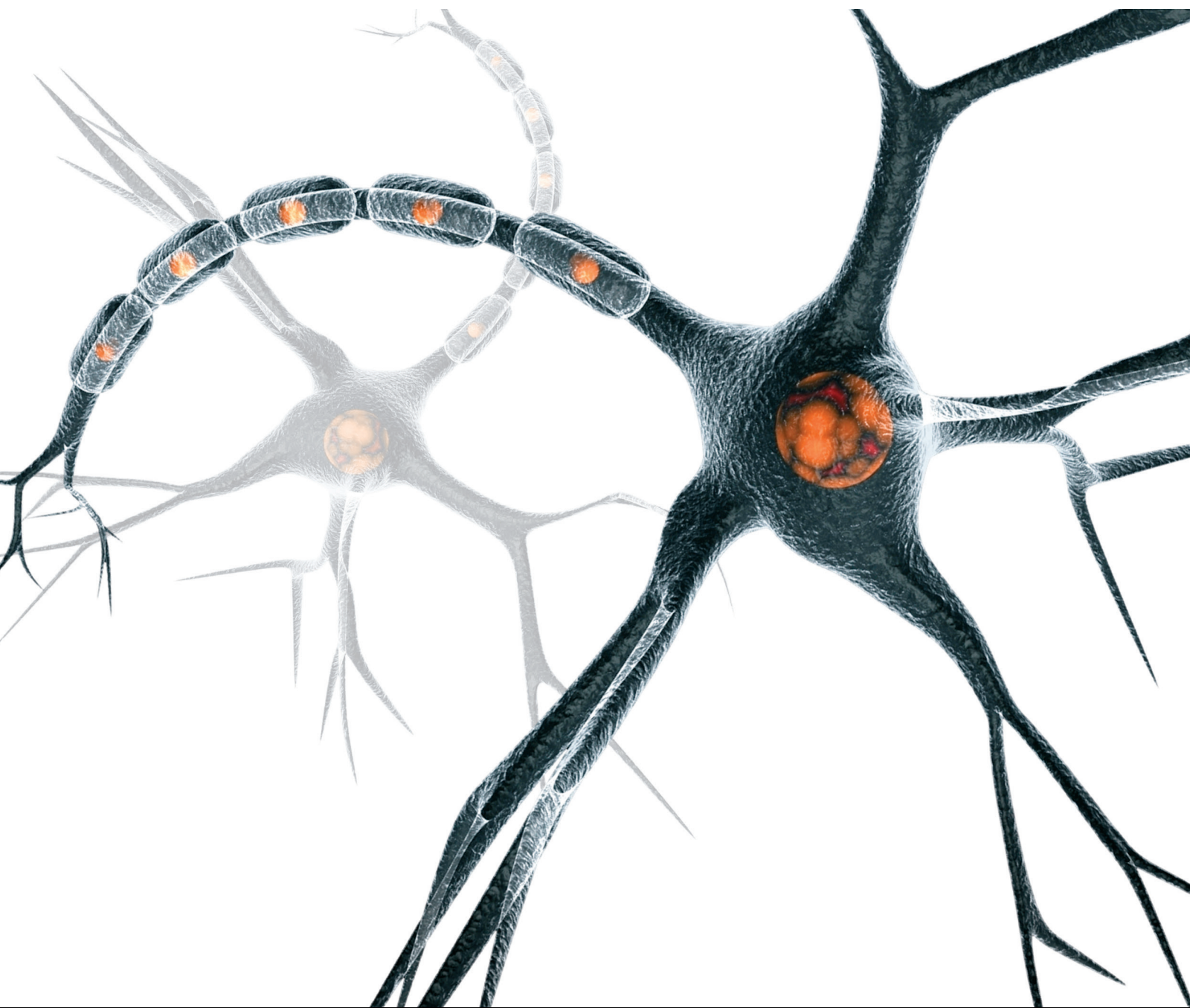
Neural Plasticity

Molecular Mechanisms of Dendritic Spine Development and Plasticity

Guest Editors: Kwok-On Lai, Bryen A. Jordan, Xin-Ming Ma, Deepak P. Srivastava, and Kimberly F. Tolias



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Editorial Board

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Hindawi Publishing Corporation
Neural Plasticity
Volume 2016, Article ID 2078121, 3 pages
http://dx.doi.org/10.1155/2016/2078121

Editorial

Molecular Mechanisms of Dendritic Spine Development and Plasticity

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Received 10 March 2016; Accepted 10 March 2016

Dendritic spines were first described by Santiago Ramón y Cajal more than one hundred years ago when he examined Golgi-stained cerebellar Purkinje cells of birds. Since then, considerable effort has been put towards understanding how these structures are formed and what their functions in the central nervous system are. It is now well established that dendritic spines represent specialized subcellular compartments on the postsynaptic neuron where the majority of excitatory synapses are located. Therefore, the density of dendritic spines is a rough indication of how much excitatory input a particular neuron receives. One notable feature of these structures is the large heterogeneity of their dimensions and shapes. They can exist as short stubby spines, long thin spines, and mushroom-shaped spines. Moreover, they are highly dynamic, such that ongoing spine growth, turnover, and morphological changes occur in both developing and adult brains. Although excitatory synapses can form and function outside of dendritic spines, their location on spine heads likely confers additional properties. For example, the presence of the spine neck is thought to create an isolated biochemical compartment on the spine head, where individual synapses of the postsynaptic neuron can function and be regulated independently of each other. Changes in the dimension and shape of individual spines also allow modulation of synaptic efficacy between specific neuronal partners and therefore contribute to synaptic plasticity and provide the cellular basis of learning and memory. Indeed, many molecular players that regulate dendritic spine morphogenesis also turn out to be essential for learning-related synaptic plasticity and memory formation. In this special issue, reviews and original research papers have been collected to address various questions on dendritic spine biology. These include the process of spine development, the functional differentiation of large and small spines, the relationships between spine changes and learning, the signaling pathways that control spine morphogenesis, and the link between spine abnormalities and brain disorders.

The formation of dendritic spines (spinogenesis) upon the initial axodendritic contact can be achieved through multiple ways as described by three different models (the Sotelo model, the Miller/Peters model, and the filopodial model). Much of the knowledge about spine formation is derived from studies on cortical, hippocampal, and cerebellar neurons. In this issue, R. Kanjhan et al. review the development of dendritic spines in motoneurons, which follows a different sequence than that described for pyramidal and Purkinje neurons in the brain. Using superoxide dismutase (SOD1) mutant mice as example, the article further discusses

increases in spine density and hyperexcitability as potential causes of motoneuron degenerative disorders.

Dendritic spines come in diverse sizes and morphologies. It is generally believed that spines with larger spine heads have greater synaptic strength than smaller spines. However, whether spines with different sizes serve distinct functions is not clear. J. J. W. Paulin et al. investigate the behavior of large and small spines within the same dendritic segments in response to tetraethylammonium chloride (TEA), which induces long-term potentiation in CA1 hippocampal neurons. They find that TEA induces opposite changes on small and large spines. The authors suggest that the immediate shrinkage of large spines is crucial for homeostatic protection whereas the subsequent enlargement of small spines is involved in synaptic strengthening.

Spine enlargement and stabilization require the synthesis of new proteins. Specific subsets of mRNAs are actively transported by specific RNA-binding proteins from the cell body to neuronal dendrites, where local protein synthesis is triggered by synaptic activity and contributes to enhanced synaptic strength in a synapse-specific manner. By pairing electric shock to whisker stimulation, M. Jasinska et al. examine the morphological changes of spines in the barrel cortex triggered by associative learning. They observe that fear conditioning increases the number of spines that contain a spine apparatus, a smooth ER-related membrane structure that may be involved in local protein synthesis and calcium buffering, as well as the number of polyribosomes in ER-free, single-synapse spines. These results support the notion that learning can increase the capacity of local protein synthesis near relevant synapses. M. E. Klein et al. review the recent advances in understanding of the role of RNA-binding proteins in neuronal targeting of mRNAs and synaptic plasticity. The authors also discuss the link between unbalanced local protein synthesis and degradation and various neurodevelopmental disorders. The article raises many open questions on this exciting and challenging field that warrant further investigation.

Although the molecular mechanisms underlying dendritic spine morphogenesis have been extensively studied in the past decade, information on this topic is still limited. Emerging studies have revealed many novel signaling mechanisms in the regulation of dendritic spine morphogenesis. G proteins are highly expressed in the brain, and specific Gα subunits are present in the postsynaptic density. V. T. Ramírez et al. demonstrate that activation of pertussis toxin-sensitive G proteins by the peptide mastoparan promotes the formation of dendritic spines and PSD-95 clusters in hippocampal neurons through a CaMKII-dependent mechanism. Numerous G-protein-coupled receptors (GPCRs) are encoded by the human genome, and one subfamily is the brain-specific angiogenesis inhibitor (BAI) subfamily of adhesion-GPCRs, which contains multiple domains in the N-terminal region that bind to other cells or the extracellular matrix. J. G. Duman et al. review recent findings that support an important role of this family of GPCRs in regulating spine morphogenesis, synaptic plasticity, and memory formation. BAI1 mediates its action through a complex of Par3 and Tiam1. Par3 belongs to the partitioning-defective proteins (Par family) that are well-known cell polarity determinants. The review by H. Zhang summarizes the emerging functions of Par proteins as well as other cell polarity complexes (the septin GTPases and Planar cell polarity proteins such as Frizzled, Dishevelled, and Van Gogh) in dendritic spine development and plasticity. Tiam1, on the other hand, is a guanine nucleotide exchange factor (GEF) for the small GTPase Rac1. The activity of GTPases is determined by GEFs, which catalyze the exchange of GDP to GTP, and GAPs, which terminate GTPase activity by hydrolyzing GTP. K. M. Woolfrey and D. P. Srivastava review how the Rho and Ras families of small GTPases and their upstream regulators act as signaling hubs to regulate dendritic spine morphogenesis in response to diverse extracellular stimuli.

Dendritic spines are supported by the actin cytoskeleton, and the function of many small GTPases on spine morphogenesis is mediated by changes in actin dynamics. Using Fluorescence Recovery After Photobleaching (FRAP), N. Domínguez-Iturza et al. examine the actin dynamics of individual spines in both cultured neurons and organotypic slices. The authors find that individual spines display specific actin dynamics independent of their positions and therefore consistent with the notion that the structure of each spine can be independently regulated. However, actin mobility within spine heads depends on contacts with astrocytes as well as spine size. Actin binds to many different proteins. One of them is the collapsing response mediator protein 2 (CRMP2), which also binds to tubulin and regulates microtubule assembly. CRMP2 can be phosphorylated by the proline-directed serine/threonine kinase Cdk5, an important kinase that regulates synapse development and function. X. Jin et al. investigate the significance of this phosphorylation event by generating CRMP2 knock-in mice, in which the Cdk5 phosphorylation site Ser-522 is substituted by Ala and therefore becomes phosphorylation-deficient. The authors observe spine loss in CA1 hippocampal neurons of the knock-in mice and suggest that it will be important to further delineate whether the CRMP2 phosphorylation mediates its effect on actin or tubulin.

Dendritic spine abnormalities have been frequently associated with various neurodevelopmental disorders such as Autism Spectrum Disorder (ASD) and intellectual disabilities. Using the heparin sulfate proteoglycan syndecan-2 and the neuron-specific F-actin regulator cortactin-binding protein 2 (CTTNBP2) as examples, H.-T. Hu et al. review how defects in neuron-specific signal transduction pathways underlying dendritic spine morphogenesis and synapse formation might contribute to the pathogenesis of these disorders. Altered spine density and morphology are also linked to psychiatric disorders such as depression. H. Qiao et al. review the three animal models of chronic stress; two of them have been widely used as an animal model of depression for recapitulating depression-like behaviors in rodents and studying the mechanisms underlying depression. Chronic stress generally causes dendritic atrophy and spine loss in the hippocampus and prefrontal cortex and yet an increase in spine density in the amygdala and nucleus accumbens. These alterations of dendritic spines are often accompanied by depression-like behaviors. The putative mechanisms that

underlie the stress-induced changes in synapse structure are also discussed.

By highlighting the molecular basis of dendritic spine morphogenesis and plasticity, we hope this special issue will provide a better understanding of learning and memory, as well as other higher-order cognitive functions of the adult brain. Insights into the mechanisms behind altered spine morphogenesis in various neurodevelopmental and psychiatric disorders may further lead to the design of potential therapeutic strategies.

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Hindawi Publishing Corporation
Neural Plasticity
Volume 2016, Article ID 3025948, 12 pages
http://dx.doi.org/10.1155/2016/3025948

Review Article

Control of Dendritic Spine Morphological and Functional Plasticity by Small GTPases

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Received 6 July 2015; Revised 6 January 2016; Accepted 19 January 2016

Academic Editor: Lin Xu

Structural plasticity of excitatory synapses is a vital component of neuronal development, synaptic plasticity, and behaviour. Abnormal development or regulation of excitatory synapses has also been strongly implicated in many neurodevelopmental, psychiatric, and neurodegenerative disorders. In the mammalian forebrain, the majority of excitatory synapses are located on dendritic spines, specialized dendritic protrusions that are enriched in actin. Research over recent years has begun to unravel the complexities involved in the regulation of dendritic spine structure. The small GTPase family of proteins have emerged as key regulators of structural plasticity, linking extracellular signals with the modulation of dendritic spines, which potentially underlies their ability to influence cognition. Here we review a number of studies that examine how small GTPases are activated and regulated in neurons and furthermore how they can impact actin dynamics, and thus dendritic spine morphology. Elucidating this signalling process is critical for furthering our understanding of the basic mechanisms by which information is encoded in neural circuits but may also provide insight into novel targets for the development of effective therapies to treat cognitive dysfunction seen in a range of neurological disorders.

