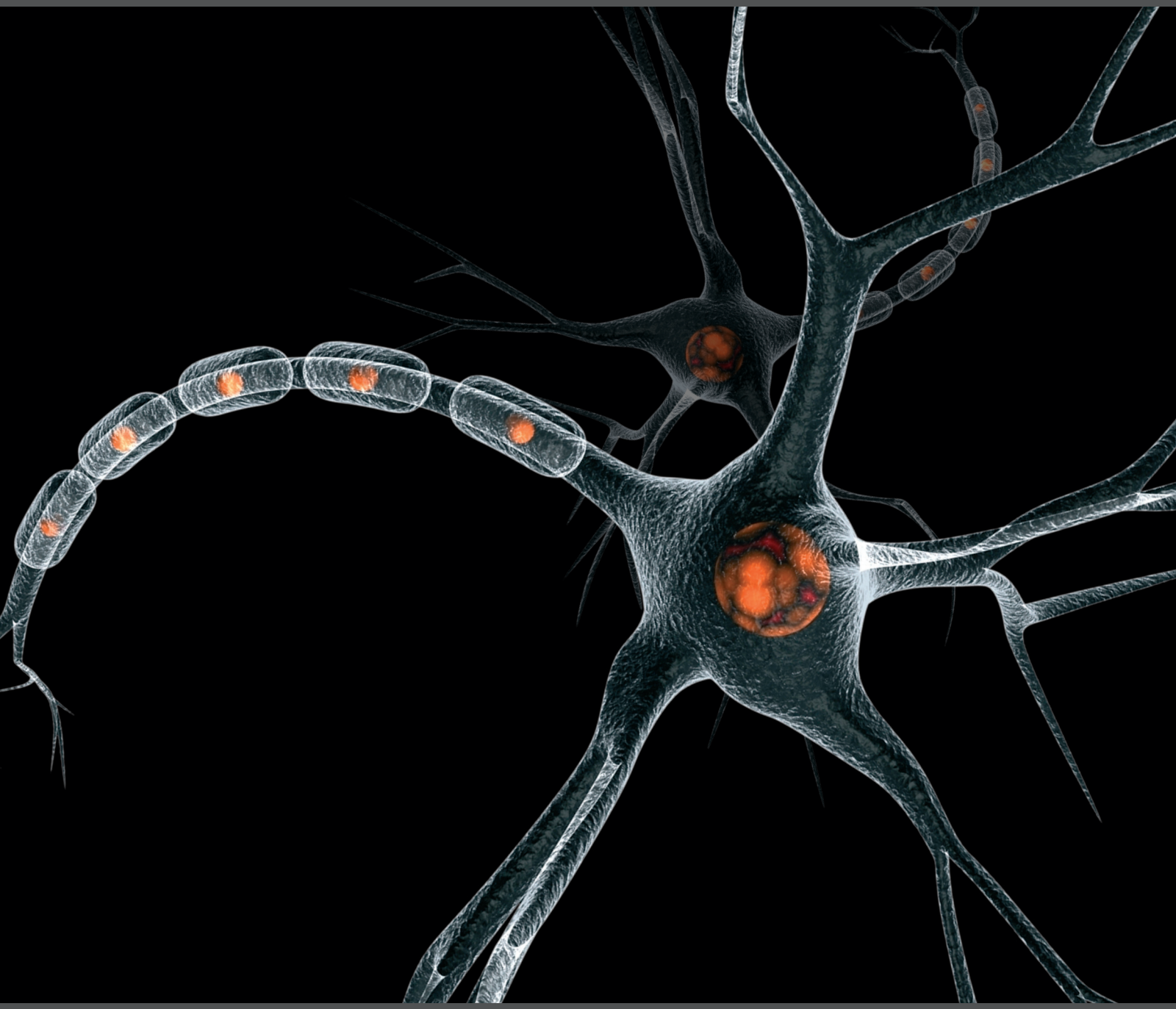
Neural Plasticity

Pathology of Synapses and Dendritic Spines

Guest Editors: Shira Knafo, Gunnar K. Gouras, Xiao-Xin Yan, and Tara Spires-Jones



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Editorial

Pathology of Synapses and Dendritic Spines

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Excitatory synapses represent the sites in which axons make a functional contact with their target neurons and they are typically located at the head of dendritic spines. Due to their critical role as mediators of interneuronal interactions, insults to synapses may result with significant clinical manifestations such as dementia or movement disorders. This special issue of Neural Plasticity discusses various aspects of synapse and spine pathology associated with Alzheimer's disease, perinatal asphyxia, and neuropathic pain.

Synapse and spine loss may represent early and profound neuropathological changes potentially underlying cognitive deficits in Alzheimer's disease (AD). In fact, synapse loss is the strongest pathological correlate of dementia in AD. The inherent plasticity of synapses makes them an attractive therapeutic target. W. Yu and B. Lu, "*Synapses and dendritic spines as pathogenic targets in Alzheimer's disease,*" review the important field of synapse and dendritic loss in AD. In their paper, the authors discuss the well-established role of oligomeric amyloid beta in causing synaptic dysfunction and loss through signaling mechanisms associated with long-term depression. They also explore the exciting new relationship between amyloid beta and tau at the postsynaptic density. Several recent studies have placed tau in dendritic spines—a surprising place for a protein that dogma places firmly on microtubules in axons—where it is turning out to mediate amyloid-beta-induced synaptic dysfunction and loss.

In another review T. Spires-Jones and S. Knafo, "*Spines, plasticity, and cognition in Alzheimer's model mice,*" provide a comprehensive analysis on recent works describing the morphological, synaptic, and behavioral characteristics of the different transgenic models of AD. Results from various models and in variety of ages show a gradual deterioration in synaptic and cognitive functions. The accumulating evidence from transgenic models discussed in this review appears to support a model of AD pathogenesis in which oligomeric amyloid beta initiates synaptic dysfunction or degeneration and induces pathological changes in tau, leading to neuronal loss and ultimately to cognitive deficits.

G. E. Saraceno et al., "*Hippocampal dendritic spines modifications induced by perinatal asphyxia,*" investigated the effect of perinatal asphyxia (PA) on the hippocampal postsynaptic density (PSD). They report an unexpected increased thickness and dispersed appearance of the PSD in the asphyctic brains. Correlative fluorescent and electron microscopy showed a decline of F-actin-stained spines in hippocampal excitatory synapses after the insult that may suggest that PA is harmful to the actin cytoskeleton. These data suggest that perinatal asphyxia may lead to long-term changes in hippocampal synapses.

NMDA receptors are located at synapses and modulate various forms of synaptic plasticity. Grin1b gene encodes the postsynaptic NMDA receptor in zebrafish. RNA and various RNA products play critical roles in regulating gene expression and production of neuroactive proteins in the nervous system. P. Pozo and B. Hoopengardner, "*Identification and characterization of two novel RNA editing sites in grin1b transcripts of embryonic Danio rerio,*" identified two novel

RNA editing events for the grin1b gene in zebrafish. This new information may have implications for transcriptional regulation of mammalian glutamate receptors, which play essential roles in neuronal transmission and plasticity, but also mediate neuronal toxicity in neurodegenerative disorders.

S. K. Kim et al., "*Synaptic structure and function in the mouse somatosensory cortex during chronic pain: in vivo two-photon imaging,*" summarize recent developments in studying *in vivo* spine dynamics in the somatosensory cortex of adult mice in a model of chronic neuropathic pain. The article highlights the importance of neural plasticity in pain research. Employing multiphoton microscopy, they describe remarkably rapid synaptic remodeling in layer 5 neurons of somatosensory cortex within days of the peripheral nerve injury (partial sciatic nerve ligation). Peripheral nerve injury via peripheral hyperactivity causes a rapid rewiring of distinct somatosensory cortex synaptic connections, leading to local somatosensory cortex hyperexcitability. The authors postulate that these local cortical changes in spine turnover following induction of neuropathic pain play an important role in chronic pain conditions.

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Research Article

Hippocampal Dendritic Spines Modifications Induced by Perinatal Asphyxia

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Perinatal asphyxia (PA) affects the synaptic function and morphological organization. In previous works, we have shown neuronal and synaptic changes in rat neostriatum subjected to hypoxia leading to long-term ubi-protein accumulation. Since F-actin is highly concentrated in dendritic spines, modifications in its organization could be related with alterations induced by hypoxia in the central nervous system (CNS). In the present study, we investigate the effects of PA on the actin cytoskeleton of hippocampal postsynaptic densities (PSD) in 4-month-old rats. PSD showed an increment in their thickness and in the level of ubiquitination. Correlative fluorescence-electron microscopy photooxidation showed a decrease in the number of F-actin-stained spines in hippocampal excitatory synapses subjected to PA. Although Western Blot analysis also showed a slight decrease in β-actin in PSD in PA animals, the difference was not significant. Taken together, this data suggests that long-term actin cytoskeleton might have role in PSD alterations which would be a spread phenomenon induced by PA.

1. Introduction

Dendritic spines are small protrusions that serve as a postsynaptic site for the 90% of the excitatory synapses in the CNS. Different kinds of dendritic spines were described based on their shape and their actin content in adult rat brains. Mushroom dendritic spines have stalks with a clear head differentiation, stubby spines are thick and have no neck, and thin spines are characterized as long and without neck [1]. Mushroom dendritic spines have a rich actin cytoskeleton network [1, 2], which is highly regulated by proteins that either stabilize the actin monomer (G-actin), such as thymosin or profiling, arp2/3, Rho-GTPase kinase contractin, or prevent polymerization and convert several polymers into small fragments of actin such as cofilin and gelsolin [3].

Several functions have been suggested for dendritic spines as they have been implicated in the mechanism of synaptic plasticity, learning and memory [4–7], and protein translocation [8]. Functional decline of dendritic spines is a consequence of synaptic loss in neurodegenerative disease and brain insults [9]. Furthermore, processes such as loss of dendritic spines, dendritic pruning, and loss of synaptic proteins precede neuronal death in many neurodegenerative disorders [10–12]. Moreover, activation and impaired function of the ubiquitin-proteasome pathway is thought to contribute to a number of neurodegenerative disorders [13]. Therefore, spine pathologies may be involved in different brain insults including hypoxia ischemia [14–17]. Perinatal asphyxia (PA) is a serious clinical complication with high mortality and morbidity [18]. Following PA, approximately 45% of newborn die and 25% have permanent neurological deficits including cerebral palsy, mental retardation and developmental delay, learning disabilities, visual and hearing problems, and different issues in school readiness [19–24].

In previous works, we have observed several alterations in striatum and hippocampal areas after PA, such as high level of ubiquitinization in dendritic spines, reactive gliosis, alterations in dendritic microtubular organization, and modification in cytoskeleton organization [17, 25, 26]. Given that numerous reports support the idea that dendritic spines are the main site damaged during brain ischemia [25, 27],

we aimed to investigate whether dendritic spine changes are a spread feature induced by PA. For this purpose, we studied dendritic spine modifications in the *Stratum radiatum* of CA1 hippocampal area.

2. Material and Methods

2.1. Animals. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Buenos Aires (School of Medicine) and conducted according to the principles of the Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80–23, revised 1996). Sprague-Dawley female rats in the fifteenth day of pregnancy were placed in individual cages and maintained on a 12 : 12 h light/dark cycle in a controlled temperature (21 ± 2°C) and humidity (65 ± 5%) environment. The animals had access to food (Purina chow) and tap water *ad libitum*. One group of animals ($n = 10$) was used as surrogate mothers, another group ($n = 10$) was assigned to PA procedures.

2.2. Materials. Eosin-phalloidin and Phalloidin-Alexa^{568} were purchased from Invitrogen (Carlsbad, CA). Secondary antibodies against mouse were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Paraformaldehyde, EM grade glutaraldehyde, sodium cacodylate, and Durcopan ACM resin were obtained from Electron Microscopy Sciences (Fort Washington, PA); special welled tissue culture plates were obtained from MatTek (Ashland, MA). β-actin antibody was purchased from Sigma-Aldrich (cat no. A5441).

2.3. Induction of Asphyxia. Ten full-term pregnant rats on gestational day 22 were anesthetized [28], rapidly decapitated, and the uterus horns were isolated through an abdominal incision and placed in a water bath at 37°C for 19 min (subsevere PA: $n = 10$ full-term pregnant rats) [25, 26, 29–31]. We have used 19 min as the maximum time of PA because more than 20 minutes result in a survival rate lower than 3% [25]. Following asphyxia, the uterus horns were rapidly opened, the pups were removed, the amniotic fluid was cleaned, and the pups were stimulated to breathe by performing tactile stimulation with pieces of medical wipes for a few minutes until regular breathing was established. The umbilical cords were ligated, and the animals were left to recover for 1 hour under a heating lamp. When their physiological conditions improved, they were given to surrogate mothers who had delivered normally within the past 24 hours. The different groups of pups were marked and mixed with the surrogate mothers' normal litters (control animals (CTL) that were left undisturbed). We maintained litters of 10 pups with each surrogate mother.

2.4. Post-Asphyctic Procedure. Four-month old male rats (6 per group) were used. Briefly, an intracardiac perfusion was performed with normal rat Ringer's at 35°C followed by fixative under deep anaesthesia (containing 50 mg/kg ketamine, 1 mg/kg rhompun and 5 mg/kg acetopromazine in sterile saline). For light microscopy analysis, rats were perfused with 4% formaldehyde (freshly made from paraformaldehyde) in 0.1 M phosphate buffer, pH 7.2. The brain was removed and fixed for 2 additional hours in the same solution at 4°C. Then, sections were embedded in Durcupan ACM resin. After removing the brain from the skull, it was postfixed in the same fixative during 2 h. Coronal or sagital sections (50–80 μm) were made with a Vibratome (Leica). Some of these sections were stained with cresyl violet according to the procedures described in Capani et al. [32].

2.5. Photooxidation. Vibratome sections were washed with 50 mM glycine-PBS containing 0.5% cold water fish gelatin to block nonspecific binding. Following 30 min of washing, the sections were incubated on a shaker, in a solution of 0.05% of eosin phalloidin-0.5% cold-water fish gelatin/50 mM glycine-PBS for 2 h at 4°C. For light microscopic studies, phalloidin conjugated to Alexa^{488} was also used because of its superior fluorescent quantum yield compared to eosin. As a negative control, eosin-phalloidin was omitted. Tissue sections stained with eosin-phalloidin were mounted on glass-welled tissue culture dishes (Mat Tek Corp) pretreated with polyethylenimine. The slices were fixed again for 2–5 min with 2% glutaraldehyde in 0.1 M cacodylate buffer, rinsed in buffer for several minutes, and placed in 50 mM glycine and potassium cyanide in cacodylate buffer for an additional 5 min to reduce nonspecific staining. Photooxidation was performed on the Zeiss Axiovert described above, equipped with a 75-W xenon arc light source. The samples were immersed in a solution of 2.8 mM diaminobenzidine in 0.1 M sodium cacodylate at 4°C bubbled with pure O_2, final pH 7.4, and then irradiated under conventional epifluorescence using a xenon lamp. After 6–8 min, a brownish reaction product began to appear in place of the fluorescence. The process was stopped by halting the excitation [1].

2.6. Electron Microscopy Procedure. Following photooxidation, tissue sections were rinsed in 0.1 M sodium cacodylate several times and incubated for 30 min with 1% osmium tetroxide in 0.1 M sodium cacodylate, pH 7.2. After several washes in double-distilled H_2O, the sections were dehydrated in an ascending ethanol series, flat-embedded in Durcopan ACM resin, and polymerized for 24 h at 60°C. Serial thin sections (80–100 nm) were cut with Reichert Ultracut E ultramicrotome using glass knives and examined using a JEOL 100CX electron microscope at 80–100 keV. One set of thin sections was poststained with a combination of uranyl acetate and lead citrate. For E-PTA staining, sections were dehydrated in an ascending series of ethanol to 100% and stained for 1 h with 1% PTA stained prepared by dissolving 0.1 g of PTA in 10 mL of 100% ethanol and adding four drops of 95% ethanol [33]. Then, sections were embedded in Durcupan ACM resin.

2.7. Morphometric Analysis of Confocal Data. The volume fraction of immunoreactive material for phalloidin was estimated using the point-counting method of Weibel [34]

and a grid delimiting 5000 μm^2 in the hippocampus. A total area of 75,000 μm^2 was evaluated in each animal. Percentage of reactive area was estimated using Image J Program (Image J 1.41^0, NIH, USA). For electron microscopy analysis sampling, procedures were adapted from Harris et al. [35] and Capani et al. [1]. For analysis, spines were sampled from *Stratum radiatum* CA1 hippocampal area. All of the synapses that have the characteristic of mushrooms dendritic spines (head larger than the neck) were used in this study since mushrooms dendritic spines are the unique F-actin positive spines [1]. Random fields of neuropil containing at least one synapse were photographed at 10000x magnification and analyzed at a total magnification of 30000x. We analyzed 643 control spines and 638 spines for tissue subjected to PA.

2.8. Quantitative Analyses of E-PTA Material. CA1 Hippocampal specimens were selected for quantitative analyses based on the quality of E-PTA staining and the degree of ultrastructural preservation, as determined from conventionally stained material from the same animals. Samples were analyzed from controls ($n = 4$) and 19 min PA animals ($n = 8$). Tissue sections were cut at thickness of 100 nm and examined and photographed at 80 keV at a magnification of 8300x with a Zeiss 109 electron microscope. For each animal, five micrographs were obtained from hippocampus. As described above, each negative was digitized into a PC computer. Using NIH Image 1.6, PSDs were first manually outlined, and then the maximal thickness, minimum thickness, length, and total area of each PSD were determined. All synapses in which the postsynaptic density, intracleft line, and presynaptic grid were clearly visible were chosen for analysis. The selection criterion resulted in the analysis of between 30 and 50 PSDs per animal for each hippocampus.

2.9. Quantitative Analysis of Dendritic Spines. For analysis, spines were sampled from hippocampus. All of the synapses that have the characteristic of mushrooms type dendritic spines (head larger than the neck) were used in this study since mushrooms spines are the unique F-actin positive spines [1]. Random fields of neuropil containing at least one synapse were photographed at 10000x magnification and analyzed at a total magnification of 30000x. We analyzed 643 control spines and 638 spines for tissue subjected to hypoxia.

