strongest evidence to support the effect of MeCP2 expression in astrocytes in RTT etiology comes from a recent study in which MeCP2 was reexpressed specifically in astrocytes of an RTT mice model. Reexpression of MeCP2 in astrocytes alone significantly improved several phenotypes including improved locomotion and prolonged lifespan. Restoration of MeCP2 on mutant astrocytes also led to a noncell autonomous effect on neighboring neurons, rescuing dendrite abnormalities and increasing the level of V-glut1 [58]. These studies show the critical role of glial cells in RTT pathology and warrant further investigation on MeCP2 function in glia.

5. MeCP2 Binds to Methylated DNA and Competes with Histone H1 for the Internucleosomal DNA

Eukaryotic DNA is compacted into chromatin, which is made up of nucleosome repeats [59, 60]. The nucleosome consists of a core particle composed of a histone octamer associated with DNA and a linker DNA that connects the core particles bound by one H1 linker histone. The histone octamer consists of two copies of each of the four histones H2A, H2B, H3, and H4. Histone H1 or linker histone seals two rounds of DNA at its entry/exit site on the surface of the nucleosome core and thereby stabilizes higher-order chromatin structure [61]. Histone H1 has many variants with specificity observed among species, tissue types, and even developmental stage.

Recent studies have suggested a possible relationship between histone H1 and DNA methylation [62, 63]. Microarray analysis of embryonic stem cells in which three H1 variants (H1c, H1d, and H1e) have been silenced revealed that approximately one third of the genes showing altered

expression pattern were regulated by DNA methylation. A quantitative reduction in the extent of DNA methylation at specific CpG dinucleotides within the imprinting control regions of the *H19-Igf2* and *Gtl2-Dlk1* gene loci was observed in these cells. It is interesting to note that most of these genes are known to be regulated by MeCP2 (Table 1). *In vitro,* MeCP2 can compete with histone H1 and bind linker DNA [64, 65]. *In vivo*, linker H1 and MeCP2 show similar mobility in the nucleus and share the same internucleosomal binding sites, evident by Fluorescence Recovery After Photobleaching (FRAP) studies [66, 67]. Furthermore, neuronal nuclei lacking MeCP2 show 2-fold upregulation of histone H1 expression [39]. These observations suggest that in neurons, MeCP2 and histone H1 may share similar functions, at least in part with respect to chromatin organization.

6. MeCP2 Genomic Distributions and Target Genes

By interpreting DNA methylation, MeCP2 modulates transcriptional repression and silencing of specific target genes. In neurons, MeCP2 closely tracks the intensity of methylated DNA [39]. Recent studies on MeCP2 genomic distribution, however, indicate that it occupies both methylated and unmethylated DNA [82]. Although DNA methylation is considered to be a stable modification, it is becoming evident that in the brain, reduction of DNMTs or reduced MeCP2 association may result in decreased DNA methylation, a process previously thought to be irreversible. To fully understand the functional role of MeCP2 in the pathobiology of RTT, and to develop effective therapeutic strategies, a comprehensive knowledge of MeCP2 genomic targets is essential. To this end, several research groups have attempted to identify global gene expression alterations caused by MeCP2 dysfunction in neuronal and nonneuronal tissues from RTT patients and mice models. However, in most cases these studies have generated only a small and mostly nonoverlapping list of target genes [23, 68–80] (Table 1). Also, direct association of these identified targets towards the pathophysiology of RTT has not been established in most of these studies. One exception to this would be Brain-Derived Neurotrophic Factor (BDNF). In 2003, two independent groups demonstrated MeCP2 binding to methylated CpG sites near the promoter III of *Bdnf* in resting neurons [70, 71]. Membrane depolarization of these neurons by KCl treatment led to the dissociation of MeCP2 from the *Bdnf* promoter. Two mechanisms have been proposed for the dissociation of MeCP2: (i) reduced CpG methylation at the MeCP2 binding site following neuronal activation [70] and (ii) phosphorylation of MeCP2 at specific lysine residues [71]. Recent studies in mice models have further provided *in vivo* evidence for functional interactions between MeCP2 and *Bdnf*. Experiments on an RTT mice model have shown that knockout of *Bdnf* exacerbated the RTT phenotypes, whereas overexpression of *Bdnf* rescued a subset of RTT-like phenotypes [83]. ChIP-based experiments in neonatal mouse brain identified two MeCP2 binding sites in an imprinted gene cluster in chromosome 6 [81]. Two genes within this cluster, *Dlx5* and *Dlx6,* showed approximately twofold increases in expression, in MeCP2-null mice brain. The study also showed alterations in histone modifications and the formation of a higher-order chromatin loop at the silenced chromatin of the *Dlx5-Dlx6* locus in wild type and the lack of formation of the chromatin loop in RTT patients. This provided evidence for a novel mode of gene repression by MeCP2, although a similar mechanism of repression has not been shown for any other MeCP2 targets.