1. Introduction

Brain function is an emergent property of the connections between neurons. Proper wiring of the brain during development is critical for cognition and memory [1–3], while, conversely, abnormal wiring due to neurological disorder, disease, or brain injury results in dysfunction [4–6]. Understanding how neural circuitry underlies information storage and processing is a fundamental challenge facing modern neuroscience [1, 3]. Though modest inroads into deciphering brain wiring have been made, very little is known about how this wiring contributes to its function. A primary obstacle to progress is the staggering complexity of neural circuits; in mammalian brains, trillions of synapses impinge on billions of neurons. One approach to managing this complexity is to limit focus to synapses of a single neurotransmitter type. Glutamatergic synapses are highly plastic, play essential roles in learning, memory, as well as cognition, and comprise the majority of the connections between pyramidal neurons in the forebrain [7–9]. A defining characteristic of these synapses is that they occur at specialized postsynaptic compartments known as dendritic spines (Figures 1(a)–1(c)). These micron-scale, actin-rich structures garnish the dendritic arbour and typically consist of a spine neck and a spine head [10, 11]. It is within the spine head that the protein-rich postsynaptic density (PSD) is found (Figure 1(c)). Embedded in the PSD are *N*-Methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type glutamate receptors which mediate excitatory synaptic transmission (Figure 1(c)) [10, 12]. Dendritic spines exhibit both transient and enduring lifetimes, persisting from minutes to years *in vivo* [7, 13]. A myriad of dendritic spine morphologies are observed in the brain and the notion that spine structure

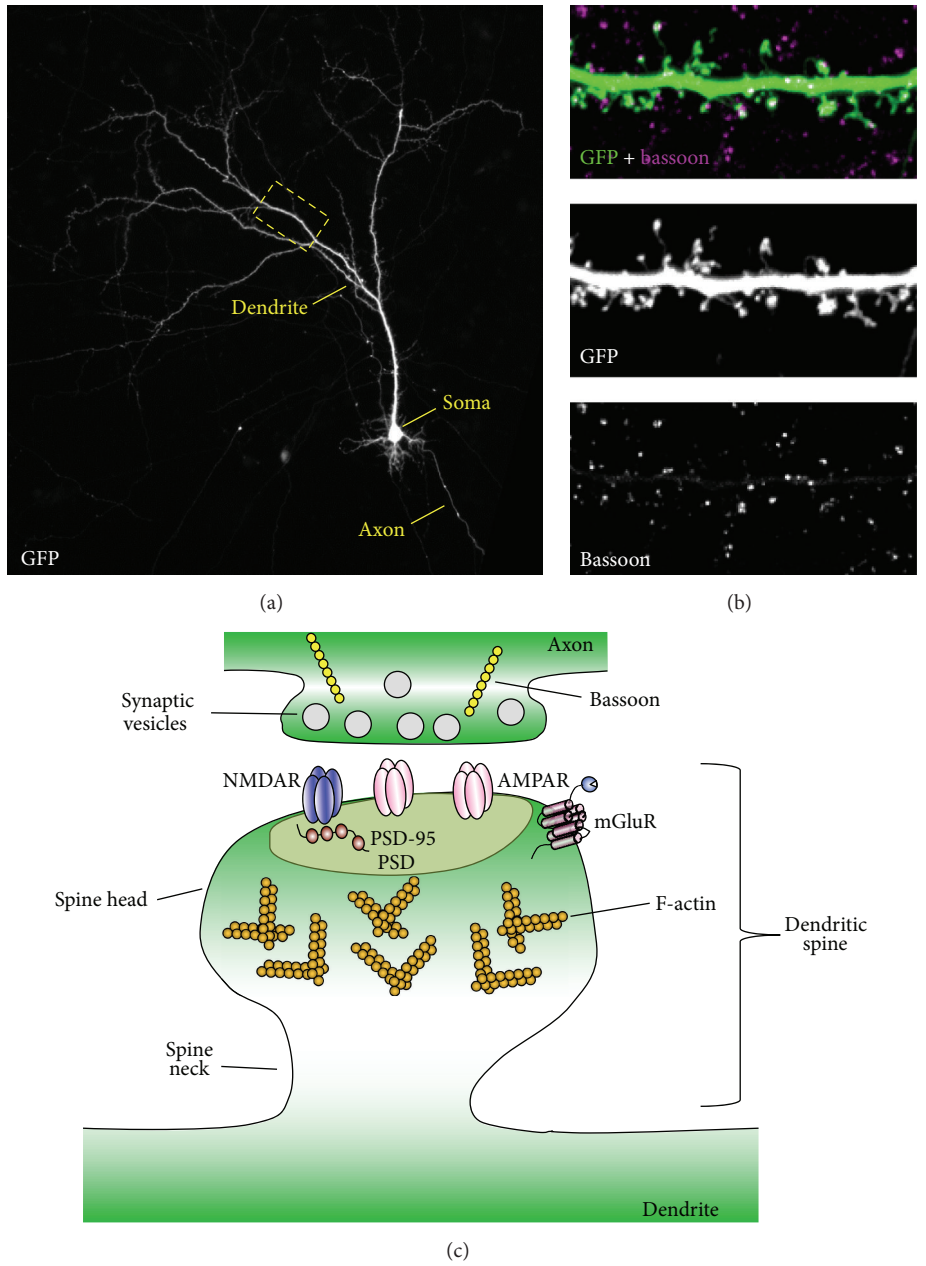

Figure 1: Dendritic spines are small protrusions along dendrites that contain postsynaptic densities. (a) Example of a cortical neuron expressing green fluorescent protein (GFP). The main dendrite is branched and has dendritic spines along its length. Dashed box indicates area magnified in (b). The neuron's axon is much thinner than the dendrite and has no spines. (b) Magnified region of dendrite of a cortical neuron expressing GFP and stained for the presynaptic protein bassoon. Dendritic spines can clearly be seen protruding from the dendrite, and many spines colocalize with bassoon, suggesting the formation of synaptic connections. In this colour scheme, colocalization is indicated by white. (c) Schematic of a mature dendritic spine making contact with an axon; note the enrichment of glutamate receptors, the scaffold protein PSD-95, and F-actin within the spine head and postsynaptic density (PSD).

is highly correlated with important synaptic properties has become a recurrent theme over the last decade [14, 15]. For example, large dendritic spines are likely to feature large PSDs and make strong connections, while small dendritic spines are indicative of weak connections and may be highly plastic [16]. Accordingly, larger spines tend to persist for long periods of time, whereas smaller, thinner spines are more transient [15, 17]. However, recent data suggests that these phenomena may be different between the cortex and hippocampus, with spines on CA1 hippocampal neurons demonstrating a more rapid turnover as compared to those found in cortical regions [18]. Nevertheless, many reports demonstrate that dendritic spines are not static structures and can rapidly reorganize in response to diverse stimuli including experience-dependent learning [19–21], as well as neuromodulatory and even hormonal signals [22–25]. One

key sequela of this structural dynamism is the ability to sample the surrounding neuropil for incident axons [19, 26, 27].

It is widely recognized that dendritic spines are an integral component in circuit formation, but the precise nature of their contribution is still a topic of inquiry and debate. Dendritic spines exhibit a wide spectrum of structural reorganization, from formation and elimination, to more subtle changes in size and shape. These structures are estimated to contain over 1000 different proteins [28], including scaffolds, receptors, adhesion proteins, signalling proteins, F-actin, and cytoskeletal proteins (Figures 2(a) and 2(b)). Current theories postulate that dendritic spines provide a chemical and electrical signalling domain that is partially discrete from their parent dendrite, thus enhancing the computational capacity of the neuron [3], and that they are sufficiently enriched with the molecular components necessary for structural and function modifications [29]. Critically, the development, refinement, and maintenance of telencephalic neural circuits are essential for sensory perception, motor control, cognition, and memory [1, 8, 30, 31]. Importantly, a better understanding of circuit dynamics can provide a bridge between plasticity phenomena observed at the synapse and animal behaviour [8, 9, 18, 19]. Thus it is essential to examine mechanisms that rewire the brain and the current review is dedicated to this purpose. In the past decade, enormous progress has been made in dissecting the molecular mechanisms that contribute to the structural plasticity of dendritic spines [10, 12, 32, 33]. A key molecular determinant of dendritic spine plasticity is the actin cytoskeleton and its regulators. Here we review recent work that has begun to unravel the complex manner in which the family of small GTPases proteins, their regulators, and effectors modulate the actin cytoskeleton to control dendritic spine morphology in support of synaptic function.

2. Actin: A Key Determinant of Dendritic Spine Morphology

The morphological malleability of dendrite spines has been shown to be due to a dynamic actin cytoskeleton [34, 35]. Spines are rich repositories of filamentous and monomeric actin and achieve both stability and dynamism through a turnover process known as treadmilling, where monomers are simultaneously added to the barbed end (at the spine periphery) and removed from the pointed end of the filament (near the spine's core) [36, 37]. A variety of proteins exhibit control over the actin cytoskeleton and many of these proteins are potent spine morphogens and synaptic modulators [23, 38–42].

Tight control of the actin cytoskeleton is crucial to proper synaptic function. Indeed, actin treadmilling controls the distribution of proteins in the postsynaptic density, including AMPA receptors, as revealed by work employing fluorescence recovery after photobleaching [43]. Thus, understanding the complex signalling pathways impinging on actin filaments is critical for revealing mechanisms underlying normal and pathological synaptic transmission. To this end, much research effort has focused on identifying and characterizing actin regulatory proteins. By considering the positioning of these proteins in signalling cascades relative to the extracellular space and the actin cytoskeleton, they can be organized into hierarchical functional groups including actin binding proteins, small GTPases, and small GTPase regulators and effectors (Figure 2(b)) [32, 44].

3. Small GTPases: Morphological Signalling Hubs in Dendritic Spines

The super family of small GTPases is classified into 5 subfamilies: the Ras, Rho, Rab, Sar1/ARF, and Ran families. Members of this superfamily regulate diverse cellular functions and are often referred to as molecular switches as they exist in binary "on" and "off" states when bound to GTP and GDP, respectively [45, 46]. The present review will be limited to members of Rho and Ras families as these proteins have been most directly linked with actin remodelling. Further, Rho- and Ras-mediated signalling pathways exhibit substantial cross talk that has important implications for spine morphological and functional plasticity. While our understanding of small GTPase control of the actin cytoskeleton has been greatly enhanced by work in nonneuronal cells, the dendritic spine represents a unique microdomain, with distinct functional requirements. As such, we will focus on studies conducted in dendritic spines unless otherwise noted.

Extensive literature links the Rho subfamily to regulation of synaptic actin structure and dynamics [47]. Perhaps best studied among these family members are Rac1 and RhoA, which have potent and opposite effects on the structure of dendritic spines [48]. Overexpression of dominant negative Rac1 leads to reduced spine density in hippocampal slices and dissociated cultures [49, 50], while overexpression of a constitutively active form or RhoA leads to spine loss [51]. It is generally accepted that Rac1 activation stimulates F-actin polymerization and stabilizes dendritic spines through the activation of downstream effectors p21-activated kinase (PAK), LIM-kinase-I (LIMK-I), and the actin binding protein cofilin [52, 53]. Conversely, RhoA activation stimulates F-actin polymerization through its downstream protein kinase ROCK, which in turn directly regulates LIMK-1 phosphorylation in nonneuronal and neuronal cells [54, 55]. Rho GTPases are rapidly and locally activated in spine heads following potentiating stimuli as revealed by two-photon fluorescence lifetime imaging of FRET-based probes [55]. Interestingly, Cdc42, a Rac-related Rho GTPase, and RhoA exhibited differential spatial activity, reflecting their unique contributions to spine morphology regulation; blockade of the RhoA signalling cascade inhibited initial spine growth while Cdc42 pathway inhibition prevented sustained spine enlargement. Reinforcing the importance of Rho GTPases in forebrain plasticity is a recent study demonstrating active Rac1-induced spine proliferation in cortical pyramidal neurons as well as enhanced plasticity of visual circuits in monocularly deprived animals [56, 57]. In concordance with this idea, disruption of signalling through Rho/Rac pathways is frequently associated with intellectual disability (ID), a condition characterized by abnormalities in dendritic spine morphology [58–60].

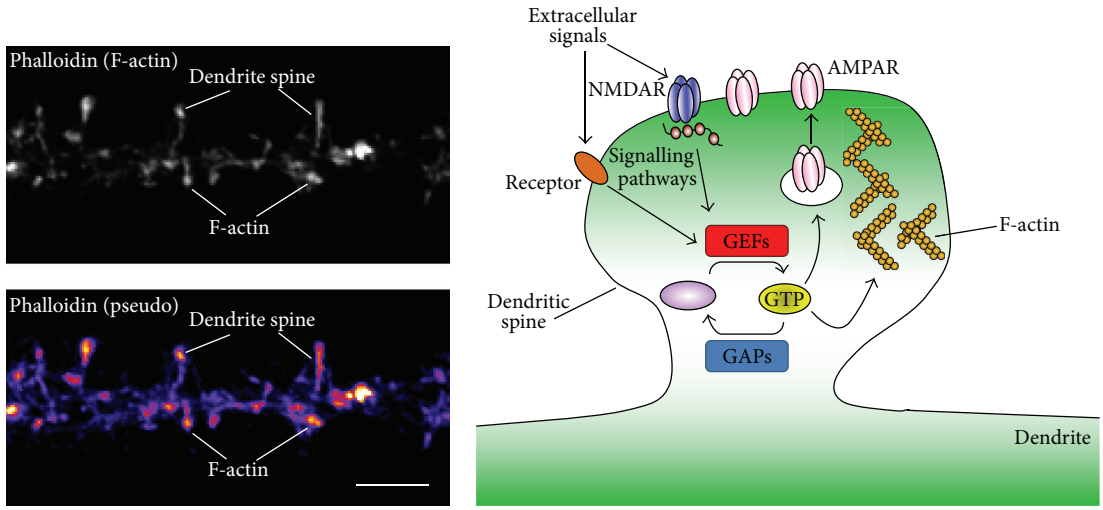

Figure 2: Dendritic spines, small GTPases, and the cytoskeleton. (a) Example of a cortical neuron immunostained with phalloidin, a marker of endogenous F-actin. Immunofluorescence reveals an enrichment of actin in dendrites and dendritic spines. (b) Schematic drawing of how extracellular signals can act via specific receptors and act via small GTPases to regulate actin dynamics and/or receptor trafficking. The dynamic actin cytoskeleton confers much of the structure of the dendritic spines, and alterations in synaptic expression of glutamate receptors (e.g., AMPA receptors) are thought to play a major role in modulating synaptic function.