2.10. Subcellular Fractionation and Preparation of PSDs. Biochemical fractionation was performed as described previously by Saraceno et al. using the whole dorsal hippocampus [17] (CTL, $n = 6$; PA $n = 6$). Dounce homogenates (H) of the pellets in ice cold TEVP buffer (10 mM Tris-HCl, pH 7.4, 5 mM NaF, 1 mM Na3VO4, 1 mM EDTA, and 1 mM EGTA, 1.25 μg/mL pepstatin A, 10 μg/mL leupeptin, 2.5 μg/mL aproptionin, 0.5 mM PMSF) containing 320 mM sucrose were centrifuged at 1000 × g to remove nuclei and large debris (P1). The supernatant (S1) was centrifuged at 10.000 × g for 10 min to obtain a crude synaptosomal fraction (P2) and subsequently was lysed hypoosmotically and centrifuged at 45.000 × g for 90 min to obtain a pellet of the synaptosomal membrane fraction (LP1). After each centrifugation, the resulting pellet was rinsed briefly with ice cold TEVP buffer before subsequent fractioning to avoid possible crossover contamination. Protein concentration was estimated by Bradford technique.

2.11. Western Blot. Western Blot analysis was carried out using LP1 fractions separated on 10% SDS-PAGE. Samples containing 50 μg of protein from each group were applied to each lane. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membrane as described previously [36–38]. The membranes were incubated with a primary antibody anti-β-actin (Sigma, 1 : 1000) overnight at 4°C. Then, after appropriate washing procedures, they were incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody for 2 hours at room temperature. The blots were developed with an ECL detection kit (Amersham). The films were scanned, and the optical density of protein bands was quantified using Gel Pro Analyzer software 3.1.00.00 (Media Cybernetics, USA). We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as load controls [17, 39–41].

2.12. Statistical Analysis. The results were expressed as the means ± SEM. Student's t-test were carried out. A probability was considered to be significant at 5% or less. Statistical analyses were performed using the GraphPad Prism 5.03 for windows statistical package (GraphPad software, Inc, San Diego, CA, USA).

3. Results and Discussion

3.1. Microscopic Analysis of Hippocampal Sections. The study of nuclear morphology by Cresyl violet staining showed that PA animals present clear nuclear condensation 4 months after injury respect to CTL animals in the *Stratum radiatum* CA1 hippocampal area sections (Figure 1). Statistical analyses showed alterations in pyramidal neurons of hippocampal CA1 area, showing an abundance of pyknotic nuclei in asphyctic animals as compared to control animals (Table 1). Then, we employed neuronal nuclei (NeuN) immunolabeling to determine the nature of the cells presenting condensed nuclei (Figure 1). Statistical analyses showed no significant difference in the number of NeuN+ nuclei in the CA1 hippocampal area of asphyctic animals respect to controls. When we analyzed the cellular distribution of NeuN labeling, it was determinate that asphyctic animals showed a significant increase in the number of abnormal NeuN+ nuclei and a significant decrease in the number of normal NeuN+ nuclei in the CA1 hippocampal area compared with CTL group (Table 1). To determine the morphology of these cells, we performed a conventional electron microscopy study. Morphological analyses showed that most cells presenting condensed nuclei evidence dark cytoplasm with rare vacuoles and compaction, a hypertrophic nucleolus, a nucleus with a festoon shape, and a twisted nuclear envelope, corresponding to neurons in degeneration [25, 32, 42, 43] (Figure 1).

Table 1: Analysis of neuron cells in CA1 hippocampal area.

Groups	Neurons with pyknotic nuclei	Neurons NeuN+	Normal neurons	Abnormal neurons
CTL	2.94 ± 0.2	75.34 ± 2.4	72.20 ± 0.5	3.04 ± 0.5
PA	6.29 ± 1.3*	71.58 ± 5.5	64.12 ± 0.4*	7.46 ± 1.2*

Data are expressed as means ± SD. Significative differences were obtain using Student t-test. $^*P < 0.05$.

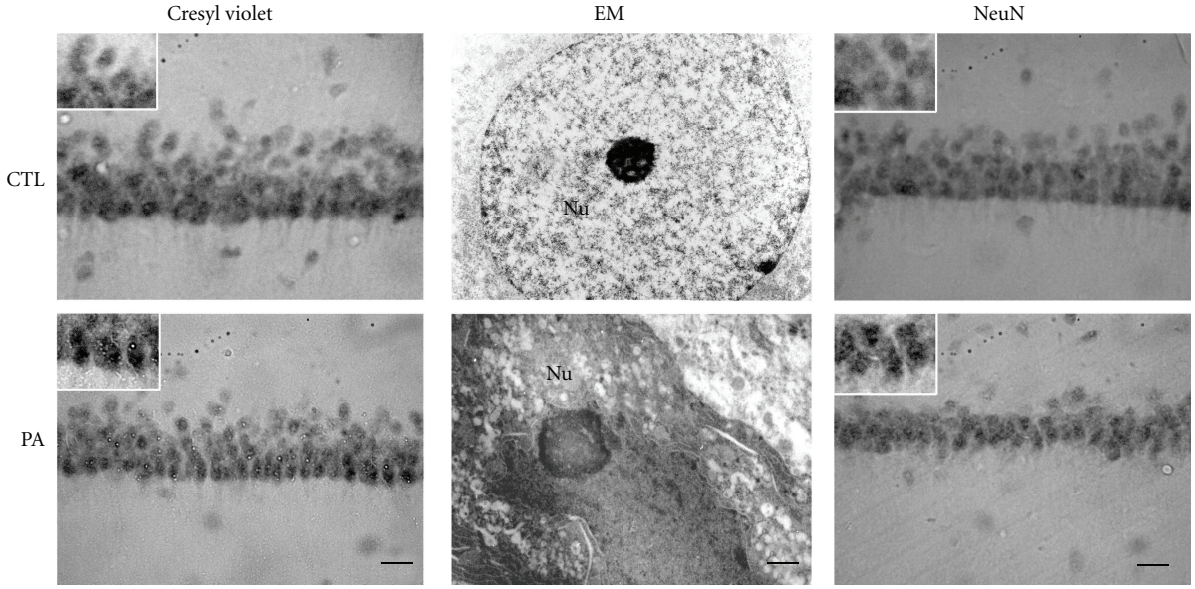

Figure 1: Micrographs of *Stratum radiatum* of CA1 hippocampal area from 4-month-old control rats and rats subjected to 19 min of PA. Vibratome sections of 50 μm were cut and stained with cresyl violet (*Left*), analysed by electron microscopy (EM) (*Middle*) and NeuN immunostaining (*Right*). A clear nuclear condensation was observed after 19 min of PA. Electron micrograph showed that most of the condensed cells correspond to neurons in degeneration. Abnormal NeuN+ nuclei were increased in asphyctic animals respect to control group. Scale bar: 30 μm and 0.5 μm for EM. Nu: nucleus.

3.2. Modification in Hippocampal PSD Stained with E-PTA. Osmium-lead-citrated staining showed no obvious alterations in the *Stratum radiatum* CA1 hippocampal area sections from 4-month-old CTL and PA rats (Figure 2). Presynaptic terminals, presynaptic vesicles, and ultrastructural organization of PSD were intact (Figure 2). On the other hand, E-PTA immunostaining showed clear alterations in synapses of rats subjected to PA (Figure 3). Following PA, the thickness of PSD increased as compared to controls (Figure 3). There was also a general increment in the amount of E-PTA-stained material in PSD of PA animals compared to controls. The statistical analysis performed confirmed these changes. t Student analyses for the area and length of the PSD and for the minimum and maximum thickness of the PSD were significant ($P < 0.05$). Post hoc tests revealed that the means of PSD area was significantly bigger as compared to the CTL group ($P < 0.01$) (Table 2). These inconsistencies between the osmium and E-PTA staining may be attributable to the fact that general heavy metal staining obscures the synaptic modifications occurring in post asphyctic tissue. In addition, it is possible that E-PTA stains different components in the PSD than osmium-uranium-lead methods. It has been known that PSDs stained with E-PTA are shorter and probably wider than those stained with the osmium-heavy metal method [44]. E-PTA preferentially stains protein(s) rich in basic amino acid residues, including lysine, arginine, and histidine, such as collagen or histones [45]. In contrast, conventional heavy metal staining stains a wide type of lipids and cytoskeletal and cytoplasmic elements [44]. Since both markers stain different components, this might explain why E-PTA staining is more effective to detect the PSDs alterations than heavy metal-stained sections.

Consistent with other studies in different models of ischemia [27, 33, 43] and using this long-term PA model [1], we did not observe any alterations in the subcellular organization of hippocampus material stained with osmium-heavy metals. However, we observed a marked increase in E-PTA-stained material in subsevere PA. Although not too much data is available about the mechanism of cell death during PA [25, 30], these findings suggest that the increased in the thickness could be related with the degradation of abnormal proteins probably before neurons trigger death mechanisms. Thus, we hypothesize that some early signals triggered in PSDs could induce late neuronal alterations in post asphyctic hippocampal tissue.

3.3. Ubiquitin-Protein Conjugates in Hippocampal PSDs. Since we and others demonstrated that E-PTA-stained

Table 2: Analysis of PSDs features in CA1 hippocampal area.

Groups	Área × 10^3 (nm^2)	Length (nm)	Minimum thickness (nm)	Maximum thickness (nm)
CTL	2.0 ± 0.2	97.1 ± 2.4	16.2 ± 0.5	43.0 ± 2.5
PA	5.7 ± 1.3**	105.1 ± 5.5	35.6 ± 0.4**	80.1 ± 2.2**

Data are expressed as means ± SD. Significative differences were obtained using Student t-test. $^{**}P < 0.01$.

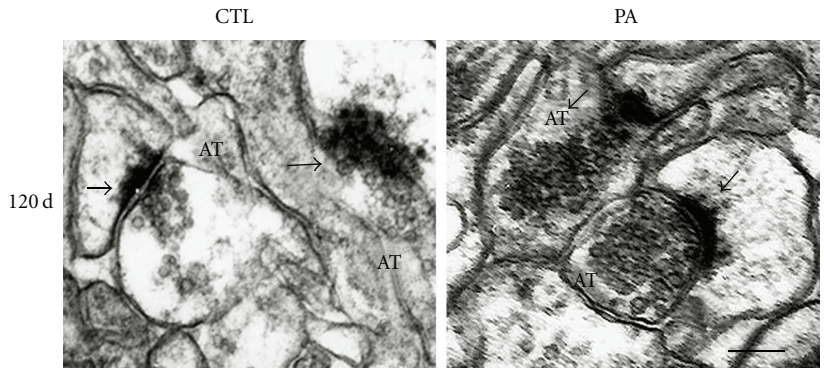

Figure 2: Electron micrographs of osmium-uranium-lead-stained synapses in *Stratum radiatum* of CA1 hippocampal area from 4-month-old control rats and rats subjected to PA. The synapses (arrows) were intact, and no obvious alterations were seen in these osmium-uranium-lead-stained synapses after PA. AT: axon terminal. Scale bar: 0.5 μm.

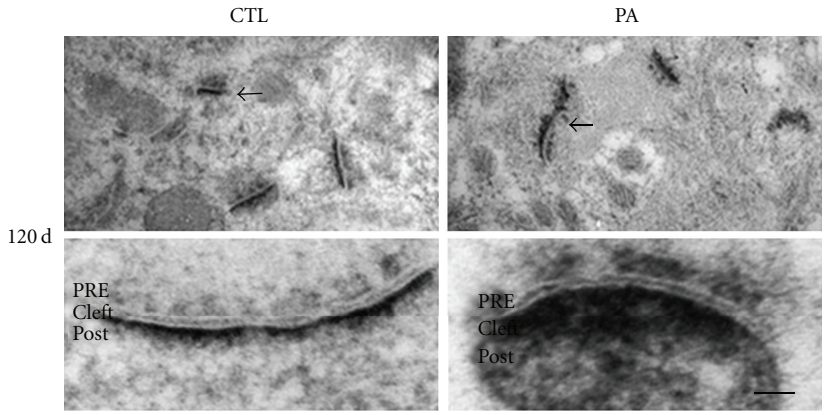

Figure 3: Electron micrographs of E-PTA-stained PSDs (arrows) in *Stratum radiatum* of CA1 hippocampal tissue section from 4-month-old control rats and rats subjected to 19 min of PA. Note the increased thickness and dispersed appearance of the PSDs in the asphyctic brains, compared with the control. Scale bar: 0.5 μm. PRE: presynapses; PSD: postsynaptic density.

aggregates could be composed of abnormal protein [25, 33, 46], we performed immunoelectron microscopy following the procedures previously described by Capani et al. [25] in order to detect ubiquitin (Figure 4). We observed ubiquitinated synaptic proteins after 19 min of PA in the *Stratum radiatum* CA1 hippocampal area sections, while negative controls, in which the primary antibody was omitted, did not show immunolabeling (data not shown). We rarely observed ubiquitin labeling in PSD of CTL animals. Taking these results into account, we could suggest that aggregates of ubiproteins are present in PSDs of asphyctic animals, as it was observed in some neurodegenerative diseases [47].

Even though data about cell death mechanisms during PA are scarce [25, 30], these findings suggest that PSD thickening could be related to degradation of abnormal proteins, probably before cell death mechanisms are triggered in neurons. Consistent with this view, persistent ubiquitination was found in the PSD of hippocampal neurons [46, 48] after transient cerebral ischemia [43], which suggests that increased ubi-protein conjugates might produce protein damage. In addition, damage in protein can be produced by the increment in ROS production and calpain activation as consequence of a rise in Ca levels after hypoxic-ischemic insult [1, 49, 50]. On the other hand, while other heat shock proteins reversibly attach to denatured proteins and help to refold or reassemble them, ubiquitin-conjugated proteins are degraded by 26 S proteasome [47]. PA insult activates the ubiquitin pathway, which might affect neuronal survival by

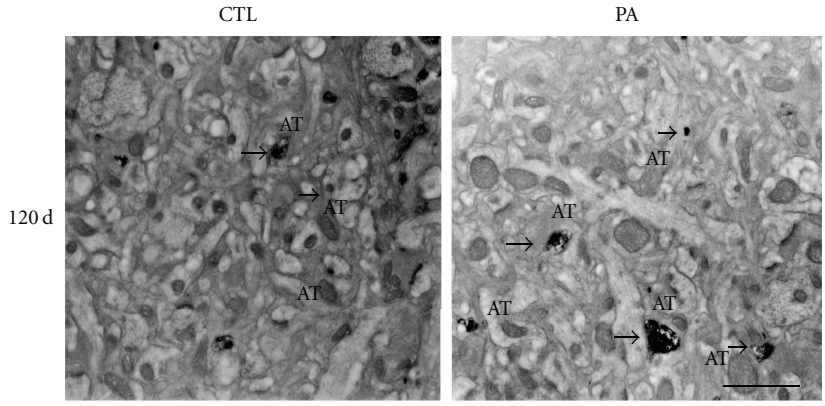

Figure 4: Electron micrographs of ubiquitin immunolabeling from *Stratum radiatum* of CA1 hippocampal tissue of 4-month-old control rats and rats subjected 19 min of PA. Strong ubiquitin stained was observed in asphyctic PSDs (arrowheads). In control animals ubiquitin staining was very weak and rare. AT: axon terminal. Scale bar: 0.5 μm.

damaged protein accumulation. Since neurons do not have the capacity to remove it, ubi-protein accumulation leads neurons to death.

3.4. F-Actin Changes in Hippocampal Dendritic Spines Induced by PA. Brain hypoxia-ischemia triggers an early increment of glutamate in the extracellular space at synaptic level [51]. High levels of glutamate produce a cascade of events in dendritic spines that lead to cell death [25, 31, 33, 43, 48, 52–55]. Since structure and function of dendritic spines are dynamically regulated by different cellular pathways acting on the actin cytoskeleton, we used light and electron microscopic techniques that had previously been used in our laboratory [1, 56] and others [4, 8] to study F-actin modifications induced by PA. By confocal microscope analyses, we observed dendritic spines represented by punctate staining using Phalloidin-Alexa^{568}. PA animals showed a decrease in punctate staining respect to CTL group (Figure 5 *Top*) ($P < 0.01$). The morphometric analysis confirmed these data. Since F-actin is mostly concentrated in mushroom dendritic spines [1], this decrement is tightly related with the F-actin contained in the dendritic spines.

Electron microscopic analyses of spine population in the photooxidated samples confirmed confocal microscopic observations. When we analyzed different dendritic spine populations, we observed that only the number of mushroom dendritic spines, the only F-actin-positive spines in control animals, showed a significant decrease after 19 min of PA ($P < 0.05$) (Figure 5 *Bottom*). In contrast, synapses did not show any sign of evident degeneration in asphyxic rats. Isolated synaptosome (LP1) fractions were analyzed by immunoblotting using anti-β-actin antibody and quantified (Figures 5 and 6). Statistical analysis showed no significant differences in mean optical densities of F-actin bands (P = n.s.) from PA and control group. However, PA animals showed a decrease in amount of β-actin with respect to CTL animals ($P = 0,058$). Both *in vivo* and *in vitro* studies showed high concentration of β-actin in dendritic spines are involved in the organization of the synapses in adult brains [57–60]. Although we observed a reduction of the number of spines F-actin positive in PA animals, the maintenance of β-actin concentration observed in synaptosomal fraction may represent the cytoskeletal support of a stable dendritic spines structure that maintains, thus, the potential morphological plasticity in circumstances where adaptive changes in synaptic connectivity are adequate [60]. Consistent with this point of view, disruption of receptor-scaffold proteins as NMDAR-PSD 95, which depends on actin polymerization interactions, can prevent cell death after ischemia [61].

Actin cytoskeleton is highly regulated by several actin binding proteins (ABP) [3]. Many ABPs have been involved in the regulation of neuronal death during ischemia. Changes in spine morphology are strongly linked with some ABP such as gelsolin. Several studies using different models of neuronal cell death have demonstrated that endogenous gelsolin's antiapoptotic properties correlated to its dynamic actions in the cytoskeleton. Gelsolin-null neurons have higher rates of cell death and a rapid and sustained elevation of Ca^{2+} levels following glucose/oxygen deprivation, as well as augmented cytosolic Ca^{2+} levels in nerve terminals following *in vitro* depolarization [52]. Gelsolin also diminishes infarct size after ischemia, preventing neuronal death [62]. Furthermore, the increment in histone acetylation induces upregulation of gelsolin, dramatically reducing the levels of actin filaments and cell death following cerebral ischemia in mice [63]. Although a recent study by Gisselsson et al. [15] has shown that actin depolymerization prevents neuronal death, we hypothesize that the decrease in β-actin in synaptosomal fraction could also be related with cell death observed after PA insult, as this process is connected with an abnormal ubi-protein increment.

