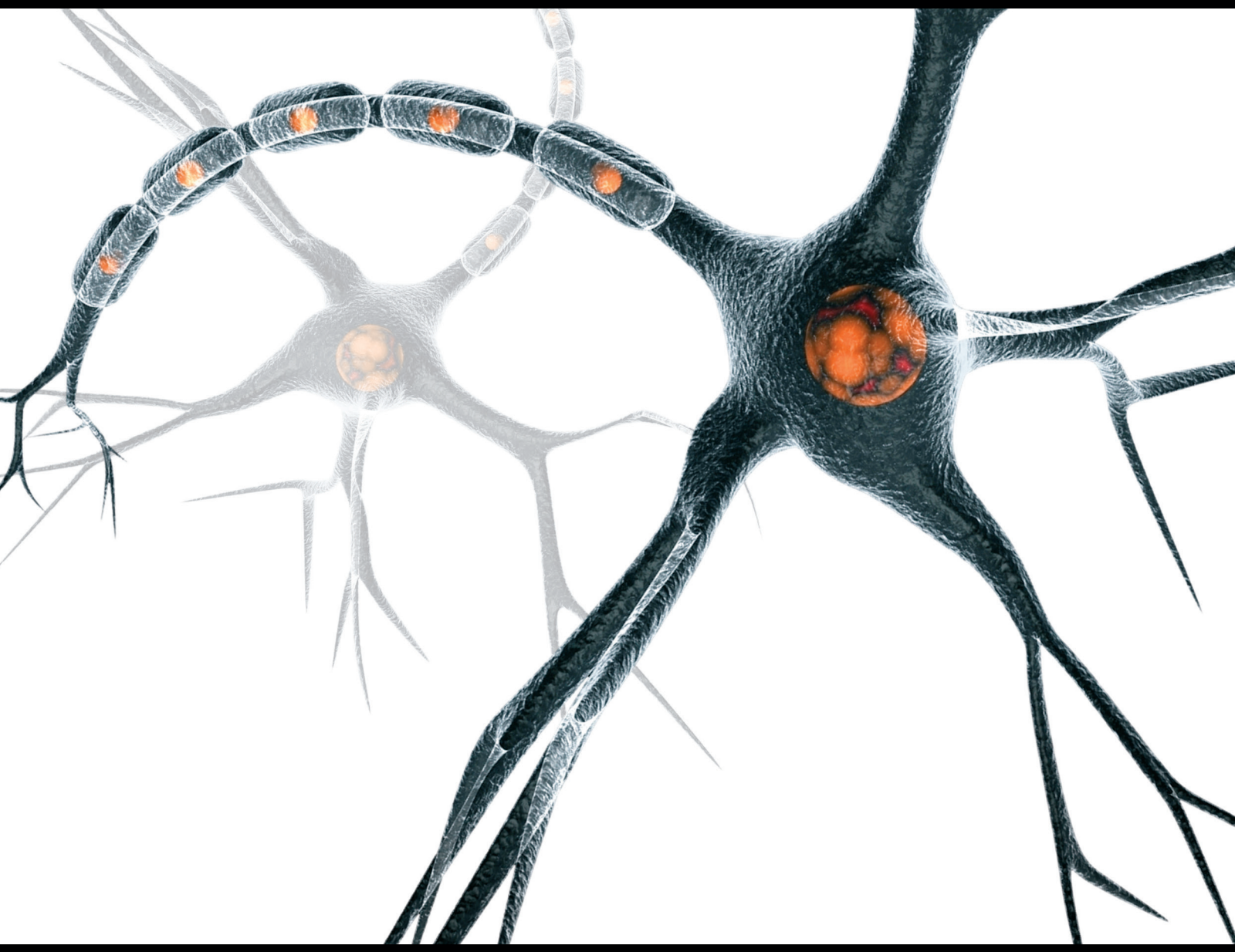
Neural Plasticity

Connecting Synaptic Activity with Plasticity-Related Gene Expression: From Molecular Mechanisms to Neurological Disorders

Guest Editors: Pablo Muñoz, Armaz Aschrafi, and Pablo R. Moya



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Editorial Board

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Editorial

Connecting Synaptic Activity with Plasticity-Related Gene Expression: From Molecular Mechanisms to Neurological Disorders

Pablo Muñoz,[1,2] Armaz Aschrafi,[3] and Pablo R. Moya[2,4]

[1]*Department of Pathology and Physiology, School of Medicine, Faculty of Medicine, University of Valparaíso, 2341386 Valparaíso, Chile*
[2]*Interdisciplinary Center for Innovation in Health (CIIS), University of Valparaíso, 8380492 Valparaíso, Chile*
[3]*Laboratory of Molecular Biology, National Institute of Mental Health, National Institutes of Health, Bethesda, MD 20819, USA*
[4]*Nucleo Milenio en Biología de Enfermedades Neurosiquiátricas nuMIND, Instituto de Fisiología, Facultad de Ciencias, Universidad de Valparaíso, 2360102 Valparaíso, Chile*

Correspondence should be addressed to Pablo Muñoz; pablo.munozca@uv.cl

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One of the most enticing endeavors in neurobiology is to examine how synapse activation leads to the expression of functionally important genes, which contribute to the maintenance and modulation of synaptic plasticity over time. To make these changes persistent, the implementation of various genetic and epigenetic mechanisms that collectively orchestrate the expression of synaptically relevant genes is critical. These findings are most gratifying as they provide an enlarging body of evidence that molecular mechanisms involving transcriptional and posttranscriptional regulation of synaptically relevant gene expression are critical for brain development, function, and neuronal plasticity. However, there is still a paucity of information on how specific molecular changes are regulated, how specific genes interact with each other during memory formation, and ultimately how these changes manifest functionally at the cellular and circuit levels. In this special issue, O. Khalaf and J. Gräff provided a comprehensive review on the mechanisms mediating the transformation from an unstable memory to a lasting one, including neural circuits and subpopulations of cells that can be recruited into a memory trace, as well as the structural changes occurring at synapses during memory formation.

We still have very limited understanding of the precise molecular mechanisms underlying the modulation of synaptic strength; over the past years a fragmentary picture is emerging through the identification of molecules whose loss of function impairs the experimental expression of synaptic plasticity. Some of these recently discovered molecules are highlighted by the authors of this special issue, representing a variety of classes of cellular functions ranging from transcription, translation, neurogenesis, gliogenesis, differentiation, Excitation/Inhibition (E/I) balance, trophic signaling, endocytosis, and neuritogenesis to synaptogenesis. Importantly, this special issue raises a series of novel hypotheses, on how single molecules contribute to precise cellular processes and how neurons collectively contribute to functional circuits, which are necessary and required for cognition. One of these hypotheses is raised by C. Engelmann and R. Haenold; in their inquiry of the transcriptional regulation of synaptic plasticity through NF-κB, they provide compelling evidence that this transcription factor may be activated synaptically, since all the machinery required for the local activation of NF-κB is concentrated in the postsynaptic density, and once activated, NF-κB is translocated to the nucleus by a cytoskeletal dependent process [1] and calcium increase [2]. This mechanism would restrict the NF-κB-dependent gene transcription only to stimulated neurons. In the case that stimulated neuron corresponds to a GABAergic neuron, NF-κB regulates the expression of the GABA synthetizing enzyme glutamate decarboxylase 65 (GAD65), by modifying the E/I

balance [3]. In the same line, the article by M. O. Caracci et al. points toward the participation of Wnt cascade, another important signaling pathway for synaptic plasticity and E/I balance. Interestingly, these authors describe alterations in both Wnt pathway and excitatory/inhibitory transmission in the etiology of autism spectrum disorders (ASDs); notably a high comorbidity of epilepsy in humans affected by ASDs is the most robust evidence of this relationship [4]. Consistent with the importance of E/I balance in epilepsy, M. Fuenzalida and C. Bonansco present a comprehensive review of the glutamatergic and GABAergic components of epilepsy but also bring to the discussion another key element involved in the synaptic function, the astrocyte, which has a major role in synaptic transmission, in what has been called the synapse tripartite [5].

Higher proportions of glial cells to neurons have been also found in both human brains and animal models of Down syndrome, another disorder with cognitive deficits. Changes in the generation rate of glial cells, therefore, might underlie such alterations in the E/I balance linked to this syndrome [6, 7]. In this regard, the article by H.-C. Lee et al. deepens on the differentiation of astrocytes, reviewing signaling pathway JAK/STAT, which is critical for gliogenesis [8] and is dysregulated in Down syndrome. From a mechanistic point of view, the STAT transcriptional activator must be phosphorylated and translocated to the nucleus to trigger gene expression required to initiate astrogliogenesis [9]. In contrast, Ngn1 activation promotes neurogenesis by competition with STAT by the binding to the coactivator p300/CBP. Complementing the topic of neurogenesis, the article by R. Ramírez-Barrantes et al. provides an overview of the involvement of the family of transient receptor potential V1 (TRPV1) in neurogenesis from neural precursors, along with cover aspects most studied in plasticity synaptic and neuronal excitability [10].

Knowledge of the molecular mechanisms that enable such activity-dependent expression of plasticity-related genes enhance our understanding of mechanisms attenuated in neurological diseases, thus opening the possibility of unraveling novel therapeutic targets to restore normal neuronal function.

In this regard, this special issue also encompasses the effect of some pathological and physiological conditions on neuronal function. P. Espinosa et al. examined the effect of neonatal exposure to sex hormones in the programming of dopaminergic neurons resulting in increased expression of tyrosine hydroxylase in ventral tegmental area and substantia nigra. On the other hand, D. A. Smagin et al. studied the effect of chronic social defeat stress in ribosomal gene expression. The authors found the greatest transcriptional effects in the hypothalamus and hippocampus, suggesting that these regions are more sensitive to stress.

Not surprisingly, in Alzheimer's disease, as well as in animal models of this disease, the hippocampus is also affected. In particular, in the article by B. Seifert et al., they elegantly demonstrated that the amyloid-beta peptide causes a dysfunction in vesicular transport of brain derived neurotrophic factor in transgenic mouse models of Alzheimer's disease. This alteration induced by amyloid-beta peptide could be due to changes in the expression of proteins involving vesicular movement and endocytosis such as Endophilins, as might suggest the study by J. Zhang et al., which showed that Endophilin 1 and 2 isoforms exhibit differential roles in synaptic vesicle endocytosis.

Understanding the precise interaction of synaptically relevant transcriptional regulation can have therapeutic potential for treatments and possibly improving these conditions, and the work by P. Lobos et al. fits within this premise: since it is known that oxidative stress contributes to the development of Alzheimer's disease, they studied the effect of astaxanthin, an antioxidant having free access to the brain, on the aberrant calcium signaling and decreased expression of type-2 ryanodine receptors (RyR2) induced by amyloid-β peptide oligomers (AβOs). They report here that astaxanthin protects hippocampal neurons in culture from the harmful effects of AβOs, preventing the generation of reactive oxygen species, activation of the transcription factor NFATc4, and RyR2 gene expression downregulation. Another example of how knowledge of the transcriptional mechanisms involved in neuronal plasticity can lead to improvement of certain conditions is the work of G. Sun et al. In this work it was found that three-dimensional cultures of spiral ganglion neurons using a combination of Matrigel and neurotrophic factors protect the culture of apoptosis and better preserve neuritic structures.

Collectively, this special issue highlights the mechanistic importance of synaptically relevant transcriptional regulation, which might open novel avenues for therapeutical approaches.

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Pablo R. Moya

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Research Article

Astaxanthin Protects Primary Hippocampal Neurons against Noxious Effects of Aβ-Oligomers

Pedro Lobos,[1] Barbara Bruna,[1] Alex Cordova,[1] Pablo Barattini,[1] Jose Luis Galáz,[1] Tatiana Adasme,[1] Cecilia Hidalgo,[1,2] Pablo Muñoz,[3] and Andrea Paula-Lima[1,4]

[1] *Biomedical Neuroscience Institute, Faculty of Medicine, University of Chile, 8380453 Santiago, Chile*
[2] *Institute of Biomedical Sciences and Center for Molecular Studies of the Cell, Faculty of Medicine, University of Chile, 8380453 Santiago, Chile*
[3] *Interdisciplinary Center for Innovation in Health (CIIS), School of Medicine, University of Valparaíso, 2341369 Valparaíso, Chile*
[4] *Institute for Research in Dental Sciences, Faculty of Dentistry, University of Chile, 8380492 Santiago, Chile*

Correspondence should be addressed to Andrea Paula-Lima; acpaulalima@u.uchile.cl

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Increased reactive oxygen species (ROS) generation and the ensuing oxidative stress contribute to Alzheimer's disease pathology. We reported previously that amyloid-β peptide oligomers (AβOs) produce aberrant Ca^{2+} signals at sublethal concentrations and decrease the expression of type-2 ryanodine receptors (RyR2), which are crucial for hippocampal synaptic plasticity and memory. Here, we investigated whether the antioxidant agent astaxanthin (ATX) protects neurons from AβOs-induced excessive mitochondrial ROS generation, NFATc4 activation, and RyR2 mRNA downregulation. To determine mitochondrial H_2O_2 production or NFATc4 nuclear translocation, neurons were transfected with plasmids coding for HyperMito or NFATc4-eGFP, respectively. Primary hippocampal cultures were incubated with 0.1 μM ATX for 1.5 h prior to AβOs addition (500 nM). We found that incubation with ATX ($\leq$10 μM) for $\leq$24 h was nontoxic to neurons, evaluated by the live/dead assay. Preincubation with 0.1 μM ATX also prevented the neuronal mitochondrial H_2O_2 generation induced within minutes of AβOs addition. Longer exposures to AβOs (6 h) promoted NFATc4-eGFP nuclear translocation and decreased RyR2 mRNA levels, evaluated by detection of the eGFP-tagged fluorescent plasmid and qPCR, respectively. Preincubation with 0.1 μM ATX prevented both effects. These results indicate that ATX protects neurons from the noxious effects of AβOs on mitochondrial ROS production, NFATc4 activation, and RyR2 gene expression downregulation.

1. Introduction

Accumulation and aggregation of the amyloid β peptide (Aβ) cause neuronal damage and death and induce the cognitive deficits that characterize Alzheimer's disease (AD) [1]. Soluble Aβ oligomers (AβOs) are among the different conformations of Aβ aggregates found in human AD brains; these neurotoxins bind to neurons and induce synaptic loss, microglia and astrocyte activation, and Tau hyperphosphorylation. Moreover, AβOs reproduce most AD pathological hallmarks when injected into animal model brains [2, 3]. The cellular and molecular mechanisms by which AβOs perturb normal neuronal function have been extensively investigated [4]. In this regard, the excessive generation of reactive oxygen species (ROS) produced by AβOs represents an important source of neuronal damage [5]. The consequent redox imbalance generated by AβOs contributes to the pathological cascade in AD and other neurodegenerative diseases, in which oxidative stress is a common pathological feature [6, 7].

Oxidative stress occurs concomitantly with the deregulation of Ca^{2+} signaling and of downstream Ca^{2+}-dependent pathways induced by AβOs [8, 9]; in particular, pathways downstream of N-methyl-D-aspartate (NMDA) receptors play a key role in AβOs induced neurotoxicity [10–12]. Aberrant activation of NMDA receptors leads to abnormal changes in gene expression that underlie AβOs-induced morphological and functional defects [13, 14]. The abnormal

NMDA receptor-mediated Ca^{2+} signaling induced by AβOs activates the protein phosphatase calcineurin, leading to downstream activation of the transcription factor NFAT [15], which promotes spine loss [11].

Astaxanthin (ATX), a red-orange carotenoid that originates the pink or red color of salmon, trout, lobster, shrimp, and other sea organisms, exhibits antioxidant, anti-inflammatory, and antiapoptotic effects. Recently, ATX was shown to protect neurons in experimental models of acute injuries, chronic neurodegenerative disorders, and neurological diseases and was proposed as a beneficial strategy to treat neurological diseases [16]. Although other antioxidants, such as resveratrol, have been shown to protect neurons from injury in similar model systems [17], ATX presents many advantages compared to other antioxidants displaying protective effects. Albeit ATX has a very similar structure to that of other carotenoids such as lutein and zeaxanthin, and it has some structural differences in the arrangement of its hydroxyl groups that provide ATX with unique characteristics. Among others properties, (i) ATX has much higher antioxidant power than other members of the carotenoid family [18]; (ii) it chelates several metal ions, preventing metal ion-induced oxidative stress [19]; (iii) it has anti-inflammatory properties [18]; (iv) it crosses the blood brain barrier, allowing free access to the central nervous system [20]; (v) it acts as damper of singlet oxygen levels [21]. The combination of these properties makes ATX a very attractive candidate for use against certain diseases of the central nervous system that are caused by increases in ROS, such as superoxide anion, hydroxyl radical, and hydrogen peroxide. Consequently, ATX has been successfully used to decrease oxidative stress in elderly patients [22] and to improve neuronal function after brain ischemia [23].

Here we investigated the possible protective effects of ATX against some of the well-known deleterious effects of AβOs on primary hippocampal neurons. Our results strongly suggest that ATX protects neurons from the noxious effects which AβOs exert on mitochondrial ROS production, NFATc4 activation, and downregulation of RyR2 gene expression, suggesting that this natural antioxidant agent may represent a future approach to treat AD.

2. Experimental Procedures

2.1. Materials. Aβ peptide (Aβ1–42) was purchased from Bachem Inc. (Torrance, CA). ATX was extracted from *Lithodes antarcticus* (BIOTEX S.A., Santiago, Chile). Hexafluoro-2-propanol (HFIP) was from Merck (Darmstadt, Germany) and dimethyl sulfoxide (DMSO) from Sigma-Aldrich (St. Louis, MO). TRIzol reagent, B27 supplement, Neurobasal medium, Dulbecco's modified essential medium (DMEM), Lipofectamine 2000, and the DNA binding dye SYBR green (Platinum SYBR Green qPCR SuperMix UDG) were from Invitrogen (Carlsbad, CA). The live/dead kit was from Molecular Probes (Chicago, IL), the Ambion DNA-free™ Kit from ThermoFisher Scientific (Chicago, IL), and the ImProm-II™ Reverse Transcriptase kit from Promega (Madison, WI). The pEGFP-C1 NFAT3 (NFATc4) (plasmid #10961; full-length human NFATc4) was a gift from Dr. J. D. Molkentin (Cambridge, MA) [24]. The HyperMito plasmid was from Evrogen (Moscow, Russia). The amplification system (MX3000P) was from Stratagene (La Jolla, CA).

2.2. Preparation of AβOs. $A\beta_{1-42}$ peptide, prepared as a dried hexafluoro-2-propanol (HFIP) film as described previously [9], was stored at −80°C for up to 4 months. Prior to use, this peptide film was dissolved in sufficient sterile DMSO to make a 5 mM stock solution. To prepare AβOs as previously described [25, 26], the 5 mM peptide solution was subsequently diluted to 100 μM with cold phosphate buffered saline (PBS), aged overnight at 4°C and centrifuged at 14,000 ×g for 10 min at 4°C to remove insoluble aggregates (protofibrils and fibrils). The supernatant containing soluble AβOs was transferred to clean tubes and stored at 4°C. Only fresh AβO preparations (2 days-old maximum) were used in all experiments.

2.3. Primary Hippocampal Cultures. Cultures were prepared from eighteen-day-old embryos obtained from pregnant Sprague-Dawley rats as previously described [25–29]. Briefly, brains were removed and placed in a dish containing HANKS-glucose solution. Hippocampi were dissected and, after stripping away the meningeal membranes, cells were gently dissociated in HANKS-glucose solution, centrifuged, and resuspended in DMEM medium supplemented with 10% horse serum. Dissociated hippocampal cells were plated on polylysine-coated plates and after 1 h DMEM was replaced by Neurobasal medium supplemented with B-27. Cultures were incubated for 15–21 days *in vitro* (DIV) at 37°C in a humidified 5% CO_2 atmosphere prior to experimental manipulations. The resulting cultures were highly enriched in neuronal cells, identified with neuronal anti-MAP-2, with a glial content <24% [25]. The Ethics Committee of the Faculty of Medicine, Universidad de Chile, approved the bioethical protocol of this study. All procedures were performed in accordance with the Guideline for the Care and Use of Laboratory Animals from the National Institutes of Health, USA. Animals were housed under a 12 h light/dark cycle in a temperature-controlled room at 24 ± 1°C with free access to food and water. Animals were euthanized under deep anesthesia to avoid animal suffering at each stage of the experiment.

2.4. Cell Viability Assay. To evaluate the effect of ATX on the cell viability of cultured hippocampal neurons maintained *in vitro* for 14 days (14 DIV), cultures were treated for 24 h with different ATX concentrations (1 nM, 10 nM, 100 nM, 1 μM, 10 μM, and 100 μM) and cell viability was evaluated by the live/dead kit following the manufacturer's instructions as previously described [29]. Briefly, after removal of the culture, medium cells were gently washed three times with warm PBS-glucose and incubated at room temperature for 30 min in the presence of 2 μM calcein AM ester and 1 μM ethidium homodimer in PBS-glucose. Live neurons were identified by green calcein fluorescence and dead neurons were identified by the red fluorescence of DNA-bound ethidium. Cells were examined and counted on a Nikon® Eclipse Ti-Eat at 20x

magnification. At least three random fields were imaged per culture well (three replicate wells were used per experimental condition in each experiment) and about 500 cells were counted in each well. Six independent experiments were performed with different neuronal cultures. Cell viability was expressed as percentage relative to the untreated control cultures, which exhibited 85% cell viability on average.

2.5. Determination of Mitochondrial Hydrogen Peroxide Generation. Cultures grown in 25 mm glass plates were transiently transfected with the HyperMito plasmid at 11–14 DIV, using a proportion of 1 : 2 DNA : Lipofectamine 2000®. HyperMito is a fusion protein of the permuted circular yellow fluorescent protein (YFP) and the regulatory domain of the transcription factor OxyR, which contains two cysteines that oxidize in response to H_2O_2 generation and form a disulfide bridge producing a conformational change that causes an increase in YFP fluorescence [30]. One-day after transfection, cultures incubated in Neurobasal medium supplemented with B-27 were treated for 1.5 h with 0.1 μM ATX, rinsed three times with modified Tyrode solution plus 0.1 μM ATX, and maintained in this solution during the experiment. At the microscope stage, cultures were stimulated with 500 nM AβOs and fluorescence signals from neuronal cells (identified as such by morphology) were recorded every 6 s in a Carl Zeiss LSM Pascal 5 confocal microscope system using 63x Oil DIC objective, excitation 488 nm, and argon laser beam. Changes in mitochondrial H_2O_2 levels are presented as F/F_0 values, where F corresponds to the experimental fluorescence and F_0 corresponds to the basal fluorescence.

2.6. Nuclear Translocation of NFATc4-eGFP. Cultures grown in 25 mm glass plates were transiently transfected with a plasmid of a fusion protein encoding a green fluorescent protein (GFP) and NFATc4 [24] at 13–15 DIV using a proportion of 1 : 2 DNA : Lipofectamine 2000®. Cultures maintained in Neurobasal medium (supplemented with B-27) were treated one day after transfection for 1.5 h with 0.1 μM ATX, previous to the addition of 500 nM AβOs for 6 h. Neurons were then fixed with 4% paraformaldehyde, washed three times with PBS, and incubated with Hoechst for 5 minutes for nuclear staining. Covers were mounted in DAKO mounting medium for microscope observation. The subcellular localization of NFATc4-eGFP was visualized in cells using a Carl Zeiss LSM Pascal 5 laser scanning confocal with the 40x objective lens. Data were analyzed using the ImageJ software (NIH). To calculate the NFATc4 ratio of nucleus versus cytoplasm, the fluorescence intensity of nuclear NFATc4 was divided by the intensity of cytoplasmic NFATc4. Nuclear translocation of NFATc4 was determined by EGFP fluorescence intensity values from a region of interest (ROI) in the nucleus, as indicated by the overlap of EFGP staining with Hoechst nuclear staining. Background fluorescence was corrected by using a ROI devoid of cells and values were normalized to their respective areas.

2.7. RNA Isolation and PCR Analysis. To determine RyR2 mRNA levels, neurons were treated for 1.5 h with 0.1 μM ATX prior to incubation with 500 nM AβOs for 6 h. To extract RNA cells were lysed as described in previous work [25]. Total RNA was isolated using TRIzol reagent. To remove any contaminating genomic DNA, a DNAase digestion step with Ambion DNA-free™ Kit was included. RNA purity was assessed by the 260/280 absorbance ratio and RNA integrity by gel electrophoresis. cDNA was synthesized from total RNA (2 μg) using the ImProm-II™ Reverse Transcriptase kit. Twenty-five ng of cDNA was used in 20 μL final volume for PCR amplification (Applied Biosystem Thermal Cycler). Amplification was performed using the primers and conditions detailed previously [25]. Real-time quantitative PCR (qPCR) was performed in an amplification system (MX3000P) using the DNA binding dye SYBR green (Brilliant III SYBER-GREEN Master Mix). Levels of RyR mRNA were calculated by the relative $2^{-\Delta\Delta Ct}$ method [31] and normalized with respect to levels of β-actin mRNA. Dissociation curves were analyzed to verify purity of products. All samples were run in triplicate.

2.8. Statistics. Results are expressed as mean ± SEM. The significance of differences was evaluated using Student's t-test for paired data and with one-way ANOVA followed by Bonferroni's *post hoc* test for multiple determinations.

3. Results

Previous studies showed that ATX, by attenuating oxidative damage, lipid peroxidation, and inhibiting the mitochondrial-related apoptotic pathway, protects hippocampal neurons against epilepsy-induced cellular loss [32]. Additionally, ATX prevents inflammation injury and improves cognition in diabetic mice [33]. To investigate the possible neuroprotective role of ATX against the toxic effects produced by AβOs, we treated primary hippocampal cultures with sublethal concentrations of AβOs (500 nM) in the presence or absence of ATX.

We first determined cell viability of primary hippocampal cultures (14 DIV) exposed for 24 h to different concentrations of ATX (ranging from 0.001 to 100 μM). Figures 1(a)–1(h) show representative live/dead images of control cultures (a), of cultures treated with different ATX concentrations ((b)–(g)) or with 250 μM H_2O_2 (h) to induce cell death. Live cells display calcein green fluorescence while dead cells exhibit punctuated ethidium red fluorescence. Figure 1(i) shows the quantitative analysis of cell viability expressed as percentages of control, determined in six independent experiments performed in six neuronal cultures. Control neurons presented at least 85% cell survival. Treatment with ATX concentrations ≤10 μM did not decrease cell viability when compared to control cultures, while treatment with 100 μM ATX induced 20% cell death. For comparison, treatment with 250 μM H_2O_2 elicited 40% neuronal death.

Considerable evidence points to brain oxidative stress as an important event in the early stages of AD [5]. In particular, enhanced generation of ROS, such as H_2O_2 and hydroxyl radicals, has been proposed as a key molecular mechanism underlying the pathogenesis of AD [34]. Since

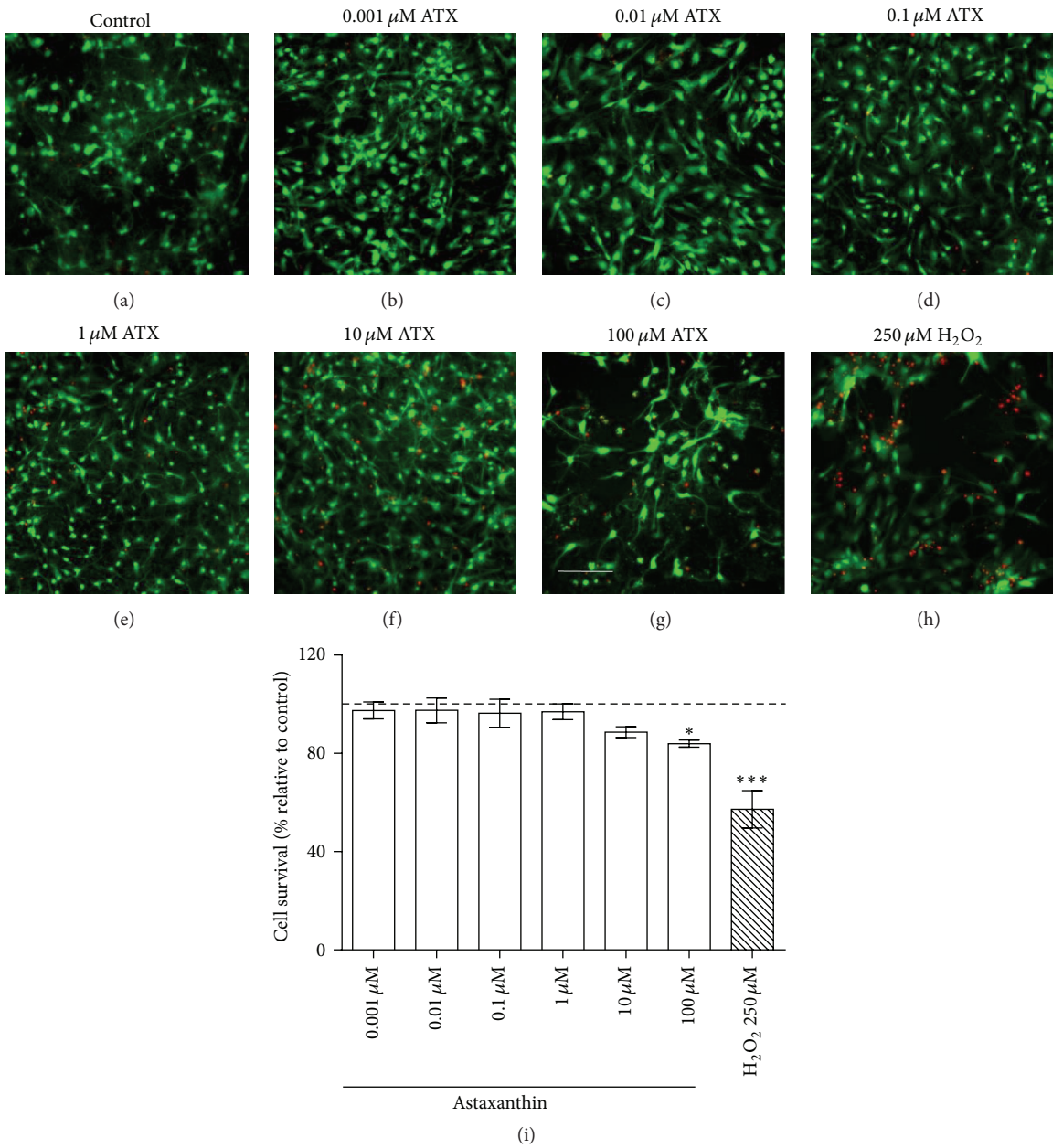

Figure 1: Lack of toxicity of ATX (≤10 μM) to primary hippocampal cultures. Representative live/dead fluorescence images ((a)–(h)) of neuronal hippocampal cultures (13–15 DIV) incubated for 24 h in the presence of vehicle (a) or treated with 0.001 μM (b), 0.01 μM (c), 0.1 μM (d), 1 μM (e), 10 μM (f), and 100 μM (g) ATX. In (h), 250 μM H_2O_2 was used to induce cell death. Live and dead neurons were identified by green calcein and red DNA-bound ethidium fluorescence, respectively. Scale bar: 50 μm. (i) shows quantitative analysis of cell survival incubated with different concentrations of ATX (white bars) and under H_2O_2 stimulation (hatched bar). Results are expressed as percentages relative to the viability of the control, untreated cultures. Values correspond to mean ± SE of six independent experiments ($n = 6$, corresponding to cultures from 6 different animals; in all experiments, each condition was tested at least in triplicate), with different neuronal cultures. Control cultures exhibited 85% of cell viability on average. Statistically significant differences among experimental conditions were evaluated by one-way ANOVA followed by Bonferroni's multiple comparison test ($^{*}p < 0.05$ and $^{***}p < 0.0001$ compared to control).

AβOs induce neuronal ROS production [9], including mitochondrial ROS production [35], we tested whether ATX prevents mitochondrial H_2O_2 generation induced by AβOs. To this purpose, we transfected neurons with a plasmid that codes for the HyperMito protein, a H_2O_2 fluorescent sensor with mitochondrial destination; 24 h after transfection we added AβOs (500 nM) to the cultures and recorded fluorescence levels for 20 minutes. The representative images illustrated in Figure 2 show that control neurons (Figure 2(a)) did not display significant fluorescence changes when comparing the image taken before vehicle addition (250 s), with the final image collected at the end of the record (1250 s).

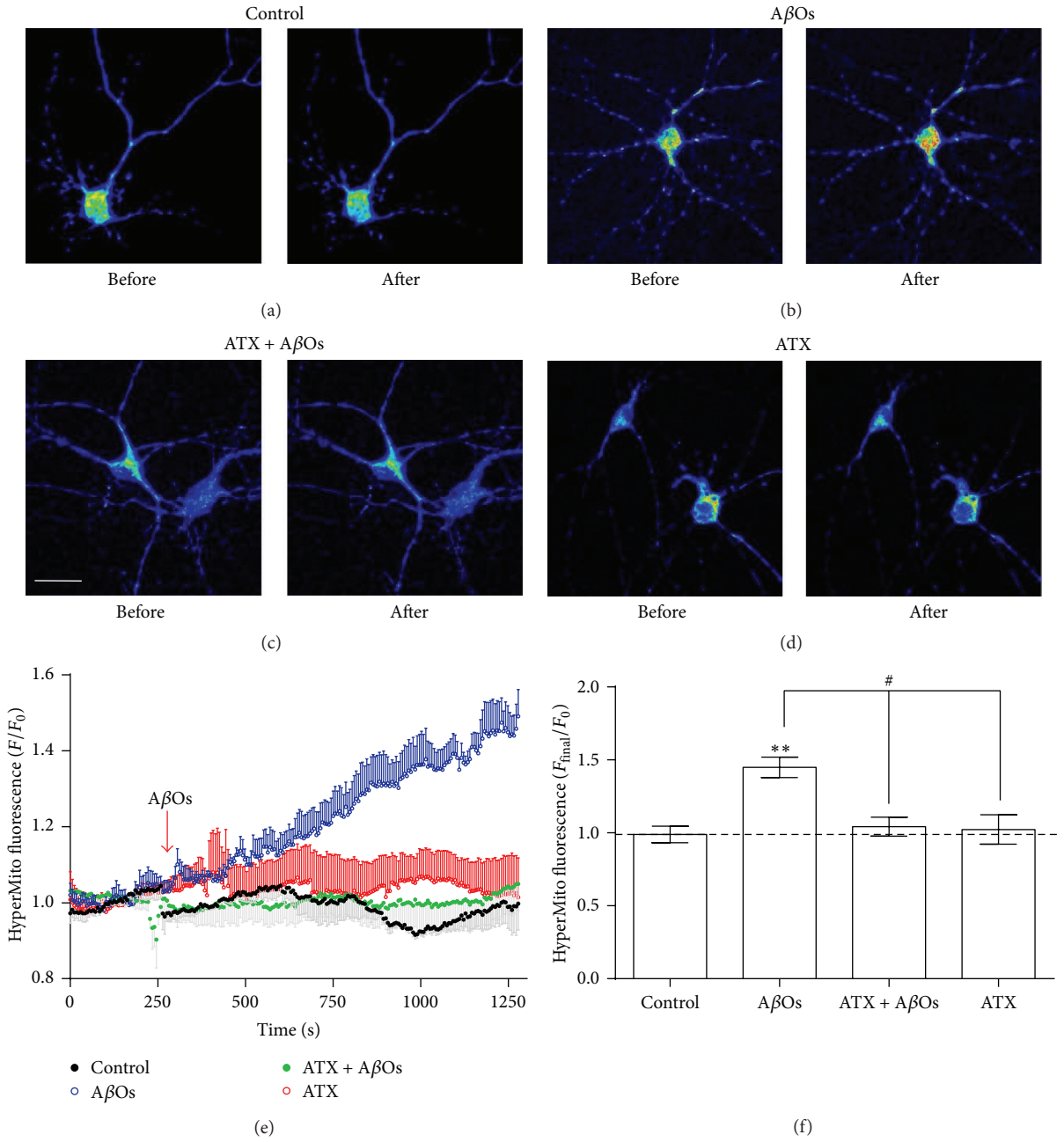

FIGURE 2: ATX prevents mitochondrial H_2O_2 generation induced by AβOs. Hippocampal neurons (13–15 DIV) were transfected with HyperMito, 24 h before the experimental maneuvers. ((a)–(d)) show representative pseudocolor images of hippocampal neurons expressing the HyperMito protein, collected before (250 s, left images) or 1000 s after (1250 s, right images) the addition of vehicle ((a) and (d)) or of 500 nM AβOs ((b) and (c)). Higher fluorescence intensity levels are expressed by the red color in a pseudocolor scale, while lower intensity levels are expressed by blue color. Scale bar: 10 μm. (e) shows representative time courses of HyperMito fluorescence, recorded in neuronal soma after the addition of vehicle (black symbols) or 500 nM AβOs in the absence (blue symbols) or presence (green symbols) of 0.1 μM ATX preincubated for 1.5 h, which alone did not induce changes in HyperMito fluorescence (red symbols). Fluorescence changes (mean ± SE) are expressed as (F/F_0), where F_0 corresponds to the basal fluorescence recorded in the soma before AβOs addition. The graph illustrates average values from 2 ROIs registered at the soma of neurons recorded in the visual field ($n = 4$). Values correspond to four different experiments performed in four cultures from four different animals; each condition was tested in duplicate. (f) shows the values of F/F_0 obtained at the end of the experiment (time 1250 s) to each condition and bars represent mean ± SE. Statistically significant differences among experimental conditions were evaluated by one-way ANOVA followed by Bonferroni's multiple comparison test ($^{**}p < 0.001$ compared to control; $^{\#}p < 0.05$ compared to indicated conditions).

Hippocampal neurons responded to AβOs with a significant increase in probe fluorescence, as illustrated by the representative images (Figure 2(b)), recorded before (250 s) and 1000 s after AβOs addition (1250 s). In contrast, neurons preincubated for 1.5 h with 0.1 μM ATX and then treated with 500 nM AβOs (Figure 1(c)) or preincubated with ATX and vehicle (Figure 1(d)) did not exhibit significant fluorescence changes when comparing the images taken at the final and the initial time point, recorded before vehicle or AβOs addition. The time courses of average fluorescence changes recorded in neurons stimulated with 500 nM AβOs or vehicle in the presence or absence of ATX, shown in Figure 2(e), indicate that AβOs addition to control neurons promoted mitochondrial H_2O_2 production within minutes (blue trace). Neurons preincubated for 1.5 h with ATX (0.1 μM) did not exhibit changes in fluorescence following addition of 500 nM AβOs (green trace) or of saline (red trace). The relative fluorescence (F_{final}/F_0) values of neurons maintained in different conditions, plotted in Figure 2(f), show that 500 nM AβOs induced a significant increase in mitochondrial H_2O_2 content, which was prevented by preincubation with 0.1 μM ATX.

A requisite step for the sustained synaptic plasticity processes underlying learning and memory is an elevation in intracellular-free Ca^{2+} concentration, which plays a central role in Ca^{2+}-dependent gene transcription [36–38]. Indeed, defective Ca^{2+} signaling is believed to underlie AD neuronal pathology [39]. The activation of calcineurin, which promotes the downstream stimulation of the transcriptional factor NFAT that is engaged in dendritic and axonal development, synaptogenesis, and neuronal survival [40], plays a prominent role among activity-dependent Ca^{2+}-signaling pathways. In particular, the isoform NFATc4 is activated by prolonged Ca^{2+} signals [41]. Moreover, activation of NFATc4 has been demonstrated *in vitro* and *in vivo* in AD [42]. Of importance, NFATc4 activation by Aβ invokes morphological changes such as neuritic dystrophy and loss of dendritic branching and spines, effects that are prevented and reverted by inhibitors of the calcineurin/NFAT pathway [42, 43]. Also *postmortem* studies showed that, in the hippocampus of patients, activation of NFATc4 correlates with cognitive deficits [44, 45]. To investigate whether preincubation with ATX prevents the activation of NFATc4 by AβOs, we determined its translocation to the nucleus in hippocampal neurons transfected with plasmid codifying for the fusion protein of NFATc4 with GFP. We preincubated neurons 24 h after transfection with 0.1 μM ATX for 1.5 h and exposed them to AβOs (500 nM) for an additional 6 h period. As illustrated in Figure 3(a), control neurons displayed GFP fluorescence mainly in the cytoplasm, indicating that in this condition NFATc4 was inactive. Incubation with AβOs for 6 h induced nuclear translocation of NFATc4/GFP, indicating that AβOs induce NFATc4 activation (Figure 3(b)). Previous incubation with ATX did not change the cytoplasmic distribution of NFATc4 in neurons incubated with AβOs for 6 h (Figure 3(c)) or in control neurons (Figure 3(d)). The average results from four experiments (Figure 3(e)) show that incubation of neurons with AβOs for 6 h increased 3-fold the nuclear/cytoplasmic ratio; this increase did not occur in neurons pretreated with ATX.

We have previously shown that brain derived neurotrophic factor (BDNF) positively regulates the expression of intracellular RyR2 Ca^{2+} channels in hippocampal cultures [46], whereas AβOs downregulate RyR2 expression during synaptotoxicity [25]. In agreement with our previous results, incubation for 6 h with AβOs (500 nM) significantly decreased RyR2 mRNA levels to approximately 54% (Figure 4(a)). Preincubation with ATX (0.10 μM) did not modify RyR2 mRNA levels but resulted in complete prevention of the reduction of RyR2 mRNA levels promoted by AβOs (Figure 4(a)). These results suggest a possible link between mitochondrial ROS generation and RyR2 expression. In addition, we found that the general antioxidant agent N-acetyl-L-cysteine (NAC), which is a cellular precursor of glutathione, also protects primary hippocampal neurons from the RyR2 mRNA decrease induced by AβOs (Figure 4(b)). Although the results presented in Figure 4 may be interpreted as an indication that RyR2 protein content was better preserved by treatment with ATX compared to NAC, these effects were not significantly different (not shown).

4. Discussion

Alzheimer's disease is the most common form of dementia worldwide. Among the most important risk factors for the development of AD are human conditions that have been associated with oxidative stress and chronic inflammation, which include aging, cardiovascular diseases, diabetes, hypertension, brain trauma, and high alcohol consumption [47, 48]. On the other hand, there are factors considered protective, as regular exercise and the consumption of diets rich in antioxidants [48, 49]. In the search for therapeutic strategies that could prevent AD, several attempts have been made to slow the disease progression with antioxidant agents [50]. Briefly, several studies carried out in *in vitro* and *in vivo* models of AD have shown some positive results of antioxidants. The main mechanisms proposed to explain these effects are (1) mimicking endogenous catalytic enzymes (mainly superoxide dismutases (SOD), catalases (CAT)) [51] and metabolic precursors of endogenous antioxidants system (Glutathione) [52]; (2) acting like ROS scavenger; and (3) causing SIRT activation [53]. Although antioxidants have been widely studied as an alternative strategy to prevent or treat AD, direct evidence is still required to support their use in the treatment of patients suffering from AD [54, 55].

Antioxidant agents are distinct chemical entities with structures that command their different modes of action; this structural diversity imparts each antioxidant agent with a unique biochemical profile, which is reflected in different sites of action and biological activities. ATX is classified as a lipophilic antioxidant of the carotenoid family, and its main protective mechanisms rely on its capacity to act as a singlet oxygen quencher and free radical scavenger. However, after scavenging reactive-free radicals, ATX is transformed into a carotenyl radical by hydrogen abstraction; this process can lead to a switch from a beneficial antioxidant agent

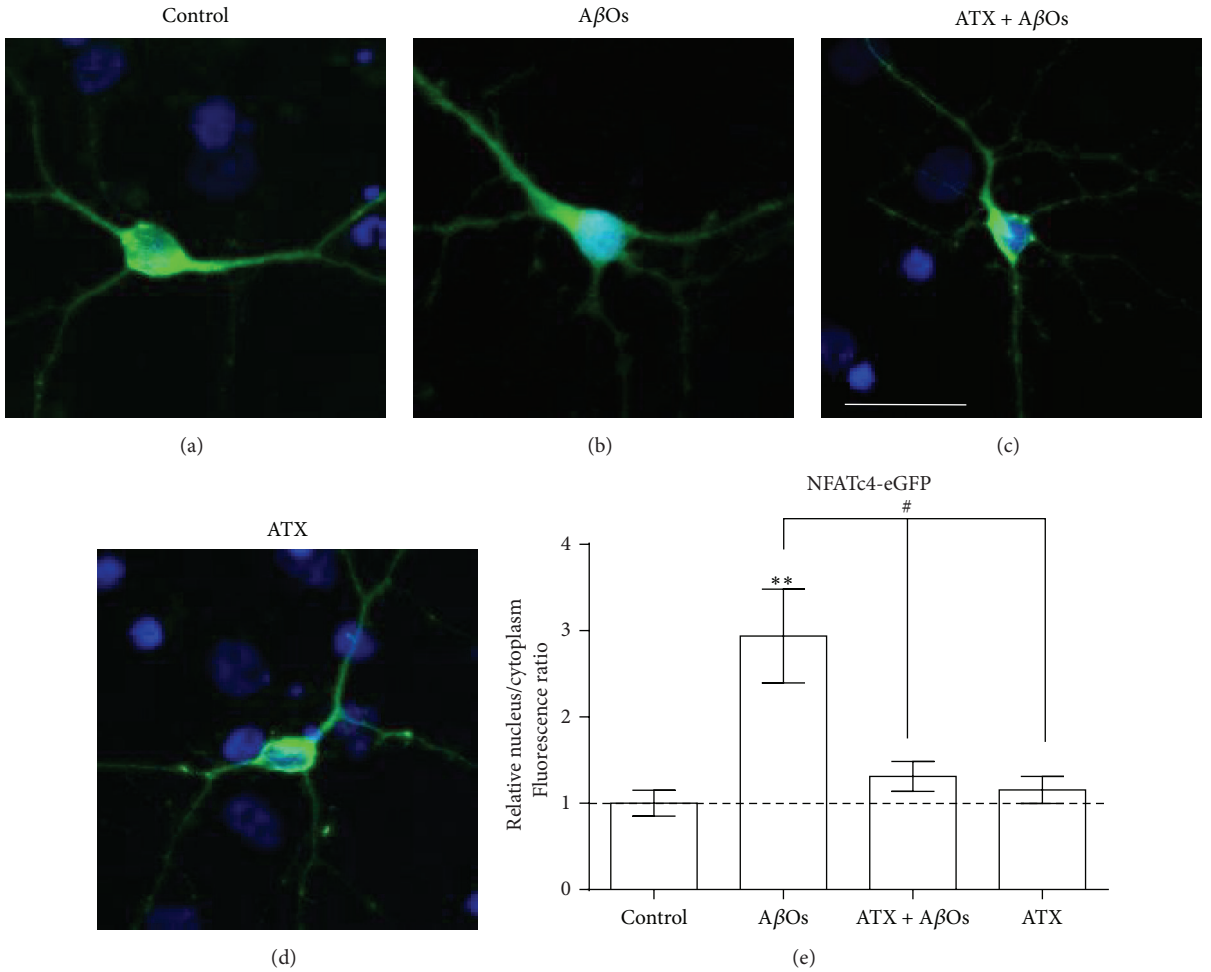

Figure 3: ATX prevents AβOs-induced NFATc4 activation. Hippocampal neurons (13–15 DIV) were transfected with EGFP-NFATc4 24 h before the experiments. ((a)–(d)) show representative images of intracellular distribution of EGFP-NFATc4 (green fluorescence) and of nuclear staining with Hoechst (blue fluorescence) in hippocampal neurons. (a) Neurons treated with vehicle, (b) stimulated with 500 nM AβOs for 6 h, (c) preincubated with 0.1 μM ATX for 1.5 h before AβOs addition, or (d) incubated with ATX for 7.5 h. Scale bar: 10 μm. (e) shows the quantification of four different experiments ($n = 4$) in cultures from four different animals; each condition was tested in duplicate (in total, 15–25 cells were analyzed per condition). The results are expressed as the mean ratio of nuclear/cytoplasmic fluorescence intensity ± SE, relative to control cells. Statistically significant differences among experimental conditions were evaluated by one-way ANOVA followed by Bonferroni's multiple comparison test ($^{**}p < 0.001$ compared to control; $^{\#}p < 0.05$ compared to indicated conditions).

to a damaging prooxidative one, which could explain ATX toxicity when used at higher concentrations (100 μM) [56]. Here, we studied the protective properties of ATX against the noxious effects of AβOs on primary hippocampal cultures and compared some of its effects with the protective actions of NAC. We also compared the effects of NAC because this is a classical and very widely studied antioxidant. Although NAC also acts as a ROS scavenger, its principal action stems from its role as a precursor of cysteine, the rate-limiting factor in the *de novo* synthesis of glutathione (GSH). In this sense, contrary to ATX, NAC is an indirect antioxidant, which, as a precursor of the antioxidant GSH, has a safer toxicity profile, allowing the use of higher concentrations [57]. Both antioxidant agents have been studied in the context of central nervous system disorders [16, 58], but evidence related to NAC is more abundant.

We have previously shown that incubation of hippocampal neurons *in vitro* with NAC, a glutathione precursor molecule with antioxidant properties, completely prevents the aberrant increase in intracellular Ca^{2+} levels and the mitochondrial fragmentation induced AβOs [25, 52]. In previous work from our laboratory, we have also shown that reducing agents suppress RyR activation by Ca^{2+} in cortical neurons [59]. Also the administration of NAC through drinking water to a transgenic mouse model of AD (mouse APP/PS-1) suppressed the protein oxidation and nitrosylation in the brains of mice aged 9 and 12 months [60]. The above results are consistent with the idea that NAC modulates the activity of RyR by avoiding the aberrant intracellular ROS increase and hence the enhanced Ca^{2+} release produced by ROS-modified RyR, induced by AβOs.

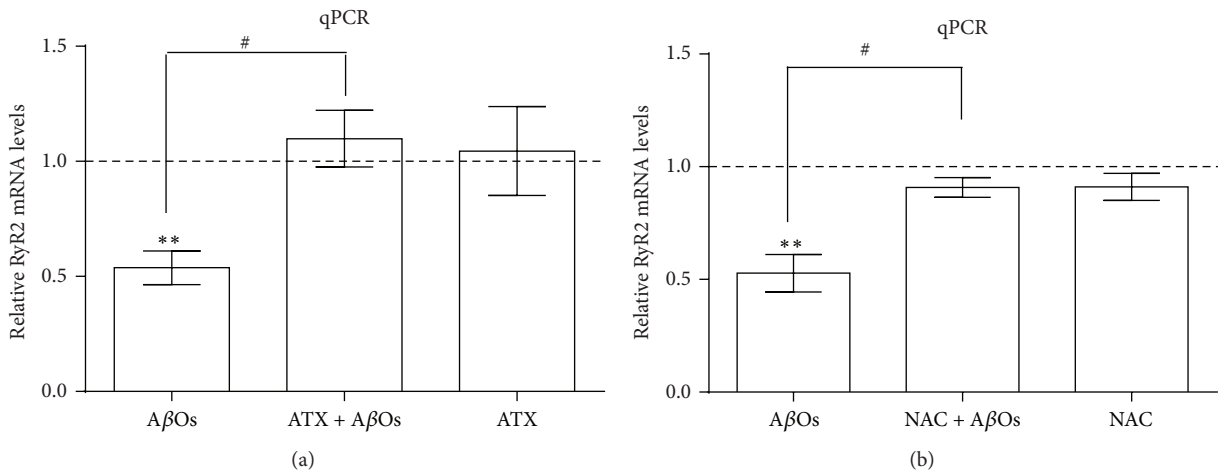

Figure 4: ATX prevents AβOs-induced RyR2 mRNA downregulation. Hippocampal cultures were preincubated with 0.1 μM ATX (a) or 10 mM of NAC (b) for 1.5 h or 30 minutes, respectively, before incubation for 6 h with 500 nM AβOs. Relative RyR2 mRNA levels were determined with qPCR, normalized to β-actin mRNA levels, and expressed as fold over control. Values represent mean ± SE ($n = 6$) from experiments performed in cultures from six different animals; each condition was tested in triplicate. Statistically significant differences among experimental conditions were evaluated by one-way ANOVA followed by Bonferroni's multiple comparison test ($^{**}p < 0.001$ compared to control; $^{\#}p < 0.05$ compared to indicated conditions).

Astaxanthin is a natural carotenoid product that is used in nutritional supplements, which can be extracted from *Lithodes antarcticus*. ATX has been shown to quench singlet oxygen and to scavenge free radicals [61]; ATX antioxidant properties reside in its polar ionic rings and nonpolar conjugated carbon–carbon bonds and are 10-fold greater than those of other carotenoids [62]. In addition to the ROS scavenging properties attributed to ATX, several studies have shown that ATX, alone or in combination with omega-3 fatty acids, protects cells by inducing antioxidant activity via the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) Nrf2/heme oxygenase-1 (HO-1) signaling pathway [63–65]. Several studies have implicated Nrf2 in the induction of HO-1, which is the enzyme that catalyzes the first and rate-limiting step of heme metabolism [66, 67]. HO-1 activity protects tissue during inflammatory stress in various conditions through the degradation of prooxidant heme and the production of carbon monoxide (CO) and bilirubin, both of which have anti-inflammatory and antiapoptotic properties, especially in ROS-dependent perturbations associated with metabolic syndrome [67].

In this work, we tested the possible neuroprotective effects of ATX on some of the noxious effects induced by AβOs on primary hippocampal cultures. We found that ATX prevented the mitochondrial generation of H_2O_2, the nuclear translocation of NFATC4, and the decrease of RyR2 mRNA levels induced by AβOs. These protective effects may result from the reduction of intracellular ROS promoted by ATX. Although ROS have important roles in cell signaling and normal neuronal function, excessive ROS generation, such as that produced by AβOs, has deleterious effects on neuronal function, which include the significant damage to DNA, RNA, proteins, and polyunsaturated fatty acids in lipids caused by irreversible oxidation. Normally, cells defend themselves against ROS damage through intracellular and extracellular defenses, in particular through enzymes such as SOD, CAT, lactoperoxidases, and glutathione peroxidases. ATX supplementation not only lowers ROS levels but also leads to an important functional recovery of the antioxidant network [68], including SOD, which catalyzes the dismutation of superoxide anion to O_2 and H_2O_2, and CAT, which protects cells from oxidative damage by catalyzing the decomposition of H_2O_2 to water and O_2.

Recent clinical studies showed that ATX promotes significant reductions in cardiovascular risk markers of oxidative stress and inflammation [69]; ATX also has considerable potential for both the prevention and treatment of various chronic inflammatory disorders, such as cancer, asthma, rheumatoid arthritis, metabolic syndrome, diabetes, and diabetic nephropathy, as well as gastrointestinal, hepatic, and neurodegenerative diseases [56]. In rats, ATX supplementation in the diet for four weeks markedly decreases the level of malondialdehyde (MDA), nitric oxide, and advanced protein oxidation products in the cortex, striatum, hypothalamus, hippocampus, and cerebellum [68]. Also ATX increases the activity of CAT and SOD enzymes as well as the level of glutathione in the brain [68]. Additionally, ATX exhibits protective effects against the neurotoxicity induced by $Abeta_{25-35}$ peptide aggregates in PC12 and neuroblastoma (SH-SY5Y) cells [70, 71]. These results are in agreement with the main conclusions presented in this work.

Besides the role of ROS and oxidative stress in AD, there are many studies linking this disease with a sustained increase in intracellular Ca^{2+} levels [39, 72, 73]. We have previously reported that AβOs addition to primary hippocampal neurons causes an increase in Ca^{2+} entry to the cytoplasm via NMDA receptors, which promotes RyR-mediated Ca^{2+} release [25]. We also showed that previous

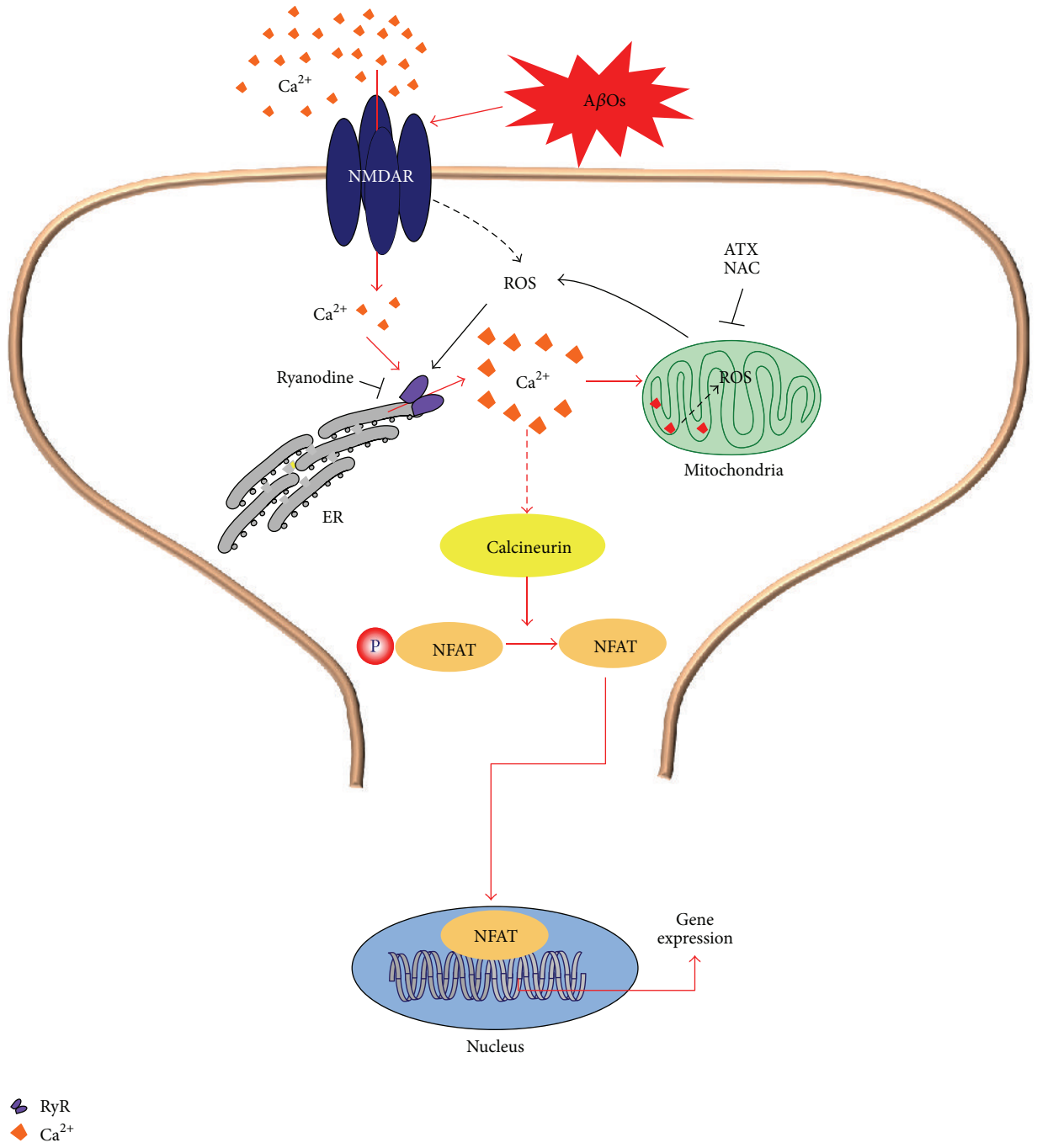

Figure 5: Scheme showing a possible mechanism to explain ATX neuroprotection over the deleterious effects of AβOs. AβOs promote increased ROS generation and induce abnormal Ca^{2+} signals in primary hippocampal neurons, which arise initially from Ca^{2+} entry through NMDA receptors; these entry signals are subsequently amplified by Ca^{2+} release through RyR channels costimulated by Ca^{2+} and the increased ROS levels generated response to AβOs [25]. Activation of RyR-mediated Ca^{2+} release by ROS [59, 77] induces mitochondrial Ca^{2+}-uptake, which is prevented by ryanodine at inhibitory concentrations [27]. These abnormal cytoplasmic Ca^{+2} signals promote NFATc4 translocation, which induces deleterious changes in gene expression and dendritic spine morphology [42]. ATX and NAC, either by scavenging ROS/RNS or by increasing antioxidant defenses, would prevent abnormal AβOs induced RyR-mediated Ca^{2+}-induced Ca^{2+} release and thus would prevent the harmful effects of enhanced NFAT nuclear translocation.

incubation of primary neurons with NAC prevents the emergence of sustained Ca^{2+} signals induced by AβOs [52]. These findings emphasize the participation of ROS in the maintenance of the Ca^{2+} signals induced by AβOs. On the other hand, intracellular Ca^{2+} chelators such as BAPTA-AM prevent the ROS generation induced by AβOs, indicating that there is a crosstalk between the ROS and Ca^{2+} signals induced by AβOs [9]. In this context, the RyR channel appears as an important actor since its activity and expression are regulated by this crosstalk [74]; thus, RyR channels act as coincident detectors of Ca^{2+} and ROS due to the presence of cysteine residues that are reversible modified by oxidants, enhancing RyR activation by Ca^{2+} [75]. We showed that incubation of primary hippocampal neurons with AβOs causes an important downregulation of RyR2 mRNA and protein contents and proposed that these reductions are crucial to the synaptotoxicity induced by AβOs [25]. Of note, *postmortem* samples of patients who died with AD display significantly reduced RyR2 expression at early stages of the disease [76].

Dysregulation of Ca^{2+}-dependent gene transcription plays a critical role in synaptic plasticity and memory defects [77]. AβOs induce calcineurin activation, which leads in turn to activation of its canonical target, the transcriptional factor NFAT. The damage observed in the cortex and hippocampus of *postmortem* AD patients during the progression of the disease, correlates with activation of the calcineurin/NFAT pathway in both glial and neuronal cells [44, 45]. Activation of this pathway, even in the absence of Aβ, is sufficient to produce a virtual phenocopy of Aβ-induced dystrophic neurites, dendritic simplification, and dendritic spine loss in both neurons in culture and in adult mouse brain [42]. Thus, AβOs appear to mediate the neurodegeneration of AD, at least in part, through calcineurin activation and subsequent stimulation of NFAT-mediated downstream cascades.

Calcineurin is susceptible to significant (up to 15 times) and reversible activation by Ca^{2+}/CaM. This activation is favored during chronic elevations of Ca^{2+} in the cytoplasm resulting from ER stress caused by exposure to misfolded proteins [78]. However, calcineurin activity is also redox-sensitive, so that oxidation of calcineurin strongly inhibits its phosphatase activity. Some possible mechanisms to explain this apparent paradox, that is, increased activity in conditions of increased ROS generation, have been discussed in studies from other groups [78, 79]. Cleaved forms of the enzyme, which were found in AD brains [79], are constitutively active.

Here, we demonstrated for the first time that ATX inhibits the nuclear translocation of NFAT induced by AβOs, suggesting that the calcineurin/NFAT pathway responds to the increased neuronal oxidative tonus induced by AβOs. Additionally, we show that ATX inhibits the downregulation of RyR2 mRNA levels promoted by AβOs. These results suggest that excessive ROS decrease RyR2 expression, although it is not known if the calcineurin/NFAT pathway mediates this decrease. Previous work indicates that the RyR2 protein plays an important role in hippocampal synaptic plasticity processes [77], so its downregulation by AβOs may also contribute to their synaptotoxic effects. We previously showed that incubation of primary hippocampal neurons with AβOs for a period of 6 h prevents the rapid spine remodeling prompted by caffeine-induced RyR-mediated Ca^{2+} release or by BDNF, which also requires RyR-mediated Ca^{2+} signals; these results suggest that the RyR2 decrease induced by AβOs produces a significant reduction of RyR2-mediated Ca^{2+} signals in response to BDNF, leading to defective synaptic remodeling [25]. Thus, decreased RyR2 protein expression may contribute to impair synaptic plasticity in AD (Figure 5).