Though most investigations of neuronal structure have focused on the Rho GTPase subfamily, other GTPases have been shown to regulate dendritic spine morphology. Members of the Ras subfamily of small GTPases have also been found to regulate dendritic spine structure and dynamics [61]. One of the first studies to link Ras with structural remodelling of dendritic spines was from a mouse model where a constitutive active form of H-Ras was overexpressed [62]. These mice displayed increased neuronal complexity, which was mirrored in subsequent studies which also revealed abnormal spine formation and connectivity [63, 64]. Consistent with a role in mediating dendritic spine plasticity, it has also been shown that Ras is activated concurrently with spine enlargement induced by uncaging of glutamate in hippocampal neurons [65]. Interestingly, the spatiotemporal dynamics of Ras activation was again different to that of the Rho GTPases, RhoA, and Cdc42, reinforcing the idea that both the temporal activation and the localization of these molecules are critical in determining their impact on cellular function [55, 65, 66]. Prior work in nonneuronal cells has also linked Rap, a member of the Ras subfamily, to cytoskeletal dynamics [67]. In neurons, activation of Rap1 by NMDA receptors in cultured cortical neurons results in a decrease in spine size [41]. Another powerful regulator of small GTPase activity in neuronal cell is the estrogen hormone, 17β-estradiol [68–70]. Interestingly, when mature cortical neurons are acutely exposed to 17β-estradiol, a rapid increase in active Rap1 is seen concurrent with an increase in spine density [25]. Critically, overexpression of RapGAP, a protein that inhibits Rap activation, blocked the effect of 17β-estradiol on spine density [25]. In contrast, overexpression of constitutively active Rap2 causes a loss of dendritic spine density and an increase in the number of filopodia-like protrusions in culture hippocampal neurons [71]. Consistent with these observations *in vitro*, mice that express a constitutively active Rap2 display fewer dendritic spines and impaired learning [72]. Collectively, these data demonstrate that Rho and Ras family GTPases have potent regulatory effects on dendritic spines which can impact cognitive function.

4. Small GTPase Regulators

GTPases are themselves tightly regulated by two classes of proteins: guanine nucleotide exchange factors (GEFs) which facilitate the binding of GTP by the GTPase and GTPase activating proteins (GAPs) which catalyze the hydrolysis of GTP to GDP. These proteins convey diverse signals from the extracellular space to GTPases and differ in their cellular expression patterns and intracellular distributions. Each GTPase can be regulated by a variety of different GEFs and GAPs, allowing for both signalling diversity and spatial specificity. Through catalyzing the exchange of the GTPase bound GDP to GTP, GEFs serve to activate GTPases. By responding to extracellular signals including neuromodulators and neuronal activity, GEFs can achieve bidirectional control over spine morphology and synaptic strength by acting through their target GTPases.

As RhoA is associated with spine shrinkage and destabilization, GEFs that activate this GTPase have similar effects on dendritic spine morphology. For example, GEF-H1 has been shown to colocalize with the AMPA receptor complex and negatively regulate spine density and length through a RhoA signalling cascade [73]. Similarly, activation of the Eph receptor A4 (EphA4) results in the retraction of dendritic spines, an effect that is dependent on activation of RhoA via its GEF, ephexin1 [74]. Another GEF involved

in the destabilization and shrinkage of spines is Epac2. This multidomain Rap1 GEF is activated by cAMP and leads to reduced spine AMPA receptor content, depressed excitatory transmission, and spine destabilization as demonstrated by live imaging studies. Conversely, inhibition of Epac2 leads to spine enlargement and stabilization [23]. Interestingly, rare *de novo* mutations of the *Epac2* gene have been found to be associated with individuals with autism spectrum disorders (ASDs) [75]. The resulting mutant Epac2 proteins displayed altered abilities to activate Rap and when expressed in primary cortical neurons, they resulted in a range of abnormal dendritic spine morphologies [23]. Analysis of *Epac2* knockout mice has further revealed deficits in social and communicative behaviours, whereas memory and leaning behaviours are seemingly unaffected [76]. Interestingly, these mice also display reduced dendritic spine turnover *in vivo*, consistent with what has been shown previously *in vitro* [23, 76]. However, it is not clear how alterations in dendritic spine plasticity are linked with altered social and communicative behaviours. More recently, using *in utero* electroporation to express an RNAi construct against Epac2 in a subset of layer 2/3 cortical neurons, a role for Epac2 in maintenance of basal, but not apical, dendrites has been revealed [77]. Interestingly, regulation of basal dendrite formation by Epac2 requires Ras signalling, as a ASD-associated mutant Epac2 protein, which has a reduced ability to bind active Ras, also induces deficits in basal dendrite maintenance [77]. This demonstrates that there can be a level of cross talk between small GTPase systems. Consistent with this, it has recently been shown that the polo-like kinase 2 (Plk2) regulates both Ras and Rap activity through directly influencing the activity regulatory proteins of each small GTPase in response to homeostatic plasticity [78]. These studies demonstrate that the synchronized regulation of both Ras and Rap small GTPases via their GEFs and GAPs plays an important role in homeostatic plasticity and in the maintenance of neuronal morphology [77, 78].

The regulation of Rac by its GEFs has also been well studied. One such GEF is kalirin-7, which is especially unique due to the fact that it is the only known Rac1 GEF expressed in the cortex of adult mice [32]. Overexpression of this kalirin-7 in cortical cultures leads to an increase in spine head area and density. Concomitantly, knockdown of kalirin-7 through an RNAi approach reduces the spine area and density [42]. Interestingly, mice in which the *kalirin* gene has been deleted exhibit many phenotypes reminiscent of schizophrenia including deficits in working memory as well as reduced dendritic spine density in the cortex [79]. In the hippocampus, the role of kalirin-7 is obscured due to the presence of two other Rac1 GTPases, Tiam1 and β-PIX [32, 52, 80]. Tiam1 is regulated by NMDA receptor activation and has also been implicated in EphB receptor-dependent dendritic spine development [80, 81]. Likewise, the Rac1 GEF β-PIX, a downstream target of NMDA receptors, has been shown to be regulated by CaM kinase kinase and CaM kinase I [52].

Select GAPs have received research attention due to their putative roles in ID. Loss of the Rho-GAP oligophrenin-1, a gene implicated in ID, disrupts activity-dependent synapse and spine maturation [82]. Another such gene is the Ras-GAP SYNGAP1, which can regulate spine morphology through its target Ras as well as downstream signalling to Rac and cofilin [83]. This study illustrates that small GTPase signalling is often complex and nonlinear and may feature cross talk between pathways. Mutations in *SYNGAP1* have also been associated with both ID and ASD [84]. Interestingly, an animal model of human *SYNGAP1* haploinsufficiency displayed accelerated dendritic spine maturation resulting in disrupted excitatory/inhibitory balance in neural networks [85]. Moreover, these mice also developed persistent behavioural abnormalities. Critically, these effects were most prominent when SYNGAP1 was disrupted during early development and minimal when disrupted in adulthood [85]. More recently, SYNGAP1 has been shown to be phosphorylated by CaMKII, resulting in the trafficking of this protein away from synapses in response to LTP stimulation. Importantly, removal of this GAP protein from synapses is thought to be required for LTP-dependent Ras activation and subsequent AMPA receptor insertion and spine enlargement [86].

A number of extracellular signals are known to exert profound influences over dendritic spine morphology, through the activation of small GTPase pathways. The predominant receptor in regulating dendritic spine plasticity in response to synaptic activity is the NMDA receptor. Following activation of NMDA receptors, dendritic spines undergo a transient increase in calcium concentration [87, 88]. This rise in calcium activates the calcium-sensing calmodulin (CaM): calcium-bound CaM subsequently activates the CaMK family of serine/threonine kinases including CaMKI, CaMKII, and CaMKIV [89]. These kinases go on to phosphorylate a variety of targets involved in spine structural plasticity, including the Rac-GEF kalirin-7, as well as other signalling and scaffolding proteins involved in plasticity [42, 90]. Aside from glutamate, other neurotransmitters have been shown to modulate dendritic spine plasticity. Activation of 5-HT2A receptors in pyramidal neurons increased spine size through a kalirin-7-Rac1-PAK-dependent mechanism [22]. This study is of particular importance as it provides a direct link between serotonergic signalling and dendritic spine morphogenesis, both implicated in schizophrenia. Another important neurotransmitter implicated in the modulation of dendritic spines and small GTPase function is dopamine [91]. For example, treatment of rats with 6-hydroxydopamine, a neurotoxin that selectively ablates dopaminergic and noradrenergic neurons, resulted in a decrease in dendritic spine density in the prelimbic cortex 3 weeks after toxin administration [92]. Intriguingly, cognitive deficits in schizophrenia have been linked with dopamine dysfunction [93, 94] and reduced dendritic spine density has been observed in postmortem tissue taken from schizophrenic patients [95–97]. Results from Solis et al. suggest that there may indeed be a pathological link between dopamine dysfunction and loss of dendritic spine density. A finding consistent with this idea is that treatment with the atypical antipsychotic olanzapine, but not the typical antipsychotic haloperidol, was able to rescue 6-hydroxydopamine-induced spine loss in the rat prefrontal cortex [98]. At the molecular level, activation of the D1/D5

receptors with the selective agonist SKF-38393 leads to spine shrinkage through activation of the Rap GEF Epac2 [23].

Less conventional neuromodulators have also been implicated in the regulation of dendritic spines. Classically defined as a hormone, estrogens have recently come into the spotlight as an important modulator of dendritic spine plasticity [99]. Treatment of primary cortical cultures with 17β-estradiol increased spine density while decreasing the AMPA receptor content of spines. These "silent synapses" were potentiated by activation of NMDA receptors, reminiscent of activity-dependent maturation of silent synapses during development [25]. These effects were mediated by the Rap/AF-6(afadin)/ERK1/2 signalling pathways, as inhibiting or interfering with the actions of these proteins was sufficient to block 17β-estradiol's effects on spines [25]. Additionally, recent studies have demonstrated that acute treatment of rat cortical cultures with 17β-estradiol leads to phosphorylation of WAVE1 and its subsequent targeting to spines, resulting in the polymerization of actin. This is thought to be required for the formation of immature dendritic protrusions in young cortical neurons [100]. Similar findings have been reported in hippocampal cultured neurons. Here, chronic treatment of hippocampal cultures with 17β-estradiol resulted in an increased number of synapses and increased localization of kalirin-7 to dendritic spines [101]. However, these actions of 17β-estradiol seem to be mediated through the estrogen receptor beta (ERβ) as activation of ERβ but not ERα agonists is able to recapitulate these effects [101–104].

5. Small GTPase Effectors and Actin Binding Proteins

Downstream of small GTPases is a series of effector proteins which convey signals to direct regulators of the actin cytoskeleton. A particularly well-described family of effectors of the Rho GTPases Rac1 and Cdc42 are the p21-activated kinases (PAKs) [105] and the Rho kinases (ROCK) [106]. The PAKs are critical for spine morphogenesis and synaptic structure, particularly in the cortex [107]. More recently, a series of studies has explored the consequences of PAK and ROCK knockout in the forebrain. Deletion of PAK1 or ROCK-2 results in the loss of F-actin from spines [108, 109]. Further, both knockout animals demonstrated deficits in hippocampal LTP, highlighting the importance of these Rho kinases for synaptic plasticity. Intriguingly, codeletion of PAK1 and PAK3 resulted in a more severe structural and functional phenotype; the PAK1/3 knockouts showed impaired bidirectional plasticity in the hippocampus, deficits in learning and memory, and gross structural abnormalities in the forebrain [110]. Shared features of these Rho kinase knockout animals include disruption of the kinase cascade downstream of the Rho GTPases, a release of cofilin from inhibition, and a subsequent loss of F-actin from dendritic spines.

More insight into the effects of PAK and ROCK family members on the actin cytoskeleton is provided by work examining LIM-kinase (LIMK). Active Pak1 can phosphorylate LIMK-1 which in turn inhibits cofilin activity [111]. As a result, genetic ablation of LIMK-1 results in elevated cofilin activity, aberrant spine morphology, and enhanced LTP [53]. Intriguingly, recent work has identified a new mechanism of regulation for LIMK-1 via lipid modification [24]. N-terminal palmitoylation of LIMK-1 targets the kinase to dendritic spines and is necessary for activity-dependent spine growth. Palmitoylation is emerging as a critical modulator of spiny synapse function [112]; small GTPases themselves are targeted to various microdomains through dynamic palmitoylation [113–115], though the implications of this signalling have yet to be explored thoroughly in neurons.

As their name suggests, actin binding proteins directly influence actin dynamics through nucleating, stabilizing, or severing actin filaments. Members of the Wiskott-Aldrich syndrome protein (WASP) family bind both monomeric and filamentous actin [116] and are relieved from autoinhibition by Rho GTPases [117]. N-WASP, a brain enriched WASP, appears to be critical for spine and excitatory synapse formation [40]. Small GTPases also exert control over a similar WASP-family verprolin-homologous protein (WAVE) family. These proteins play a role in spine maintenance [118] and formation [119]; deficient WAVE1 expression is accompanied by spatial memory deficits in mice [120].

The Arp2/Arp3 complex is a well-studied actin nucleator and facilitator of actin branching [121]. The Arp2/Arp3 complex is downstream of Rho family GTPases, WASP, and WAVE proteins [122] and is likely to be instrumental in dendritic spine remodelling during spine growth [123]. Inhibition of the Arp2/Arp3 complex by protein kinase C binding protein (PICK1) is necessary for spine shrinkage during LTD [124]. More recently, PICK1 has been shown to signal downstream of AMPARs to inactivate Cdc42 [125]. As mentioned above, cofilin is another critical determinant of actin skeletal dynamics and competes with the Arp2/Arp3 complex by severing and debranching actin filaments [126]. Though prolonged cofilin activation promotes a reduction in spine size [127], it appears that a transient burst of cofilin activity is required for spine growth during chemically induced LTP [128]. A recent review of small GTPase control of the actin cytoskeleton covers these pathways in greater detail [44].