In addition, our group has previously observed learning; reference and working spatial memory impairments in the Morris water maze in 3-months-old rat, subjected to acute asphyxia immediately after birth, using the hypoxic-ischemia model described in the present manuscript [31]. It is well known that the performance in these spatial tests is disrupted after hippocampal damage [64]. Moreover, deficits were observed in the exploration of a novel environment. Therefore, synaptic modifications observed in asphyctic

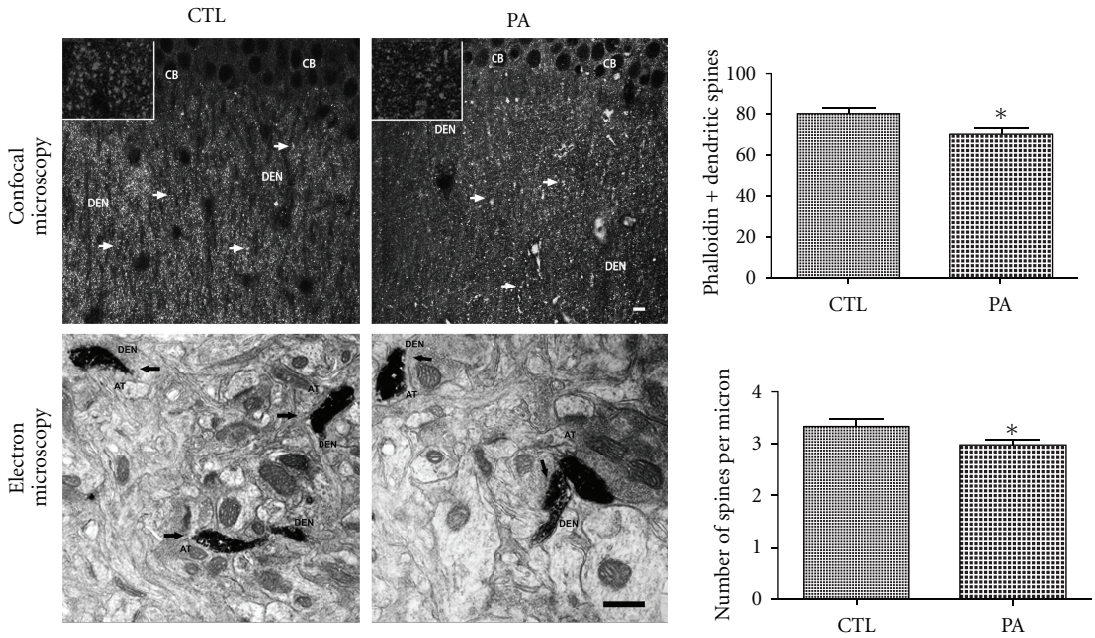

Figure 5: *Top*: confocal microscope images of Phalloidin-Alexa^{568} from *Stratum radiatum* of CA1 hippocampal tissue from 4-month-old control and asphyctic rats. A decrease in the punctate staining was observed after 19 minutes of PA (arrows). The assessment of the percentage of the reactive area from *Stratum radiatum* of CA1 hippocampal Phalloidin-Alexa^{568} staining in PA rats showed a decrement in the reactivity area staining with phalloidin. $^*P < 0.01$. Bars and error bars represent mean + SEM. DEN: dendrites; CB: cell body. Scale bar: 10 μm. *Bottom*: electron micrograph of photooxidated area in the *Stratum radiatum* of CA1 hippocampal tissue of 4-month-old rats. Arrows point out the dendritic spines stained. A decrement in the number of the F-actin-positive spines was observed after 19 min of PA. AT: axon terminal; DEN: dendritic shaft. Scale bar: 1 μm. The graph shows the assessment of the number of spines per field from *Stratum radiatum* of CA1 hippocampal slices. A significant decrement in the number of positive F-actin spines was observed in the PA group in comparison with the CTL group. $^*P < 0.01$. Bars and error bars represent mean + SEM.

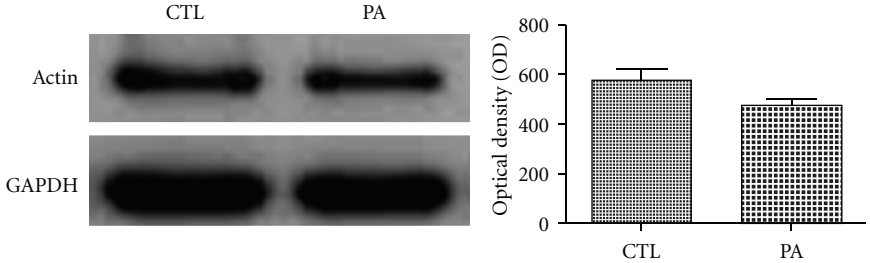

Figure 6: Immunoblots of hippocampal sinaptosomal fractions of 4-month-old CTL and PA rats. We used the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading controls. The assessment of the percentage of optical density of immunoblots from the 4-month-old CTL and PA rats showed no significant difference in the optical density with respect to control group (CTL). $P = 0,058$. Bars and error bars represent mean + SEM.

animals could be related with behavioral deficits previously described by our group [31].

4. Conclusions

These findings suggest that excessive protein ubiquitination in hippocampal PSD, 4 months after a subsevere PA insult, seems to be related to the increment in protein accumulation in this area. In spite of this increment, we observed a decrease in β-actin which suggests that PA is damaging the actin cytoskeleton. Moreover, the amount of β-actin in PA animals is correlated with the decrement in the number of mushroom-shaped dendritic spines. Although further studies will be necessary to determine the role of ubiprotein accumulation in PSD, we could speculate that PSD alterations might be involved in the generation of an aberrant biochemical pathway leading to long-term modifications in the brain of PA animals, as we described in a previous paper [25]. In agreement with this point of view, Alzheimer's disease has a deleterious action on the actin cytoskeleton linked with PSD, leading to dendritic spine dysfunction and synaptic degeneration [65].

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Research Article

Identification and Characterization of Two Novel RNA Editing Sites in *grin1b* Transcripts of Embryonic *Danio rerio*

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Discovering RNA editing sites in model organisms provides an insight into their adaptations in addition to finding potential sites for the regulation of neural activity and the basis of integrated models of metazoan editing with a variety of applications, including potential clinical treatments of neural dysregulation. The zebrafish, *Danio rerio*, is an important vertebrate model system. We focused on the *grin1b* gene of zebrafish due to its important function in the nervous tissue as a glutamate receptor. Using a comparative sequence-based approach, we located possible RNA editing events within the *grin1b* transcript. Surprisingly, sequence analysis also revealed a new editing site which was not predicted by the comparative approach. We here report the discovery of two novel RNA editing events in *grin1b* transcripts of embryonic zebrafish. The frequency of these editing events and their locations within the *grin1b* transcript are also described.

1. Introduction

Adenosine-to-inosine RNA editing occurs primarily in components of neural function and synaptic transmission; adenosines within the targeted transcript are converted to inosine which is interpreted as guanosine during translation and manifests as A/G mixed signals in sequence chromatograms. Several editing sites have been reported in zebrafish (*Danio rerio*) including one located in the *GRIA2* gene [1]. There are several NMDA receptors coded in the zebrafish genome [2], and we have discovered editing in a member of this group, *grin1b* (*NMDAR1.2*).

Grin1b is an ionotropic NMDA glutamate receptor located on zebrafish chromosome 5. The coding sequence of the mature *grin1b* transcript is 2,814 nucleotides. Translation of this mature mRNA produces a protein product made up of 937 amino acids and serves as a postsynaptic glutamate receptor and ligand-gated ion channel.

NMDA receptors are important in neural plasticity and long-term potentiation; hyperexcitation of the receptors can lead to neuronal death. Understanding the factors that influence the regulation of these receptors is therefore important for the treatment of a variety of human neurological disorders.

2. Materials

2.1. RNA Isolation and RT-PCR. RNA was extracted from wild-type *Danio rerio* EK strain embryos 60–72 hours old using the TRI reagent protocol (MRC, Cincinnati, OH); the nomenclature of the editing sites incorporated an "E" designation to emphasize that embryonic tissue was used. All Danio research was done in accordance with institutional IACUC guidelines.

Reverse transcription was performed using Invitrogen MMLV-RT and associated components (Carlsbad, CA), primed with a polythymidylate (polyT) primer. Subsequent PCR was performed using Promega GoTaq (Madison, WI) and specific primer sets (see Tables 1 and 2) from IDT (Coralville, IA). DNA for genomic controls was isolated using the Qiagen (Valencia, CA) DNA mini procedure from the same tissue sample set used for RNA.

2.2. Sequencing and Restriction Digests. Electrophoresis was done on 1.2% gels and gel extraction as per Qiagen (Valencia, CA) gel extraction kit instructions.

Sequencing services were provided by SeqWright (Fisher, Houston, TX). Restriction digests were performed with NEB (Ipswich, MA) enzymes *MluI* and *BstNI*, following NEB

recommendations regarding temperature and inclusion of bovine serum albumin (BSA).

Intensity values based on ethidium bromide fluorescence were acquired using Kodak Gel Logic (Rochester, NY) software and adjustments as described in the text.

3. Results

A BLAST search was performed using *grin1b* coding sequence (CDS) against NCBI databases; substantial regions of sequence identity were detected only against Danio sequences. Danio *grin1b* CDS was then compared against EST sequences, and upon filtering though several hits we selected a sequence comparison containing several A–G mismatches (Figure 1). These A–G mismatches were interpreted as potential A-to-I RNA editing sites.

The sequences of interest selected in this study involved RNA : RNA comparisons of curated zebrafish *grin1b* transcripts, many from the anterior segment of the eye and possibly encompassing the nervous tissue of the retina (Figure 1) (see also [2]: NR1.2 expression pattern). As a result, the sequences compared aligned perfectly to one another except for five A-G mismatched positions, hereafter referred to as E1, E2, E3, E4, and E5 (Figure 1, Section 2). This predictive result was encouraging and prompted us to continue our research using the sites identified by this BLAST search.

We mapped editing sites E1 and E2 to *grin1b* exon 15, E3 to exon 16, and E4 and E5 to exon 17 (Figure 1(b), Table 1) (as per zfin.org, ensembl.org). As shown in Figure 1, all five candidate editing sites were located in adjacent exons within a 468 nucleotide region in the mature *grin1b* mRNA.

We were then able to design oligonucleotides aimed at amplifying the regions of the *grin1b* transcript containing the potential editing sites using Accelrys DS Gene software. Primer pairs (Figure 2) were chosen for subsequent RT-PCR reactions. The primer combination Forward3/Reverse3 (F3/R3) produced a strong band at 311 nucleotides (Figure 3); this product represented a region encompassing all five putative editing events (Figure 3) and was extracted for further sequence analysis of the predicted RNA editing sites within the *grin1b* transcript.

Upon sequencing, the extracted product showed no chromatographic evidence of A/G mixed signals for sites E1, E2, E3, and E4; however, the predicted site E5 showed a double peak corresponding to an A/G mixed signal (Figure 4(a)). Surprisingly, upon further analysis of the chromatogram sequence, we were able to detect another A/G mixed signal which we named E6 (based on the chronology of our predictions and not transcript position) (Figure 4(b)), although E6 did not show up as an A–G mismatch in the original BLAST comparisons. This result illustrates the limitations of comparative approaches in editing site predictions. We interpret the false positives in the initial screen as rare single nucleotide polymorphisms or polymerase errors in cloned transcripts.

We proceeded to replicate our results by conducting two separate, additional RT-PCR reactions (combined total of 3 independent RT-PCR reactions) under the same conditions to verify the occurrence of these editing sites. As expected, these produced robust bands at the size predicted and confirmed A/G mixed signals corresponding to E5 and E6 in both new reactions (data not shown).

Table 1

Predicted site	Exon	Location in transcript	Predicted recoding?
1	15	(Nucleotide 2630)	Yes (G**A**G/G**G**G) (E/G)
2	15	(Nucleotide 2659)	Yes (**A**GG/**G**GG) (R/G)
3	16	(Nucleotide 2690)	Yes (U**A**C/U**G**C) (Y/C)
4	17	(Nucleotide 2823)	No (AA**A**/AA**G**)
5*	17	(Nucleotide 2832)	No (GC**A**/GC**G**)
6* (see Figures 1(a) and 1(b))	16	(Nucleotide 2736)	No (CA**A**/CA**G**)

* Confirmed sites.

Table 2

Primer List.ZFGrin1b:
F1 (CTGCGAAACCCATCAGATAAG)
R1 (AACTCCAACACTGCTGAATC)
F2 (AACTCCGGCATTGGAGAAGG)
R2 (TTCACTGTGGCGTAGATGAAC)
F3 (AATTTGGCAGCCTTCCTAGTG)
R3 (AACAGCTCGCCCGTAGTAAC)
E5.F1 (TATGATGTGGGGGTGGAGAC)
E5.R1 (TGCCGATACCGAATCCAGAG)
E6.F1 (GGAATTGCAGACACCAAACAC)
E6.R1 (TGGCGGTACATGGTGCTAAG)

The next step in our investigation was aimed at confirming these signals as editing sites, rather than single nucleotide polymorphisms, in the *grin1b* transcript of zebrafish. For this purpose we amplified the genomic region of *grin1b* in the region of our candidates E5 and E6. Since single nucleotide polymorphisms (SNPs) can often be misinterpreted as RNA editing events, it was necessary to amplify and sequence the genomic *grin1b* regions to distinguish between these explanations for the mixed chromatographic signals; ADAR editing enzymes do not edit DNA. Therefore, we designed two new sets of primers for subsequent zebrafish PCR reactions using genomic DNA; these primers included regions from the introns bracketing the regions corresponding to E5 and E6, separately, and do not generate products when used in RT-PCR (data not shown). We conducted separate PCR reactions for E5 and E6. Figure 5 shows the results of these PCR reactions where two distinct bands are visible at around 200 base pairs (bp) and 223 bp for E5 and E6, respectively; after sequencing, only adenosine signal (no detectable guanosine above background, later confirmed by restriction digests) was observed at either site (Figure 5).

We chose to use restriction digestion and densitometry to quantitate levels of RNA editing. Editing occurs within a transcript population and may result in the creation or

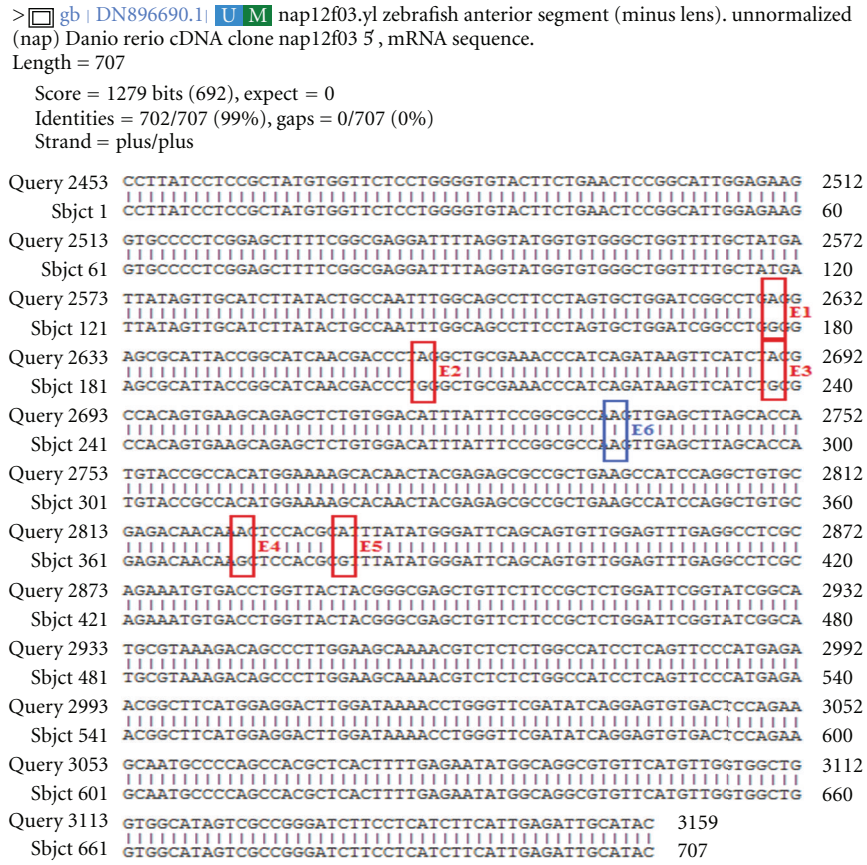

Figure 1: Alignments. A schematic showing the relative (a) and genomic (b) locations of the 5 initially predicted RNA editing events obtained from BLAST searches within the *grin1b* transcript. Predicted sites E1–E5 are located in a stretch of about 468 bases in the mature mRNA corresponding to exons 15 through 17 (as per zfin.org, ensembl.org). Schematic corresponds to NCBI representation of the *grin1b* gene in 2011. *The E6 site was discovered only after direct sequencing.

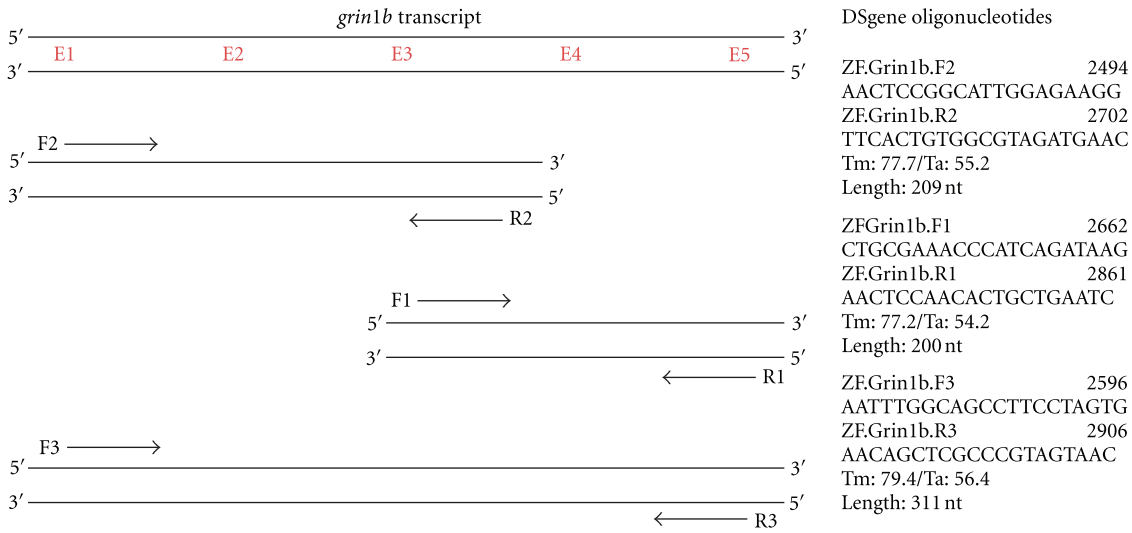

Figure 2: Editing locations. Using the DS Gene computer program (Accelrys), primer pairs were designed to amplify the *grin1b* transcript regions containing the predictive editing events. A total of three primer pairs were constructed, two of which (F1/R1 and F2/R2) amplified a region containing at least three of the five possible edits, and one primer pair (F3/R3) targeted to amplify a region containing all five possible edits.