The present results indicate that ATX, via its antioxidant properties, may prevent important deleterious effects of AβOs on gene expression, which might be controlled at least in part by the calcineurin/NFAT pathway. Taken together, our results demonstrate the potential of ATX to prevent synaptotoxic effects of AβOs in an *in vitro* model of AD. Given the neuroprotective effects of ATX against different neurological disorders, the results presented here support the idea that daily consumption of ATX may be a beneficial strategy in human health management of AD and possibly of other neurological disorders as well.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Plasticity of Hippocampal Excitatory-Inhibitory Balance: Missing the Synaptic Control in the Epileptic Brain

Christian Bonansco[1] and Marco Fuenzalida[1,2]

[1]*Centro de Neurobiología y Plasticidad Cerebral (CNPC), Instituto de Fisiología, Facultad de Ciencias, Universidad de Valparaíso, Gran Bretaña Avenida 1111, 2360102 Valparaíso, Chile*
[2]*Núcleo Milenio en Biología de Enfermedades Neurosiquiátricas, Instituto de Fisiología, Facultad de Ciencias, Universidad de Valparaíso, Gran Bretaña Avenida 1111, 2360102 Valparaíso, Chile*

Correspondence should be addressed to Christian Bonansco; christian.bonansco@uv.cl and Marco Fuenzalida; marco.fuenzalida@uv.cl

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Synaptic plasticity is the capacity generated by experience to modify the neural function and, thereby, adapt our behaviour. Long-term plasticity of glutamatergic and GABAergic transmission occurs in a concerted manner, finely adjusting the excitatory-inhibitory (E/I) balance. Imbalances of E/I function are related to several neurological diseases including epilepsy. Several evidences have demonstrated that astrocytes are able to control the synaptic plasticity, with astrocytes being active partners in synaptic physiology and E/I balance. Here, we revise molecular evidences showing the epileptic stage as an abnormal form of long-term brain plasticity and propose the possible participation of astrocytes to the abnormal increase of glutamatergic and decrease of GABAergic neurotransmission in epileptic networks.

1. Introduction

Epilepsy is characterized by spontaneous recurrent seizures and comprises a diverse group of syndromes with different aetiologies [1]. Epilepsy is the second most common brain disorder, affecting about 1% of the world's population [2]. Temporal lobe epilepsy (TLE) remains as one of the most severe and frequent pharmacoresistant types of focal acquired epilepsies. The recurrent seizure is an electrographic hallmark of several types of epilepsy, which consist in an excessive synchronous discharge of cerebral neurons, generated in one or more neuron populations (i.e., epileptic focus) [3]. The electrical activity in epileptic network is associated with an E/I synaptic imbalance, which promotes neuronal hyperexcitability and hypersynchronization, through an increase in excitatory neurotransmission as well as decrease of inhibitory neurotransmission and/or GABA-mediated hyperexcitability [4–6]. During the seizures, associated with heightened neuronal excitability and abnormal synchronization of discharge in the epileptic focus, the disruption of brain functions occurs [7]. Much of the knowledge about neurobiology of epilepsy has been attained from resected temporal lobe tissues from patients, whereas the cellular basis of epilepsy has been obtained from acute experimental models of seizures (i.e., ictogenesis) [8, 9], which contrasts with the limited understanding of neurobiological mechanisms of epilepsy development (epileptogenesis) [10, 11] (see Box 1). Both inhibitory and excitatory synapses are found to exhibit important changes that can mediate the initiation and evolution of self-sustaining seizures. The synaptic plasticity is essential to normal brain function such as our ability to learn and modify our behaviour. Several evidences have showed that astrocytes can modulate the synaptic plasticity and excitability in both excitatory and inhibitory synaptic circuits [12–14]. Currently, experimental evidence suggests that the start, progress, and consolidation of epileptic stage could overlap with the mechanisms underlying the long-term plasticity, learning, and memory [15, 16], which could be explained by an alteration of the factors that regulate the synaptic plasticity of excitatory and inhibitory circuits. Here, we will review the main

evidences in those cellular and molecular alterations with focus on the synaptic plasticity that conducts to E/I imbalance and a pronounced vulnerability of the brain to epilepsy.

Box 1

Kindling and Long-Term Plasticity in Hippocampal Formation. Epilepsy encloses a set of neurological disorders of diverse aetiology, characterized by the development of gradual and progressive spontaneous seizures, which increase in recurrence and severity with time. To study epilepsy, both acute and chronic models have been developed [17]. Kindling, one of the chronic models of experimental epileptogenesis more extensively used, can be induced either *in vivo* (i.e., freely moving rats) [8, 18–20] or *in vitro* (i.e., brain slices) [21–23], allowing reproducing the progressive development of disease. The kindling protocol consists in the repetitive presentation of stimuli (either chemical or electrical) on a nervous structure, usually amygdala or hippocampus, at subconvulsive intensities, which elicits gradual and progressive augmentation of electroencephalographic (EEG) activity after stimuli and behavioural. In several pharmacological and acute models of epileptogenesis, the epileptic state is reached after spontaneous recurrent seizures [24, 25]. EEG activity corresponding to tonic-clonic firing of population spikes, namely, after discharge (AD), can be detected in stimulated structure as well as in projection areas. This AD or electrographic seizure (i.e., EGSs *in vitro*) has been attributed to ictal discharges as product to the increase of synchronous activity and hyperexcitability of a large group of neurons [26]. The long-term changes in the synaptic efficacy are activity-dependent of network and can produce either facilitation or depression, depending on the stimulus parameters and repetition [27]. In hippocampal neurons, long-term potentiation (LTP) of glutamatergic synapses produces the strengthening of synaptic efficacy, which can be induced by high-frequency stimulation or by coincidence between pre- and postsynaptic activity. As well as in excitatory synapses, the neuronal activity can trigger LTP or LTD of GABAergic synaptic strength. The strength of GABAergic inhibition can regulate the ability of excitatory synapses to undergo long-term plasticity, a key mechanism underlying the hippocampal circuit excitability and modifying the learning and memory process. Interestingly, a similar repetitive stimulation protocol used to induce LTP at glutamatergic can also trigger LTD at GABAergic synapses in CA1 pyramidal neurons [28, 29]. Such studies have allowed revealing the progress and consolidation of the epileptic stage as an abnormal form of long-term plasticity [15, 16], which seems to require simultaneous increase of excitatory and decrease of inhibitory neurotransmission.

2. Is the Glutamatergic Plasticity Different in Epileptic Brain Compared to Normal One?

Long-term changes in synaptic efficacy of glutamatergic neurotransmission are a most widely studied model of learning and memory [30]. Depending on stimulus trend, synaptic changes can increase or decrease the synaptic efficacy, leading to long-term potentiation (i.e., LTP) or depression (LTD), respectively. Experimentally, LTP results in a synaptic facilitation, lasting hours to months according to parameters and repetition of stimuli. In the hippocampal formation, one of brain structures involved in the storage of long-term memory and that seems to play a major role in declarative memory, the glutamatergic strengthening is activity-dependent and input-specific and requires temporal coincidence between pre- and postsynaptic depolarization due to voltage dependence of N-methyl-D-aspartate receptors (NMDARs) activation. The resulting calcium (Ca^{2+}) influx can activate a plethora of signalling that promotes the protein synthesis, translocation of receptors, and gene expression, leading to structural and molecular long-lasting synaptic changes [30].

Several lines of evidences indicate that an abnormally enhanced glutamatergic activity, often referred to as the "*glutamate hypothesis*," is one of the key alterations in the pathophysiology of epilepsy. Pioneering studies carried out in a chronic model of epilepsy both *in vivo* [18, 31] and *in vitro* [21, 22] showed that repeated electrical tetanizing stimulation produces burst of population spikes, whose duration and numbers progress with repetitive presentation of stimuli (see Box 1). Similarly, spontaneous recurrent seizures can be observed in other pharmacological epileptogenesis models (i.e., pilocarpine and pentylenetetrazol), which reproduces the repetitive neuronal activation evoked by stimulation [24, 25, 32]. In several models the progress of neural hyperexcitability is inhibited by NMDAR antagonists (i.e., APV and MK801). Interestingly, the progressive increasing of seizures is insensitive to APV once they were established, and their developing disrupts the hippocampal LTP [33, 34]. Like Hebbian synaptic plasticity, the activation of NMDARs is necessary to induce the long-term synaptic changes and developing synchronous firing, but not for the maintenance of epileptic seizures [22]. Overexpression of NMDARs and AMPARs in hippocampal formation has been widely documented both from resected tissue of TLE patients and in several animal epilepsy models [32, 35]. Indeed, the immunoreactivity patterns for AMPARs and NMDARs subunits GluR1, GluR2/3, or NR1 and NR2, respectively, showed alterations in all hippocampal subfields obtained from TLE patients, with differential distributions depending on subtype TLE (i.e., TLE sclerotic v/s TLE nonsclerotic) [36]. In particular, NR1 immunoreactivity was increased in the CA3-CA1 *Stratum radiatum*, while GluR2/3 was expressed strongly in soma and proximal dendrites on both pyramidal neurons and dentate granule cells [32, 35]. It has been suggested that such expression as well as reorganization of the glutamate receptors is a feature of the epileptic hippocampus already remodelled. Like in NMDARs-dependent synaptic plasticity (i.e., LTP), these changes may provide one of the molecular substrates that supports the enhancement of glutamatergic activity in the pathophysiology of epileptogenesis. In addition, the most commonly used anticonvulsant drugs exert their effects by decreasing glutamatergic transmission and/or neuronal excitability (i.e., levetiracetam, oxcarbazepine, and lamotrigine) [37, 38] or by increasing GABAergic inhibition (i.e., vigabatrin, tiagabine, and valproate). Also, the induction of experimental epilepsy is inhibited by some drugs that bind selectively to proteins of neurotransmitter release machinery

(i.e., levetiracetam), reducing the glutamatergic transmission, and is commonly used as antiepileptic [39].

Extracellular glutamate, measured by *in vivo* microdialysis, is elevated fivefold in the epileptogenic human hippocampus during interictal state and increases 30 times higher than normal during the seizure [40]. Moreover, the content as well as activity of glutamine synthase (GS), the enzyme responsible of glutamate-glutamine conversion within the astrocytes, is decreased in brain tissue of TLE patients [41, 42]. Indeed, L-methionine sulfoximine (MSO), a specific inhibitor of GS, is an effective seizure inductor [43], via reduction in the amplitude of the inhibitory GABA-mediated postsynaptic current (IPSC) in hippocampal neurons and changing the astroglial and/or the extracellular accumulation of glutamate. Two glial specific transporters, EAAT1 and EATT2, which are largely responsible for glutamate clearance from extracellular space, are expressed in astrocytes [44]. The inhibition of glial EATT2 induces epileptic bursts [45], while knockout mice for EAAT1/EAAT2 showed spontaneous epileptic seizures and profound hyperexcitability compared to wild type mice. [45]. In addition, in a drug-resistant form of human epilepsy, a reduced level of EAAT2 mRNA has been reported [46]. Conversely, no differences were detected in EAAT1 or EAAT2 expression levels between control and sclerotic (i.e., like TLE) human hippocampus [47, 48] suggesting the participation of additional nonmetabolic factors.

Other molecular targets that are overexpressed in both epileptic patients and experimental models are the metabotropic glutamate receptors (mGluRs). mGluRs form a family of eight subtypes, classified into three groups, where group I and group II include mGluR1/5 and mGluR2/3, respectively. These receptors are widely expressed in both neurons and astrocytes through the brain and have been implicated in the modulation of both glutamatergic and GABAergic neurotransmission as well as in glia-neuron crosstalk [49–51]. Indeed, we and others have recently demonstrated that the glutamate spontaneously released from astrocyte sets the basal probability of glutamate release via group I mGluRs activation [13, 52–54] and that their overactivation could be implicated in the glutamate upregulation on epileptic brain (see below).

These long-term changes in the expression/reorganization of the glutamate receptors, transporters, and/or metabolic enzymes represent plastic changes at synaptic level that contributes to progression and development of epilepsy. Like NMDARs-dependent synaptic plasticity (i.e., LTP), morphological and functional changes in the postsynaptic/presynaptic compartment and neuron-glia signalling would be providing one of the molecular substrates that supports the enhancement glutamatergic activity required to develop epilepsy.

3. Is the GABAergic Synaptic Plasticity Implicated in the Epilepsy?

The GABAergic interneurons play an essential role in the synchronization of local networks and functional coupling in different brain [55]. Given the crucial role of inhibitory synapses in regulating both neuronal excitability and excitatory synaptic plasticity, changes in GABAergic synaptic efficacy can have important functional and pathological consequences [56]. As in excitatory neurotransmission, changes in GABA (γ-aminobutyric acid) receptor composition, expression, cellular distribution, and function, therefore, have profound consequences for neural excitability, and they are associated with the etiology of several neurological and mental diseases, including epilepsy [57].

4. GABA Plasticity: Synaptic versus Extrasynaptic GABA Receptors, Synaptic Efficacy, and Epilepsy

The regulation of relative strengths of excitatory and inhibitory synapses is a powerful way to stabilize network activity. Synaptic communication requires constant adjustments of pre- and postsynaptic efficacies, to optimize their function and/or adapt to a changing environment [58, 59]. Research carried out during the last two decades has made it clear that inhibitory synapses undergo short- and long-term forms of synaptic plasticity [56, 60]. The activity-dependent changes in inhibitory neurotransmission are typically accompanied by alterations in GABAergic efficacy and synapse structure that range from morphological reorganization of postsynaptic density to *de novo* formation and elimination of inhibitory contacts [61]. Depending on the inhibitory interneuron cell type and the brain region, the inhibitory plasticity is dependent on changes in either GABA release or the number/sensitivity/responsiveness of postsynaptic GABA receptors [56]. Inhibitory neurotransmission in the mammalian brain is largely a result of GABA signaling. GABA acts on two main classes of receptors, the type-A ionotropic GABA receptors ($GABA_A$Rs) and the type-B metabotropic GABA receptors ($GABA_B$Rs). Similar to nicotinic receptors, $GABA_A$Rs are composed of different subunits assembled in a pentameric structure [62–64]. Native heteropentameric $GABA_A$Rs subtypes have a high structural diversity, being divided into classes based on sequence identity: α(1–6); β(1–3), γ(1–3), δ, ε, π, θ, and ρ(1–3). $GABA_A$Rs comprising $\gamma 2$ and α 1–3 subunits are most common type of receptor at synapses sites. These $GABA_A$Rs are ligand-gated channels permeable to chloride and bicarbonate that produce minimal direct change in the membrane potential but generate a large conductance that shunts the excitatory depolarization [65]. Furthermore, the extrasynaptic $GABA_A$Rs comprise $\alpha 4$ and $\alpha 6$ subunits combined to δ subunit, and they are responsible for tonic inhibition [66]. In addition to subunit composition and localization, other mechanisms exist that control $GABA_A$Rs on a rapid time-scale, such as regulation of receptor trafficking, clustering, and surface expression. At synapses, $GABA_A$Rs constitutively undergo significant rates of endocytosis, being rapidly recycled or targeted for lysosomal degradation [67, 68]. Therefore, changes in the rates of $GABA_A$Rs endocytosis and/or endocytic sorting represent potentially powerful mechanisms to regulate $GABA_A$Rs cell surface number and inhibitory synaptic transmission [67, 69]. A direct relationship between the number of postsynaptic $GABA_A$Rs and the strength of the synapse has been

demonstrated [70, 71]. Therefore, to maintain a stable cell-surface receptor number, continual membrane insertion of newly synthesized or recycled receptors is required [72]. Therefore, changes in the trafficking of these receptors could regulate neuronal plasticity and contribute to the manifestation of a wide range of brain disorders [72–74]. *Postmortem* studies in epileptic patients have revealed severe alteration in the number and expression of extrasynaptic $GABA_A$Rs [75]. The trafficking of $GABA_A$Rs to and from the membrane is altered during prolonged seizures and has been suggested to contribute to benzodiazepine pharmacoresistance in patients with status epilepticus (SE) [72, 76]. Interestingly, the epileptiform activity alters intracellular Ca^{2+} concentrations and calcineurin activity, which correlates with the decrease of $GABA_A$Rs from the surface, possibly contributing to pathological signaling during SE [77]. The loss of GABAergic interneurons and/or a reduction in the GABAergic synapses could result in a decrease of GABA release, a decrease of extracellular GABA availability, and a reduction of tonic inhibition. In rat hippocampal culture model it has observed a downregulation of tonic GABA inhibition after chronic epileptogenic stimulation [78]. On the other hand, experimental evidence indicates that, in epilepsy, at least epileptogenic period the tonic GABAergic current are maintained or augmented in several hippocampal neurons [79, 80]. Reduction of several subtypes of extra and perisynaptic $GABA_A$Rs has been reported in hippocampus of animals with TLE. A molecular and pharmacological study shows that the overexpression of two subtypes of extrasynaptic $GABA_A$Rs ($\alpha5\beta3\gamma2$ and $\alpha6\beta3\delta$) can enhance the tonic inhibition and reduce the epileptiform activity [81]. In addition, mice lacking the $GABA_A$Rs δ subunit exhibit an impaired GABAergic efficacy and increased seizure susceptibility, and mice lacking the $GABA_A$Rs $\alpha5$ subunit exhibit a diminished tonic inhibition and elevated hyperexcitability [82]. Therefore, the physiological consequences of these changes depend not only on the subunits of $GABA_A$Rs, but also on somatodendritic localization, as well as the presynaptic, perisynaptic, or extrasynaptic sites composition of $GABA_A$Rs [83].

The functional interaction between dendritic ionic channels and neurotransmitter receptors (i.e., $GABA_A$Rs) plays a determinant role in neural integrating and dendritic excitability. Because of its characteristic biophysical properties, some cationic current, as noninactivating, mixed Na^+-K^+ current, Ih can shape both hyperpolarizing and depolarizing inputs. It has been demonstrated that after febrile seizures the dendritic Ih current is upregulated, which results in general enhancement of hippocampal dendritic excitability [84]. The febrile seizures induce a PKA-dependent presynaptic potentiation of GABAergic IPSC, $GABA_A$Rs mediated IPSCs in CA1 pyramidal cells [5]. After febrile seizures the burst of IPSP can activate the Ih current inducing a postinhibitory rebound and can result in pyramidal-cell discharges following the inhibitory barrage, which can be prevented by application of selective h channel blocker ZD-7288 [5].

Recently, an endocannabinoids-dependent presynaptic long-term depression (LTD) has been described in different brain regions [28, 85, 86]. Typically, endocannabinoids (eCBs) can transiently or permanently reduce the GABA neurotransmission by activation of type 1 eCBs receptors (CB1Rs) [87–90]. Different pattern of neuronal activity can induce simultaneously LTP at excitatory glutamatergic synapses and presynaptic form of LTD at inhibitory GABAergic synapses in hippocampal CA1 PNs [29, 91]. Cannabinoids have been proposed as a "circuit breaker," because of their ability to stop the progress of seizures and limit neuronal degeneration [92, 93]. After a brain insult that induces a cellular depolarization, glutamate release, and increase of intracellular Ca^{2+}, neurons can release eCBs, which can damp the seizures and reduce the neuronal cell death as a consequence of SE. Several data obtained from both human and animal models of epilepsy showed changes in CB1R expression at hippocampal GABAergic synapses [94, 95]; alterations in the production and breakdown of eCBs could thus have profound effects on excitability and synaptic transmission in the hippocampus [93]. While eCBs are sufficiently powerful to silence a synapse, the activation of CB1Rs, generally confined to the synaptic axonal terminal [92, 96] (but see below), does not influence somatic action potential firing. As a result, excitation of dendrites can trigger repetitive somatic action potentials that readily travel to the synaptic terminals where they can reduce and/or eliminate the CB1R-mediated inhibition of release. By decreasing inhibition, the increase of CB1Rs on inhibitory terminals will increase the E/I ratio and shift the system toward hyperexcitability. Interestingly, upregulation of CB1Rs is itself dependent on CB1R activation during the seizures and can be prevented by CB1R antagonists [97]. In contrast, decreased activation of the CB1 receptor, through either genetic deletion of the receptor or treatment with a CB1 antagonist, can increase the pilocarpine seizure severity without modifying seizure-induced cell proliferation and cell death [98]. Recently, it has been shown that inhibition of hydrolase α/β-hydrolase domain 6 (ABHD6), which is involved in eCBs metabolism, can protect against seizures in mouse models of epilepsy [99].

In addition, CB1 receptor in specific neuronal has provided functional and anatomical evidence that CB1 receptors on hippocampal glutamatergic neurons are necessary for the CB1-dependent protection against acute excitotoxic seizures [100]. Dentate gyrus mossy glutamatergic cells, where CB1 receptors are present at low but detectable levels, are the central mediators of on-demand endocannabinoid-dependent protection against excitotoxicity seizures in the adult mouse brain [100]. Moreover the activation of CB1R present on glutamatergic terminals can suppress recurrent excitation in the dentate gyrus of mice with TLE, suggesting an anticonvulsive role of cannabinoids [101]. It has demonstrated that status epilepticus can selectively compromise GABA release at synapses from a subtype of hippocampal interneurons dentate accommodating interneurons to fast-spiking basket cells interneurons. The functional decrease in CB1R-sensitive inhibition of FS-BCs resulted from enhanced baseline $GABA_B$Rs-mediated suppression of synaptic release after SE [102]. Recently, it has shown that block of monoacylglycerol lipase and the subsequent increase of 2-arachidonoylglycerol (2-AG) can delay the development of generalized seizures and

decrease the seizures and postdischarge duration in the kindling model of TLE [103]. Taken together these data indicate that the endocannabinoids signaling might be a promising target to control neuronal excitability during seizure activity.

As we have highlighted before, $GABA_A$Rs in the CNS mediate both fast synaptic and tonic inhibition. The phasic inhibition is characterized by a short-lasting inhibitory postsynaptic potential (IPSP) and tonic inhibition is characterized by persistent, long-lasting one (IPSP). $GABA_A$Rs mediating tonic inhibition are different from those mediating phasic inhibition. They are located outside the synapse and hence are referred to as perisynaptic or extrasynaptic receptors [63]. The effect of extracellular GABA on high affinity, slowly desensitizing extrasynaptic $GABA_A$Rs, is termed "tonic inhibition." This tonic activation of extrasynaptic and perisynaptic $GABA_A$Rs provides a powerful means of regulating neuronal excitability [79]. Several polymorphisms and mutations in genes encoding extrasynaptic $GABA_A$Rs have been associated with several types of human epilepsies, implying that dysfunction of extrasynaptic $GABA_A$R-mediated currents has dramatic effects on neuronal excitability [104, 105]. In addition, application of tiagabine (or EF1502), a GABA transporter inhibitor, enhanced the anticonvulsant effect of $GABA_A$Rs agonist gaboxadol [106]. The tonic inhibition mediated by extrasynaptic $GABA_A$Rs is dependent on the GABA availability, whose modification may play a prominent role during SE. Tonic GABAergic signaling, extracellular GABA availability, and inactivation of GABA neurotransmission are highly sensitive to changes in the efficacy of GABA uptake transporter (GATs 1–4) located in the presynaptic nerve ending as well as in astrocytic processes ensheathing synapses [106, 107]. Also, recaptured GABA by the axon terminals is mostly reused to fill vesicles via vesicular GAT [108]. According to their essential function within the control of synaptic and extrasynaptic GABA levels, GATs have been linked to epilepsy [109]. Drugs acting either selectively or nonselectively at GATs are used for antiepileptic medication [110, 111]. GAT-1 inhibitors are effective against the kindled focal and secondary generalized seizures [110].

Astrocytes can set the tone of GABAergic inhibition in local neural circuits [63]. In the neocortex, GAT-1 and GAT-3 are the most abundantly expressed ones, with GAT-1 mainly expressed in GABAergic interneurons and less on astrocytes, while GAT-3 is mainly expressed in astrocytes [112]. Recent works show that astrocytic GAT-3 is important to control the excitability of hippocampal cells when network activity is increased [112]. Several studies showed that astrocytes can release GABA and activate extrasynaptic high affinity GABA receptor to mediate tonic inhibition in neighboring neurons and modulate the brain physiology [113].

As we already highlighted, in adult brain the activation of $GABA_A$R causes neuronal membrane hyperpolarization due to increased chloride permeability. This hyperpolarizing response critically depends on chloride extrusion via the K-Cl-cotransporter KCC2. The role of KCC2 is critical in order to maintain the equilibrium potential of GABA (E_{GABA}) at a sufficiently negative level to prevent the neuron from firing action potentials [114, 115]. The downregulation of KCC2 in response to trauma and/or intense seizure activity leads to a long-lasting decrease in the efficacy of both shunting and voltage inhibition and results in the development of network hyperexcitability. Decreased KCC2-mediated chloride extrusion and impaired hyperpolarizing $GABA_A$R-mediated currents have been implicated in TLE, as well as other types of epilepsy [116, 117]. Seizure-induced downregulation of KCC2 activity depends on posttranscriptional mechanisms [115, 118] including protein phosphatase 1-mediated dephosphorylation of KCC2 at serine 9 and cleavage by the protease calpain, which is activated by Ca^{2+} and/or BDNF [115].

On the other hand, functional $GABA_B$Rs are formed by heterodimeric assembly of two subunits $GABA_B$1 and $GABA_B$2 [119, 120]. $GABA_B$Rs are coupled to different effectors via GTP binding protein [121]. Postsynaptic $GABA_B$Rs are coupled to Gi subtype of G-protein protein which downregulated cyclic AMP production and promoted activation of inwardly rectifying potassium channels resulting in a slow and sustained neuronal hyperpolarization [122]. Presynaptically located $GABA_B$Rs inhibit transmitter release by inhibiting activation of voltage-gated Ca^{2+} channels [123, 124]. Several studies have shown that $GABA_B$Rs can modulate cell survival, migration, and neuronal differentiation, as well as regulating synaptogenesis, maturation, and plasticity of synaptic connections [120]. $GABA_B$Rs are essential for the stability of cortical network activity [125]. Thus, high doses of $GABA_B$Rs antagonist disrupt the normal hippocampal and cortical oscillations including delta waves and sleep spindles, as well as fast gamma oscillations, and lead to epileptiform activity [126]. Also, $GABA_B$Rs knock-out mice are prone to developing spontaneous seizures [127, 128]. Moreover, the $GABA_B$R agonist baclofen can also promote excitability and seizure generation in both human patients and epilepsy animal models [129]. In addition, it has been shown that $GABA_B$R expression is altered in both TLE patients and animal models [130, 131].

The persistent $GABA_B$R activation in epileptic mice can suppress the inhibitory output from hippocampal cholecystokinin basket cell interneurons, which leads to disinhibition in hippocampal networks, enhances gamma activity, and promotes the transition to pathological hyperexcitability These data suggest an important role of $GABA_B$Rs in the generation and control of epileptiform activity and act as a promising therapeutic target for the treatment of seizures.

In parallel to functional changes, multiple morphological changes are found in human and animal epilepsy models. Axonal sprouting of excitatory and inhibitory neurons of hippocampal formation is frequently observed in temporal lobe epilepsy [132, 133]. The loss of GABAergic interneurons and compensatory axonal sprouting are the main inhibitory reasons for GABAergic neuron decrease, restoration, and potentiation. The inhibitory neurons exhibit similar axonal growth and synaptogenesis, which has been suggested as an explanation for the persistence or increase in labeling of GABAergic axons and terminals in human temporal lobe epilepsy and related animal models [134, 135]. It has been observed that hippocampal SOM/GABA neurons can undergo substantial axonal reorganization, project beyond their normal innervation territory, and form functional but

aberrant circuitry in a mouse model of epilepsy [135, 136]. Recently, in a rat model of TLE it has showed a loss of CCK-containing GABAergic terminals and synapses in the inner molecular layer of the dentate gyrus causes the reduction of CCK-containing GABAergic synaptic transmission to DGCs, tending to reduce seizure threshold [137].

5. Astrocyte: The Third Element in the Abnormal Plasticity of Epilepsy

It is well known that astrocytes form a "tripartite" functional unit with presynaptic and postsynaptic structures, which regulates synaptic transmission and neuronal plasticity [13, 138]. This astrocyte-neuron communication allows that Ca^{2+}-dependent glutamate release from astrocytes can increase the glutamatergic neurotransmission through metabotropic glutamate receptors (mGluRs) activation located in the presynaptic terminal [13, 139–141]. While brain disease mechanisms are largely considered to have a neuronal origin, growing evidence suggests that disturbances of astrocyte-neuron cross-talk are related to brain disorders including epilepsy [54, 142–144]. As consequence of neuropathological conditions including epilepsy, reactive astrocytes exhibit several changes in the expression rate of proteins, including cytoskeleton proteins, transporters, enzymes, and receptors. Moreover, proinflammatory molecules can induce the releases ATP from microglia, which, via gliotransmitter release from neighbours astrocyte, modify the synaptic efficacy [145].

Several evidences suggest that mGluRs would be a molecular key in the alteration of synaptic plasticity in an epileptic network, where glutamate-mediated gliotransmission is a putative signal that contributes to the increased excitability and neuronal hypersynchronicity [146].

Overexpression of mGluR group I/II in reactive astrocytes and neurons in hippocampal tissue from both TLE patients and epilepsy experimental models has been widely reported [51, 146, 147]. These findings have been also corroborated in a kainate-induced model of epilepsy, in which mGluRs are also overexpressed and colocalized in hippocampal GFAP-positive astrocytes [148]. The kindling-induced enhancement of LTP and maintenance population spike was prevented in presence of specific mGluR group I antagonists [149, 150]. In acute epilepsy model increasing of the astrocytic Ca^{2+} waves correlates with increase in frequency of synchronic neuronal depolarizations [151]. This TTX-insensitive increase in astrocytic Ca^{2+} wave preceded or occurred concomitantly with paroxysmal depolarization shift (PDS). Moreover, several anticonvulsive agents potently reduced astrocytic Ca^{2+} signalling and removed the epileptic activity [152]. Interestingly, this epileptic activity was inhibited by the application of antagonists NMDARs and AMPARs providing concrete evidence about the role played by the astrocytes as a new source of glutamatergic excitation to epileptic activity. Taken together, glutamate release from astrocyte has been implicated in the glutamatergic imbalance described in epileptic networks, maintaining a high glutamatergic tone and setting excitatory transmission near to seizure threshold [40, 144]. Recently, we showed that astrocytes from epileptic hippocampus display Ca^{2+}-dependent hyperexcitability, through a mechanism that requires the activation of astroglial P2Y1R which increases glutamate-mediated gliotransmission, upregulating the synaptic efficacy in the CA3-CA1 circuit via presynaptic mGluR5 activation [54]. At postsynaptic level, glutamate release from astrocytes induces slow inward current (SIC) in adjacent neurons, mediated by extrasynaptic NMDARs activation [138, 153]. The functional role of SICs is involved in the synchronicity of neuronal networks due to their capacity to induce SIC-dependent depolarization in pyramidal neurons distant by ~100 μm, which would allow for simultaneously controlling the excitability at a group of neighboring pyramidal cells [151, 154]. Several evidences have described that an increase of astrocytic Ca^{2+} transients during acute epileptiform activities is correlated to an increase in frequency of SIC [153, 155] and preceded or occurred concomitantly with paroxysmal depolarization shift (PDS) [152]. However, the SIC contribution in the hypersynchronic neuronal discharges that characterize the ictal-interictal activities is still unknown.

Recently, it has been demonstrated that astrocytes are involved in the eCBs system, responding to exogenous cannabinoids ligands as well as eCBs through activation of CB1R [156]. This activation increased the astrocytes Ca^{2+} levels through the mobilization of Ca^{2+} from internal stores and stimulates the release of glutamate that modulates synaptic transmission and plasticity. While a study reported a proconvulsive effect of cannabinoids ligands, another showed that activation of CB1Rs have a potent antiepileptic activity [97, 157]. However, a recent report has shown that CB1R antagonist reduces the maintenance of epileptic discharges, which can be abolished when the intracellular astrocyte Ca^{2+} increase is prevented [158] suggesting that gliotransmission triggered by astroglial Ca^{2+} elevation is involved in the hippocampal epileptic activity.

The role of astrocyte in modulation of GABAergic transmission is less understood. Like glutamate release from neurones, GABA also evokes Ca^{2+} oscillations in astrocytes via $GABA_B$ receptors [159]. Glutamate release from astrocytes can mediate either depression or potentiation [160, 161] of inhibitory transmission, contributing to E/I imbalance on the projection neurons. In particular, varied mechanisms can contribute to glutamate depressor effects on GABAergic interneurons, including decrease of amplitude of miniature IPSC and action potential-dependent GABA release by kainate receptors activation [161]. Also it has been demonstrated that activation of presynaptic mGluR group III can depress the GABAergic transmission to identify interneurons [162–164] as well as to pyramidal cells of hippocampus [165].

These evidences suggest that the activation of presynaptic mGluR group III in GABAergic presynaptic terminals added to activation of presynaptic mGluR group I in glutamatergic presynaptic terminals may be, at least in part, explaining the simultaneous E/I imbalance exhibited in epileptic brain (Figure 1). Other gliotransmitters released from astrocytes also have been associated with changes in the synaptic efficacy and excitability in E/I circuit. ATP increased astroglial

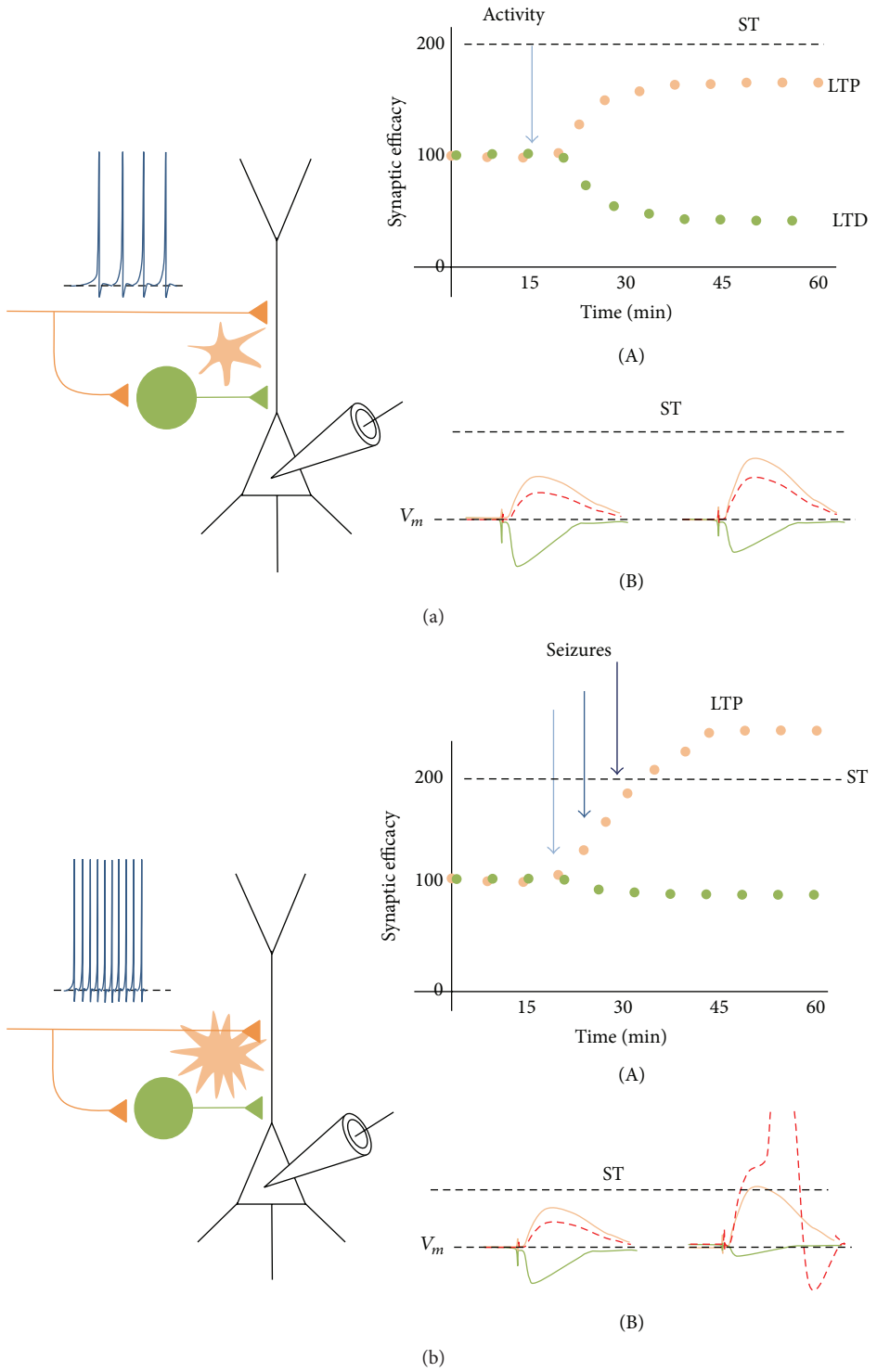

Figure 1: Glutamatergic and GABAergic long-term plasticity and tripartite neuronal-astroglial network in normal and epileptic brain. (a) During physiological neuronal activity, coincidence between postsynaptic depolarization and glutamatergic (orange) and GABAergic interneuron (green) simultaneously activated induces increase of synaptic efficacy (i.e., mean amplitude of postsynaptic response) in both glutamatergic [CB1] synapses (LTP) at the same time of a decrease of efficacy of GABAergic transmission (LTD; A). (B) By GABAergic and glutamatergic input integration, the net increase in membrane potential falls below the seizure threshold (ST). (b) During epileptiform neuronal activity, astroglial hyperexcitation through $GABA_AR$s, GluRs, and/or eCBsRs activation, which increases the intracellular Ca^{2+} release of astroglial glutamate, increasing the excitatory neurotransmission while inhibitory transmission remains unchanged (A). (B) In this condition, glutamatergic/GABAergic rate results in an excitatory imbalance, exceeding the seizure threshold.

Ca^{2+} elevations and depolarized the GABAergic interneurons, enhancing the inhibition onto projections neurons of hippocampus [12, 166]. Similarly, D-serine released from astrocytes controls the NMDA receptor-mediated synaptic potentiation. Because astrocyte-neuron communication is a form of communication cell and synapse specific, astrocyte may represent glutamatergic sources to modulate the E/I balance. However, it is unknown if astroglial glutamate, ATP, or D-serine can simultaneously regulate the glutamatergic and GABAergic plasticity.

6. Conclusions and Future Directions

The cellular basis of learning and memory is believed to depend on short- and long-lasting changes in synaptic plasticity. Typically, changes in the strength and plasticity of excitatory synaptic transmission have been assumed to underlie learning and memory processes. More recent investigation has demonstrated that inhibitory transmission is not only plastic; it also modulates the efficacy and threshold of excitatory synaptic plasticity. In several brain areas, the excitatory and inhibitory synaptic plasticity can occur simultaneously [29, 56]. The same patterns of activation that trigger LTP in excitatory synapses can also induce short- or long-lasting plasticity in inhibitory synapses. This functional balance between excitatory and inhibitory synapses is established during development and maintained throughout life and is essential to brain function [114]. The astrocytes are strategically positioned, close to excitatory and inhibitory synapses, allowing them to integrate the adjacent synaptic activity via gliotransmitters release, including control of synchronous depolarization of groups of neurons [153], reducing the threshold of synaptic plasticity or suppressing the synaptic transmission [13, 81]. Through these coordinating actions the astrocytes can contribute to the excitatory/inhibitory balance, modulating the neural network operations in a specific-cells manner.

As in a variety of neural disorders, deregulation of the E/I balance of synaptic transmission has been associated with epilepsy. It is still unknown whether this imbalance is causative for the disease or a consequence of pathological pattern of network activity. Even though the long-lasting molecular changes that lead to LTP/LTD in normal brain seem to be the same required to turn into an epileptic one, astrocyte-neurons networks represent a new pathological key to explain the concerted alterations in synaptic plasticity to generate the E/I imbalance. Therefore, future experimental approaches should give consideration to such astroglial-neuronal network in the brain, which may offer new therapeutic target for treatment of nervous system disorders.

Conflict of Interests

The authors declare no competing financial interests.

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Review Article

Potential Role of JAK-STAT Signaling Pathway in the Neurogenic-to-Gliogenic Shift in Down Syndrome Brain

Han-Chung Lee,[1,2] Kai-Leng Tan,[1,3] Pike-See Cheah,[1,3] and King-Hwa Ling[1,2]

[1]*NeuroBiology & Genetics Group, Genetics & Regenerative Medicine Research Centre, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia*
[2]*Medical Genetics Laboratory, Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia*
[3]*Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia*

Correspondence should be addressed to King-Hwa Ling; lkh@upm.edu.my

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Trisomy of human chromosome 21 in Down syndrome (DS) leads to several phenotypes, such as mild-to-severe intellectual disability, hypotonia, and craniofacial dysmorphisms. These are fundamental hallmarks of the disorder that affect the quality of life of most individuals with DS. Proper brain development involves meticulous regulation of various signaling pathways, and dysregulation may result in abnormal neurodevelopment. DS brain is characterized by an increased number of astrocytes with reduced number of neurons. In mouse models for DS, the pool of neural progenitor cells commits to glia rather than neuronal cell fate in the DS brain. However, the mechanism(s) and consequences of this slight neurogenic-to-gliogenic shift in DS brain are still poorly understood. To date, Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling has been proposed to be crucial in various developmental pathways, especially in promoting astrogliogenesis. Since both human and mouse models of DS brain exhibit less neurons and a higher percentage of cells with astrocytic phenotypes, understanding the role of JAK-STAT signaling in DS brain development will provide novel insight into its role in the pathogenesis of DS brain and may serve as a potential target for the development of effective therapy to improve DS cognition.

1. Introduction

It is well known that the presence of all or part of an extra copy of chromosome 21 (HSA21) causes Down syndrome (DS). The worldwide prevalence of DS is about 1 in 1000 live births [1]. An increased HSA21 copy number results in DS phenotypes, such as upward slanting eyes, flat facial features, and intellectual disability [2]. Individuals with DS also develop hypotonia, congenital heart defects, cognitive impairment, and early onset of the Alzheimer disease (AD). Approximately 50–70% of DS individuals develop dementia before the age of 60 [3]. The clinical features vary; however, intellectual disability remains an invariable hallmark of this syndrome and may be related to impairment of neurogenesis.

Individuals with DS demonstrate central nervous system abnormalities, such as reduced brain size, weight, volume, neuronal density, and neuronal distribution as well as increased synaptic abnormalities [4–10]. Studies to date using DS mouse models and aborted human DS fetuses have revealed defective cell proliferation and neurogenesis in several brain regions, such as the cerebellum and hippocampus [11, 12], which are critical for motor movement, learning, and memory. Notably, the reduced number of neurons is due to severely impaired proliferation of cerebellar cells and an increased number of apoptotic cells in the hippocampal region of human fetuses with DS. In contrast, the number of glial cells, especially astrocytes, has been shown to be increased in the DS brain [13, 14]. In the mammalian brain, astrocytes are the predominant cell type and are essential for regulating synapse formation [15], synaptic plasticity [16], maintaining the blood brain barrier, regulating neurotransmitters, and preserving ion homeostasis [17]. The

consequences of gliogenic shift in the DS brain remain unknown. It has been postulated that the shift potentially causes neurogenesis and proliferation defects, which likely is due to reduction of neuronal precursor specification or overall cell-cycling speeds [18]. Therefore, a gliogenic shift in the DS brain may disturb homeostasis and affect the brain development, which may be a major factor contributing to the intellectual disability observed in DS individuals.

The discovery of neural progenitor cell bias towards glial lineages has been shown to be consistent in the brain of both human and mouse model for DS. In human DS fetuses, the percentage of astrocytes in the hippocampal region has been shown to be significantly higher compared to control fetuses [13]. A similar observation was also found in other brain regions, such as the frontal lobe of human DS fetuses [19]. In a DS mouse model, Contestabile and colleagues [14] also reported a comparable observation in Ts65Dn versus disomic mice where the number of cells with an astrocytic phenotype in the hippocampal dentate gyrus was larger in Ts65DN. Neurosphere cultures derived from Ts1Cje mouse models of DS further demonstrated a reduction in the number of neurons, whereas the number of astrocytes was increased [20]. Moreover, a twofold increase in the number of astrocytes derived from human DS-induced pluripotent stem cell (iPSC) cultures was also reported [18]. Recent evidence has shown that a gliocentric shift in DS astrocytes caused a reduction of neurogenesis and neuronal cell death via the release of S100B, which resulted in elevated nitric oxide (NO) generation [21]. Therefore, understanding the mechanism(s) underlying the neurogenic-to-gliogenic shift and the consequences in DS brain may shed light on the etiology of early neurodegeneration as well as neuronal reduction, which may contribute to the intellectual disability seen in individuals with DS.

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway is one of the major gliogenic pathways [22]. Upon activation by gliogenic factor/cytokines, the JAK-STAT signaling pathway specifies glial differentiation. Importantly, genes encoding receptors for interferons (interferon-α receptor 1 (IFNAR1), IFNAR2, and IFN-γ R2 (IFNGR2)) responsible for activating JAK-STAT signaling cascades were found to be located on HSA21 and are triplicated in DS [23], suggesting a potential dysregulation of the downstream JAK-STAT signaling leading to activation of gliogenesis in DS brain. Herein, we review the association of IFNs with JAK-STAT signaling and highlight the potential role of this pathway in promoting the neurogenic-to-gliogenic shift in DS brain, which may lead to the development of novel therapeutics for DS.

2. The Canonical JAK-STAT Signaling Pathway

The first evidence of JAK-STAT signaling pathway involvement in brain development was based on primary cultures of embryonic cortical precursor cells derived from rats [22]. However, JAK-STAT signaling is not limited to brain development, as it is also involved in the development of hematopoietic cells and regulatory immune responses [24]. More importantly, normal function of this pathway is necessary for neural stem cell maintenance, growth, and renewal as well as overall cell survival and apoptosis [25].

In mammals, JAK proteins are comprised of four members (JAK1–3 and tyrosine kinase (TYK) 2), while the STAT proteins consist of seven (STAT1–4, STAT5a, STAT5b, and STAT6) [26]. The JAK-STAT signaling pathway is activated when various ligands bind to their corresponding receptor (Table 1). The receptors in the JAK-STAT pathway do not have tyrosine kinase activity and therefore are not able to activate any signaling cascades. JAK members initiate the signaling cascade by phosphorylating downstream transcription factors. In general, the receptor associates with JAK; the JAKs are brought into close proximity when ligands bind their corresponding receptor, leading to dimerization of receptor subunits and allowing the JAKs to phosphorylate each other [27]. Transphosphorylation results in JAK activation, which allows them to phosphorylate the receptor and create a binding site for SH2 domains of STATs [28].

The STATs are latent transcription factors that reside in the cytoplasm but become activated when STATs bind to the receptor and JAKs phosphorylate the conserved Tyr residue near the C-terminus of STATs [28, 29]. Upon activation by Tyr phosphorylation, members of the STAT family then interact with each other and dimerize through their conserved SH2 domains. Consequently, phosphorylated STATs are transported from the cytoplasm into the nucleus. Once in the nucleus, dimerized STATs bind to the promoters of target genes to initiate transcription (Figure 1) [28]. The well-known downstream target of JAK-STAT signaling cascade is glial fibrillary acidic protein (*Gfap*), which is required for astrocyte differentiation [30].

3. Expression Patterns of JAK-STAT during Mouse Brain Development

Messenger RNA expression of *Stat*s varies and is dependent on different brain developmental stages (Figure 2). Gene expression data mining from the Allen Developing Mouse Brain Atlas (http://developingmouse.brain-map.org/) showed low expression of *Stat1* in all brain regions from embryonic day (E) 11.5 to postnatal day (P) 4 with a gradual increase in expression from P14 to P28. *Stat1* was highly expressed in the pontine and pontomedullary hindbrain at P14 and also prosomere 2 at P28 compared to other brain regions. Similar to *Stat1*, *Stat3* was highly expressed between P4 and P28. *Stat4* had low expression throughout all developmental stages, except the telencephalic vesicle region at P14 compared to other brain regions. *Stat5a* expression wasalso low throughout all developmental stages, except the medullary hindbrain region during E18.5. Although *Stat5b* exhibited generally low expression in all brain regions throughout development (E11.5–P14), it was found to be highly expressed only at P28 throughout various regions in the brain. No *in situ* hybridization of *Jak* and *Stat6* was found in the Allen Developing Mouse Brain Atlas.

At the protein level, members of the JAK-STAT pathway are expressed in different regions of the developing and mature brain, such as the basal forebrain, striatum,

Table 1: Cytokines and their corresponding specific receptors in JAK-STAT signaling activation.

	Ligands	Receptor	JAK kinases	STATs
IFN family	IFN-α/β	IFNAR	JAK1, TYK2	STAT1, STAT2, STAT3, STAT5a/5b
	IFN-γ	IFNGR	JAK1, JAK2	STAT1, STAT3, STAT5a/5b
	IL-10	IL-10R	JAK1, TYK2	STAT1, STAT3
gp 130 family	IL-6	gp130	JAK1, JAK2	STAT1, STAT3
	IL-11	gp130	JAK1	STAT1, STAT3
	IL-12	IL-12R	JAK2, TYK2	STAT4
	CNTF	gp130 and LIFRβ	JAK1, JAK2	STAT1, STAT3
	LIF	gp130 and LIFRβ	JAK1, JAK2	STAT1, STAT3
	OSM	gp130 and OSMR	JAK1, JAK2	STAT1, STAT3
	CT-1	gp130 and LIFRβ	JAK1, JAK2	STAT3
	G-CSF	G-CSFR	JAK1, JAK2	STAT3
	Leptin	LEPR	JAK2	STAT3
βc family	IL-3	IL-3R	JAK2	STAT5a/5b
	IL-5	IL-5R	JAK2	STAT5a/5b
	GM-CSF	GM-CSFR	JAK2	STAT5a/5b
γ-chain (gC) family	IL-2	IL-2R	JAK1, JAK3	STAT1, STAT3, STAT4, STAT5a/5b
	IL-7	IL-7R		
	IL-9	IL-9R		
	IL15	IL-15R		
	IL-4	IL-4R	JAK1, JAK3	STAT6
	IL-13	IL-13R	JAK1, JAK2, TYK2	STAT6

hippocampus, and cerebral cortex [31]. Their expression is also found to be differentially regulated, depending on the stages of brain development, and is summarized in Figure 3. JAK1 expression was relatively low compared to JAK2 and its expression was consistent across all developmental stages of rat brain [31]. Expression of JAK2 was higher in the developing brain, specifically at E14 and E18, and gradually diminished towards adulthood [31]. Using Western blot, De-Fraja and colleagues failed to detect expression of JAK3 in selected brain regions as well as in whole brain. Recently, Kim and colleagues [32] found that JAK3 expression was increased in embryonic (E11 and E15) and postnatal brains (P6), but its expression diminished towards adulthood. Expression of another JAK member, TYK2, was not detected in both developing and mature brain [31].

Activated JAK members trigger expression of STAT proteins. STAT1 has a complex expression pattern that varies at different brain developmental stages. STAT1 expression levels have been shown to gradually decrease from E14 to P2, with an increase in expression in adulthood [31]. Later in the aging brain (26 months), expression of STAT1 remained invariant [33]. STAT3 was found to be constitutively expressed in the cerebral cortex and the hippocampus of both embryonic and adult brains. Its expression in the striatum and the basal forebrain was higher at E14 and gradually decreased from E18 towards adulthood [31]. STAT3 is needed for pleiotropic action, such as determination of neuronal cell fate, survival, regeneration, and apoptosis throughout brain development. In 2000, De-Fraja and colleagues observed that expression of STAT3 was markedly downregulated in the aging brain (26 months) [33].

In general, expression of STAT5 is low in the cortex and basal forebrain of immature brain, and its expression becomes gradually more pronounced towards adulthood. Interestingly, STAT5 in the striatum showed a reverse expression pattern. Furthermore, STAT5 has been shown to be weakly expressed in the hippocampus in both embryonic and postnatal brains [31]. STAT6 has been shown to be consistently expressed across all brain regions throughout the embryonic stages (E14–E18). Its expression then progressively decreased in more developed stages (P2, P10, and adult) [31]. The STAT4 protein, however, was not detectable in any brain region (cerebral cortex, striatum, basal forebrain, and hippocampus) [31]. Therefore, JAK-STAT mRNA and proteins are spatiotemporally expressed and function in the regulation of neurodevelopment in both developing and mature brain.

4. The Role of JAK-STAT Pathway in Neuronal Differentiation and Gliogenesis

JAK-STAT signaling is essential for gliogenesis, rather than promoting neurogenesis. During the neurogenic phase, JAK-STAT signaling is tightly regulated by DNA methylation;

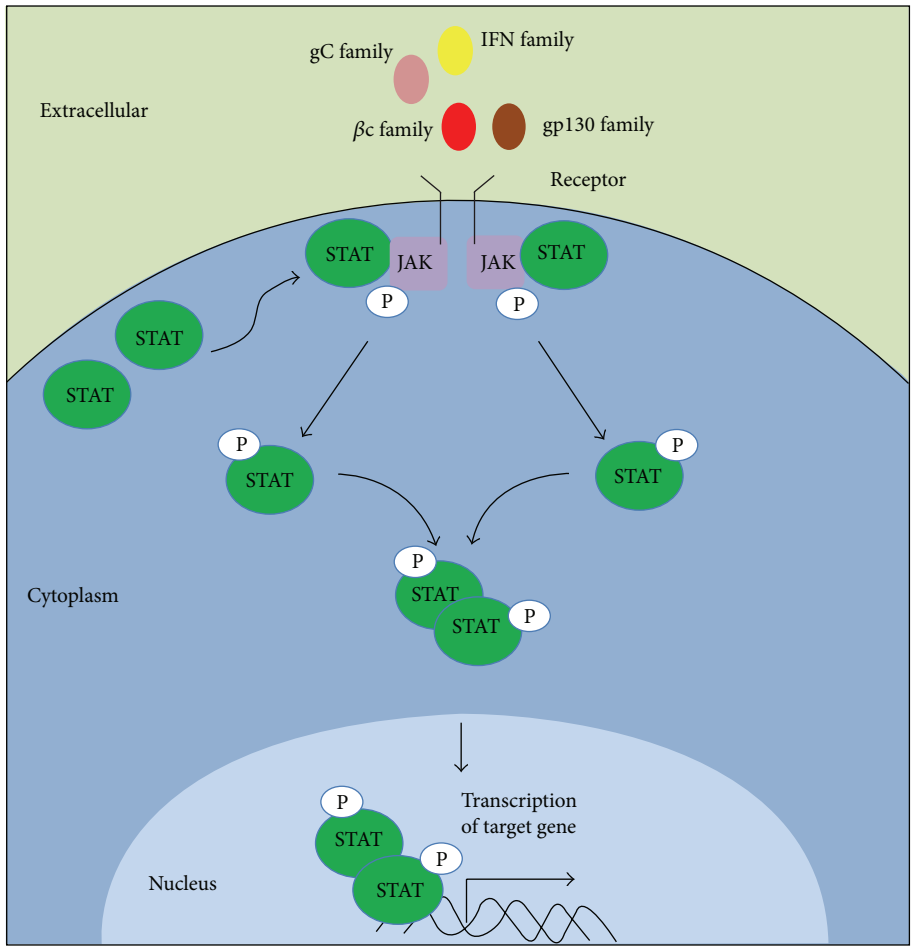

Figure 1: The canonical JAK-STAT signaling pathway. Ligands bind to corresponding receptors and allow the appropriate JAKs to phosphorylate each other, leading to their activation. Activated JAKs phosphorylate STATs, followed by subsequent dimerization of the STATs. STAT dimers are transported into the nucleus and bind the promoters of target genes to initiate transcription. The ligands include interleukins (IL-10, IL-19, IL-20, and IL-22), IFNs (IFNα, IFNβ, and IFNγ), gp130 family (IL-6, IL-11, oncostatin M, leukemia inhibitory factor, cardiotrophin-1, granulocyte colony-stimulating factor, IL-12, Leptin, and ciliary neurotrophic factor), and γ-chain (gC) family (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21).

however, JAK-STAT pathway activity becomes robustly activated during the transition from neuronal to glial differentiation. Moreover, various intrinsic and extrinsic gliogenic signals dictate neuroepithelial cells to switch from a neuronal to glial differentiation, such as epigenetic signals and transcription factors (intrinsic) as well as cytokines and growth factors (extrinsic) [34].

In the canonical JAK-STAT signaling pathway, cardiotrophin-1 binds to gp130 and LIFβ coreceptors and activates the JAKs. The STAT3 transcription factor is then activated through phosphorylation by JAKs. Active STAT3 then binds the p300/CBP coactivator proteins and forms a larger complex with Smad, which is a downstream effector of bone morphogenic protein signaling [35]. This Smad:p300/CBP:STAT3 complex then translocates into the nucleus, which specifies glial cell fate by transcriptional activation of astrocytic genes, such as *Gfap* and *S100β*. However, STAT3 can also be activated by different ligands, such as IFN and interleukins (Table 1) [26, 36–38].

Activation of JAK-STAT signaling alone is insufficient to initiate gliogenesis. Other factors that promote gliogenesis, such as Notch signaling, are also required; however, the gliogenic action of Notch signaling must coincide with activation of JAK-STAT signaling (Figure 4). At the same time, neurogenesis must be inhibited via recombination signal sequence-binding protein Jκ (RBP-Jκ). RBP-Jκ binds to the repressive cofactor protein nuclear receptor corepressor to suppress gliogenic genes and inhibit glial cell differentiation when the JAK-STAT pathway is not activated [39].

Epigenetic alteration of chromatin structure by the polycomb group complex during the transition to gliogenesis leads to suppression of *Ngn1* and *Ngn2* and promotes glial differentiation [40]. In addition, Notch effector protein nuclear factor I/A binds to astrocytic gene promoters, such as *Gfap*, to induce dissociation of the DNA methylating enzyme [41]. Consequently, the chromatin enters a relaxed state, allowing for the transcription of gliogenic genes, such as *Gfap*, *Stat1*, and *S100β* [42].

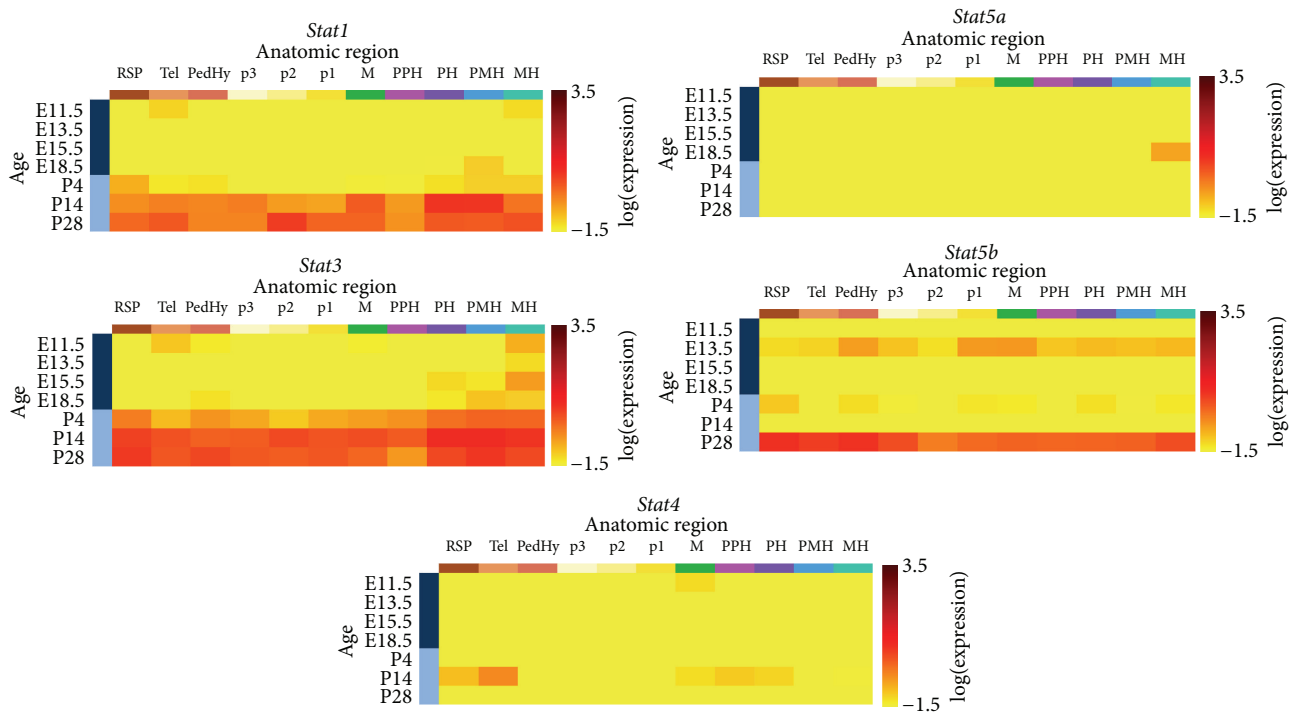

Figure 2: *Stat* (*Stat1*, *Stat3*, *Stat4*, *Stat5a*, and *Stat5b*) gene expression in C57BL/6J mouse whole brain throughout development presented in each anatomical region with log (expression). *Jak* gene expression data are not available. RSP: rostral secondary prosencephalon; Tel: telencephalic vesicle; PedHy: peduncular (caudal) hypothalamus; p3: prosomere 3; p2: prosomere 2; p1: prosomere 1; M: midbrain; PPH: prepontine hindbrain; PH: pontine hindbrain; PMH: pontomedullary hindbrain; MH: medullary hindbrain (medulla). Allen Developing Mouse Brain Atlas 2013 (http://developingmouse.brain-map.org/).

5. Potential Roles of JAK-STAT Signaling in Promoting Gliogenesis in DS Brain

The overproduction of several cytokines has been reported to be associated with the pathophysiology of DS. Gliogenic shift in the DS brain may be modulated by the different ligands and receptors that activate the JAK-STAT signal transduction pathway. For example, the level of IFNγ is markedly increased in trisomy 16 (Ts16) mouse fetus brain [43]. Together with the overexpression of the IFN receptor gene [44], this may sensitize the cells to interferon interaction and lead to activation of the JAK-STAT signaling pathway.

Gliogenic shift has been observed in both DS human and mouse brains. In the hippocampal region of human fetal DS brains at 17–21 weeks of gestation, a significantly higher number of astrocytes and lower percentage of neurons have been shown [13]. There was also a reduction in proliferating cells in the hippocampal germinal layer and parahippocampal gyrus [13]. The frontal lobe of 18–20-gestational-week-old human DS fetuses also showed a significantly higher number of radial glial cells and mature astrocytes compared to age-matched controls [19]. Furthermore, precursor cells from cerebellar neurogenic regions (external granular and ventricular zones) of human DS fetuses were proliferation-impaired [12]. Briggs and colleagues demonstrated a twofold increment of increase glial lineages in DS iPSC culture [18]. Interestingly, a neurosphere culture of stem/progenitor cells from the subventricular zone of Ts1Cje mice at P84 showed an increase in astrogliogenesis and reduced neurite outgrowth in differentiated neurons when compared to the age-matched controls despite no differences in the pool of neural stem cells [20]. These results suggest that, in early stages, the neural stem cell pool in the brain of the DS mouse model may not differ from their euploid controls but tends to differentiate into glial lineages and defective neurons as the brain matures or regenerates itself in the adult stage.