Among the list of Rap effectors are a number of actin cytoskeleton regulators. Rap1 binds directly to afadin, also known as AF-6 [129] which is a multidomain scaffolding protein instrumental in cell-cell adhesion [130]. Indeed, active Rap was responsible for the subcellular targeting of afadin in neurons under basal and after NMDA receptor activation [41, 131]. Intriguingly, following activation of NMDA receptors, afadin translocates to both synapses and the nucleus in a time-dependent manner. At synapses, afadin is required for activity-dependent and Rap-dependent spine modifications [41], whereas in the nucleus, afadin is required for the time-dependent phosphorylation of H3 histones, suggesting a potential role in regulating activity-dependent gene transcription [131]. Afadin also directly interacts with the actin-polymerizing protein profilin [129] and with the adhesion protein, N-cadherin [132], and the AMPA receptor subunit, GluA2 [133]. Consistent with these interactions, afadin is required for linking N-cadherin with the kalirin-7, therefore allowing regulation of Rac activation and linking

N-cadherin with the dynamic modulation of dendritic spine morphology [132]. Moreover, knockdown of afadin using an RNAi approach results in a loss of dendritic architecture, dendritic spine density, and AMPA receptor mediated transmission [133]. Rap has also been shown to interact with and activate the Rac-GEFs Vav2 and Tiam1 [134], providing another example of small GTPase pathway cross talk.

Thus, a stereotyped spine-morphogenic signalling cascade begins with an extracellular signal that is conveyed to GEFs or GAPs that control small GTPase activity, which in turn influences actin binding proteins through small GTPase effectors. It is now emerging that, in addition to activity-dependent signalling via NMDA receptors, other extracellular signals, including neuromodulators [22, 23] and neurosteroids, may act via similar pathways.

6. Conclusions

Understanding how neurons encode information is a fundamental challenge in determining how we store and retrieve information about our surrounds, allowing us to adapt at a behavioural level. Growing evidence indicates that a key cellular correlate of information encoding is the regulation of dendritic spines and thus excitatory synaptic connections [1, 3]. In this review, we have presented recent evidence that places small GTPase proteins as an important intermediate between extracellular signals and the actin cytoskeleton, allowing for the regulation of synapse structure and function. Important advances have been made in our understanding of the molecules that exert a tight regulation of small GTPase function in neurons [32, 61], and it is also emerging that these molecules have unique spatiotemporal dynamics that are critical to their cellular functions [55, 65, 66]. Our current understanding suggests that small GTPases can act independently, via their effectors, directly regulating the actin cytoskeleton, to exert effects of dendritic spine structure and numbers, as well as on synaptic function. However, several studies have now demonstrated that multiple small GTPases can act in cooperation to bring about changes in dendritic spine, or on the maintenance of overall neuronal morphology [77, 78]. Moreover, it is also emerging that a wide range of extracellular signals also signal via small GTPases to exert morphogenic actions [22, 25, 42, 47, 50, 65, 74, 80, 81]. Many of these extracellular signals can activate the same small GTPases, suggesting that within a single neuron multiple factors can modulate the activity of a single subfamily of small GTPase. Elucidating how neurons integrate multiple signals and how they in turn summate impacting the function of the cell and ultimately affect cognition is fast emerging as another challenge. It is likely that gaining a greater understanding of the spatiotemporal dynamics of small GTPase signalling will provide an insight into how neurons handle this amount of information. In addition, further determining the complex manner in which regulators of small GTPase signalling interact and determining the nonlinear manner in which multiple pathways are activated by the same signals will provide a more comprehensive understanding of how multiple factors regulate spine plasticity.

It is also of note that multiple neurodevelopmental, psychiatric, and neurodegenerative disorders have been strongly associated with disruptions of neural circuits [6, 135]. Indeed, numerous neuropathological postmortem studies have strongly linked abnormal spine morphology with the pathogenesis of a number of neuropsychiatric, neurodevelopmental, and neurodegenerative disorders [135, 136], such as ID [137], fragile-X [138], Down's syndrome [139], autism spectrum disorders (ASDs) [140–142], schizophrenia [96, 143], depression [144], and Alzheimer's disease [145, 146]. It is currently posited that dendritic spine dysmorphogenesis can lead to defective or excessive synapse function and connectivity, resulting in disruptions in neural circuitry. This topic has recently been reviewed in depth [2, 6, 135]. Dysregulation of the complex mechanisms that control dendritic spine structure and function may contribute to these synaptic irregularities. Understanding the cellular mechanisms by which dendritic spine morphogenesis occurs will expand not only our knowledge of normal brain function, but that of abnormal brain function as well. Though a greater understanding of the cellular mechanisms that underpin cortical plasticity will be required, harnessing structural plasticity may offer a powerful future therapeutic avenue for neuropathologies.

Conflict of Interests

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

Acknowledgments

Research described in the text has been funded by grants from the Medical Research Council (MRC), UK, Royal Society, UK, Brain and Behaviour Foundation (formally NARSAD), Psychiatric Research Trust to Deepak P. Srivastava, and American Heart Association (AHA) to Deepak P. Srivastava and Kevin M. Woolfrey.

References

[1] D. B. Chklovskii, B. W. Mel, and K. Svoboda, "Cortical rewiring and information storage," *Nature*, vol. 431, no. 7010, pp. 782–788, 2004.

[2] G. Z. Tau and B. S. Peterson, "Normal development of brain circuits," *Neuropsychopharmacology*, vol. 35, no. 1, pp. 147–168, 2010.

[3] R. Yuste, "Dendritic spines and distributed circuits," *Neuron*, vol. 71, no. 5, pp. 772–781, 2011.

[4] Y. Bernardinelli, I. Nikonenko, and D. Muller, "Structural plasticity: mechanisms and contribution to developmental psychiatric disorders," *Frontiers in Neuroanatomy*, vol. 8, article 123, 2014.

[5] P. Penzes, A. Buonanno, M. Passafaro, C. Sala, and R. A. Sweet, "Developmental vulnerability of synapses and circuits associated with neuropsychiatric disorders," *Journal of Neurochemistry*, vol. 126, no. 2, pp. 165–182, 2013.

[6] M. Van Spronsen and C. C. Hoogenraad, "Synapse pathology in psychiatric and neurologic disease," *Current Neurology and Neuroscience Reports*, vol. 10, no. 3, pp. 207–214, 2010.

[7] D. H. Bhatt, S. Zhang, and W.-B. Gan, "Dendritic spine dynamics," *Annual Review of Physiology*, vol. 71, pp. 261–282, 2009.

[8] M. Fu and Y. Zuo, "Experience-dependent structural plasticity in the cortex," *Trends in Neurosciences*, vol. 34, no. 4, pp. 177–187, 2011.

[9] A. Holtmaat and K. Svoboda, "Experience-dependent structural synaptic plasticity in the mammalian brain," *Nature Reviews Neuroscience*, vol. 10, no. 9, pp. 647–658, 2009.

[10] K. M. Harris and R. J. Weinberg, "Ultrastructure of synapses in the mammalian brain," *Cold Spring Harbor Perspectives in Biology*, vol. 4, no. 5, 2012.

[11] J. Tønnesen, G. Katona, B. Rózsa, and U. V. Nägerl, "Spine neck plasticity regulates compartmentalization of synapses," *Nature Neuroscience*, vol. 17, no. 5, pp. 678–685, 2014.

[12] T. Tada and M. Sheng, "Molecular mechanisms of dendritic spine morphogenesis," *Current Opinion in Neurobiology*, vol. 16, no. 1, pp. 95–101, 2006.

[13] L. Gu, S. Kleiber, L. Schmid et al., "Long-term in vivo imaging of dendritic spines in the hippocampus reveals structural plasticity," *Journal of Neuroscience*, vol. 34, no. 42, pp. 13948–13953, 2014.

[14] H. Kasai, M. Fukuda, S. Watanabe, A. Hayashi-Takagi, and J. Noguchi, "Structural dynamics of dendritic spines in memory and cognition," *Trends in Neurosciences*, vol. 33, no. 3, pp. 121–129, 2010.

[15] J. Noguchi, A. Nagaoka, S. Watanabe et al., "In vivo two-photon uncaging of glutamate revealing the structure-function relationships of dendritic spines in the neocortex of adult mice," *Journal of Physiology*, vol. 589, no. 10, pp. 2447–2457, 2011.

[16] M. Matsuzaki, G. C. R. Ellis-Davies, T. Nemoto, Y. Miyashita, M. Iino, and H. Kasai, "Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons," *Nature Neuroscience*, vol. 4, no. 11, pp. 1086–1092, 2001.

[17] A. J. G. D. Holtmaat, J. T. Trachtenberg, L. Wilbrecht et al., "Transient and persistent dendritic spines in the neocortex in vivo," *Neuron*, vol. 45, no. 2, pp. 279–291, 2005.

[18] A. Attardo, J. E. Fitzgerald, and M. J. Schnitzer, "Impermanence of dendritic spines in live adult CA1 hippocampus," *Nature*, vol. 523, no. 7562, pp. 592–596, 2015.

[19] J. T. Trachtenberg, B. E. Chen, G. W. Knott et al., "Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex," *Nature*, vol. 420, no. 6917, pp. 788–794, 2002.

[20] D. Tropea, A. K. Majewska, R. Garcia, and M. Sur, "Structural dynamics of synapses in vivo correlate with functional changes during experience-dependent plasticity in visual cortex," *The Journal of Neuroscience*, vol. 30, no. 33, pp. 11086–11095, 2010.

[21] G. Yang, F. Pan, and W.-B. Gan, "Stably maintained dendritic spines are associated with lifelong memories," *Nature*, vol. 462, no. 7275, pp. 920–924, 2009.

[22] K. A. Jones, D. P. Srivastava, J. A. Allen, R. T. Strachan, B. L. Roth, and P. Penzes, "Rapid modulation of spine morphology by the 5-HT2A serotonin receptor through kalirin-7 signaling," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 46, pp. 19575–19580, 2009.

[23] K. M. Woolfrey, D. P. Srivastava, H. Photowala et al., "Epac2 induces synapse remodeling and depression and its disease-associated forms alter spines," *Nature Neuroscience*, vol. 12, no. 10, pp. 1275–1284, 2009.

[24] C. Liston, J. M. Cichon, F. Jeanneteau, Z. Jia, M. V. Chao, and W.-B. Gan, "Circadian glucocorticoid oscillations promote learning-dependent synapse formation and maintenance," *Nature Neuroscience*, vol. 16, no. 6, pp. 698–705, 2013.

[25] D. P. Srivastava, K. M. Woolfrey, K. A. Jones et al., "Rapid enhancement of two-step wiring plasticity by estrogen and NMDA receptor activity," *Proceedings of the National Academy of Sciences of the United States*, vol. 105, no. 38, pp. 14650–14655, 2008.

[26] S. Konur and R. Yuste, "Imaging the motility of dendritic protrusions and axon terminals: roles in axon sampling and synaptic competition," *Molecular and Cellular Neuroscience*, vol. 27, no. 4, pp. 427–440, 2004.

[27] H.-B. Kwon and B. L. Sabatini, "Glutamate induces de novo growth of functional spines in developing cortex," *Nature*, vol. 474, no. 7349, pp. 100–104, 2011.

[28] R. D. Emes, A. J. Pocklington, C. N. G. Anderson et al., "Evolutionary expansion and anatomical specialization of synapse proteome complexity," *Nature Neuroscience*, vol. 11, no. 7, pp. 799–806, 2008.

[29] L. A. Colgan and R. Yasuda, "Plasticity of dendritic spines: subcompartmentalization of signaling," *Annual Review of Physiology*, vol. 76, pp. 365–385, 2014.

[30] J. L. Chen and E. Nedivi, "Neuronal structural remodeling: is it all about access?" *Current Opinion in Neurobiology*, vol. 20, no. 5, pp. 557–562, 2010.

[31] C. Sala and M. Segal, "Dendritic spines: the locus of structural and functional plasticity," *Physiological Reviews*, vol. 94, no. 1, pp. 141–188, 2014.

[32] P. Penzes, M. E. Cahill, K. A. Jones, and D. P. Srivastava, "Convergent CaMK and RacGEF signals control dendritic structure and function," *Trends in Cell Biology*, vol. 18, no. 9, pp. 405–413, 2008.

[33] Y. Yoshihara, M. De Roo, and D. Muller, "Dendritic spine formation and stabilization," *Current Opinion in Neurobiology*, vol. 19, no. 2, pp. 146–153, 2009.

[34] M. Fischer, S. Kaech, D. Knutti, and A. Matus, "Rapid actin-based plasticity in dendritic spines," *Neuron*, vol. 20, no. 5, pp. 847–854, 1998.

[35] E. F. Spence and S. H. Soderling, "Actin out: regulation of the synaptic cytoskeleton," *The Journal of Biological Chemistry*, vol. 290, no. 48, pp. 28613–28622, 2015.

[36] E. N. Star, D. J. Kwiatkowski, and V. N. Murthy, "Rapid turnover of actin in dendritic spines and its regulation by activity," *Nature Neuroscience*, vol. 5, no. 3, pp. 239–246, 2002.

[37] N. A. Frost, H. Shroff, H. Kong, E. Betzig, and T. A. Blanpied, "Single-molecule discrimination of discrete perisynaptic and distributed sites of actin filament assembly within dendritic spines," *Neuron*, vol. 67, no. 1, pp. 86–99, 2010.

[38] M. Bosch, J. Castro, T. Saneyoshi, H. Matsuno, M. Sur, and Y. Hayashi, "Structural and molecular remodeling of dendritic spine substructures during long-term potentiation," *Neuron*, vol. 82, no. 2, pp. 444–459, 2014.

[39] J. George, C. Soares, A. Montersino, J. C. Beique, and G. M. Thomas, "Palmitoylation of LIM kinase-1 ensures spine-specific actin polymerization and morphological plasticity," *eLife*, vol. 4, 2015.