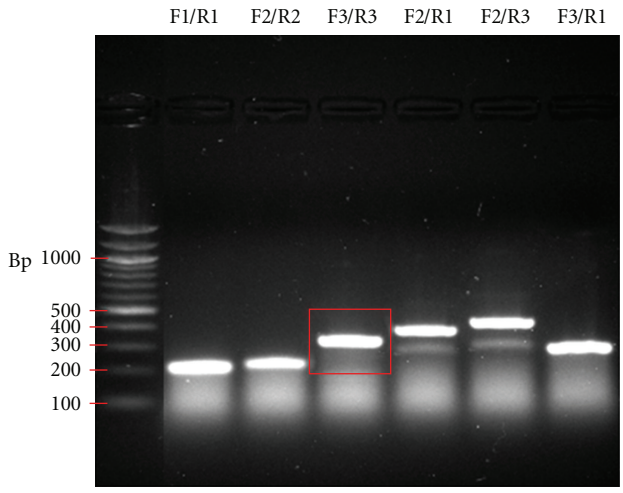

Figure 3: F3/R3 product. RT-PCR products (Invitrogen MMLV-RT, GoTaq Green Polymerase). Several reactions successfully produced robust bands at the predicted sizes: 200 and 209 nucleotides for F1/R1 and F2/R2, respectively, and 311 nucleotides for F3/R3. Since the product of primer pair F3/R3 (outlined in a red box) encompassed all 6 possible editing sites, this RT-PCR band product was extracted for sequencing (100 bp ladder, New England Biolabs, Ipswich, MA).

destruction of a restriction enzyme site. The unedited and edited transcript forms were analyzed using New England Biolabs (NEB) and Accelrys DS Gene software, and restriction enzymes were chosen that could differentiate between editing and lack of editing in a transcript at either site 5 or site 6, separately. *MluI* (A/CGCGT) was chosen for site 5, and *BstNI* (CC/WGG) was chosen for site 6. Editing at site 5 creates an *MluI* restriction enzyme site (ACGC**A**T to A/CGC**G**T), while editing at site 6 creates a *BstNI* restriction site (CCA**A**G to CC/A**G**G). No editing at site 5 prevented *MluI* restriction and therefore gave a full-length 311 bp product; editing at site 5 produced 2 bands (78 bp, 233 bp). No editing at site 6 produced 3 bands after *BstNI* digestion (22 bp, 81 bp, 208 bp; the 208 bp band was used as diagnostic for lack of editing), while editing followed by *BstNI* digestion produced 4 bands (22 bp, 69 bp, 81 bp, 139 bp; the 69 bp and 139 bp bands were treated as diagnostic for the presence of editing). Incomplete editing at either site manifested as a mix of full-length and cut products for each restriction.

Three identically primed (F3/R3; a 311 bp amplicon) PCRs from each of three independent, oligo dT-primed RTs (9 total amplifications) were used for these analyses: from each set of three reactions, one was used for *MluI* digestion, one for *BstNI*, and one for an untreated control. The products were extracted from an agarose gel and purified using a Qiagen gel extraction kit and protocol. Presence of the extracted band was confirmed via gel electrophoresis, and 25 uL restriction digests were performed as per NEB recommendations. Restriction digests were analyzed following gel electrophoresis using a Kodak Gel Logic imaging station and Kodak software. The intensity of the diagnostic versus uncut bands (also compared to unrestricted control bands) was analyzed and corrected for band size (as per [3]). The results confirmed approximate frequencies of editing that were initially predicted by sequence chromatograms (Figure 4) and integration of chromatographic signals (data not shown). Editing at site 5 was 26.98% with a standard deviation of 4.10%, and editing at site 6 was 21.36% with a standard deviation of 4.47% (Figure 6).

4. Discussion

The positions edited occur within the reading frame of the gene at 3rd codon positions and do not result in amino acid

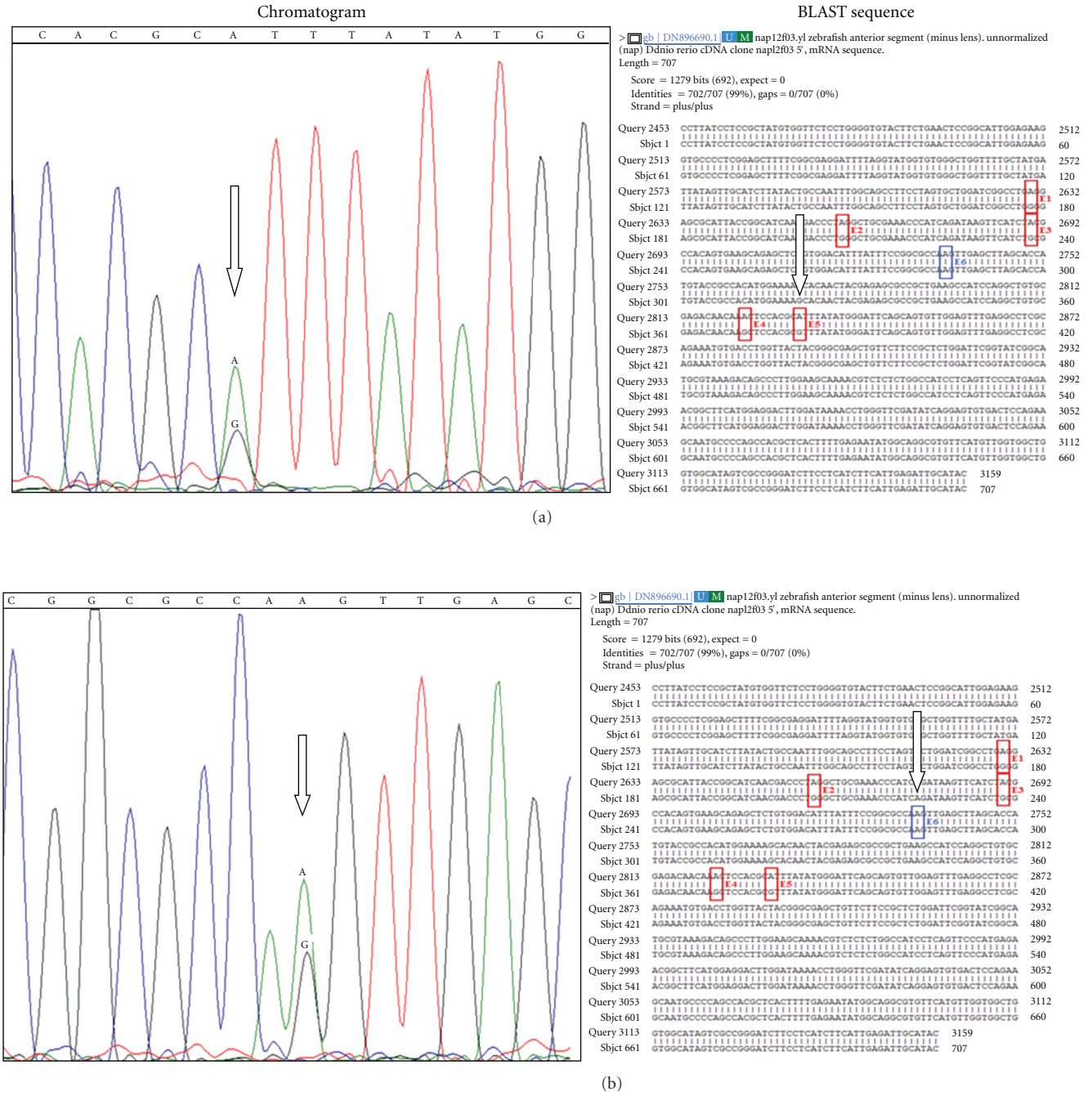

Figure 4: Sequencing results; E5, E6. Chromatogram sequence analysis of the RT-PCR product obtained using the F3/R3 primer pairs. The sequences show double peaks representing A/G mixed signals corresponding to position E5 ((a) open black arrow) and to a new position, E6 ((b) open black arrow and outlined in blue), in the BLAST sequence.

substitutions. The positions of these edits indicate that they do not result in transcript recoding. This considerably complicates an analysis of the purpose of ADAR regulation at these loci; however, an intriguing possibility is that nonrecoding edits affect the binding of additional factors such as micro RNAs or positioning cues for ADARs. Searches of existing microRNA databases such as miRBase (http://www.mirbase.org/) for *D. rerio* miRNAs targeting the E5 and E6 region reveal that there are no known zebrafish miRNAs that bind in this area (within 100 bases 5′ of E6 and greater than 100 bases 3′ of E5), although we suggest the presence of additional unidentified novel miRNAs (or other small RNA species) could have an effect. A number of such possibilities for non-recoding edits are discussed by Morse and colleagues [4].

There are several avenues of future research that may elucidate the function of non-recoding editing at these positions. Secondary structures of these regions were predicted

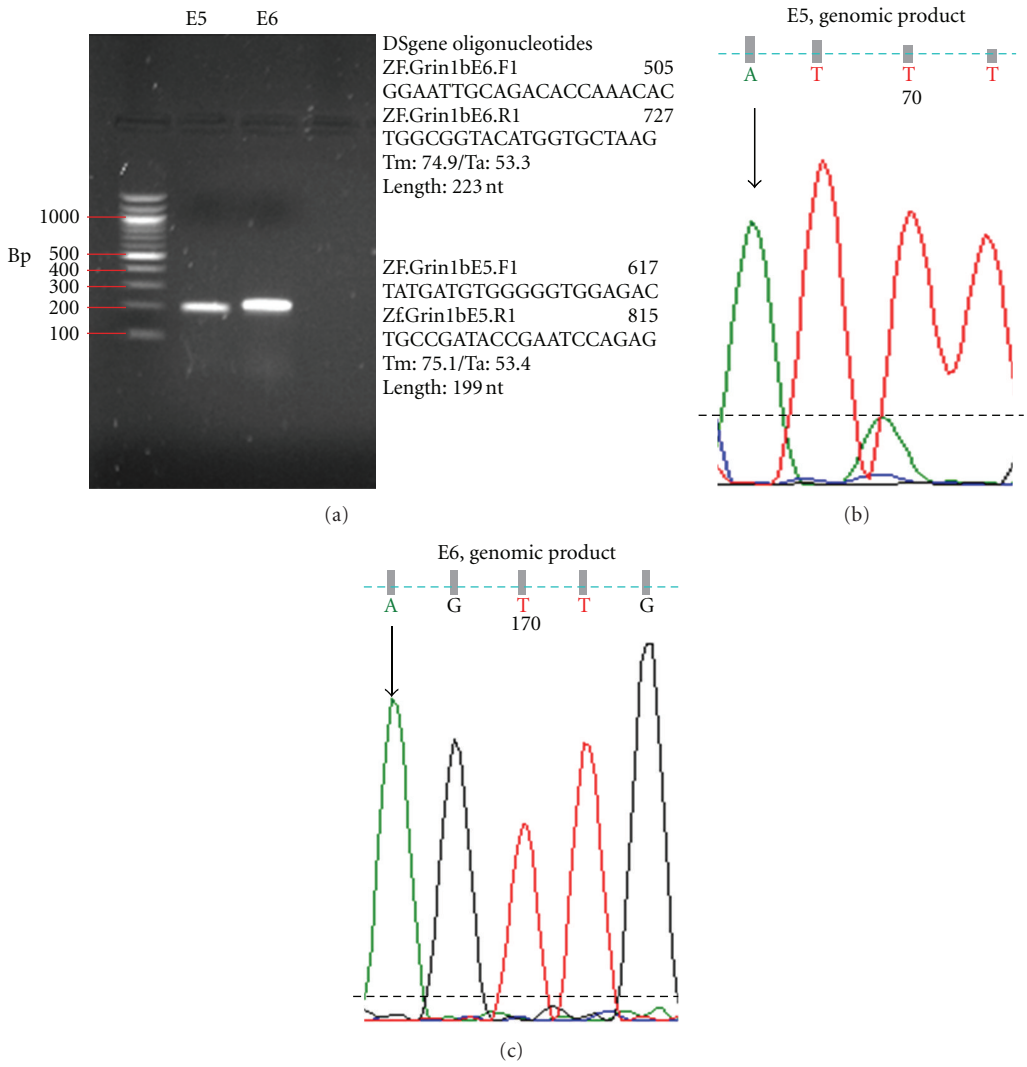

Figure 5: Genomic products; E5, E6. PCR amplification of regions within the *grin1b* gene corresponding to E5 and E6 potential RNA editing sites. As predicted by the DS Gene computer program (Accelrys), primers designed to target the genomic E5 sequence (ZF.Grin1bE5.F1 and ZF.Grin1bE5.R1) produced a strong band at around 200 nucleotides. Primers aimed at amplifying the genomic E6 sequence (ZF.Grin1bE6.F1 and ZF.Grin1bE6.R1) produced a strong band at around 220 nucleotides. Both of these bands were extracted for sequencing reactions. Amplicons from genomic DNA show no evidence of A/G polymorphism (A only). Dashed black lines represent noise levels per sample.

using the mfold program developed by Zuker [5] in consideration of future structural confirmations (see [3]). Constructs with these changes can be made (separately or coordinately) with plasmid mutageneses and the affect on editing assayed in cell culture and transgenic animals. Examinations of binding affinities of zebrafish ADARs with edited versus unedited constructs can be pursued, as well as searches for differences in miRNA binding caused by these editing changes. This editing may also be coincidental, rather than functional, through mimicking the structure of editing enzyme substrates.

The *grin1b* ortholog in humans is the *GRIN1* NMDA receptor gene. Several mutations in *GRIN1* are associated with severe mental retardation. When tested in *Xenopus* oocyte systems, increased calcium entry occurred with one mutated form [6] and the authors of this study point out the potential pathogenicity of the resultant increased calcium influx. Such results highlight the clinical value and human relevance of the study of *grin1b* in model vertebrates, such as Danio.

RNA, in its many forms, plays a pivotal role in cellular processes. The processes of RNA editing and splicing along with micro RNAs, RNA interference, snRNAs, ribozymes, and so forth certainly point to RNA as a fundamental conductor and orchestrator of genetic instructions. Although RNA editing has been widely observed in many model organisms, finding a specific editing site is still a daunting task. We are interested in discovery of new editing sites as well as regulation of those sites, in a variety of model organisms. The Department of Biomolecular Sciences contains faculty who work collaboratively with a variety of experimental systems, and the record of Kung and colleagues' discovery of RNA editing in the *gria2* transcript of zebrafish [1] was a prompt

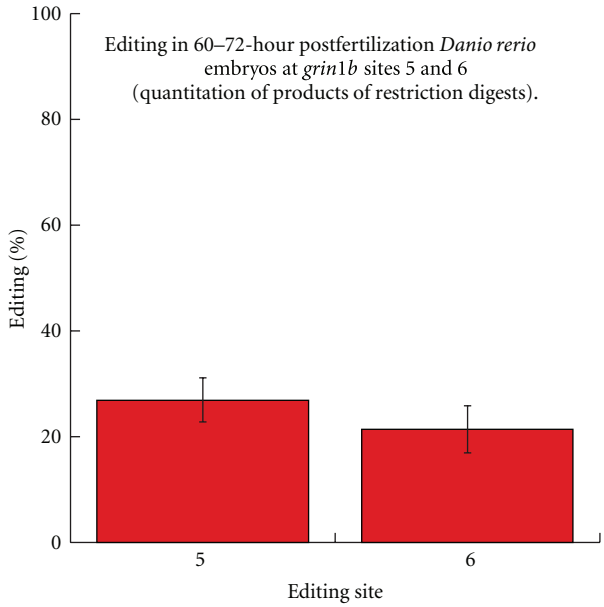

Figure 6: Editing frequencies and error bars (variation). Percent editing at sites 5 and 6 as measured by quantitation of the products of restriction digestion. Bars are standard deviation. Plotted with KaleidaGraph 4.

and a clue as to what additional targets of editing might exist in transcripts from gene families in Danio. We were able to demonstrate the presence of two new sites of RNA editing in the *grin1b* gene transcript of zebrafish. We also call attention to the fact that site E6 was detectable only by direct sequencing and not by comparative methods; many editing sites, especially those that are unique to a single species, may remain to be discovered. Moreover, by characterizing editing in terms of frequency and location we hope to contribute to the current knowledge of RNA editing with the goal of participating in the full elucidation of this intriguing molecular process.

Acknowledgment

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Review Article

Synaptic Structure and Function in the Mouse Somatosensory Cortex during Chronic Pain: *In Vivo* Two-Photon Imaging

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Recent advances in two-photon microscopy and fluorescence labeling techniques have enabled us to directly see the structural and functional changes in neurons and glia, and even at synapses, in the brain of living animals. Long-term *in vivo* two-photon imaging studies have shown that some postsynaptic dendritic spines in the adult cortex are rapidly eliminated or newly generated, in response to altered sensory input or synaptic activity, resulting in experience/activity-dependent rewiring of neuronal circuits. *In vivo* Ca^{2+} imaging studies have revealed the distinct, input-specific response patterns of excitatory neurons in the brain. These updated *in vivo* approaches are just beginning to be used for the study of pathophysiological mechanisms of chronic diseases. In this paper, we introduce recent *in vivo* two-photon imaging studies demonstrating how plastic changes in synaptic structure and function of the mouse somatosensory cortex, following peripheral injury, contribute to chronic pain conditions, like neuropathic and inflammatory pain.