Efforts to unravel the disrupted molecular mechanisms that lead to DS learning and memory deficits have been carried out in various studies on human samples as well as mouse models. Sturgeon and colleagues postulated on pathways and HSA21-encoded genes and proteins that may cause intellectual disability through a meta-analysis of databases comprising protein-coding genes, human pathways, and protein-protein interactions [45]. Based on their pathway analysis of HSA21 genes, they reported that JAK-STAT is an enriched pathway with HSA21 protein associations, including IFNAR1, IFNAR2, IFNGR2, and IL10RB [45]. Moreover, RT-qPCR of whole brain samples from the DS mouse model Ts1Cje demonstrated overexpression of *Ifnar1*, *Ifnar2*, and *Il10rb* genes [46]. In addition, global gene expression analysis performed on the cerebral cortex, cerebellum, and hippocampus of Ts1Cje mice at four different postnatal time points (P1, P15, P30, and P84) showed *Stat1* upregulation in Ts1Cje cerebellum and cerebral cortex at P84 as confirmed by RT-qPCR and Western blot [47]. Supporting evidence from

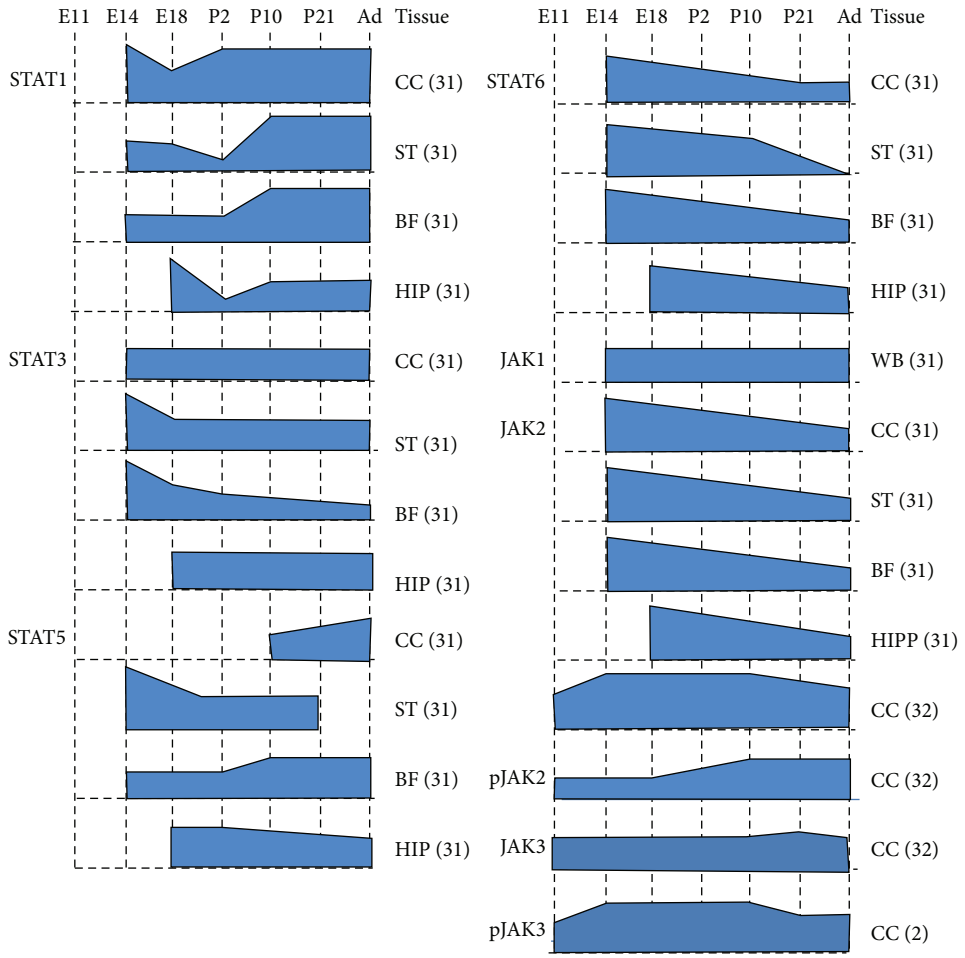

Figure 3: JAK (JAK1, JAK2, and JAK3), phosphorylated JAK (pJAK2 and pJAK3), and STAT (STAT1, STAT3, STAT5, and STAT6) protein levels in the mammalian nervous system throughout various developmental stages until adulthood. Drawing is not to scale. BF: basal forebrain; CC: cerebral cortex; HIP: hippocampus; ST: striatum; WB: whole brain. (1) Sprague Dawley rat [31]; (2) mouse [32].

a bioinformatics analysis using the Database for Annotation, Visualization, and Integrated Discovery also identified JAK-STAT signaling as a major pathway dysregulated in the Ts1Cje cerebral cortex and hippocampus [48]. There was also a significant increase of IFNAR2 proteins in the cerebral cortex of DS fetuses at 19–21 weeks of gestational age [49].

Impairment of neurogenesis and enhancement of glial cell generation in the DS brain may be the main factors contributing to intellectual disability associated with the syndrome. Although the precise mechanisms have not yet been fully defined, it has been proposed that the action of IFNs (IFN-α/β) is related to trisomy 21 and related phenotypic anomalies [50]. An extra copy of an IFN receptor (IFNR) gene within the triplicated region leads to a 1.5-fold increase in gene expression, which subsequently increases cellular responsiveness to IFNs. These receptors activate JAK-STAT signaling cascades after binding to their ligands. It is posited that IFNs are involved in this mechanism because their receptors (IFNAR1, IFNAR2, and IFNGR2) are located on the extra copy of the genomic segment of chromosome 21 [51].

The JAK-STAT pathway is canonically induced by IFNs [52], which are key mediators of astrogliogenesis in neural stem cells [53]. IFNγ treatment of proliferative wild-type murine E14 neurosphere-derived neural precursor cells showed reduced proliferation but upregulated GFAP and βIII-tubulin expression with simultaneous sonic hedgehog and *Stat1* activation [54]. Moreover, IFNβ treatment of human SH-SY5Y cells and mouse primary cortical neurons was recently shown to negatively regulate brain-derived neurotrophic factor signaling and action via prevention of tropomyosin-related kinase receptor type B activation. IFNβ activation of JAK-STAT signaling resulted in downregulation of tropomyosin-related kinase receptor type B, which led to a reduction of neurite outgrowth and neuronal differentiation [55]. Results of these *in vitro* studies suggest that the IFNs and IFNRs are inducers of JAK-STAT signaling in driving gliogenesis of neuronal cell cultures. Unfortunately, information on the source of cytokines or IFNs that result in increased gliogenesis in DS subjects remains limited and was not studied or reported in the aforementioned references. Thus, the mechanism of action whether via paracrine or autocrine is not well described. However, IFN and other

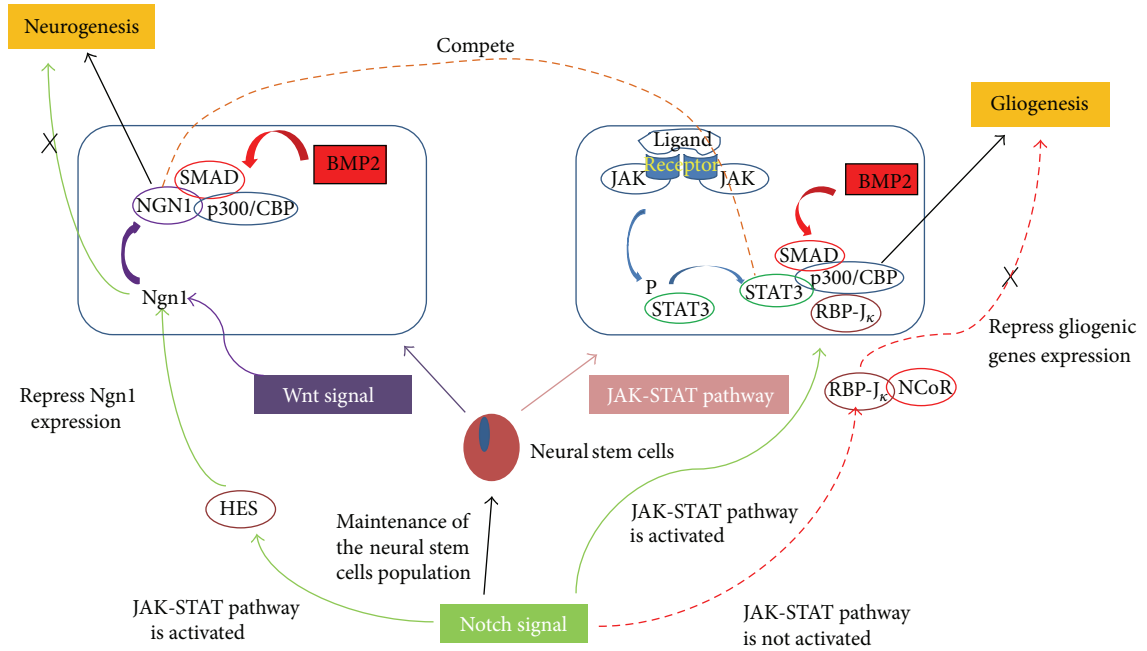

Figure 4: Crosstalk between Wnt, Notch, and JAK-STAT signaling pathways. Wnt signaling activates Ngn1, which then binds p300/CBP coactivator proteins to promote neuronal differentiation. Ngn1 competes with STAT3 to inhibit gliogenesis during neurogenic phases. During gliogenesis, the STAT3 transcription factor is activated through phosphorylation by JAKs. The active form of STAT3 binds the p300/CBP coactivator proteins and promotes gliogenesis. Notch signaling activates gliogenic genes; HES inhibits neurogenesis via inhibition of proneural genes. At the same time, RBP-Jκ is also activated to promote gliogenesis. RBP-Jκ binds a repressive cofactor protein, termed nuclear receptor corepressor, to suppress gliogenic genes and inhibit glial cell differentiation when the JAK-STAT pathway is not activated.

proinflammatory cytokines are potentially secreted from DS astrocytes, since *in vitro* studies have demonstrated that media collected from DS astroglia cultures exhibit a neurotoxic effect on neural progenitor cells (NPCs) [21]. In 2014, Ling and colleagues suggested that overexpression of IFNR may increase responsiveness to IFN, thus leading to activation of downstream targets, namely, the JAK-STAT signaling pathway [47]. Therefore, overstimulation of JAK-STAT signaling due to overexpression of IFNRs may promote a neural progenitor cell fate toward gliogenic pathways in the DS brain.

The fact that IFNR genes are triplicated and upregulated in DS individuals and mouse models may predispose DS brain to greater IFN sensitivity. Therefore, IFN-JAK-STAT activation in the neurogenic-to-gliogenic shift should be further investigated, since it may represent a potential therapeutic target for preventing and/or reversing this shift in DS individuals.

6. Gain- or Loss-of-Function Mutations in JAK-STAT Genes among DS Individuals

To date, the effect of mutations in JAK-STAT genes among DS individuals on the neurogenic-to-gliogenic shift in the brain has not been clearly delineated. Most of the mutations within these genes were investigated in relation to leukemia. DS individuals have 10- to 20-fold higher risk in developing leukaemia [56], which is a disorder that constitutes 60% of all malignancies in DS individuals [57].

Genetic mutations in JAK kinases (except TYK2) have been highlighted in various diseases, including myeloproliferative disorders and cancers. Mutation of the JAK domains can result in gain- or loss-of-function in the activity of the JAK-STAT pathway. Somatic mutations of JAK1 are more prevalent in adult acute lymphoblastic leukaemia (ALL) patients, especially in those with T cell precursor ALL (T-ALL) [58]. However, these JAK1 mutated cases were also reported to have mutations in NOTCH1 [58], which is the favourable prognosis of childhood T-ALL [59]. The reported mutations were missense and some of them were predicted to have the ability to control the kinase activity through destabilising interdomain interactions [58]. Hornakova and colleagues reported that different ALL-associated JAK1 mutations can differentially potentiate responses to type I interferons [60]. They also showed that an *in vivo* leukaemia model with cells expressing a JAK1 mutation was hypersensitive to the antiproliferative effect of type I interferon [60]. The mechanism for this observation may occur through the proliferative potential of STAT5 signaling and the antiproliferative potential of STAT1 signaling in hematopoietic precursor cells [61]. JAK2 mutations, especially the V617F point mutation that causes a constitutively active kinase [62], are implicated in myeloproliferative diseases [61]. Laurence and colleagues have suggested that the V617F mutation alters the interdomain interaction between the kinase and pseudokinase domain, which is needed to control JAKs in their inactivate state [61]. This causes spontaneous autophosphorylation and activation of the mutant kinases

in hematopoietic precursor cells, thus preventing cytokine-mediated control of growth and survival [63, 64]. Moreover, this mutation may disrupt the binding of JAK2 protein to suppressor of cytokine signaling 3 (SOCS3), thus allowing JAK2 protein to circumvent the second regulatory constraint [65].

Interestingly, most of the mutations reported within JAK kinases that are associated with DS-related malignancies were gain-of-function mutations. Activating somatic JAK2 mutations were reported in 20% of DS ALL patients and nearly all of the JAK2 point mutations happened at a common site, an arginine-to-guanine residue at position 683 (A683G) [66]. Functional studies on the mutation in murine Ba/F3 cells showed cytokine-independent growth and constitutive activation of JAK/STAT signaling pathway [67]. Another mutation that involved a five-amino-acid deletion within the JH2 pseudokinase domain in JAK2 (JAK2DeltalREED) was found in DS B-ALL patients [68]. It has been suggested that the location of the JAK2 mutation may affect different downstream signaling cascades in a cell-dependent context [69]. The JAK mutations reported in DS patients were also shown to affect myeloid progenitors, suggesting that these mutations are the secondary acquired genetic events in trisomic progenitor cells [69]. Walters and colleagues identified activating JAK3 mutations in a small subset of DS acute megakaryocytic leukaemia (AML) cases [70, 71]. They showed that the *in vivo* cell model expressing the JAK3 mutants demonstrated cytokine-independent growth and a particular JAK3 mutation (A572V) conferred characteristics of megakaryoblastic leukaemia in C57BL/6 mice [70]. Genomic rearrangement of cytokine receptor-like factor 2 (CRLF2) has been reported to be associated with JAK kinases mutations in childhood B-ALL cases, including DS patients [72, 73]. Although various gain-of-function mutations within JAK kinases have been strongly associated with the development of leukemias in DS individuals, none of these events were described in relation to dysregulated brain development and function. These observations, however, suggest that JAK-STAT signaling as an important pathway within the brain that is potentially associated with the proposed model of neurogenic-to-gliogenic shift.

7. IFN-JAK-STAT Targeting as a Potential Therapeutic for DS

Modulation of the level of IFNs or expression/phosphorylation of IFNR-JAK-STAT candidates may reduce JAK-STAT signaling activation, leading to the modification of cell fate determination in DS brain development. Mäkelä and colleagues have reported that IFN-JAK-STAT pathway-activated caspase-3 functions in apoptosis [74]. Although their study was not conducted with neural progenitor cells derived from DS brain, it was suggested that IFN affected cell proliferation and survival. Remarkably, neural progenitor cells derived from E17 rat brain treated with IFNγ induced phosphorylation of Stat1, which in turn, activated p21, Bcl-2 family proteins, and caspase-3 [74]. These proteins also affect cell proliferation and promote cell death. In contrast, partial knockout of Ifnar2 and Ifngr2 in neuronal cultures derived from trisomy of chromosome 16 mouse fetuses showed improved cell growth and viability [75]. Moreover, addition of anti-IFNγ IgG to the culture medium significantly increased the viability of cortical neurons derived from trisomy of chromosome 16 mouse fetuses but had no effect on euploid neurons [76].

A subset of DS patients exhibit neuropathological features and suffer from early onset Alzheimer's disease (AD). IFN has been shown to activate JAK-STAT signaling, and its proinflammatory effect caused neuronal cell death in AD [77]. Therapeutically, it was suggested that the progression of AD can be reduced by blocking IFNAR1 [77]. Therefore, administration of IFN antagonists may have a therapeutic benefit in DS. Collectively, inhibiting both IFNs and their receptors can improve the viability of neurons and may restore neurogenesis in the DS brain.

The IFN inhibitor Normferon was developed by Dr. Maroun following the positive outcome of targeting IFN and INFR in a DS mouse model [75, 76, 78]. In contrast to IFN inhibitors, there are currently more than ten JAK inhibitors currently being assessed in clinical trials, such as ruxolitinib, SAR302503, lestaurtinib, CYT-387, pacritinib, LY2784544, XL019, AZD1480, NS-018, and BMS-911543 [79]. These drugs were tested in hematologic cancers, such as leukemia and myelodysplastic syndrome. Ruxolitinib, a selective JAK1 and JAK2 inhibitor, is the only JAK inhibitor approved by the US Food and Drug Administration and is mainly used for patients with myelofibrosis. IFN is known to activate JAK1 and JAK2 kinases, which subsequently activate STAT1 [80]. Therefore, inhibitors of JAK1 or JAK2 kinases, such as ruxolitinib, may serve as a potential treatment for DS to restore neurogenesis. Laboratory analysis of low-level administration of ruxolitinib should be carried out to ascertain the effect on neurogenesis in DS mice and/or humans.

Another potential therapeutic for DS is nucleic acid-based therapy. The nucleic acid/oligonucleotide can be antisense, ribozymes, short interfering RNA, microRNA, and aptamers that inhibit gene expression and function at transcriptional or translational levels [81]. Currently, use of the nucleic acid-based approach for targeting STAT focuses on cancer treatment and therapy [82]. However, several limitations have been reported, mainly regarding degradation of the nucleic acid/oligonucleotide when delivered into biological systems [82]. Therefore, chemical modification of the nucleic acid/oligonucleotide, termed locked nucleic acid (LNA), has been proposed to increase its stability. The LNA name stems from the ribose ring of the nucleic acid/oligonucleotide being locked by a methylene linkage between the $2'$-oxygen and $4'$-carbon [83]. Therefore, incorporating LNA can increase resistance of the nucleic acid/oligonucleotide to nuclease degradation. Its low toxicity also makes it as a potential therapeutic tool [84]. LNA-based nucleic acid targeted-inhibition has specifically emerged as an important platform for drug development. A clinical trial conducted using LNA-antisense oligonucleotide EZN-2968, which targets hypoxia inducible factor-1 (HIF-1), found that overexpression of HIF-1 in cancer cells leads to upregulation of genes important for cancer cell survival [85]. Two out of 10

patients were found to have reduced expression and levels of HIF-1α mRNA and protein, respectively, after administration of EZN-2968. A similar strategy may be applied to target the IFN-JAK-STAT signaling pathway for DS therapy by inhibiting expression of IFNRs or JAK-STAT. For example, *miR-9* was shown to directly target mRNAs of *Lif*, *gp130*, and *Jak1* by downregulating these crucial upstream elements of the JAK-STAT signaling pathway, thus leading to decreased phosphorylation of STAT and suppression of astrogliogenesis [86]. Therefore, a *miR-9* LNA mimic could be developed into a potential therapy to regulate the level of JAK-STAT activation. JAK-STAT signaling, however, has pleiotropic functions: inhibiting expression of JAK-STAT may improve the DS phenotype, at least short-term, while it may also cause secondary adverse effects on other organs or systems. Therefore, careful experimental design and result interpretation are crucial for developing an effective targeted therapy for DS individuals.

8. Concluding Remarks

JAK-STAT signaling is one of the most important pathways determining gliogenic cell fates. In this review, the potential role of JAK-STAT signaling in neurological diseases, such as DS, has been highlighted. It is believed that neuropathological and cognitive impairment in DS patients may be attributed to, at least in part, defective neurogenesis and a reduction in the number of neurons in several brain regions, including the cerebrum, hippocampus, and cerebellum. In addition, the increased number of astrocytes in DS brain may also be a potential factor leading to intellectual disability in DS individuals.

The bias of neuroepithelial cells towards gliogenesis also indicates dysregulation of JAK-STAT signaling during brain development. The Notch signaling pathway coincides with the JAK-STAT pathway to bring about the gliogenic shift. Without JAK-STAT signaling, the Notch pathway instead represses gliogenic genes. Defective JAK-STAT signaling may contribute to the overproduction of glial cells. Therefore, it is crucial to understand the role of JAK-STAT signaling pathways in controlling astrocytic fate in DS as a potential therapeutic target for improving cognitive function in DS individuals.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Programming of Dopaminergic Neurons by Neonatal Sex Hormone Exposure: Effects on Dopamine Content and Tyrosine Hydroxylase Expression in Adult Male Rats

Pedro Espinosa,[1] Roxana A. Silva,[1] Nicole K. Sanguinetti,[1] Francisca C. Venegas,[1] Raul Riquelme,[1] Luis F. González,[1,2] Gonzalo Cruz,[3] Georgina M. Renard,[1] Pablo R. Moya,[2] and Ramón Sotomayor-Zárate[1]

[1]*Laboratorio de Neuroquímica y Neurofarmacología, Centro de Neurobiología y Plasticidad Cerebral, Instituto de Fisiología, Facultad de Ciencias, Universidad de Valparaíso, 2360102 Valparaíso, Chile*
[2]*Laboratorio de Neurogenética, Centro de Neurobiología y Plasticidad Cerebral, Instituto de Fisiología, Facultad de Ciencias, Universidad de Valparaíso, 2360102 Valparaíso, Chile*
[3]*Laboratorio de Alteraciones Reproductivas y Metabólicas, Centro de Neurobiología y Plasticidad Cerebral, Instituto de Fisiología, Facultad de Ciencias, Universidad de Valparaíso, 2360102 Valparaíso, Chile*

Correspondence should be addressed to Ramón Sotomayor-Zárate; ramon.sotomayor@uv.cl

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We sought to determine the long-term changes produced by neonatal sex hormone administration on the functioning of midbrain dopaminergic neurons in adult male rats. Sprague-Dawley rats were injected subcutaneously at postnatal day 1 and were assigned to the following experimental groups: TP (testosterone propionate of 1.0 mg/50 μL); DHT (dihydrotestosterone of 1.0 mg/50 μL); EV (estradiol valerate of 0.1 mg/50 μL); and control (sesame oil of 50 μL). At postnatal day 60, neurochemical studies were performed to determine dopamine content in substantia nigra-ventral tegmental area and dopamine release in nucleus accumbens. Molecular (mRNA expression of tyrosine hydroxylase) and cellular (tyrosine hydroxylase immunoreactivity) studies were also performed. We found increased dopamine content in substantia nigra-ventral tegmental area of TP and EV rats, in addition to increased dopamine release in nucleus accumbens. However, neonatal exposure to DHT, a nonaromatizable androgen, did not affect midbrain dopaminergic neurons. Correspondingly, compared to control rats, levels of tyrosine hydroxylase mRNA and protein were significantly increased in TP and EV rats but not in DHT rats, as determined by qPCR and immunohistochemistry, respectively. Our results suggest an estrogenic mechanism involving increased tyrosine hydroxylase expression, either by direct estrogenic action or by aromatization of testosterone to estradiol in substantia nigra-ventral tegmental area.

1. Introduction

Different adverse stimuli in early life produce alterations in normal development that persist until adulthood and may be risk factors for diseases in adult life. Lucas defined the concept of *programming* as "the physiological redirection of a tissue or organ by a stimulus, in a sensitive period of development, that produces adverse functional changes in adulthood" [1]. Animal research initially focused on fetal exposure (*fetal programming*), but recent research has expanded the concept of programming to include early postnatal exposure (*neonatal programming*).

In 1959, Phoenix et al. [2] first reported the long-term effects produced by androgens on the central nervous system (CNS) and their behavioral implications in reproduction [2]. This early research determined that the maximum sensitivity to the effects of androgens occurs during the gestational period, childhood, and puberty [3, 4]. In recent years, it has been shown that environmental pollutants act as endocrine disruptors capable of producing a myriad of effects in

the brain (for review see [5]). For example, some pollutants, such as polychlorinated biphenyls (administered in neonatal period, through lactation), produce learning deficits and changes in spatial orientation tasks in monkeys and rats (for review see [6]). Moreover, neonatal exposure to polybrominated diphenyl ether (PBDE), another endocrine disrupter, increases spontaneous locomotor behavior in adult rats [7]. Interestingly, the authors showed that different doses of PBDE administered during neonatal period can result in either an increase or decrease in nicotine-induced locomotor activity [7]. Exposure to environmental pollutants affects specific neuronal groups including midbrain dopaminergic neurons. For example, neonatal or postnatal administration of bisphenol A (BPA) in rats produces an increase in spontaneous locomotion behavior, which was associated with decreased immunoreactivity for tyrosine hydroxylase (TH) in substantia nigra (SN) and a decrease in gene expression of dopamine transporter (DAT) in midbrain nuclei [8]. Therefore, the evidence in the literature shows that the neonatal period is a window of sensitivity to the effects of hormonally active compounds, when exposure to compounds such as endocrine disrupter or sex hormones can generate long-term effects on the functioning of neural circuits.

In CNS, the physiological effects of testosterone (T) are mediated through its reduction to dihydrotestosterone (DHT) by cytochrome P450 5-α-reductase [9] or its aromatization to estradiol (E_2) by cytochrome P450 aromatase [10]. In the brain, cytochrome P450 5-α-reductase exists in two isoforms [11, 12]; type-1 isoform is expressed in similar levels in females and males [12], while the type-2 isoform is only expressed in males in the late stage of fetal development and early stage of postnatal period [10]. On the other hand, cytochrome P450 aromatase is highly expressed in males in the hypothalamus during gestational development and then progressively decreases during the neonatal stage and through childhood and adulthood [10]. The same pattern is observed for cytochrome P450 aromatase activity in hypothalamus of male rats [13]; however, in the female brain, cytochrome P450 aromatase activity is steady throughout all life stages [13]. Interestingly, in midbrain, cytochrome P450 aromatase activity is highest during the first two weeks of neonatal stage in both male and female rats [13].

Dopaminergic neurons are one of the major groups of cells in the midbrain and belong to nigrostriatal and mesocorticolimbic circuits [14]. The nigrostriatal pathway is formed by dopaminergic projections from the SN *pars compacta* to the striatum [15–18], while dopaminergic projections from ventral tegmental area (VTA) to nucleus accumbens (NAcc) and prefrontal cortex (PFC) form the mesocorticolimbic circuit, or reward system [19–22]. In the latter, natural rewarding cues such as sex [23] or food [24], as well as drugs of abuse, [25] increase extracellular dopamine (DA) levels in NAcc.

Sex hormone receptors are expressed in both nigrostriatal and mesocorticolimbic circuits [26–30] and regulate the expression of critical proteins for dopaminergic function, such as tyrosine hydroxylase (TH), the rate-limiting enzyme of catecholamine synthesis [29, 31–34]. Sex hormone exposure during early developmental stages or sensitive period of life has been associated with some CNS disorders. In humans, research has shown that increased androgen levels during adolescence are associated with risky behavior, such as development and maintenance of alcohol dependence [35]. Also, studies have shown that increased levels of sex hormone in amniotic fluid during the fetal stage are positively related with autism [36]. Animal studies have shown that androgen exposure during early development might play a role in the development of attention deficit hyperactivity disorder [37]. At neurochemical level, neonatal testosterone exposure decreases extracellular serotonin levels in the amygdala [38] and increases noradrenaline (NA) and glutamate in ventromedial hypothalamus [39] of adult female rats. In regard to estrogens, we previously found that neonatal exposure to estradiol valerate (EV) in female rats increases DA content in the tuberoinfundibular area [39] and nigrostriatal pathway [40] in adulthood. However, the long-lasting effects of neonatal exposure to different sex hormones on midbrain dopaminergic neurons have been scarcely studied. Thus, the aim of the present study was to determine potential changes induced by neonatal administration of testosterone propionate (TP), DHT, or EV on DA content and expression of TH mRNA in midbrain dopaminergic neurons in adult male rats.

2. Materials and Methods

2.1. Animals. 93 male Sprague Dawley pups from fifteen litters were used. The remaining female pups were assigned to other studies. All animals were housed in a temperature-controlled room ($21 \pm 2^{\circ}$C) under a 12 h light cycle with lights on at 08:00 h, with food and water *ad libitum*. All experimental procedures were approved by Ethics Committee of the Faculty of Science, Universidad de Valparaíso, and the Institutional Animal Experimentation Ethics Board and the Science Council (FONDECYT) of Chile. Efforts were made to minimize the number of animals used and their suffering.

2.2. Drugs and Reagents. Testosterone propionate (TP), dihydrotestosterone (DHT), estradiol valerate (EV), sesame oil, dopamine standard, EDTA, and 1-octanesulfonic acid were purchased from Sigma-Aldrich, Inc. (St. Louis, Missouri, USA). All other reagents were of analytical and molecular grade.

2.3. Experimental Procedure. Each group of animals was single-injected at postnatal day (PND) 1 with 1.0 mg TP, 1.0 mg DHT, 0.1 mg EV, or sesame oil (control) per pup. TP, DHT, and EV were dissolved in 50 μL of sesame oil. Pups were divided randomly into four groups of animals: control ($n = 29$), TP ($n = 32$), DHT ($n = 27$), and EV ($n = 32$). The doses of TP, DHT, and EV used were previously published [39, 40, 42, 43]. All the pups were raised with a lactating mother until the weaning age at PND 21. After weaning, animals were housed in groups according to gender and treatment in standard cages. At PND 60, the groups of rats were randomly assigned for the following experimental protocols.

(i) Determination of DA Content. Rats were decapitated with a guillotine and the brain was removed. We microdissected at 4°C SN and VTA (which were dissected as one tissue) and striatum; these brain tissues were weighed on analytical balance (model JK-180, Chyo, Japan) as previously described [40, 44, 45]. Brain tissues were stored at −80°C for further analysis.

(ii) Determination of TH mRNA. Rats were decapitated and the brain was removed. SN and VTA were separately microdissected at 4°C using micropunch (Harris Micro-Punch, tip diameter of 2.0 mm, Ted Pella Inc., CA, USA). Brain tissues were weighed on an analytical balance and stored at −80°C for further analysis.

(iii) Determination of TH Protein. Rats were anesthetized and transcardially perfused. Brains were removed and cut into coronal slices for immunohistochemistry for TH.

(iv) Determination of NAcc DA Release. Using *in vivo* brain microdialysis in anesthetized animals, basal and stimulated-K^+ DA extracellular levels were measured through HPLC coupled to electrochemical detection. After completion of *in vivo* experiments, rats were euthanized by decapitation.

2.4. Real-Time PCR. Real-time PCR was used to determine whether the mRNA encoding TH changed in the SN and VTA in adulthood of male rats exposed to sex hormones at PND 1. Total RNA was extracted using RNeasy Mini Kit (number 74104, Qiagen, Valencia, CA, USA) following manufacturer instructions. The quantification of total RNA was made in NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and 4 ng of total RNA was reverse-transcribed using QuantiTect Reverse Transcription Kit (number 205314, Qiagen, Valencia, CA, USA). The reaction was made in a master mix including 8.0 μL of total RNA (genomic DNA free), 1.0 μL of Quantiscript Reverse Transcriptase, 4.0 μL of Quantiscript RT Buffer, and 1 μL of RT Primer Mix. The reaction was terminated by heating the samples at 95°C for 3 min.

For TH mRNA quantification, all samples were analyzed in triplicate in 10 μL reaction, and a standard real-time PCR reaction mix was prepared containing the following components: 5.0 μL of QuantiTect SYBR Green PCR Kit (number 204143, Qiagen, Valencia, CA, USA), 2.8 μL of nanopure and sterile water, 0.1 μL of each primer, and 2 μL of cDNA. For specific gene amplification, a standard protocol of 45 cycles was used in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA). After initial polymerase activation at 95°C for 10 min, primer-specific amplification and quantification cycles were run at 95°C for 15 sec and 61.7°C for 20 sec. The TH primer was designed from data published in GenBank, access number NM_012740: forward 5′-GGT-CTA-CTG-TCC-GCC-CGT-GAT-T-3′ and reverse 5′-GAG-CTT-GTC-CTT-GGC-GTC-ATT-G-3′. To normalize TH mRNA content, ribosomal 18s mRNA was measured in each protocol, using primers previously published [42] and commercially available, GenBank access number X01117 (for 18s, forward 5′-TCA AGA ACG AAA GTC GGA GG-3′ and reverse 5′-GGA CAT CTA AGG GCA TCA CA-3′). Amplification of 18s RNA was performed in a different tube to avoid interference with the amplification of the mRNAs. Reaction tubes lacking RT enzyme were used as PCR-negative controls. Specificity of generated amplicons was verified by performing melting curves at the end of each reaction. To verify the products from the RT-PCR reaction, they were separated on 2.0% agarose gels, stained with ethidium bromide, and compared to a 100 bp standard (data not shown).

2.5. Immunohistochemistry for Tyrosine Hydroxylase (TH). Fifteen male rats were anesthetized with chloral hydrate (400 mg/Kg i.p.) and transcardially perfused with saline (0.9% p/v NaCl), followed by ice-cold fixative solution (4% p/v paraformaldehyde in phosphate buffered saline solution (PBS) 0.1 M with pH 7.4). Brains were removed from the skull and postfixed for 30 min. Brains were then dehydrated in 20% p/v sucrose solution for 48 h at 4°C. Afterwards, 30 μm thick coronal slices were prepared on a cryostat (model KD-2950, Kedee, China). Interest slices were selected from SN-VTA located −5.4 mm from bregma according to the atlas of Paxinos and Watson [46]; these slices were put on 24-well plate and washed with PBS 0.01 M for 10 minutes following incubation with H_2O_2 (0.3% v/v in PBS) for 30 minutes.

Coronal slices were washed twice again with PBS 0.01 M for 10 minutes and then were incubated for 1 h in blocking solution (Triton X-100 0.4% v/v and NGS 3% v/v in PBS). The incubation of the first anti-TH rabbit antibody (catalog number 657012, Calbiochem, Merck Millipore, Merck KGaA, Darmstadt, Germany) it has been made with dilution 1 : 5000 in blocking solution over night with soft agitation at 4°C [47]. After incubation, slices were washed 4 times with PBS 0.01 M for 10 min each time. The second incubation it has been made for 2 h with second biotinilated anti-rabbit antibody (catalog number BA-1000, Vector Laboratory Inc., Burlingame, CA, USA) at ambient temperature, diluted 1 : 1000 in PBS 0.01 M solution with BSA 0.2% p/v. Afterwards, the slices were washed 4 times with the same protocol mentioned above. Then slices were incubated for 1 h with ABC kit (Kit Vectastain ABC, Vector Laboratory Inc., Burlingame, CA, USA) and then were washed twice. For the chromogenic stain, slices were incubated with DAB (diaminobenzidine) 0.05% p/v with H_2O_2 0.025% v/v in PBS (DAB: catalog number D-5905, Sigma-Aldrich). At the end of the reaction (5–10 min), the slices were washed 2 times and finally were put on slide and fixed with Eukitt (catalog: 03989, Sigma-Aldrich, Inc., St. Louis, Missouri, USA).

The slices were photographed bilaterally with a microscope with Motic camera (BA-210, Motic, British Columbia, Canada) at 4x objective for area determination of SN and VTA; the following photographs were taken at 10x for TH-positive cells counting with ImageJ software (http://rsbweb.nih.gov/ij/. For each rat, SN and VTA were selected in 4 slices, then manual counts were performed in blind by three independent investigators using as reference the medial terminal nucleus of accessory optic tract (MT).

2.6. Dopamine Content in the SN-VTA. Tissue homogenization was performed according to Chi et al. [48] and our previous work [40]. Briefly, the tissue was collected in 400 μL of 0.2 M perchloric acid and then homogenized in a glass-glass homogenizer in ice. The homogenate was centrifuged at 12,000 ×g for 15 minutes at 4°C (model Z233MK-2, Hermle LaborTechnik GmbH, Wehingen, Germany) and the resultant supernatant was filtered (0.2 μm HPLC Syringe Filters disposable filter PTFE, model EW-32816-26, Cole-Parmer Instrument Company, USA). The filtered supernatant was injected into a HPLC coupled to electrochemical detection for determination of DA content. The pellet was resuspended in 1 N NaOH for protein quantification by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Richmond, CA, USA) using bovine serum albumin as standard. The DA content was expressed as picograms per milligram of total protein.

2.7. In Vivo Brain Microdialysis. At PND 60, the animals were deeply anesthetized with choral hydrate (400 mg/Kg, i.p.) and placed in a stereotaxic apparatus (model 68002, RWD Life Science Co. Ltd., Shenzhen, China). Body temperature of the animals was maintained at 37°C with an electrical blanket controlled by a thermostat. A quarter of the initial dose of choral hydrate was given every hour to maintain the animal anesthetized during the course of the experiments. Concentric brain microdialysis probes (2 mm membrane length, model CMA 11, 6,000 Daltons cut-off, Solna, Sweden) were implanted in NAcc using the coordinates according to the atlas of Paxinos and Watson [41] (NAcc: 1.56 mm posterior, 1.50 mm lateral, and 7.8 mm ventral to bregma). Microdialysis probes were perfused with Krebs-Ringer's phosphate buffer (KRP in mM: NaCl 120; KCl 2.4; Na_2HPO_4 0.9; NaH_2PO_4 1.4; pH = 7.4) at a rate of 1 μL/min using an infusion pump (model RWD 210, RWD Life Science Co. Ltd., Shenzhen, China). After a stabilization period of 90 min, two perfusion samples were collected every 20 min in 3 μL of 0.2 M perchloric acid. At 40 min, KRP was changed for 70 mM KRP-potassium (K^+) during 20 min. After those 20 min (between 60 and 100 min of perfusion protocol), KRP solution was again perfused through the microdialysis probe. All the perfusion samples were maintained on ice during the experiment and stored at −80°C until analysis. At the end of each experiment, animals were euthanized by decapitation and brains were quickly removed and stored in formalin. Brain sections of 50 μm were stained with cresyl violet to verify microscopically probe location. One example of probe placement is shown in Figure 2(a).

2.8. DA and DOPAC Quantifications. Ten microliters of each cleaned supernatant or dialysate samples were injected to the HPLC system with the following setting: an isocratic pump (model PU-2080 Plus, Jasco Co. Ltd., Tokyo, Japan), a UniJet microbore column (MF-8912, BAS, West Lafayette, IN, USA), and an electrochemical detector (set at 650 mV, 0.5 nA; model LC-4C, BAS, West Lafayette, IN, USA). The mobile phase, containing 0.05 M NaH_2PO_4, 1.0 mM 1-octanesulfonic acid, 0.27 mM EDTA, and 4.0% (v/v) CH_3CN (pH adjusted to 2.5), was pumped at a flow rate of 80 μL/min. DA levels were assessed by comparing the respective peak area and elution time of the sample with a reference standard and the quantification was performed using a calibration curve for each neurotransmitter (Program ChromPass, Jasco Co. Ltd., Tokyo, Japan).

2.9. Statistical Analysis. Data were expressed as mean ± SEM. One-way ANOVA followed by Newman-Keuls post hoc test was used to determine eventual significant differences between groups. The statistical analyses were carried out with GraphPad Prism v5.0 (GraphPad Software, San Diego, CA).

3. Results

The aim of our work was to determine if neonatal sex hormone exposure causes long-term changes in dopaminergic neural circuits associated with locomotion (nigrostriatal pathway) and motivation (reward system). This exposure to sex hormones during early developmental stages could be vulnerability factors predisposing to developing neuropsychiatric disorders in adulthood. To accomplish this aim, we used high performance liquid chromatography (HPLC) coupled to electrochemical detection to determine neurotransmitter levels in biological samples obtained from dissected brain (for tissue content) or microdialysates (for extracellular levels). We also used real-time PCR and immunohistochemistry to determine changes in gene and protein expression of TH.

3.1. Long-Lasting Effects of Neonatal Sex Hormones Administration on TH mRNA Expression in SN-VTA. In our model, neonatal exposures to EV and TP in male pups produced a significant increase in TH mRNA expression at PND 60 in SN (Figure 1(a) [$F_{(3,13)} = 29.31$, $P < 0.0001$]) and VTA (Figure 1(b) [$F_{(3,13)} = 483.3$, $P < 0.0001$]).

3.2. Long-Lasting Effects of Neonatal Sex Hormones Administration on TH Protein Expression in SN-VTA. Figure 2 shows low and high magnification photomicrographs of TH-immunoreactive neurons in SN and VTA of control, TP, DHT, and EV male rats. Neonatal exposures to EV and TP increase TH protein expression at PND 60 in SN (Figure 3(a) [$F_{(3,11)} = 15.22$, $P = 0.0003$]) and VTA (Figure 3(b) [$F_{(3,11)} = 280.4$, $P < 0.0001$]).

3.3. Long-Lasting Effects of Neonatal Sex Hormones Administration on DA and DOPAC Content in SN-VTA and Striatum. Figure 4 shows the effects of neonatal administration of TP, DHT, or EV on DA content in SN-VTA (panel (a)) and striatum (panel (b)) of adult male rats. Neonatal exposure to TP or EV produced a significant increase on DA content in SN-VTA of adult male rats [$F_{(3,20)} = 5.206$, $P = 0.0081$]. However, the neonatal administration of DHT (a nonaromatizable androgen) did not affect the DA content (Figure 4). On the other hand, striatal DA content was unaffected by neonatal sex hormones administration compared to control rats (Figure 4(b)) [$F_{(3,20)} = 2.884$, $P = 0.0613$].

Table 1: Substantia nigra-ventral tegmental area (SN-VTA) DA and DOPAC levels from control, TP, DHT, and EV adult male rats.

Group	DA (pg/mg protein)	SEM	DOPAC (pg/mg protein)	SEM	DOPAC/DA	SEM	P value	n
C	7985.3	2029.6	2450.9	603.6	0.3532	0.0491	—	6
TP	17407.5	4388.6	3175.9	538.5	0.2913	0.0654	0.457	6
DHT	5765.8	858.8	1889.2	277.3	0.3121	0.0329	0.574	6
EV	20744.0	4007.3	4109.1	635.3	0.2117	0.0208	0.030*	6

DA: dopamine; DOPAC: 3,4-dihydroxiphenylacetic acid. * P value < 0.05 when comparing the ratio (DOPAC/DA) to the control group.

Table 2: Striatal DA and DOPAC levels from control, TP, DHT, and EV adult male rats.

Group	DA (pg/mg protein)	SEM	DOPAC (pg/mg protein)	SEM	DOPAC/DA	SEM	P value	n
C	122001	18034	12970	2854	0.1004	0.014	—	7
TP	78322	8136	10714	1706	0.1359	0.013	0.0875	6
DHT	103276	9689	27071	2737	0.2707	0.023	0.0001*	6
EV	143159	23860	15583	3034	0.1156	0.024	0.5790	6

DA: dopamine; DOPAC: 3,4-dihydroxiphenylacetic acid. * P value < 0.05 when comparing the ratio (DOPAC/DA) to the control group.

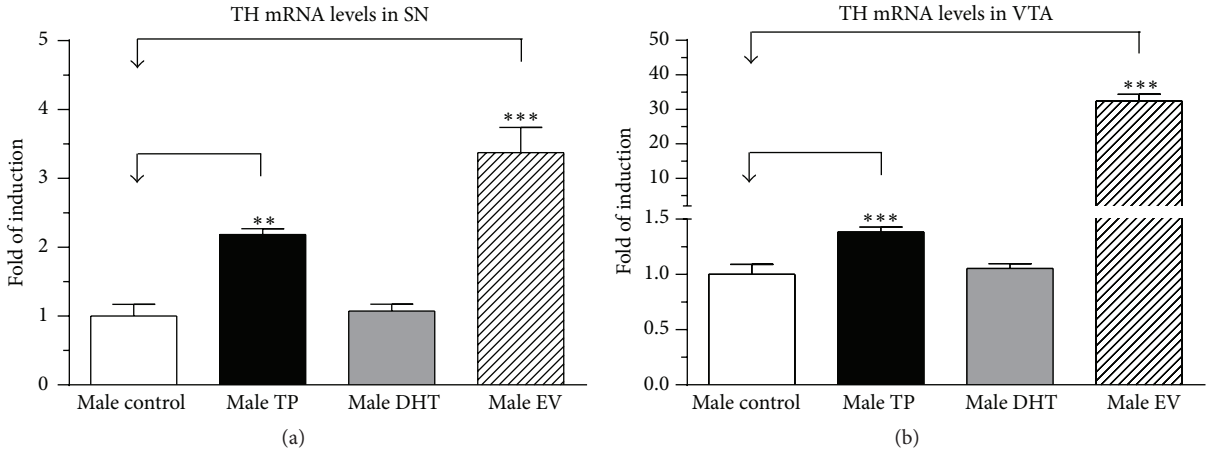

Figure 1: Effect of neonatal exposure to testosterone propionate (TP), dihydrotestosterone (DHT), or estradiol valerate (EV) on TH mRNA levels in substantia nigra (SN) (a) and ventral tegmental area (VTA) (b) of adult male rats. All data have been normalized for levels of 18s expression within the same sample. Results are expressed as fold of induction regard control group and represent the mean ± SEM (SN: $n = 4$, 4, 5, and 4 per male control, TP, DHT, and EV, resp.; VTA: $n = 4$, 3, 6, and 4 per male control, TP, DHT, and EV, resp.) (*** $P < 0.0001$, ** $P < 0.01$; one-way ANOVA followed by the Newman-Keuls multiple comparison test).

When we analyzed the ratio between the main metabolite of DA (DOPAC) and DA in SN-VTA, we observed a significant reduction in this value in EV versus control rats (Table 1, * $P < 0.05$). On the contrary, the ratio (DOPAC/DA) in striatum increases significantly in DHT versus control rats (Table 2, * $P < 0.0001$).

3.4. Long-Lasting Effects of Neonatal Sex Hormones Administration on DA Release in NAcc. Figure 5(b) shows the effects of neonatal exposure to TP, DHT, or EV on NAcc DA release induced by depolarizing stimulus in adult male rats. For each experimental group, the perfusion of KRP-K^+ produced an increase in NAcc DA release with respect to its own baseline levels. However, when comparing the magnitudes of the NAcc DA release induced by KRP-K^+ at 60 min, we observed a greater effect in DA releasability in TP and EV rats versus DHT and control rats [$F_{(3,17)} = 6.031$, $P = 0.0054$].

4. Discussion

4.1. Long-Lasting Effects of Neonatal Sex Hormones Administration on mRNA and Protein Expression of TH and DA Content in SN-VTA. Our work demonstrates that DA content in SN-VTA in adult male rats is affected by early exposure to E_2—either directly by EV exposure or indirectly through the partial aromatization of T to E_2 in TP male—which produces an increase in mRNA and protein expression of TH (Figures 1–3), which results in an increase in DA content in SN-VTA.

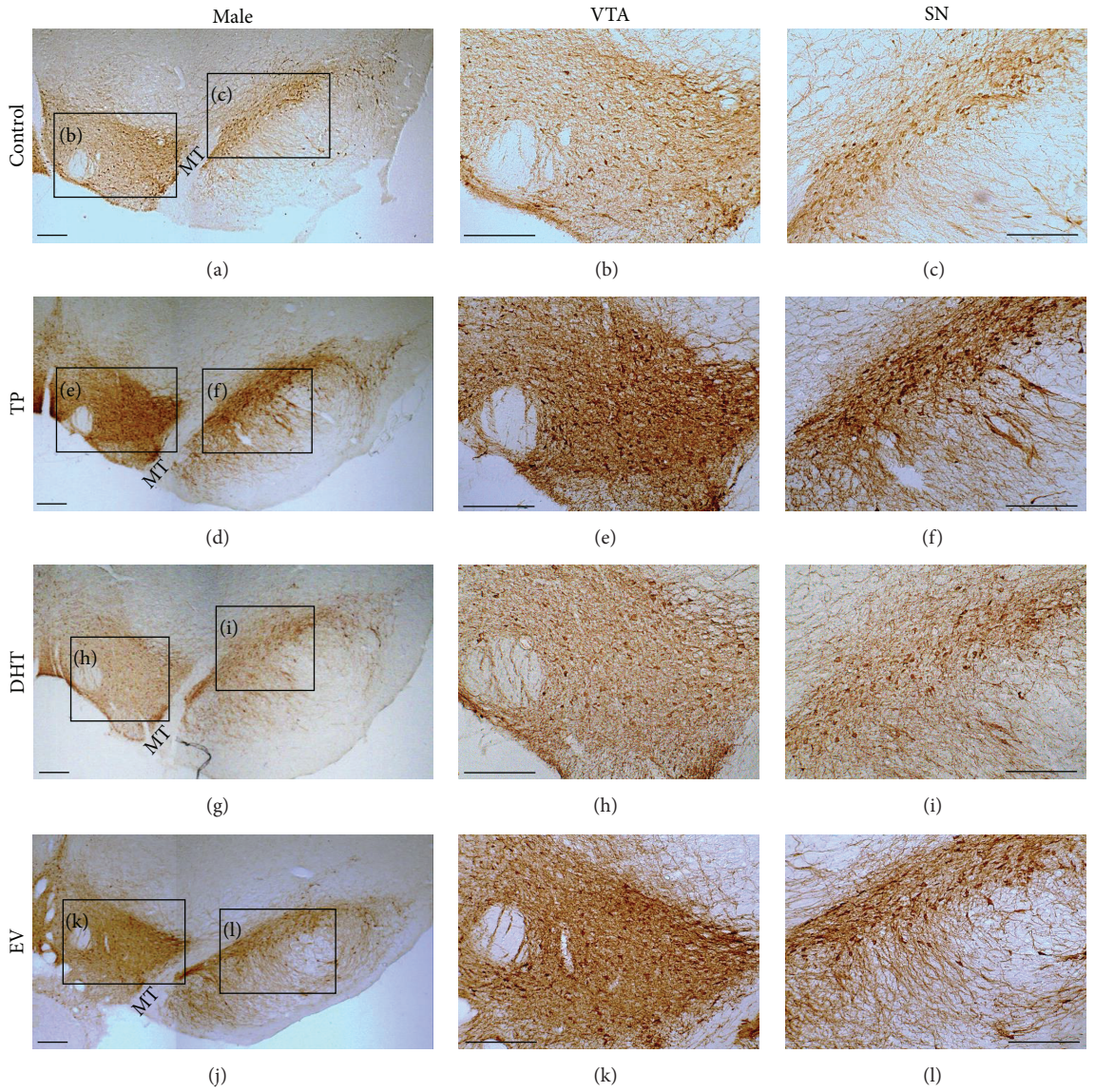

Figure 2: Effect of neonatal exposure to sesame oil (control: a, b, c), testosterone propionate (TP: d, e, f), dihydrotestosterone (DHT: g, h, i), or estradiol valerate (EV: j, k, l) on tyrosine hydroxylase-immunoreactive neurons in ventral tegmental area (VTA: b, e, h, j) and substantia nigra (SN: c, f, i, l) of adult male rats (control $n = 3$, TP = 4, DHT = 2 and EV = 5). Scale bars = 400 μm. MT: medial terminal nucleus of accessory optic tract.

The fact that we did not find similar effects with neonatal exposure to DHT validates our "aromatization hypothesis." Thus, present results show that DHT, a nonaromatizable androgen, did not cause changes in the CNS parameters measured but that there were long-lasting effects observed in peripheral tissues. Although the doses of TP or DHT (1 mg/Kg s.c.) used in our study could be considered high, to the early life stage, similar doses have been previously reported in previous studies by our [39] and other research groups [49, 50], aimed at triggering evident neuroendocrine changes in the brain.

Our proposed "aromatization hypothesis" for neonatal exposure to TP at PND 1 in males rats is based on the high levels of cytochrome P450 aromatase expression and activity in rat midbrain [51], which would promote the conversion of T to E_2. Although it has been shown that E_2, through binding to estrogen receptors (ERs), is able to increase TH expression in midbrain dopaminergic neurons of adult female rodents [13, 27–29], it is unlikely that testosterone and estradiol administered at PND 1 remain in serum until PND 60 and cause a direct effect on dopaminergic neurons in adulthood. Therefore, TP and EV increased estradiol levels in the brain within an early window of development, which indeed could alter the patterns of neurogenesis and apoptosis of neural dopaminergic progenitors through modulating neurotrophic factors (for review see [28]). This probably is the cause for the increased number of TH-positive neurons demonstrated in our study. In addition to this, we also observed an increase

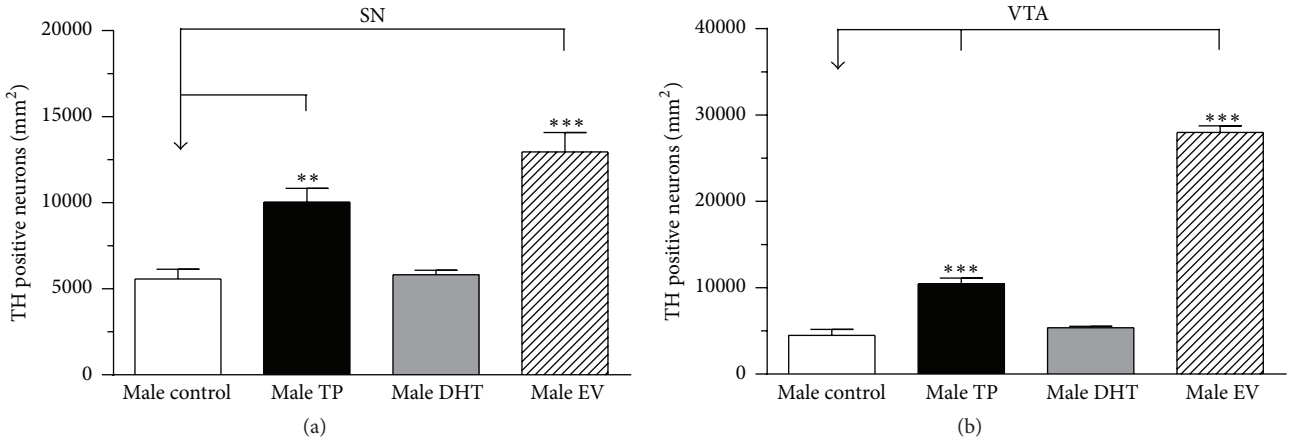

Figure 3: Quantitative analysis of tyrosine hydroxylase- (TH-) positive neurons in SN (a) and VTA (b) of adult male rats (PND 60) Data are expressed as the mean ± SEM (control $n = 3$, TP = 4, DHT = 3, and EV = 5) ($^{***}P < 0.0001$, $^{**}P < 0.01$, and $^{*}P < 0.05$; one-way ANOVA followed by the Newman-Keuls multiple comparison test).

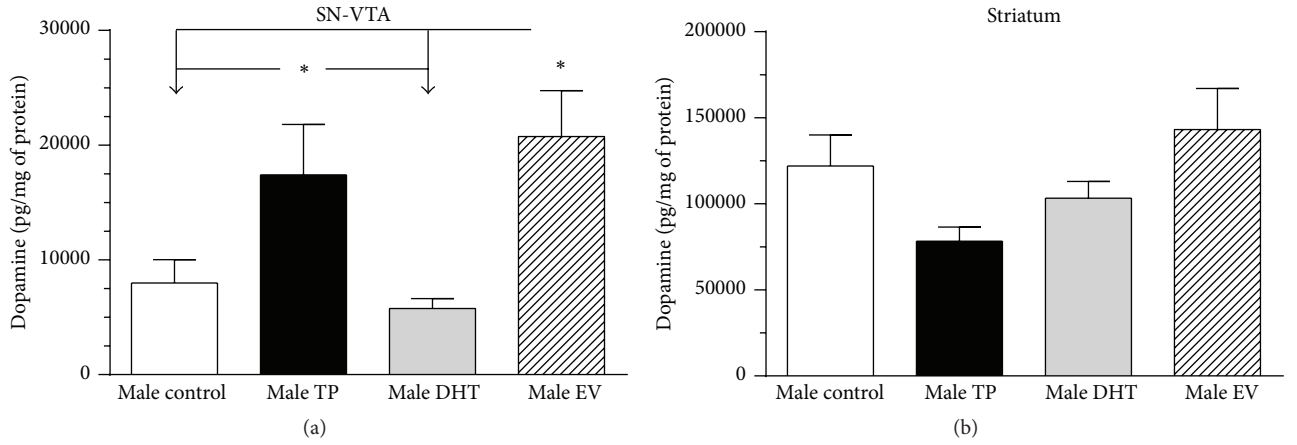

Figure 4: Dopamine content in the substantia nigra-ventral tegmental area (SN-VTA) (a) and striatum (b) of adult male with neonatal exposure to testosterone propionate (TP), dihydrotestosterone (DHT), or estradiol valerate (EV). Results are expressed as pg/mg of protein and represent the mean ± SEM ($^{*}P < 0.05$; one-way ANOVA followed by the Newman-Keuls multiple comparison test; $n = 6$ per male in each condition).

in TH expression in adulthood. This is consistent with the fact that ERα knockout mice have decreased TH protein levels in midbrain dopaminergic neurons [52] compared to wild type mice. The regulation of TH by estrogens occurs mainly by the genomic pathway and involves the binding of the ligand-receptor complex to estrogen response elements in the TH gene [53]. We think that the long-term effect of this early increase in serum estradiol on TH expression could involve long-term epigenetic regulation of gene expression, as observed in multiple genes in different models of estrogenic exposure [54]. However we cannot exclude the possibility that the neonatal administration of TP or EV may cause permanent changes in the cytochrome P450 aromatase expression in the brain or at testicular level such as previously demonstrated by Persky et al. [55].

The increased mRNA and protein expression of TH observed in our work could be responsible for the increased DA content observed in SN-VTA in adult male rats exposed early to EV or TP (Figure 4). In this regard, the magnitude of the increased TH expression in TP rats was lower than in EV rats, possibly due to the partial aromatization of exogenous T to E_2. In this sense, we previously demonstrated that neonatal exposure to EV increases DA content in the ventromedial hypothalamus [39] and SN-VTA in adult female rats [40].

4.2. Long-Lasting Effects of Neonatal Sex Hormones Administration on DA Release in NAcc. Dopamine release induced by a depolarizing stimulus in NAcc of adult male rats exposed to EV and TP during first hours of life was greater than in DHT and control rats. This effect is directly related to the high mRNA and protein expression of TH (Figures 1, 2, and 3) and DA content (Figure 4(a)) in SN-VTA of EV and TP rats. As discussed previously, greater NAcc DA release induced by KRP-K^+ perfusion is produced by neonatal exposure to high levels of E_2 that programs midbrain dopaminergic neurons in adult male rats.

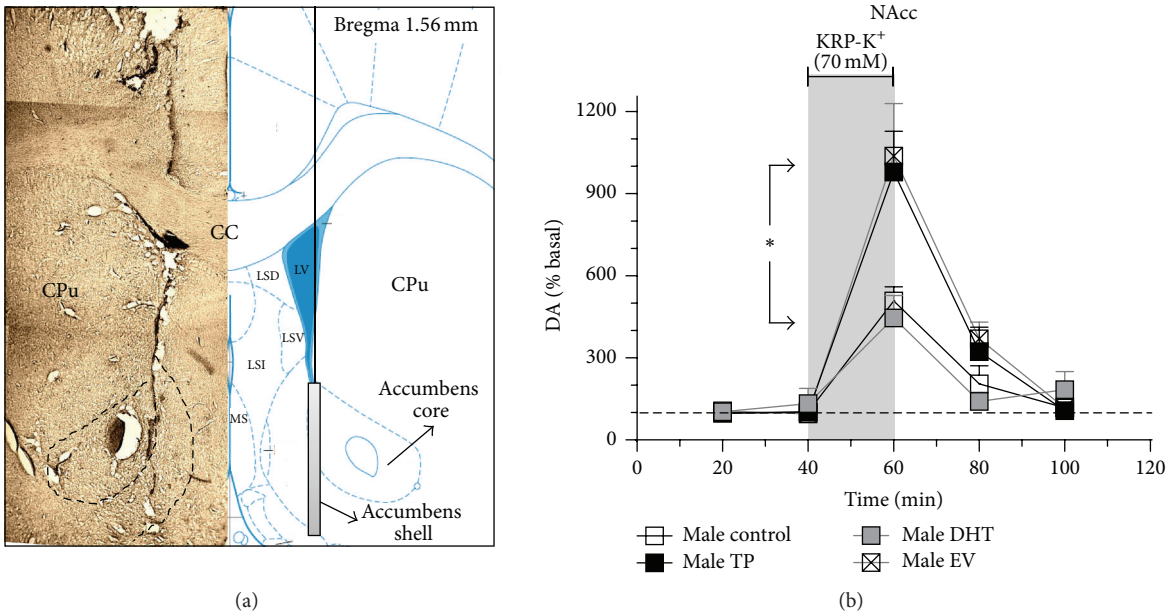

Figure 5: Dopamine release in nucleus accumbens (NAcc) of adult male with neonatal exposure to testosterone propionate (TP), dihydrotestosterone (DHT), or estradiol valerate (EV). Panel (a) (left) shows a typical placement of microdialysis probe in NAcc. Panel (a) (right) shows a scheme of NAcc extracted from the rat brain atlas [41] with an example of the theoretical probe position. Panel (b) shows extracellular dopamine levels in the NAcc and the grey bar indicates the time during which Krebs-Ringer's phosphate (KRP) buffer-K^+ [70 mM] was perfused. Asterisk indicates a significant difference when comparing the effect of KRP-K^+ between the respective experimental groups ($^*P < 0.05$; one-way ANOVA followed by the Newman-Keuls multiple comparison test). Results are expressed as percentage of the respective basal levels (mean) ± SEM ($n = 6$ for male control and $n = 5$ for each treatment group). MS, medial septum; LSI, lateral septum intermediate; LSV, lateral septum ventral; LSD, lateral septum dorsal; cc, corpus callosum; LV, lateral ventricle; CPu, caudate putamen.

Another possibility to explain the effect of early sex hormone administration on increased DA release has been suggested from *in vitro* experiments using superfusion chambers with slices of hypothalamus. Becker and Ramirez showed an increase of amphetamine-induced DA release in hypothalamus slices in castrated adult male rats compared to castrated adult male rats supplemented with TP [30]. This is an interesting finding since it has been shown that castrated adult male rats have higher TH expression levels in SN-VTA than intact male rats [33] or castrated male rats with TP replacement [30]. In this sense, we observed a reduction in T serum levels in adult male rats treated neonatally with TP, EV, or DHT, which could be associated with a reduction in the size of the testes in the adulthood (see Supplementary Figure 1B in Supplementary Material available online at http://dx.doi.org/10.1155/2016/4569785). Possibly the reduction of T serum levels observed in adult males treated neonatally with DHT is due to the high doses of DHT used by us, since, in the work of Persky et al., they used lower doses of DHT and did not observe changes in T serum T levels in the adulthood [55]. This study suggests that early exposure to EV or TP produces an increase in NAcc DA release induced by depolarizing stimulus through an estrogenic effect produced by neonatal exposure to E_2 or by and increased endogenous production of E_2 in adulthood as demonstrated by Persky et al. using the neonatal administration of TP [55].

At the behavioral level, we previously reported that, in EV female rats, a single dose of amphetamine (1 mg/Kg i.p.) did not produce a significant increase in locomotor activity when compared with control female rats [40]. This is interesting, as in the current study we observed a reduction in the ratio of DOPAC to DA in male rats that could reflect a reduction in DA uptake, suggesting a reduction in DAT levels. In this sense, DAT is the main pharmacologic target of amphetamine and a reduction in DAT expression could be related with a reduction in pharmacological effect of amphetamine. In the literature, this hypothesis is supported in a reduction of DAT expression in the NAcc of ovariectomized rats [56] and a reduction of DAT expression in striatum of rats exposed to BPA during prenatal and postnatal stages [57].

Neonatal exposure to sex hormones, particularly during critical periods of development, may induce long-lasting changes in the CNS. In this sense, E_2 administered in the neonatal period shows an important effect on dopaminergic circuitry; as demonstrated in the current work, a single dose of EV or TP increases DA content and TH expression (limiting enzyme of DA synthesis) in mesolimbic areas. These changes also involve an increase in DA release in NAcc. These effects are produced by a positive modulation on TH expression produced by estrogens, suggesting that there may be a role of aromatization of E_2 to T, as a nonaromatizable androgen such as DHT did not produce

the same effect. We strongly think that our "aromatization hypothesis" could be ratified in future studies by using the coadministration of a selective cytochrome P450 aromatase inhibitor such as letrozole or anastrozole along with TP.