[40] A. M. Wegner, C. A. Nebhan, L. Hu et al., "N-WASP and the Arp2/3 complex are critical regulators of actin in the development of dendritic spines and synapses," *Journal of Biological Chemistry*, vol. 283, no. 23, pp. 15912–15920, 2008.

[41] Z. Xie, R. L. Huganir, and P. Penzes, "Activity-dependent dendritic spine structural plasticity is regulated by small GTPase Rap1 and its target AF-6," *Neuron*, vol. 48, no. 4, pp. 605–618, 2005.

[42] Z. Xie, D. P. Srivastava, H. Photowala et al., "Kalirin-7 controls activity-dependent structural and functional plasticity of dendritic spines," *Neuron*, vol. 56, no. 4, pp. 640–656, 2007.

[43] J. M. Kerr and T. A. Blanpied, "Subsynaptic AMPA receptor distribution is acutely regulated by actin-driven reorganization of the postsynaptic density," *Journal of Neuroscience*, vol. 32, no. 2, pp. 658–673, 2012.

[44] P. Penzes and M. E. Cahill, "Deconstructing signal transduction pathways that regulate the actin cytoskeleton in dendritic spines," *Cytoskeleton*, vol. 69, no. 7, pp. 426–441, 2012.

[45] Y. Takai, T. Sasaki, and T. Matozaki, "Small GTP-binding proteins," *Physiological Reviews*, vol. 81, no. 1, pp. 153–208, 2001.

[46] J. Cherfils and M. Zeghouf, "Regulation of small GTPases by GEFs, GAPs, and GDIs," *Physiological Reviews*, vol. 93, no. 1, pp. 269–309, 2013.

[47] T. Saneyoshi and Y. Hayashi, "The Ca^{2+} and rho GTPase signaling pathways underlying activity-dependent actin remodeling at dendritic spines," *Cytoskeleton*, vol. 69, no. 8, pp. 545–554, 2012.

[48] A. Tashiro and R. Yuste, "Regulation of dendritic spine motility and stability by Rac1 and Rho kinase: evidence for two forms of spine motility," *Molecular and Cellular Neuroscience*, vol. 26, no. 3, pp. 429–440, 2004.

[49] A. Y. Nakayama, M. B. Harms, and L. Luo, "Small GTPases Rac and Rho in the maintenance of dendritic spines and branches in hippocampal pyramidal neurons," *Journal of Neuroscience*, vol. 20, no. 14, pp. 5329–5338, 2000.

[50] P. Penzes, A. Beeser, J. Chernoff et al., "Rapid induction of dendritic spine morphogenesis by trans-synaptic ephrinB-EphB receptor activation of the Rho-GEF kalirin," *Neuron*, vol. 37, no. 2, pp. 263–274, 2003.

[51] A. Tashiro, A. Minden, and R. Yuste, "Regulation of dendritic spine morphology by the Rho family of small GTPases: antagonistic roles of Rac and Rho," *Cerebral Cortex*, vol. 10, no. 10, pp. 927–938, 2000.

[52] H. Zhang, D. J. Webb, H. Asmussen, S. Niu, and A. F. Horwitz, "A GIT1/PIX/Rac/PAK signaling module regulates spine morphogenesis and synapse formation through MLC," *Journal of Neuroscience*, vol. 25, no. 13, pp. 3379–3388, 2005.

[53] Y. Meng, Y. Zhang, V. Tregoubov et al., "Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice," *Neuron*, vol. 35, no. 1, pp. 121–133, 2002.

[54] M. Maekawa, T. Ishizaki, S. Boku et al., "Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase," *Science*, vol. 285, no. 5429, pp. 895–898, 1999.

[55] H. Murakoshi, H. Wang, and R. Yasuda, "Local, persistent activation of Rho GTPases during plasticity of single dendritic spines," *Nature*, vol. 472, no. 7341, pp. 100–106, 2011.

[56] C. Cerri, A. Fabbri, E. Vannini et al., "Activation of Rho GTPases triggers structural remodeling and functional plasticity in the adult rat visual cortex," *Journal of Neuroscience*, vol. 31, no. 42, pp. 15163–15172, 2011.

[57] J. G. Duman, S. Mulherkar, Y. K. Tu, J. X. Cheng, and K. F. Tolias, "Mechanisms for spatiotemporal regulation of Rho-GTPase signaling at synapses," *Neuroscience Letters*, vol. 601, pp. 4–10, 2015.

[58] N. Nadif Kasri and L. Van Aelst, "Rho-linked genes and neurological disorders," *Pflügers Archiv—European Journal of Physiology*, vol. 455, no. 5, pp. 787–797, 2008.

[59] E.-E. Govek, S. E. Newey, C. J. Akerman, J. R. Cross, L. Van der Veken, and L. Van Aelst, "The X-linked mental retardation protein oligophrenin-1 is required for dendritic spine morphogenesis," *Nature Neuroscience*, vol. 7, no. 4, pp. 364–372, 2004.

[60] S. Bassani, J. Zapata, L. Gerosa, E. Moretto, L. Murru, and M. Passafaro, "The neurobiology of x-linked intellectual disability," *Neuroscientist*, vol. 19, no. 5, pp. 541–552, 2013.

[61] X. Ye and T. J. Carew, "Small G protein signaling in neuronal plasticity and memory formation: the specific role of ras family proteins," *Neuron*, vol. 68, no. 3, pp. 340–361, 2010.

[62] R. Heumann, C. Goemans, D. Bartsch et al., "Transgenic activation of Ras in neurons promotes hypertrophy and protects from lesion-induced degeneration," *Journal of Cell Biology*, vol. 151, no. 7, pp. 1537–1548, 2000.

[63] T. Arendt, U. Gärtner, G. Seeger et al., "Neuronal activation of Ras regulates synaptic connectivity," *European Journal of Neuroscience*, vol. 19, no. 11, pp. 2953–2966, 2004.

[64] G. Seeger, U. Gärtner, and T. Arendt, "Transgenic activation of Ras in neurons increases synapse formation in mouse neocortex," *Journal of Neural Transmission*, vol. 112, no. 6, pp. 751–761, 2005.

[65] C. D. Harvey, R. Yasuda, H. Zhong, and K. Svoboda, "The spread of Ras activity triggered by activation of a single dendritic spine," *Science*, vol. 321, no. 5885, pp. 136–140, 2008.

[66] H. Murakoshi and R. Yasuda, "Postsynaptic signaling during plasticity of dendritic spines," *Trends in Neurosciences*, vol. 35, no. 2, pp. 135–143, 2012.

[67] J. L. Bos, "Linking Rap to cell adhesion," *Current Opinion in Cell Biology*, vol. 17, no. 2, pp. 123–128, 2005.

[68] A. H. Babayan and E. A. Kramár, "Rapid effects of oestrogen on synaptic plasticity: interactions with actin and its signalling proteins," *Journal of Neuroendocrinology*, vol. 25, no. 11, pp. 1163–1172, 2013.

[69] I. S. Nethrapalli, M. Singh, X. Guan et al., "Estradiol (E2) elicits src phosphorylation in the mouse neocortex: the initial event in E2 activation of the MAPK cascade?" *Endocrinology*, vol. 142, no. 12, pp. 5145–5148, 2001.

[70] D. P. Srivastava, E. M. Waters, P. G. Mermelstein, E. A. Kramár, T. J. Shors, and F. Liu, "Rapid estrogen signaling in the brain: implications for the fine-tuning of neuronal circuitry," *The Journal of Neuroscience*, vol. 31, no. 45, pp. 16056–16063, 2011.

[71] Z. Fu, S. H. Lee, A. Simonetta, J. Hansen, M. Sheng, and D. T. S. Pak, "Differential roles of Rap1 and Rap2 small GTPases in neurite retraction and synapse elimination in hippocampal spiny neurons," *Journal of Neurochemistry*, vol. 100, no. 1, pp. 118–131, 2007.

[72] J. Ryu, K. Futai, M. Feliu, R. Weinberg, and M. Sheng, "Constitutively active Rap2 transgenic mice display fewer dendritic spines, reduced extracellular signal-regulated kinase signaling, enhanced long-term depression, and impaired spatial learning and fear extinction," *Journal of Neuroscience*, vol. 28, no. 33, pp. 8178–8188, 2008.

[73] M.-G. Kang, Y. Guo, and R. L. Huganir, "AMPA receptor and GEF-H1/Lfc complex regulates dendritic spine development

through RhoA signaling cascade," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 9, pp. 3549–3554, 2009.

[74] W.-Y. Fu, Y. Chen, M. Sahin et al., "Cdk5 regulates EphA4-mediated dendritic spine retraction through an ephexin1-dependent mechanism," *Nature Neuroscience*, vol. 10, no. 1, pp. 67–76, 2007.

[75] E. Bacchelli, F. Blasi, M. Biondolillo et al., "Screening of nine candidate genes for autism on chromosome 2q reveals rare nonsynonymous variants in the cAMP-GEFII gene," *Molecular Psychiatry*, vol. 8, no. 11, pp. 916–924, 2003.

[76] D. P. Srivastava, K. A. Jones, K. M. Woolfrey et al., "Social, communication, and cortical structural impairments in Epac2-deficient mice," *Journal of Neuroscience*, vol. 32, no. 34, pp. 11864–11878, 2012.

[77] D. P. Srivastava, K. M. Woolfrey, K. A. Jones et al., "An autism-associated variant of Epac2 reveals a role for Ras/Epac2 signaling in controlling basal dendrite maintenance in mice," *PLoS Biology*, vol. 10, no. 6, Article ID e1001350, 2012.

[78] K. J. Lee, Y. Lee, A. Rozeboom et al., "Requirement for Plk2 in orchestrated ras and rap signaling, homeostatic structural plasticity, and memory," *Neuron*, vol. 69, no. 5, pp. 957–973, 2011.

[79] M. E. Cahill, Z. Xie, M. Day et al., "Kalirin regulates cortical spine morphogenesis and disease-related behavioral phenotypes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 31, pp. 13058–13063, 2009.

[80] K. F. Tolias, J. B. Bikoff, C. G. Kane, C. S. Tolias, L. Hu, and M. E. Greenberg, "The Rac1 guanine nucleotide exchange factor Tiam1 mediates EphB receptor-dependent dendritic spine development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 17, pp. 7265–7270, 2007.

[81] K. F. Tolias, J. B. Bikoff, A. Burette et al., "The Rac1-GEF Tiam1 couples the NMDA receptor to the activity-dependent development of dendritic arbors and spines," *Neuron*, vol. 45, no. 4, pp. 525–538, 2005.

[82] N. N. Kasri, A. Nakano-Kobayashi, R. Malinow, B. Li, and L. Van Aelst, "The Rho-linked mental retardation protein oligophrenin-1 controls synapse maturation and plasticity by stabilizing AMPA receptors," *Genes and Development*, vol. 23, no. 11, pp. 1289–1302, 2009.

[83] H. J. Carlisle, P. Manzerra, E. Marcora, and M. B. Kennedy, "SynGAP regulates steady-state and activity-dependent phosphorylation of cofilin," *The Journal of Neuroscience*, vol. 28, no. 50, pp. 13673–13683, 2008.

[84] F. F. Hamdan, H. Daoud, A. Piton et al., "De novo SYNGAP1 mutations in nonsyndromic intellectual disability and autism," *Biological Psychiatry*, vol. 69, no. 9, pp. 898–901, 2011.

[85] J. P. Clement, M. Aceti, T. K. Creson et al., "Pathogenic SYNGAP1 mutations impair cognitive development by disrupting maturation of dendritic spine synapses," *Cell*, vol. 151, no. 4, pp. 709–723, 2012.

[86] Y. Araki, M. Zeng, M. Zhang, and R. L. Huganir, "Rapid dispersion of SynGAP from synaptic spines triggers AMPA receptor insertion and spine enlargement during LTP," *Neuron*, vol. 85, no. 1, pp. 173–190, 2015.

[87] A. Sobczyk and K. Svoboda, "Activity-dependent plasticity of the NMDA-receptor fractional Ca^{2+} current," *Neuron*, vol. 53, no. 1, pp. 17–24, 2007.

[88] B. L. Bloodgood, A. J. Giessel, and B. L. Sabatini, "Biphasic synaptic Ca influx arising from compartmentalized electrical signals in dendritic spines," *PLoS Biology*, vol. 7, no. 9, Article ID e1000190, 2009.

[89] S. S. Hook and A. R. Means, "Ca^{2+}/CaM-dependent kinases: from activation to function," *Annual Review of Pharmacology and Toxicology*, vol. 41, no. 1, pp. 471–505, 2001.

[90] T. R. Soderling, "CaM-kinases: modulators of synaptic plasticity," *Current Opinion in Neurobiology*, vol. 10, no. 3, pp. 375–380, 2000.

[91] P. Penzes, K. M. Woolfrey, and D. P. Srivastava, "Epac2-mediated dendritic spine remodeling: implications for disease," *Molecular and Cellular Neuroscience*, vol. 46, no. 2, pp. 368–380, 2011.

[92] O. Solis, D. I. Limón, J. Flores-Hernández, and G. Flores, "Alterations in dendritic morphology of the prefrontal cortical and striatum neurons in the unilateral 6-OHDA-rat model of Parkinson's disease," *Synapse*, vol. 61, no. 6, pp. 450–458, 2007.

[93] M. Akil, J. N. Pierri, R. E. Whitehead et al., "Lamina-specific alterations in the dopamine innervation of the prefrontal cortex in schizophrenic subjects," *The American Journal of Psychiatry*, vol. 156, no. 10, pp. 1580–1589, 1999.

[94] A. F. T. Arnsten, "Adrenergic targets for the treatment of cognitive deficits in schizophrenia," *Psychopharmacology*, vol. 174, no. 1, pp. 25–31, 2004.

[95] J. E. Black, I. M. Kodish, A. W. Grossman et al., "Pathology of layer V pyramidal neurons in the prefrontal cortex of patients with schizophrenia," *The American Journal of Psychiatry*, vol. 161, no. 4, pp. 742–744, 2004.