1. Introduction

Chronic pain initiated by tissue or nerve injury is a major challenge to clinical practice as well as basic neuroscience [1]. Peripheral neuropathic or inflammatory injury triggers structural and functional plastic changes in the cortical pain neuromatrix including the primary somatosensory cortex (S1) and anterior cingulate cortex (ACC), which results in altered nociceptive signal processing, such as mechanical allodynia (painful response to innocuous mechanical stimuli) [2, 3]. In previous brain imaging studies, for example, patients and animals under chronic neuropathic or inflammatory pain states showed increased activation and somatotopic reorganization in the S1, the extent of which was highly correlated with the pain intensity levels [4, 5]. Changes in gray matter density and in cortical thickness of the pain-related areas including the S1, ACC, and insula cortex were also found in chronic pain subjects [6, 7]. Further, several strategies to reduce the S1 hyperexcitation and reorganization showed benefits against chronic pain [8–11]. Although much is now known about such macroscopic changes in the cortex, it remains to be elucidated how and to what extent cortical connections are remodeled during chronic pain, and how such remodeling affects pain behaviors. This paper focuses on the recent findings from *in vivo* two-photon imaging studies to address the aforementioned questions: (1) the rapid and phase-specific remodeling of synaptic structures in the S1 of neuropathic pain mice following peripheral nerve injury [12] and (2) the enhanced activity of the S1 neurons affecting ACC neuronal function during inflammatory pain [13].

2. Structural Remodeling of Synapses in the Mouse S1 during Neuropathic Pain

Based on static measurements between different groups and on macroscopic observations, it has been believed that

structural rewiring of neuronal connections in the cortex during chronic pain following injury takes much longer periods of time (i.e., months or years) than the development of allodynia and functional changes in cortical excitation, such as long-term potentiation (LTP), that occur within days or weeks [3, 14]. Recent long-term *in vivo* two-photon imaging studies have revealed that novel sensory experiences, or motor learning, can however induce rapid structural reorganization of synaptic connections in the related sensory or motor cortex that occur within days and are temporally correlated with functional plasticity of cortical circuits [15–19]. Given the high similarity of the mechanisms between chronic pain and learning and memory, as exemplified by the two forms of use-dependent synaptic plasticity "central sensitization" and "LTP", respectively, [3, 20–22], it seemed reasonable to hypothesize that neuronal circuits in the S1 of intact brain would be remodeled following peripheral nerve injury with a similar time scale of the development of neuropathic pain behaviors and S1 hyperexcitability. Supporting this idea, several *in vitro* studies using intracellular filing of neurons in rat brain slices with biocytin suggested that dendritic structures in the S1 and medial prefrontal cortex were significantly changed at one or two weeks after peripheral nerve injury [23, 24]. A recent long-term *in vivo* two-photon imaging approach [12], described below, has now shown that in living mice structural changes in cortical circuits can indeed occur within the same rapid time scale as functional changes, indicating that the previous notion about only slow and chronic changes in cortical connections occurring in chronic pain states should be modified.

2.1. Time Course of the Development of Mechanical Allodynia and the S1 Hyperexcitability following Neuropathic Injury. Neuropathic pain following partial sciatic nerve ligation (PSL) is a well-characterized mouse model [25, 26] that can be subdivided, based on the behavioral signs of mechanical allodynia, into an early "development" phase (~post-PSL to day 6) and a later "maintenance" phase (day 6 onwards) (Figure 1(a)). Hind paw stimulation-evoked cortical field potentials recorded in the S1 layer 1 *in vivo* [27] significantly increase in the development phase, and to an even greater extent in the maintenance phase (Figure 1(a)). From these behavioral and electrophysiological results, it might be expected that spine turnover in the S1 of neuropathic mice might be enhanced in a phase-dependent manner as well. To test this prediction, we utilized a transgenic mouse that sparsely expresses enhanced green fluorescent protein (GFP-M line) in cortical neurons [28] and set about to repeatedly image with the two-photon laser scanning microscope the same apical dendrites of functionally identified adult S1 hind-paw layer 5 pyramidal neurons, before and after PSL injury (Figure 1(b)). Layer 5 pyramidal neurons are the major output cells in the S1 and their distal tuft dendrites that are innervated by thalamocortical and corticocortical long-range projections as well as local circuit inputs encode information about somatosensory stimuli [29]. However, some consideration had to first be given about the most appropriate imaging procedure.

2.2. Chronic Cranial Window for Long-Term In Vivo Two-Photon Imaging during Chronic Pain. For long-term high-resolution imaging of synaptic structures in the cortex of living adult mice, the overlying opaque skull bone should be partially removed to make a cranial window. There are broadly two types of cranial window, namely, the "thinned-skull" window and the "open-skull" glass window [30–32]. The thinned-skull preparation is achieved by thinning the skull bone over a small area (about 1 mm in diameter) to be less than 30 μm thick, whereas in the open-skull preparation a piece of the cranial bone is removed (about 2–5 mm in diameter), leaving intact the dura, and the exposed brain is covered with a thin glass coverslip (*for detailed methods and their pros and cons, see protocol articles,* [33–36]). Although thinned-skull preparation has many advantages (e.g., less invasive), it is difficult to image the same area more than 4 times, and rethinning procedure is required every imaging session, which is not necessary in the open-skull preparation. However, the mechanical sensitivity of the hind paw moderately increased for 2 weeks after an open-skull glass window implantation procedure, before completely returning to normal at 4 weeks after implantation [12]. Thus, long-term *in vivo* imaging experiments during neuropathic pain could only commence from 1 month after the cranial window implantation.

2.3. Dendritic Spine Dynamics Strikingly Increased during the Development Phase of Neuropathic Pain, But Were Restored in the Maintenance Phase. High-magnification successful repeated imaging of individual dendritic spines (Figure 1(b)) revealed the unexpected result that there was a marked increase in spine turnover rate ($N_{\text{gain}} + N_{\text{loss}}/2N_{\text{total}}$), an excellent indicator of structural synaptic plasticity, during the development phase of neuropathic pain, but a turnover just rapidly decreased back to normal during the maintenance phase. The observed spine turnover changes in the PSL mice are region- and injury-specific, because little change was found in the barrel cortex of PSL mice and in the S1 hind paw area of sham control mice [12]. Considering the time-course of mechanical allodynia and S1 hyperexcitability together (Figure 1(a)), these spine turnover data may provide the structural and temporal correlates of neuropathic pain at the level of cortical synapse. It also suggests that neuropathic pain-specific formation of new connections and elimination of preexisting circuits occur mainly within the early phase of neuropathic pain. Even though large scale sprouting or retraction of axonal and dendritic arbors of pyramidal cells in the adult cortex of living animals is rarely seen in imaging over a few weeks [30, 37, 38], even after neuropathic injury [12], a minor fraction of persistent synapses added or subtracted by neuropathic injury or novel sensory experiences can sufficiently store specific long-term information [39, 40].

The rate of spine gain following PSL injury showed a striking increase during the development phase, together with relatively moderate increase in spine loss rate, resulting in significant increase in spine density at the end of the development phase (Figure 1(c)). Such an increase in spine density was mainly due to a significant upregulation of

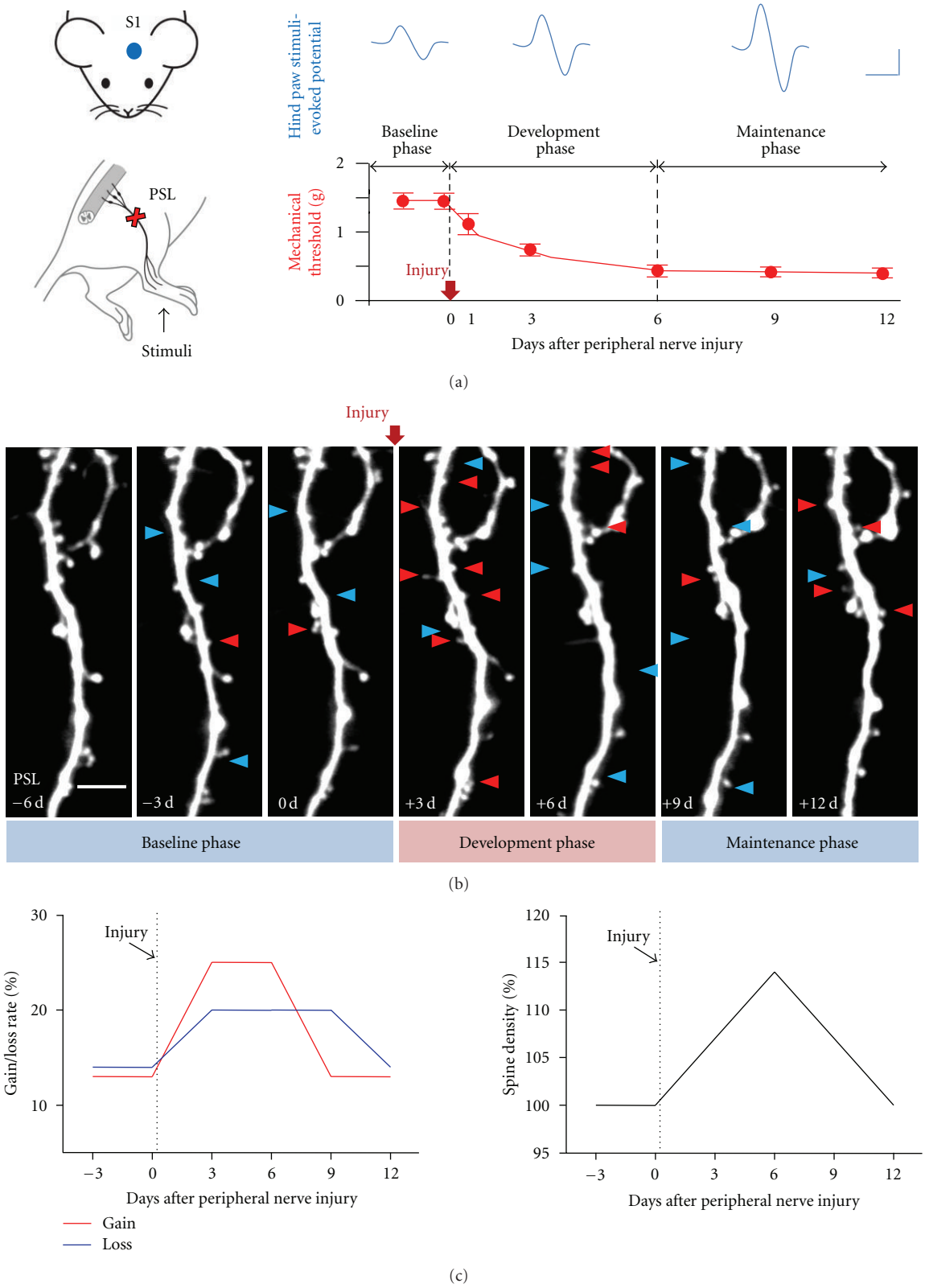

Figure 1: Rapid and phase-specific structural plasticity of dendritic spines in the S1 following peripheral nerve injury. (a) Left panels: schematic diagram of the PSL injury model and associated investigations in the S1. Bottom graph outlines the development and maintenance phases of mechanical allodynia following PSL injury with the upper panels showing the concurrent phase-dependent increases in somatosensory-evoked potentials in the S1. Scale bars, 50 ms (horizontal) and 50 μV (vertical). (b) *In vivo* two-photon time-lapse images of the same S1 dendritic segment following PSL injury. Arrowheads indicate the spines that generated (red) and eliminated (blue) when compared with the previous imaging session. Scale bar, 5 μm. (c) Schematic representation of the time course of changes in spine gain/loss rates (left) and overall spine density (right) during neuropathic pain. (a–c) Reproduced and adapted, with permission, from [12].

thin spines [41]. Interestingly, increased spine elimination following injury continued up to post-PSL 9 days, whereas the new spine formation rate was reduced to normal baseline levels from the beginning of the maintenance phase. As a result, spine density returned to control level on post-PSL 12 days (Figure 1(c)). Since the major fraction of new spines was transient in the S1 [12] and in other sensory cortex areas [17, 18, 42], irrespective of injury and novel experience, subsequent elimination of new spines that had been generated during the development phase contributes to the above result, reflecting the refinement process of new connections.

2.4. Early Afferent Hyperactivity Is the Main Cause of Mechanical Allodynia and of S1 Synapse Remodeling. Preemptive or perioperative analgesia is based on the "pain memory" concept, in which an injury-induced afferent barrage can initiate the development of subsequent sensitization in the central nervous system that in turn contributes to the persistence of chronic pain [20–22, 43]. Analgesics and local nerve blockade before or during injury, but not after, can prevent or reduce pain, analgesic requirements, and abnormal changes in the spinal dorsal horn [44–46]. Similarly, the development of mechanical allodynia and upregulation of spine turnover following nerve injury were completely inhibited by local blockade of afferent activity in the injured sciatic nerve throughout the development phase [12]. However, the same nerve blockade, if begun in the maintenance phase, showed only a transient and moderate reduction in allodynia [12, 44]. These findings not only suggest the important role of early afferent hyperactivity-induced remodeling of the S1 synaptic structures in maintaining neuropathic pain, but also extend the pain memory hypothesis to the individual synapse level in the cortex.

2.5. Neuropathic Injury-Specific Formation of New Persistent Spines and Elimination of Preexisting Spines. The increased number of new persistent spines that are generated during sensory manipulation or motor training has been considered as representing long-term memory traces [47]. Monocular deprivation [17], motor learning [19], and partial whisker trimming [42], or an enriched environment for whisker stimulation [18], all increased the number of new persistent (NP) spines on layer 5 pyramidal cells in the mouse visual, motor and barrel cortex, respectively. Consistent with those findings, the number of NP spines that appeared during the development phase of neuropathic pain was significantly higher than that of NP spines that appeared both before PSL, and in time-matched control groups (Figures 2(a) and 2(b)). Notably, the volume of NP spines that appeared during the development phase of neuropathic pain was substantially increased in the maintenance phase [12]. Since the spine volume is positively correlated with synaptic strength [40, 48], this result, together with increased number of NP spines (Figures 2(a) and 2(b)), probably indicates the encoding and subsequent enhancement of a neuropathic pain memory at single synapses, underlying the long-lasting nature of neuropathic pain.

Although sensory manipulation or motor learning upregulates NP spines in the relevant cortical area [12, 17–19, 42] as mentioned above, such manipulations did not change the final overall spine density with one exception [17], perhaps reflecting a limitation of the brain's capacity to accumulate all NP and previously persistent spines in response to each new experience or each new incident of learning. Thus, cortical circuit rewiring requires the removal of unnecessary preexisting connections at the same time as NP synapse formation. Indeed, a significant proportion of previously persistent spines were selectively eliminated over 2 weeks following PSL injury (Figures 2(a) and 2(c)) and simple extrapolation of those results with a single exponential fit estimated that 2/3rds of previously persistent spines in PSL mice might be completely eliminated [12]. This suggests a significant impact of neuropathic injury on cortical circuits throughout the whole life of chronic pain subjects.

3. Functional Plasticity of Intra- and Interregional Cortical Circuits during Persistent Inflammatory Pain

As mentioned above, peripheral injury induces functional plastic changes in the cortical pain neuromatrix including the S1 and ACC, where the integration and processing of pain signals might occur. Although it has been suggested that the S1 and ACC play a major role in the sensory and emotional aspects of pain, respectively [7, 49, 50], little is known about if and how the two cortical regions interact with each other under chronic pain conditions, and whether such interactions contribute to pain behaviors. Since layer 2/3 (L2/3) excitatory neurons in the S1 integrate sensory information originating from peripheral nerves via L4 neurons and transmit these signals to other pain-related cortical areas [51, 52], it would be a good strategy to determine the plastic changes in the S1 L2/3 neurons' function during chronic pain and then investigate how these changes may affect the ACC activity and pain behavior.

In vivo two-photon Ca^{2+} imaging in living transgenic animals expressing a fluorescence only in inhibitory neurons (Venus) [53], combined with a multicell bolus loading of Ca^{2+} indicators (fura-2) [54, 55] and the astrocyte-specific dye (Sulforhodamine 101, SR101) [56] allows us to distinguish the response of astrocytes, excitatory neurons, and inhibitory neurons (Figure 3(a)). Furthermore, the neuronal activity in tens or hundreds of each cell type can be monitored at the same time during peripheral sensory stimulation [54]. Such experiments, using the Complete-Freund's-Adjuvant-(CFA-) induced inflammatory pain model in mice, showed that the probability and amplitude of Ca^{2+} transients in the S1 L2/3 excitatory neurons, and the number of cells activated by either low-intensity hind paw stimulation or electrical stimulation of the L4 region, are significantly increased (Figures 3(b) and 3(c)). Considering that the amplitude of evoked Ca^{2+} transients reflects the number of action potentials [55], these results suggest that the excitability of the S1 L2/3 neurons in response to mechanical stimulation of the hind paw is enhanced under inflammatory pain condition, at least in part through an amplified synaptic transmission from L4.