Finally, neonatal administration of estradiol, or testosterone through its aromatization to E_2, produced long-lasting effects in midbrain dopaminergic neurons that are associated with persistent alteration of sex hormones. The current results are of importance, as exposure to hormone-like compounds in the environment during critical developmental periods could have long-term effects on brain function. In this sense, different environmental pollutants acting as endocrine-disrupting chemicals could permanently modify neurotransmitter levels in the brain and produce long-lasting changes. Further research in this arena is warranted, provided the involvement of dopaminergic circuits in Parkinson's disease, psychiatric disorders, and addiction.

Conflict of Interests

The authors of this work declare that they have no conflict of interests.

Authors' Contribution

Pedro Espinosa and Roxana A. Silva contributed equally and are first authors of this work.

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Review Article

Synaptic Wnt/GSK3β Signaling Hub in Autism

Mario O. Caracci,[1,2] Miguel E. Ávila,[1,2] and Giancarlo V. De Ferrari[1,2]

[1] *Center for Biomedical Research, Faculty of Biological Sciences and Faculty of Medicine, Universidad Andres Bello, P.O. Box 8370134, Santiago, Chile*
[2] *FONDAP Center for Genome Regulation, Santiago, Chile*

Correspondence should be addressed to Giancarlo V. De Ferrari; gdeferrari@unab.cl

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Hundreds of genes have been associated with autism spectrum disorders (ASDs) and the interaction of weak and *de novo* variants derive from distinct autistic phenotypes thus making up the "spectrum." The convergence of these variants in networks of genes associated with synaptic function warrants the study of cell signaling pathways involved in the regulation of the synapse. The Wnt/β-catenin signaling pathway plays a central role in the development and regulation of the central nervous system and several genes belonging to the cascade have been genetically associated with ASDs. In the present paper, we review basic information regarding the role of Wnt/β-catenin signaling in excitatory/inhibitory balance (E/I balance) through the regulation of pre- and postsynaptic compartments. Furthermore, we integrate information supporting the role of the glycogen synthase kinase 3β (GSK3β) in the onset/development of ASDs through direct modulation of Wnt/β-catenin signaling. Finally, given GSK3β activity as key modulator of synaptic plasticity, we explore the potential of this kinase as a therapeutic target for ASD.

1. Introduction

Autism spectrum disorders (ASDs) are highly heterogeneous, pervasive developmental disorders characterized by impaired social communication skills, repetitive behaviors, and a restricted range of interests [1]. The wide range of phenotypical traits regarding comorbidities and various degrees of cognitive and language impairments makes up the "spectrum" and adds complexity to the determination of genetic markers associated with a distinct phenotype [2]. ASDs have a strong genetic component as ascertained by a 90% concordance among monozygotic twins [3]. Significant advancements have been made in identifying molecular mechanisms involved in ASDs by studying disorders with Mendelian inheritance patterns such as Tuberous Sclerosis complex (*TSC1* and *TSC2*), Rett syndrome (*MECP2*), Fragile X syndrome (FXS; which results from mutated Fragile X mental retardation-1, *FMR1*), and Cowden syndrome (*PTEN*), but, altogether, these disorders do not account for more than 10% of cases [4]. In the last few years, efforts have focused on understanding the genetic contribution of single nucleotide variants (SNVs) and copy number variants (CNVs) in ASD [5, 6]. While genome wide association studies (GWAS) have identified over 100 genes associated with ASDs, most of the variants identified have a weak effect suggesting a greater contribution for rare variants [7]. Rare variants and *de novo* occurring SNVs and CNVs have a larger contribution to the onset of ASD [6]. Indeed, *de novo* CNVs are significantly enriched in individuals affected with the disorder and it is estimated that 8% of cases that carry these variants are likely to be pathogenic [8, 9]. On the other hand, 9% of *de novo* SNVs in affected individuals are disruptive or frameshift mutations that generate nonconserved amino acid changes such as premature stop codons or alternative splice sites ultimately affecting the normal biological function of the resulting protein [10, 11]. Overall, it is estimated that these deleterious *de novo* variants affect ASD susceptibility in 10–15% of probands [10, 11]. Nevertheless, exomic data suggests that no single gene could account for more than 1% of ASD cases, which makes it difficult to target a single protein to treat autistic behaviors. More recently, the integration of these genes into functional networks has allowed the identification of specific molecular pathways that could be disrupted in ASD [12, 13]. In this regard, recent exome sequencing studies

in family trios identified that 39% of the more disruptive *de novo* mutations are part of an interconnected network of chromatin remodeling, synaptic plasticity, and Wnt/β-catenin signaling genes [13–15].

Through the analysis of biochemical and pharmacological data, animal models of the disease, and genetic association studies, we predicted earlier that the onset/development of ASDs might involve the additive effect of genetic variants within Wnt/β-catenin signaling components and/or genes coding for molecules that modulate its functional activity [16], and such hypothesis has received considerable attention recently [6, 17, 18]. Wnts are lipid modified secreted glycoproteins that signal through three major pathways: the Planar Cell Polarity (PCP), Wnt/Ca^{+2}, and the canonical Wnt/β-catenin signaling pathway [19]. Wnt/β-catenin signaling is the most well understood cascade and it starts via binding of the Wnt ligand to cell membrane receptors Frizzled (*FZD*), belonging to the 7-transmembrane domains family of proteins and to members of the low density lipoprotein receptor related proteins 5 and 6 (*LRP5/6*), which act as coreceptors [20]. Wnt binding to its membrane receptor activates intracellular signaling leading to the dissociation of β-catenin from the degradation complex consisting of Axin and adenomatous polyposis coli (APC) scaffolds [21], and the serine-threonine kinases casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β) [22]. As a net result, β-catenin accumulates in the cytosol and translocates to the nucleus where it interacts with T-cell factor/lymphoid enhancing factor (TCF/LEF) transcription factors to activate transcription of target genes [23]. Conversely, in the absence of a Wnt ligand, Axin and APC facilitate CK1 and GSK3β sequential phosphorylation of β-catenin [22] targeting the protein for ubiquitination by the β-transducing repeat-containing protein (β-TrCP) and subsequent proteasome degradation [24].

It is interesting to note that the tumor suppressor complex formed by TSC1 and TSC2 interacts with the β-catenin degradation complex and thus modulates the action of Wnt signaling [25, 26]. Other genetic elements associated with ASDs are the canonical Wnt2 ligand [27], the hepatocyte growth factor receptor (MET) [28, 29], which is a target gene of Wnt/β-catenin signaling [30], and several genes encoding for cadherins, including *CDH5*, *CDH8*, *CDH9*, *CDH10*, *CDH13*, *CDH15*, *PCDH10*, *PCDH19,* and *PCDHb4* [31], some of which may interact with β-catenin in cell-cell adhesion complexes. More recently, the chromo-helicase domain protein 8 (CHD8) [13, 14, 32], which inhibits β-catenin through direct binding [33], and DYRK1A that modulates Wnt signaling through interaction with the p120 catenin [34] have been found to be associated with ASDs. Interestingly, these genes harbor recurrent disruptive mutations and display a high correlation with head size abnormalities [14], which is a feature commonly observed during the first 2-3 years of life of an ASD individual [35]. Finally, rare *de novo* genetic variants in the β-catenin (*CTNNB1*) gene itself have been implicated in severe intellectual disability [36]. Therefore, the convergence of genetic markers in synaptic components opens a therapeutic window that aims not only to correct developmental brain abnormalities, but also to compensate the inherent plasticity through modulation of the highly dynamic synapse. In the present paper, we review current knowledge of synaptic transmission leading to excitatory and inhibitory (E/I) imbalance commonly seen in ASD and how this phenomenon relates to dysfunction of the Wnt/β-catenin pathway. Furthermore, we trace functional defects to GSK3β activity and explore its pharmacological regulation as a potential therapeutic target for ASD, particularly in relation to synaptic plasticity.

2. Wnt/β-Catenin Signaling and Synaptic Transmission Defects in ASDs

The inherent ability of the brain to process information is accomplished by a highly sophisticated network that allows long-distance communication between cells and which is largely based on the E/I balance from neuronal connections. Genetic, functional, and structural information suggests that the E/I balance may underlie the symptomatology of ASDs [37–39]. This idea has been examined through optogenetic methods in the medial prefrontal cortex of mice, and it was found that the elevation, but not the reduction, of cellular E/I balance (i.e., increase in excitatory transmission) induced cellular defects in information processing, leading to behavioral and social deficits [39]. E/I balance anomalies have similarly been observed in several ASD animal models, including the neuroligin 3 (*NLGN3*) mutant mice, and the models for Rett, Fragile X, and Angelman syndromes (Rev. in [40]). In humans, one of the most relevant evidence associating the E/I balance with ASDs is its high comorbidity with epilepsy (30% comorbidity with ASDs) [41, 42]. Epileptic activity can be triggered by blocking synaptic inhibitory transmission or by activating excitatory transmission linking the E/I imbalance in the establishment of epileptiform seizures [43].

Wnt signaling has been widely acknowledged during patterning, development, and maturation of functional synapses within the CNS [16, 44–48]. Wnt1, Wnt3a, Wnt7a, and Wnt8 are ligands known to activate Wnt/β-catenin signaling and are involved in brain development and synaptogenesis [49–51]. Wnt7a and Wnt8a have also been shown to regulate excitatory synaptic formation [45, 52]. Furthermore, a recent study suggests that LRP6, Wnt/β-catenin signaling coreceptor, is critical for the development of functional synapses *in vivo* [52], which further supports the involvement of Wnt/β-catenin signaling in synaptic development. Interestingly tetanic stimulation induces the release of the Wnt3a ligand from the postsynaptic terminal [53]. We demonstrated later that treatment with purified Wnt3a protein of cultured hippocampal neurons enhanced a fast influx of Ca^{2+} in the presynaptic terminal and enhanced mEPSC frequency at the postsynaptic terminal, in an LRP6-dependent mechanism [54]. Hence, the data suggests a prominent role for Wnt/β-catenin signaling in the regulation of excitatory synaptic transmission in pre- and postsynaptic compartments, thus ascribing a role for the signaling cascade in E/I balance regulation (Figure 1).

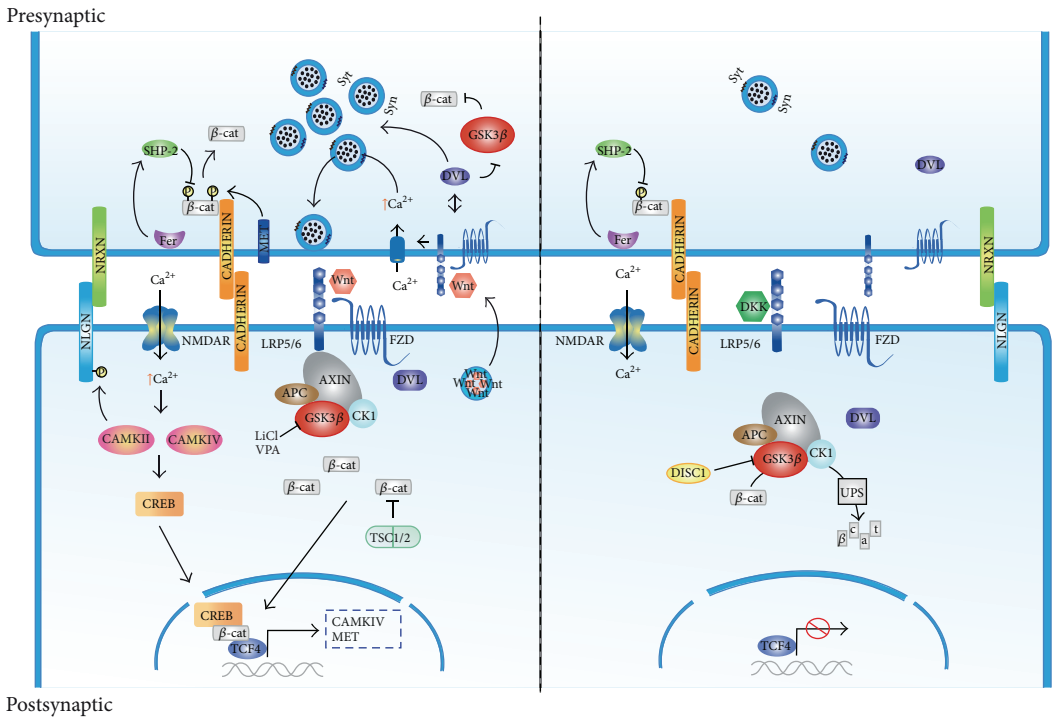

Figure 1: Wnt/β-catenin signaling in ASDs. Wnt binding to FZD-LRP5/6 complex receptor at the membrane recruits the destruction complex and inhibits GSK3β activity thus stabilizing β-catenin in the cytoplasm and nucleus. Activation of the Wnt/β-catenin pathway facilitates synaptic plasticity through the activation of voltage gated ion channels that allows activation of CAMK and CREB mediated transcription. Mutations in TSC associated with ASD prevent β-catenin degradation which results in a gain of function of the Wnt pathway. In the presynaptic terminal cadherin mediated cell adhesion between synapses is weakened by phosphorylation of β-catenin and synaptic vesicle clustering is enhanced through DVL1. Clustering is also dependent on NLGN/NRXN cell adhesion complexes. Both lithium (LiCl) and VPA activate Wnt/β-catenin signaling through inhibition of GSK3β activity. Conversely, in the absence of a Wnt ligand, activated GSK3β targets β-catenin for proteosome-mediated degradation. Mutations associated with DISC1 fail to inhibit GSK3β and thus activate Wnt/β-catenin pathway. In the presynaptic side Wnt signaling buffering of synaptic vesicles is inhibited and adherens junctions mediated by cadherins are strengthened.

3. ASDs and Wnt Signaling at the Presynaptic Terminal

At the presynaptic region, canonical Wnt signaling has a major role in clustering and recycling of synaptic vesicles (SVs). Conditioned media containing Wnt7a, and to a lesser extent Wnt3a, were found to enhance SVs recycling in primary cultures of rat hippocampal neurons [55]. Similarly, loss of Wnt7a function inhibits SVs clustering, an effect that is mimicked by loss of function of Dishevelled 1 (*DVL1*) signaling downstream of Wnt ligands [47]. Interestingly, Dvl1 knockout mice exhibit social interaction and sensorimotor abnormalities [56]. Moreover, the Wnt7a/Dvl1 double mutant mice show defects in spine morphogenesis and excitatory synaptic neurotransmission [45], which parallels behavioral abnormalities with a disrupted presynaptic assembly and E/I balance, as it is likely observed in ASDs.

Wnt/β-catenin signaling also seems to trigger neurotransmitter release and SV trafficking by modulating the function of SVs-associated phosphoproteins, including membrane-trafficking proteins such as synapsin and synaptotagmin. While all three members of the synapsin (SYN) gene family (*SYN1-3*) [57] have been associated with ASDs [58–60], it has been shown that canonical Wnt ligands such as Wnt7a and Wnt3a enhance the clustering [61] and phosphorylation [54] of Syn1 at the synaptic button prior to neurotransmitter release. Likewise, *SYN2* is predicted as a Wnt/β-catenin target gene [62] and is upregulated as a consequence of enhanced Wnt signaling activity in hippocampal neurons from APC conditional knockout mice that has impaired learning and memory and that displays ASD-like behaviors [63]. Finally, it was shown that the Wnt signaling component Dvl1 is involved in neurotransmitters release at the tip of neurites of differentiated neurons through direct binding to the presynaptic protein synaptotagmin I [64].

Other mechanisms modulating the activity of the presynaptic terminal involve the function of cell adhesion proteins, most notably trans-synaptic cadherin interactions. It is widely accepted that cadherin-β-catenin adhesion complexes have an essential function during the recruitment and clustering of SVs to synapses [65–69]. Indeed, ablation of β-catenin results in the mislocalization of SVs along the axon, while clustering of active zone proteins like Bassoon is unchanged [68]. Tyrosine 654 phosphorylation of β-catenin weakens cadherin-catenin interactions [70]. Interestingly, the tyrosine kinase FER, which is an ASDs' candidate gene [71],

activates the tyrosine phosphatase SHP-2 which removes β-catenin phosphorylation and strengthens cadherin mediated adhesion [72]. Among other proteins modulating β-catenin dissociation from cell adhesion complexes that have been genetically linked with ASD is the MET receptor tyrosine kinase [30], which phosphorylates Tyr142 in β-catenin and promotes its dissociation from cadherins [73], thus linking regulation of cell adhesion by catenins in the pathophysiology of ASDs. In sum, the data available indicates an essential role for Wnt/β-catenin signaling in synaptic structure stability and function through modulating cell adhesion, vesicle exocytosis, and clustering well beyond β-catenin functioning solely as a TCF/LEF transcriptional coactivator.

4. ASDs and Wnt Signaling at the Postsynaptic Terminal

Experience driven plasticity is highly dependent on proper synaptic transmission and is mainly modulated by Ca^{2+} related pathways. Canonical and noncanonical Wnt pathways have been extensively related to Ca^{2+} homeostasis and signaling [47, 54, 74, 75]. Ligands such as Wnt3a [54], Wnt5a [75], and Wnt7a [47] have all been shown to increase Ca^{2+} influxes in neurons. It is accepted that activation of L-type voltage sensitive Ca^{2+} channels (L-VSCCs) or NMDA receptors allows the entrance of Ca^{2+} which in turn activate CAMKII triggering actin cytoskeleton reorganization to regulate dendritic growth [76]. In this regard, CAMKII and the Wnt target gene CAMKIV [77] activate transcription factors such as CREB to start activity dependent transcription to further promote synaptic development [78]. CAMKIV has been associated with ASD [79] and additionally it mediates β-catenin dependent dendritic growth upon Ca^{2+} influx [78, 80].

Activation of CAMKII and other kinases through NMDAR-mediated Ca^{2+} influx is an event preceding the establishment of long-term synaptic potentiation (LTP) that allows the recruitment of AMPARs at the postsynaptic terminal, which in turn enhances long lasting excitatory transmission [81]. Additionally, CAMKII robustly phosphorylates the cell adhesion neuroligin 1 (*NLGN1*) protein increasing its surface expression [82]. Notably, suppression of Wnt/β-catenin signaling impairs LTP and conversely its activation facilitates it [53], and both enhanced and diminished LTP have been observed in animal models of ASD. For instance, given that enhanced LTP has been observed in TSC2 mutant model [83] and that *TSC2* missense mutations fail to inhibit the Wnt pathway [26], it is likely that overactivation of the signaling cascade may enhance LTP in this specific model. In contrast, mutant models for Fragile X mental retardation-1 (*FMR1*), and also for the disrupted in schizophrenia 1 (*DISC1*) genes, exhibit diminished capacity to establish LTP [84, 85]. Besides their putative role in schizophrenia, ASDs and other neurological diseases [86–88], common *DISC1* genetic variants, directly impact Wnt/β-catenin signaling function (see below) [89]. Altogether, the data suggest that the Wnt/β-catenin pathway plays a central role in Ca^{2+} homeostasis at postsynaptic terminals, which is commonly disrupted in ASD. In addition, abnormal establishment of LTP, phenomenon in which the signaling cascade plays an important role, has profound effects in activity driven plasticity affecting efficient synaptic transmission and disrupting the E/I balance.

LTP is the most well understood paradigm of activity driven plasticity and is considered to be one of the synaptic mechanisms underlying learning and memory [81]. In turn, several aspects of the ASD core symptomatology and the high comorbidity with intellectual disability disorder could be explained by defective memory mechanisms [90]. Indeed, diminished episodic memory has been reported for high functioning ASD individuals and is thought to impair the relational binding of elements comprising complex stimuli [91]. Therefore, rescuing defects in LTP that appears to be highly regulated by the Wnt/β-catenin pathway specifically through the modulation of GSK3β could improve core ASD symptomatology and open a therapeutic window for the treatment of ASD through the fine-tuning of synaptic plasticity.

5. Synaptic Wnt/GSK3β Signaling Hub in ASD

GSK3 is an evolutionary conserved serine/threonine kinase highly abundant in the brain. Two homologous isoforms, GSK3α and GSK3β, have been described in mammals and are involved in multiple cellular processes including glycogen metabolism, gene transcription, microtubule stability, and apoptosis [92]. GSK3β is as a convergence point of major prevalent neurological disorders, including Alzheimer's disease, schizophrenia, and bipolar disorder [93–95], and its activity is negatively regulated by Wnt signaling. As mentioned before, the *DISC1* gene has an essential role in modulating brain structure and function and when mutated leads to neuropsychiatric behavior. DISC1 inhibits GSK3β activity by direct physical interaction resulting in reduced β-catenin phosphorylation and activation of Wnt/β-catenin signaling cascade [96] and common genetic variants affecting the coding sequence of the gene were found to suppress Wnt/β-catenin signaling activity [89]. Regarding ASDs, hyperactivation of GSK3β has been documented in animal models of FXS [97–99]. For instance, knock in mice expressing constitutively active form of GSK3β displays similar social preference abnormalities as FMR1 KO mice [99].

Mouse models for Fragile X, Phellan-McDermid, and Angelman syndromes, as well as for Tuberous Sclerosis, all present an abnormal number of dendritic spines that suggest a dysregulation in synaptic turnover [100–102]. In this regard, postnatal ablation of GSK3β in mice forebrain has anxiolytic and prosocial effects [103] and its overexpression accounts for spatial learning deficits in the Morris water maze paradigm [104]. Interestingly, forebrain deletion of GSK3β leads to reduced spine density where persistent spines are lost and newly formed spines are unstable [105]. These structural abnormalities are accompanied by a drop in AMPA dependent mEPSC and the effect is mimicked by the expression of constitutively active β-catenin [105]. Furthermore, pharmacological inhibition of GSK3β has been shown to increase internalization of NMDA and AMPA receptors, effect that is mainly observed for NR2B containing receptors [106].

Conversely, activation of GSK3β impairs the establishment of LTP [107] and high frequency stimulation inhibits GSK3β in a Ca^{2+} dependent mechanism [108]. Given that increased abnormal spine density is a pathological hallmark in ASD that may lead to brain hyperconnectivity underlying the basis for E/I balance, the data suggests that inhibition of the Wnt/β-catenin signaling through hyperactivation of GSK3β might help to explain transmission anomalies as it is observed in ASD.

6. Pharmacological Regulation of GSK3β in ASD

Due to its high heterogeneity, genetic factors cannot be held accountable for the entire spectrum of autism suggesting a role for environmental factors in the onset of ASD. *In utero* exposure to anticonvulsive medication is known to cause neurodevelopmental abnormalities [109]. The most well studied anticonvulsive agent in these subjects is valproic acid (valproate, VPA), a known inhibitor of GSK3β [110] and of histone deacetylase (HDAC) [111] activities. As an inhibitor of GSK3β, VPA induces the stabilization of β-catenin and the activation of Wnt target genes, though the exact mechanism of GSK3β is not currently understood. Indeed, *in utero* exposure to VPA increases the incidence of autism in the offspring [112, 113] and mice models, which have been prenatally exposed to VPA exhibiting ASD-like behaviors and morphological brain abnormalities [112, 114]. Currently, mice prenatally exposed to VPA (VPA mice) are widely used as animal models to understand the onset/development of ASDs [115]. This VPA mouse model results from intraperitoneal injection in embryonic stages E12–E17, which is a critical period in forebrain development, where dysregulation of Wnt signaling (different time points) induces morphological abnormalities in the brain [116].

While several molecular mechanisms regarding the onset of ASDs in VPA mice have been reported, the activation of Wnt/β-catenin signaling is central through the regulation of GSK3β. VPA mice exhibit elevated NMDA receptor levels and enhanced LTP [117] and inhibition of GABA transporter VGAT expression in cortical cultures [118], suggesting an important enhancement in excitatory neurotransmission. Likewise, VPA mice induce demethylation of *WNT1* and *WNT2* genes further enhancing Wnt/β-catenin signaling [119]. In this regard, sulindac treatment, an anti-inflammatory drug that downregulates Wnt/β-catenin signaling by enhancing GSK3β expression in the prefrontal cortex or the hippocampal region of VPA mice [120], improved repetitive stereotypic activity, learning and memory, as well as behavioral abnormalities [120, 121]. Interestingly, the VPA transcriptome revealed enhanced expression of multiple genes involved in Wnt/β-catenin, neurotrophin, and LTP signaling, the same pathways which also appear enriched in the transcriptome of lithium [122], which mimics Wnt/β-catenin signaling by inhibiting GSK3β [123]. Nonetheless, although prenatal treatment with VPA appears to enhance the expression of Wnt/β-catenin signaling, most of the data comes from *in vitro* cell cultures exposed to VPA and not from *in vivo* studies using mice prenatally exposed to the drug. In this context, it is interesting to note that chronic VPA treatment in mice has been shown to correct dendritic spine deficits and to improve novel object recognition [124]; thus, the postnatal basal activity of the Wnt/β-catenin pathway is still unknown. In this context, it is interesting to note that ASD could result from a transient gain of function of the Wnt/β-catenin pathway during embryonic development and a subsequent decline after birth.

Lithium has been widely used to manage mood disorders, such as bipolar disorders, and it is not uncommon for ASD children to feature symptoms within this spectrum such as euphoria, mania, or paranoia [125]. Few studies have documented the effects of lithium in ASDs but overall they show promising results as a therapeutic agent. For instance, lithium administration to 30 children and adolescents diagnosed with ASD through DSM-IV-TR criteria improved the symptomatology on 43% of patients [125]. Likewise, chronic administration of lithium to neonatal rats who exhibit ASD-like behaviors abolished their symptoms and improved defects in neurogenesis and E/I balance [126]. Additionally, chronic lithium treatment reversed the increase in cerebral protein synthesis and ameliorates behavioral abnormalities commonly observed in FXS mice models [127], probably through inhibitory GSK3β phosphorylation (phosphor-Ser9 and phosphor-Ser21) [128]. Interestingly, pharmacological inhibition of GSK3β rescues LTP and hippocampal neurogenesis defects in FMR1 knockout mice and improves cognitive tasks [97, 103]. Furthermore, GSK3β inhibition similarly rescues dendritic spines deficit observed in FXS mice suggesting that inhibition of this kinase and thus activation the Wnt/β-catenin play a role in reactivating synaptic plasticity and these effects might play an important role in the behavioral and learning improvements observed.

Antagonists for metabotropic glutamate receptor (mGluRs) are up to date the most successful pharmacological modulators improving ASD symptomatology probably through regulation of abnormal mRNA translation at synapses [129]. In this context, the use of MPEP (2-methyl-6-phenylethynyl-pyridine), mGluR5 antagonists, increases inhibitory GSK3β phosphorylation selectively in *FMR1* knockout mice [130], effect that is mimicked by chronic lithium treatment. Moreover, this compound corrects dendritic spine deficits through upregulation of PSD-95 and learning impairment in FXS mice model [131], further ascribing a regulatory function directly at the synapses as the underlying mechanism for the therapeutic effect. Finally, MPEP treatment induces the expression of several pathways, including those governed by Wnt signaling in the frontal cortex of rats [132].

7. Concluding Remarks

ASD displays a high genetic heterogeneity that results in a wide range of abnormal phenotypes and settling a unified paradigm that accounts for the gain or loss of function of genetically associated genes has been an elusive task. Currently, most elements associated with ASDs converge in signaling pathways important for synaptic plasticity, where Wnt/β-catenin signaling plays a central role. As described in this review, several lines of evidence indicate that Wnt

signaling regulation of serine/threonine kinase GSK3β has profound effects in activity dependent synaptic plasticity and thus in the regulation of the E/I balance. Through dissecting Wnt/GSK3β activity and pharmacology in cells and animal models of ASDs, it seems plausible that there may be differential effects driven by Wnt/β-catenin signaling activity during the initial patterning of brain structures and later on when these structures have been established. Overall, the therapeutic value of GSK3β modulation that seems to rescue synaptic plasticity events that could be disrupted in ASD brains warrants further basic and clinical investigation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Mario O. Caracci and Miguel E. Ávila contributed equally to this work.

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Research Article

Amyloid-Beta Induced Changes in Vesicular Transport of BDNF in Hippocampal Neurons

Bianca Seifert,[1] Robert Eckenstaler,[1] Raik Rönicke,[2,3] Julia Leschik,[4] Beat Lutz,[4] Klaus Reymann,[3] Volkmar Lessmann,[1,5] and Tanja Brigadski[1,5]

[1]*Institute of Physiology, Medical Faculty, Otto-von-Guericke-University, 39120 Magdeburg, Germany*
[2]*Institute of Clinical Chemistry and Pathobiochemistry, Medical Faculty, Otto-von-Guericke-University, 39120 Magdeburg, Germany*
[3]*German Center for Neurodegenerative Diseases (DZNE), 39120 Magdeburg, Germany*
[4]*Institute of Physiological Chemistry, University Medical Center of the Johannes Gutenberg University, 55128 Mainz, Germany*
[5]*Center of Behavioral Brain Sciences (CBBS), 39106 Magdeburg, Germany*

Correspondence should be addressed to Tanja Brigadski; tanja.brigadski@med.ovgu.de

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The neurotrophin brain derived neurotrophic factor (BDNF) is an important growth factor in the CNS. Deficits in transport of this secretory protein could underlie neurodegenerative diseases. Investigation of disease-related changes in BDNF transport might provide insights into the cellular mechanism underlying, for example, Alzheimer's disease (AD). To analyze the role of BDNF transport in AD, live cell imaging of fluorescently labeled BDNF was performed in hippocampal neurons of different AD model systems. BDNF and APP colocalized with low incidence in vesicular structures. Anterograde as well as retrograde transport of BDNF vesicles was reduced and these effects were mediated by factors released from hippocampal neurons into the extracellular medium. Transport of BDNF was altered at a very early time point after onset of human APP expression or after acute amyloid-beta(1-42) treatment, while the activity-dependent release of BDNF remained unaffected. Taken together, extracellular cleavage products of APP induced rapid changes in anterograde and retrograde transport of BDNF-containing vesicles while release of BDNF was unaffected by transgenic expression of mutated APP. These early transport deficits might lead to permanently impaired brain functions in the adult brain.

1. Introduction

The neurotrophin BDNF is an important growth factor supporting the function of the developing and the adult central nervous system. Depending on cell type and expression pattern BDNF coordinates a multitude of biological functions, like growth of neurites, synaptogenesis, and neuroprotection [1–5]. A lack of BDNF has long-range consequences on cellular as well as on systemic level and therefore could underlie different neurodegenerative diseases [6–8]. Deficits in BDNF transport and thus a lack of BDNF support in the respective target area could initiate neurodegenerative processes in the brain [8–12]. Although many previous studies addressed the neuroprotective signalling of neurotrophins, the regulation of neurotrophic support by the different transport processes of BDNF-containing vesicles is not well understood.

So far, the transport of BDNF was investigated by immunohistochemical staining, radioimmunoassay, live cell imaging of fluorescently labeled BDNF, or quantum-dot labelled BDNF [13–15]. The injection of radioactively labeled recombinant BDNF in different brain regions uncovered an extensive anterograde and retrograde transport of BDNF in the central nervous system [13, 16–18]. Live cell imaging of neuronal cultures expressing fluorescently labeled BDNF revealed a velocity of BDNF-containing vesicles of 0.1–1.4 μm/s [14, 19–21]. In both directions, the transport of BDNF-containing vesicles is dependent on the dynactin-motor complex [14, 19, 20]. In transgenic mouse models of Alzheimer's disease, an impaired retrograde transport of endocytosed BDNF was described by Poon and colleagues. They observed a deficit in retrograde transport of endocytosed BDNF/TrkB-signalling endosomes which was

associated with amyloid-beta induced downregulation of ubiquitin C-terminal hydrolase [22, 23]. A similar influence of amyloid precursor protein (APP) or cleavage products of APP on activity-dependent secretion of BDNF has not yet been described.

In this study, we demonstrate a fast action (within 5 min) of amyloid-beta(1-42) on the transport of BDNF-containing vesicles. BDNF and APP colocalized with low incidence in vesicular structures and the degree of colocalization increased with higher APP expression level in a cell. However, deficits in anterograde as well as retrograde BDNF transport were not correlated with APP expression at the single cell level but were rather induced by soluble factors released from hippocampal neurons into the extracellular medium. Furthermore, short application of amyloid-beta(1-42) but not amyloid-beta(3(pE)-42) induced a reduction in average speed of retrogradely directed vesicles. Anterogradely directed vesicles were affected by transgenic expression of APP as well as by amyloid-beta treatment at later time points of culturing. Nevertheless, activity-dependent release of BDNF was not affected by any of these manipulations affecting the transport of BDNF vesicles.

2. Materials and Methods

2.1. Cell Culture. All experiments were performed in accordance with the ethical guidelines for the use of animals in experiments and were approved by the local animal care committee (Landesverwaltungsamt Sachsen-Anhalt).

Hippocampal neurons of C57BL/6 mice were isolated at postnatal day 2 and prepared as described previously [4, 24] with minor modifications: primary postnatal (P2) neocortical astrocytes were isolated and cultured for 2-3 weeks in BME medium, containing 10% FCS until being expanded to confluence. Astrocytes were passaged and seeded on glass coverslips at a density of 80 000 cells per 3.5 cm dish in BME/10% FCS to yield astrocyte islands of 100–300 μm in diameter after 7–14 days *in vitro* (DIV). β-D-Arabinofuranosylcytosine (3 μM) was added 4 d after seeding of astrocytes to avoid further growth of astrocyte islands. Dissociated postnatal (P2) hippocampal neurons were plated in BME/10% FCS at a density of 1–10 neurons per astrocyte island onto the coverslips. After 20 h, the plating medium was exchanged to serum-free medium (Neurobasal with 2% B27 supplement; Invitrogen, San Diego, CA).

Hippocampal neurons from 5xFAD mice were isolated at postnatal day 2 and plated at a density of 70 000 cells per cm^2 on a polyornithine-coated cover slip. After 20 h, the plating medium was exchanged to serum-free medium (Neurobasal with 2% B27 supplement; Invitrogen, San Diego, CA).

2.2. Transfection. Hippocampal cultures were transfected with expression vectors coding for BDNF-mCherry, HA tagged proBDNF-GFP, APP-YFP, APPmCherry, or GFP, respectively [25, 26], using the Ca^{2+}-phosphate precipitation method described previously [25] with minor modifications: hippocampal cultures of C57BL/6 mice were transfected at 9 DIV and hippocampal cultures of transgenic 5xFAD mice were transfected at DIV 10 with the respective expression plasmids. Cells were used for experiments 1–3 days after transfection.

2.3. Live-Cell Imaging and Image Analysis. Coverslips were transferred into Petriperm dishes (Greiner Bio-One, Frickenhausen, Germany) containing HEPES buffered saline (20 mM HEPES, 4 mM KCl, 100 mM NaCl, 1 mM Na_2HPO_4, 4 mM $CaCl_2$, 10 mM glucose, 10 μM glycin, and 1 mM $MgCl_2$; pH 7.4) and imaged with an inverted epifluorescence microscope (IX70, Olympus, Hamburg Germany) using high aperture oil immersion objectives (40x, n.a.: 1.0) and a CCD camera (Sensys 1401E, Photometrics) as described previously [27] with minor modifications: cells were kept at a constant temperature of 30°C using a plate warmer (Minitüb, Tiefenback, Germany). Time-lapse images were acquired at a frequency of 2 Hz with an exposure time of 300 ms. 1-minute time-lapse recordings were analyzed with MetaMorph software (Molecular Devices, Downington, PA, USA). Dynamics of BDNF-containing vesicles were analyzed in individual thin neurites by manual tracking using the "track points" function of MetaMorph. Vesicles were tracked for 1 min or until they disappeared out of view. BDNF-containing vesicles were considered as immobile when the percentage of motion during observation time was lower than 15% or the minimal distance of movement during observation time was below 8 μm. Vesicles which showed diffusing behavior estimated by the ratio of displacement and distance (>4) were excluded. Otherwise the vesicles were defined as mobile.

Colocalization of proteins was examined with a confocal laser scanning microscope (LSM 780, Zeiss, Jena, Germany) using a 20x water immersion objective (W Plan-ACHROMAT, numerical aperture: 1.0). Images were acquired and analyzed with the ZEN 2010 software. Weighted colocalization coefficient was calculated by the Zen software.

2.4. Immunohistochemistry. Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 20 min and permeabilized with 0.3% Triton X-100 in PBS. Unspecific binding was inhibited by incubation with blocking solution (1x PBS, 10% BSA and 0.1% Triton X-100) for 15 min. Incubation with primary and secondary antibodies was done for 2 h in PBS containing 1% BSA and 0,1% Triton X-100. Cells were fixed on a glass slide with Immumount (Thermo Scientific, Waltham, MA, USA) and stored at 4°C until analysis. All steps were performed at room temperature. Primary antibodies used were anti-APP (rabbit, 1 : 1000, Upstate, Millipore Corporation, USA) and anti-HA (mouse, 1 : 1000, Covance, New Jersey, USA). Secondary antibodies used were Cy3 coupled anti-rabbit IgG (1 : 1000, Dianova, Hamburg, Germany) and Alexa Fluor 350 coupled anti-mouse IgG (1 : 1000, Moleculare Probes, Life Technologies, Carlsbad, California, USA). Colocalization analysis was performed by MetaMorph software (Molecular Devices, Downingtown, PA).

2.5. Preparation of Amyloid-Beta Oligomers. Amyloid-beta oligomers were generated as described in [28]. Briefly, the lyophilized peptides (amyloid-beta(1-42), MoBiTec) were

dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma, St. Louis, MO) to a concentration of 1 mM. The solution was aliquoted, HFIP was evaporated, and the peptide film was stored at −80°C. Twenty-four hours before use, amyloid-beta peptides were dissolved under sterile conditions in dimethylsulfoxide (DMSO; Sigma; 100 μM) and sonicated. Amyloid-beta oligomers were obtained by diluting the stock solution to 5 μM in NB/B27 and incubating at 4°C for 24 h. In previous experiments, the quality of the oligomer preparation was controlled by negative stain electron microscopy and with sodiumdodecylsulfate-polyacrylamidgelelectrophoresis (SDS-PAGE) [29]. Early neuronal dysfunction by amyloid-β oligomers depends on activation of NR2B-containing NMDA receptors. We already investigated this oligomer preparation for detrimental effects on neuronal function and neuronal structural integration. Using primary neuronal cell culture and hippocampal slices from rat and mouse, we found that administration of submicromolar concentrations of Aβ oligomers readily impairs long-term potentiation, reduces baseline synaptic transmission, decreases neuronal spontaneous network activity, and induces retraction of synaptic contacts [29].

Short-term treatment of hippocampal neurons with 500 nM amyloid-beta peptides (dissolved in HBS) was performed in the recording chamber for 5–30 min. Long-term incubation of hippocampal neurons with 500 nM amyloid-beta peptides was performed in NB/B27 culture medium for 24 h in the cell culture incubator.

2.6. Release Measurements. Primary hippocampal neurons were transfected with BDNF-GFP expressing plasmid at 10 DIV and imaged essentially as described previously [24]: three days after transfection, cover slips with hippocampal neurons were transferred into a bath chamber (Luigs & Neumann) and inspected with an upright fluorescence microscope (Olympus BX51W) using a 60x water immersion objective (LUMFI, Olympus, Hamburg, Germany, NA: 1.1). Image capture was performed using a cooled CCD camera (CoolSnap HQ^2, Photometrics, Huntington Beach, CA, 14 bit dynamic range), controlled by VisiView software (Visitron Systems, Puchheim, Germany). The exposure times for time-lapse recordings (between 0.3 and 1.5 sec) were adjusted for each cell such that vesicles were clearly distinguishable from the background without driving the CCD chip into saturation. BDNF-GFP release was stimulated by applying 50 mM KCl containing HEPES buffer with a local perfusion system. To estimate the incidence of vesicle fusion, 0.3 mM bromphenol blue (BPB; compare [30]) containing HEPES buffer was superfused after the release experiment. Analysis of images was performed by MetaMorph software (Molecular Devices, Downingtown, PA). In brief, single vesicles were selected to measure the average fluorescence kinetics of single cells. Background fluorescence intensities were subtracted for vesicle and the average intensity was normalized to the time point before fusion event. A monoexponential extrapolation of the photo bleaching observed during baseline recordings was applied to correct the normalized fluorescence data [30]. For analysis of release amplitude maximum fluorescence intensity of each single secretory granule after fusion event (time point = 0) was set to 100% and relative fluorescence decrease 300 s after fusion was analyzed. The percentage of fusion events was calculated by the fraction of vesicles disappearing during BPB application after release experiment. This fraction of vesicles could be also detected by the change in fluorescence intensity after fusion event.

2.7. Statistics. Statistical analysis was performed using SPSS version 21 software (IBM Corp., Armonk, NY, USA) using either one-way or two-way ANOVA following *post hoc* Tukey's or pairwise multiple comparisons as indicated in the text and figure captions. The level of significance was set at $p < 0.05$.

3. Results

3.1. Motional Properties of BDNF-Containing Vesicles In Transgenic Mouse Models of Alzheimer's Disease. To analyze the role of BDNF transport in Alzheimer's disease, live cell imaging of fluorescently labeled proteins was performed in dissociated hippocampal neurons from an Alzheimer's disease mouse model (5xFAD) [31–33]. This transgenic mouse line was engineered to overexpress Aβ(1-42) [32, 34] by coexpressing amyloid precursor protein (APP) with three familial Alzheimer's disease (FAD) mutations and presenilin 1 (PS1) with two FAD mutations which additionally increases Aβ(1-42) production. Both transgenic proteins are under the control of the Thy1 promoter which drives early postnatal neuron-specific transgenic expression [35, 36]. Dissociated hippocampal cultures derived from these 5xFAD animals and their wild type littermates showed similar cell density and cell survival after 12 days *in vitro* (DIV) (Supplementary Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/4145708). These cultures were transfected with BDNF-mCherry at 10 DIV (Figure 1(a)) and transport dynamics of single BDNF-containing vesicles in thin processes with axonal morphology were analyzed by time-lapse video microscopy. During the observation time BDNF-containing vesicles either showed stop-and-go movements or were immobile (Figure 1(b)). Analyzing the motility of BDNF-containing vesicles, as quantified by the percentage of motions during observation, we observed a significant reduction in vesicular motility in 5xFAD derived hippocampal neurons compared to wild-type littermates. While the mean motility of BDNF-containing vesicles was 59.7 ± 1.3% under control conditions, the mean motility of BDNF-containing vesicles was significantly reduced to 49.1 ± 1.3% in 5xFAD transgenic hippocampal neurons ($p < 1 * 10^{-7}$; Figure 1(c)). A similar result was obtained by analyzing the fraction of mobile and immobile vesicles. While 76.0 ± 2.0% of BDNF-containing vesicles displayed a directed movement in wild-type neurons, only 65.8 ± 2.4% of BDNF-containing vesicles were actively transported in 5xFAD transgenic neurons. Accordingly, a larger proportion of vesicles was immobile in these transgenic neurons showing no active transport at all (WT: 24.0 ± 2.0%; 5xFAD: 34.2 ± 2.4% $p < 0.0013$; Figure 1(d)). Altogether, these results indicate a transition of BDNF-containing vesicles from mobile to an immobile state in 5xFAD mice derived neurons.

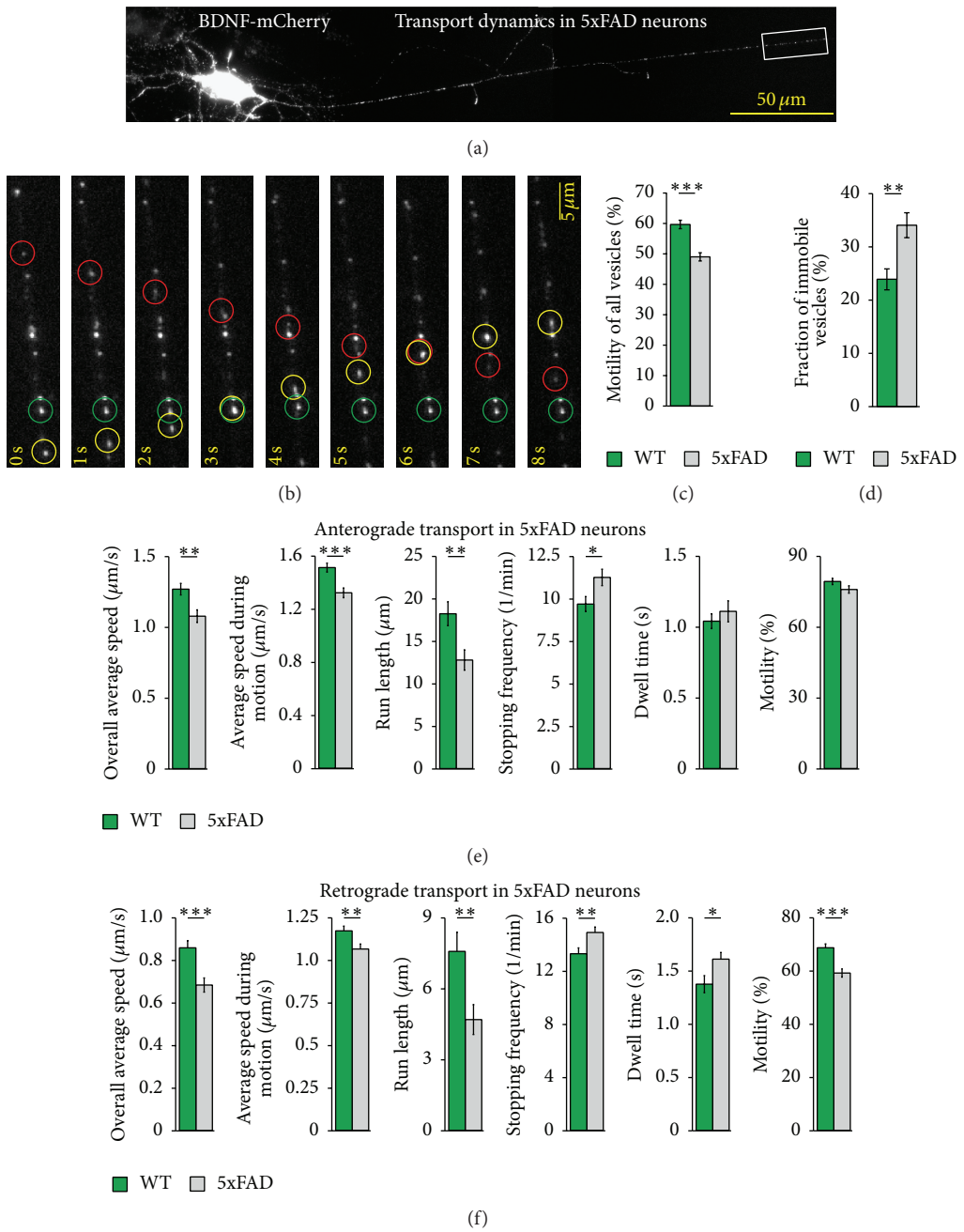


Figure 1: Transport dynamics of BDNF-containing vesicles in hippocampal neurons of 5xFAD mice. Dissociated hippocampal neurons of transgenic mice with five familial Alzheimer's disease mutations (5xFAD) and wild-type littermates were transfected at 10 DIV with fluorescently labeled BDNF. The dynamic behavior of BDNF-containing vesicles was analyzed by live cell imaging. (a) Representative picture of a hippocampal neuron transfected with BDNF-mCherry. (b) Boxed area from (a) at higher magnification and at different time points of time-lapse recording. Red and yellow circles mark BDNF-containing vesicles showing a directional movement. Red circle marks a vesicle which moves in anterograde direction from the soma to distal part of the neurite, while yellow circle marks a vesicle which moves in retrograde direction from distal part of the neurite to the soma of the neuron. Green circle marks a vesicle which was immobile during observation. (c) Bar diagram showing the mean motility of BDNF-containing vesicles in hippocampal neurons of 5xFAD mice compared to wild-type littermates. Note that the motility of BDNF-containing vesicles was significantly reduced in hippocampal neurons from 5xFAD mice compared to wild-type littermates. (d) Bar diagram indicates the percentage of immobile BDNF-containing vesicles. Note that there are significantly more immobile vesicles in neurons of transgenic animals compared to wild-type littermates. (e) Transport characteristics of mobile anterogradely directed BDNF-containing vesicles. Bar diagrams show motional properties of BDNF-containing vesicles as indicated. The overall average speed and the average speed during motion, run length, and stopping frequency of BDNF-containing vesicles were significantly changed in neurons of transgenic animals compared to wild-type littermates. (f) Motional properties of retrogradely directed BDNF-containing vesicles. All motional properties of BDNF-containing vesicles moving in retrograde direction were significantly changed in neurons of transgenic animals compared to wild-type littermates. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; one-way ANOVA). Error bars represent SEM.

We next asked whether, in addition to the increased number of immobile vesicles, also motional properties of dynamic vesicles were affected by the transgene expression. Since the vesicle motion was rather characterized by a stop-and-go behavior than by moving at constant speed, different motional properties of BDNF-containing vesicles were analyzed. To this end, we determined the stopping frequency which is defined by the number of stops per unit of time (Supplementary Figure 2). Furthermore, we analyzed the dwell time, expressed by the time the vesicles spent in the same position during one stop. Both stopping frequency and dwell time characterize the stopping phase of a vesicle. During the mobile phase (go), the vesicles move with a certain speed which is described by the average speed of the vesicles during motion, omitting the stops of the vesicles. The covered distance of vesicles between two stops is described by the run length (cf. [37]). The overall average speed of BDNF-containing vesicles which differs from the average speed during motion was defined as the ratio of distance covered to the observation time (cf. [38]) (Supplementary Figure 2). Finally, the percentage of motions occurring during the observation time was determined as a read-out of the motility of one vesicle. While the motility as well as the dwell time was similar for anterogradely directed BDNF-containing vesicles under both conditions (motility: 5xFAD: 75.50 ± 1.57%; Ctrl: 78.91 ± 1.33%; dwell time: 5xFAD: 1.10 ± 0.07 s; Ctrl: 1.04 ± 0.05 s), the stopping frequency of anterogradely directed vesicles was significantly increased in transgenic neurons as compared to wild-type neurons (Figure 1(e)). In addition to the stopping frequency of anterogradely directed vesicles, the run length as well as the average speed during motion was significantly decreased in transgenic neurons resulting in a reduced overall average speed of BDNF-containing vesicles (Figure 1(e)). All motional properties of retrogradely directed BDNF-containing vesicles were significantly changed in transgenic neurons, compared to wild-type neurons (Figure 1(f)). Altogether, the overall average speed of BDNF-containing vesicles was reduced in transgenic neurons due to a lower speed during motion and a higher number of stops during the active transport of BDNF-containing vesicles.

3.2. The Deficits in BDNF Transport Are Also Observed upon Acute Overexpression of hAPP but Occur Independently of Intracellular Levels of APP. The Thy1 promoter in 5xFAD mice initiates transgene expression in neuronal cells at postnatal day 6 [35, 36]. To determine how fast the expression of human APP (hAPP) can negatively influence the transport of BDNF-containing vesicles, we cotransfected hippocampal neurons from C57BL/6 animals with BDNF-mCherry and GFP or APP-YFP, respectively, to mimic an early increased expression of hAPP. Again, the number of immobile vesicles was increased in hippocampal neurons transfected with BDNF-mCherry and hAPP-YFP compared to neurons transfected with BDNF-mCherry and GFP as a control (Figure 2(b)). In addition, the transport dynamics of mobile BDNF-containing vesicles were significantly changed in hippocampal neurons expressing hAPP compared to control neurons (Figures 2(b)–2(d), Supplementary Figure 3). Expression of hAPP in hippocampal neurons revealed similar transport deficits for anterograde and retrograde motion as observed in 5xFAD mice-derived neurons. The onset of BDNF transport deficits occurred three days after the initiation of hAPP expression. Since the expression level of APP varied among the cells investigated, we asked whether the magnitude of transport deficits correlated with the cellular content of APP. Therefore, the fluorescence intensity of APP-YFP in the cell body of the investigated cells (which estimates the individual intracellular APP-level) was plotted against the motional properties of the respective individual cell. However, no correlation between the level of APP and transport deficits was obvious (Supplementary Figure 4). Thus, although the extent of transport deficits increases with the duration of APP expression in hippocampal cultures, there was no evidence that higher amounts of cellular APP negatively influence the transport of mobile BDNF-containing vesicles in the same cell. However, the overall intracellular APP expression level does not uncover the involvement of APP or APP-derived molecules in different aspects of cellular functions at the subcellular level. For example, previous studies suggested a function of APP as an adapter protein for cargo transport ([39, 40] but see [41]). Therefore, single APP molecules might directly interact with BDNF-containing vesicles on the subcellular level. Thus, to examine a function of APP as adapter protein for BDNF vesicle transport, we investigated whether both proteins colocalize at the subcellular level. Hippocampal neurons transfected with proBDNF-GFP were immunostained with an antibody directed against the N-terminal part of APP. The mature domain of BDNF colocalized with low frequency with the N-terminal part of endogenous APP in vesicular-like structures (Figure 3(a)). In addition, also the prodomain of BDNF which predominantly colocalizes with the mature domain of BDNF reveals clearly detectable but relatively low levels of colocalization of ProBDNF and APP (Supplementary Figure 5). These results suggest modest localization of BDNF with APP within the same structure in hippocampal neurons. Nevertheless, APP might act as an adapter protein for a subpool of BDNF-containing vesicles. However, the absence of any correlation between the strength of APP expression and the motional properties in individual cells indicated that not the levels of overexpressed hAPP in a given cell were responsible for impairing transport properties, suggesting rather that APP cleavage products in the culture medium are more likely to be the trigger for the impaired transport properties.

3.3. Aβ Peptides Induce Transport Deficits on a Short Time Scale. To test whether the disturbance of BDNF transport is mediated by factors released into the extracellular medium, hippocampal neurons cotransfected with BDNF-mCherry and GFP were cultured after transfection together with hippocampal neurons cotransfected with BDNF-mCherry and hAPP-YFP (Supplementary Figure 6). Neurons cotransfected with BDNF-mCherry and GFP which were transferred into a culture dish with neurons also cotransfected with BDNF-mCherry and GFP served as control. Transport dynamics of single BDNF-containing vesicles were analyzed by time-lapse

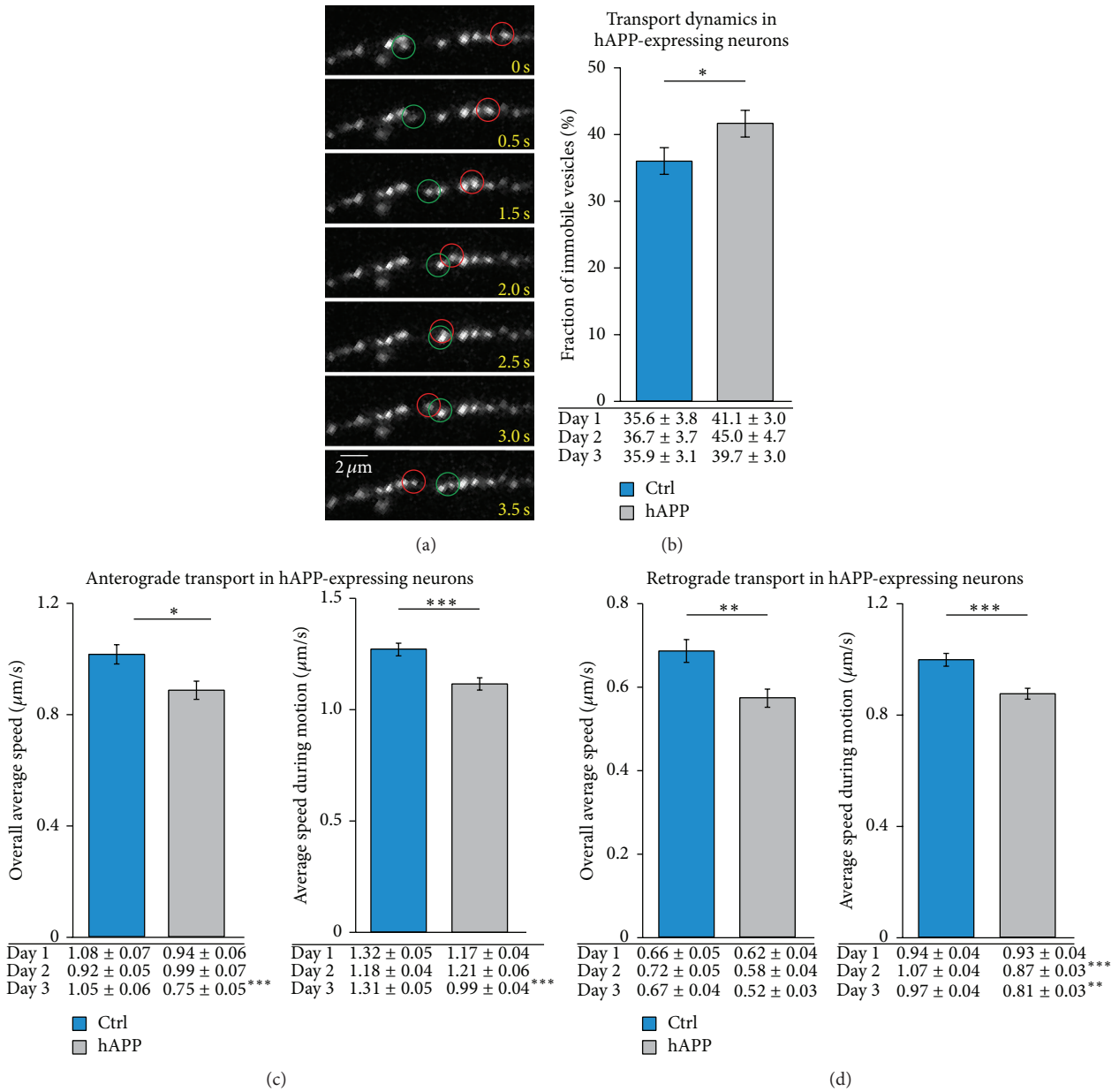


Figure 2: Expression of hAPP reduced vesicular transport of BDNF. Dissociated hippocampal neurons of C57BL/6 mice were cotransfected at 10 DIV with BDNF-mCherry and hAPP-YFP. The dynamic behavior of BDNF-containing vesicles was analyzed by live cell imaging 1–3 days after transfection. (a) Representative picture of BDNF-containing vesicles at different time points of time-lapse recording. Red and green circles mark BDNF-containing vesicles showing a directional movement. (c) Bar diagram indicates the mean percentage of immobile BDNF-containing vesicles in control neurons or in neurons transfected with hAPP during the whole observation period. Tables below bar diagram give the mean value of the incidence of immobile vesicles for day 1, day 2, and day 3. Transient expression of hAPP significantly changed the proportion of immobile vesicles. (d) Transport dynamics of anterogradely directed BDNF-containing vesicles. Bar diagrams show the mean value of the indicated motional properties during the whole observation period. Tables below bar diagrams give the mean value of the indicated motional property for day 1, day 2, and day 3. The overall average speed as well as the average speed during motion was significantly reduced in neurons transfected with hAPP compared to control neurons. While the characteristics of BDNF-containing vesicles were similar in neurons expressing hAPP and control neurons at day 1 after expression of hAPP, the average speed of BDNF-containing vesicles was significantly reduced in neuronal cultures transfected with hAPP at day 3. (e) Transport dynamics of retrogradely directed BDNF-containing vesicles. The motional properties as indicated were significantly changed in neurons transfected with hAPP compared to controls ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; two-way ANOVA followed by pairwise multiple comparison). Error bars represent SEM.

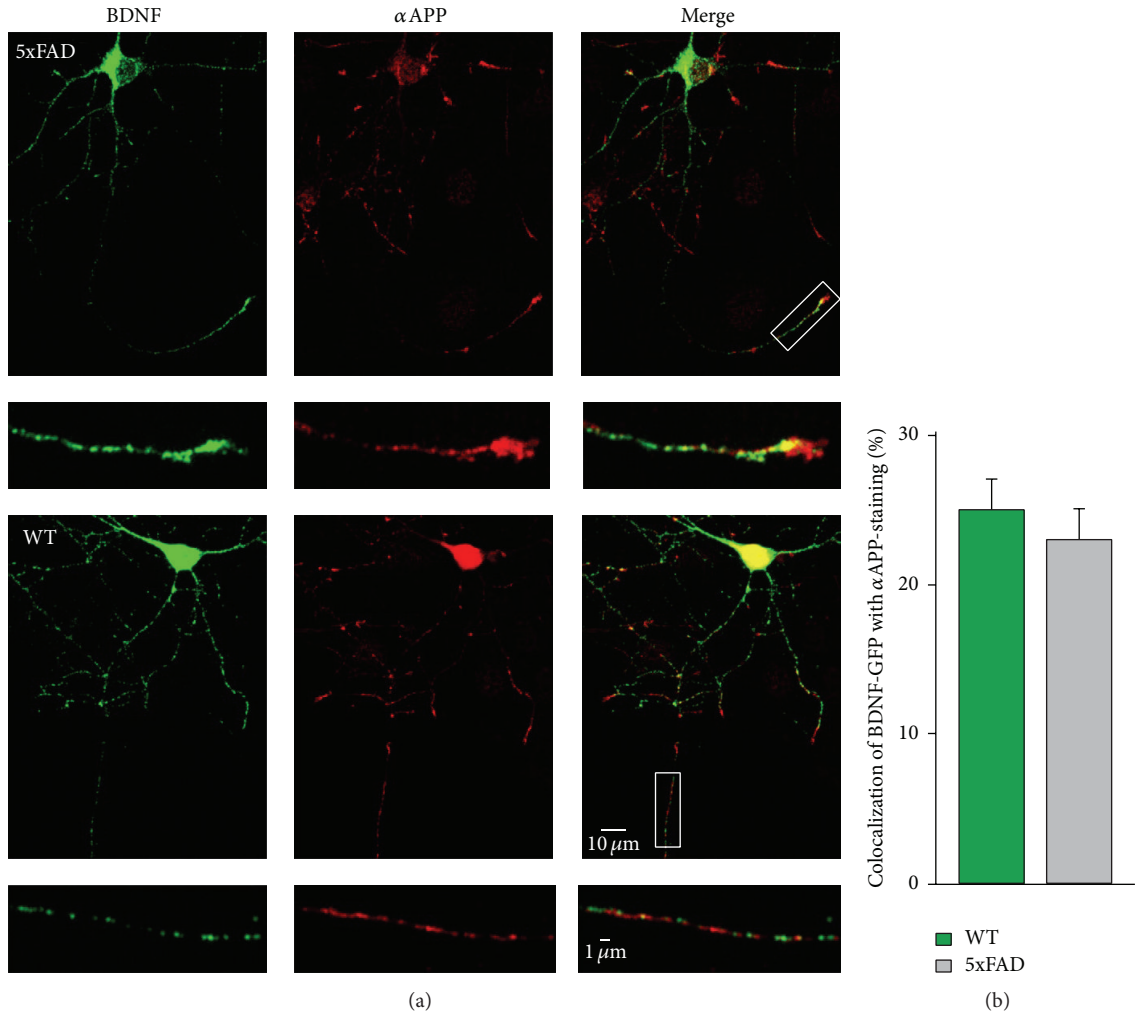
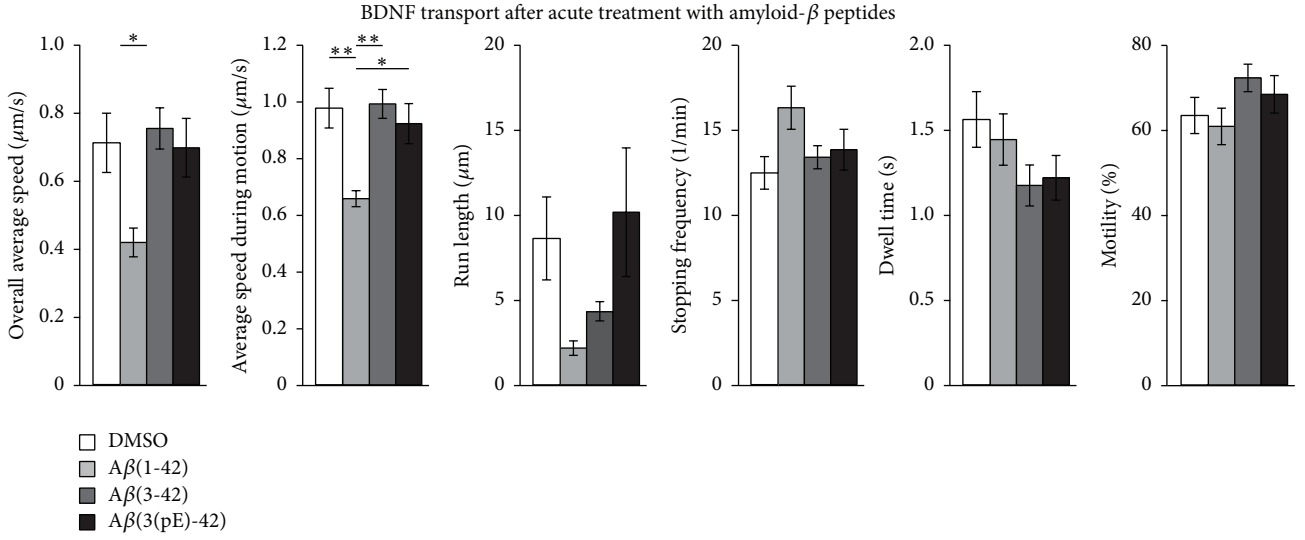


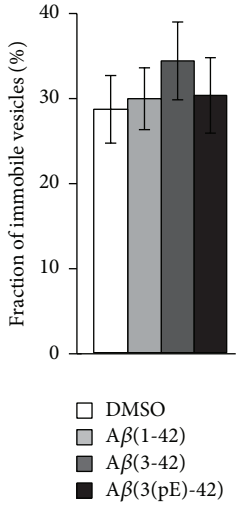
Figure 3: A subpopulation of BDNF-containing vesicles colocalize with APP. (a) Dissociated hippocampal cultures of 5xFAD mice and wild-type littermates were transfected with BDNF-GFP (green) and immunostained with an antibody directed against the N-terminus of endogenous APP (red). Representative pictures showing a transfected and immunostained hippocampal WT neuron (upper part) and 5xFAD neuron (lower part) and higher magnification of boxed areas. Mature BDNF colocalized with APP in vesicular structures. (b) Percentage of αAPP staining colocalizing with total BDNF-GFP area. Error bars represent SEM.

video microscopy. Interestingly, the fraction of immobile vesicles was similar in both conditions (Ctrl in hAPP media: 31.2 ± 2.4%; Ctrl in Ctrl media: 27.8 ± 3.0%; $p = 0.34$, two-way ANOVA). However, the transport deficits of mobile BDNF-containing vesicles observed for control neurons in hAPP medium (Ctrl in hAPP) were comparable to the transport deficits seen after hAPP expression in hippocampal neurons (cf. Figure 2). The average speed during motion and the overall average speed for anterogradely directed BDNF-containing vesicles were significantly reduced in hippocampal neurons transferred into culture medium of hippocampal neurons transfected with hAPP (Supplementary Figure 6). Thus, an extracellular factor which is produced in hippocampal neurons transfected with hAPP and released into extracellular medium might be responsible for the observed deficits in transport of BDNF (Supplementary Figure 6).