[96] L. A. Glantz and D. A. Lewis, "Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia," *Archives of General Psychiatry*, vol. 57, no. 1, pp. 65–73, 2000.

[97] J. J. Hill, T. Hashimoto, and D. A. Lewis, "Molecular mechanisms contributing to dendritic spine alterations in the prefrontal cortex of subjects with schizophrenia," *Molecular Psychiatry*, vol. 11, no. 6, pp. 557–566, 2006.

[98] H.-D. Wang and A. Y. Deutch, "Dopamine depletion of the prefrontal cortex induces dendritic spine loss: reversal by atypical antipsychotic drug treatment," *Neuropsychopharmacology*, vol. 33, no. 6, pp. 1276–1286, 2008.

[99] D. P. Srivastava, K. M. Woolfrey, and P. Penzes, "Insights into rapid modulation of neuroplasticity by brain estrogens," *Pharmacological Reviews*, vol. 65, no. 4, pp. 1318–1350, 2013.

[100] A. M. Sanchez, M. I. Flamini, X.-D. Fu et al., "Rapid signaling of estrogen to WAVE1 and moesin controls neuronal spine formation via the actin cytoskeleton," *Molecular Endocrinology*, vol. 23, no. 8, pp. 1193–1202, 2009.

[101] X.-M. Ma, J.-P. Huang, E.-J. Kim et al., "Kalirin-7, an important component of excitatory synapses, is regulated by estradiol in hippocampal neurons," *Hippocampus*, vol. 21, no. 6, pp. 661–677, 2011.

[102] D. P. Srivastava, K. M. Woolfrey, F. Liu, N. J. Brandon, and P. Penzes, "Estrogen receptor β activity modulates synaptic signaling and structure," *Journal of Neuroscience*, vol. 30, no. 40, pp. 13454–13460, 2010.

[103] C. Galvin and I. Ninan, "Regulation of the mouse medial prefrontal cortical synapses by endogenous estradiol," *Neuropsychopharmacology*, vol. 39, no. 9, pp. 2086–2094, 2014.

[104] K. J. Sellers, F. Erli, P. Raval, I. A. Watson, D. Chen, and D. P. Srivastava, "Rapid modulation of synaptogenesis and spinogenesis by 17β-estradiol in primary cortical neurons," *Frontiers in Cellular Neuroscience*, vol. 9, article 137, 2015.

[105] E. Manser, T. Leung, H. Salihuddin, Z.-S. Zhao, and L. Lim, "A brain serine/threonine protein kinase activated by Cdc42 and Rac1," *Nature*, vol. 367, no. 6458, pp. 40–46, 1994.

[106] Z.-S. Zhao and E. Manser, "PAK and other Rho-associated kinases—effectors with surprisingly diverse mechanisms of regulation," *Biochemical Journal*, vol. 386, no. 2, pp. 201–214, 2005.

[107] M. L. Hayashi, S.-Y. Choi, B. S. Shankaranarayana Rao et al., "Altered cortical synaptic morphology and impaired memory consolidation in forebrain-specific dominant-negative PAK transgenic mice," *Neuron*, vol. 42, no. 5, pp. 773–787, 2004.

[108] S. Asrar, Y. Meng, Z. Zhou, Z. Todorovski, W. W. Huang, and Z. Jia, "Regulation of hippocampal long-term potentiation by p21-activated protein kinase 1 (PAK1)," *Neuropharmacology*, vol. 56, no. 1, pp. 73–80, 2009.

[109] Z. Zhou, Y. Meng, S. Asrar, Z. Todorovski, and Z. Jia, "A critical role of Rho-kinase ROCK2 in the regulation of spine and synaptic function," *Neuropharmacology*, vol. 56, no. 1, pp. 81–89, 2009.

[110] W. Huang, Z. Zhou, S. Asrar, M. Henkelman, W. Xie, and Z. Jia, "p21-activated kinases 1 and 3 control brain size through coordinating neuronal complexity and synaptic properties," *Molecular and Cellular Biology*, vol. 31, no. 3, pp. 388–403, 2011.

[111] D. C. Edwards, L. C. Sanders, G. M. Bokoch, and G. N. Gill, "Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics," *Nature Cell Biology*, vol. 1, no. 5, pp. 253–259, 1999.

[112] Y. Fukata and M. Fukata, "Protein palmitoylation in neuronal development and synaptic plasticity," *Nature Reviews Neuroscience*, vol. 11, no. 3, pp. 161–175, 2010.

[113] Y. Uechi, M. Bayarjargal, M. Umikawa et al., "Rap2 function requires palmitoylation and recycling endosome localization," *Biochemical and Biophysical Research Communications*, vol. 378, no. 4, pp. 732–737, 2009.

[114] J. G. T. Wurtzel, P. Kumar, and L. E. Goldfinger, "Palmitoylation regulates vesicular trafficking of R-Ras to membrane ruffles and effects on ruffling and cell spreading," *Small GTPases*, vol. 3, no. 3, pp. 139–153, 2012.

[115] A. Nishimura and M. E. Linder, "Identification of a novel prenyl and palmitoyl modification at the CaaX motif of Cdc42 that regulates RhoGDI binding," *Molecular and Cellular Biology*, vol. 33, no. 7, pp. 1417–1429, 2013.

[116] C. Egile, T. P. Loisel, V. Laurent et al., "Activation of the CDC42 effector N-WASP by the *Shigella flexneri* IcsA protein promotes actin nucleation by Arp2/3 complex and bacterial actin-based motility," *Journal of Cell Biology*, vol. 146, no. 6, pp. 1319–1332, 1999.

[117] A. S. Kim, L. T. Kakalis, N. Abdul-Manan, G. A. Liu, and M. K. Rosen, "Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein," *Nature*, vol. 404, no. 6774, pp. 151–158, 2000.

[118] S. H. Soderling, E. S. Guire, S. Kaech et al., "A WAVE-1 and WRP signaling complex regulates spine density, synaptic plasticity, and memory," *The Journal of Neuroscience*, vol. 27, no. 2, pp. 355–365, 2007.

[119] Y. Kim, J. Y. Sung, I. Ceglia et al., "Phosphorylation of WAVE1 regulates actin polymerization and dendritic spine morphology," *Nature*, vol. 442, no. 7104, pp. 814–817, 2006.

[120] S. H. Soderling, L. K. Langeberg, J. A. Soderling et al., "Loss of WAVE-1 causes sensorimotor retardation and reduced learning and memory in mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 4, pp. 1723–1728, 2003.

[121] E. D. Goley and M. D. Welch, "The ARP2/3 complex: an actin nucleator comes of age," *Nature Reviews Molecular Cell Biology*, vol. 7, no. 10, pp. 713–726, 2006.

[122] T. Takenawa and S. Suetsugu, "The WASP-WAVE protein network: connecting the membrane to the cytoskeleton," *Nature Reviews Molecular Cell Biology*, vol. 8, no. 1, pp. 37–48, 2007.

[123] P. Hotulainen, O. Llano, S. Smirnov et al., "Defining mechanisms of actin polymerization and depolymerization during dendritic spine morphogenesis," *Journal of Cell Biology*, vol. 185, no. 2, pp. 323–339, 2009.

[124] Y. Nakamura, C. L. Wood, A. P. Patton et al., "PICK1 inhibition of the Arp2/3 complex controls dendritic spine size and synaptic plasticity," *The EMBO Journal*, vol. 30, no. 4, pp. 719–730, 2011.

[125] D. L. Rocca and J. G. Hanley, "PICK1 links AMPA receptor stimulation to Cdc42," *Neuroscience Letters*, vol. 585, pp. 155–159, 2015.

[126] C. Chan, C. C. Beltzner, and T. D. Pollard, "Cofilin dissociates Arp2/3 complex and branches from actin filaments," *Current Biology*, vol. 19, no. 7, pp. 537–545, 2009.

[127] Y. Shi, C. G. Pontrello, K. A. DeFea, L. F. Reichardt, and I. M. Ethell, "Focal adhesion kinase acts downstream of EphB receptors to maintain mature dendritic spines by regulating cofilin activity," *Journal of Neuroscience*, vol. 29, no. 25, pp. 8129–8142, 2009.

[128] J. Gu, C. W. Lee, Y. Fan et al., "ADF/cofilin-mediated actin dynamics regulate AMPA receptor trafficking during synaptic plasticity," *Nature Neuroscience*, vol. 13, no. 10, pp. 1208–1215, 2010.

[129] B. Boettner, E.-E. Govek, J. Cross, and L. Van Aelst, "The junctional multidomain protein AF-6 is a binding partner of the Rap1A GTPase and associates with the actin cytoskeletal regulator profilin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 16, pp. 9064–9069, 2000.

[130] K. Mandai, H. Nakanishi, A. Satoh et al., "Afadin: a novel actin filament-binding protein with one PDZ domain localized at cadherin-based cell-to-cell adherens junction," *Journal of Cell Biology*, vol. 139, no. 2, pp. 517–528, 1997.

[131] J.-E. Van Leeuwen, I. Rafalovich, K. Sellers et al., "Coordinated nuclear and synaptic shuttling of afadin promotes spine plasticity and histone modifications," *The Journal of Biological Chemistry*, vol. 289, no. 15, pp. 10831–10842, 2014.

[132] Z. Xie, H. Photowala, M. E. Cahill et al., "Coordination of synaptic adhesion with dendritic spine remodeling by AF-6 and kalirin-7," *Journal of Neuroscience*, vol. 28, no. 24, pp. 6079–6091, 2008.

[133] D. P. Srivastava, B. A. Copits, Z. Xie et al., "Afadin is required for maintenance of dendritic structure and excitatory tone," *The Journal of Biological Chemistry*, vol. 287, no. 43, pp. 35964–35974, 2012.

[134] W. T. Arthur, L. A. Quilliam, and J. A. Cooper, "Rap1 promotes cell spreading by localizing Rac guanine nucleotide exchange factors," *The Journal of Cell Biology*, vol. 167, no. 1, pp. 111–122, 2004.

[135] P. Penzes, M. E. Cahill, K. A. Jones, J.-E. VanLeeuwen, and K. M. Woolfrey, "Dendritic spine pathology in neuropsychiatric disorders," *Nature Neuroscience*, vol. 14, no. 3, pp. 285–293, 2011.

[136] J. C. Fiala, J. Spacek, and K. M. Harris, "Dendritic spine pathology: cause or consequence of neurological disorders?" *Brain Research Reviews*, vol. 39, no. 1, pp. 29–54, 2002.

[137] M. Dierssen and G. J. A. Ramakers, "Dendritic pathology in mental retardation: from molecular genetics to neurobiology," *Genes, Brain and Behavior*, vol. 5, supplement 2, pp. 48–60, 2006.

[138] S. A. Irwin, R. Galvez, and W. T. Greenough, "Dendritic spine structural anomalies in fragile-X mental retardation syndrome," *Cerebral Cortex*, vol. 10, no. 10, pp. 1038–1044, 2000.

[139] S. Takashima, A. Ieshima, H. Nakamura, and L. E. Becker, "Dendrites, dementia and the Down syndrome," *Brain and Development*, vol. 11, no. 2, pp. 131–133, 1989.

[140] J. Pickett and E. London, "The neuropathology of autism: a review," *Journal of Neuropathology and Experimental Neurology*, vol. 64, no. 11, pp. 925–935, 2005.

[141] H. Y. Zoghbi, "Postnatal neurodevelopmental disorders: meeting at the synapse?" *Science*, vol. 302, no. 5646, pp. 826–830, 2003.

[142] J. J. Hutsler and H. Zhang, "Increased dendritic spine densities on cortical projection neurons in autism spectrum disorders," *Brain Research*, vol. 1309, pp. 83–94, 2010.

[143] D. A. Lewis and R. A. Sweet, "Schizophrenia from a neural circuitry perspective: advancing toward rational pharmacological therapies," *Journal of Clinical Investigation*, vol. 119, no. 4, pp. 706–716, 2009.

[144] J. M. Gorman and J. P. Docherty, "A hypothesized role for dendritic remodeling in the etiology of mood and anxiety disorders," *Journal of Neuropsychiatry and Clinical Neurosciences*, vol. 22, no. 3, pp. 256–264, 2010.

[145] S. T. DeKosky and S. W. Scheff, "Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity," *Annals of Neurology*, vol. 27, no. 5, pp. 457–464, 1990.

[146] D. J. Selkoe, "Alzheimer's disease is a synaptic failure," *Science*, vol. 298, no. 5594, pp. 789–791, 2002.

Hindawi Publishing Corporation
Neural Plasticity
Volume 2016, Article ID 3857934, 11 pages
http://dx.doi.org/10.1155/2016/3857934

Review Article

Proteostasis and RNA Binding Proteins in Synaptic Plasticity and in the Pathogenesis of Neuropsychiatric Disorders

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Received 6 July 2015; Accepted 30 September 2015

Academic Editor: Christian Wozny

Decades of research have demonstrated that rapid alterations in protein abundance are required for synaptic plasticity, a cellular correlate for learning and memory. Control of protein abundance, known as proteostasis, is achieved across a complex neuronal morphology that includes a tortuous axon as well as an extensive dendritic arbor supporting thousands of individual synaptic compartments. To regulate the spatiotemporal synthesis of proteins, neurons must efficiently coordinate the transport and metabolism of mRNAs. Among multiple levels of regulation, transacting RNA binding proteins (RBPs) control proteostasis by binding to mRNAs and mediating their transport and translation in response to synaptic activity. In addition to synthesis, protein degradation must be carefully balanced for optimal proteostasis, as deviations resulting in excess or insufficient abundance of key synaptic factors produce pathologies. As such, mutations in components of the proteasomal or translational machinery, including RBPs, have been linked to the pathogenesis of neurological disorders such as Fragile X Syndrome (FXS), Fragile X Tremor Ataxia Syndrome (FXTAS), and Autism Spectrum Disorders (ASD). In this review, we summarize recent scientific findings, highlight ongoing questions, and link basic molecular mechanisms to the pathogenesis of common neuropsychiatric disorders.