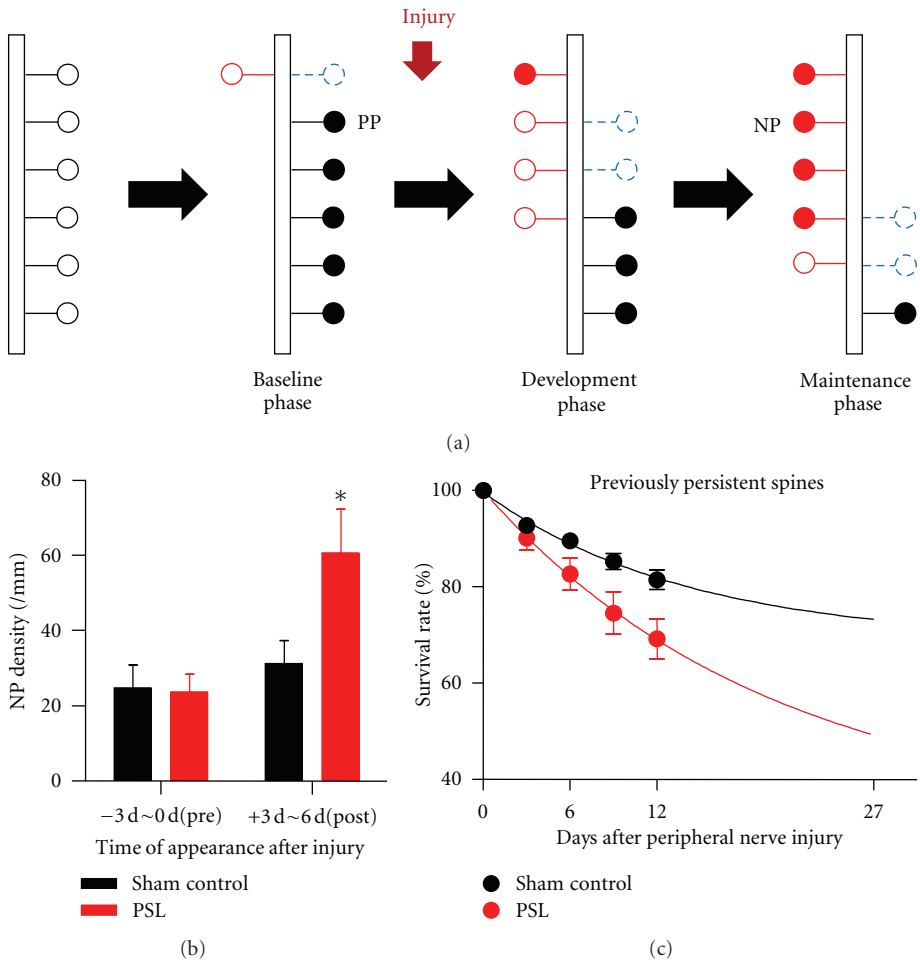

Figure 2: Neuropathic pain injury increases the formation of new persistent spines and increases elimination of previously persistent spines. (a) Simplified model for spine formation (red open circles) and elimination (blue, dashed open circles) under basal conditions and during neuropathic pain. PP: previously persistent spines (black filled circles). NP: new persistent spines (red filled circles). Note the increase in NP spines (b) and decrease in PP spines (c) following PSL injury. (b and c) Reproduced and adapted, with permission, from [12].

Since the experience of pain is related to activation of both sensory and emotional aspects, which are thought to be differentially processed in the S1 and ACC, respectively [49, 50], the two cortical areas are expected to interact with each other [57]. Pharmacological inhibition of the S1 L2/3 neuronal activity in CFA-injected mice, but not in normal control mice, significantly attenuated the ACC activity evoked by hind paw stimulation, as well as significantly attenuating allodynia [13]. Conversely, pharmacological activation of the S1 L2/3 enhanced the ACC activity and induced an allodynic behavior in normal mice [13]. Therefore, there are considerable interactions between the S1 and ACC when the S1 L2/3 excitatory synaptic transmission is abnormally enhanced, which contribute to chronic pain behavior.

4. Concluding Remarks

In conclusion, we propose the following working hypothesis of the cortical mechanisms of chronic pain (Figure 4): peripheral nerve or tissue injury induces peripheral hyperactivity [21, 58], which causes a rapid rewiring of S1 synaptic connections [12]. Such synaptic remodeling, including an increased synaptogenesis and synapse elimination, and an enhanced strength of persisting synapses, causes local hyperexcitability of S1 to peripheral stimulation and might also affect the ACC or other pain-related cortical areas, finally leading to chronic pain behavior (allodynia) [12, 13].

The applications of *in vivo* two-photon imaging to pain research, as described above, are still at an early stage. There remain many unsolved questions regarding the pathophysiological changes of cortical synaptic structures and neuronal functions during chronic pain. For example, what happens to cortical inhibitory neurons and their synapses during chronic pain? How do cortical glial cells, such as astrocytes and microglia, contribute to plastic changes in synaptic structure and function during peripheral injury-induced chronic pain? What is the causal relationship between chronic pain and cortical synaptic remodeling? How do several cortical and subcortical regions comprising the pain

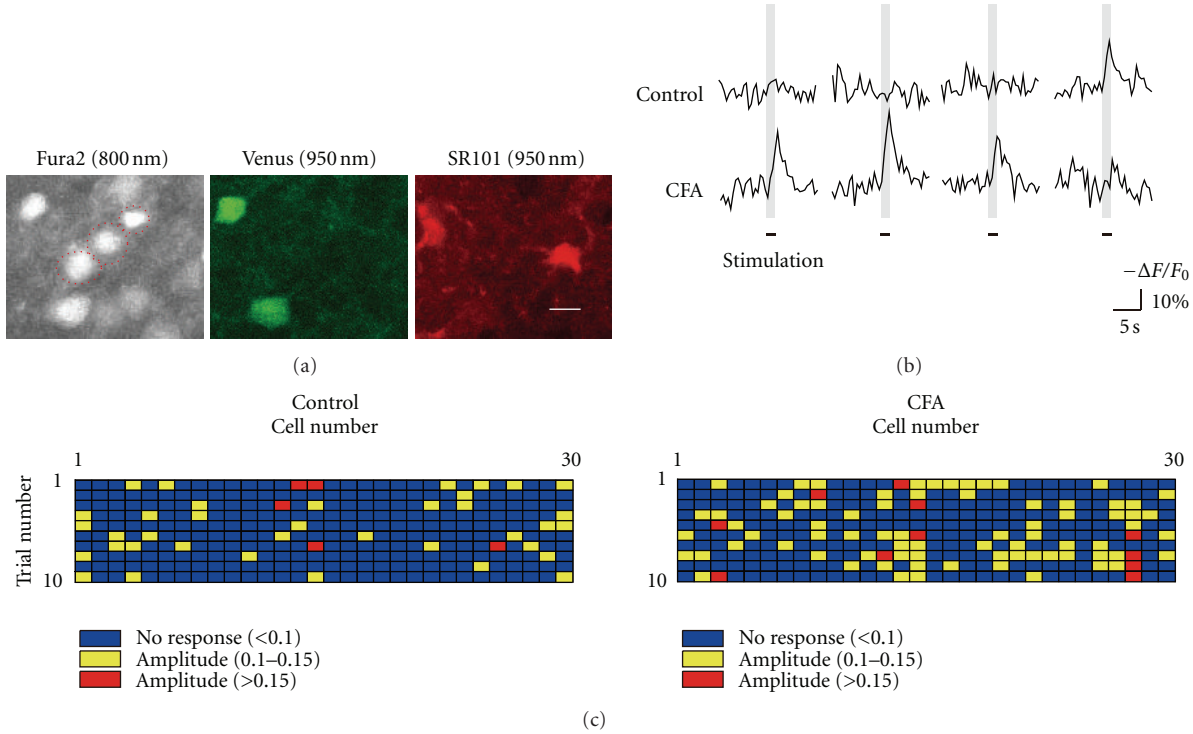

Figure 3: Enhanced activation of L2/3 excitatory neurons in response to hind paw stimulation and in response to stimulation of L4 neurons. (a) Identification of the S1 L2/3 excitatory neurons (red dotted circles in the Fura2 image), inhibitory neurons (green, Venus image), and astrocytes (red, SR101 image). Ca^{2+} indicator (Fura2) was excited at 800 nm two-photon laser, whereas Venus and SR101 were excited at 950 nm laser. Only cells that were positive for Fura2, but not for Venus and SR101, (i.e., excitatory neurons) were included in analysis. Each subpanel shows the same imaging area of the mouse S1 L2/3. (b) Representative traces of Ca^{2+} transients in identified L2/3 excitatory neurons during the same intensity stimuli (mechanical stimuli of hind paw or electrical stimuli of L4 cells) under control conditions (top traces) and following CFA-induced inflammatory pain (lower traces). (c) Distribution of the amplitude of Ca^{2+} responses to 10 successive stimuli in a sample of 30 L2/3 excitatory neurons under control conditions (left) and following CFA-induced inflammatory pain (right). (a–c) Reproduced and adapted, with permission, from [13].

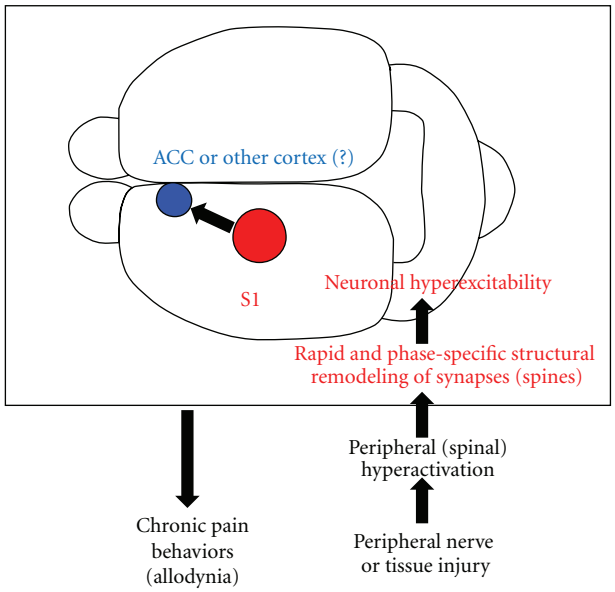

Figure 4: Working hypothesis for the cortical mechanism of peripheral injury-induced chronic pain. We propose that peripheral injury (nerve ligation or inflammation) induces rapid structural and function remodeling of S1 cortical synapses as described in the text. This, alongside possible other contributions of inhibitory interneurons and glia, results in hyperexcitability of excitatory S1 cortical neurons. These may project and interact with other regions within the pain "neuromatrix", such as the ACC, to result in chronic pain behaviors such as allodynia.

neuromatrix, including not only S1 and ACC, but also insular cortex and thalamus, interact with each other? We are optimistic that these and other important questions will be resolved in the near future.

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Review Article

Synapses and Dendritic Spines as Pathogenic Targets in Alzheimer's Disease

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Synapses are sites of cell-cell contacts that transmit electrical or chemical signals in the brain. Dendritic spines are protrusions on dendritic shaft where excitatory synapses are located. Synapses and dendritic spines are dynamic structures whose plasticity is thought to underlie learning and memory. No wonder neurobiologists are intensively studying mechanisms governing the structural and functional plasticity of synapses and dendritic spines in an effort to understand and eventually treat neurological disorders manifesting learning and memory deficits. One of the best-studied brain disorders that prominently feature synaptic and dendritic spine pathology is Alzheimer's disease (AD). Recent studies have revealed molecular mechanisms underlying the synapse and spine pathology in AD, including a role for mislocalized tau in the postsynaptic compartment. Synaptic and dendritic spine pathology is also observed in other neurodegenerative disease. It is possible that some common pathogenic mechanisms may underlie the synaptic and dendritic spine pathology in neurodegenerative diseases.

1. Introduction

The number of neurons in the human brain approximates the number of stars in the galaxy. Each of these neurons makes an average of 1000 contacts with other neurons. The result is an incredibly complex and sophisticated network made of roughly 100 trillion synapses. Communications between neurons in the brain occur primarily through synapses formed between presynaptic and postsynaptic partners. For fast synaptic transmission, there are two types of synapses: type I synapses use glutamate as the neurotransmitter and are excitatory, whereas type II synapses use gamma-amino butyric acid (GABA) as the major neurotransmitter and are inhibitory. While dendritic shafts are the main location for the inhibitory GABAergic synapses, dendritic spines, which are small membrane protrusions from dendritic shafts that contain glutamate receptors and postsynaptic density components, are the primary locations of excitatory synapses. A functional balance between neuronal excitation and inhibition is established during development for homeostatic control of neuronal excitability and is maintained into adulthood [1–4]. On the other hand, imbalances between neuronal excitation and inhibition have been associated with many neurological disorders including epilepsy [5], schizophrenia [6], fragile X syndrome [7], and autism [8].

Information can be stored in the brain by multiple synaptic mechanisms, including altered structure and chemistry of existing synapses, formation of new synapses, or elimination of old ones. Such synaptic plasticity is thought to be fundamental to learning and memory in the brain [9]. At the electrophysiological level, synaptic plasticity is reflected in processes known as long-term potentiation (LTP) and long-term depression (LTD) [10]. Excitatory synapses contain AMPA and NMDA ionotropic glutamate receptors localized on dendritic spines, with basal synaptic transmission largely mediated by the AMPA receptors. High synaptic activity opens NMDA receptors, leading to long-lasting changes in postsynaptic AMPA receptor number and LTP of synaptic transmission [11]. Alternatively, low levels of synaptic stimulation can activate NMDA receptors to produce LTD [12]. At the morphological level, LTP is generally associated with dendritic spine growth, whereas LTD can induce the removal of postsynaptic AMPA receptors and loss of spines [13–19]. It is thus not surprising that synaptic development, maintenance,

and plasticity under normal physiological conditions are frequently associated with changes in the morphology and number of dendritic spines [20].

In many neurodegenerative diseases, particularly those exhibiting cognitive impairments such as Alzheimer's disease (AD) and Parkinson's disease (PD), dendritic spines are altered in number and shape before eventual neuronal death is observed. Changes in dendritic spine number and morphology are also found in other disease conditions such as autism, Down syndrome, drug addiction, fragile X syndrome, and schizophrenia [20–24]. It is worth emphasizing that degeneration of synapses and dendritic spines is one of the earliest features in those neurodegenerative disease conditions, prior to subsequent loss of neurons. Interventions aimed to protect the nervous system from the ravages of these disease would therefore seem more effective when the synaptic and spine pathology are prevented as early as possible.

In this review article, we will summarize recent advances in our understanding of the molecular mechanisms underlying synaptic and dendritic spine pathology in neurodegenerative diseases, particularly in AD and PD. The readers are referred to some excellent previous reviews on the observation of synaptic and dendritic spine pathology in neurological disorders [22–25].

2. Synapse and Dendritic Spine Pathology in AD

AD is the most common neurodegenerative disease and the leading cause of dementia in the elderly. Decades of intensive research have uncovered amyloid plaque and neurofibrillary tangle (NFT) as the pathological hallmarks, and soluble amyloid-β (Aβ) oligomers as the leading candidate for the causative agent of AD [26, 27]. However, the mechanistic link between amyloid plaque and NFT and the mechanism by which Aβ oligomer may cause cognitive impairments remains poorly defined, and there is no effective treatment for this devastating disease. Substantial evidences have accumulated indicating that the memory deficits in AD patients do not correlate well with amyloid plaque burden; instead, the loss of synaptic markers is a better predictor of clinical symptoms and disease progression [28]. Together with studies using animal AD models, these studies have lent support to the hypothesis that AD could be conceptualized as a disease of synaptic failure [28].

Early structural studies of postmortem tissues showed that when compared with age-matched control brains, AD brains had reduced synapse density and number of dendritic spines in the cortex and hippocampus, principal brain areas affected by the disease, and that greater loss of dendritic spines was associated with lower mental status [29, 30]. These findings suggested that progressive loss of dendritic spines is directly related to the pathogenesis of AD and represents a good indicator of disease progression. Studies of transgenic mouse models of AD have shown that, in the vicinity of amyloid plaques, there were dramatic spine loss and neurite dystrophy, structural changes that could lead to altered neuronal circuits and brain functions [31–33]. Further studies showed that the accumulation of soluble Aβ might be the culprit that leads to dendritic spines loss [34]. Aβ is the proteolytic product of a large protein called amyloid precursor protein (APP), which is cleaved by beta- and gamma-secretases to produce Aβ and other fragments of the precursor protein [35]. Interestingly, the formation and secretion of Aβ peptides are positively regulated by neuronal activity, and excess Aβ peptide can in turn depress excitatory synaptic transmission onto neurons that produce Aβ as well as nearby neurons that do not produce Aβ [36]. Thus, activity-dependent modulation of Aβ production may normally participate in a negative feedback regulatory loop to restrain neuronal hyperactivity, the impairment of which could contribute to AD pathogenesis [36]. Under normal conditions, Aβ monomers could be cleared by proteolytic enzymes like neprilysin, chaperone molecule ApoE, or the lysosomal and proteasomal pathways. However, under pathological conditions, soluble Aβ levels are increased, leading to the buildup of Aβ oligomers, which can be further sequestered into protofibrils and fibrils as seen in plaques [27].

Several lines of evidence support that Aβ is the primary causative agent of AD. First, genetic studies of familial forms of AD have identified rare genetic mutations that cause AD by altering the production or metabolism of Aβ peptides, leading to their aberrant accumulation [27, 37]. Soluble Aβ levels have been found to better correlate with disease progression and severity than amyloid plaques or NFTs [34]. Second, Aβ oligomers formed *in vitro* from synthetic peptides, purified from cultured cells expressing APP, or from cortex of AD patient brains can induce synaptic dysfunction and neuritic degeneration [38–41]. Third, the reduction of soluble Aβ levels using an immunization method in mouse AD models rescued the cognitive deficits [42]. However, despite the overwhelming supporting evidences, the Aβ hypothesis of AD as described above still faces challenge, since several highly publicized clinical trials targeting Aβ had failed.

3. Mechanisms Underlying the Synapse and Dendritic Spine Pathology in AD

The molecular mechanisms through which Aβ might cause synaptic loss and neuronal death remain uncertain. Aβ has been found to form pore-like structures with calcium channel activity, which could interfere with calcium signaling [43, 44]. Aβ can also affect LTP and LTD by modulating glutamate receptor-dependent signaling pathways [45–47] and trigger aberrant patterns of neural network activity [48]. Aβ may also cause mitochondrial dysfunction [49] and lysosomal failure [50].

One of the earliest clues about the mechanisms of Aβ-induced synaptic dysfunction came from studies of cultured neurons derived from Tg2576 mutant APP transgenic mice [51]. Among the synaptic changes observed were fewer and smaller postsynaptic compartments and fewer and enlarged active presynaptic compartments. Notably, the earliest observable change in synaptic components was the reduction of PSD-95, which is a master regulator of the assembly and

anchoring of postsynaptic density components such as glutamate receptor subunits [52]. Aβ was shown to be the toxic agent causing these synaptic changes since the effects were blocked by gamma-secretase inhibitor treatment and recapitulated by application of synthetic Aβ to wild-type neurons [51]. Similar PSD-95-related synaptic defects were also observed in human AD brain samples [53]. The molecular mechanisms through which Aβ influences PSD-95 remain to be determined. Studies in *Drosophila* models showed that PAR-1 kinase, the fly homologue of mammalian microtubule affinity regulating kinases (MARKs), can directly phosphorylate the fly PSD-95 homologue Dlg, and this phosphorylation event caused the delocalization of Dlg from the postsynaptic membrane [54]. PAR-1/MARK kinases are known to be activated by APP or Aβ in *Drosophila* or mammalian neurons [55, 56]. It would be interesting to test whether MARKs are critical mediators of Aβ toxicity on mammalian synapses and dendritic spines.