The proteolytic products of APP which are secreted into extracellular space play an important role in the pathogenesis of Alzheimer's disease. One of the best studied and most toxic amyloid-beta peptides is amyloid-beta(1-42) which is a major component of amyloid plaques. Another amyloid species, the N-terminally truncated and modified amyloid-beta(3pE-42), was also described to be upregulated in Alzheimer's disease brains. Both peptides are known for their high toxicity as well as for rapid accumulation in brains of 5xFAD mice [32, 42] and are important players in the pathogenesis of Alzheimer's disease. To analyze whether one of these proteolytic products is responsible for the observed deficits in BDNF transport, hippocampal neurons transfected with BDNF-mCherry were treated with different amyloid-beta oligomers. Amyloid-beta treatment was performed either acutely (5–30 min) or for a time period of 24 h. Acute treatment with amyloid-beta(1-42) but not the N-terminally truncated amyloid-beta(3-42) or the truncated and modified amyloid-beta(3(pE)-42) significantly decreased the average speed of retrogradely directed BDNF-containing vesicles (Figure 4(a)). All other motional



(a)



BDNF transport after long-term treatment with amyloid-β peptides

	Anterograde					
	Overall average speed (μm/s)	Average speed during motion (μm/s)	Run length (μm)	Stopping frequency (1/min)	Dwell time (s)	Motility (%)
DMSO	1.48 ± 0.08	1.63 ± 0.07	29.4 ± 3.2	6.98 ± 0.70	0.78 ± 0.06	87.0 ± 1.9
Aβ(1-42)	0.89 ± 0.06	1.15 ± 0.05	14.4 ± 3.3	12.49 ± 0.70	1.32 ± 0.12	71.3 ± 2.3
Aβ(3-42)	0.96 ± 0.09	1.17 ± 0.08	15.5 ± 3.8	11.89 ± 1.15	1.09 ± 0.10	75.4 ± 3.2
Aβ(3(pE)-42)	0.88 ± 0.08	1.07 ± 0.07	9.7 ± 2.0	12.30 ± 1.04	0.99 ± 0.09	75.9 ± 3.0
	Retrograde					
DMSO	0.95 ± 0.07	1.17 ± 0.06	11.8 ± 1.8	12.4 ± 0.7	1.09 ± 0.07	73.9 ± 2.2
Aβ(1-42)	0.63 ± 0.06	0.93 ± 0.05	6.0 ± 1.4	15.6 ± 0.7	1.46 ± 0.08	60.1 ± 2.3
Aβ(3-42)	0.60 ± 0.08	0.86 ± 0.06	5.0 ± 1.7	14.8 ± 1.0	1.41 ± 0.12	61.8 ± 4.1
Aβ(3(pE)-42)	0.62 ± 0.06	0.89 ± 0.06	5.0 ± 1.2	14.5 ± 0.9	1.47 ± 0.14	63.5 ± 3.3

(b)

Figure 4: Application of amyloid-beta(1-42) altered BDNF transport with faster onset than observed for other Aβ peptides. Dissociated hippocampal neurons derived from 5xFAD wild-type littermates were transfected at 10 DIV with fluorescently labeled BDNF. The dynamic behavior of BDNF-containing vesicles was analyzed by live cell imaging after (a) acute and long-term (b) treatment with different amyloid-beta peptides. (a) Acute treatment with amyloid-beta(1-42) influenced average speed of retrogradely directed BDNF-containing vesicles. Bar diagrams show the mean values of the indicated motional properties after acute treatment with different amyloid-beta peptides. The average speed during motion was significantly reduced in neurons treated with amyloid-beta(1-42) compared to neurons treated with solvent (0,1% DMSO), amyloid-beta(3-42), or amyloid-beta(3(pE)-42), respectively ($^{*}p < 0.05$, $^{**}p < 0.01$, one-way ANOVA followed by *post hoc* Tukey Test). (b) Long-term treatment with amyloid-beta (24 h) influenced different motional properties of BDNF transport. (left) Bar diagram shows the percentage of immobile BDNF-containing vesicles in hippocampal neurons after treatment with different amyloid-beta peptides. (right) Table shows the mean values for the different motional properties of anterogradely and retrogradely directed BDNF-containing vesicles in hippocampal neurons. Note that all motional properties were significantly changed after 24 h treatment with amyloid-beta(1-42) compared to control conditions. Long-term incubation with amyloid-beta(3-42) or amyloid-beta(3(pE)-42) also significantly influenced some motional properties of BDNF-containing vesicles ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; one-way ANOVA followed by *post hoc* Tukey's test). Error bars represent SEM.

properties were not significantly altered after this short time period of amyloid-beta treatment (Supplementary Figure 6). However, these motional properties were significantly altered after incubation with amyloid-beta(1-42) for 24 h (Figure 4(b)). Likewise, BDNF transport dynamics were also affected by long-term incubation with amyloid-beta(3-42) or amyloid-beta(3pE-42). Nevertheless, although all motional properties of mobile BDNF-containing vesicles were significantly changed by amyloid-beta(1-42), there was no change in the number of immobile vesicles (Figure 4(b)) which suggest

a possible role of intracellular APP on detachment of moving vesicles from microtubules. Taken together, these data reveal a pronounced slowing for most aspects of BDNF-vesicle movement by the different soluble amyloid-beta peptides tested with earliest onset for amyloid-beta(1-42) induced effects on average speed of BDNF-containing vesicles.

3.4. Release of BDNF Is Not Affected in Neurons from 5xFAD Mice. Reduced transport efficiency of BDNF vesicles could eventually decrease the number of BDNF-containing vesicles ready for exocytosis. Therefore, we analyzed whether activity-dependent release of BDNF was affected by transgenic expression of mutated hAPP. Hippocampal neurons from 5xFAD mice were transfected with BDNF-GFP and depolarized by local superfusion with 50 mM potassium in extracellular solution [24]. The depolarization-induced release of BDNF was analyzed by monitoring GFP fluorescence intensity of BDNF-GFP-containing vesicles using time-lapse video microscopy (all experimental conditions as in Figure 1). Changes in fluorescence intensity became evident 10–50 s after onset of depolarization (Figure 5(b)). Increase in fluorescence intensity due to neutralisation of intravesicular pH after fusion pore opening of BDNF-GFP containing vesicles was followed by decrease in fluorescence intensity indicating depolarization-induced release of BDNF (Figures 5(b)-5(c)). Increase in fluorescence intensity as well as decrease in fluorescence intensity after fusion event was similar in both groups. Furthermore, the density of BDNF-GFP containing vesicles (data not shown) as well as number of vesicular fusion events was similar under both conditions (Figures 5(d)-5(e)). Therefore, neither percentage of fusion events nor release of BDNF from single vesicles was changed in hippocampal neurons derived from 5xFAD mice. Thus, although the transport of BDNF vesicles was affected very early after transgenic expression of hAPP, activity-dependent release of BDNF was not affected.

These results suggest that APP plays a role in transport of BDNF rather than in exocytosis of BDNF-containing vesicles.

4. Discussion

In the present study, we show that different manipulations increasing either APP protein expression or the level of APP-derived molecules in the extracellular medium disturbed retrograde and anterograde transport of the neurotrophin BDNF. Thus, transgenic overexpression of mutated hAPP in 5xFAD mice, plasmid-driven overexpression of hAPP in hippocampal cultures, or extracellular application of amyloid-beta peptides all decreased anterograde as well as retrograde transport of BDNF vesicles. The effects of soluble Aβ peptides could be shown to occur on a fast time scale (within 30 min). Although, the proteins APP and BDNF colocalize with low probability in vesicular structures, the deficits in BDNF transport were not correlated with APP levels at the single cell-level but were mediated by factors released from hippocampal neurons into the extracellular medium. Retrograde transport was affected prior to the anterograde direction, suggesting different mechanisms being responsible for the direction specific deficits. Despite reduced transport efficiency, activity-dependent release of BDNF was not affected by transgenic expression.

The neurotrophic factor BDNF is a very important growth factor for the development of synapses and the survival of neurons [2, 3, 5, 43–45]. A lack of BDNF in the target area can lead to neuronal loss [6–8]. In addition to a reduced level of BDNF, a deficit in transport of BDNF can also result in a lack of neurotrophic support in the respective target areas [6, 8, 9, 11]. BDNF is stored in secretory vesicles which are sorted to either the constitutive or the activity-dependent pathway of secretion [24, 46, 47]. Proteolytic cleavage events of the proBDNF precursor can take place either in intracellular compartments or in the extracellular space, following secretion [25, 47–49]. Our results confirm that the prodomain of BDNF and mature BDNF colocalize very well in hippocampal neurons (Supplementary Figure 5). Nevertheless, colocalization of both domains cannot rule out proteolytic processing which takes place inside these secretory vesicles. The protein APP has also been shown to undergo proteolytical processing by different secretases and is sorted into secretory granules [50–55]. Our results now reveal for the first time that BDNF and APP can colocalize, albeit with low abundance, in vesicular structures of hippocampal neurons (Figure 3). This observation is consistent with previous reports indicating kinesin-1-dependent transport of both cargoes in neurons [26, 56–62] although kinesin-1 is predominantly described for transport of APP while BDNF transport is also known to depend on kinesin-3 [19, 63, 64].

Furthermore, our experiments revealed a higher incidence of immobile vesicles in 5xFAD mouse derived neurons. There was no similar change in vesicle motility in wild-type C57BL/6 neurons which were cultured in medium conditioned by wild-type neurons transfected with hAPP-YFP or in hippocampal neurons which were treated with amyloid-beta (cf. Supplementary Figure 6 and Figure 4; see also [65]). Therefore, the change in fraction of immobile vesicles may be dependent on elevated intracellular APP level rather than on extracellular amyloid-beta peptides. APP has been described to act as an adaptor protein interacting with kinesin-1 thereby connecting vesicles to axonal transport ([39, 40] but see [41]). Consequently, APP could be responsible for the transport of a small pool of BDNF-containing vesicles to defined subcellular target compartments. A direct or indirect interaction of APP with kinesin-1 could be responsible for such a detachment of moving vesicles [66–68]. Nevertheless, the low degree of APP and BDNF colocalization argues for a largely independent transport of both proteins. On the same vein, the velocity of anterogradely directed vesicles containing APP was reported to reach up to 9 μm/s (mean velocity: 4,7 μm/s) [26, 69, 70], whereas we observed an average speed of BDNF-containing vesicles of 1 μm/s (max 4 μm/s), being consistent with previous findings [20–22, 71, 72]. This comparison of vesicle velocities speaks in favour of largely independent transport of both proteins in neurons.

One of the first changes in dynamic transport of BDNF-containing vesicles we observed was the reduction of average speed during motion of retrogradely directed BDNF-containing vesicles (Figure 4). Other deficits in transport of BDNF-containing vesicles occurred only later, suggesting

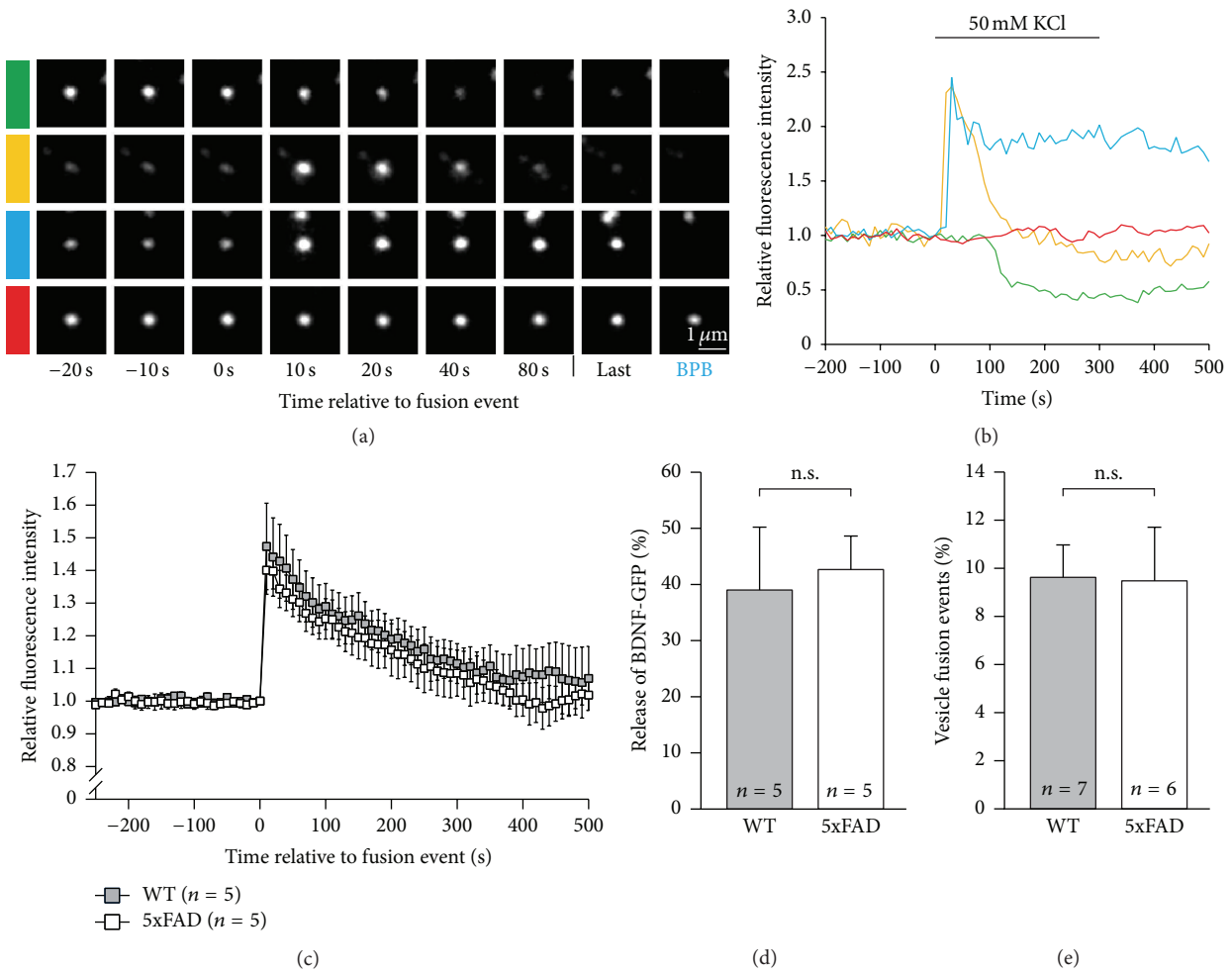


Figure 5: Release of BDNF was not affected after expression of mutated hAPP. Dissociated hippocampal neurons from 5xFAD mice and wild-type littermates, respectively, were transfected at 10 DIV with a BDNF-GFP plasmid. Depolarization-induced release of BDNF-GFP as well as the incidence of fusion event of BDNF-containing vesicles was analyzed by live cell imaging. (a) Representative single vesicles of a hippocampal neuron transfected with BDNF-GFP showing fusion events as indicated by the change in fluorescence intensity (green, yellow, and blue) compared to a single vesicle showing no fusion pore opening (red). For most events, BDNF-GFP fluorescence increased due to the neutralisation of the intragranular pH after fusion. BDNF release was observed as decrease in fluorescence intensity after fusion event. At the end of the release measurement, neurons were superfused with BPB (0.3 mM) to differentiate between vesicles in an open or closed state at this time point (scale bar = 1 μm). (b) Graph shows time course of fluorescence intensity of color coded vesicles shown in (a). Change in fluorescence intensity indicates neutralisation of intravesicular pH after fusion pore opening of BDNF-GFP-containing vesicles (fluorescence increase) and depolarization-induced release of BDNF-GFP (fluorescence decrease), respectively. (c) Average time course of GFP-fluorescence intensity of single hippocampal neurons. Note the absence of any difference in release efficiency or time course between neurons from 5xFAD animals and wild-type littermates. (d) Quantification of the fluorescence decrease after fusion event. The maximal fluorescence intensity after fusion was set to 100% and the remaining fluorescence intensity 300 s after fusion pore opening was analyzed. BDNF release amplitude was unaltered in hippocampal neurons derived from 5xFAD mice. (e) Quantification of depolarization-induced fusion events. Bar diagram shows the percentage of fusion events of vesicles in hippocampal neurons from 5xFAD animals and wild-type littermates (n.s. = no significant difference; one-way ANOVA). Error bars represent SEM.

that distinct mechanisms are responsible for these distinguishable deficits in BDNF transport. Fast changes in retrograde transport of BDNF-containing vesicles were described to be mediated by inhibition of vesicular ATP production [73]. However, at variance with our findings in the present study, anterograde and retrograde transport of BDNF were affected on a similar time scale as well as to a similar extent after inhibiting a key step of glycolysis [73], suggesting that short-term application of amyloid-beta(1-42) does not perturb glycolysis. Furthermore, short-term treatment with amyloid-beta(1-42) has been described to rapidly induce changes in synaptic function [74, 75]. Although electrical activity can modify vesicular trafficking, these changes are not restricted to retrograde transport [76–79], again suggesting that these cellular events are not upstream of the early deficits in retrograde transport that we observe. Acute treatment with amyloid-beta is known to modulate GSK3-signalling. GSK3-signalling can modify specifically

anterograde but not retrograde transport [80, 81] suggesting a different unknown mechanism to underlie the amyloid-beta induced fast changes in retrograde transport in our study, where retrograde transport is affected first.

Long-term treatment (24 h) with amyloid-beta(1-42) as well as long-term treatment with amyloid-beta(3(pE)-42) which has been discussed recently to be a potential player in triggering Alzheimer's disease [82] affected anterograde as well as retrograde transport of BDNF (Figure 4). Similar changes were observed in hippocampal cultures of 5xFAD transgenic animals (Figure 1). 5xFAD mice produce very high levels of amyloid-beta(1-42) as well as amyloid-beta(3(pE)-42) that rapidly accumulate intraneuronally starting at the age of 1.5 months, while cognitive impairments are obvious at 4-5 months [32, 42]. Therefore, the observed deficits in transport of BDNF which occurred shortly after initiation of transgene expression in our experiments are likely to take place very early in development of 5xFAD mice and might permanently impair brain functions. Long-term treatment with amyloid-beta peptides has been described to induce several changes (for recent reviews see, e.g., [74, 83, 84]), including effects of amyloid-beta(1-42) on synaptic cargo transport [23, 65, 71, 72, 85]. Transport of synaptic cargoes is influenced by amyloid-beta oligomers in a NMDAR- and GSK-3-dependent fashion [65, 71, 72]. While Tang and colleagues [65] described an amyloid-beta induced NMDAR and GSK-3-dependent mechanism which affects velocity of synaptic cargo vesicles, Silverman's group uncovered an amyloid-beta induced NMDAR and GSK-3-dependent mechanism completely disrupting vesicle trafficking [71, 72]. A similar disruption of BDNF transport after amyloid-beta treatment could not be confirmed for the investigation period analyzed in our study (Figure 4(b)). In the 3 different assays which we used to analyze BDNF transport, we did not observe such a drastic reduction in the fraction of mobile vesicles. In our measurements, the moving or mobile vesicles were significantly slower after acute amyloid-beta(1-42) treatment or after 24 h treatment with amyloid-beta(1-42), respectively, but they were still moving. We did not observe disruption of BDNF transport and thereby increase in immobile vesicle pool after amyloid-beta treatment at all. The small but significant increase in immobile vesicle pool which we observed was only evident when hippocampal neurons overexpressed APP suggesting a function of APP and not amyloid-beta in detaching mobile vesicles from microtubules. Culturing conditions but also the way of analysis (manual versus automated tracking, e.g., [86]) could account for these different results. Another amyloid-beta induced mechanism being responsible for delayed deficits in transport of endocytosed BDNF/TrkB signaling endosomes was described by Poon and colleagues [22]. Long-term treatment with amyloid-beta oligomers decreased cell body accumulation of extracellularly applied BDNF-GFP [23] suggesting impaired retrograde transport of endocytosed BDNF/TrkB-complex. The amyloid-beta induced deficits in retrograde transport of these signaling endosomes were mediated by an ubiquitin C-terminal hydrolase dependent mechanism [22]. An additional influence of ubiquitination pathways on anterograde transport seems to be likely [87] so that also ubiquitination pathways may be upstream of the late deficits in transport deficits that we observe. Furthermore, amyloid-beta treatment has been described to induce generation of ROS [88, 89] which downregulate histone deacetylation by inhibiting histone deacetylase [90]. Deacetylation of microtubules was described to destabilize microtubule networks thereby leading to an increased number of stopping events or prolonged dwell time [60], which is in line with our observations (Figures 1 and 4).

Activity-dependent release of BDNF depends on ATP production, Calcium influx, and efficiency of glutamatergic synaptic transmission (reviewed in [91]). In addition, it is conceivable that BDNF release is also limited by the availability of BDNF vesicles ready for exocytosis at release sites. Interestingly, although the transport of BDNF was affected by amyloid-beta application as well as by transgenic expression on different levels, the release of BDNF was similar in hippocampal neurons of 5xFAD mice as compared to their control littermates. Thus, although the protein APP and the proteolytic cleavage products of APP have been shown to affect neuronal function on different levels, the depolarization induced release of BDNF was unchanged in the investigation period covered by our experiments. Recently, the role of intracellular amyloid-beta oligomers in synaptic vesicle release was investigated [92, 93]. Yang and colleagues showed that amyloid-beta monomers as well as oligomers which accumulated intraneuronally directly bind to intracellular syntaxin 1A. Furthermore, this cytosolic interaction of amyloid-beta oligomers significantly inhibited SNARE-mediated exocytosis. In contrast, we did not observe any influence on BDNF release in hippocampal neurons under these conditions. One possible explanation for this opposing result might be the importance of different protein isoforms in the release process of neurotransmitter and neuropeptide vesicles. In general, release of neurotransmitters and release of neuropeptides share similar mechanism. However, syntaxin 1A is known to be important for presynaptic exocytosis of neurotransmitter in neurons. Furthermore, it is known that syntaxin 1B and not syntaxin 1A plays an important role in release of BDNF in glial cells. Whether the identical protein machinery is important for BDNF release in neuronal cells is unknown until now. Besides the different protein isoforms, intraneuronal accumulation of amyloid-beta oligomers which is a prerequisite for direct interaction of amyloid-beta and intracellular domain of syntaxin might be a mechanism which follows deficits in transport processes. Intraneuronal accumulation of amyloid-beta(1-42) has been reported in the 5xFAD mouse model starting at 1.5 months of age. However, the transgene expression is under the regulation of the Thy1 promoter in 5xFAD mice starting with expression of mutated APP and presenilin within the first days after birth. This speaks for a delayed intraneuronal accumulation of amyloid-beta oligomers. In our hippocampal cultures derived from newborn 5xFAD mice we cannot exclude intraneuronal accumulation of amyloid-beta at this early time point. Nevertheless, we observed an effect of exogenous amyloid-beta(1-42) on transport kinetics of BDNF-containing granules within minutes. For intracellular accumulation, endocytosis of amyloid-beta and leakage of

endocytosed amyloid-beta out of endosome or lysosomes are necessary, which makes it unlikely that intraneuronal accumulation of exogenous amyloid-beta is important for the fast effect on BDNF transport we observed. Furthermore, the observation that BDNF release is unaffected in 5xFAD cultures might give a hint that deficits in transport occur before intracellular accumulation of amyloid-beta. Retrograde transport deficits could influence endosomal or lysosomal membrane trafficking. These transport deficits may end in a lysosomal leakage [94] thereby resulting in an intraneuronal accumulation of Abeta and an ongoing of pathophysiological processes.

Our study reveals that the neurotrophic factor BDNF shows modest colocalization with the protein APP in vesicular structures of hippocampal neurons. The anterograde as well as retrograde transport of BDNF-containing vesicles was impaired in different model systems of Alzheimer's disease. Interestingly, short-term treatment of hippocampal neurons with amyloid-beta(1-42) resulted in a reduced average speed of BDNF-containing vesicles already after acute application. Since retrograde transport was affected prior to the anterograde direction, this suggests different mechanisms being at work for the direction-specific deficits in BDNF transport.

5. Conclusion

The neurotrophin brain derived neurotrophic factor (BDNF) is an important growth factor in the central nervous system. Deficits in transport of this secretory protein could underlie neurodegenerative diseases. We discovered that extracellular cleavage products of APP induced rapid changes in transport kinetics of BDNF-containing vesicles. Our data furthermore suggest a possible role of APP on detachment of moving vesicles from microtubules thereby reducing the number of mobile vesicles while release of BDNF was unaffected by transgenic expression of mutated APP.

Conflict of Interests

The authors declare no competing financial interests.

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Review Article

Structural, Synaptic, and Epigenetic Dynamics of Enduring Memories

Ossama Khalaf and Johannes Gräff

Laboratory of Neuroepigenetics, Brain Mind Institute, Faculty of Life Sciences, École Polytechnique Fédérale de Lausanne (EPFL), 1015 Lausanne, Switzerland

Correspondence should be addressed to Johannes Gräff; johannes.graeff@epfl.ch

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Our memories are the records of the experiences we gain in our everyday life. Over time, they slowly transform from an initially unstable state into a long-lasting form. Many studies have been investigating from different aspects how a memory could persist for sometimes up to decades. In this review, we highlight three of the greatly addressed mechanisms that play a central role for a given memory to endure: the allocation of the memory to a given neuronal population and what brain areas are recruited for its storage; the structural changes that underlie memory persistence; and finally the epigenetic control of gene expression that might regulate and support memory perseverance. Examining such key properties of a memory is essential towards a finer understanding of its capacity to last.

1. Introduction

Based on experience, memory is the capacity of an individual to acquire, store, and retrieve information. The physical substrate of such memories in our brains is known as memory trace or as first coined by the German biologist Semon (1859–1918) as "engram" [1–3]. One of the fundamental questions in memory research is how the experiences that we acquire transform into engrams that persist over time. It is generally acknowledged that the records we form from our daily experiences are not stored instantaneously but rather retained in an initially labile state that gradually transforms into a more stable trace or engram that is characterized by resistance to disruption [4–6]. Although this view has been challenged by the reconsolidation hypothesis, stipulating that even a stably stored memory could become transiently sensitive to disruption upon recall [6, 7], it is evident that not all forms of memories are amenable to disruption [8]. This is particularly relevant for strong memories, induced by an intensive training protocol, and long-lasting forms of memories, ranging from several weeks to months [9, 10] in age. Based on these grounds, but notwithstanding several studies testifying to the amenability of even long-term memories to disruption [11, 12], in this review we focus on 7-day-old—and older—memories as being remote and with the potential to endure, and we outline three mechanisms that might contribute to such endurance: first, memory allocation and storage; second, structural neuronal changes; and third, nuclear epigenetic dynamics (Figure 1).

Memory allocation refers to an early process by which certain neural circuits are assigned to stow a specific memory and what might favor the allocation of a memory into a specific population of neurons over others. In this review, we focus on some of the well-described elements that govern such allocation; still it is clear that we are only at the beginning of understanding the entire process of memory allocation, and many more aspects thereof remain to be identified. Once allocated, the question of where the memory is stored and what brain regions upkeep the memory is another one of utmost importance. The whereabouts of a specific memory is thought to be dependent on how old this memory is. The more nascent it is, the more it will be hippocampal-dependent, but as it matures it will change such dependence to higher cortical regions [13, 14]. Here, we describe brain areas that have been defined to be essential for the support of a long-lasting memory.

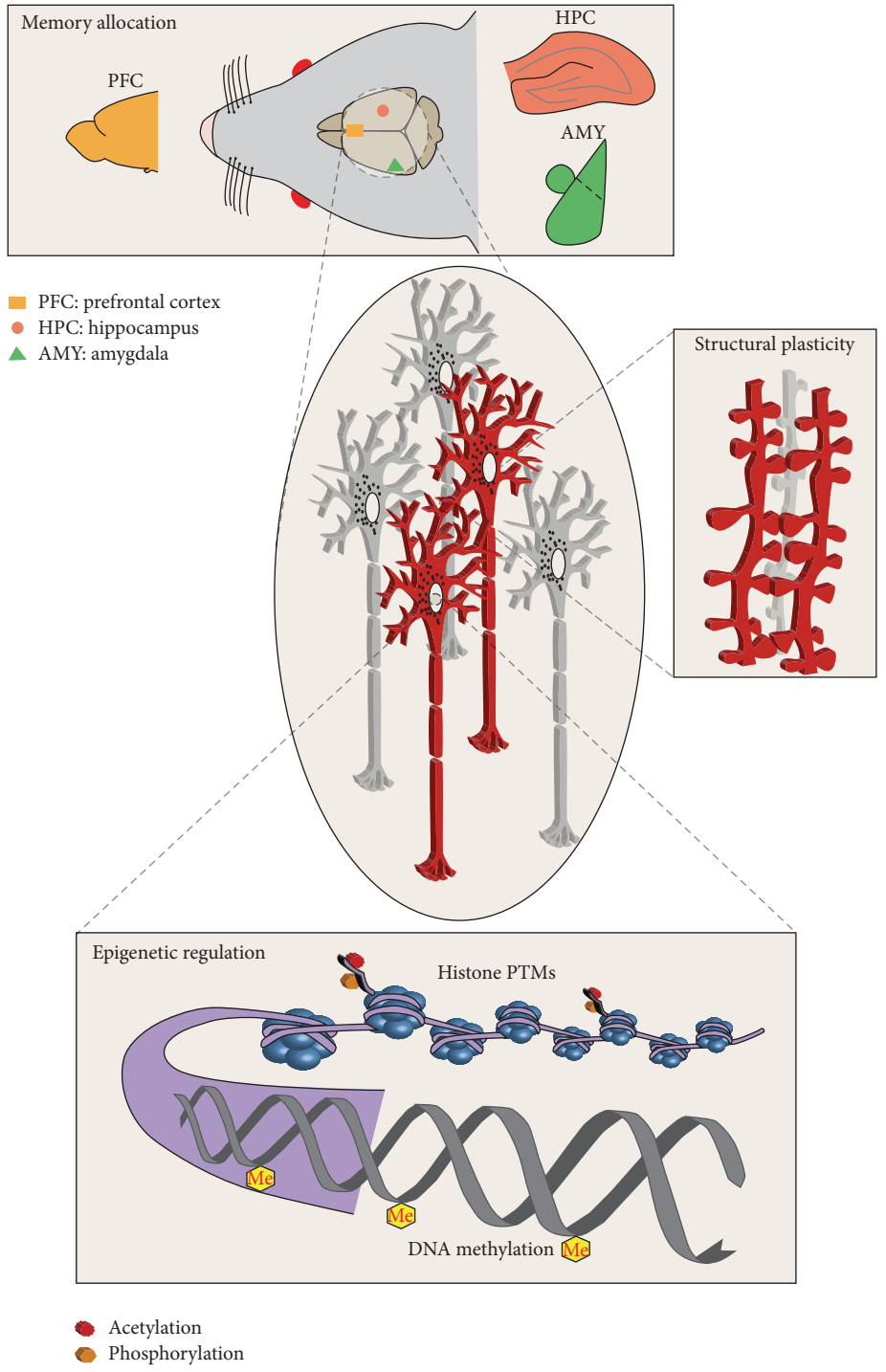

Figure 1: Schematic illustrating three essential mechanisms that might contribute to remote memory storage and thus memory endurance in the (rodent) brain, which are discussed in this review. First, during memory allocation, learning induces the activity of a specific subpopulation of cells—likely spread across different brain areas—which will become recruited into the memory trace. The amygdala (AMY), the hippocampus (HPC), and the prefrontal cortex (PFC) are known to be activated during memory allocation (for details see text). Second, in cells allocated to a specific memory—also known as the memory engram [1–3]—structural changes at the level of dendritic spines have been demonstrated by several studies. These changes are exclusive to the cells of the memory trace or engram (red) but not observed in other cells (grey) [53]. Third, memory engram cells are also likely to be characterized by epigenetic changes, such as posttranslational modifications (PTMs) on histone proteins, and methylation of the DNA, the core chromatin constituents. Note, however, that such engram-specific engagement of epigenetic mechanisms remains to be experimentally demonstrated.

Furthermore, many neuroscientists believe that memories are encoded into neurons as structural changes in synaptic connections. Indeed, such structural plasticity is under comprehensive study in order to understand how brain circuits are modifying themselves in terms of number and strength of synaptic connections that correlate with the persistence of a memory [15–17]. We discuss these physical changes in synapses and their potential to support enduring memories.

Lastly, we also discuss the epigenetic modifications that are associated with long-lasting memories. We shed light on such modifications to the DNA or the histone tails that could lead to a cascade of changes in gene expression, a key feature of long-term memories [18], and which might thereby be able to assist memories to persist throughout the life of an individual.

2. Memory Allocation and Storage

Once formed, memories gradually transform from an initially vulnerable state to a more permanent state that is increasingly persistent to disruption. Such process of postexperience memory stabilization was first described by Müller and Pilzecker referring to it as "memory consolidation" [4, 5]. Later, two different types of memory consolidation have been distinguished: cellular/synaptic and systems consolidations. Cellular consolidation is a rather fast process taking place within the first few hours following learning and necessary for the initial stabilization of memories in hippocampal circuits [13]. In contrast, the systems consolidation process is slower and involves a time-dependent, gradual reorganization of the brain regions that support the memory, with the memory dependence shifting from the hippocampus to cortical regions [14]. This led to the contemporary view of systems consolidation which states that the hippocampus (HPC) is merely a temporary store for new information, while its permanent storage depends on largely distributed cortical networks [14].

In this section, we review what molecular and cellular events govern memory allocation in or to a certain neuronal population and then what brain regions support long-lasting memory storage.

2.1. Memory Allocation. By definition, memory allocation is the set of processes that determine where information is stored in a particular neural circuit [19]. Several studies showed that such allocation is not random but rather dependent on specific molecular mechanisms [20–22]. In one of these studies [20], using a viral vector Han et al. artificially increased the levels of CREB (cAMP responsive element-binding protein), a transcription factor important for the stability of synaptic potentiation and memory [23] in neurons of the lateral amygdala (LA), a subcortical brain structure implicated in emotional memories [24, 25], in mice. Twenty-four hours after a tone fear conditioning training, the mice were tested for the tone and sacrificed 5 min later. Using cellular compartment analysis of temporal activity by fluorescence *in situ* hybridization (catFISH), LA neurons transfected with CREB—identified by its GFP fluorescent tag—were found to be three times more likely than their neighboring nontransfected cells to express activity-regulated cytoskeletal (*Arc*), a gene required for synaptic function and memory [26, 27]. This suggests that CREB levels bias neurons to become part of the engram and to be encoded by the tone conditioning in the amygdala.

In a subsequent loss-of-function study, cells that were virally transfected with CREB in the same behavioral paradigm were ablated using diphtheria toxin receptor (DTR). In this system, the expression of the DTR is inducible by the Cre-recombinase, which is also found in the same viral construct, making all the cells that receive the construct eventually express the DTR. Following the tone test (24 h after training), the mice were injected with the diphtheria toxin (DT) that will only interact with the cells expressing the DTR and kill them. The experimental group (CREB viral vector transfected and DT injected) showed a significant impairment in tone conditioning when tested 2 days after the DT injection [21]. Similar results were obtained using a different approach that allows for reversible neuronal activation instead of permanently killing the cells [22]. There, the *Drosophila* allostatin inhibitory receptor was delivered to the LA through the same viral construct providing CREB, and pronounced amnesia for tone conditioning was obtained as a result of inactivating these cells by allostatin peptide treatment. This amnesia was reversed upon retesting the mice one day later without the allostatin peptides demonstrating the reversibility of the allostatin effects and the link between activity in the CREB cells and recall [22]. Despite the exclusive focus on CREB in the previous studies, the convergent findings using three different strategies strongly support its important role in memory allocation in the amygdala.

Another influential factor that determines the allocation process appears to be neurogenesis in the dentate gyrus (DG). Using 5-bromo-2′-deoxyuridine (BrdU), a permanent stain that intercalates with dividing DNA allowing the tracing of newly born neurons, a recent study showed that 4- to 8-week-old DG neurons are preferentially recruited after spatial learning [28]. In contrast, 2-week-old neurons integrated with lower efficiency and 1-week-old neurons did not integrate at all [28]. In line with a recent study showing that 4-week-old (but not 1-week-old) neurons have the essential synaptic structure and physiology to support the appropriate connections with hippocampal circuits [29], this suggests that the timing of neuronal development relative to training is indeed vital in the memory allocation process. Nevertheless, the nature of memory allocation processes that take place in brain areas devoid of neurogenesis and outside the amygdala remains to be determined.

2.2. Memory Storage. After the initial allocation of a memory to a specific neural circuit begins the more prolonged process of systems consolidation that involves gradual reorganization of the brain regions that support memory formation and storage [13, 14]. Classical studies characterizing memory loss in patients with lesions of the medial temporal lobe (MTL) [30, 31] revealed that the hippocampus serves as a temporary store for new information, but that permanent information storage depends on a broadly distributed cortical

network [14]. These human data are indeed consistent with observations that hippocampal lesions in the first week after training, but not thereafter, disrupt contextual fear memories in rats, and thus, maintaining a proper hippocampal trace is crucial to establish remote memories in the cortex [32]. From more refined studies, several molecules have in the meantime been identified that maintain the hippocampal trace of a memory in the days following training for the persistence into a remote memory [33, 34] (for a more detailed overview of other molecules that are involved in memory storage, but that have not been specifically assessed for remote memory storage, the reader is referred to [19]). For instance, when NMDA (*N*-methyl-D-aspartate) receptor (NMDAR) function was inducibly suppressed in the CA1 region in the week following the training of two hippocampal-dependent tasks (Morris Water Maze and contextual fear conditioning), remote memory formation for these tasks was blocked. However, when done at later time points, the suppression of the NMDAR function did not affect the remote memory formation [33]. Similar results were obtained when levels of α-calcium/calmodulin kinase II (α-CaMKII), a signaling enzyme mainly expressed in the excitatory neurons of the forebrain and essential for neuronal plasticity [35], were altered [34]: overexpressing a dominant-negative form of α-CaMKII in the week after training, but not afterwards, blocked the formation of remote contextual fear memories [34]. Together, these results support the importance of the HPC, especially during the first week following encoding, for memory consolidation in cortical networks and furthermore suggest that there is a crucial week-long window during which normal hippocampal activity is needed for the memory to be consolidated.

However, several studies found that cortical regions are also implicated in the initial phase of memory formation [36–39], thus challenging the idea that the HPC is solely involved in this process. In one of the recent studies in this regard [38], real-time optogenetic inhibition of excitatory medial prefrontal cortex (mPFC) neurons during contextual fear conditioning showed that such temporally precise inhibition impaired the formation of long-term associative memory, tested 30 d after of acquisition [38]. In another recent study [39], using a doxycycline-inducible mouse line (TetTag) to tag the activated neurons [40], optogenetic stimulation of the activated neural population during contextual fear memory training in the retrosplenial cortex (RSC), a cortical region implicated in episodic memories and emotional associations [41–44], was sufficient to produce fear memory retrieval even when tested until 2 d after acquisition [39]. These results are in line with previous studies [36, 37] showing that the PFC is critically involved in memory encoding and that its inactivation by local infusion of NMDAR antagonist could block contextual memory acquisition in mice [36] and learning of new paired-associates in rats [37].

In another intriguing study, Lesburguères et al. used a social transmission of food preference (STFP) test, where an associative olfactory memory develops after a study animal (observer) learns about the safety of a certain food (novel odor for the observer) from an interaction session with another animal that has already tasted the food (demonstrator). Then the observer shows reduced fear towards this novel food upon the first encounter and significant consumption thereof. The authors first showed that the acquisition of such food preference memory is dependent on the orbitofrontal cortex (OFC) only for 30-day-old remote memory, but not for recent memory (24 h after training), and that for the first period after training (7 d) it is mainly HPC-dependent [45]. Nevertheless, the authors then went on to show that there is an intricate interplay between the HPC and the OFC for such memory to endure. Using the excitatory glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) to block the activity of the OFC during the 2-week period following training, an unexpected memory loss to a novel odor test was observed 30 d later. Likewise, inactivating the OFC immediately before training blocked the memory after 30 d, and not after 7 d, indicating that early cortical activity is required for subsequent stabilization of such memory [45].

Beyond memory formation, several studies investigated the role of extrahippocampal structures in remote memory storage, from which the anterior cingulate cortex (ACC) emerges to play a key role at least in remote contextual fear memory storage [46–49]. Thus, lidocaine-mediated pharmacological inactivation of the ACC disrupts the retrieval of remote contextual fear memory in mice 18 d and 36 d after training, while inactivating the prelimbic cortex (PL)—a region located near the ACC in the mPFC—at the same time points did not disrupt the very same memory [46]. Similarly, the lidocaine-mediated inactivation of the PFC and the ACC was shown to impair remote spatial memory retrieval when tested 30 d after acquisition [47]. These results are in line with previously reported data from a study using noninvasive functional brain imaging to examine the metabolic activity of different brain regions underlying spatial discrimination memory storage in mice [48]. In this study, increased metabolic activation in the frontal cortex, together with the recruitment of the ACC and temporal cortices, was observed 25 d—but not 5 d—after acquisition [48]. Together, these findings indicate a high level of involvement of cortical areas during the retrieval of remote memories, postulating these areas to be vital structures for remote memory storage.

Finally, from a reconsolidation point of view and how memory storage could affect such process, it has been previously demonstrated that infusing anisomycin (ANI), a protein synthesis inhibitor, to the dorsal HPC (dHPC) or the ACC after contextual fear memory recall (45 d or 30 d after acquisition, resp.) disrupts the memory when tested 1 d after anisomycin treatment [11, 49]. Collectively, these results highlight an equal importance of hippocampal and cortical regions in remote memory reconsolidation, which suggest that probably the process of memory formation and storage does not depend solely on a single brain area but is more distributed among different structures that share the upkeep of the trace.

3. Structural Changes

Amongst many aspects that categorize a memory to be remote is persistence, yet how this property is achieved

is still enigmatic. The strength and number of synaptic connections that are formed after an experience offer one possible explanation as to how remote memories could endure and last throughout life [18]—since we know that such processes—such as increased dendritic spine density—are indeed implicated in 1-day-old memories [15, 50, 51]. In this section, we shed light on the structural changes that modify the connectivity of brain networks and that might underlie remote memory perseverance.

A few years ago, Restivo and colleagues used contextual fear conditioning as a behavioral paradigm to show that recent and remote memory formation trigger region-specific and time-dependent morphological changes in hippocampal and cortical networks of mice [16]. Right after fear conditioning, there was a significant increase in spine density in the CA1 field of the hippocampus compared to the naïve or even pseudoconditioned groups. 36 days later, in contrast, this increase in spine density had developed sequentially when it reached the cortical regions, specifically the ACC. Thus, hippocampal plasticity *per se* is seemingly crucial in driving the structural changes that were observed at a remote time point, yet its role was merely time limited, an observation that was recently confirmed using time-lapse two-photon microendoscopy [52]. To further prove this assumption, a hippocampal lesion was generated early at the day of conditioning, where it abolished the growth of significant spine density in the ACC (36 d after training) compared to the sham group [16]. In contrast, when this lesion was introduced at a later time point (24 days after conditioning), it did not prevent the spine density changes in the ACC neurons. The detected structural changes in either region were directly correlated to the strength of the conditioned memory: an absence of these structural changes in the hippocampal or the cortical regions was accompanied by memory impairments for recent and remote memories, respectively. This is in line with a recent demonstration that such increase in synaptic density and plasticity occurs exclusively in engram cells, but not in nonengram cells, in the DG 24 h after encoding [53].

Importantly, such structural remodeling in hippocampal and cortical regions is essential for memory stabilization and afterwards for remote memory expression. The spine growth at the hippocampal neurons is important at an early time point after conditioning, yet this importance starts to fade with time, when a more permanent trace is formed in the cortex [17], as illustrated by the following study. To inhibit the structural changes that occur in the cortex, a transcription factor that is known to negatively regulate spine growth, myocyte enhancer factor 2 (MEF2), was overexpressed through a viral vector to increase the MEF2-dependent transcription in ACC neurons at 2 different time points, either 1 day or 42 days after conditioning. At the earlier time point, the stabilization of the conditioned memory and the associated increase in spine growth was blocked, whereas no effect was observed at the later time point [17]. This suggests that the increase in spine growth at the ACC following conditioning happens in a time-dependent manner and that it is central for the stabilization and persistence of such memory.

In contrast to the abovementioned studies, another study showed a rapid formation of new spines in the motor cortex of mice following a novel motor skill learning task [54]. Using *in vivo* superficial dendrites imaging, they demonstrated that there is an immediate formation of spines in the motor cortex following a novel motor learning task (within 1 h after learning initiation) and that these spines are preferentially stabilized upon subsequent training and endure long after training stops (up to 120 d) [54]. This suggests that the early cortical structural changes during motor learning and the subsequent stabilization over months subserve as long-lasting structural basis for memory maintenance and persistence of a motor skill. Similarly, a more recent study reported that the encoding of a long-term episodic memory itself elicits early structural changes in neocortical regions. In this study, structural plasticity in the mPFC was significantly increased 1 h following contextual fear conditioning [38]: investigating the morphology of individual dendritic spines on mPFC pyramidal neurons revealed that the ratio of the thin spines to mushroom spines was significantly increased following conditioning. This suggests that dendritic spine plasticity in the mPFC circuit also contributes to memory encoding, which is surprising as the remodeling of the cortex was traditionally thought to be limited to the later stages of memory processing that promote remote memory storage [55]. Further investigations are now needed to have a better understanding of these structural changes and how they are employed to serve memory lasting or extinction (Box 1).

4. Epigenetic Regulation

Remote memories persist throughout the life of individuals, whereas the protein molecules that may subserve these memory traces are thought to turn over on the order of days [56]. To address such unanswered questions dealing with the molecular basis for a lifelong memory, it has been proposed by Crick (1916–2004) in 1984 and later on by the molecular biologist Holliday (1932–2014) in 1999 that epigenetic mechanisms—particularly DNA methylation—could partly explain the persistence of memories over a lifetime [57, 58]. Epigenetics has long been heralded as a stable and self-perpetuating regulator of cellular identity through establishing persistent and heritable changes in gene expression across cell divisions [20]. Although the nervous system is essentially composed of nondividing cells, the recent decade has shown that epigenetic mechanisms could nevertheless play a fundamental role in forming lasting memories.

Commonly, DNA is packaged into chromatin through its wrapping around octamers of histone proteins. Chromatin can exist either as heterochromatin or as euchromatin: heterochromatin is characterized by condensed chromatin and subsequent transcriptional repression, whereas euchromatin is characterized by a relaxed chromatin state that allows the transcriptional machinery to access the DNA for gene expression [59]. Apart from short interfering RNA molecules that mediate posttranscriptional gene silencing [60] and induce epigenetic changes in gene expression via modifications of chromatin [61], the switch between both states of chromatin

In addition to remote memory storage, memory extinction—in the case of remote fearful memories—also alters structural spine plasticity. For instance, remote memory extinction was found to diversely alter the spine density and spine size in the ACC and infralimbic cortex (ILC) in mice [78]: extinction of a 31-day-old contextual fear memory decreased the density of dendritic spines in the ACC significantly, but not the size. In contrast, the spine density remained elevated in the ILC but the size of spines decreased dramatically. The persistence of spine enlargement in the ACC upon extinction could be essential to warrant that the consolidated fear and the extinction memory traces are kept in a dormant state to allow their reactivation long after training. This may indicate that the extinction *per se* partially remodels the neuronal network supporting the original memory representation. Intriguingly, another study described the opposite effects of fear conditioning and extinction on dendritic spine remodeling in the frontal association cortex (FrA) of rats [79]. Using two-photon microscopy to examine the formation and elimination of postsynaptic dendritic spines of the FrA, the cued fear conditioning caused rapid and long-lasting spine elimination that was significant over 2 and 9 days. After 2 days of extinction training, the spine formation was significantly increased and its degree predicted the effectiveness of the extinction to reduce the conditioned freezing response. These results paradoxically conclude that fear conditioning mainly promotes spine elimination, whereas extinction essentially induces spine formation. More studies in different brain areas will be of high interest to corroborate these findings.

Box 1: Recent insights into structural plasticity and remote fear memory extinction.

is governed by two major epigenetic modifications: DNA methylation and posttranslational modifications (PTMs) on histone tails. DNA methylation refers to the covalent addition of a methyl group to the cytosine base by DNA methyltransferases (DNMTs), while PTMs are the addition and removal of chemical moieties to histone tails, which are dynamically regulated by chromatin-modifying enzymes [22]. These modifications include—but are not limited to—histone acetylation, phosphorylation, and methylation [62] (see Tweedie-Cullen et al., for a complete overview of recently identified PTMs in the brain [63]). Both types of epigenetic modifications are associated with learning and memory, and many recent studies have shown that these epigenetic changes could support memory formation and maintenance through a cascade of specific changes to gene expression including enduring memories.

4.1. DNA Methylation. The first study to investigate the potential role of DNA methylation in regulating memory formation by Sweatt and colleagues showed that *Dnmt* gene expression is upregulated in the adult rat hippocampus following contextual fear conditioning and that its inhibition blocks memory formation [64]. Accordingly, fear conditioning was associated with an upregulation of mRNA levels of the DNMT subtypes that are responsible for *de novo* methylation, DNMT3A and DNMT3B, in the CA1 region 30 min after training. Then, to show that the hippocampal DNMT activity is necessary for memory consolidation, DNMT inhibitors—5-azadeoxycytidine (5-AZA) or zebularine (zeb)—were locally infused right after the training, where they abolished the freezing response of the injected group 24 h after (test day 1). Interestingly, when retrained immediately after test day 1 and retested 24 h later (test day 2), the DNMT inhibitor-treated group showed significantly higher freezing than on test day 1, and when retrained and retested 24 h later (test day 3), they showed equivalent freezing to the vehicle-treated group. But when 5-AZA was infused 6 h after training and animals were tested 18 h later (24 h after training), the inhibitor-injected group displayed normal fear memory indicating that the effect of DNMT inhibition is merely due to blocking consolidation and not due to any other effects on the retrieval or the performance of the animals [64]. These experiments suggest that the transient inhibition of DNMT in the hippocampus following training blocks memory consolidation in a resilient manner that could be reverted as soon as the inhibitor clears off and that the necessary DNA methylation states for consolidation could be reestablished.

In a follow-up study, Miller et al. found a rapid increase in methylation of a memory-suppressor gene in the hippocampal CA1 region 1 h after contextual fear conditioning. Using quantitative real-time PCR, the methylation levels of protein phosphatase 1 (*PP1*), a memory-suppressor gene that is suggested to promote memory decline [65], were dramatically higher in the fear-conditioned group compared to the control group. This increase in methylation was associated with lower levels of *PP1* mRNA, yet the increase in methylation was attenuated and associated with a twofold increase in the mRNA levels when 5-AZA was infused locally 1 h after training. Conversely, a demethylation of a memory-promoting gene was found in the CA1 region 1 h after contextual fear conditioning. The demethylation of *reelin*, a gene that enhances long-term potentiation and the loss of function of which results in memory formation deficits [66, 67], was pronounced in the trained group with its mRNA levels being significantly higher than the control group. DNMT inhibition using 5-AZA led to further demethylation of *reelin* and even higher levels of its mRNA. These data suggest that the DNA methylation is dynamically regulated and that it is a crucial step in memory formation.

Importantly, cortical DNA methylation also seems to support remote forms of memories [68]. The cortical DNA methylation of the memory-suppressor *calcineurin* (*CaN,* also known as *Ppp3ca*), a gene that downregulates pathways supporting synaptic plasticity and memory storage, was investigated using methylated DNA immunoprecipitation (MeDIP) in rats. *CaN*'s cortical DNA methylation persisted for at least 30 d after contextual fear conditioning, and its mRNA levels were significantly reduced in the trained group 2 h after retrieval 30 d after training. Importantly, when

the NMDA receptor antagonist (AP5) was infused into the dorsal hippocampus (CA1) just before training, *CaN* methylation in the dorsal medial prefrontal cortex (dmPFC) 7 d after training was blocked, indicating that a single hippocampus-dependent learning experience is sufficient to drive lasting, gene-specific methylation changes in the cortex. Moreover, intra-ACC infusions of DNMT inhibitors (5-AZA or zeb or RG108) 30 d after training disrupted fear memory and were associated by a significant reduction in the *CaN* methylation levels. However, the infusion of these inhibitors 1 d after training had no effect on fear memory 30 d later [68]. These results indicate that cortical DNA methylation is indeed triggered by a learning experience, and most importantly, its perpetuation supports long-lasting, persistent memories. More detailed studies including investigating DNA methylation changes on a genome-wide scale or within engram-bearing cells are clearly warranted to deepen our knowledge of the implication of these changes in remote memory storage.

4.2. Histone PTMs. Newly formed hippocampus-dependent memories need to be stabilized into a long-lasting ACC-dependent memory trace [46, 69, 70]. Several studies demonstrated that changes in gene expression in both brain regions accompany such stabilization [46, 47]. This differential gene expression has recently been associated with epigenetic modifications in terms of histone PTMs [71]. Using a novel object recognition task on mice, serine (S) 10 phosphorylation on histone (H) 3, lysine (K) 14 acetylation on H3 as well as H4K5 acetylation, and H3K36 trimethylation in the PFC associated with remote (7 d after training) memory consolidation. Importantly, the doxycycline-inducible selective inhibition of the memory-suppressor gene *PP1* in a transgenic mouse line showed improved remote memory performance accompanied by increased histone PTMs. In contrast, blocking the occurrence of these PTMs using a cocktail of inhibitors targeting the epigenetic enzymes responsible thereof impaired remote object memory, suggesting that these histone PTMs are essential for memory consolidation and retention. Finally, these histone PTMs were increased in the promoter region of *Zif268*—an immediate early gene important for memory formation and storage [72]—and its expression levels shift from the hippocampus to the PFC as the memory matures [71]. This study shed light on the spatiotemporal dynamics of these histone PTMs in the hippocampus and cortex and demonstrated that they could act as molecular marks subserving memory consolidation—at least up to 7 d after training.

Similar results were obtained for memory consolidation of social transmission of food preferences [45]. There, associative olfactory memory was linked to a marked increase in H3 acetylation in the OFC 1 h after training, but such increase disappeared upon inactivating the OFC using tetrodotoxin or CNQX. Additionally, increasing the OFC histone acetylation by infusing HDAC inhibitors (sodium butyrate or trichostatin A) was associated by an increase in memory robustness at the remote time point (30 d) [45]. Together, these results stipulate that this cortical epigenetic mark observed very early during training might be essential for tagging these neurons to allocating them to the long-term olfactory memory and that thereafter these neurons will participate in the system consolidation process driven by the HPC-OFC circuitry in order to help this memory to endure. It would be highly interesting to repeat this study with CREB-transfected OFC neurons in order to test this hypothesis.

In addition to histone PTMs, a recent study by Zovkic et al. has shown that a variant of histone H2A (H2A.Z) is actively exchanged in the hippocampus and cortex in response to fear conditioning in mice [73]. H2A.Z is known to be associated with nucleosomes adjacent to the transcription start site (TSS) of a gene, and its presence has been strongly linked to dynamic changes in gene expression [74]. To investigate its effect on transcriptional changes associated with learning, chromatin immunoprecipitation (ChIP) was used. Binding of H2A.Z was reduced at the +1 nucleosome (first nucleosome downstream of the TSS) of memory-promoting genes (*Npas4*, *Arc*, *Egr1*, *Egr2*, and *Fos*), and there was an increase in the expression of those genes 30 min after the contextual fear training. In contrast, H2A.Z binding was increased for the memory-suppressor gene *CaN* and associated with reduced expression of this gene. This suggests that H2A.Z at the +1 nucleosome restricts memory-related gene transcription [73]. Furthermore, the methylation of the promoter region of the gene encoding H2A.Z (*H2afz*) was shown by MeDIP to be increased 30 min after contextual fear conditioning, when it was accompanied by reduced H2A.Z protein expression throughout the hippocampus, whereas the expression levels of H2A.Z returned to baseline after 2 h [73].

To assess a causal involvement of H2A.Z in memory consolidation, an adenoassociated virus (AAV) depleting H2A.Z in the dorsal CA1 region of the hippocampus was used. This approach improved fear memory 24 h and 30 d after training compared to a scramble-injected control group. In contrast, when H2A.Z was depleted from the mPFC, there was no effect on fear memory at the hippocampus-dependent 24 h time point, yet the freezing was significantly higher at remote time points 7 and 30 days after training [73]. Moreover, a genome-wide transcriptional analysis was carried out to evaluate the impact of H2A.Z depletion on training-induced gene expression in CA1 and mPFC 30 min after training. The analysis showed a differential expression—between the trained and untrained groups—in many genes including a number of the early learning-related genes: *Arc*, *Fos*, *Egr1*, and *Egr2* [73]. Although the study did not ascertain the specific target genes through which H2A.Z regulates memory, it clearly demonstrated that H2A.Z is dynamically regulated during learning and memory and that it could be an important epigenetic contributor to the complex coordination of gene expression in memory. Future, more refined studies will certainly help to elucidate the role of histone exchange and histone PTM processes associated with remote memory storage or extinction (Box 2).

5. Summary

The allocation of a memory to a particular neural circuit is a critical step in memory formation. We reviewed how CREB is involved in such process highlighting its important

In addition to memory formation and storage, a recent study also showed an epigenetic involvement into remote fear memory attenuation [80]. In this study, permanent attenuation of remote fear memories was achieved by using a histone deacetylase-2 inhibitor (HDAC2i) in combination with reconsolidation-updating paradigms, which increased the acetylation levels of histone H3K9/14 (AcH3). In contrast to a vehicle-treated control group that was resistant to remote memory attenuation, a significant increase in AcH3 was noticed 1 h after remote fear memory recall in the ACC, which stayed elevated even after the extinction training. In the HPC, no change was observed in the acetylation levels of AcH3 1 h after recall, yet a significant increase was seen in the HDAC2i-treated group after extinction training. More specifically, this observed increase in acetylation in the HDAC2i-treated group was detected in the promoter region of neuroplasticity-related genes such as *cFos*, *Arc*, and *Igf2*, which showed a concomitant increase in expression [81]. This clearly displays that attenuating remote fear memories using an HDAC2i promotes increased histone acetylation-mediated neuroplasticity and in turn demonstrates an epigenetic contribution to this process.

Box 2: Recent insights into epigenetic dynamics of remote memory attenuation.

role. Additionally, electrophysiological studies showed that cells transfected with CREB viral vectors are more excitable compared to the neighboring cells or even those transfected with the control vector [22]. This could partially address the preference of allocating the memory to CREB cells since their increased excitability might render them more responsive to sensory inputs and therefore more likely to get activated during conditioning training. However, it could still be possible that there are other molecular determinants and processes that are important for memory allocation. Indeed, although CREB is ubiquitously expressed, it seems unlikely that memory allocation depends solely on this transcription factor. Likewise, adult neurogenesis is restricted to only certain brain regions, and the data showing that new granule cells when mature are increasingly likely to be incorporated into circuits supporting spatial memory [28, 29] is not necessarily the sole determinant of allocating a memory to a specific neural population.

Another important aspect of memory persistence is which brain regions maintain its storage and what supports such perseverance. We highlighted the importance of the ACC in the upkeeping of remote memories since its inactivation prevents the recall of remote contextual fear memory as well as the reconsolidation of such remote memory 24 h after its retrieval [46, 49]. Intriguingly, a recent study identified for the first time monosynaptic projections from the ACC to the hippocampal CA fields that controls memory retrieval in mice [75]. Using retrograde tracers, this study characterized novel connections between ACC and CA fields (AC-CA) that subserve a potential bidirectional communication between the ACC and the hippocampus. Manipulating these projections optogenetically demonstrated a causal top-down control on memory retrieval, where the cells contributing to the AC-CA projection can activate contextually conditioned fear behavior (3-day-old memory), whereas their inhibition impaired the retrieval of such memory [75]. Nevertheless, further investigations are still needed to elucidate the role of these projections on the regulation of different memory processes.

In fact, the cellular reconsolidation of a remote memory might not solely depend on the ACC since it has been shown previously that infusing anisomycin in the dHPC blocks the reconsolidation of remote contextual fear memory and that optogenetically inactivating the CA1 region would even impair recalling it [12]. Contradictorily, another study did not find any evidence that neither the ACC nor the dHPC is involved in the cellular reconsolidation of remote contextual fear memory following retrieval [76]. More studies are highly anticipated to resolve these divergent findings, although such discrepancy could be partly attributed to the difference in the strength and length of the training and retrieval sessions used or in the inactivation method and its efficiency, since it has been demonstrated that these experimental conditions significantly affect the behavioral outcome [10, 77].