1. Ribonucleoproteins and RBPs

The majority of cytoplasmic mRNAs in neurons are associated with RBPs and other accessory proteins as part of large macromolecular complexes termed ribonucleoprotein (RNP) granules [1]. Granules are diverse in composition and function, mediating many aspects of posttranscriptional RNA regulation including cellular transport, protection from nucleases, and translational control [2–4]. The modular domain structure of RBPs facilitates granule formation and enables auxiliary interactions with other factors necessary to ensure precise localization and metabolism of mRNA cargos [5]. Recent evidence suggests that only one mRNA is present per RNP granule [6–9]. This stoichiometry may be achieved through cotranscriptional packaging of the RNP complex as many RBPs contain nuclear localization sequences and undergo nucleocytoplasmic transport [10, 11]. Following transcription, RBPs are thought to spontaneously couple to mRNA targets to facilitate processing of pre-mRNAs through splicing, editing, polyadenylation, and granule formation (Figure 1). In addition, many RBPs contain prion-like low complexity sequences that may allow for spontaneous self-aggregation. Thus, cargo mRNAs could act as scaffolds/nucleators for aggregation by recruiting and increasing the local concentration of RBPs to catalyze spontaneous granule formation [12]. Low complexity sequences may also promote heteroaggregation of accessory proteins into granules and allow for the dynamic disassembly and reformation of RNP granules in response to synaptic activity [7, 12, 13].

2. RBPs and Neuronal Dendritic Targeting

The control of RNA distribution is a fundamental mechanism underlying localized expression of proteins [14]. This is especially pertinent among the intricate dendritic arbors of

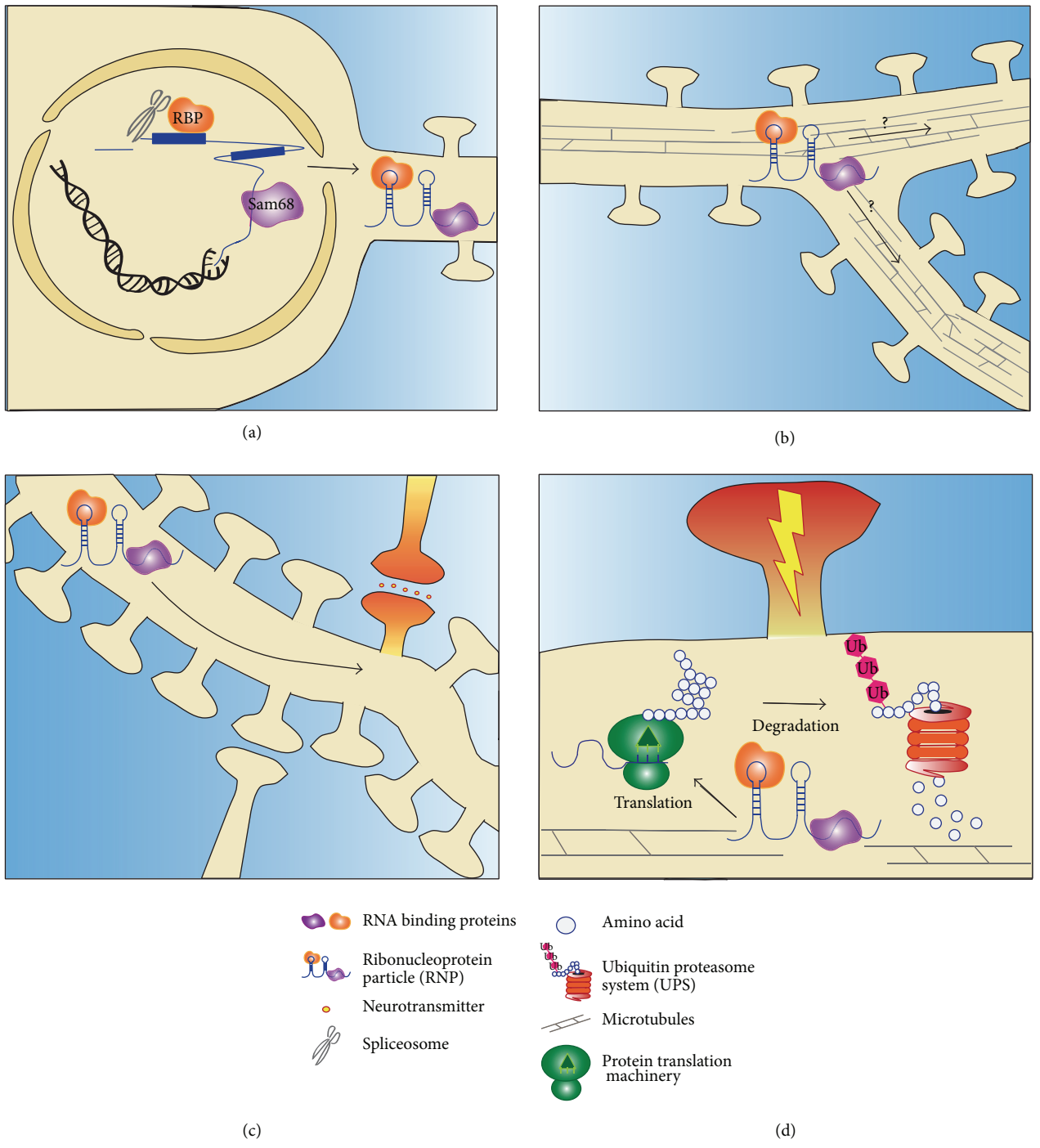

Figure 1: The trials and tribulations of RBPs and RNPs. (a) RBPs assemble cotranscriptionally and regulate mRNA splicing and modification preventing the coassembly of multiple mRNAs per RNP. Motifs in the 5′UTR and 3′UTR as well as retained intronic sequences facilitate dendritic targeting of RNPs. (b) RBPs transport mRNAs along microtubules to destinations dictated by the cargo mRNA sequence. Through input-specific events, synapses or dendritic branches may autonomously regulate their mRNA content. (c) Excitatory synapses at dendritic spines greatly outnumber mRNAs in dendrites and even more so counting inhibitory synapses. Despite being sparsely distributed, local mRNAs contribute significantly to synaptic function. (d) Upon synaptic stimulation, RBP function determines mRNA fate. Derepression by translational repressors can be followed by promotion of translation by RBPs like Sam68 (purple). Translation is counterbalanced by proteasomal or lysosomal degradation.

neurons where synapses are thought to independently alter their morphology and function in response to neuronal activity via spatial restriction of gene expression. In addition to diffusion and transport of somatically generated proteins, synthesis from dendritically targeted mRNAs shapes the local proteome around synaptic contacts. The active transport of mRNAs into neuronal processes requires their association with RNA transport particles, which contain (1) specific RBPs to prevent translation prior to delivery, (2) adaptors for association with cytoskeletal translocation machinery, and (3) molecular motors [15] (Figure 1). A number of groups have identified dendritic targeting elements (DTEs) in the 3′ untranslated regions (3′UTR) of mRNAs present in synaptodendritic compartments including CaMKIIα, beta actin, MAP2, ARC, and BDNF [16–24]. BDNF and CaMKIIα isoforms with shortened 3′UTRs lacking DTEs are not dendritically targeted, suggesting that alternative splicing of cis-elements can regulate mRNA localization [16, 25]. One of the best-studied DTEs is the "zipcode" found in the 3′UTR of beta-actin mRNA, which is recognized by the RBP Zipcode Binding Protein 1 (ZBP1) and is necessary for its transport and translational regulation [19]. Interestingly, the beta-actin 3′UTR contains an additional nonoverlapping DTE recognized by the RBP Src-Associated in Mitosis 68 KDa (Sam68) [26]. We have shown that Sam68 is crucial for the dendritic transport and translation of beta-actin mRNA, similar to ZBP1 [27]. Whether multiple DTEs allow for concurrent binding of RBPs is an outstanding question in the field and will be addressed in more detail later in this review.

Diverse mRNA targeting mechanisms have been identified in neurons. Retained introns in cytoplasmic mRNAs represent an additional and surprising class of cis-acting dendritic targeting elements that are regulated by the spliceosome. Cytoplasmic intron-sequence retaining transcripts (CIRTs) contain intronic elements that are sufficient to target these mRNAs to dendrites [28–30]. The presence of spliceosome constituents in dendrites raises the intriguing possibility that localized splicing may represent a previously unappreciated activity-dependent cytoplasmic process [31]. In this manner, incompletely processed CIRTs could be transitionally repressed until fully spliced and made competent by the dendritic spliceosome [32]. Indeed, several RBPs implicated in splicing have been observed in synaptodendritic compartments [11, 33] including Sam68 [34–38].

Many of the over 2550 mRNAs present in dendrites [39, 40] lack any known dendritic targeting element, suggesting the existence of an alternative targeting mechanism. Recent evidence implicates nonsequence specific, structural elements in both the 3′UTRs and 5′UTRs in mRNAs in dendritic and synaptic localization [23]. Structural G-quartet stem loops, rather than sequence recognition, have been implicated in how the RBP Fragile X Mental Retardation Protein (FMRP) binds to mRNA cargos [41–43]. However, recent additional evidence indicates binding sites may be present throughout the entire sequence [44] or at specific 3′UTR sites [45]. Altogether, these findings suggest the existence of functionally distinct mechanisms to localize RNAs within neuronal processes and highlight the role of noncoding mRNA sequences in subcellular targeting. Recent genome-wide association studies suggest that mutations in noncoding targeting elements are linked with disease phenotypes, underscoring the importance of mRNA transport in supporting neuronal function [46, 47].

3. RBPs and Input-Specific Translation

Input specificity requires the localized expression of proteins following mRNA transport. Dendritic translation in neurons was first evidenced by the presence of ribosomes [48, 49] and mRNAs [50, 51] closely associated with postsynaptic contacts. This was later confirmed by studies demonstrating that mechanically isolated dendrites are translation-competent [24, 52] and can sustain protein synthesis-dependent forms of synaptic plasticity [24, 53, 54]. Furthermore, the transport and localized translation of mRNAs are both synapse- and stimulus-specific [24].

RBPs like FMRP and ZBP1 mediate translational repression and release cargos locally in response to particular stimulus-driven posttranslational modifications. For example, locally active Src kinase phosphorylates ZBP1 upon arrival at the synapse, which reduces its affinity for beta-actin mRNA and liberates it for subsequent translation [55]. Other RBPs are thought to directly promote protein synthesis. Sam68, for example, has been recently shown to positively regulate translation in neurons [34, 36, 37, 56–58]. Our group found that Sam68 regulates the levels of beta-actin mRNA and protein in synaptodendritic compartments [27]. We found that Sam68 regulates the loading of beta-actin mRNA onto polysomes isolated from synaptic fractions, suggesting that Sam68 promotes local protein synthesis. Sam68 was originally identified as Src binding partner [59] and phosphorylation of Sam68 by both tyrosine and serine-threonine kinases has been shown to regulate its affinity for RNA [60–62]. Therefore, synaptic activity may trigger downstream signaling cascades that affect local Sam68-dependent protein synthesis. New research aimed at disentangling the molecular mechanisms that regulate the spatial and temporal derepression and translation of mRNAs will help to further our understanding of RBPs in synaptic function and brain disorders.

4. Balancing Protein Synthesis and Degradation in Synaptic Plasticity and Disease

Protein translation and degradation both contribute to proteostasis and are essential for proper synaptic function. Aberrant protein levels at synapses are thought to be pathogenic primarily by affecting the expression and/or maintenance of synaptic transmission and plasticity. Several ASD susceptibility genes encode for proteins that regulate translation, or proteasomal degradation (outlined below). These genes are also involved in the induction and expression of mGluR-mediated long-term depression (mGluR-LTD), a type of synaptic plasticity that requires protein synthesis [63–65]. Deficits in mGluR-LTD have been observed in numerous

mouse models of ASDs and other cognitive and neurodegenerative disorders such as Alzheimer's and Parkinson's disease [65–70]. One of the most widely studied examples is that of the translational repressor FMRP [44], whose absence leads to Fragile X Syndrome (FXS), the most common form of inherited intellectual disability in boys. FMRP binds to a plethora of mRNAs suggesting that the underlying pathology of FXS may result from broad translational dysregulation of the neuronal transcriptome [44]. Mice null for FMRP display general increases in basal protein synthesis [71] but lack translation in response to activation of mGluRs [72]. Paradoxically, FMRP KO mice exhibit exaggerated mGluR-LTD despite a lack of mGluR-triggered protein synthesis. One theory is that elevated basal levels of plasticity-related proteins in the FMRP null mice bypass the need for mGluR-triggered translation, thus resulting in enhanced mGluR-LTD, which may contribute to the neurological symptoms of FXS [73]. However, recent work from our group demonstrates that the magnitude of mGluR-LTD is not necessarily correlated with synaptic protein abundance [56]. Other deficits, including altered neuronal excitability [74–76] or decreased proteasome function [77, 78], could underlie the exaggerated LTD observed in FMRP null mice.

In addition to FXS and ASD, RBP dysfunction may also play a role in other disorders including schizophrenia and amyotrophic lateral sclerosis. The protein disrupted in schizophrenia-1 (DISC-1) [79] was recently identified as a novel RBP and a component of RNP granules [80]. Unregulated expression of DISC-1 has been associated with schizophrenia and clinical depression. DISC-1 appears to be important for dendritic mRNA transport and for maintenance of late phase long-term potentiation (L-LTP) [80]. Accumulation of the transactive response DNA-binding protein-43 (TDP-43) in the cytoplasm is evident in sporadic forms of amyotrophic lateral sclerosis, frontotemporal dementia, and Alzheimer's disease [81]. TDP-43 binds to DNA and RNA and has been shown to regulate splicing, mRNA stability, and mRNA transport and translation as well as synaptic function in motor neurons [82, 83]. These results suggest a broad role for RBP in normal and pathological brain function.