7. Human Diseases Associated with *MECP2* Mutations

MeCP2 mutations are mostly sporadic, occurring preferentially as C > T transitions of CpG dinucleotides and mostly on the paternal X chromosome [84, 85]. As mentioned earlier, *MECP2* mutations are mainly associated with Rett Syndrome, a progressive postnatal neurological disorder predominantly affecting females with an incidence of 1 in 10,000 [86]. RTT is characterized by an apparently nonsymptomatic phase for the first 6–18 months of age followed by apraxia, deceleration of head growth, gait abnormalities, stereotypic hand movements, and mental retardation. The lifespan of RTT patients is variable, and some patients survive up to 70 years of age [87, 88]. In male individuals, *MECP2* mutation leads to fatal neonatal encephalopathy [89], Rett syndrome-like features, and familial X-linked mental retardation with or without motor abnormalities [89–91]. Male patients with RTT usually have a short lifespan and very often develop congenital encephalopathy [92, 93]. *MECP2* mutations have been detected in more than 90% of classical RTT patients. Approximately 65% of *MECP2* mutations causing RTT can be attributed to 8 recurrent missense or nonsense mutations within the MBD (R106W, R133C, T158M, and R168X) or TRD (R255X, R270X, R294X, and R306C) [94, 95].

Previous studies have implicated possible correlations between these mutations and RTT phenotypes [14, 96]. MeCP2 mutations have also been detected, albeit in very few patients, with Prader-Willi syndrome [97], Angelman syndrome [98], nonsyndromic mental retardation [99], and autistic patients [100]. Currently, Rett Syndrome has no effective treatment. However, in RTT mice lacking *Mecp2*, reactivation of the *Mecp2* gene after the onset of disease can rescue the disease phenotype [101, 102]. This demonstrates the possibility of RTT gene therapy strategies, where delivering *MECP2* into the affected neurons may indeed improve RTT symptoms. Creating the first generation of *MECP2* isoform-specific retroviral and lentiviral gene therapy vectors, we showed their efficient and long-term expression in the adult brain-derived neural stem cells, in their neuronal progenies, and in the brain microenvironment [56]. However, the *in vivo* rescue effect of the gene therapy delivery of these viruses remains to be elucidated. Our studies also showed the feasibility of using the endogenous *Mecp2* promoter for transgenic *MECP2* expression. This is significant, since one of the critical concerns towards the design of RTT gene therapy strategy is the prevention of *MECP2*

Table 1: Known targets of MeCP2.