Structural plasticity is another key point towards understanding the endurance of some memories. It provides a physical substrate for the storage of memories. We highlighted the synaptic plasticity that follows memory formation at hippocampal dendrites and that such plasticity reaches cortical areas in a time-dependent manner [16, 17]. Nonetheless, we also shed light on two interesting studies supporting the view of an early cortical reorganization during motor skill learning [54] as well as episodic memory acquisition [38], which demonstrated the importance of such structural changes for lasting memories. The reduced density of spines in cortical areas upon remote fear extinction is in line with these findings and suggests remodeling in the cortical circuit of the original memory [78]. However, a contradicting study showed that it is rather fear memory formation that is accompanied by spine elimination and that extinction involves spine formation [79]. These results are quite confusing, and although they could also be reflecting that opposite processes are at play in different cortical areas, they need to be addressed properly soon.

The epigenetic regulation was the final point we highlighted in this review, and the data we reviewed—collectively—support a dynamic pattern of epigenetic modifications including both DNA methylation [68] and histone PTMs [71] that subserve a spatiotemporal shift of the memory trace from the HPC to higher cortical regions during the process of memory consolidation. Also, the early tagging of certain neurons with epigenetic marks during encoding is central for the memory to be allocated to the tagged neurons and for the subsequent participation of these neurons in the circuit supporting such memory [45]. Furthermore, the extinction of remote fear memories with an HDAC2i increased histone acetylation-mediated neuroplasticity [80], and the lack of such plasticity from the hippocampus upon

remote memory recall supports the idea of hippocampal disengagement for remote memories [46, 48, 55]. Nevertheless, whether memories might indeed be "coded in particular stretches of chromosomal DNA" as originally proposed by Crick [57] and if so what the enzymatic machinery behind such changes might be remain unclear. In this regard, cell population-specific studies are highly warranted.

Taken together, we find ourselves in an exciting period witnessing an increasing number of studies, which dare to investigate remote memory formation, storage, and persistence. Yet it is clear that we are still in need of further investigations to unveil the dynamics of neuronal circuits and molecular mechanisms mediating such persistence. Ultimately, deciphering these processes would definitely contribute to the understanding, and possibly dulling, of abnormally long-lasting fear memories like those underlying anxiety disorders or posttraumatic stress disorder.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

The Three-Dimensional Culture System with Matrigel and Neurotrophic Factors Preserves the Structure and Function of Spiral Ganglion Neuron *In Vitro*

Gaoying Sun,[1,2] Wenwen Liu,[1,2] Zhaomin Fan,[1] Daogong Zhang,[1] Yuechen Han,[1] Lei Xu,[1] Jieyu Qi,[3,4] Shasha Zhang,[3,4] Bradley T. Gao,[5] Xiaohui Bai,[1,2] Jianfeng Li,[1,2] Renjie Chai,[3,4] and Haibo Wang[1,2]

[1] *Otolaryngology-Head and Neck Surgery, Provincial Hospital Affiliated to Shandong University, Jinan 250022, China*
[2] *Shandong Provincial Key Laboratory of Otology, Jinan 250022, China*
[3] *Key Laboratory for Developmental Genes and Human Disease, Ministry of Education, Institute of Life Sciences, Southeast University, Nanjing 210096, China*
[4] *Co-Innovation Center of Neuroregeneration, Nantong University, Nantong 226001, China*
[5] *College of Medicine, The University of Tennessee Health Science Center, Memphis, TN 38163, USA*

Correspondence should be addressed to Renjie Chai; renjiec@seu.edu.cn and Haibo Wang; wang.hb7585@hotmail.com

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Whole organ culture of the spiral ganglion region is a resourceful model system facilitating manipulation and analysis of live sprial ganglion neurons (SGNs). Three-dimensional (3D) cultures have been demonstrated to have many biomedical applications, but the effect of 3D culture in maintaining the SGNs structure and function in explant culture remains uninvestigated. In this study, we used the matrigel to encapsulate the spiral ganglion region isolated from neonatal mice. First, we optimized the matrigel concentration for the 3D culture system and found the 3D culture system protected the SGNs against apoptosis, preserved the structure of spiral ganglion region, and promoted the sprouting and outgrowth of SGNs neurites. Next, we found the 3D culture system promoted growth cone growth as evidenced by a higher average number and a longer average length of filopodia and a larger growth cone area. 3D culture system also significantly elevated the synapse density of SGNs. Last, we found that the 3D culture system combined with neurotrophic factors had accumulated effects in promoting the neurites outgrowth compared with 3D culture or NFs treatment only groups. Together, we conclude that the 3D culture system preserves the structure and function of SGN in explant culture.

1. Introduction

In mammalian cochleae, spiral ganglion neurons (SGNs) are specialized bipolar neurons that transmit auditory information from ear to brain. SGNs originate from the cochleovestibular ganglion [1, 2] and then mature along with the specialization of hair cells (HCs) within the prosensory domain of the cochlea. Finally, the SGNs extend peripheral axons that couple to HCs at the ribbon synapse [3], through which the SGNs transmit auditory information from HCs to the central nervous system. Therefore, the survival of SGNs is indispensable for the preservation of hearing, and damage to SGNs exacerbates hearing loss [4]. However, the mechanisms underlying the stimulation and guidance of neurite outgrowth from SGNs to their targets are still unclear and are in need of further exploration.

The two traditional approaches of dissociated cell culture and conventional organotypic culture have been commonly used for culturing SGNs in previous research. Dissociated cell culture has been widely used to study the survival [5, 6] and neurite regeneration [7] of SGNs. Although the dissociated spiral ganglion cell culture contains all of the cell types in the spiral ganglion region and might also contain the soluble factors that are provided by glia cells (GCs) for

the support of the SGNs, the cell-cell adhesions are disrupted and the physiological cell-cell interaction between SGNs and GCs is destroyed. SGNs are arranged in a three-dimensional (3D) structure in the cochlea *in vivo*; thus, it is necessary to maintain this environment if one wants to observe how SGNs act normally in biological processes and how they react to stress [3]. The conventional organotypic culture involves adhering the freshly dissected spiral ganglion organ onto the dish surface, and this type of culture is also called two-dimensional (2D) culture [8, 9]. Conventional 2D organotypic culture has been used to explore the effects of small molecules on the development of SGN neurites [9] and the changes of gene expression in response to injury and hypoxia [10] and so on. However, because it is unable to provide the physical scaffold needed for the outgrowth of SGNs, neurites extending from spiral ganglions under 2D conditions are very few and very short [11, 12], and this makes the 2D culture a less than ideal culture system to study the structure and function of SGNs. Therefore, the 3D culture system, which can encapsulate SGNs to mimic the normal 3D environment *in vivo*, is regarded as the ideal model to preserve the delicate structure and function of SGN explants in culture.

Among all kinds of 3D culture systems, matrigel, which is a reconstituted basement-membrane-like matrix (BM), has been successfully used as a 3D culture model in inner ear research. For instance, Edin et al. cultured human superior vestibular ganglions in 3D-matrigel *in vitro* to investigate inner ear neuron regeneration [13], and Spencer et al. have used matrigel as a scaffold for chicken cochlear cultures to develop an HC regeneration model [14]. These studies have demonstrated the bioactivity and tissue compatibility of matrigel in inner ear tissues. However, the effect of 3D-matrigel culture in maintaining the structure and function of SGNs in explant culture remains uninvestigated.

In this study, we succeeded in establishing the 3D culture system with matrigel to encapsulate the spiral ganglion region isolated from neonatal mice. This system was able to protect the SGNs against apoptosis, promote the growth of growth cone, boost the neurite sprouting and outgrowth, and elevate the synapse density of SGNs. When combined with neurotrophic factors, 3D system also has accumulated effect to significantly promote the neurites outgrowth. In conclusion, the 3D-matrigel culture system preserved the structure and function of SGN in short-term culture, which may be applicable to the *in vitro* study of the physiology and pathophysiology of the inner ear.

2. Materials and Methods

2.1. Animals and SGN Dissection. All experiments were approved by the Animal Care Committee of Shandong University, China, on the care and use of Laboratory Animal for Research Purposes. Postnatal 3-day-old (P3) C57BL/6 mice were purchased from the Animal Center of Shandong University (Jinan, China).

The dissection procedures were performed as described in previous studies with slight modifications [9, 15]. P3 C57BL/6 mice were decapitated and skulls were opened midsagittally with a pair of small surgical scissors. Immediately, temporal bones of two sides were cut off and placed into sterile Hank's Balanced Salt Solution (Hyclone, USA) on a flat ice pack. The cochlear capsule was removed by fine forceps to expose the membranous labyrinth under a dissecting microscope. The stria vacularis and the organ of Corti were removed and the middle turn was chosen to keep sampling consistence between groups. After discard of the modiolus, the remnant SGN bulk was cut into 4 equal portions about 200 μm in diameter each.

2.2. Organotypic Cell Culture. There were two different media used in the study; the primary growth medium (PGM) consists of 10% fetal bovine serum (Gibco, USA) and 50 μg/mL ampicillin in DMEM/F12. The full medium (FM) was supplemented with N2 (1 : 100, Invitrogen, USA), B27 (1 : 50, Invitrogen), epidermal growth factor (EGF, 20 ng/mL, Sigma, USA), basic fibroblast growth factor (bFGF, 10 ng/mL, Sigma), insulin-like growth factor-1 (IGF-1, 50 ng/mL, Sigma), heparan sulfate (50 ng/mL, Sigma), and ampicillin (50 μg/mL, Sigma) in DMEM/F12 (Gibco) and should be used within two weeks.

Isolated SGN explants were adhered onto a 10 mm glass coverslip precoated with CellTak (BD Biosciences, USA) and cultured in PGM in 4-well dish (Greiner Bio-One, Germany) overnight without any treatments. Then, the next day, the media were changed into FM combined with different treatments as follows.

For 2D culture, explants were kept in FM without any additives, as the uniform control of the other treated groups.

For 3D culture, the concentrations of matrigel added in FM include 2%, 10%, 20%, and 50%. Correspondingly, 2 μL, 10 μL, 20 μL, or 50 μL ice-cold matrigel was mixed together with 98 μL, 90 μL, 80 μL, or 50 μL FM and dropped on to the tissue explants directly. The gelation was initiated to solidify immediately at room temperature and was completed 10 min later. Then, the culture dish was transferred to the incubator.

For neurotrophic factor- (NF-) treated groups, 10 ng/mL BDNF (R&D Systems, USA) and 10 ng/mL NT3 (R&D Systems, USA) were added in the 2D culture or 20% matrigel 3D culture system, which were regarded as the 2D-NF or 3D-NF group, respectively.

All treated explants were then cultured for 48 h or 7 days at 37°C, 5% CO_2, and 95% humidity.

2.3. Immunostaining. After organotypic culture, SGN explants were incubated in ice-cold PBS at 4°C for 30 min and washed twice with PBS. In order to keep the architecture structure of SGNs, the coated matrigel was carefully removed except that the surrounded cultured tissue, followed by the fixation with 4% paraformaldehyde and permeabilization with 1% TritonX-100 in PBS (Sigma), samples were immersed in blocking solution (0.1% TritonX-100, 8% donkey serum, 1% bovine serum albumin, and 0.02% sodium azide in PBS) at room temperature for 1 h. Then, samples were incubated with different primary antibodies: β-tubulin (1 : 1000, Neuromics, USA), neurofilament (1 : 1000, Millipore, USA), and synaptophysin (1 : 1000, Millipore), diluted in blocking solution, respectively, at 4°C overnight. The next day, tissues were incubated with FITC-conjugated or TRITC-conjugated (1 : 1000,

Table 1: PCR primer sequences used in the experiments.

Gene	Forward sequence	Reverse sequence
Casp8	GCTGTATCCTATCCCACG	TCATCAGGCACTCCTTT
Casp9	GGACCGTGACAAACTTGAGC	TCTCCATCAAAGCCGTGACC
Casp3	GGAGCAGCTTTGTGTGTGTG	CTTTCCAGTCAGACTCCGGC
Apaf1	TGTGTGAAGGTGGAGTCAAGG	CCTCTGGGGTTTCTGCTGAA
Bcl-2	TGACTTCTCTCGTCGCTACCG	GTGAAGGGCGTCAGGTGCAG
Bax	CGTGGTTGCCCTCTTCTACT	TTGGATCCAGACAAGCAGCC

Invitrogen) secondary antibody along with DAPI (1 : 800, Sigma-Aldrich) or phalloidin (1 : 1000, Sigma-Aldrich) in 0.1% TritonX-100 and 1% BSA in PBS at room temperature for 1 h. Then, the coverslips were mounted and observed under a laser scanning confocal microscope (Leica, Germany).

2.4. Terminal Deoxynucleotidyl Transferase dUNT Nick End Labeling (TUNEL) Detection. Cell apoptosis was studied by DNA fragmentation with a TUNEL staining kit (Click-iT Plus TUNEL Assay for *In Situ* Apoptosis Detection, Invitrogen) according to the manufacturer's instruction. The nucleus was stained with DAPI, and the explants were evaluated using the confocal microscopy (Leica, Germany).

2.5. RNA Extraction and Real-Time Polymerase Chain Reaction (RT-PCR). After being cultured in the 3D-matrigel for 48 h, SGN explants were firstly incubated in ice-cold PBS at 4°C for 30 min and the coated matrigel turned into liquid form; then, the explant was washed three times with ice-cold PBS to remove the matrigel clearly. Then, 20 explants were collected into TRIzol (Life Technologies, USA) to obtain the total RNA following the manufacturer's instructions. The RNA concentration was measured with a Bio-Rad spectrophotometer. cDNA was synthesized from 1 μg total RNA by reverse transcription using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the manufacturer's protocols. Quantitative real-time PCR (RT-PCR) was performed using SYBR Premix Ex Taq (TaKaRa, Japan). GAPDH was used as housekeeping gene. Each 25 μL PCR reaction mixture contained 12.5 μL 2X SYBR Green PCR Master Mix, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 2 μL template, and 9.5 μL sterilized distilled water. Each group contained three samples and each PCR was carried out in triplicate. The conditions of PCR were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 30 s followed by dissociation at 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. All data were analyzed using the Eppendorf Realplex 2. PCR primers for the genes were listed in Table 1.

2.6. Image Analysis. Confocal images of SGNs were taken using a Leica SPE confocal fluorescence microscope (Leica). Z-stack images were taken at 0.2 μm intervals to span the samples. The number, length, height, and area of the neurite outgrowth were evaluated separately. As shown in the schema diagram of Figures 2(a)–2(d), the number of SGN neurites directly extended from each explant was counted, the length of neurite was measured from the base of the neurite at the explant edge to its farthest end, and the distance between the upper and the lower planes of stained neurites was regarded as the average height of neurites in SGN explant. As for the area measurement, all the far ends of neurites of each explant were connected to form an irregular closed shape, whose area was calculated by Image J software.

2.7. Statistical Analysis. For each processing condition, at least three individual experiments were conducted. Data were presented as mean ± SEM (standard error of the mean), and comparisons between groups were tested by Student's t-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Establish the 3D Culture System and Optimize the Concentration of Matrigel. After being cultured *in vitro* for 7 days, explants were immunostained with the SGNs marker β-tubulin and observed under the confocal microscope. As illustrated in Figure 1(c), in the 2D cultured group, only several neurite outgrowths were extended from the explant, and with limited length. The 3D culture with matrigel promoted the growth of SGN neurites remarkably (Figure 1(c)). We compared four parameters of the neurite outgrowth (the number, length, height, and area) to analyze the effect of 3D culture statistically, and as shown in Figures 2(e)–2(h), 2% matrigel did not increase the growth of neurite compared with the 2D culture control group, except for slight increase in the number of neurites (data not shown here), while 10% matrigel culture significantly promoted the number, height, and area of the neurites to 2.57-fold, 1.17-fold, and 1.25-fold ($p < 0.01$, $p < 0.05$, and $p < 0.05$) that in control group. Remarkably, 20% matrigel dramatically increased all four parameters of SGN neurite outgrowth, as the average number, length, height, and area of neurite have been raised to 9.31-fold, 1.50-fold, 1.60-fold, and 2.00-fold of control ($p < 0.001$, $p < 0.05$, $p < 0.001$, and $p < 0.001$, resp.), while 50% matrigel increased the number, height, and area to 8.87-fold, 1.58-fold, and 1.50-fold ($p < 0.001$, $p < 0.001$, and $p < 0.001$), but the length of neurites has no significant difference compared with the control group. Thus, these data suggested that 20% matrigel could benefit the cultured SGN explants the most as they grew much more and longer neurites, as well as extended larger height and area, and was chosen for the 3D culture in the following experiments.

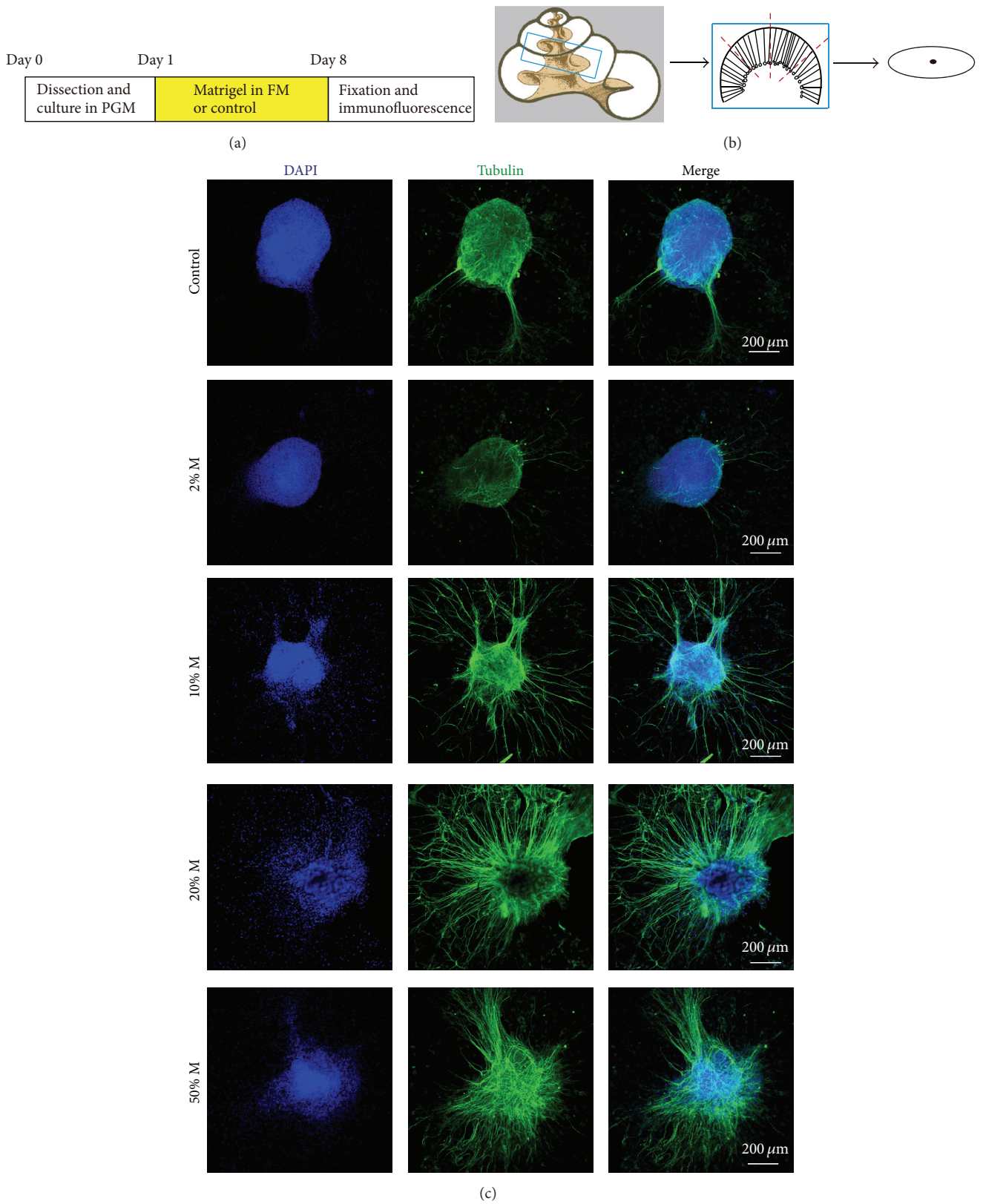


Figure 1: Immunofluorescence imaging of SGN explants cultured with different concentrations of matrigel. (a) The diagram of the assay. (b) The schematic of dissection and culture of SGN explants from neonatal mice cochleae. The middle turn of P3 cochlea was dissected out, the stria vascularis, the organ of Corti, and modiolus were discarded, and remnant SGN bulk was cut into four equal pieces, attached to coverslips, and cultured. (c) Representative SGN explants stained with DAPI (blue) and anti-β-tubulin (green) antibody for each experimental condition.

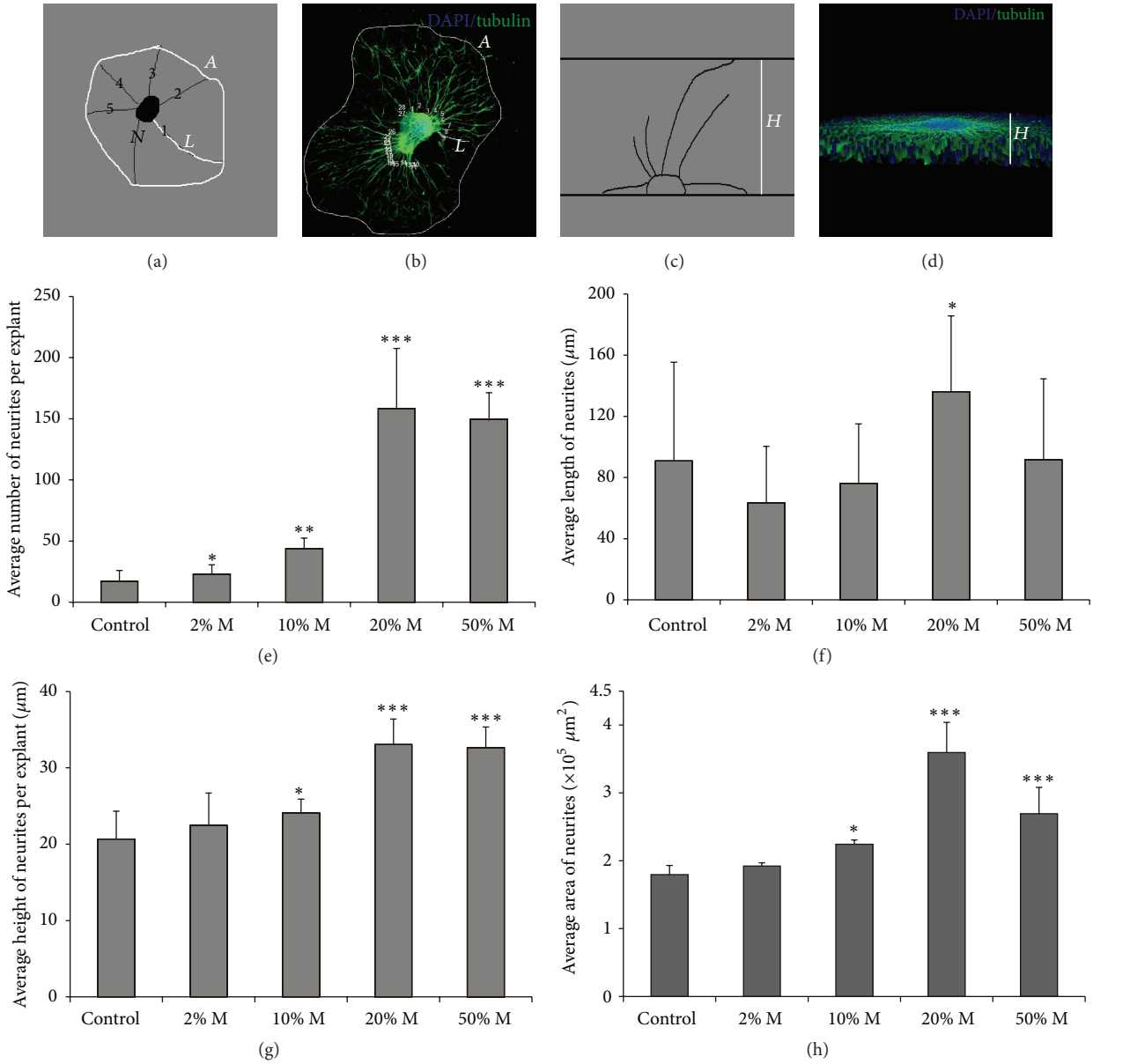


Figure 2: 3D-matrigel culture promoted neurite outgrowth of SGN explants. (a) The planar characteristics of SGN explants, the number (N), the length (L), and the area (A) of neurites, were measured. (b) SGN explant as the illustration for number, length, and area of neurites. (c) The schematic of the height (H) of neurites in SGN explant. (d) SGN explant as the illustration for the height of neurites. (e–h) Quantifications of the average number, length, height, and area of neurites. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3.2. 3D Culture Protects the SGN Explants against Apoptosis. Taking advantage of TUNEL assay to detect the apoptosis, we observed significant amount of apoptotic SGNs in 2D group after being cultured for 48 h *in vitro* and found that the 3D culture system had significant protective effect against apoptosis on the SGN explants. As evidenced in Figure 3, apoptotic SGNs showed an abnormal or irregular morphology such as the breakage of nerve fibers, shrinkage of neurons, and nuclear condensation. The TUNEL assay confirmed that 2D culture caused apoptotic death in SGNs as there were 10.17 ± 2.87 TUNEL-β-tubulin double positive neurons out of one explant. However, very few TUNEL-β-tubulin double positive cells were found in 3D culture system with 20% matrigel (Figure 3(c)), suggesting that the 3D condition could be helpful in reducing apoptosis and surviving longer for the SGN explants.

Furthermore, RT-PCR data showed that SGN explants cultured in 3D-matrigel system had significantly lower expression of proapoptotic genes Casp8, Casp9, Casp3, Apaf1, and Bax compared to the 2D group ($p < 0.01$, Figure 3(d)), while they had significantly higher mRNA expression of anti-apoptotic gene Bcl-2 in the 3D group ($p < 0.001$, Figure 3(d)).

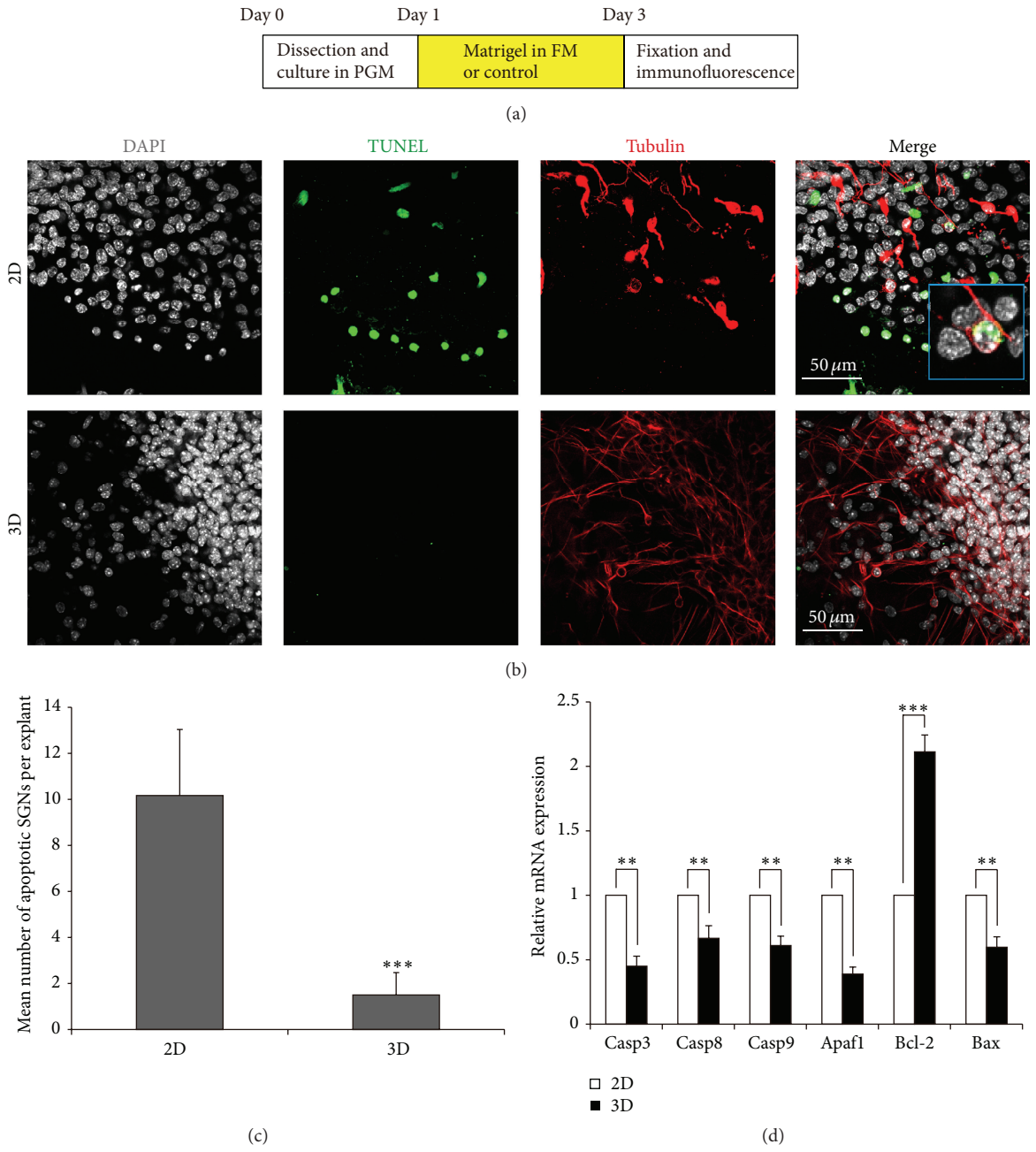


FIGURE 3: 3D culture protected SGN explants from apoptosis. (a) The diagram of the assay. (b) Apoptotic SGNs (white arrow) were observed in 2D culture, characterized by DAPI (grey), TUNEL (green), and β-tubulin (red) costained. (c) Compared with 2D culture, 3D culture reduced the average number of apoptotic SGNs. $^{***}p < 0.001$. (d) 3D culture regulated the mRNA expressions of apoptotic genes. $^{**}p < 0.01$ and $^{***}p < 0.001$.

These data demonstrated that 3D culture with matrigel could regulate the expression of apoptosis related genes and protected the SGN explants from apoptosis.

3.3. 3D Culture Preserves the Delicate Structure of SGN Explants. We found some abnormal growth patterns of SGN neurites in the 2D cultured group after being cultured for 7 days *in vitro*, such as reversal, fasciculation, curling, and swelling, which were absent in the 3D cultured groups. Figure 4(c) showed the reversal performance of the neurites, instead of extending out from the edge of explant and developing in a radial structure as in the 3D group, some neurites grew back to the original explant and in a disordered model. Sometimes, neurites changed their radial growth direction from the edge of the explant and clumped into fascicles (Figure 4(b)), some curved in the process (Figure 4(d)), which were regarded as fasciculation and curling, respectively. Moreover, a prominent swelling could

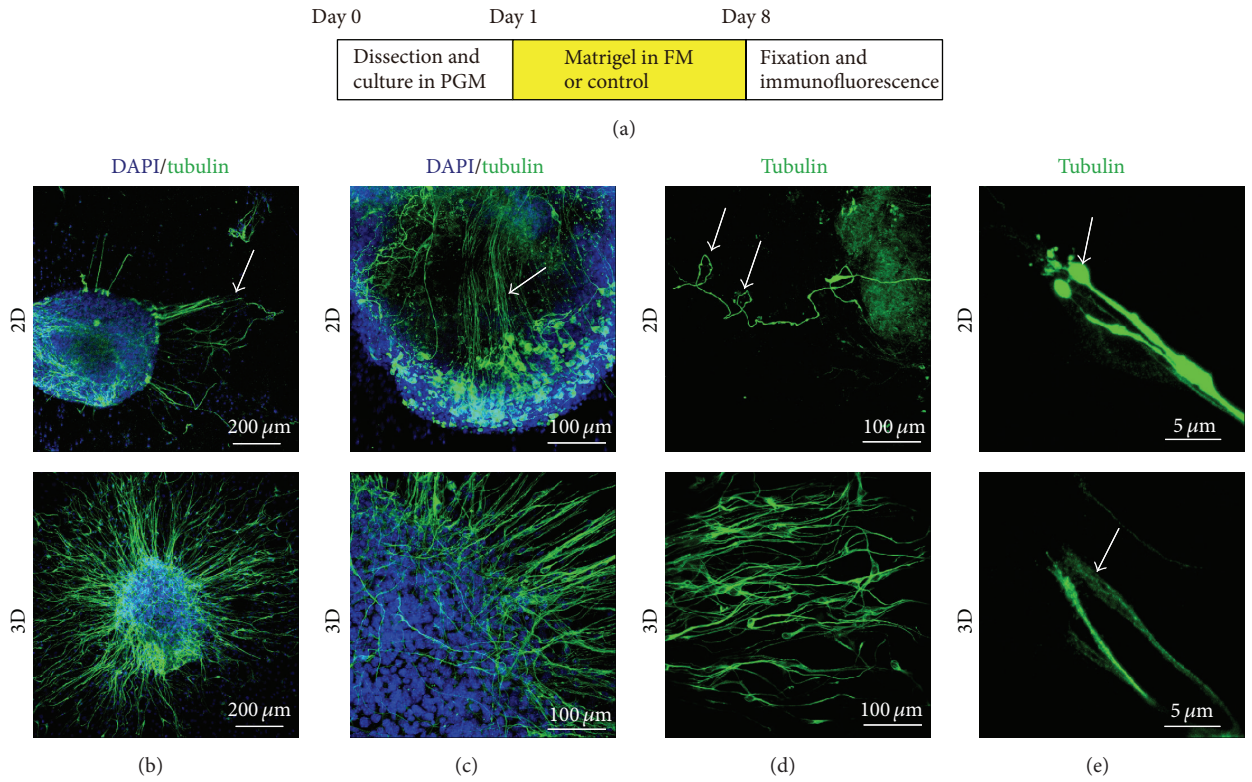


Figure 4: 3D culture preserved the delicate structure of neurites in SGN explants. (a) The diagram of the assay. Fasciculation (b), reversal (c), curling (d), and swelling (e) observed on SGN explants in 2D group. DAPI, blue; β-tubulin, green.

be seen at the termination of some neurites in 2D culture group (Figure 4(e)). However, none of these patterns was found in the 3D cultured groups. These results indicated that 3D culture preserved the delicate structure in the extension of neurites of SGN explants.

3.4. 3D Culture Promotes the Growth of Growth Cones. Next we investigated the effect of 3D culture on the SGN growth cones by comparing the number, length of filopodia, and the area of growth cone after being cultured for 48 h *in vitro*. We stained the cultured SGN with phalloidin and β-tubulin and found the average number of filopodia emerging from the growth cones was greater when cultured in 3D culture system compared to the 2D group ($p < 0.01$, Figure 5(c)). Similarly, the average filopodia length from the tips of individual filopodia to the edge of the growth cone was longer ($p < 0.05$, Figure 5(d)), and the growth cone area of neurites was larger when cultured under 3D condition than those under 2D system ($p < 0.01$, Figure 5(e)). Altogether, the 3D culture condition facilitated the growth of SGN growth cones and the development of filopodia compared to the 2D group.

3.5. 3D Culture Enhances the Sprouting of Neurites in SGN Explants. The sprouting of neurites in SGN explants was measured after being cultured for 7 days *in vitro*, and Figure 6(b) illustrated that more dendrites were extended from the cell bodies of SGNs in explants in 3D culture systems than in the 2D group (Figure 6(b)). Statistical analysis showed that the number of primary dendrites, the total branch length, the number of branch tips, and the number of branch points were all elevated significantly in 3D group compared to the 2D culture group (Figures 6(c)–6(f), $p < 0.05$, $p < 0.001$, $p < 0.01$, and $p < 0.001$, resp.), suggesting that 3D culture promoted neurite sprouting and outgrowth of SGN explants.

3.6. 3D Culture Elevates Synapse Density in SGN Explants. To further investigate the effect of 3D culture on the function of organotypic SGNs, we also detected the synaptophysin expression in neurites to analyze the synapse density. Synaptophysin is a membrane protein specific to synaptic vesicles and correlates directly with the presence of neurotransmitter [16, 17]. Therefore, it also serves as a specific presynapse marker. Representative images after culture in 20% matrigel system for 7 days were shown in Figure 7. 3D system significantly elevated the synapse density during culture compared to 2D controls (Figures 7(b) and 7(c)). Statistical analysis proved that synaptophysin puncta density was significantly increased in 3D system compared to 2D group ($p < 0.01$, Figure 7(d)), indicating that 3D system promoted synapse maturation and synaptic plasticity in SGN explant culture.

3.7. Synergistic Influence of Matrigel 3D System with BDNF and NT3 on Neurite Outgrowth. In the auditory system, diverse factors are reported to play important roles in the development and morphology of SGNs, including brain-derived neurotrophic factor (BDNF) and neurotrophin-3

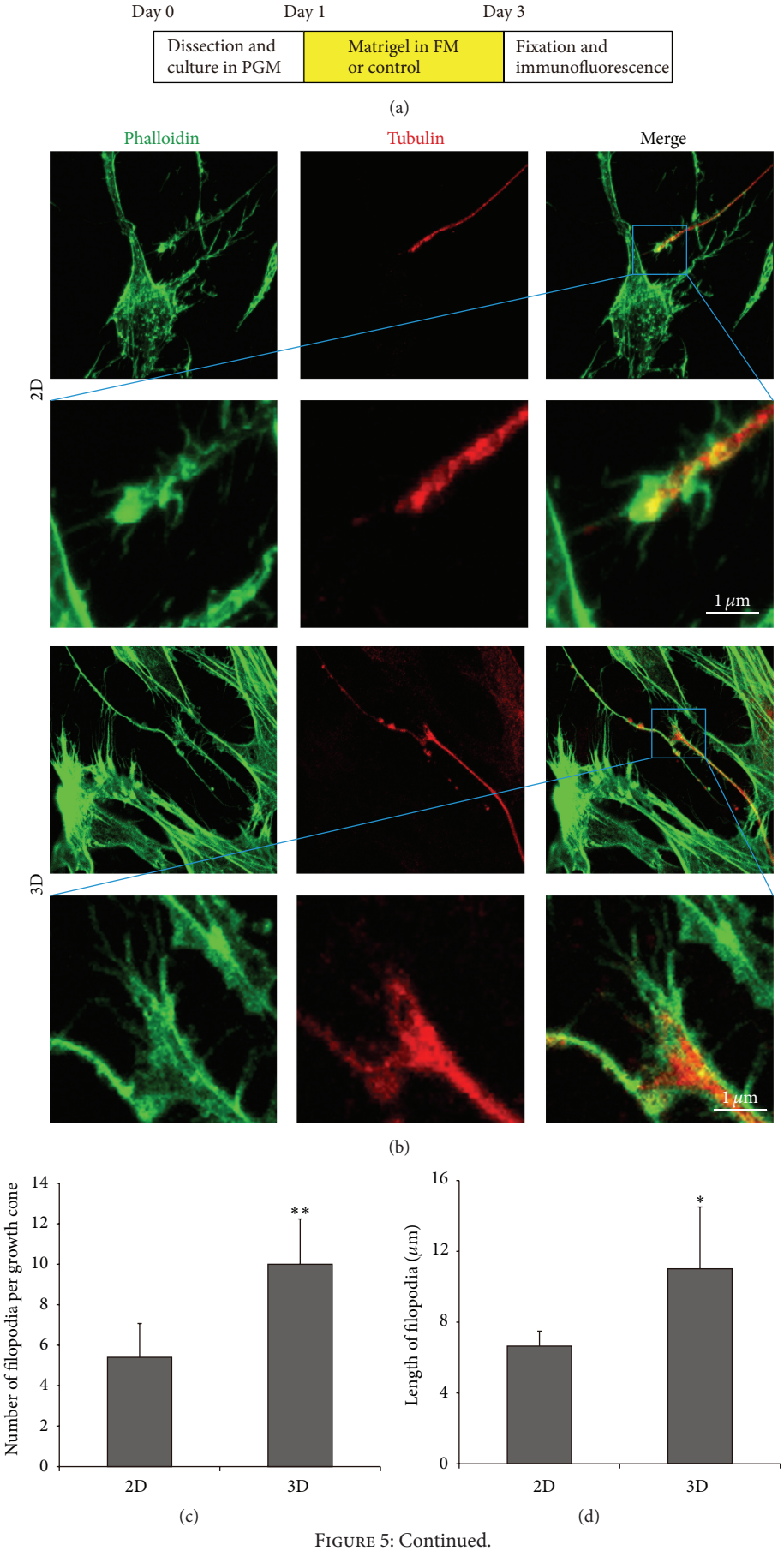


FIGURE 5: Continued.

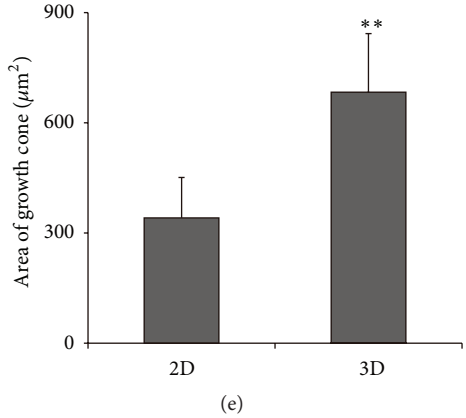


Figure 5: 3D culture affected the growth of growth cones. (a) The diagram of the assay. (b) Morphology of the SGNs growth cone cultured in 2D and 3D systems. Phalloidin, green; β-tubulin, red. (c) Quantification of the average number of filopodia per growth cone. (d) Quantification of the average length of filopodia from the edge of the growth cone to tips of each filopodia. (e) Quantification of the average area per growth cone. $^{*}p < 0.05$ and $^{**}p < 0.01$.

(NT3). Based on the above findings that 3D-matrigel culture system could promote the outgrowth of SGNs, we further explored the combined effects of 3D culture system together with NFs on SGN explants after 7 days in culture. As shown in Figure 8, treatment with BDNF and NT3 in 3D system significantly increased the number and area of neurites compared to NFs-only and 3D-only groups ($p < 0.01$, $p < 0.001$, $p < 0.001$, and $p < 0.001$, resp.), but not the length of SGNs. Moreover, the number, height, and area of neurites were significantly enhanced in the 3D-NF group when compared with the 2D-NF group ($p < 0.001$, $p < 0.001$, and $p < 0.001$, resp.). Altogether, these results demonstrated that 3D culture system combined with NFs has accumulated effects in promoting the neurites outgrowth, indicating the BDNF and NT3 have synergistic influence on the neurite outgrowth of SGN explants, especially under the 3D-matrigel culture condition.

4. Discussion

Whole-organ culture of the organ of Corti has been used as the popular model to study both the biology and physiology of cochlear cell types and also the pathologic processes affecting them. Specifically, the 2D culture has been used to examine the effects of growth factors on SGNs [18–21]. Although this method is simple and provides a viable culture for SGN explant, it does not adequately preserve the natural complex 3D structure of the cochlea. In this study, we used the matrigel to construct a 3D culture system to wrap up the SGN tissue bulk *in vitro*. To our knowledge, this is the first report about the 3D culture system that supplied physical support for the murine SGN explant and maintained its physiological structure and function.

Matrigel is a commercially available analog of physiological BM, which has been used to make different kinds of 3D culture models [22–24]. In the present study, the 3D-matrigel culture system promoted the neurite outgrowth of SGN explant significantly compared with the 2D group; not only were the planar characteristics, including the length and number of SGNs neurites, increased, but also the spatial attributes, such as height and area of the neurite outgrowth, were enlarged remarkably, and the latter factors were thought to be more important for maintaining the space structure of spiral ganglion region. Meanwhile, the 3D culture preserved the delicate structure of SGN explants as no abnormal growth patterns of SGN neurites were observed in the 3D cultured group. Thus, these results suggested that the culture system built by matrigel could be used to mimic the 3D organization of SGNs under physiologic conditions. Furthermore, our results also revealed that the effect on neurite outgrowth of SGN explants was dependent on concentrations of matrigel, and the 20% matrigel was considered as the most effective concentration as it promoted all the four parameters tested in the experiment. One possible reason for this is that the physical property of matrigel is related to its concentration: in our study, 20% matrigel became jelly-like substance at 37°C, while 2% matrigel was still liquid and cannot provide a 3D condition, or 50% matrigel was too thick which might have limited the tension and spreading of SGN neurites.

Previous studies reported that SGNs in 2D cultured explants inevitably degenerate in a few days [25], and 3D system is thought to be the potential way to improve the culture condition and to preserve the structure and function of cultured explants. In our research, we also observed significant amount of apoptotic SGNs in the 2D culture group as evidenced by TUNEL assay and RT-PCR results (Figure 3), while in 3D culture system very few apoptotic SGNs were observed, significant lower expression of proapoptotic genes and higher expression of antiapoptotic gene were detected (Figure 3(c)), suggesting that the 3D condition significantly reduced the apoptosis and enhanced the survival of SGN explants. These findings therefore allow us to speculate that the 3D environment not only provided the scaffold to support the SGN explant but also conducted the protective action by building up a 3D structure to provide larger surface area and better nutrient transmission. For the matrigel 3D culture

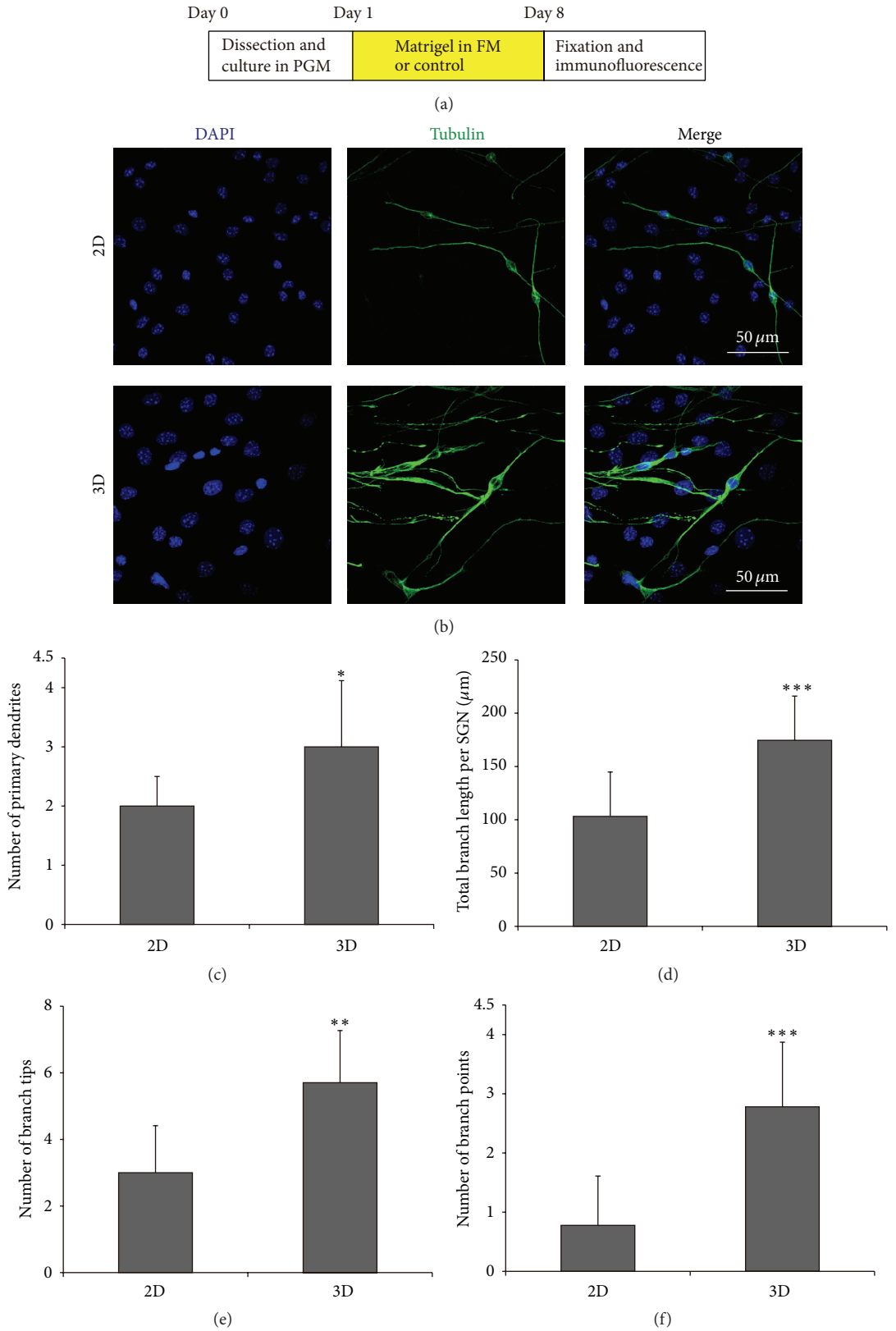
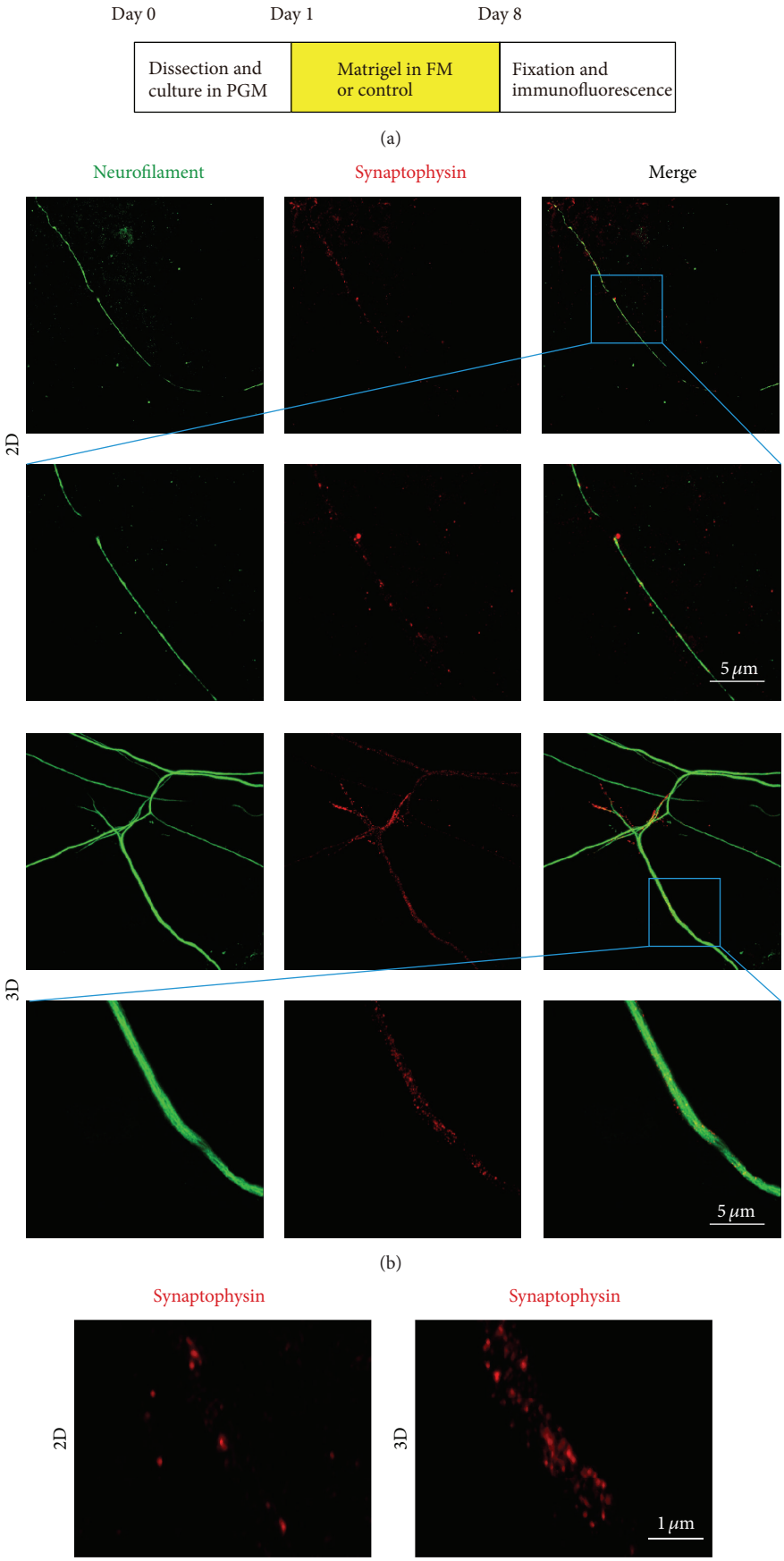


FIGURE 6: 3D culture improved the sprouting of neurites in SGN explants. (a) The diagram of the assay. (b) Representative images of SGNs explants cultured in 2D and 3D systems. DAPI, blue; β-tubulin, green. (c–f) Quantifications of the average number of primary dendrites, total branch length, number of branch tips, and number of branch points in 2D and 3D systems. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.



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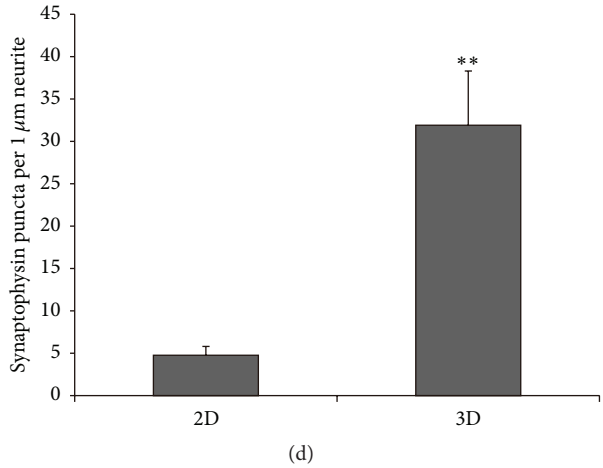


(d)

Figure 7: 3D culture elevated synapse density in SGN explants. (a) The diagram of the assay. (b) Representative images of SGNs explants immunostained with neurofilament (green) and synaptophysin (red) cultured in 2D and 3D systems. (c) Representative images in high magnification of synaptophysin (red) immunostained SGN explants in 2D and 3D systems. (d) Quantification of the number of synapse puncta in 2D and 3D systems. $^{**}p < 0.01$.

system, the major component of matrigel is laminin, but the culture media also contain EGF, IGF-1, bFGF, and other growth factors. Since the 3D structure system provided larger surface and better nutrient transmission, the growth factors in the culture media could be better taken inside into the cultured organs. It has been reported that the FGF participated in SGN development and support their postnatal survival following differentiation [26, 27]; the FGF/FGF receptor signaling affected the C-Jun-N-terminal kinase- or caspase-dependent pathways to alter apoptosis in cochlea [28]. EGF receptor has been found to be expressed in both the neonatal and adult mice spiral ganglion [29, 30], and IGF-1-deficient cochlear neurons showed increased caspase-3-mediated apoptosis [31]. Therefore, the better uptake of these growth factors in 3D-matrigel system might regulate the above signaling pathways in SGNs and might be responsible for the downregulation of proapoptotic factors and the increased survival ratio, but this still needs to be further explored in the future.

Growth cone is dynamic, actin-supported extension of a developing neurite seeking its synaptic target. Filopodia are the dominant structures in growth cones. In the present study, after being cultured in the 3D-matrigel culture system, the number, length, and area of filopodia in growth cones were enhanced significantly compared to the 2D culture group (Figure 5). In addition, the number of primary dendrites, the total branch length, the number of branch tips, and the number of branch points were also significantly elevated in 3D-matrigel culture system. Altogether, these results suggest that the 3D culture condition facilitated the growth of growth cones, the development of filopodia, and the neurite sprouting and outgrowth compared to the 2D system.

In mammals, sound information is detected by mechanosensitive HCs and transmitted to SGNs through ribbon synapses. By examining somatic presynaptic and postsynaptic protein content in SGNs new insights into the functional organization of the auditory primary afferents can be provided. In our research, synaptophysin was used as the presynapse marker, and the expression was enhanced in the 3D culture group compared to the 2D group, demonstrating that the synapse density was significantly improved under the 3D culture condition, which further suggests that the 3D-matrigel culture system has the potential to promote the synapse maturation and synaptic plasticity of SGNs.

In the auditory system, BDNF and NT3 are predominant NFs, which have distinct functions in the development and maintenance of SGNs in developing inner ear [32]. In our study, treatment with BDNF and NT3 to SGN explants increased the number and area of neurites both in 2D and 3D systems compared with those of the NF-only and 3D-only groups separately. More importantly, the number, height, and area of neurites were significantly enhanced in the 3D-NF group when compared with the 2D-NF group, indicating that 3D culture system has accumulated effects in promoting the neurites outgrowth when combined with NFs. However, the length of neurite was not affected by the administration of BDNF and NT3 in both 2D and 3D systems, which is consistent with the previous studies in 2D culture system [8].

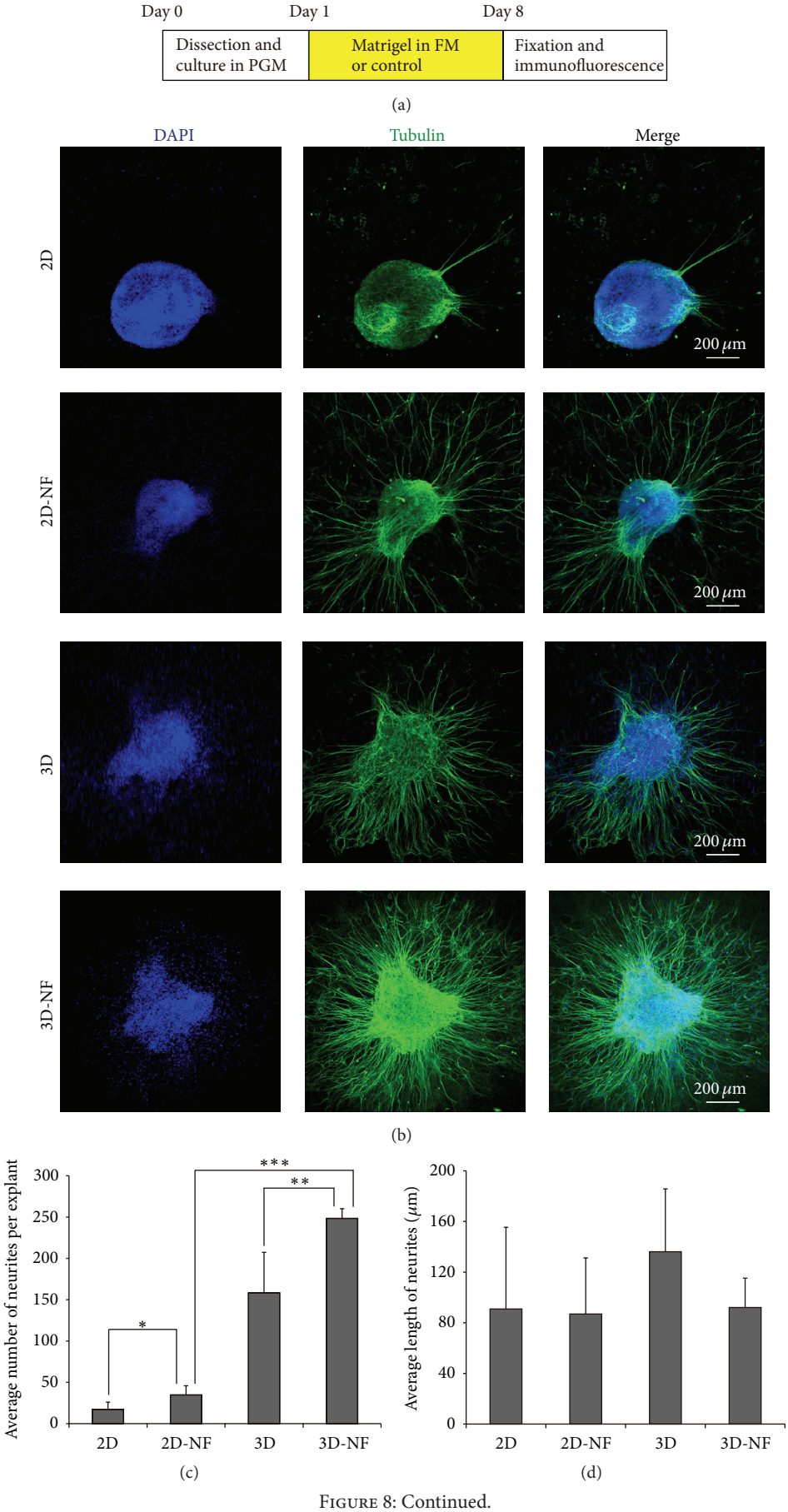
Taken together, 3D culture system with matrigel exhibited excellent biocompatibility and promoted the survival, structure, outgrowth, and function of SGNs throughout the culture period compared to the 2D culture. The present study highlighted the potential of matrigel as a 3D culture system applied to the *in vitro* study of the physiology and pathophysiology of the inner ear.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Gaoying Sun and Wenwen Liu contributed equally to this work.



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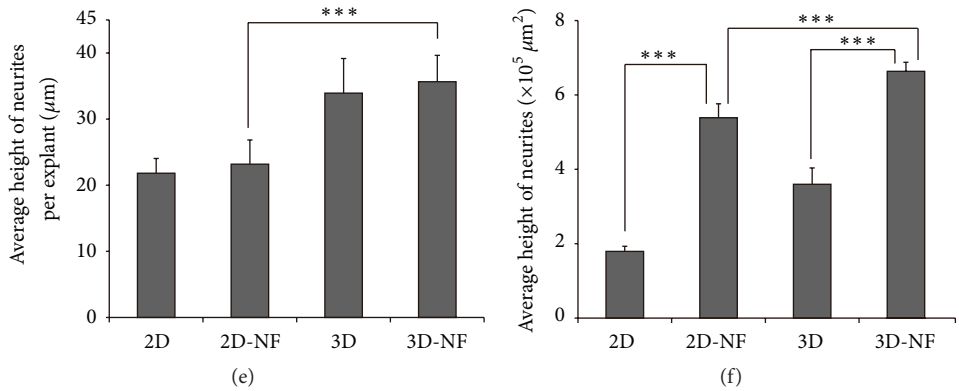

Figure 8: Synergistic influence of matrigel with BDNF and NT3 on neurite outgrowth. (a) The diagram of the assay. (b) Representative staining of neurites observed on SGN explants treated with 2D 2D-NF, 3D, and 3D-NF. DAPI (blue), β-tubulin (green). (c–f) Quantifications of the average number, length, height, and area of neurites in treated groups. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Acknowledgments

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Review Article

Transcriptional Control of Synaptic Plasticity by Transcription Factor NF-κB

Christian Engelmann and Ronny Haenold

Leibniz Institute on Aging-Fritz Lipmann Institute (FLI), Beutenbergstrasse 11, 07745 Jena, Germany

Correspondence should be addressed to Ronny Haenold; ronny.haenold@leibniz-fli.de

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Activation of nuclear factor kappa B (NF-κB) transcription factors is required for the induction of synaptic plasticity and memory formation. All components of this signaling pathway are localized at synapses, and transcriptionally active NF-κB dimers move to the nucleus to translate synaptic signals into altered gene expression. Neuron-specific inhibition results in altered connectivity of excitatory and inhibitory synapses and functionally in selective learning deficits. Recent research on transgenic mice with impaired or hyperactivated NF-κB gave important insights into plasticity-related target gene expression that is regulated by NF-κB. In this minireview, we update the available data on the role of this transcription factor for learning and memory formation and comment on cross-sectional activation of NF-κB in the aged and diseased brain that may directly or indirectly affect κB-dependent transcription of synaptic genes.