A significant recent advance in our understanding of the mechanisms of the synaptic toxicity of Aβ has been the finding that Aβ uses LTD-related signaling mechanisms to affect synaptic function and dendritic spine morphology [45]. One of the principle mechanisms of LTD induction is the removal of AMPA receptors from the postsynaptic membrane through endocytosis. Significant parallels were found between Aβ-induced synaptic changes and LTD. Overexpression of Aβ resulted in decreased spine density and postsynaptic AMPA receptor number, through signaling molecules implicated in LTD, such as p38 MAP kinase and calcineurin. Importantly, expression of a mutant form of AMPA receptor that resists LTD-driven endocytosis blocked the morphological effects and synaptic depression induced by Aβ [45]. This study implicated the endocytosis of AMPA receptors as a major mechanism through which Aβ causes synaptic dysfunction and subsequent degeneration, but the detailed molecular mechanisms remain unclear.

Recent studies using transgenic mouse models of AD have implicated the microtubule-binding protein tau as a major mediator of the toxicity of Aβ at the postsynaptic compartment and dendritic spines. Although tau abnormality has long been observed in AD, as exemplified by the formation of NFTs by tau that accompany plaque pathology, and tau abnormality can cause neurodegeneration in the absence of plaque pathology as in frontotemporal dementia cases [57, 58], the direct involvement of tau in Aβ-induced synaptic and dendritic spine pathology may initially appear surprising, since tau is generally considered a presynaptic protein that is primarily localized to axons. In fact, the relationship between NFTs and amyloid plaques in disease pathogenesis has long been a source of considerable debate [37, 59, 60]. Studies in mice suggested that the two lesions might be causally linked. In transgenic mouse models, intracranial injection of synthetic Aβ, or crossing of APP transgenic mice with tau transgenic mice, promoted NFT pathology [61–63], and immunization of APP/Psn/tau triple transgenic mice with antibodies against Aβ reduced the levels of hyperphosphorylated tau [64]. This was consistent with earlier studies showing that the removal of tau could relieve Aβ-induced neurotoxicity in cultured neurons [65]. Together, these studies support the notion that the initiating event in AD is the accumulation of the toxic Aβ peptides, and that tau abnormality is a major downstream molecular event that contributes to disease pathogenesis [37].

How tau abnormality arises in AD is not well understood. Current efforts have focused on the role of aberrant phosphorylation of tau [27]. Previous studies have shown that Aβ could lead to abnormal activation of a number of kinases, including cyclin-dependent kinase-5 (CDK5) [66, 67], Fyn kinase [68, 69], glycogen synthase kinase-3beta (GSK3β) [70], and MARK [71–73], all of which promote tau hyperphosphorylation and could potentially affect synaptic structure and function. However, very few *in vivo* studies have been done to assess the roles of tau kinases or phosphatases in conferring tau toxicity and in causing AD-related memory deficit. Identification of the relevant kinases or phosphatases will provide attractive therapeutic targets for AD.

Recently studies have shown that removing endogenous tau can prevent Aβ-induced behavioral deficits in a mouse AD model expressing human APP, and block excitotoxin-induced neuronal dysfunction in both transgenic and non-transgenic mice [74]. Since current data support postsynaptic toxicity as a primary mechanism of Aβ action in causing learning and memory deficits in AD, this study raised the possibility that tau may also act in the postsynaptic compartment. Indeed, under both physiological and pathological conditions, tau was found in dendrites [75, 76], albeit the level of dendritic tau was much higher under disease conditions. Tau was known to interact with microtubules through its microtubule-binding domain to stabilize microtubule and regulate axonal transport. It has many putative phosphorylation sites and becomes hyperphosphorylated in AD patients and transgenic animal models [57, 58]. Apart from the notion that phosphorylation can lead to the dissociation of tau from the microtubules, other pathophysiological effects of this molecular event are unknown.

A recent study has indicated that phosphorylated tau could accumulate in dendritic spines, where it may affect the synaptic trafficking and/or anchoring of glutamate receptors, thereby influencing postsynaptic function [76]. Interestingly, this effect of tau on synaptic function occurred without causing the loss of synapses or dendritic spines. This study thus revealed a critical role for tau phosphorylation in causing tau mislocalization and subsequent synaptic impairment, and it established dendritic spines as pathogenic targets of tau action. Another study provided further mechanistic insights into the dendritic function of tau [75]. Tau interacts with fyn [77], a protein tyrosine kinase that can phosphorylate tau and whose activity is increased in AD brain [78]. Ittner et al. showed that the interaction of tau with fyn leads to the targeting of fyn to dendritic spines, where fyn can phosphorylate NMDA receptor subunit 2 (GluR2), resulting in stabilization of the interaction between GluR2 and PSD-95 and enhanced excitotoxicity. Tau also shows strong interaction with PSD-95, providing further support for a dendritic role of tau besides its known axonal function. Importantly, the toxic effects of APP/Aβ were attenuated by interfering with GluR2/PSD-95 interaction with a cell-permeable peptide [75], supporting that dendritic

tau-mediated fyn recruitment and GluR2/PSD-95 interaction confer Aβ toxicity at the postsynapse.

Thus, a "tau hypothesis" has been put forward based on these recent results; Aβ triggers the phosphorylation of tau, causing tau to dissociate from the microtubules and accumulate at the dendritic compartments. Phosphorylated tau exhibits stronger interaction with Fyn and thus facilitates the targeting of fyn to dendritic spines. The targeting of fyn to postsynaptic density sensitizes the NMDA receptors and renders neurons more vulnerable to the toxicity of Aβ in the postsynaptic compartment [79]. It remains to be determined whether tau becomes hyperphosphorylated *in situ* in the dendritic spines as a result of altered kinase/phosphatase activities there, or that it becomes hyperphosphorylated elsewhere and is then transported to the dendritic spines. Nevertheless, targeting the tau-dependent pathway, for example, by reducing tau protein level, inhibiting tau kinase activities, or increasing phosphatase activities, would represent suitable new ways of treating AD.

In summary, we can consider the toxic effect of Aβ on neuronal synapses and dendritic spines as a normal physiological process gone awry, instead of some pathological process unique to the disease process. Aβ is continuously produced in the brain, and its production can be stimulated by neuronal activity. Aβ can then feedback on the hyperactive neuron using a LTD-related mechanism to tune down neuronal activity, for example, by promoting AMPAR removal. This process normally acts as a homeostatic mechanism to restrain neuronal hyperactivation. In the disease process, however, the buildup of Aβ tips the balance of this process toward excessive synaptic depression and AMPAR removal, resulting in synapse and spine loss (Figure 1). The molecular mechanisms involved in Aβ toxicity on synapses and dendritic spines are just beginning to be elucidated. We propose that a signaling cascade from Aβ to tau and PSD-95, involving tau kinases such as PAR-1/MARK and its activating kinase LKB1, might be involved (Figure 1).

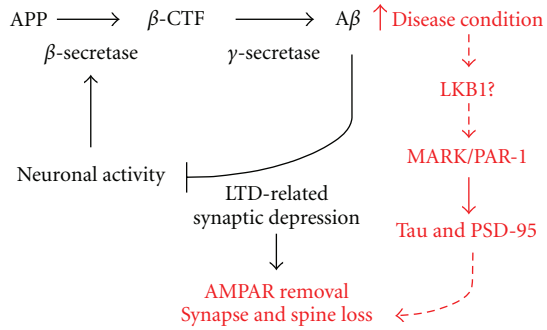

Figure 1: A diagram depicting the physiological and pathological roles of Aβ. The pathway in black represents the normal function of Aβ in restraining neuronal hyperactivation. In response to neuronal activation, there is upregulation of BACE, leading to overproduction of Aβ, which then acts through LTD-related mechanism involving AMPAR removal to tune down neuronal activity. In disease condition (depicted in red), however, the excessive accumulation of Aβ leads to excessive synaptic depression and AMPAR removal, which eventually results in synapse and spine loss. Based on our unpublished work (Yu et al., manuscript submitted), we propose that Aβ can act through the LKB1 → MARK → tau/PSD-95 signaling cascade to cause synapse and spine loss.

4. Possible Nonneuronal Contribution to Synapse and Spine Pathology in AD

Although much of the research on the mechanisms of Aβ toxicity to synapses and spines has taken a "neuron-centric" approach, it is worth noting that other nonneuronal cell types in the brain play critical roles in the formation and maturation of synapses during development, and similar mechanisms may operate in the adult brain to mediate the effects of Aβ on neuronal synapses and dendritic spines.

Besides providing trophic factors for neurons, glial cells have been shown to play key roles in regulating neuronal migration, axon guidance, and synapse formation [80]. In one of the better-characterized cases, astrocytes were shown to secret signals that induce synapse formation by retinal ganglion cells (RGCs). A family of extracellular matrix proteins called thrombospondins (TSPs) was identified as the synaptogenic signals coming from astrocytes [81]. The TSP receptor from the neuronal side involved in synaptogenesis was found to be the calcium channel subunit $\alpha 2\delta$-1 [82]. Interestingly, the synapses formed by TSPs are postsynaptically silent due to the lack of surface AMPA receptors, whereas those formed by astrocyte conditioned medium are postsynaptically active, suggesting that additional factors are secreted by the astrocytes to control synaptic strength and plasticity [83]. The identity of these additional factors is currently unknown. Also, synapses are made in excess during development, and the extra synapses or weak synapses are eliminated by a process involving signals from astrocytes that induce the classical complement pathway protein C1q in neurons [84]. In addition to secreted factors, astrocytes can regulate synapse formation using contact-mediated mechanisms. Astrocytes also regulate dendritic spine morphology through a contact-mediated mechanism involving bidirectional ephrin/EphA signaling. In the hippocampus, for example, astrocytes express ephrin A3, whereas neurons express the ephrin receptor EphA4. Perturbing ephrin/EphA signaling results in defects in spine formation and maturation [85]. One can imagine that disruption of astrocyte-neuron interaction by Aβ could affect synapse and spine morphology through the above-mentioned mechanisms. In this respect, it is interesting to note that a recent study has shown that lentiviral-mediated delivery of EphB2 expression constructs in the dentate gyrus of hAPP transgenic mice reversed deficits in NMDA receptor-dependent LTP and memory impairments [86]. Whether there is glial involvement in this experimental setting has not been examined.

The other abundant glial cells in the brain are microglia. Unlike the astrocytes, these cells are of mesodermal origin. The roles of microglia in disease pathogenesis in AD and other neurodegenerative diseases are very complex and controversial [87, 88]. This probably has to do with the diverse activities of these cells in the brain. Relevant to AD pathogenesis, microglia can promote Aβ clearance, release

anti-inflammatory cytokines and neurotrophic factors on one hand, and they can also affect the activation of complement systems and elimination of synapses and spines on the other hand [88]. Thus microglia can exert neuroprotective as well as neurodegenerative effects, depending on the strength, timing, and duration of their activation. Imaging studies showed that activated microglia were found in patients with MCI [89], suggesting that neuroinflammation is an early event in the disease process. Consistent with this finding, microglial activation was observed early in a tauopathy mouse model, preceding NFT formation and roughly concurrent with synapse loss and impairment of synaptic function [90]. Interestingly, supplement of immunosuppressant FK506 to young mice attenuated tau pathology and increased lifespan, suggesting that microglia activation may contribute to disease. In another AD mouse model expressing the E693Δ mutation that causes AD by enhanced Aβ oligomerization without fibrillization, it was found that the mice displayed age-dependent accumulation of intraneuronal Aβ oligomers at around 8 months, when abnormal tau phosphorylation, and impairments of hippocampal synaptic plasticity and memory were observed. However, microglial activation was observed from 12 months, astrocyte activation from 18 months, and neuronal loss at 24 months [91]. It is not known in this case whether microglial and astrocyte activation plays a neurodegenerative role or as part of a neuroprotective, compensatory response.

Despite the large amount of literature documenting a detrimental role for microglia and astrocyte activation in the disease process, these cells are important for neuronal heath during development and later in adult life. For example, microglia are proposed to play a surveillance role by constantly monitoring and sensing synaptic health [92], and, in addition to the critical roles, astrocytes play in synapse formation as mentioned earlier, and these cells can also control extracellular glutamate levels, remove excess extracellular K^+, release gliotransmitters, store glucose and transform it into lactate as energy source of neurons, and scavenge ROS to protect against oxidative damages [88]. Given these essential roles of glia to neuronal function and health, it is possible that damaging of glial cells by Aβ may have equally harmful effect on the neurons eventually. In fact, there is evidence that glial cells can release ROS upon Aβ exposure [93], and glial-released cytokines may even trigger a signaling process that promotes tau hyperphosphorylation [94]. Thus, a possible role of dysfunction glial cells in AD pathogenesis should be considered, especially in the early stages of the disease process (Figure 2).

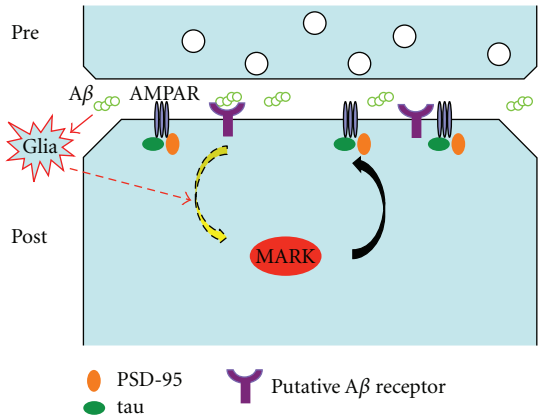

Figure 2: A diagram depicting a potential role of glia in mediating the synaptic toxicity of Aβ. Aβ oligomers presumably secreted from the presynaptic neuron could bind to its putative receptor on the postsynaptic cell, and this could then initiate a signaling cascade leading to activation kinases such as MARK, which then acts on tau, PSD-95, and possibly other synaptic substrates to affect AMPAR removal from the synaptic surface, leading to synapse and spine loss. Alternatively, Aβ could act on glial cells near neuronal synapses, which then release factors such as cytokines to activate signaling molecules such as MARK and cause synapse and spine loss. These two mechanisms are not mutually exclusive and could in fact occur simultaneously to mediate Aβ toxicity.

5. Conclusions and Future Directions

Synapse and dendritic spine pathology have been observed in the early stages of neurodegenerative diseases before neuronal death is evident, suggesting that these cellular locations represent pathogenic sites of action by the disease-causing agents early in the disease process. At least in the case of AD, there is compelling evidence supporting a pathogenic role for the synaptic and dendritic spine abnormalities. An intriguing possibility is that, as in AD, defects in the morphology and function of synapses and dendritic spines may play a critical role in the pathogenesis of PD. In fact, alterations in synaptic plasticity as represented by LTP and LTD are observed in PD, and some familial PD-associated genes have been shown to affect synapse and dendritic spine morphology and function [95, 96]. It would thus be interesting to examine whether LTP- and LTD-related signaling mechanisms are involved in PINK1/Parkin-induced synapse and dendritic spine changes. In this respect, it would also be interesting to test the potential role of dendritic tau in mediating the synaptic effects of the FPD genes. This is particularly relevant, given the identification of tau as a susceptibility factor for PD [97]. Future studies along these directions could lead to the identification of common molecular mechanisms underlying the pathogenesis of AD, PD, and possibly other neurological disorders and offer new therapeutic strategies.

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Review Article

Spines, Plasticity, and Cognition in Alzheimer's Model Mice

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The pathological hallmarks of Alzheimer's disease (AD)—widespread synaptic and neuronal loss and the pathological accumulation of amyloid-beta peptide (Aβ) in senile plaques, as well as hyperphosphorylated tau in neurofibrillary tangles—have been known for many decades, but the links between AD pathology and dementia and effective therapeutic strategies remain elusive. Transgenic mice have been developed based on rare familial forms of AD and frontotemporal dementia, allowing investigators to test in detail the structural, functional, and behavioral consequences of AD-associated pathology. Here, we review work on transgenic AD models that investigate the degeneration of dendritic spine structure, synaptic function, and cognition. Together, these data support a model of AD pathogenesis in which soluble Aβ initiates synaptic dysfunction and loss, as well as pathological changes in tau, which contribute to both synaptic and neuronal loss. These changes in synapse structure and function as well as frank synapse and neuronal loss contribute to the neural system dysfunction which causes cognitive deficits. Understanding the underpinnings of dementia in AD will be essential to develop and evaluate therapeutic approaches for this widespread and devastating disease.

1. Introduction

Alzheimer's disease (AD) is a devastating progressive neurodegenerative disease characterized by cognitive decline, brain atrophy due to neuronal and synapse loss, and the formation of two pathological lesions, extracellular amyloid plaques composed largely of amyloid-beta peptide (Aβ), and neurofibrillary tangles, intracellular aggregates of hyperphosphorylated tau protein [1, 2].

The brain is a remarkably adaptable network of neurons sharing information through approximately 10^{14} synaptic connections. The plasticity of this network in response to environmental stimuli enables the brain to adapt to new demands and allows learning and the formation of new memories. Changes of synapses and dendritic spines, the postsynaptic element of most excitatory synapses, in response to stimulation are thought to underlie the brain's plasticity [3]. It follows that disruption of neural circuits due to both synapse loss and decline in the ability of remaining spine synapses to change in response to stimuli likely contribute to the disruption of cognition observed in neurodegenerative diseases such as Alzheimer's disease. Indeed, it is known that synapses are lost during AD and that, in AD tissue, synapse loss correlates strongly with cognitive decline, arguing the importance of this process as causative to dementia [4–7].

Rare familial forms of AD occur in which amyloid precursor protein (APP), the precursor to the Aβ peptide, or presenilin (PS) 1 or 2, the catalytic subunit of the gamma-secretase complex which cleaves APP to form Aβ, are mutated and result in an autosomal dominant, early-onset form of the disease [8]. Mutations in the tau protein have not been found to cause AD but can lead to familial frontotemporal dementia with Parkinsonism linked to chromosome 17 [9, 10]. These mutations strongly implicate amyloid processing as an instigating factor in the disease and also provide genetic tools for the construction of transgenic mouse models of the disease which recapitulate many of its pathological features [11]. In contemporary AD research, these transgenic models are being used to characterize

the physiological and behavioral consequences of AD neuropathology in order to investigate the fundamental question of the underlying anatomical causes of dementia. APP and APP/PS1 transgenic mice express high levels of amyloid beta (Aβ) and progressively develop many of the pathological phenotypes of AD, including abundant extracellular Aβ plaques, synaptic dysfunction and loss, astrocytosis, activation of microglia, and cognitive deficits [12]. For decades, Aβ plaques were thought to cause dementia in AD patients by physically interrupting normal neural connectivity and function. However, the lack of correlation between Aβ plaque load and the degree of cognitive impairment in AD patients [4] and the fact that Aβ plaques occupy a negligible fraction (less than 5%) of the neuropil [13–15] in cognitively impaired transgenic mice [15] raised the possibility that fibrillar Aβ in plaques does not contribute significantly to dementia in AD patients. Instead, soluble Aβ species (i.e., monomeric, oligomeric, and protofibrillary Aβ species that linger in aqueous solution after high-speed centrifugation) seem to be the main culprits of the functional deficits in these mice and probably also in initiating disease in AD patients.