Gene target	Function	Cell/tissue type studied	Direct association with MeCP2 (cell line used for ChIP)	Reference
PCDHB1	Cell adhesion	Oral cancer cell lines (ZA, KOSC2, HSC5, NA)	Yes (SH-SY5Y)	[68]
PCDH7	Cell adhesion		Yes (SH-SY5Y)	
APBP3	Intracellular signal transduction		Yes (SH-SY5Y)	
CLU	Extracellular molecular chaperone	RTT patient brain (frontal cortex)	No (SH-SY5Y)	[69]
CRMP1	Component of semaphoring signal transduction pathway		Yes (SH-SY5Y)	
DNMI	Vesicular trafficking, production of microtubule bundles, hydrolyzes GTP		Yes (SH-SY5Y)	
GNBI	Integrates signals between receptor and effector proteins		Yes (SH-SY5Y)	
APLP1	Enhancer of neuronal apoptosis		No (SH-SY5Y)	
CO1	Mitochondrial respiratory chain		No (SH-SY5Y)	
GDI1	Regulates GDP/GTP exchange		No (SH-SY5Y)	
Bdnf	Neuronal plasticity and survival	Mouse E14 cortical culture Rat E18 cortical neurons	Yes (mouse E14 cortical culture Rat E18 cortical Neurons)	[70, 71]
Fxyd1	Ion transport regulator for Na, K-ATPase	RTT mice cerebellum RTT patient's brain—superior frontal gyrus	Yes (adult mice brain, *Mecp2* wt and *Mecp2* null mouse; HEK293T cells)	[72, 73]
Reln	Neuronal layer formation, cell-cell interactions	RTT mice cerebellum	Yes (adult mice brain)	[72]
Gtl2	Growth suppressor			
ID1 *ID2* *ID3* *ID4*	Regulation of neuronal differentiation	SH-SY5Y	Yes (SH-SY5Y)	[74]
IGFBP3	Modulation of IGF functions	RTT mice model	Yes (HeLa cells; mice cortices)	[75]
UBE3A	Ubiquitin ligase	Brain cerebral samples of RTT, AS, and autism patients	No (adult mouse cerebellum samples)	[76]
GABRB3	GABA-A receptor			
Sst	Regulation of cell migration	RTT mice models (*Mecp2* null and *Mecp2* Tg) and control mice; Hypothalamus	Yes (RTT mice models (*Mecp2* null and *Mecp2* Tg) and control mice; Hypothalamus)	[23]
Oprk1	Signal transduction			
Gamt	Organ morphogenesis			
Gprin1	Neurite development			
Mef2c	Neuron development and differentiation			
A2bp1	RNA splicing and mRNA processing			
xHairy2a	Neuronal differentiation	Xenopus embryos	Yes (xenopus neurula stage embryos)	[77]
Sgk1	Cellular stress response	RTT mice model; brain samples	Yes (mice brain tissue)	[78]
Fkbp5	Hormone signalling			
Uqcrc1	Mitochondrial respiratory chain	RTT mice model; brain samples	Yes (adult mice; whole brain)	[79]
Crh	Stress response	RTT mice model; brain samples	Yes (RTT mice brain samples)	[80]
Dlx5	Transcription factor	Not done	Yes (mouse brain)	[81]

overexpression. In humans, overexpression of *MECP2* caused by duplication of the *MECP2* locus leads to a variety of neurological symptoms including seizures and mental retardation [103–105]. Alternatively, drug treatments can be designed to target proteins, which may compensate for MeCP2 loss in neurons. One study, in particular, has provided great hope towards pharmacological treatment of RTT in the future. Treatment of an RTT mice model with the active peptide fragment of IGF-1 significantly improved many disease phenotypes and extended the overall lifespan of the mice

[106]. The generation of RTT-specific iPS (induced Pluripotent Stem) cells has provided an ideal platform to analyze potential pharmacological treatments for Rett Syndrome [107, 108].

8. Closing Remarks

One of the most studied genes to link epigenetics to human disease is the X-linked *MECP2* gene. *MECP2* mutations lead to Rett Syndrome and are also associated with a broad spectrum of neurological disorders. Despite the impressive progress on our understanding about MeCP2, there are still many fundamental questions remaining to be addressed; at the methylated DNA, does MeCP2 dimerization require hetero- or homodimerization? Do MeCP2 isoforms show differential expression and/or function in CNS and are they developmentally regulated? What are the factors that regulate MeCP2 expression and splicing within various tissues? And finally, does MeCP2 act as a nonspecific DNA methyl binding protein on methylated DNA or does it recognize and prefer particular sites within the genome and what is the contribution of its interacting protein partners towards defining the specificity and sensitivity of such genomic distribution? A comprehensive knowledge of these unanswered questions will help to understand how the products of a single gene, such as *MECP2*, have such vast functional properties.

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