1. Introduction

Acquisition and consolidation of new information by neuronal networks often referred to as learning and memory formation depend on the instant alterations of electrophysiological parameters of synaptic connections (long-term potentiation, long-term depression), on the generation of new neurons (neuroneogenesis), on the outgrowth of axons and dendrites (neuritogenesis), and on the formation/remodulation of dendritic spines (synaptogenesis). The transmission of active synapses becomes potentiated by additional opening of calcium channels and incorporation of preexisting channel proteins, that is, during the induction of long-term potentiation. In contrast, long-term structural reorganization of the neuronal network depends on the induction of specific gene expression programs [1]. The transcription factor NF-κB has been shown to be involved in all of the aforementioned processes of learning-associated neuronal plasticity, that is, long-term potentiation, neuroneogenesis, neuritogenesis, and synaptogenesis (for review, see [2]). With respect to the diverse functions of NF-κB in neuroneogenesis and neuritogenesis, and to its local function as structural protein at the postsynaptic membrane, we refer to specialized review articles [3, 4].

In mammals, NF-κB consists of five subunits (RelA, RelB, c-Rel, p105/50, and p100/52), possessing either transcriptional activator (Rel proteins) or repressor (p50, p52) functions. Within the CNS, NF-κB signaling encompasses activation of mainly RelA, c-Rel, and p50 containing heterodimers (canonical pathway). In addition to an inducible form in neurons and glial cell populations where NF-κB becomes rapidly activated under metabolic or traumatic stress [5, 6], the transcription factor also possesses constitutive activity in subsets of neuronal cell populations [7]. This has been convincingly presented in brains of *κB-lacZ* reporter mice that express the enzyme beta-galactosidase in dependence on NF-κB transcriptional activation [8, 9]. It has been generally assumed that spontaneous NF-κB activation in the absence of any obvious stimulation exerts a function for neuronal development and maintenance of the mature CNS. However, converse studies on primary neurons, in which such an activation was nearly absent [10], evoke questions on unknown physiological activation mechanisms under steady-state-conditions. One reasonable explanation for

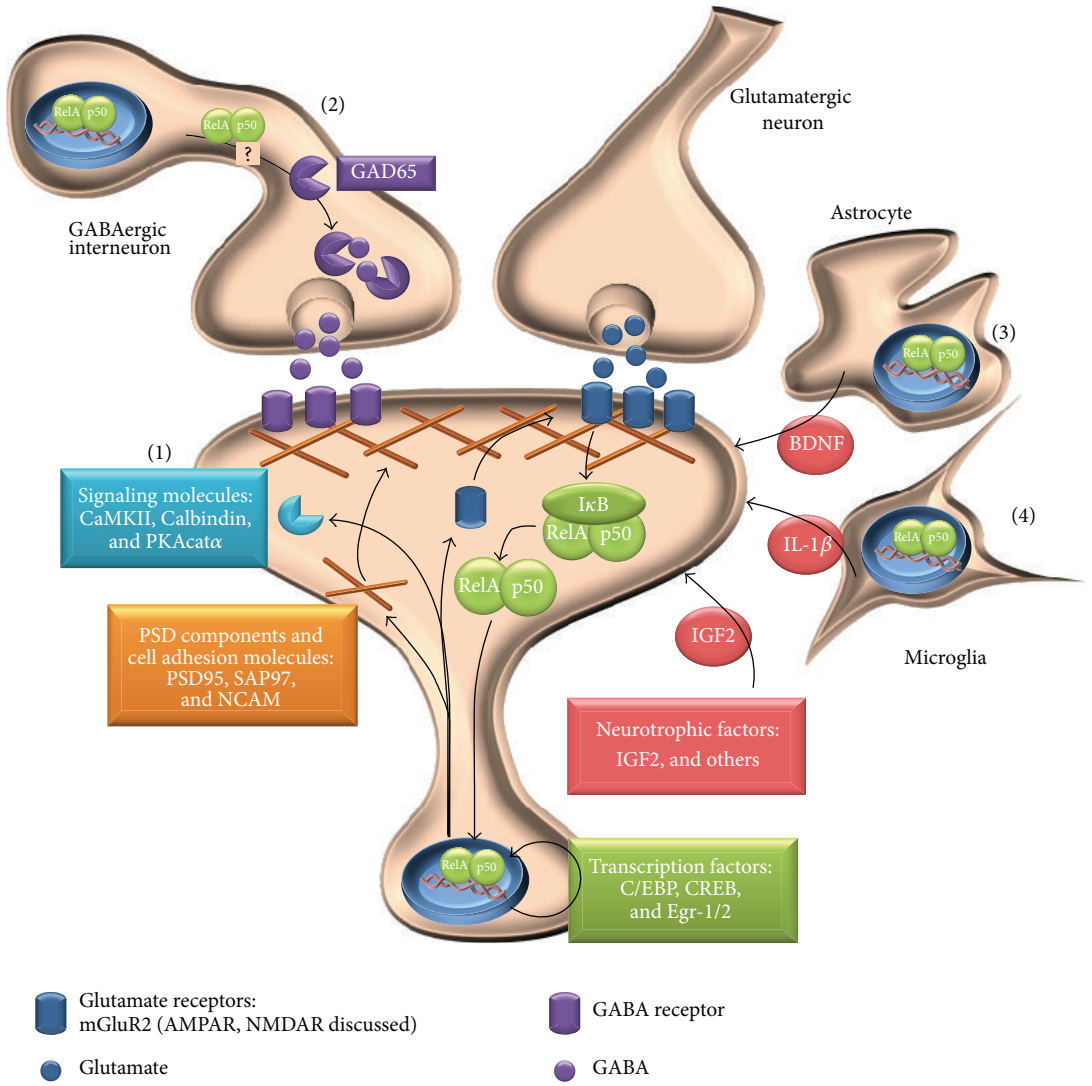

Figure 1: Overview of NF-κB regulated components in synaptic plasticity. (1) At the postsynapse NF-κB regulates the expression of various components of the postsynaptic density, ion channels/receptors, signaling molecules, and transcription factors that are involved in the modulation of synaptic transmission. (2) In inhibitory neurons, NF-κB regulates the expression of GAD65 that is required for GABA synthesis. (3) Synaptic plasticity is further modulated by paracrine release of neurotrophic factors from adjacent cells. In astrocytes, activation of NF-κB led to the secretion of BNDF, and (4) microglia express the NF-κB target gene IL-1β. For references, see Table 1.

the aforementioned constitutive NF-κB activity lies in a synaptic plasticity associated activation that occurs during basal neuronal function [11]. Such activity-dependent activation is supported by a number of observations and facts leading to the conclusion that NF-κB signaling is directly involved in spinogenesis and strengthening of synaptic connections during learning and memory formation (Figure 1).

(i) All NF-κB Pathway Proteins Are Present at the Synapse. This has been shown by immunochemistry and biochemical analysis of isolated synaptosomes (for recent review, see [12]). Within the synaptosome, NF-κB is localized to a free synaptoplasmic pool and a membrane-anchored pool, with a dynamic exchange between both pools. This further led to the hypothesis for a local role of NF-κB at the synapse in addition to its nuclear function as a transcription factor, that is, for labeling and preparing the synapse for remodeling and plastic changes [13].

On the subcellular level, NF-κB is concentrated at the postsynaptic density and dendritic cytoplasm to induce gene expression within the signal-receiving neuron. Immunohistochemical analysis of brain sections revealed also an axonal localization of NF-κB proteins *in vivo* [14, 15]. Although the functional relevance of such axoplasmic NF-κB requires further investigation, it points to a presynaptic feedback mechanism that might respond to synaptic stimulation by retrogradely transported NF-κB. For example, NF-κB regulates presynaptic transmitter production of γ-aminobutyric acid releasing (GABAergic) interneurons by direct or indirect transcriptional upregulation of the GABA synthetizing

Table 1: Overview of NF-κB target genes in synaptic plasticity.

Target gene	Location	Effect	Gene description	Reference
		Neurotrophic factors		
IGF2	Postsynaptic	Direct	Insulin-like growth factor 2	[34]
BDNF	In astrocytes	Direct	Brain-derived neurotrophic factor	[41]
		Structural/adhesion/scaffolding proteins		
PSD95	Postsynaptic	Indirect	Postsynaptic density compartment (also SAP90) membrane-associated guanylate kinase	[23, 34]
SAP97	Postsynaptic	Indirect	Synapse-associated protein 97 or disks large homolog 1 (DLG1)	[34]
NCAM	Postsynaptic	Direct	Neuronal cell adhesion molecule	[42]
ICAM3	Postsynaptic	Direct	Intercellular adhesion molecule 3	[43]
Slitrk1	Postsynaptic	Direct	SLIT and NTRK-like family member 1	[44]
Tiam1	Postsynaptic	Direct	T-cell lymphoma invasion and metastasis-inducing protein 1	[44]
		Receptor and signaling proteins		
Calbindin	Postsynaptic	Indirect	Calcium binding protein -D28k and -D9k	[45, 46]
CaMKII δ	Postsynaptic	Direct	Ca(2+)/calmodulin-dependent protein kinase type II alpha chain	[47]
CREB	Postsynaptic	Indirect	cAMP response element-binding protein	[21]
C/EBP	Postsynaptic	Direct	CCAAT/enhancer-binding protein transcription factor	[48–50]
Egr-1	Postsynaptic	Direct	Early growth response protein 1 or NGFI-A (nerve growth factor-induced protein A)	[51]
Egr-2	Postsynaptic	Direct	Early growth response protein 2	[52]
Fos	Postsynaptic	c-Rel binding sites identified	Transcription factor	[53, 54]
GAD65	Presynaptic	Indirect	Glutamic acid decarboxylase function in GABA synthesis	[16]
NMDA1 receptor subunit 1 (Grin1)	Postsynaptic	κB binding sites identified	Subunit 1 of N-methyl-D-aspartate glutamate receptor	[55]
NMDA2A receptor subunit 2A (Grin2A)	Postsynaptic	κB binding sites identified	Subunit 2A of N-methyl-D-aspartate glutamate receptor	[56]
mGluR2	Postsynaptic	Direct	Metabotropic glutamate receptor 2	[57]
PKAcatα	Postsynaptic (hippocampus)	Direct	Protein kinase A catalytic subunit α	[21]

enzyme glutamate decarboxylase 65 (GAD65) [16]. Thereby, changes in GABAergic NF-κB activity can affect the magnitude of inhibitory transmission. Functionally, transgenic mice with overexpression of the NF-κB superrepressor IκBα-SR in GABAergic neurons display enhanced excitatory signal transduction, increased long-term potentiation, and enhanced spatial learning and memory [16]. Overall, presynaptic functions of κB-dependent gene expression for synaptic plasticity require further investigations.

(ii) NF-κB Becomes Activated at Active Synapses. According to its function as a synapse-to-nucleus signal transducer in the postsynaptic neuron NF-κB is locally activated by synaptic transmission and retrogradely transported into the neuronal soma. Stimulation of various receptors leads to the activation of NF-κB within the postsynaptic compartment (for review, see [17]). One mechanism that has been described in detail involves the activation of group I metabotropic glutamate receptors (GpI-mGluRs) in excitatory neurons. Stimulation of hippocampal neurons with a GpI-mGluR agonist results in nuclear translocation of NF-κB within one hour as detected by a temporary increase in DNA binding activity of p50, RelA, and c-Rel [18]. Three major signaling pathways involving protein kinase C (PKC), calmodulin-derived, and Ras/PI3K/Akt cascades link postsynaptic receptor stimulation to local phosphorylation of IKK kinases, which represents the key regulatory step for NF-κB activation. They all involve the opening of calcium channels at the plasma membrane and intracellular calcium stores to increase synaptoplasmic calcium levels, which represents a specific

feature of local NF-κB activation at neuronal synapses [19]. Degradation of inhibitory IκBα mobilizes the NF-κB dimer and exposes the nuclear localization sequence, which is required for its dynein/dynactin-dependent transport along microtubules into the nucleus [12, 20]. The exact dynamics of subcellular redistribution of NF-κB from the synaptosomal cytoplasm and membrane to the nucleus during memory consolidation is currently a matter of investigation [13].

(iii) NF-κB Induces Expression of Target Genes for Synaptic Plasticity. Studies on knockout mice have facilitated the search for NF-κB target genes linked to synaptic plasticity. One of the first genes identified is the α catalytic subunit of protein kinase A (PKAcatα) whose expression is pivotal for the induction of synaptic plasticity and spatial learning in mice. The promoter region of the *PKAcatα* gene contains one NF-κB binding site that is conserved in several species and binding of RelA/p50 to this site has been demonstrated by band shift assays, thus indicating a direct transcriptional regulation of PKAcatα by NF-κB [21]. There is a growing list of genes that are induced by NF-κB in the context of synaptic plasticity albeit the direct or indirect transcriptional regulation mechanism has not always been determined (for recent review, see [22]). These targets represent a wide range of functions such as scaffolding and cell adhesion proteins, neurotrophic factors, neurotransmitters, ion channels, and signaling molecules (Table 1). The identification of further NF-κB target genes and the exploration of their subunit and context-specific upregulation during different learning paradigms will be the real challenge to fully understand the diversity of target genes orchestrated by NF-κB. Moreover, there is growing evidence for the requirement of κB-dependent gene expression in nonneuronal cells that locally support synaptic plasticity (Figure 1). For example, inhibition of NF-κB specifically in astrocytes by overexpressing a dominant-negative form of IκBα (*GFAP-IκBα-dn*) impaired spatial and nonspatial learning in female mice [23]. This was accompanied by a reduced expression of neuron-specific PSD95 and mGluR5. Despite the fact that astrocyte-specific target genes of NF-κB have not been investigated in this study, the data strongly suggest that astrocytes positively modulate the expression of synaptic proteins by neurons. To investigate the contribution of microglial NF-κB to learning and memory formation in mice, IKK2 has been deleted in myeloid cells including microglia (*mIKK2KO*) recently [24]. Among various well-known target genes of NF-κB only transcript levels for *Interleukin-1β* were altered in the brains of *mIKK2KO* mice, which coincided with transiently increased short-term fear memory of the transgenic mice. This observation reveals a novel and unexpected role for microglial IKK2/NF-κB in the homeostatic regulation of synaptic plasticity [24]. Taken together, synaptic plasticity requires the adaptive regulation of κB-responsive genes not only within the pre- and postsynaptic neuron, but also within adjacent astro- and microglial cells.

(iv) Activation of NF-κB Is Required for Learning and Memory Formation. Notably, NF-κB signaling is obviously dispensable for normal CNS development. This has been shown in a number of mouse lines with congenital impaired NF-κB signaling. Transgenic mice with CNS-specific deletion of abundantly expressed RelA or inactivated upstream regulators of NF-κB (IκBα, IKK) in the neuroglial compartment are indiscernible regarding overall neuroanatomical and behavioral features [5, 6, 25, 26]. However, a large number of behavioral studies on animals with inactivated NF-κB provide convincing evidence for its requirement specifically in learning and memory formation. These experiments have been performed on different species (crab [27], mouse [28], and rat [29]), with different approaches for NF-κB inactivation (pharmacological, NF-κB decoy, and genetic knockout) and by testing different forms of learning conditions (long-term habituation, fear conditioning, and spatial learning) (for review on evolutionarily conserved roles in synaptic plasticity, see [22, 30]). Altogether, they implicate that among the subunits investigated (p50, RelA, and c-Rel) all are required for the establishment of learning and memory formation. However, it has to be stated that the specific learning mechanisms in these species, that is, synaptic plasticity, have not always been explicitly investigated. Moreover, there exists only very limited data on systematic knockout studies that allow for conclusions on NF-κB subunits in individual tests. Recently, we have addressed this question by studying cortical plasticity of monocular deprived p50 knockout ($p50^{KO}$) mice and mice with CNS-restricted deletion of RelA ($RelA^{CNSKO}$). The results show a nonredundant requirement of both subunits of the classical NF-κB pathway, RelA, and p50, for the establishment of synaptic plasticity (unpublished data). Functionally, this obligation stands in contrast to the injury-induced activation of NF-κB in the lesioned brain, such as after stroke and axonal fiber injury. Here, antagonistic effects of the transactivator subunit RelA and the transcriptional repressor p50 for neuronal survival have been observed [5, 6].

The aforementioned results implicate that canonical NF-κB acts as a positive regulator of synaptic plasticity by transcriptional upregulation of synaptic proteins. In the past, this role of NF-κB has been addressed almost exclusively using loss-of-function mutants with deletion of specific NF-κB subunits (p50, c-Rel, and RelA), or by pan-inhibition of NF-κB following overexpression of inactive IKK or non-degradable forms of the NF-κB inhibitor IκBα. Recently, studies on NF-κB gain-of-function mutants have been performed providing additional and exciting insights into the pivotal role of NF-κB as an enhancer for synaptic plasticity thereby demonstrating its potential for clinical applications. According to its proposed function as a transcriptional activator of activity-dependent gene expression genetically induced hyperactivation of NF-κB signaling should maximize synaptic plasticity by reinforcing κB-dependent gene expression of synaptic proteins. This assumption has been tested in mice with disinhibited NF-κB signaling in which autoinhibitory upregulation of IκBα is impaired by the mutation of the *ikba* promoter ($I\kappa B\alpha^{M/M}$) [31]. Primary neurons from $I\kappa B\alpha^{M/M}$ mice stimulated with TNF displayed a sustained NF-κB activation compared to WT neurons. During neuronal culture dissociated hippocampal neurons form

excitatory and inhibitory synaptic contacts that can be stained with antibodies against the glutamatergic presynaptic marker VGLUT or the GABAergic presynaptic marker VGAT. Quantitative analysis of the synaptic puncta revealed an increase in excitatory synapse density and a decrease in inhibitory synapse density on transgenic neurites suggesting an altered synaptic connectivity within $I\kappa B\alpha^{M/M}$ hippocampal neurons [31]. Functionally, the imbalance of glutamatergic and GABAergic synaptic transmission leads to spontaneous burst firing and hyperexcitability of $I\kappa B\alpha^{M/M}$ neurons. Notably, when tested for learning and memory formation young adult $I\kappa B\alpha^{M/M}$ mice performed better for hippocampus-dependent contextual fear memory as compared to their littermate controls. The cognitive enhancement in $I\kappa B\alpha^{M/M}$ mice was further confirmed in the acquisition of spatial memory. Here, $I\kappa B\alpha^{M/M}$ mice revealed an enhanced retention of the memory. The cognitive improvements occurred independent of any changes in general behavior; that is, the transgenic mice showed normal levels of activity, anxiety-like behavior, or nociception [31].

Another study examined the synaptogenetic effect of hyperactivated NF-κB in a model of drug-addicted behavioral sensitization in mice. First, mice received a viral-mediated gene transfer to express a constitutively active IKK (*IKKca*) in the nucleus accumbens (NA) [32]. Then, they were administered with chronic cocaine, which induced dendritic spine changes. Despite the fact that the rewarding behavior of cocaine-sensitized *IKKca* mice was not changed as tested by conditional place preference (CPP) training, the number of dendritic spines on NA neurons was significantly increased under constitutive activation of NF-κB by *IKKca*. Among the tested factors with known potential for spinogenesis, only BDNF, but not GDNF, was enhanced in *IKKca* mice, suggesting that *bdnf* is a transcriptional target of NF-κB in the NA. Recently, the fibroblast growth factor homologous factor 1 (FHF1/FGF12) has been identified as a physiological "break" of NF-κB activity that restricts neurite and spine formation in mature cortical neurons. *Fhf1* gene silencing strongly activated neuronal NF-κB activity and, thereby, significantly increased spine densities in a NEMO-dependent manner [33]. These results certainly warrant behavioral studies on FHF1-deficient mice in the future. Taken together, these experiments show that hyperactivation of NF-κB can amplify synaptic plasticity by promoting κB-dependent expression of the aforementioned structural, signaling, or neurotrophic factors.

(v) Open Questions and Future Directions. While there is convincing evidence for a role of NF-κB in synaptic plasticity, a number of crucial questions remain to be answered. First, only a limited number of studies have examined subunit-specific transcriptional activator and repressor functions of NF-κB. With an increasing list of κB-regulated target genes expressed during synaptic plasticity, this might shed light on the gene-regulatory mechanisms required for the selected expression. Along this line, the role of RelB-dependent NF-κB signaling for synaptic plasticity might be underestimated. Schmeisser et al. presented evidence for robust presence of RelB protein particularly at the synapse by analyzing crude homogenates, synaptosomes, and PSD fraction from adult mouse forebrain for the subcellular localization of NF-κB family members. Interestingly enough, among the Rel proteins only RelB showed an enrichment in the synaptosomal fraction, in contrast to equally distributed RelA or c-Rel, which was almost exclusively present in the PSD [34]. Future studies are required to address synaptogenetic functions of alternative NF-κB signaling.

Second, the influence of NF-κB as a mediator of cellular stress, inflammation, and neurosenescence on κB-dependent gene expression in synaptic plasticity warrants further investigations. Dysregulation of NF-κB signaling by inflammation or environmental factors interferes with neurogenesis in both the developing and the adult brains. For example, cell-intrinsic activation of NF-κB in neural stem cells by acute stress suppresses hippocampal neurogenesis in adult rats [35]. Likewise, maternal infection during pregnancy can affect foetal brain development via the release of proinflammatory activators of NF-κB [36, 37], which might cause neurological disorders like depression and learning disabilities. Finally, constitutive activation of NF-κB is a hallmark of aging leading to its hyperactivation and proinflammatory gene expression in neurons [38, 39]. Indeed, the NF-κB motif has been identified as the most upregulated gene expression program in aged tissues including the brain [40]. It seems plausible that such changes in NF-κB activity might directly affect κB-dependent transcription of synaptic genes and, thus, might contribute to the impairments in synaptic plasticity observed in the aging brain. Vice versa, lifelong attempts in learning and memory formation, which have been demonstrated as a protective factor against premature cognitive impairments, might imply selective activation patterns of NF-κB. Given their high social relevance, the regulation of synaptic plasticity and learning and memory formation by NF-κB warrants further investigations.

Abbreviations

BDNF:	Brain-derived neurotrophic factor
CNS:	Central nervous system
FHF1/FGF12:	Fibroblast growth factor homologous factor 1
GABA:	γ-Aminobutyric acid
IκBα-dn:	Dominant-negative IκBα
IκBα-SR:	IκBα superrepressor
IKK:	IκBα kinase complex
mGluR5:	Metabotropic glutamate receptor type 5
NF-κB:	Nuclear factor kappa B
PKAcatα:	α Catalytic subunit of protein kinase A
PSD95:	Postsynaptic density protein 95
VGAT:	Vesicular GABA transporter
VGLUT:	Vesicular glutamate transporter
WT:	Wild type.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Perspectives of TRPV1 Function on the Neurogenesis and Neural Plasticity

R. Ramírez-Barrantes,[1] C. Cordova,[1] H. Poblete,[2] P. Muñoz,[1] I. Marchant,[1] F. Wianny,[3,4] and P. Olivero[1]

[1]*Escuela de Medicina, Universidad de Valparaíso, Hontaneda 2664, 2341386 Valparaíso, Chile*
[2]*Institute of Computational Comparative Medicine, Nanotechnology Innovation Center of Kansas State, Department of Anatomy and Physiology, Kansas State University, Manhattan, KS 66506-5802, USA*
[3]*INSERM, U846, Stem Cell and Brain Research Institute, 18 Avenue Doyen Lépine, 69500 Bron, France*
[4]*Université de Lyon, 69003 Lyon, France*

Correspondence should be addressed to P. Olivero; pablo.olivero@uv.cl

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The development of new strategies to renew and repair neuronal networks using neural plasticity induced by stem cell graft could enable new therapies to cure diseases that were considered lethal until now. In adequate microenvironment a neuronal progenitor must receive molecular signal of a specific cellular context to determine fate, differentiation, and location. TRPV1, a nonselective calcium channel, is expressed in neurogenic regions of the brain like the subgranular zone of the hippocampal dentate gyrus and the telencephalic subventricular zone, being valuable for neural differentiation and neural plasticity. Current data show that TRPV1 is involved in several neuronal functions as cytoskeleton dynamics, cell migration, survival, and regeneration of injured neurons, incorporating several stimuli in neurogenesis and network integration. The function of TRPV1 in the brain is under intensive investigation, due to multiple places where it has been detected and its sensitivity for different chemical and physical agonists, and a new role of TRPV1 in brain function is now emerging as a molecular tool for survival and control of neural stem cells.

1. Introduction

Repairing and Renewing the Brain from the Inside. Brain development implicates cell migration, differentiation, and plasticity to configure an efficient neuronal network. While neurons can live long periods of time, a large number of neurons die during developmental and pathophysiological processes in a lifetime. The loss of neurons in adulthood can lead to nervous system disorders such as neurodegenerative diseases, which involve cognitive and motor alterations causing severe disability and generally death. While pharmacological treatment for this kind of diseases may attenuate symptoms and disease progression at initial stages, pharmacological efficacy gradually decreases over time [1]. New experimental approaches must be developed to design efficacious therapies for repairing and renewing the neuronal network to restore lost functions in order to expand possibilities of cures for brain diseases.

Neural stem cells (NSCs) can generate different types of neurons. In order to generate sensory neurons, motor neurons, or interneurons, NSCs in adequate microenvironment must receive cellular context-specific molecular signals to determine fate and location. These newly formed neurons establish new circuits and modify existing pathways connecting neuron to neuron. In this context, NSC graft appears to be a promising strategy to recover lost functions due to neurons death in the brain. Unfortunately, neural grafts have not been as successful as expected, due to poor survival of grafted cells and the inability of surviving cells to connect with central network [2, 3]. The control of stem cells differentiation into specific types of neurons as well as their survival and connectivity may enable the establishment of a renewal

supply to replace dead or damaged neurons. Recently, the controlled expression of the nonselective cationic channel TRPV1, which is widely expressed in peripheral and central nervous system [4–9], has proven effective on the control of many functions in brain neurons [10–12]. The regulated activity of TRPV1 promotes migration [13], axon growth [14], cell-to-cell extension [15], and release of neurotransmitters (GABA, dopamine, and glutamate) [6, 16], and more interestingly a novel function has been reported which is the ability to control brain neurogenesis [10, 17, 18]. In addition, the regulated activation of the channel is also involved in cell resistance against local oxidative environment in brain regions and other tissues [11, 19–21].

In this review we explore the contribution of TRPV1 channel function in NSC fate, discussing possible roles of the channel in neurogenesis and network integration, and eventually we propose the use of TRPV1 control as a new clinically promising strategy to improve the plasticity of newborn neural network derived from grafted neural precursors in the damaged brain.

2. Polymodal TRPV1: An Environmental Signal Integrator

Transient receptor potential ion channel (TRP) family consists of a number of nonselective cationic channels capable of integrating environmental physicochemical signals and coupling their activity to downstream amplification of cellular signal through cation permeation and membrane depolarization [4, 9, 22–24]. In cellular context, the activity of TRPs is modulated by several molecular mechanisms such as phosphorylation, reactive oxygen species (ROS), membrane lipid composition, intracellular calcium, and ATP levels [8, 24–26]. Among the six members (TRPV1-6) of TRPV (vanilloid) subfamily, TRPV1 was the first identified and best characterized [8, 27]. TRPV1 is a homotetrameric nonselective cation channel ($P_{Ca}/P_{Na} = 9.6$) with the same characteristics as other TRPs [28]. It is activated by several physical stimuli such as temperature, voltage, protons, osmolarity, pH [4, 23, 27], chemical ligands such as PIP_2 or endocannabinoids like anandamide, and exogenous ligands as resiniferatoxin and capsaicin [27, 29, 30] (Figure 1(a)).

New evidence links a specific hydrophobic pocket near the S5 helix that contains amino acids R579, F582, and L585 to the binding of PIP_2 [31] and cholesterol [32] (Figure 1(b)). The binding of these two molecules to the hydrophobic pocket may either potentiate or inhibit TRPV1 activity. More interestingly, the α-3-OH diastereoisomer of cholesterol epicholesterol has no effects on TRPV1-mediated currents, suggesting the existence of a stereospecific binding site [32]. 17β-Estradiol increases currents evoked by capsaicin in dorsal root ganglion neurons [33] and capsaicin-induced nociception, whereas these capsaicin effects are reduced by testosterone [34]. Thus, modification of TRPV1 hydrophobic environment may alter its biophysical properties and contribute to functional coupling. Several mechanisms show intrinsic cooperative regulation, suggesting allosteric modulation of these ion channels, although there is no definite evidence on the potential binding site. It is noticeable that, in particular context, TRPV1 could work as ionotropic receptor of cholesterol-derived molecules with opposite actions. This feature allows them to act as signal integrators [31], playing critical roles in excitable and nonexcitable cell functions underlying sensory physiology, proliferation, growth, male fertility, and neuronal plasticity [7, 35–37].

3. TRPV1 in the Brain

TRPV1 was first described in peripheral afferent fibers and identified as a detector of harmful signals in primary sensory neurons [27]. The currently known distribution of TRPV1 includes testis, heart, lung, stomach, and brain [8, 36, 38–40]. Particularly, in peripheral nervous system (PNS) TRPV1 is highly expressed in dorsal root ganglia (DRG), trigeminal ganglia, and primary sensory neurons, which are sensory neuronal components of nociceptive Aδ and C-fibers' circuits [4, 8]. In PNS TRPV1 was primarily studied for its anti-inflammatory and antinociceptive functions [20, 27, 37], but currently a more general function has been attributed to TRPV1; this is an integrator of several noxious stimuli such as low pH (pH < 6.0) or high temperature (>43 degrees Celsius) [41]. In central nervous system (CNS) the expression of TRPV1 is still controversial. Whereas some seminal reports showed very low or no expression of the channel in CNS [27, 42], recent reports have shown (1) that well-recognized endogenous activators such as N-arachidonoyldopamine (NADA) or exogenous activators such as capsaicin (CAP) or even potent TRPV1-specific inhibitors like capsazepine (CPZ) or resiniferatoxin (I-RTX) can modulate the activity of neurons in CNS [11, 36, 43, 44] and (2) direct evidence on the expression of TRPV1 by immunohistochemistry, PCR, autoradiography, and *in situ* hybridization in mammalian brain [5, 39, 45, 46]. The amount of expression of TRPV1 differs importantly between central and peripheral nervous system. In the brain, it is 20- to 30-fold lower than in DRG [27, 47]. The poor TRPV1 expression in CNS has demanded greater precision and refinement of experimental methods in order to increase the reliability of localization of the channel in the brain and its significance. In addition, the existence of TRPV1 alternates which are heterogeneously distributed throughout the nervous system [48] complicates the interpretation of the results from several expression studies. However, a remarkable study using mice with genetically modified TRPV1 reporter protein along with other techniques such as *in situ* hybridization, calcium-imaging, RT-PCR, and slice electrophysiological recordings provided definite evidence on the expression of functional TRPV1 in primary afferent neurons while low levels of expression were found in entorhinal cortex, olfactory bulb, hippocampus, and hypothalamus [43], which are nevertheless active enough to modulate excitability in hypothalamus [43]. More intriguingly, TRPV1 can be transiently expressed during brain development. In some brain regions the expression can suffer postnatal restriction depending on age, physiological, or pathological condition [45], suggesting that TRPV1 functional expression might be modulated by the metabolic cell state.

The number of reports addressing the functional effect of activation/suppression of TRPV1 channel expressed in

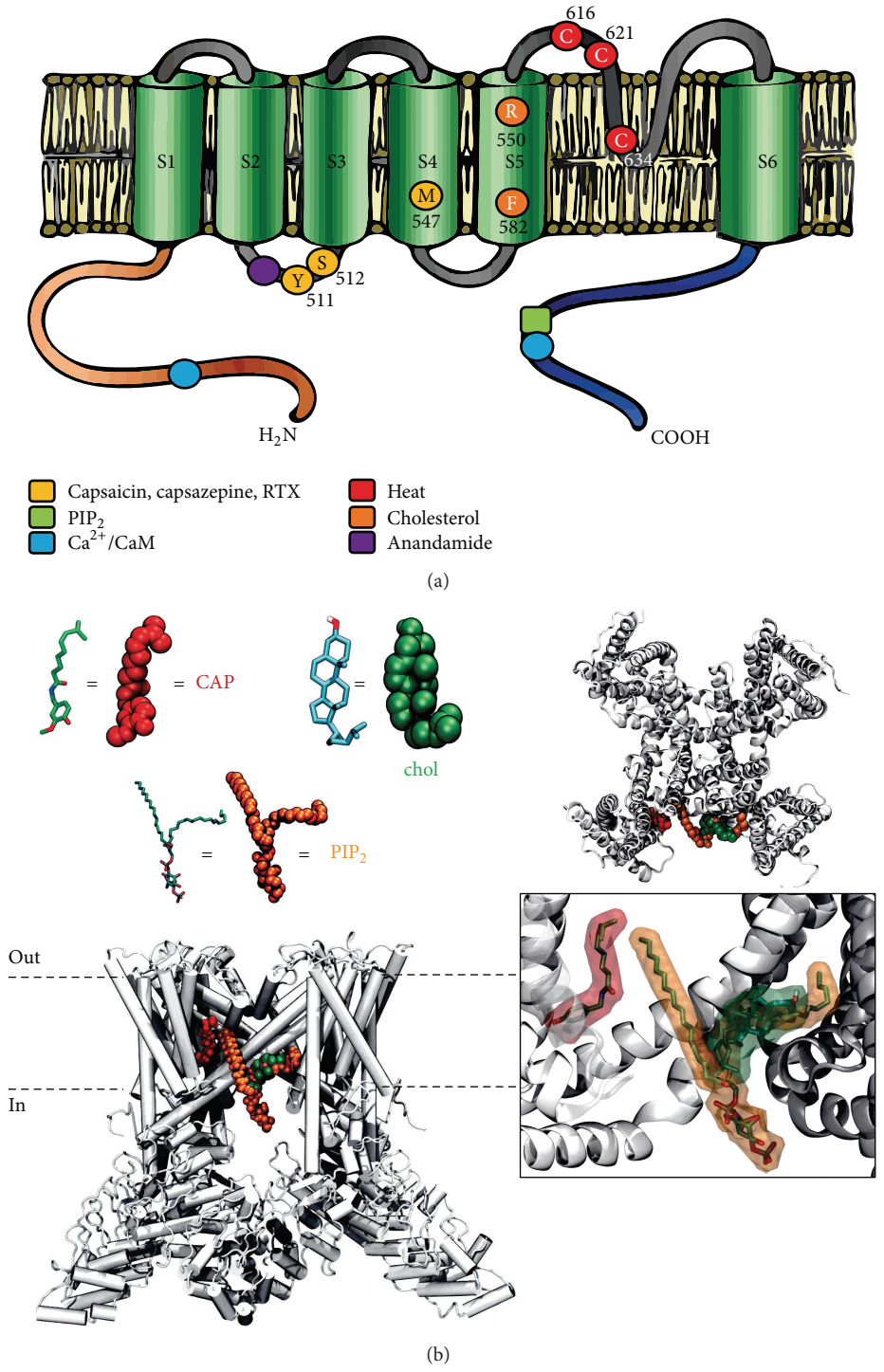

Figure 1: Diagram of regions involved in TRPV1 function. (a) The primary structure involves six transmembrane segments (S1–S6) with a pore domain between the fifth (S5) and sixth (S6) segment, and both C and N termini are located intracellularly. The functional TRPV1 receptor is believed to form a homotetramer. Amino acid residues involved in the binding of chemical and physical activation/modulation of TRPV1 activity are indicated in a color scheme. Vanilloid compounds, as the activators capsaicin and resiniferatoxin, as well as inhibitor capsazepine share the same binding site, while cholesterol-binding site is composed of a promiscuous hydrophobic pocket in S5. (b) Model for hydrophobic pocket in S5 linker with the binding of lipidic molecules such as cholesterol (chol), PIP_2, and capsaicin (CAP) generated by molecular dynamics. In this binding conformation, all the molecules occupy a groove formed between S5 and C-terminal of the subunit.

several brain regions increases each year. To date, both TRPV1 mRNA and protein have been found mainly in cortical structures and hippocampal pyramidal neurons in areas CA1, CA3, and dentate gyrus but have also been found in the locus coeruleus, cerebellum, thalamic and hypothalamic nuclei, periaqueductal grey, and limbic structures including the caudate putamen, the central amygdala, and the substantia nigra pars compacta [5, 45, 49]. With regard to the cell type where TRPV1 is expressed, it has been reported in different lineages, most commonly neurons. For instance, in hippocampal dentate gyrus many pyramidal neurons throughout the CA1–CA3 areas express TRPV1 receptor on cell bodies. In thalamus, TRPV1 expression has been found in neuronal cytoplasmic and axonal staining; in cerebellum TRPV1 channels surround several Purkinje cell bodies, especially on basal areas corresponding to the initial axonal segment; in cortex the expression also surrounds the nucleus; and in substantia nigra double labelling immunofluorescence shows a complete overlap between TRPV1 and tyrosine hydroxylase, confirming the presence in dopaminergic neurons [5, 46, 50]. We assayed our experimental strategy to identify the expression of TRPV1 by immunofluorescence in heterologous system and in neurons of primate prefrontal cortex, confirming the expression of TRPV1 in neurons and glia in mammalian brain (Figure 2).

4. TRPV1 Expression in Neural Progenitors

Recent publications add a novel cell lineage to the vast list of cell types that express this channel in the brain. TRPV1 is expressed in neurogenic brain regions, in particular, in the hippocampal dentate gyrus subgranular zone (SGZ) as well as the subventricular zone in telencephalon (SVZ). In adult rat, TRPV1 is colocalized with nestin, a marker of NSCs. Since postnatal neurogenesis occurs up to day 21 and declines afterwards in mice, the expression of TRPV1 was measured in postnatal days 7, 14, 21, and 39, being positive at the time points that corresponded to the time course of postnatal neurogenesis p7, p14, and p21. More interestingly, TRPV1 was no longer detected from p39, when postnatal neurogenesis had declined [18]. Additionally to the expression of TRPV1 detected in early neural precursors, stimulating neurogenesis by exercise paradigm upregulates TRPV1 expression above baseline in the adult hippocampus [18]. In the same line, we induced *in vitro* differentiation of monkey embryonic stem (ES) cells to neural precursor and explored the expression of TRPV1 at different stages of differentiation. We used the LYON-ES1 cell line that stably expresses Tau-GFP, isolated at SBRI (Stem Cell and Brain Research Institute, INSERM, France). The primate pluripotent markers-expressing LYON-ES1 cells [51] are indefinitely self-renewable and have the capability of multilineage differentiation [51, 52]. We examined the expression of TRPV1 in LYON-ES1 cells, NSCs, glial cells, and neurons derived from LYON-ES1 cells. We used Pax6 as a marker for NSCs, β-III-tubulin, and glial fibrillary acidic protein (GFAP) as markers for neurons and glial cells [51, 52], respectively. We found that TRPV1 was expressed in NSCs (Figure 3) with cytoplasmic signal accompanied by a nuclear mark (Figure 3), as described previously [46, 53, 54]. In contrast, we did not detect TRPV1 in pluripotent stem cells, neurons, or glial cells.

The expression of TRPV1 has also been characterized in specific regions of the brain as previously mentioned [16, 36, 39], with particular microenvironment or extracellular pathways engaged in neurogenesis. The expression of TRPV1 in NSCs was evident in all experiments supporting the *in vivo* results previously reported [18].

Up until now, the TRPV1 expression pattern in neural precursors and its role in neurogenesis have been poorly studied and a new field on TRPV1 research is open with interesting implications in tissue regeneration.

5. TRPV1 Functions in the Brain

The function of TRPV1 in the brain has been subjected to exhaustive investigation, because of the multiple places where it has been detected, its sensitivity to different chemical and physical agonists, and its versatility as calcium channel. The intracellular calcium concentration modulated by TRPV1 is capable of triggering various processes such as excitability, proliferation, synaptic plasticity, resistance to oxidative stress, and cell death, depending on the concentration, timing, and transience of the signal [49, 55].

The most studied aspect of TRPV1 function relates to its activity on synaptic plasticity and excitability in the brain. The control of TRPV1 activity has proven effective to modulate the excitability in neurons [56–58]. In particular, peripheral nerve endings increase glutamate release following the activation of TRPV1 by heat [27]. Capsaicin, NADA, and endocannabinoids increase release of neurotransmitter in the central nervous system, the basal ganglia, hypothalamus [6, 12, 59], and cranial visceral afferent terminals in caudal solitary tract nucleus (NTS), in brainstem [60]. TRPV1 is involved in hippocampal long-term potentiation (LTP) [61] and depression (LTD) mediated, respectively, by vanilloids and endocannabinoids like anandamide [36, 59]. In addition, it could induce release of GABA in dentate gyrus depressing excitatory synaptic transmission [62]. Lastly, in the neighboring ventral tegmental area, capsaicin also increased the firing rate of dopamine neurons [16] as it did in excitatory synapses in the substantia nigra. Facilitated spontaneous excitatory postsynaptic current frequency by capsaicin and NADA without affecting amplitude suggested a presynaptic mechanism [63, 64].

TRPV1 has recently been demonstrated to have an important role in the regulation of cortical excitability by modulation of synaptic transmission in the human brain [58]. It would be interesting to understand how several physical and chemical activators or modulators interact to enhance or inhibit TRPV1 activity, because an allosteric coupling has been demonstrated with distinct agonist, increasing the effect of the channel activation and stimulating neuroplasticity [29, 31, 32].

6. TRPV1 and Neurogenesis

Spontaneous calcium oscillations play an important role in nervous system development, neural induction, axon

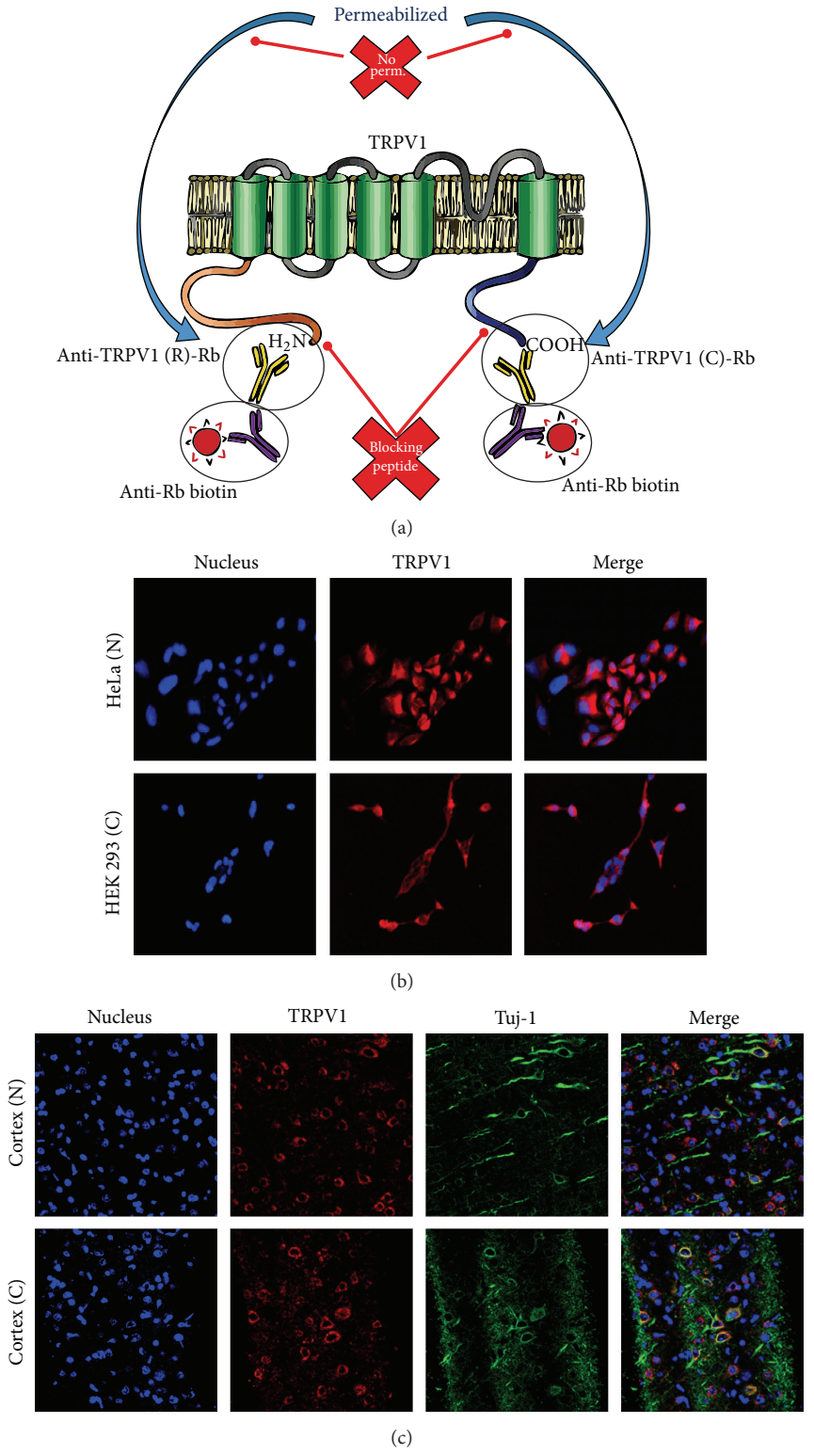

Figure 2: TRPV1 detected by immunofluorescence. (a) Methodology proposed for detection of TRPV1 by immunofluorescence. Using two antibodies against different epitopes of the channel allows corroborating the expression of the channel. In this case, we showed an antibody against the C-terminal and another against the N-terminal. As both antibodies bind to intracellular epitopes, it is advisable to use as internal control of the technique a sample without permeabilization of the plasma membrane, which prevents the entry of the antibody into the cell. One added strategy to improve signal sensitivity was the use of a blocking peptide, in this case, for the C-terminal or N-terminal. The competition of the blocking peptide with the epitope of the channel should diminish the intensity of the signal indicating the specificity of the technique. (b) Detection of TRPV1 in heterologous expression system using antibodies against the N-terminal and C-terminal of TRPV1. (c) Detection of TRPV1 in primate prefrontal cortex, using an antibody against the N-terminal and another against the C-terminal.

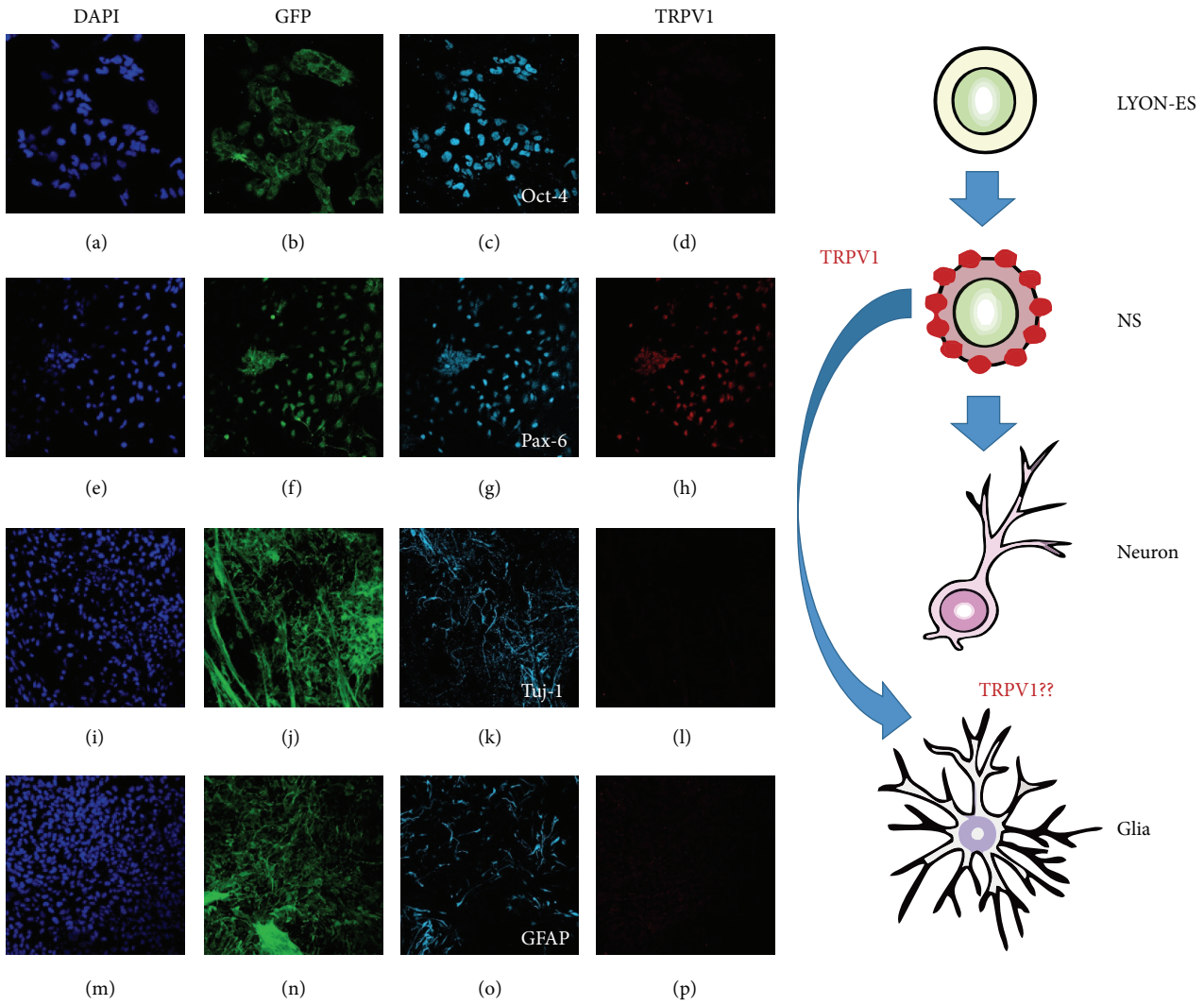

Figure 3: Expression of TRPV1 in neuronal differentiation process derived from LYON-ES. Determination of expression of TRPV1 by immunofluorescence staining for undifferentiated monkey ESCs stably expressing Tau-GFP (TAU-GFP LYON-ES1 line, a–d), NSCs (NS, e–h), neurons (i–l), and glial cells (m–p) derived from TAU-GFP-LYON-ES1 cells. At each stage, we performed immunofluorescence with antibodies against Oct4 (c) to identify LYON-ESCs, Pax6 for NSCs (g), β-III-tubulin for neurons (k), glial fibrillary acid protein (GFAP) for glial cells (o), and TRPV1 (d, h, l, p). Each experiment was accompanied by nuclear staining with DAPI (a, e, i, m), all the cells having a GFP fused to the microtubule-associated protein tau (b, f, j, n). The magnification of images was 20x for LYON-ESCs and neurons, and 40x for NSCs and glia cells.

guidance, growth cone morphology, migration, and proliferation [15, 65–67]. These oscillations are a combination of extracellular influx mediated by ionic channels and release of intracellular store from endoplasmic reticulum and/or mitochondria. TRP channels like TRPC and TRPV family have been related to control of neuronal differentiation and activity in CNS. TRPC channels mediate cortical neural precursor proliferation induced by bFGF [68] and TRPV1 differentiation triggered by retinoic acid [10] and rimonabant [17]. These two families also modulate the excitability in well-differentiated neurons and their precursors.

The first signs that linked TRPV1 with neurogenesis were indirect; in transiently transfected F11 cells and embryonic DRG neurons that endogenously express the channel, it was localized in neurites and growth cones where it regulates motility [15]. Dynamic processes such as growth cone motility and the direction of neurites involve calcium signals via cell surface receptors. The guidance of developing axons requires an active growth cone, and a localized calcium signal in the growth cone is sufficient for both attraction and repulsion [69]. TRPV1 located in the growth cone has been involved in the formation of filopodia in neurons [15]. In addition, TRPV1 contributes to cytoskeleton reorganization [70], cell migration [13], and regeneration of injured neurons [53].

These early discoveries indicate a key role of TRPV1 during neuronal differentiation. More specifically, TRPV1-expressing SHSY5Y neuroblastomas induced to differentiate

by retinoic acid showed upregulation of total and cell surface TRPV1 protein expression. Specifically, these upregulated channels were localized in cell bodies and the new neurites. Besides, retinoic acid increased both the intracellular free calcium concentration and the relative calcium influx induced by capsaicin [10]. Moreover, rimonabant, an antagonist of cannabinoid receptor 1 (CB1), was evaluated as inducer of neurogenesis in dentate gyrus and subventricular zone (SVZ), expecting that the inhibition of CB1 triggered the generation of neurons [17]. Neurogenesis was increased in both wild-type and knockout mice for CB1, but the neurogenesis-promoting effect of rimonabant disappeared in TRPV1 knockout mice [17]. Until now the mechanisms remain unexplored, although the interaction of rimonabant and TRPV1 has been associated with other processes as neural cell survival in a global cerebral ischemia model [21]. The hypothesis is that the quick activation of TRPV1 followed by desensitization could induce transient increase of calcium signal activating survival pathways and others associated with neurogenesis such as ERK pathway [17]. At least in the case of neuroprotection, the effect over TRPV1 appears to be direct since it is abolished by the application of CPZ [21].

These findings added evidence on the TRPV1 involvement in neurogenesis and the interaction between vanilloids and the endocannabinoid system during the generation of new neurons. However, in dentate gyrus and the subventricular zone, loss of TRPV1 expression promotes proliferation of neural precursors. The TRPV1 knockout mice exhibited substantial rise in postnatally proliferating cells in both stem cell niches, but lesser differentiation to neurons or glia [18]. The primary neural precursors originated from newborn TRPV1 knockout mice expressed stem cell genes like nestin or Sox2 and no differentiation markers for astrocytes (GFAP) or neurons (β-III-tubulin). Thus, the loss of TRPV1 in neural precursors disturbs differentiation and the growth potential. These data confirm the role of TRPV1 in orchestrating proliferation/differentiation of neural precursors, which has already been reported in other cell types.

Finally, another interesting and not well-understood function of TRPV1 is the control of cell death. Micromolar concentrations of capsaicin and acid solution (pH 5.5) induce a cytosolic calcium increase, mitochondrial membrane depolarization, ROS production, and cell death via TRPV1 activity [71]. In rat cortical neurons TRPV1 activation by capsaicin induces apoptotic cell death through L-Type Ca^{2+} channels, provoking Ca^{2+} influx, ERK phosphorylation, ROS production, and caspase-3 activation [72]. However, similar results have been reported for capsaicin without TRPV1 participation [73, 74] suggesting both dependent and independent effects of this vanilloid. TRPV1 knockout (KO) mice present a testis, brain, and heart phenotype much more susceptible to cell death by oxidative stress stimuli compared to wild-type mice [40, 75]. Pretreatment with capsaicin can prevent cell death induced by ischemia/reperfusion in lung in rabbits with concomitant diminishing of lipid peroxidation [76]. Besides, in hippocampus subjected to 10 min ischemia, CA1 neurons pretreated with capsaicin were less susceptible to cell death and the effect was inhibited with capsazepine antagonist of TRPV1. The use of rimonabant, the same compound that induces neurogenesis by TRPV1, as a postischemic treatment facilitated neuroprotection independent of CB1 receptor and inhibited by capsazepine. The same effect was measured in a model of temporary global cerebral ischemia by pretreatment with capsaicin in Mongolian gerbils [20]. The mechanism suggested involves a moderate increase in Ca^{2+} influx via TRPV1. This transitory influx may induce tolerance to subsequent calcium overload, preconditioning the response and inducing neuroprotection. However, capsaicin administrated 5 minutes after recirculation had no effect [20]. A possible explanation is that capsaicin and other pharmacological agonists of TRPV1 induce activation of the channel followed by acute desensitization. In the case of vanilloids, this could occur after the first 20 seconds following the addition of vanilloid compounds [77]. The molecular mechanism includes several pathways related with intracellular Ca^{2+} concentration. One of them is dependent on the balance between phosphorylation and dephosphorylation of TRPV1 triggered by Ca^{2+}-calmodulin pathway. Moreover, the dephosphorylation of the amino acid Ser502 and Thr704 by CaMKII has been associated with desensitization of the channel [77]. On the other hand, it has been demonstrated that calcium may induce TRPV1-caveolar endocytosis and lysosomal degradation [78, 79]. Independently of the mechanisms, the controlled activity of this polymodal receptor (activation-desensitization) seems to be critical for the cellular homeostasis in oxidative environment, acting as a modulator of cell viability. The precise cellular mechanism underlying TRPV1 activation-modulating cell homeostasis and viability remains unclear. Current information on cell death relates the activation of TRPV1 with abnormal function of the mitochondria. Mitochondrial dysfunction is frequently observed in cell death induced by high doses of capsaicin through TRPV1 activation. A significant portion of calcium entering the cytoplasm after the activation of TRPV1 is accumulated by mitochondria. Uptake of calcium by DRG neurons rises up to 20-fold compared to controls in the presence of 1 μM capsaicin, without observable cytotoxic effect; however, pretreatment with the mitochondrial uncoupler almost stopped capsaicin-dependent accumulation of calcium [80, 81]. Furthermore, TRPV1 expression diminishes the damage produced by high salt-diet in mouse heart compared to knockout maintaining the mitochondrial function [82]. Particularly, the controlled activation of TRPV1 induces an increase in expression of sirtuin 3, a protein that regulates the activity of Complex I, ATP production, and increases ROS clearance through deacetylation of Mn-SOD [83]. It seems that capsaicin activation of TRPV1 can prevent cardiac mitochondria dysfunction caused by high salt intake [82]. Apparently, the activity of TRPV1 is coupled to mitochondrial function, regulating the calcium buffering and the clearance of mitochondrial ROS [84]. The deregulated activation of TRPV1 by high doses of chemical activators, for instance, could induce cell death possibly mediated by overstimulation of mitochondrial function.

The control of differentiation and of cell death could become interesting targets in the regulation of survival in

neural precursors. Intense neuronal activity or neurodegenerative diseases increase oxidative environment finely modulated through TRP-induced homeostatic stability [76]. Then, the control of TRPV ion channels expression and activity on neural progenitors could trigger efficient signaling crosstalk mechanisms in response to oxidative stress, dysfunction, and damage of neural network [85, 86]. Specifically, the excitotoxicity might be modulated by calcium-dependent receptors internalization mediated by TRP ion channels. Thus, TRP expression and function might stimulate cell protection and regeneration on oxidative-stressed tissues [76, 87].

The transmission of electrical signals from neuron to neuron in complex networks and circuits is central to brain function. Cultured neural stem/progenitors may differentiate into neurons as a consequence of external neural activity [88]. This activity-dependent neurogenesis requires calcium channels in others, in proliferating stem/progenitor cells. Control of proliferation, survival, and connection on the brain network in neural precursors is key to obtain "excitation-neurogenesis coupling" [89], a perfect merge of the stem cell-derived newborn neurons with the remaining neurons in the brain. Thus, the controlled activation of TRPV1 might offer an innovative strategy to cover all these important aspects of well-functioning neural precursors.

7. Perspectives: Control of Neural Stem Cells through TRPV1

In the last years new techniques have been developed to monitor the activity of neural and nonneuronal cells remotely controlling ionic channels. The activation of specific cells through ionic channels triggers gene expression and peptide release *in vivo*. This constitutes a valuable research tool and a novel strategy for controlling cellular activity through regulated protein expression with potential applications in clinical settings.

The control of ionic channels depends on their biophysical properties as gating, desensitization, or allosteric coupling [90, 91]. These properties may be perturbed using chemicals ligands, voltage and light activation (optogenetics), physical modulation by temperature in specific points, or even magnetic fields [57, 92–94]. Some of these mechanisms are more convenient than others; chemical drug use is the simplest but the interaction with the entire organism in *in vivo* experiments leads to secondary effects or nonspecific results. On the other hand, the control of complex networks in animals through electrical or optical methods is technically challenging, because the depth of the tissues strongly attenuates electrical fields and light emission [92]. Magnetic fields were described as a specific remote control method because they have good penetration in biological tissues due to weak interaction with biological molecules [92, 94]. This weak interaction implies that in cells the magnetic fields have to be converted into a different stimulus such as aggregation of particles or mechanical force to act on their targets. One of the most common uses is the coupling of metal nanoparticles to ionic channels; these nanoparticles absorb energy and heat in response to radiofrequencies [94–96]. Using a temperature-sensitive channel, the heating can be converted into a cellular signal to allow ion influx and control of cellular functions [94].

TRPV1 have been used to control specific functions in neuronal and nonneuronal context in culture and in behaving animals [56, 57, 91, 97, 98]. In pancreas, a modified TRPV1 with antibody-coated iron oxide nanoparticles has been used as a temperature sensor that gates calcium to stimulate synthesis and release of bioengineered insulin via a promoter sensitive to calcium when heated in a low-frequency magnetic field [94]. This is particularly interesting because radio waves can be used to remotely activate insulin secretion by heating both externally applied and endogenously synthesized nanoparticles. Nowadays it is possible to generate cells with the ability to produce nanoparticles and of being controlled by TRPV1 activation, at least in nonneural context. However, the selective TRPV1 expression-mediated activation of neurons has shown to produce fast activity onset and consequent behavioral responses that depend on the specified neural population [57]. A paradigm of control where TRPV1-induced neural activity reached peaks within 7 minutes lasted only 10 minutes and was repeated immediately, stimulating brain plasticity through neurotransmitter release of dopaminergic or serotonergic neurons in freely moving mice [57].

Theoretically, selective control of stem cells could be exploited to investigate mechanistic pathways of excitation-neurogenesis coupling [99]. The development of functional TRPV1 channels in our stem cells-derived neurons or in neural precursors may provide a strategy to remotely control the survival, differentiation, and plasticity of these cells.

Imagine there is a new regeneration medicine of stem cells with genetically modified low radiofrequency-sensitive TRPV1 channels to control the survival and integration of stem cells grafts in the human brain.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Dysfunction in Ribosomal Gene Expression in the Hypothalamus and Hippocampus following Chronic Social Defeat Stress in Male Mice as Revealed by RNA-Seq

Dmitry A. Smagin,[1] Irina L. Kovalenko,[1] Anna G. Galyamina,[1] Anatoly O. Bragin,[2] Yuriy L. Orlov,[2,3] and Natalia N. Kudryavtseva[1]

[1]*Modeling of Neuropathology Laboratory, Institute of Cytology and Genetics, Siberian Department of Russian Academy of Sciences, Novosibirsk 630090, Russia*
[2]*Laboratory of Behavioral Neuroinformatics, Institute of Cytology and Genetics, Siberian Department of Russian Academy of Sciences, Novosibirsk 630090, Russia*
[3]*Novosibirsk State University, Novosibirsk 630090, Russia*

Correspondence should be addressed to Natalia N. Kudryavtseva; n.n.kudryavtseva@gmail.com

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Chronic social defeat stress leads to the development of anxiety- and depression-like states in male mice and is accompanied by numerous molecular changes in brain. The influence of 21-day period of social stress on ribosomal gene expression in five brain regions was studied using the RNA-Seq database. Most *Rps, Rpl, Mprs*, and *Mprl* genes were upregulated in the hypothalamus and downregulated in the hippocampus, which may indicate ribosomal dysfunction following chronic social defeat stress. There were no differentially expressed ribosomal genes in the ventral tegmental area, midbrain raphe nuclei, or striatum. This approach may be used to identify a pharmacological treatment of ribosome biogenesis abnormalities in the brain of patients with "ribosomopathies."

1. Introduction

Chronic social defeat stress (CSDS) can lead to the development of behavioral psychopathology, which is accompanied by anxiety- and depression-like states in male mice [1–3] similar to those in humans. It has been shown that, under CSDS, the adult brain undergoes numerous changes, including changes in gene expression in different brain regions [3, 4], DNA methylation, histone acetylation, and chromatin remodeling [5, 6] as well as decreases in hippocampal neurogenesis [7–9].

Analyzing the whole transcriptome using RNA-Seq in five brain regions of depressive mice with chronic social defeats experience, we observed changes in the expression of numerous genes. This report is concentrated on the analysis of genes encoding ribosomal and mitochondrial ribosomal proteins (*Rps* and *Rpl, Mrpl* and *Mrps*) which are responsible for translation, transcription, and proliferation and are involved in neural plasticity in healthy cells.