Mice expressing dementia-associated tau mutations have also been developed to study the contributions of neurofibrillary pathology to dementia and the interplay between Aβ and tau [16, 17]. While genetic studies clearly implicate amyloid as the initiating factor in AD, the correlation of tangles with neuronal loss in AD brain, together with the lack of neuronal loss and tangle formation in APP transgenic models, and the lack of efficacy with Aβ-directed therapeutics have contributed to the idea that tau pathology is an important contributor to dementia downstream of Aβ [18].

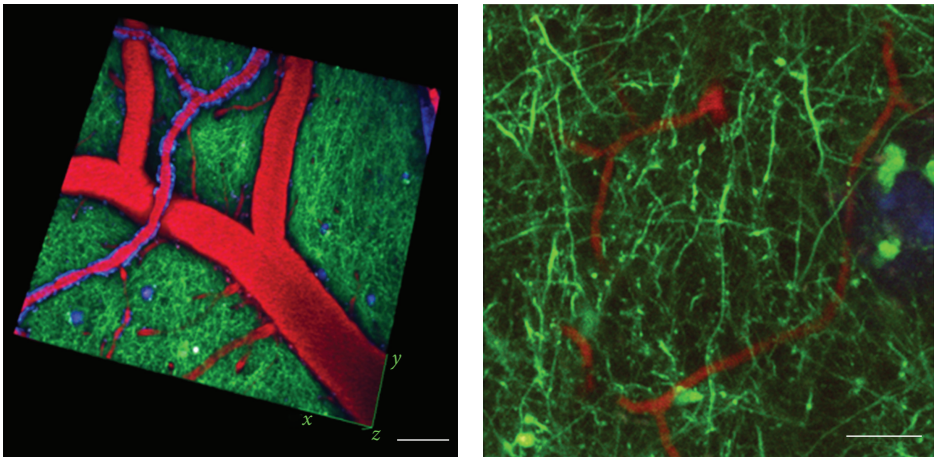
This paper will review the work on dendritic spine changes and their contribution to functional changes in synapses and behavioral deficits in AD models. It is important to address these questions because the ability of synapses and spines to change even in aged brain and the strong correlation between synapse loss and cognitive decline in AD indicate that enhancing spine plasticity could prevent or even reverse cognitive deficits associated with neurodegenerative disease.

2. *In Vivo* Imaging Reveals Dendritic Spine Loss and Plaque-Associated Structural Plasticity Deficits in AD Models

Dendritic spines form the postsynaptic element of the vast majority of excitatory synapses in the cortex and hippocampus brain regions important for learning and memory. Changes in spines are thought to be a structural basis for these processes [3]. Loss of dendritic spines similar to the synapse loss observed in human AD has been reported in several mouse models that develop amyloid and tau pathology [13, 19–25]. The use of mouse models for *in vivo* multiphoton imaging allows longitudinal investigations to determine the temporal sequence of pathological events and to answer "chicken-or-egg" questions such as which comes first, spine loss or plaques? In order to perform these experiments, it is first necessary to fluorescently label dendritic spines and pathological lesions such as plaques and tangles. Spines can be imaged with transgenic expression of fluorophores such as GFP and YFP [26–29] or through filling neurons with fluorescent dextrans [30] or fluorescent proteins expressed in adeno-associated virus or lentivirus [21, 31]. To label plaque pathology in AD models, the blood-brain barrier-penetrable compounds, Pittsburghs compound B and methoxy-XO4 (developed by William Klunk), have been used in conjunction with *in vivo* multiphoton imaging to observe amyloid plaques and their clearance after treatment with immunotherapy [32–35].

Imaging of amyloid plaques together with imaging dendrites and dendritic spines filled with fluorescent proteins can be used to assess the effects of pathology on the surrounding neuropil (Figure 1). This technique shows that plaques form rapidly, over the course of one day, and that within one week of plaque formation, surrounding dendrites begin to curve and exhibit dystrophic swellings [36]. Spine loss around plaques was determined to be due to a loss of stability of spines in the vicinity of plaques with more spine elimination than that in control brain, reflecting dysfunctional structural plasticity [37]. These structural plasticity changes contribute to functional deficits around plaques. In one study, neural circuit function was assessed using a fluorescent reporter of neuronal activation (the coding sequence of Venus, flanked by short stretches of the 5′ and 3′ untranslated regions from CamKIIα) which gets transported to dendrites and locally translated in response to activity resulting in increased fluorescence in dendrites after neuronal activation. APP/PS1 transgenic mice have greatly reduced levels of this reporter in dendritic segments surrounding plaques, and they failed to upregulate its expression in response to environmental stimulation, a phenomenon which was robust in wild-type animals [38]. Resting intraneuronal calcium levels are also disrupted around plaques, indicating dysfunction [39].

From the above studies, it is clear that plaques affect local dendrites and dendritic spines, but the precise bioactive molecule around plaques which induces spine loss was not clear for many years. The strongest candidate for the synaptotoxic molecule around plaques arose as soluble oligomeric Aβ due to work by William Klein, Dennis Selkoe, and other groups who reported that soluble Aβ causes dendritic spine collapse and impairs synaptic plasticity in culture [40–44], correlates with memory loss in transgenic mice [45, 46], and impairs memory and synaptic plasticity *in vivo* [47, 48]. In further support of the synaptotoxic role of Aβ, both active and passive immunotherapy to remove Aβ have favorable effects on memory, plaque clearance, and neurite architecture in AD models [33, 49–53]. The first direct assessment of whether oligomeric Aβ is present at synapses in the brain came from application of the array tomography technique to AD mouse brain tissue. Array tomography, developed by Micheva et al., overcomes the axial resolution limitation of confocal microscopy by physically sectioning tissue into 70 nm ribbons of serial sections which can then be used for immunofluorescent analysis to accurately quantify the contents of small structures such as synapses [54, 55].



(a) (b)

Figure 1: *In vivo* multiphoton imaging of plaques (labeled with methoxy X-O4, blue), vasculature (labeled with Texas red dextran, red), and dendrites (transgenically expressing YFP, green) in mice transgenic for mutant human APP and PS1 crossed with YFP transgenic mice allow examination of dendritic spine plasticity and loss. Low-resolution three-dimensional image stacks (a) are used to repeatedly find the same imaging sites. Higher-resolution image stacks (b) are used for spine analysis. Scale bars 100 μm (a) 10 μm (b).

In APP/PS1 mice, this technique shows that oligomeric Aβ is in fact present at a subset of shrunken excitatory postsynaptic densities, particularly in a halo of oligomeric Aβ surrounding the dense cores of plaques [56]. As would be predicted from the association of dendritic spine changes with physiological plasticity [57–59] and the presence of oligomeric Aβ at shrinking spines, dendritic spines can recover with therapeutic interventions aimed at removing oligomeric Aβ or inhibiting calcineurin which is activated downstream of Aβ-associated increases in intracellular calcium [30, 39, 60–62]. Removing soluble Aβ with the topical application of the 3D6 antibody allows rapid increases in the structural plasticity of dendritic spines within one hour, before any clearance of fibrillar Aβ occurs [30].

Tau overexpression has also been associated with spine loss in postmortem studies of human tau transgenic animals [25]. In rTg4510 mice, pyramidal cells have reduced spine density compared to wild-type animals, but tangle-bearing neurons have no more loss than their non-tangle-bearing neighbors [25]. Similarly rTg4510 hippocampal circuits are deficient in experience-dependent upregulation of immediate early genes compared to wild-type mice, but tangle-bearing neurons are not impaired compared to non-tangle-bearing cells in rTg4510 brain [63]. *In vivo* and array tomography imaging of tangles in rTg4510 mice has been developed and is demonstrating similar indications that soluble tau may be more toxic than fibrillar tau in terms of axonal transport and neuronal death [64–68]. In cultured neurons and transgenic mice overexpressing tau, mislocalization of tau to dendritic spines disrupts synaptic function [69].

Very recent data elegantly link Aβ, tau, and dendritic spine loss [70, 71]. Ittner et al. established that tau has a dendritic function in targeting the Src kinase Fyn to dendritic spines. Fyn phosphorylates NMDA receptor NR2 subunits mediating their interaction with the postsynaptic scaffolding protein PSD95 and disrupting this interaction of tau, and Fyn prevents Aβ toxicity in APP transgenic mice [70]. Similarly, Roberson et al. found that Aβ, tau, and Fyn jointly impair synaptic network function in electrophysiological studies of APP and Fyn overexpressing mice on a tau null background [71]. In culture, oligomeric Aβ was found to cause tau mislocalization to dendrites which was associated with local calcium elevation and dendritic spine loss [72].

3. Synaptic Plasticity Is Severely Impaired in AD Mouse Models

It is widely accepted that, in early stages of AD, synaptic dysfunction is the cause of dementia [84, 85]. Synaptic plasticity provides a neurophysiological substrate for learning and memory and is, therefore, often used to evaluate the phenotype of transgenic mice. In APP transgenic AD mouse models, there are significant alterations in hippocampal synaptic transmission and plasticity at excitatory glutamatergic synapses that sometimes appear in young animals long before Aβ is deposited in plaques (see Table 1). Most studies performed before mice reached 6 months of age report intact basal synaptic transmission [75, 78, 81, 83] although some exceptions were also reported [74, 86]. It should be noted that the lack of detectable changes in basal synaptic transmission in the majority of studies does not rule out synaptic dysfunction that has been overcome by functional compensation. Indeed, evidence of functional compensation in response to spine loss induced by Aβ has been observed in several models [87–89]. From 6 months on, most of the AD transgenic mice show significant deficits in basal synaptic transmission [75, 78, 79, 81–83, 90]. This age-related deterioration in synaptic transmission in AD transgenic mice is unlikely to result from a decreased transmitter release probability because paired-pulse facilitation (PPF), which correlates inversely with the probability of transmitter release

Table 1: Progressive synaptic malfunction AD transgenic mice.

Model	Mutations	Age (months)	Basal synaptic transmission	Long-term potentiation	Paired-pulse facilitation
Tg2576	APPswe	2-3		Impaired [73]	
		4–6	Impaired [74]	Normal [74]/impaired [73]	
		6–12	Impaired [75]		Normal [75]
		>12	Normal [76, 77]/Impaired [75]	Normal [75]/impaired [76, 77]	Normal [4, 6]
PDAPP	APP (V717F)	<6	Normal [78]	Impaired [78]	Impaired [78]
		6–12			
		>12	Impaired [78]	Normal [78]	Impaired [78]
APP/PS1	APPswe/PS1dE9	<6	Normal [79]	Impaired [79, 80]	
		6–12	Impaired [79, 80]	Impaired [79, 80]	Normal [80]
		>12			
3xTg-AD	APPSwe, PS1M146V, and tauP301L	1-2	Normal [81]	Normal [81]	Normal [81]
		3–6			
		6–12	Impaired [81]	Impaired [81]	Normal [81]
		>12			
5XFAD	$APP_{swe/lnd/fl}$ and a PS1 transgene carrying double FAD mutations (M146L and L286V)	<6	Normal [82]	Normal [82]	Normal [82]
		6–12	Impaired [82, 83]	Impaired [82, 83]	Normal [82]
		>12			

[78], remains intact in most of AD transgenic mice, even at advanced ages [78, 86] (see Table 1). Impairments in long-term potentiation (LTP) were shown both *in vitro* and *in vivo*, in the CA1 as well as dentate gyrus regions of the hippocampus [76, 91]. Failure of LTP expression is detected in AD mice in some cases before 4 months of age [73, 78] but usually appears later [75, 76, 81–83, 86, 92], when Aβ load is higher. These findings emphasize the fact that extracellular deposition of fibrillar Aβ is not required for the development of severe functional deficits in AD models. This conclusion is strengthened by the observation that direct application of Aβ oligomers into the brain prevents LTP [48, 93, 94].

Studies of the mechanisms of Aβ-mediated synaptic dysfunction converge on the theme of increased postsynaptic calcium concentrations leading to internalization of NMDA and AMPA receptors via mechanisms similar to those seen in long-term depression [40, 42, 95, 96]. Overall, these findings suggest that synaptic dysfunction is an early event in AD pathogenesis and may play a role in the disease process.

4. Impaired Cognition in AD Mouse Models

Learning and memory processes are believed to depend on changes of synaptic transmission in certain areas of the brain, including the hippocampus. Most studies done with AD transgenic mice assess spatial navigation capability (e.g., Morris water maze, radial maze, Barnes maze) since this memory system depends on the hippocampus and is highly conserved in mammals [97]. The onset of cognitive decline is difficult to define in humans, particularly without a reliable biomarker. Thus, the use of transgenic mouse models to address this question is particularly useful, since the early cognitive changes can be identified and correlated with molecular and cellular changes. The implication of Aβ in the cognitive decline in AD transgenic mice is no longer controversial. However, there were contrasting reports regarding the onset of cognitive decline in different AD models (Table 2). In some studies, deficits in learning and memory were observed at 3 months, implicating soluble Aβ assemblies [98, 99], while other studies have shown onsets at intermediate ages [76, 98, 100–104] or at advanced ages [100, 101, 105, 106], invoking insoluble Aβ plaques. Moreover, in the 3XTg-AD mouse model, spatial long-term retention memory deficits were found to correlate with intraneuronal Aβ at 4 months [81], an age when these transgenic mice do not have Aβ plaques [107]. A similar observation has been shown for 5x FAD mice, which also accumulate high amounts of intraneuronal Aβ peptides [108] and present with significant impairment in the working memory already at 4-5 months of age [102, 103]. Due to this controversy, the Aβ species responsible for the cognitive decline in these mice was under debate for many years. Strong evidence for the toxicity of soluble Aβ came from a study showing that naturally secreted soluble Aβ oligomers administrated into the rat's lateral cerebral ventricles at picomolar concentrations disrupt the memory of a complex learned behavior [47]. This suggests that soluble Aβ oligomers, rather than Aβ plaques, may be responsible for the cognitive impairment in the absence of Aβ plaques or neuronal death.

Although aged AD mice are impaired at learning several tasks that depend on the hippocampus, the performance of these mice on tasks requiring an intact amygdala, such as cued-fear conditioning, has been thoroughly established only for Tg2576 mice [112, 119] and aged APP/PS1 mice [15]. In these models amygdala-dependent learning is severely impaired at advanced ages, implying that neurons of

Table 2: Progressive cognitive impairments in APP AD transgenic mice.

Model	Age (months)	Spatial task		Working memory	Fear conditioning	
		Learning	Probe test		Contextual	Cued
Tg2576	<6	Impaired [109]/normal [98, 100, 110]	Normal [100, 111]	Normal [110]/impaired [98]	Impaired [74, 109, 112–118]	Normal [119]
	6–12	Normal [101]/impaired [110, 120]	Normal [120]/ impaired [111]	Normal [98, 101]/impaired [110]	Impaired [115, 121]/normal [122]	Normal [122]
	>12	Impaired [101, 110]	Impaired [111]	Impaired [76, 98, 101, 110]	Impaired [112, 115, 117]	Normal [112, 119]/impaired [119]
APP/PS1	<6	Normal [123–128]	Normal [126, 127]/ impaired [128]	Normal [124, 127, 129, 130]/impaired [80]	Normal [131–133]/impaired [79, 117]/enhanced [127]	
	6–12	Impaired [79, 80, 105, 126–128, 134]	Normal [105, 127, 134]/impaired [79, 80, 126, 128]	Impaired [80, 127, 129, 130]	Impaired [79]/normal [127]	
	>12	Impaired [105, 106, 124, 128, 135]	Impaired [105, 106]	Impaired [124, 129]		Impaired [15]
3xTg-AD	1-2	Normal [107]	Normal [107]	Normal [107, 136]		
	3– 6	Impaired [137–139]	Impaired [107, 137, 138]	Normal [136]		
	6–12	Impaired [107, 140]	Impaired [107, 139, 140]	Impaired [136, 139, 141]	Impaired [139]	
	>12		Impaired [142]	Impaired [141, 143]		
5XFAD	<6		Impaired [108, 144]	Normal [102, 108]	Normal [82, 145]	Normal [144]
	6–12	Impaired [144]		Impaired [102, 103]	Impaired [82, 144, 145]	
	>12			Impaired [102, 103, 146]		

the amygdala, similar to hippocampal neurons, are susceptible to the toxic effect of Aβ.

At later stages of the disease, widespread synaptic and neuronal death probably contribute greatly to dementia. These effects are likely mediated by tau downstream of the initiating amyloid pathology [18]. Reflecting this later stage of dementia, tau-expressing mouse lines which undergo neuronal loss develop behavioral deficits. Interestingly, two of these mouse lines which have reversible expression of pathological tau exhibit recovery of cognition after transgene suppression even after extensive neuron loss [147, 148]. These studies point to the powerful ability of synapses to regenerate and allow functional recovery of neural circuits if the toxic insult in the disease can be removed.

5. Conclusions

The data presented in this paper are from a strong body of literature supporting the hypothesis that oligomeric Aβ accumulation in the brain initiates the disease process in AD by impairing structural and functional plasticity of synapses. This underlies behavioral deficits observed in APP mouse models which begin before Aβ deposition in plaques and continue after plaque deposition when the plaques appear to be a reservoir of oligomeric Aβ causing local structural and functional disruptions. Downstream of the initial amyloid insult, tau pathology contributes to synapse and neuronal loss and consequent cognitive decline. AD transgenic mice are characterized by a number of specific cognitive deficits, compatible with AD, which makes them indispensable for testing of novel anti-AD drugs. Finally, the plastic nature of synapses and their clear involvement in both early and late stages of cognitive decline in these AD models highlight the importance of synaptic targets for therapeutic approaches.

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