Two groups of animals were analyzed: male mice in a depression-like state following CSDS over a 21-day period of agonistic interactions and control mice. Choice of the brain regions selected for testing was based on their functions, localization of neurons of some neurotransmitter systems, and differential involvement in the mechanisms of a depression-like state in our experimental paradigm [1, 10]. These regions are as follows: the midbrain raphe nuclei, a multifunctional brain region, which contains the majority of serotonergic neuronal bodies; the ventral tegmental area (VTA), which contains the bodies of dopaminergic neurons, is widely implicated in natural reward circuitry of the brain, and is important in cognition, motivation, drug addiction, and emotions relating to several psychiatric disorders; the striatum, which is responsible for the regulation of motor

activity and stereotypical behaviors and is also potentially involved in a variety of cognitive processes; the hippocampus, which belongs to the limbic system, is essential for memory consolidation and storage, and plays important roles in the neurogenesis and emotional mechanisms; and the hypothalamus, which regulates the stress reaction and many other physiological processes.

2. Materials and Methods

2.1. Animals. Adult male mice of C57BL/6J were obtained from Animal Breeding Center in Pushchino (Moscow region, Russia). Animals were housed under standard conditions (12:12 hr light/dark regime; switch-on at 8.00 a.m.: at temperature of 22 ± 1°C; and food (pellets) and water available *ad libitum*). Experiments were performed on 10–12-week-old animals. All procedures were in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC). The study was approved by Scientific Council number 9 of the Institute of Cytology and Genetics SB RAS of March, 24, 2010, N 613.

2.2. Chronic Social Defeat Stress. Prolonged negative social experiences (defeats) in male mice were induced by daily agonistic interactions with an aggressive partner [1, 10]. Pairs of weight-matched animals were placed in steel cages (14 × 28 × 10 cm) bisected by a perforated transparent partition, which allowed the animals to see, hear, and smell each other but prevented physical contact. The animals were left undisturbed for two days to allow for adaptation to the new housing conditions and sensory contact before they were exposed to encounters. Every afternoon (14:00–17:00 p.m. local time), a transparent cage lid was placed on the cage, and, 5 min later (the period necessary for individual activation), the partition was removed for 10 minutes to encourage agonistic interactions. The superiority of one of the mice was firmly established within two or three encounters with the same opponent. The winning mouse would attack, bite, and chase the losing mouse, which would display only defensive behavior (sideways posture, upright posture, withdrawal, lying on the back, or freezing). As a rule, aggressive confrontations between males were discontinued by lowering the partition if the sustained attacks had lasted 3 min or less to prevent damage to the losers. Each defeated mouse (loser or defeater) was exposed to the same winner for three days; afterwards, each loser was placed, once a day after the fight, in an unfamiliar cage with an unfamiliar winner behind the partition. Each winning mouse remained in its original cage. This procedure was performed for 21 days and yielded an equal number of winners and losers. Two groups of animals were analyzed in this experiment: (1) depressive mice: groups of chronically defeated mice on 21st days of agonistic interactions and (2) controls: the mice without any consecutive experience of agonistic interactions. The detailed description of this behavioral method has been previously published [10].

All of the mice were decapitated simultaneously: the 21-time defeated mice were sacrificed 24 hours after the last agonistic interaction and the control animals. The brain regions from both experimental groups were dissected by one experimenter according to the map presented in the Allen Mouse Brain Atlas (http://mouse.brain-map.org/static/atlas). All of the biological samples were placed to the RNAlater solution (Life Technologies, USA) and stored at −70°C until sequencing.

2.3. RNA-Seq Method. The collected samples were sequenced at JSC Genoanalytica (http://genoanalytica.ru/, Moscow, Russia), where the mRNA was extracted using the Dynabeads mRNA Purification Kit (Ambion, USA). cDNA libraries were constructed using NEBNext mRNA Library PrepReagent Set for Illumina (NEB, USA) following the manufacturer's protocol and were subjected to Illumina sequencing. More than 20 million reads were obtained for each sample. The resulting "fastq" format files were used to align all of the reads to the GRCm38.p3 reference genome using the TopHat aligner [11]. The Cufflinks program was used to estimate the gene expression levels in FPKM (fragments per kilobase of transcript per million mapped reads) and then to detect the differentially expressed genes (DEGs) in the analyzed and control groups. Each brain region was considered separately for 3 versus 3 animals. Only annotated gene sequences were used in the following analysis. Genes were considered to be differentially expressed at $P < 0.01$.

3. Results and Discussion

Gene expression levels were compared between mouse groups affected by social stress, depressive mice, and the control animals. Analysis of differentially expressed genes showed their dependence on the brain regions (Table 1): in the hypothalamus, 3703 genes changed their expression pattern under CSDS (ratio of up/down is 2244/1459, resp.); in the striatum, 931 genes changed their expression pattern under CSDS (up/down, 770/161); in the hippocampus, 841 genes changed their expression pattern under CSDS (up/down, 423/418); in the VTA, 549 genes changed their expression pattern under CSDS (up/down, 229/320); and, in the raphe nuclei, 453 genes changed their expression pattern under CSDS (up/down, 104/349) at the chosen level of statistical significance ($P < 0.01$). Thus, the largest number of differentially expressed genes in depressive mice was observed in the hypothalamus, and approximately 4 times fewer genes were observed in the striatum and hippocampus and approximately 7 times fewer genes were observed in the VTA and midbrain raphe nuclei. In the hypothalamus and striatum, the number of upregulated genes was higher than number of downregulated genes. In the VTA and midbrain raphe nuclei area, most of the differentially expressed genes were downregulated. In the hippocampus, the numbers of upregulated and downregulated genes were approximately equal. We can assume that the number of differentially expressed genes and direction of change (up or down) may be used as marker of more or less intensive involvement of any brain area into molecular mechanisms of depression-like state in mice. These changes may depend on function

Table 1: The number of genes that changed their expression in brain regions of depressive male mice.

	Raphe nuclei	Hippocampus	VTA	Striatum	Hypothalamus
All genes	453	841	549	931	3703
Upregulated	104	423	229	770	2244
Downregulated	349	418	320	161	1459
Ribosome genes	Up/down	Up/down	Up/down	Up/down	Up/down
RPS	0	0/2	0	0	13/2
RPl	0	2/3	0	0	21/1
Mrps	0	0	0	0	2/0
Mrpl	0	0	0	0	8/2

of brain regions and different mechanisms regulating CSDS. Other authors have demonstrated changes of numerous genes expression in the nucleus accumbens under CSDS [3].

In the midbrain raphe nuclei, VTA, and striatum, the *Rps, Rpl, Mrpl,* and *Mrps* genes did not change their expression under CSDS. Because ribosomes are responsible for protein synthesis in all cells, we suspect that the translation, transcription, and proliferation of proteins are not significantly disturbed in these brain regions.

In the hippocampus and hypothalamus, the major components of ribosomes—the small ribosomal subunit that reads the RNA (*Rps*) and the large subunit that connects amino acids to form a polypeptide chain (*Rpl*)—changed their expression under CSDS. In the hippocampus of depressive mice, the largest number of ribosomal genes (*Rpl7, Rpl36a, Rpl39, Rps4x,* and *Rps27a*) was downregulated and only 2 genes (*Rpl35* and *Rpl18*) were upregulated. We can assume that downregulation of the ribosomal genes may be associated with a decrease of proliferation in the hippocampal dentate gyrus under CSDS in mice, as described by many authors using a similar experimental paradigm [7–9].

In the hypothalamus, numerous ribosomal genes changed their expression under CSDS (14 *Rps* and 22 *Rpl* genes). *Rps14, Rps8, Rps6ka1, Rps9, Rps5, Rps19, Rps16, Rps3, Rpsa, Rps2, Rps26,* and *Rps10* and *Rpl37a, Rpl41, Rpl19, Rpl23a, Rpl37, Rpl8, Rpl10a, Rpl36, Rpl7a, Rpl12, Rpl35, Rpl34, Rplp0, Rpl6, Rpl28, Rpl18, Rplp2, Rpl13, Rpl18a, Rpl29,* and *Rplp1* were upregulated, and *Rpl22l1, Rps6ka3,* and *Rps6ka6* were downregulated (Figures 1 and 2). Enhanced expression of the *Rpl18* and *Rpl35* genes was overlapped in the hippocampus and hypothalamus.

The hypothalamus is responsible for production of numerous hormones that are involved in the regulation of many physiological functions and psychoemotional states. Many diseases are connected with abnormal hypothalamic function, such as changed stress reactions, metabolism, loss or increase of appetite, changed emotional behavior, memory loss, sleep disorders, and affective and somatic states. Because decreased stress reactivity, weight loss, and development of pronounced anxiety- and depression-like state were observed in the mice after CSDS [1, 12], we suggest a significant involvement of the hypothalamus in these pathological processes. Support for this hypothesis comes from the observation that the largest number of all differentially expressed genes was observed in this region. The majority of these genes (60%),

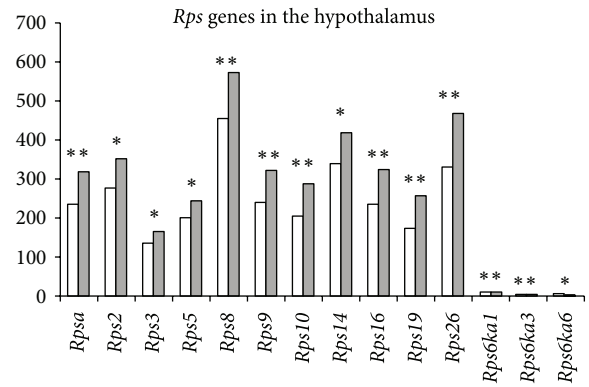

Figure 1: The differentially expressed ribosomal *Rps* genes in the hypothalamus of mice following CSDS. The Cufflinks program was used to estimate the gene expression levels in FPKM. The levels of the *Rps* gene expression are presented in the control (left columns) and depressive mice (right columns). The *Rps14, Rps8, Rps6ka1, Rps9, Rps5, Rps19, Rps16, Rps3, Rpsa, Rps2, Rps26,* and *Rps10* genes were upregulated, whereas *Rps6ka3* and *Rps6ka6* were downregulated under CSDS in depressive mice. Statistical significance $P < 0.01$ and $q < 0.05$. $^{*}P < 0.01$; $^{**}P < 0.001$.

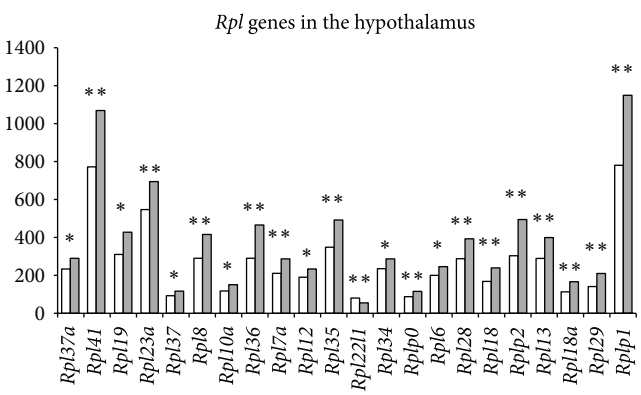

Figure 2: Differentially expressed ribosomal *Rpl* genes in the hypothalamus of mice following CSDS. The Cufflinks program was used to estimate the gene expression levels in FPKM. The levels of the *Rpl* genes expression are presented in the control (left columns) and depressive mice (right columns). The *Rpl37a, Rpl41, Rpl19, Rpl23a, Rpl37, Rpl8, Rpl10a, Rpl36, Rpl7a, Rpl12, Rpl35, Rpl34, Rplp0, Rpl6, Rpl28, Rpl18, Rplp2, Rpl13, Rpl18a, Rpl29,* and *Rplp1* genes were upregulated, whereas the *Rpl22l1* gene was downregulated. Statistical significance $P < 0.01$ and $q < 0.05$. $^{*}P < 0.01$; $^{**}P < 0.001$.

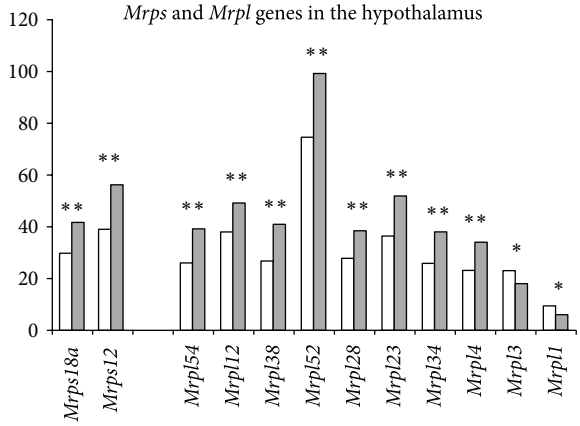

Figure 3: Differentially expressed mitochondrial ribosomal *Mrps* and *Mrpl* genes in the hypothalamus of mice following CSDS. The Cufflinks program was used to estimate the gene expression levels in FPKM. The levels of the *Mrps* and *Mrpl* gene expression are presented in the control (left columns) and depressive mice (right columns). The *Mrpl54, Mrpl12, Mrpl38, Mrpl52, Mrpl28, Mrpl23, Mrpl34, Mrpl4, Mrps18a, Mrps12, Mrps18a,* and *Mrps12* genes were upregulated, whereas *Mrpl1 and Mrpl3* were downregulated. Statistical significance $P < 0.01$ and $q < 0.05$. $^{*}P < 0.01$; $^{**}P < 0.001$.

including ribosomal genes, were upregulated in depressive mice. However, does upregulation of numerous ribosomal genes present a feedback mechanism in response to hypothalamic activation under CSDS, or is this a result of ribosomal gene dysfunction developing in depressive mice?

In recent years a number of human diseases have been identified and categorized as "ribosomopathies" [13, 14] caused by alterations in either the structure or function of ribosomal components, which are associated with distinct mutations in the ribosomal biogenesis pathway. These diseases include Diamond-Blackfan anemia, Shwachman-Diamond syndrome, and dyskeratosis congenita. The *Rps10* and *Rps26* genes are commonly mutated in Diamond-Blackfan anemia and have been associated with mutations in seven other ribosomal protein genes (*Rps19, Rps24, Rps17, Rpl35A, Rpl5, Rpl11*, and *Rps7*) in approximately 43% of patients [15, 16]. Interestingly, increased expression of the *Rps19, Rps14, Rps10,* and *Rps26* genes, which are involved in Diamond-Blackfan anemia, was observed in depressive mice. We did not find literature data concerning ribosome dysfunction during depression; however, our observation concerning changes in the expression of ribosomal genes in the hippocampus and hypothalamus in mice indicates developing ribosomal dysfunction under CSDS.

There were no mitochondrial ribosomal genes found that changed expression under CSDS in the hippocampus. However, in the hypothalamus the results obtained indicate the development of possible mitochondrial protein dysfunctions: the mitochondrial ribosome genes *Mrpl54, Mrpl12, Mrpl38, Mrpl52, Mrpl28, Mrpl23, Mrpl34, Mrpl4 Mrps18a,* and *Mrps12* were upregulated in depressive mice, whereas *Mrpl1* and *Mrpl3* were downregulated (Figure 3). Thus, we can assume a strong link between CSDS leading to the development of a depression-like state in mice and the activation of mitochondrial ribosomal genes in the hypothalamus. These suppositions are confirmed indirectly by experimental data that have demonstrated the upregulation of mitochondrial genes in the amygdala of rats in a depression-like state induced by inescapable tail shock [17]. In a genetic model of depression, changes in the number and morphology of mitochondria in the hippocampus were shown [18]. Another author group observed the influence of chronic unpredictable stress on the serotonin levels in the raphe nuclei and hippocampus and overactivation of mitochondria in the raphe nuclei of mice [19]. Earlier we found decreased brain serotonergic activity in depressive mice as shown by decreased serotonin levels and/or 5-hydroxyindoleacetic acid and tryptophan hydroxylase activity, the key limiting enzyme of serotonin synthesis, in different brain areas [2, 20], as well as downregulation of serotonergic *Tph2, Sert, Maoa,* and *Htr1a* gene expression, which are associated with the synthesis, inactivation, and reception of serotonin, respectively, in the midbrain raphe nuclei [21]. We can suggest that overactivation of mitochondria, determined by the respiratory control ratio, ATP synthesis rate, and activities of superoxide dismutase and glutathione peroxidase shown by authors [19] in the raphe nuclei may be result of feedback mechanisms on the development of hypofunction of serotonergic activity [2, 20, 21] in this brain region of stressed mice. Conversely, in the hypothalamus, activation of tryptophan hydroxylase was observed in depressive mice [2, 20]. It could be assumed that the development of mitochondrial dysfunction in depressive mice is associated with activation of serotonergic system, at least in the hypothalamus. This conclusion is indirectly confirmed by observations that patients with mitochondrial disorders can show primary psychiatric symptomatology, including mood disorder, cognitive impairment, psychosis, and anxiety [22].

Mitochondrial disorders may be caused by either acquired or inherited mutations in the mitochondrial DNA or in nuclear genes that code for mitochondrial components [23]. These disorders may also be the result of acquired mitochondrial dysfunction due to adverse effects of drugs, infections, or other environmental causes. The majority of mitochondrial disorders are associated with neurological abnormalities, including seizures and myoclonus, psychomotor retardation, dementia, ataxia, motor neuron disease, weakness, and chronic fatigue [24]. Depressive mice have been shown to demonstrate also motor retardation, immobility, and helplessness in any situations [1, 2, 12].

Undoubtedly it is difficult to find a direct association between the overexpression of ribosomal genes and mitochondrial ribosomal genes in the hypothalamus and the depression-like state in mice, which would help to understand causes and consequences of these processes. At this stage of research, it is impossible to elucidate the detailed sequence of neurochemical events involved, and, as a result, the molecular changes that occur due to restructuring brain regulation in male mice under CSDS. However, it is clear that, starting with a change in social behavior and psychoemotional state under CSDS, at certain stages this process

launches a cascade of systemic changes at the whole brain level, its regions, and specific neurons following changes in metabolism and reception of neurotransmitter systems. As a result, it leads to the changes in the expression of genes involved in the development of affective disorders. The changes observed in ribosomal and mitochondrial ribosomal gene expression may indicate ribosome dysfunction. Our model, which induces a mixed anxiety/depression-like state [1, 2] in male mice following CSDS may be used to identify a pharmacological treatment of ribosome biogenesis abnormalities in the brain.

Conflict of Interests

None of the authors have any conflict of interests to report.

Authors' Contribution

Natalia N. Kudryavtseva performed study design, analyzed and interpreted data, and wrote the main paper text. Dmitry A. Smagin, Irina L. Kovalenko, and Anna G. Galyamina contributed substantially to behavioral data acquisition, received brain materials, and analyzed the RNA-Seq database. Yuriy L. Orlov and Anatoly O. Bragin revised statistics critically. All authors gave final approval.

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Research Article

Distinct Functions of Endophilin Isoforms in Synaptic Vesicle Endocytosis

Jifeng Zhang,[1,2] Minghui Tan,[1,3] Yichen Yin,[4] Bingyu Ren,[2] Nannan Jiang,[2] Guoqing Guo,[1] and Yuan Chen[2]

[1]*Department of Anatomy, Medical College of Jinan University, Guangzhou 510630, China*
[2]*Center for Neurobiology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510080, China*
[3]*Department of Orthopedics, The First Affiliated Hospital of Jinan University, Guangzhou 510630, China*
[4]*Department of Neurology, Guangzhou Red Cross Hospital, The Fourth Affiliated Hospital of Jinan University, Guangzhou 510220, China*

Correspondence should be addressed to Guoqing Guo; tgqguo@yahoo.com and Yuan Chen; cheny33@mail.sysu.edu.cn

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Endophilin isoforms perform distinct characteristics in their interactions with N-type Ca^{2+} channels and dynamin. However, precise functional differences for the endophilin isoforms on synaptic vesicle (SV) endocytosis remain unknown. By coupling RNA interference and electrophysiological recording techniques in cultured rat hippocampal neurons, we investigated the functional differences of three isoforms of endophilin in SV endocytosis. The results showed that the amplitude of normalized evoked excitatory postsynaptic currents in endophilin1 knockdown neurons decreased significantly for both single train and multiple train stimulations. Similar results were found using endophilin2 knockdown neurons, whereas endophilin3 siRNA exhibited no change compared with control neurons. Endophilin1 and endophilin2 affected SV endocytosis, but the effect of endophilin1 and endophilin2 double knockdown was not different from that of either knockdown alone. This result suggested that endophilin1 and endophilin2 functioned together but not independently during SV endocytosis. Taken together, our results indicate that SV endocytosis is sustained by endophilin1 and endophilin2 isoforms, but not by endophilin3, in primary cultured hippocampal neurons.

1. Introduction

Clathrin-mediated endocytosis (CME) is an evolutionarily conserved process that cells use to internalize specific components of the plasma membrane [1, 2]. In neurons, CME plays a particularly important role, functioning both on the presynaptic and postsynaptic sides of neuronal synapses [3, 4]. Numerous studies have revealed that CME regulates the recycling of synaptic vesicles (SVs) at presynaptic membranes [4, 5], mediates the internalization of neurotransmitter receptors, and contributes to synaptic plasticity by controlling postsynaptic excitability at postsynaptic membranes [5–8].

Endophilin is one of the major endocytic proteins important in CME [9–11]. At least three isoforms of endophilin have been identified and distributed in various tissues. For instance, endophilin1 is expressed only in the brain, endophilin2 exists in multiple tissues, and endophilin3 is found mainly in the brain and testis [12–14]. Endophilin dysfunction has been linked to both cancer and neurodegenerative diseases [9, 15–17]. Microinjection of endophilin antibodies into giant axons causes a stimulus-dependent depletion of SV in lamprey [10]. Mutant *Drosophila* larvae lacking endophilin fail to take up FM1-43 dye, indicating an inability to retrieve synaptic membranes [18]. Endophilin1 assists in downregulating epidermal growth factor receptor (EGFR) and other growth factor receptors [15]. Similarly, overexpression of endophilin2 in 293T cells increases EGF-induced endocytosis of EGFRs [19]. However, endophilin3 reportedly inhibits receptor-mediated endocytosis. The COS-7 cells transfected with endophilin1 do not affect transferrin uptake efficiency,

whereas transfection with full-length endophilin3 strongly reduces it [20].

At the subcellular level, all three endophilin isoforms are concentrated at synaptic terminals and in the cytosol of neurons [13]. Electron microscopy of immunogold-labeled endophilin shows that endophilin is localized at both presynaptic and postsynaptic membranes. Endophilin functions as a membrane-bending molecule and is delivered to endocytic zones by exocytosis [21]. Endophilin2 and endophilin3, but not endophilin1, are involved in Arc/Arg3.1-mediated AMPA receptor endocytosis on postsynaptic membranes, suggesting that isoform specificity can confer particular properties to specific endocytic pathways [21]. At the presynaptic terminal, a series of studies revealed that the functional domains of endophilin affect several stages of CME [9, 10]. An amino acid sequence analysis showed that all three endophilin isoforms consist of an N-terminal Bin/amphiphysin/Rvs (BAR) domain, a variable middle region, and a C-terminal SRC homology-3 (SH3) domain [22]. The BAR domain mainly participates at the base of the membrane invagination, an early step in endocytosis of the membrane [23]. The SH3 domain is involved in the interaction of endophilin with other endocytic machinery molecules that contain a proline-rich domain (PRD), such as synaptojanin and GTPase dynamin [12–14]. Disrupting the interactions perturbs the fission and uncoating of synaptic clathrin-coated vesicles [24]. Our previous studies found an unconventional PRD embedded within endophilin2, and the intramolecular interaction between this unconventional PRD and the C-terminal SH3 domain is Ca^{2+}-dependent [25]. Further biochemical studies demonstrated that endophilin1 has a similar Ca^{2+}-dependent interaction with voltage-gated Ca^{2+} channels and dynamin as endophilin2, whereas endophilin3 does not [26]. These findings imply that these structurally similar endophilin isoforms may have distinct characteristics in interactions with other endocytic machineries. However, the precise functional differences of each endophilin isoform on presynaptic SV endocytosis remain unknown.

Here, we report that endophilin isoforms have different effects on SV endocytosis. We found that SV endocytosis was impaired in endophilin1 or endophilin2 knockdown neurons but was unaffected in endophilin3 knockdown neurons. Our results indicate that endophilin1 or endophilin2, but not endophilin3, is the main regulatory machinery involved in clathrin-mediated SV endocytosis.

2. Materials and Methods

2.1. Ethics Statement. The experiments were conducted with one-day-old Sprague Dawley rats. All animal procedures were performed in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* produced by the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at Zhongshan School of Medicine in Sun Yat-Sen University. All efforts were made to minimize the suffering and number of animals used.

2.2. Hippocampal Neuronal Culture and Transfection. Rat hippocampal neurons were cultured as described previously [27]. Neurons cultured in 24-well culture plates at 12–14 days *in vitro* were used to perform the transfection. Endophilin siRNA (100 pmol) was combined with 37 μL of 2.5 M $CaCl_2$ solution in sterile, deionized water for a final volume of 300 μL and then mixed well with 300 μL of 2x HEPES-buffered saline. The mixture was vortexed and incubated at 25°C for 15 min. In each well, 30 μL of the mixture was added dropwise to the cells and allowed to incubate for 40 min. A GFP expression plasmid was cotransfected with the siRNAs to mark the transfected cells.

2.3. Plasmids. The full-length endophilin1, endophilin2, and endophilin3 cDNA fragments were subcloned into pEGFP-C1 plasmids (Clontech). All constructs were verified by sequencing. A detailed description of the methods used for constructing cDNA plasmids is available in our previous studies [25, 26].

2.4. RNA Interference. The 19-nucleotide siRNAs for each endophilin isoform and their related negative controls (NC, scrambled sequence) were designed using online software (http://www.promega.com/siRNADesigner/) and were synthesized by Shanghai Gene-Pharma Co., Ltd. (Shanghai, China). These included the si1-1- and si1-2-targeted sequences 5′-GGGCTAAACTCAGTATGAT-3′ and 5′-CCGACGCTTAGACTTTGAT-3′ of endophilin1 mRNA (NM_053935), si2-1- and si2-2-targeted sequences 5′-GAGGTTCTATTACCTTTCT-3′ and 5′-GCTTCGTCATCATTTAGAT-3′ of endophilin2 mRNA (NM_031239), and si3-1- and si3-2-targeted sequences 5′-GCCGAAAGAAGCATGTTTA-3′ and 5′-GCTTCGTCATCATTTAGAT-3′ of endophilin3 mRNA (NM_031238). The targeted regions showed no significant homology with any other genes using BLAST.

2.5. HEK293 Cell Culture and Transfection. Human embryonic kidney (HEK) 293 cell culture was performed as described previously [27]. To determine the efficacy and specificity of siRNAs, cotransfection of 100 pmol of siRNAs or NC together with 2 μg of the corresponding endophilin-pEGFPC1 plasmids into HEK293 cells was performed using calcium phosphate.

2.6. Western Blotting. The cells were lysed and separated 48 h after cotransfection using 10% SDS-PAGE and electrophoretically transferred to PVDF membranes (Pierce, Rockford, IL). The membranes were blocked with 5% nonfat milk in TBS and 0.1% Tween-20 and then incubated with an isoform-specific endophilin antibody or anti-endophilins1–3 antibody (Santa Cruz). After three to four washes with TBS and 0.1% Tween-20, the membranes were incubated with HRP-conjugated secondary antibodies (Jackson ImmunoResearch). The protein bands were detected after developing the blots using an ECL kit (Pierce).

2.7. Immunostaining. Three days after siRNA transfection, the hippocampal neurons were fixed with 4% paraformaldehyde (Sigma). Immunostaining was then performed using

a standard protocol described previously [27]. The primary antibodies, anti-endophilin1, 2, or 3, were used at a dilution of 1 : 100, and donkey anti-goat IgG (H + L) Dylight 549 (Jackson ImmunoResearch) was used at a dilution of 1 : 500. After staining, the cells were mounted on glass slides using Fluoro Gel II with DAPI (EMS) and imaged with a Carl Zeiss LSM 710 confocal microscope. Images were acquired with the same optical slice thickness in every channel using a 63x oil objective and a resolution of 1024 × 1024 pixels. The RNA interference efficiency in hippocampal neurons was determined by calculating the percentage of endophilin-positive cells, as previously described [28].

2.8. Electrophysiological Recordings. Excitatory postsynaptic currents (EPSCs) were recorded in cultured hippocampal neurons using dual whole-cell recordings by evoking an action potential (AP) or a train of APs in transfected neurons. The APs were evoked in transfected neurons by delivering brief (3 ms) and large (300–400 pA) depolarizations in current-clamp mode, and EPSCs were recorded in voltage-clamp (−70 mV) mode in a nearby, nontransfected neuron. For the input-output relationship of APs, APs were evoked using a 400 ms current injection at various intensities (0–200 pA). To measure the depletion of the readily releasable pool (RRP) of SVs, the transfected neurons were challenged with high-frequency stimulation (100 pulses at 5 Hz). To compare the size of the frequency-dependent depression of EPSCs during high-frequency stimulation, the 2nd to 100th responses were normalized to the 1st response, and the last 20 responses were averaged and normalized to the 1st response to compare among the groups. To measure the replenishment of the RRP, the transfected neurons were stimulated with consecutive high-frequency stimulation. For consecutive high-frequency stimulation, four trains of stimulation (5 Hz, 100 stimuli) were applied with a 3 min interval, during which the transfected neuron was stimulated at 0.1 Hz. To compare the recoveries of the RRP, the EPSC amplitudes recorded at 0.1 Hz after the 1st, 2nd, 3rd, and 4th train were normalized to the amplitude recorded before the 1st train. The experiment was conducted at room temperature using an EPC-10 patch-clamp amplifier and Patchmaster software (Heka Electroniks). The EPSCs were recorded with a patch electrode (3–5 MΩ tip resistance) and filtered at 2 kHz. The extracellular bath solution contained (in mM) NaCl, 128; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 15; and HEPES, 20 (pH 7.3). The pipette solution contained (in mM): KCl, 147; Na_2-phosphocreatine, 5; EGTA, 2; MgATP, 2; Na_2GTP, 0.3; and HEPES, 10 (pH 7.2).

2.9. Statistical Analysis. Data are presented as the mean ± SEM. The statistical significance of the differences was analyzed using Student's *t*-test between two groups and one-way ANOVA with Newman-Keuls *post hoc* tests for comparisons among more than two groups.

3. Results

3.1. Knockdown of Endophilin Isoforms Does Not Affect SV Exocytosis. The efficacy of the designed siRNAs against the endophilin isoforms was tested in HEK293 cells. At least one interference fragment selectively inhibited the expression of its corresponding endophilin (Figure 1(a)). The specificity of each endophilin isoform siRNA was also determined. As shown in Figure 1(b), isoform-specific siRNAs did not reduce the expression of other endophilin isoforms. We next confirmed the effectiveness of the siRNAs in cultured hippocampal neurons. As shown in Figure 1(c), compared with the NC, the expression of endogenous endophilin1 was significantly knocked down in the Endo1 siRNA-transfected neurons. Similar results for the siRNA effect were also observed with the other two endophilin isoforms (data not shown). Isoform-specific siRNA resulted in 70–80% knockdown of the corresponding endogenous endophilin (Figure 1(d)). These results indicate that the designed endophilin isoform-specific siRNAs are effective.

To examine whether endophilin isoforms affected SV endocytosis, we performed dual-cell patch-clamp recordings in cultured hippocampal neurons. Neurons transfected with siRNA or NCs (Figure 2(a) left, green) were challenged with various stimulation patterns, and EPSCs were recorded from neighboring nontransfected cells (Figure 2(a) right, gray). We first assessed whether the intrinsic electrophysiological features of the transfected neurons were altered by transfection of siRNA or NC. The number of APs induced under the same stimulation parameters in neurons transfected with either siRNA ($n = 16$, including five Endo1 siRNAs, five Endo2 siRNAs, and six Endo3 siRNAs) or NC ($n = 16$) was consistent with that in nontransfected neurons ($n = 18$; $p > 0.05$; Figure 2(c)), indicating that introducing siRNA or NC into cultured neurons did not change the intrinsic electrophysiological features of neurons. When stimulating at a low frequency (0.1 Hz for 3 min), neurons transfected with either siRNA or NC released neurotransmitters in response to brief depolarizing pulses (Figure 2(b), right). The EPSC amplitude evoked from NC-transfected neurons was 435.492 ± 86.872 pA ($n = 19$). The EPSC amplitudes in endophilin isoform knockdown groups were 569.328 ± 89.497 pA for Endo1 siRNA ($n = 13$), 478.991 ± 97.81 pA for Endo2 siRNA ($n = 11$), and 615.125 ± 129.694 pA for Endo3 siRNA ($n = 10$), which were not significantly different from that in the NC group ($p > 0.05$, Figure 2(d)). These results suggest that knocking down endophilin isoforms has no apparent effect on SV exocytosis, consistent with a report that ablation of endophilin in *Drosophila* yields no significant evoked excitatory junctional potential changes [29].

3.2. Endophilin1 and Endophilin2, but Not Endophilin3, Are Involved in SV Endocytosis. When stimulating at high frequency, a reduction in the synaptic response (called short-term synaptic depression, STD) was observed as a result of depleting the RRP of the SV (Figure 3(a), NC). At a stimulating intensity of 5 Hz (100 stimuli), synaptic depression was enhanced in neurons transfected with Endo1 siRNA compared with that in neurons transfected with NC (Figures 3(a) and 3(b)). Similar results were also observed for Endo2 siRNA-transfected neurons (Figures 3(a) and 3(c)). The marked STD in Endo1 siRNA- and Endo2 siRNA-transfected

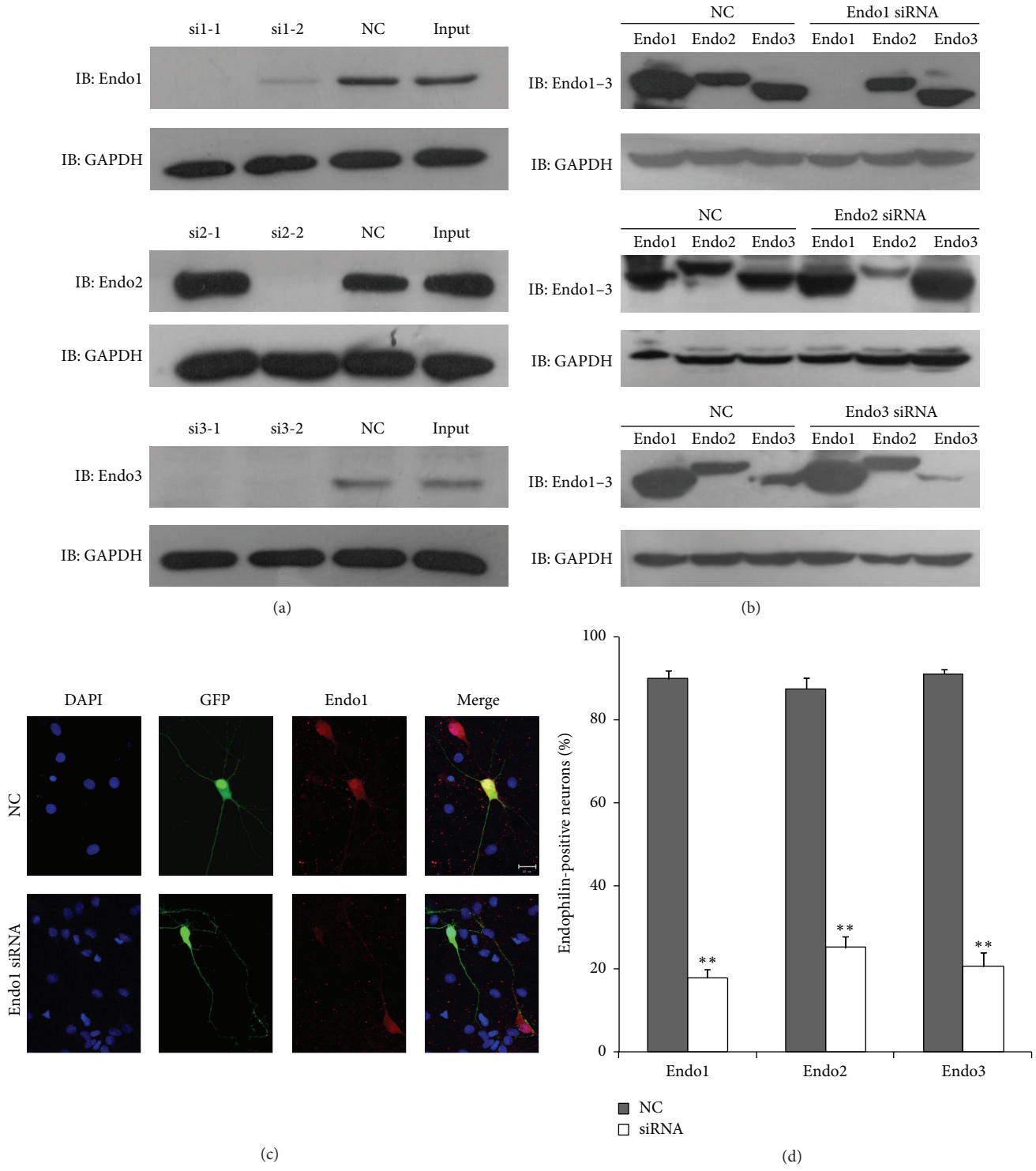

Figure 1: Efficacy and specificity of the designed siRNAs. (a) Immunoblotting detection of endophilin isoforms and GAPDH in HEK293 cells cotransfected with endophilin-pEGFPC1 plasmids and their corresponding siRNAs or negative control (NC). (b) Effect of each endophilin isoform-specific siRNA on the expression of other endophilin isoforms in HEK293 cells. (c) Neurons cotransfected with GFP (green) and NC or Endo1 siRNA. Cultures were stained using an endophilin1 antibody (red). Scale bar, 20 μm. (d) The percentages of endophilin-positive neurons with each treatment were quantified as the mean ± SEM of three independent experiments. $^{**}P < 0.005$.

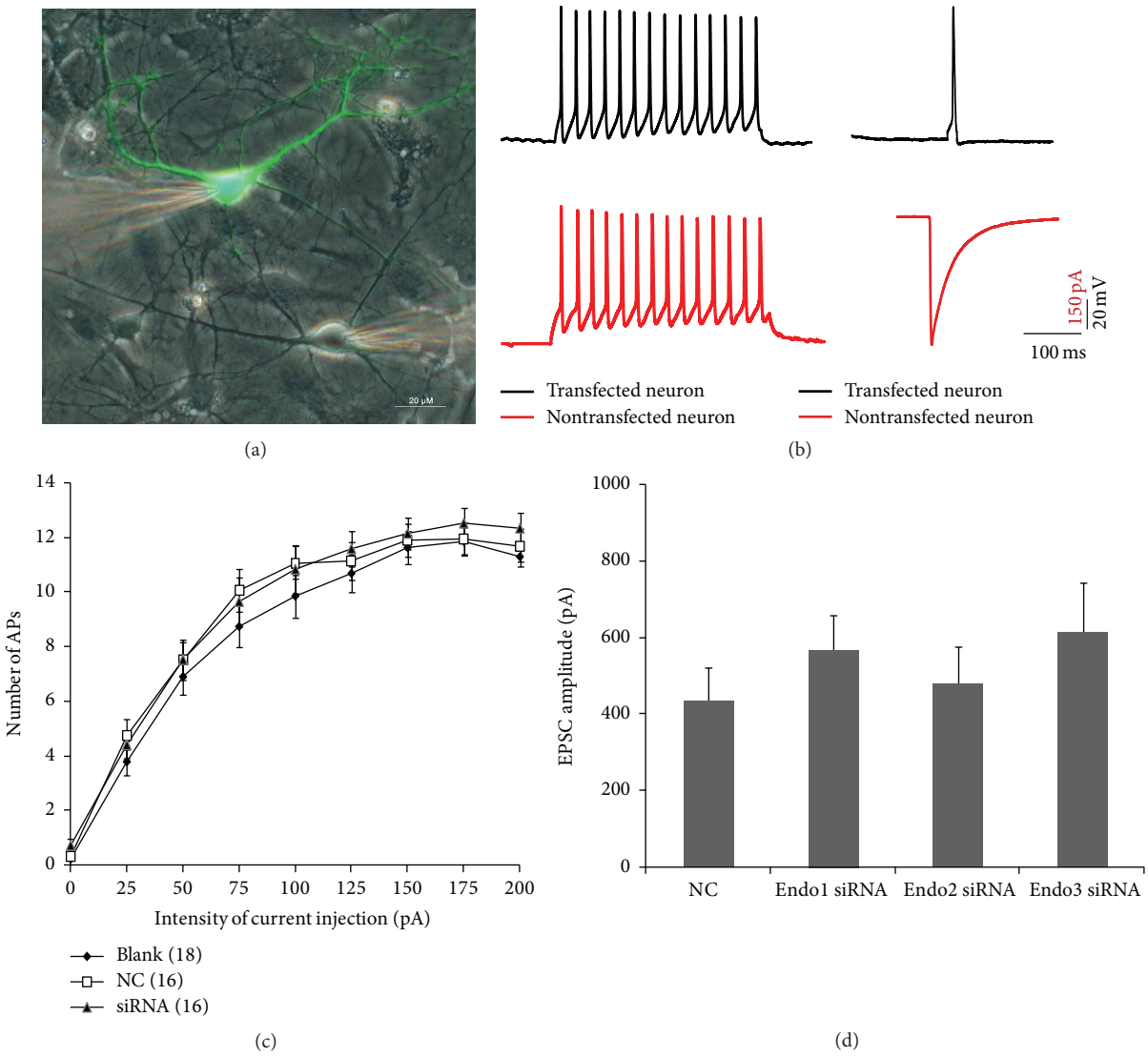

Figure 2: Basic electrophysiological characteristics and evoked excitatory postsynaptic currents (EPSCs) of transfected neurons. (a) Dual-cell patch-clamp recorded hippocampal neurons transfected with siRNA or a scrambled sequence (negative control, NC) (left, green) and nontransfected neurons (right, gray). (b) Representative traces from neurons. Transfected and nontransfected neurons exhibit many action potentials (APs) following 400 ms, 200 pA current injection (left panel). EPSC evoked in a nontransfected neuron by a single AP from a transfected neuron (right panel). (c) Input-output relationship of APs evoked by 400 ms current injection at various intensities in normal neurons (blank, $n = 18$), NC ($n = 16$), and siRNA-transfected neurons ($n = 16$, including five Endo1 siRNAs, five Endo2 siRNAs, and six Endo3 siRNAs). (d) EPSC amplitude evoked in a nontransfected neuron by a single AP in a neuron transfected with NC ($n = 19$), Endo1 siRNA ($n = 13$), Endo2 siRNA ($n = 11$), and Endo3 siRNA ($n = 10$).

neurons suggested that SV endocytosis was impaired. These results were consistent with previous observations when SV endocytosis was impaired [27, 29]. However, the EPSC amplitude in Endo3 siRNA-transfected neurons was not decreased compared with that in NC-transfected neurons (Figures 3(a) and 3(d)). The mean normalized EPSC amplitudes for the last 20 responses in neurons transfected with Endo1 siRNA and Endo2 siRNA were 0.266 ± 0.029 ($n = 13$) and 0.281 ± 0.012 ($n = 8$), respectively, significantly lower than that in the NC group (0.586 ± 0.034, $n = 8$, $^{**}p < 0.005$). However, there was no significant difference in the mean normalized EPSC amplitude between the Endo3 siRNA (0.566 ± 0.027, $n = 12$) and NC groups ($p > 0.05$, Figure 3(e)).

When SV endocytosis is impaired, replenishment of the RRP is also hindered. Thus, to further confirm that these three endophilin isoforms played distinct roles in SV endocytosis, presynaptic neurons were challenged with consecutive high-frequency stimulation. Four trains of stimulation (5 Hz, 100 stimuli) were applied with a 3 min interval, during which time the presynaptic neuron was stimulated at 0.1 Hz. When multiple train stimulations were applied, a steady decrease was observed in the averaged EPSC amplitude evoked at 0.1 Hz

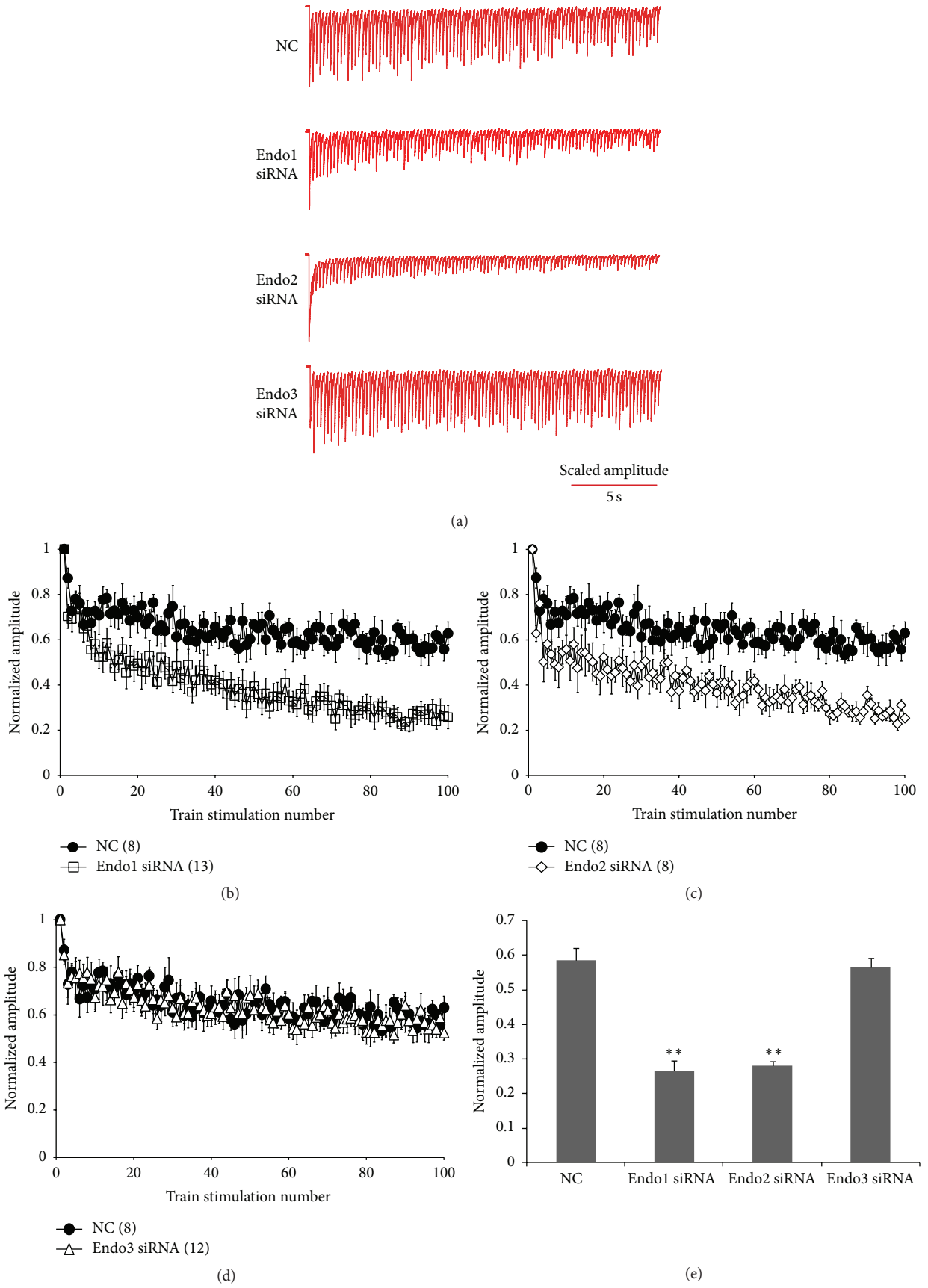

Figure 3: Continued.

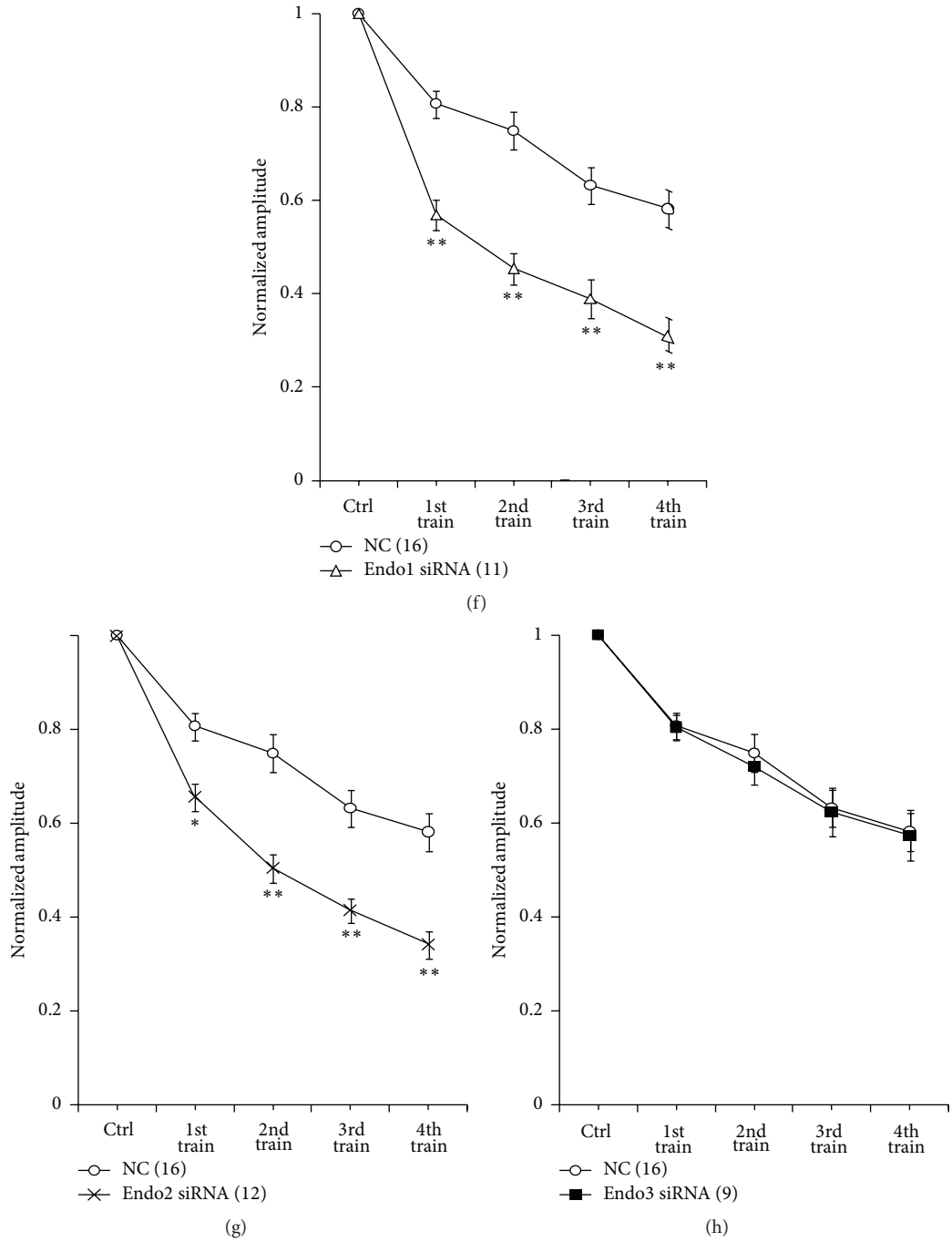

Figure 3: Quantitative analysis of endophilin isoform knockdown on sustained neurotransmitter release. (a) Representative traces of excitatory postsynaptic currents (EPSCs) in nontransfected neurons evoked by 100 pulses at 5 Hz in neurons transfected with control or endophilin siRNA. (b–d) Changes in short-term synaptic depression (STD) in nontransfected neurons evoked by stimulation of 100 pulses at 5 Hz in neurons transfected with endophilin siRNA ((b), Endo1 siRNA, $n = 13$; (c), Endo2 siRNA, $n = 8$; and (d), Endo3 siRNA, $n = 12$). (e) Average normalized amplitude of the last 20 responses in endophilin siRNA-transfected neurons compared with that in scrambled negative control- (NC-) transfected neurons; $^{**}p < 0.005$. (f–h) Normalized amplitude of EPSCs evoked by multiple train stimulations in neurons transfected with NC or endophilin siRNA. (f), Endo1 siRNA, $n = 11$; (g), Endo2 siRNA, $n = 12$; and (h), Endo3 siRNA, $n = 9$. $^{*}p < 0.05$, $^{**}p < 0.005$.

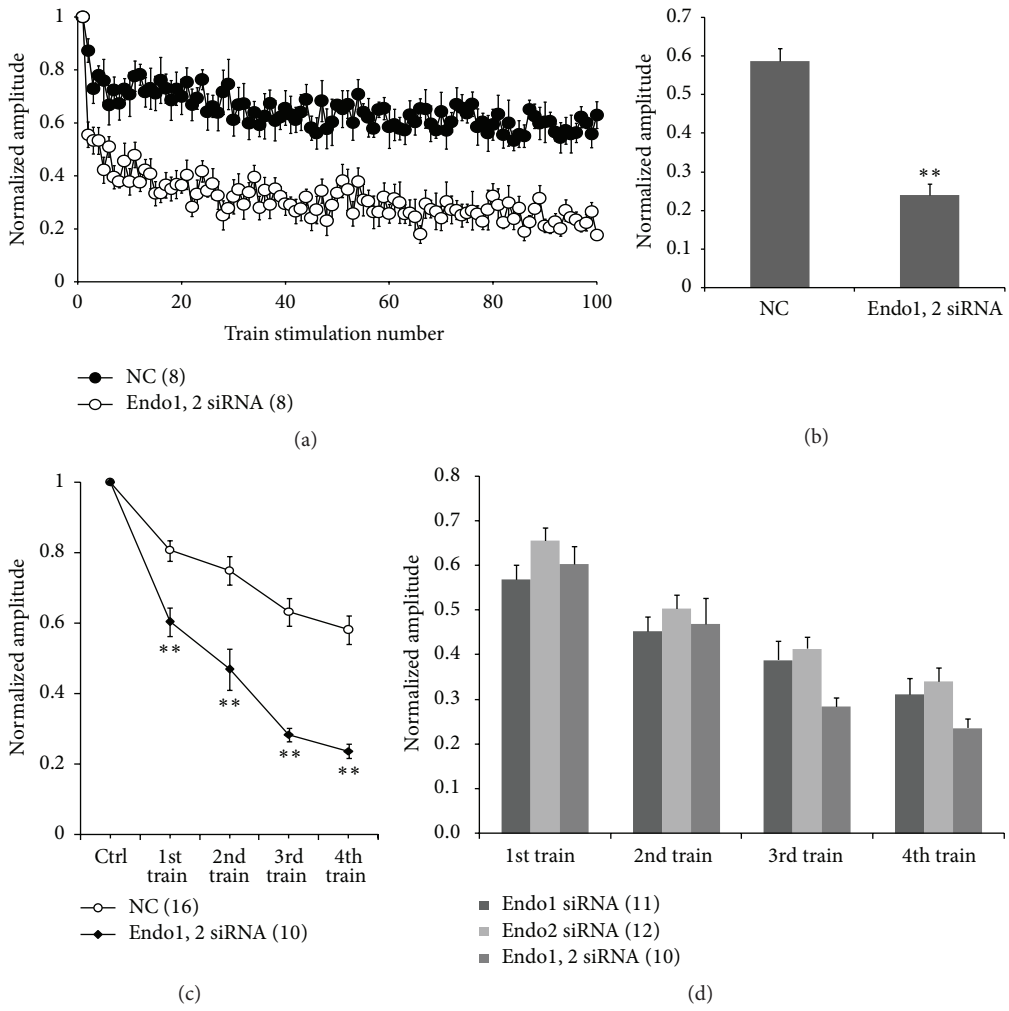

Figure 4: Quantitative analysis of sustained neurotransmitter release in endophilin1, endophilin2 (Endo1, 2) double-knockdown neurons. (a) Changes in short-term synaptic depression (STD) in nontransfected neurons evoked by 100 pulses at 5 Hz in neurons cotransfected with Endo1 and Endo2 siRNAs ($n = 8$). (b) Average normalized amplitude of the last 20 responses in Endo1, 2 siRNA-transfected and scrambled negative control- (NC-) transfected neurons; $n = 8$, $^{**}p < 0.005$. (c) Normalized amplitude of excitatory postsynaptic currents (EPSCs) evoked by multiple train stimulation in neurons transfected with control or Endo1, 2 siRNA; $^{**}p < 0.005$. (d) Normalized amplitudes compared among Endo1 siRNA ($n = 11$), Endo2 siRNA ($n = 12$), and Endo1, 2 siRNA ($n = 10$) after the 1st, 2nd, 3rd, and 4th train stimulation; $p > 0.05$.

during the interval between single train stimulations. Neurons transfected with Endo1 siRNA exhibited an accelerated decrease in the EPSC amplitude from the beginning of the 1st train to the 4th train compared with neurons transfected with NC ($n = 11$, $^{**}p < 0.005$; Figure 3(f)). A similar result was also found in the Endo2 siRNA group ($n = 12$, $^{*}p < 0.05$ after the 1st train, $^{**}p < 0.005$ after the 2nd, 3rd, and 4th trains; Figure 3(g)). However, no significant difference was detected in the EPSC amplitude between the Endo3 siRNA group and the NC group ($n = 9$, $p > 0.05$; Figure 3(h)). These electrophysiological results suggest that replenishment of the RRP is hindered in Endo1 siRNA- and Endo2 siRNA-transfected neurons but not in Endo3 siRNA-transfected neurons.

3.3. Endophilin1 and Endophilin2 Function Together, Not Independently, in SV Endocytosis. To investigate the relationship between endophilin1 and endophilin2 in SV endocytosis, neurons cotransfected with Endo1 siRNA and Endo2 siRNA (Endo1, 2 siRNA) were stimulated using single high-frequency stimulation and consecutive high-frequency stimulation. The response of the Endo1, 2 siRNA knockdown neurons during a single high-frequency stimulation revealed that the EPSC amplitude rapidly decreased to reach a significantly lower plateau (approximately 30% of the control) compared with that in the NC group ($n = 8$, Figure 4(a)). The mean normalized EPSC amplitude for the last 20 responses in the Endo1, 2 siRNA group was significantly lower than that in the control group (0.240 ± 0.023 versus 0.586 ± 0.034, $n = 8$, $^{**}p < 0.005$; Figure 4(b)). However, no significant difference was detected for this measure among the Endo1 siRNA, Endo2 siRNA, and Endo1, 2 siRNA groups (Figures 3(e) and 4(b)). When given multiple train stimulations, neurons transfected with Endo1, 2 siRNA exhibited an accelerated decrease in the EPSC amplitude from the beginning of the 1st train to the 4th train compared with neurons in the NC group ($n = 10$, $^{**}p < 0.005$; Figure 4(c)). The normalized EPSC

amplitudes after the 1st and 2nd trains in the Endo1, 2 siRNA cotransfected group were consistent with those in both Endo1 siRNA and Endo2 siRNA groups. Although the Endo1, 2 siRNA group exhibited lower EPSC amplitudes than either Endo1 siRNA or Endo2 siRNA groups after the 3rd and 4th trains, there was no significant difference among them ($p > 0.05$, Figure 4(d)). These results suggest that endophilin1 and endophilin2 influence SV endocytosis, functioning together, but not independently, to mediate SV endocytosis.

4. Discussion

In our previous study, we found that all endophilin isoforms formed endophilin-Ca^{2+} channel complexes in neurons. Endophilin1 and endophilin2 demonstrated clear Ca^{2+}-dependent interactions with the Ca^{2+} channel, whereas endophilin3 did not [26]. However, it remained unknown whether this characteristic would affect SV endocytosis. To address this issue in the present study, we generated endophilin isoform-specific knockdowns in cultured hippocampal neurons. Each endophilin siRNA successfully reduced the expression of its corresponding isoform approximately 70–80% (Figure 1). Neither transfected siRNA nor NC affected the tested intrinsic electrophysiological properties of the neurons. As shown in Figure 2(d), the EPSC amplitude evoked at low frequency in each siRNA group exhibited no significant difference from that in neurons transfected with NC, suggesting that none of the endophilin isoform knockdowns affected SV exocytosis, consistent with results from a study that ablated endophilin in *Drosophila* [29].

By microinjecting anti-endophilin antibodies into the giant axon of the lamprey, scientists first obtained evidence that endophilin is involved in SV endocytosis [10]. Fly and worm mutants lacking endophilin also demonstrated that endophilin is an important part of the machinery driving SV recycling [29, 30]. Using cultured cortical neurons derived from endophilin triple knockout (TKO) mice, Milosevic et al. showed that endophilin was implicated in SV endocytosis at mammalian central synapses, particularly in the process of clathrin uncoating [9]. In our study, through selectively knocking down endophilin isoforms in cultured hippocampal neurons, we found that neurons with endophilin1 or endophilin2 knockdowns exhibited synaptic depression, similar to the results in flies and TKO mice [9, 29], whereas neurons with the endophilin3 knockdown were not different from control neurons in this regard. This result demonstrates that SV endocytosis is sustained by endophilin1 or endophilin2 isoforms, but not by endophilin3. Although all three endophilins contain BAR and SH3 domains, which are considered the molecular basis of the endophilin involvement in SV endocytosis [13, 23], they do not exhibit the same functions in SV endocytosis. This may be related to differences in their variable regions between the BAR and SH3 domains. Previous analysis on the secondary structure of the proteins indicated that the variable regions in endophilin1 and endophilin2 carry short α-helices, whereas a β-turn was detected in the N-terminal region of endophilin3 [20]. Our previous study showed that the variable regions of endophilin1 and endophilin2 also harbor a calcium binding site (E264), but no calcium binding site was found in endophilin3 [26]. The calcium binding site enables endophilin to interact with other proteins at resting Ca^{2+} levels and dissociate at high Ca^{2+} levels [25, 27]. In the present study, compared with endophilin3, the amplitude of the EPSC evoked from endophilin1 or endophilin2 isoform-specific knockdown neurons rapidly decreased after stimulation (Figure 3). In this situation, neurons are depolarized and the Ca^{2+} concentration is considered to be at a high level (over 1 μM). These data suggest that although the BAR and SH3 domains are necessary for the role of endophilin in SV endocytosis, the contribution of the calcium binding sites in endophilin1 and endophilin2 may be more important for endophilin function.

Reducing the endogenous levels of either endophilin1 or endophilin2 through RNA inference impedes SV endocytosis (Figure 3). However, the response of cotransfected Endo1, 2 siRNA neurons to single high-frequency or multiple high-frequency stimulation displayed no obvious difference compared with those of neurons transfected with Endo1 or Endo2 siRNA alone. This suggests that the two endophilins do not have overlapping roles in SV endocytosis. Studies have shown that endophilin1 and endophilin2 are found predominantly as stable dimers through a coiled-coil domain in their conserved NH2-terminal moiety [31]. This suggests that endophilin1 and endophilin2 may function together, not independently, to mediate SV endocytosis. Dimerization may allow endophilins to link a number of different cellular targets to the endocytic machinery.

In conclusion, our study suggests that, in cultured mammalian primary neurons, endocytosis of presynaptic vesicles is sustained by endophilin1 or endophilin2 isoforms, but not by endophilin3, suggesting that isoform specificity confers particular properties on specific endocytic pathways. The mechanism for this isoform specificity remains to be determined in future studies.

Conflict of Interests

The authors declare that they have no conflict of interests with the contents of this paper.

Authors' Contribution

Jifeng Zhang and Minghui Tan contributed equally to this work.

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