Consistent with links between unbalanced protein levels and disease, several neuropsychiatric disorders have been associated with mutations in components of the ubiquitin proteasome system (UPS) [84–86] including Parkinson's disease, spinocerebellar ataxia, X-Linked Mental Retardation, and Angelman Syndrome [87–92]. Evidence for transport of proteasomal subunits and E3 ligases into dendritic spines [93] and activity-dependent ubiquitination of the synaptic proteome [94, 95] suggests that local effects of the UPS contribute to synaptic proteostasis and input specificity. Since the UPS degrades proteins, one might expect that pathologies associated with impaired UPS function would arise from a toxic accumulation of substrates. However, this is not always the case, suggesting that UPS function is more complex than initially imagined [96]. For example, monoubiquitination of diverse synaptic proteins can regulate synaptic transmission independent of protein degradation [97, 98]. Nonproteolytic monoubiquitination of the RBP CPEB3 regulates dendritic spine growth and AMPA receptor abundance and ultimately regulates learning and memory [99]. Moreover, monoubiquitination of PSD95 [100, 101] and PICK1 by Parkin [102] may regulate the surface expression of AMPA subunits and the acid sensing channel, respectively. Several groups have demonstrated acute inhibition of the proteasome affects long-term plasticity, but there is some disagreement in the literature on the nature of the disruptions [56, 103–106].

In the case of FXS, studies demonstrate that FMRP is rapidly degraded by the proteasome during the induction of synaptic plasticity [105, 107]. Therefore, mutations that inhibit proteasomal degradation of FMRP may lead to altered plasticity and result in neuropsychiatric symptoms. Consistent with this hypothesis, a recent study discovered that loss of the E3 ubiquitin ligase Cdh1-APC prevents FMRP degradation, as well as the induction of mGluR-LTD [108]. Furthermore, previous work in the Cdh1 knockout mouse identified a deficit in late phase LTP and contextual fear conditioning [109]. Therefore, the interaction between FMRP and Cdh1-APC may be essential for multiple forms of plasticity and memory formation. Presumably, degradation of FMRP would positively impact the translation of its cargo mRNAs to support long-term postsynaptic changes, though the direct effect of FMRP degradation on protein levels remains to be addressed.

Fragile X Tremor Ataxia Syndrome (FXTAS) is a neurodegenerative disorder characterized by adult-onset ataxia and cognitive decline. In FXTAS, a pathogenic premutation trinucleotide repeat expansion (50–200 repeats) in the 5′UTR of the FMR1 gene (FMRP) does not result in transcriptional repression but rather causes a toxic gain-of-function through the formation of intranuclear inclusions containing the FMR1 mRNA and sequestered RBPs [110]. Recent work suggests that the sequestration of crucial cellular factors, including Sam68, into these intranuclear inclusions contributes to the cognitive deficits observed in FXTAS [111]. Indeed, Sam68 is functionally impaired in FXTAS patient tissue [111] and accumulation at intranuclear inclusions precedes other deficits, suggesting that loss of Sam68 function plays a causal role in FXTAS [111]. Sam68 KO mice display ataxia [57, 112] and both Sam68 KO mice and primary neurons lacking Sam68 display deficits in dendritic spine morphology [27], which is also seen in other FXTAS models using expanded CGG repeats [113].

We have recently shown that Sam68 is critically involved in coordinating mRNA translation and degradation via the proteasome during the induction of synaptic plasticity. Sam68 is likely necessary to promote the rapid translation of several plasticity-related proteins in response to mGluR activation. In Sam68 KO animals, the balance of proteostasis is abnormal and tipped towards degradation. Interestingly, Sam68 KO mice display impaired mGluR-LTD that can be rescued by blocking the proteasome [56]. In our model, rapid proteasomal degradation acts as a homeostatic scaling mechanism to prevent the accumulation of plasticity-related proteins and thus the induction of further rounds of mGluR-LTD, independent of mGluR activation. Our recent research has put together a more nuanced view of synaptic proteostasis in synaptic plasticity, as a push-pull between RBP-mediated translation and proteasomal degradation. Disruptions to this

balance may underlie the pathogenesis of neuropsychiatric disorders including FXTAS.

5. Unanswered Questions

5.1. Is There Presynaptic Protein Synthesis? In accordance with current scientific research, the bulk of this review has focused on the mechanisms by which RBPs regulate *post*synaptic sites. However, many forms of synaptic plasticity are presynaptically expressed in vertebrate and invertebrate preparations and may require protein synthesis [114–116]. Over 300 mRNA species identified in mature axons include transcripts encoding for components of the translation machinery [117]. During development, the RBPs ZBP1, FMRP, and CPEB respond to stimulus-specific cues in axonal growth cones to mediate mRNA translation [41, 118–121] and ZBP1 localizes at axonal branch points where it mediates beta-actin mRNA translation and branch stabilization [122]. In mature axons, it was recently shown that amyloid beta peptides stimulate the axonal synthesis of the transcription factor ATF4 among other proteins, which can shuttle into the presynaptic nucleus and initiate cell death [123]. It has been postulated that axons use a different type of translation machinery (e.g. monosomes v. polysomes) [124, 125] and that ribosomes are localized to electron-dense regions and/or tethered to the cell membrane within the axon [125, 126]. This may explain why structural evidence for presynaptic/axonal ribosomes is scarce. As many neuronal subtypes have highly branched axonal projections that synapse on multiple neurons, one would also expect input-specific regulation of presynaptic function. RBPs provide a plausible mechanism by which this regulation could be accomplished in adult CNS axons. The development and implementation of new strategies to isolate and visualize axons and presynaptic compartments should inform this line of study.

5.2. How Is Specificity Achieved Given the Ratio of Synapses to RNAs? The breadth of the dendritic transcriptome supports an important role for local translation in long-term plasticity [39]. However, the mRNAs for many important synaptic proteins such as BDNF, GluA2, SHANK, and ARC are conspicuous in their scarcity or absence in the dendrite [6, 9, 13, 23]. Furthermore, the number of even the most abundant dendritic mRNAs (beta-actin and CaMKIIα) is typically an order of magnitude less than the number of synapses. This discrepancy poses the simple mechanistic problem that there are not nearly enough mRNAs to supply proteins on a one to one basis with synapses as required. This problem may be circumvented by a high translational efficiency of synaptic mRNAs, with each mRNA being translated many times to produce an adequate number of proteins. Newly synthesized proteins would then traffic towards the appropriate synapse and thus few mRNAs could supply proteins to an entire dendritic branch, rather than a single dendritic spine or synapse. To our knowledge, direct measurements of mRNA translational efficiency at synapses have been prohibitively difficult to obtain. Exciting new developments in fluorescent tagging, including spaghetti monster fluorescent proteins [127] and SunTag [128], may soon allow for the direct visualization and measurement of local translation.

Additionally, electron microscopy studies in adult hippocampus reveal that there are far fewer dendritic polysomes (the presumed sites of local translation) than synapses [129–131]. Perhaps only a subset of synapses undergo long-term morphological and functional changes or require protein synthesis to do so. Large dendritic spines containing the spine apparatus and endoplasmic reticulum [132] could comprise this group. Alternatively, the rapid and bidirectional transport of mRNAs by RBPs towards active synapses along neuronal dendrites may be an ongoing process long after transcription [133]. In this case, synapses undergoing plasticity might physically capture transporting mRNP granules through unknown mechanisms. Indeed, a portion of dendritic beta-actin mRNAs display active and bidirectional transport [134] and polysomes themselves can redistribute from dendritic shafts into spines in response to a plasticity-inducing stimulus [135].

As we propose in the previous paragraphs, the discovery of motile dendritic RNPs suggests that the local area served by a single mRNA may well be a dendritic branch rather than a single spine. Recent work suggests CaMKIIα mRNA and protein demonstrate branch specificity in response to mTOR activity [136]. If the dendritic branch rather than individual synapses represents the consolidated integrative unit underlying translation-dependent forms of plasticity as previously suggested [137, 138], then input specificity might refer to a branch, rather than an individual synaptic junction. Under these conditions, having few dynamically transported mRNAs at each dendritic branch may be sufficient for plasticity. New massively multiplexed, FISH-based techniques to localize all the mRNAs in a neuron will allow for the determination of the spatial relationship of mRNAs to synapses and branch points [139, 140]. In concert with these new techniques, further experiments using more physiological inductions of plasticity along with mRNA visualization will be of great benefit in elucidating the movements of dendritic mRNAs and the spatial extent of "local" translation.

5.3. What Is the Contribution of Locally Translated Protein to the Existing Local Pool? To our knowledge, accurate numbers of actin molecules at neuronal dendritic spines have not been calculated and likely vary substantially based on conditions. However, an estimate based on studies of stereocilia of the inner ear [141], which are actin-rich protrusions of similar size, suggests 10^5 actin molecules per spine (each stereocilia contains ~100–700 actin filaments on average and 370 actin molecules per micron of actin filament). Considering ribosomal processing speeds (6–9 amino acids/sec) [142] and the size of beta actin, we estimate that one beta-actin protein can be produced every 50 seconds, or 36 beta-actin molecules can be produced in 30 minutes per mRNA and per ribosome. Even considering multiple mRNAs and polyribosomes, the amount of newly synthesized beta-actin would likely represent only a small fraction of available synaptic molecules. If the transport and translation of beta-actin mRNAs into dendritic spines contribute to morphological

plasticity following synaptic stimulation, then a rationale must be found for why this population must be *newly* made as opposed to being recycled from large synaptic pools or transported from the cell body. Temporally regulated irreversible posttranslational modifications may functionally distinguish beta-actin molecules. Indeed, newly synthesized beta-actin localizes at the leading edge of filament formation, perhaps through fast arginylation at the N-terminus [143], which has been previously shown to increase actin polymerization [144, 145]. In addition, spatially regulated posttranslational modifications may also confer functional distinctions on newly synthesized proteins. BDNF synthesized at dendrites has been implicated in spine head growth and pruning, whereas BDNF synthesized in the cell body promotes spine formation [146]. Thus, though the contribution of local translation to total dendritic protein for highly abundant proteins may be small, the functional distinction of newly and locally synthesized proteins may be the primary effector of synaptic alterations. The recent development of techniques that enable the visualization and quantification of newly synthesized proteins, such as fluorescent noncanonical amino acid tagging (FUNCAT) [147], may provide new insight into the contribution of RBP-mediated local translation to the total protein pool.

5.4. Do Multiple RBPs Bind to RNAs? Several mRNAs contain nonoverlapping binding sites for diverse RBPs, suggesting complex and multifactorial regulation of mRNA metabolism. Beta-actin mRNA itself contains nonoverlapping binding sites for ZBP1 [19], Sam68 [26], and FMRP [45], although how these RBPs combine to regulate beta-actin metabolism is unknown. We compared Sam68 mRNA cargos identified using UV-crosslinking techniques [36] and found that 83.7% of these mRNAs also bound to FMRP [44, 45]. As loss of protein synthesis *promoted* by Sam68 leads to *impaired* mGluR-LTD and loss of FMRP *repression* leads to *enhanced* mGluR-LTD [148], Sam68 and FMRP could bind cooperatively to bidirectionally regulate RNA cargo metabolism. Thus, Sam68 and FMRP may differentially regulate a common pool of dendritically expressed neuronal mRNAs to regulate synaptic function, although whether they bind at the same time is unclear. It is interesting to speculate how the opposing actions of these RBPs coordinate the metabolism of single mRNA. Perhaps diverse RBPs regulate stimulus-specific synaptic activity. In this way, a different translational response could be activated after a weak or strong stimulus, or from different types of synaptic activity (i.e., excitatory versus inhibitory, metabotropic versus ionotropic). Perhaps multi-RBP regulation of mRNAs provides additional layers of regulation for fine-tuning spatial and temporal protein expression. Answers to these questions remain unclear and will require additional experimentation.

6. Conclusion

To achieve proteostasis, neurons must spatially coordinate multiple cellular processes across thousands of synapses. In the cellular milieu, mRNAs are always packaged into ribonucleoprotein (RNP) granules, which coordinate the transport and translation of their cargo mRNAs. Disease causing mutations in mRNAs or RBPs may lead to disruption of mRNA packaging into granules and alter subsequent transport and translation. These pathological mechanisms underscore the importance of mRNA being in the right place at the right time, as well as the importance of mRNA as a structural platform to coordinate interactions between RBPs and associated proteins. However, many unanswered questions remain, including a lack of sufficient mRNA particles for synapses, the contribution of local translation to existing pools of protein, and the interactions and complex regulation of multiple RBPs per single mRNA. There are numerous limitations in studying mRNA trafficking and translation in neurons, such as the lack of an assay to determine the exact localization and timing of synthesized proteins (however, see [149]). Moreover, most transcriptional and mRNA transport processes have been studied in the context of strong and non-physiological stimuli, such as bath application of neurotransmitters in cellular cultures. As techniques for single synapse stimulation and single molecule imaging of mRNA in live tissue improve, we may observe different behavior of mRNA transport under more physiological stimulation paradigms.

Conflict of Interests

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

References

[1] A. R. Buxbaum, G. Haimovich, and R. H. Singer, "In the right place at the right time: visualizing and understanding mRNA localization," *Nature Reviews Molecular Cell Biology*, vol. 16, no. 2, pp. 95–109, 2015.

[2] G. Elvira, S. Wasiak, V. Blandford et al., "Characterization of an RNA granule from developing brain," *Molecular and Cellular Proteomics*, vol. 5, no. 4, pp. 635–651, 2006.

[3] R. Fritzsche, D. Karra, K. L. Bennett et al., "Interactome of two diverse RNA granules links mRNA localization to translational repression in neurons," *Cell Reports*, vol. 5, no. 6, pp. 1749–1762, 2013.

[4] Y. Kanai, N. Dohmae, and N. Hirokawa, "Kinesin transports RNA: isolation and characterization of an RNA-transporting granule," *Neuron*, vol. 43, no. 4, pp. 513–525, 2004.

[5] B. M. Lunde, C. Moore, and G. Varani, "RNA-binding proteins: modular design for efficient function," *Nature Reviews Molecular Cell Biology*, vol. 8, no. 6, pp. 479–490, 2007.

[6] M. Batish, P. van den Bogaard, F. R. Kramer, and S. Tyagi, "Neuronal mRNAs travel singly into dendrites," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 12, pp. 4645–4650, 2012.

[7] A. R. Buxbaum, B. Wu, and R. H. Singer, "Single beta-actin mRNA detection in neurons reveals a mechanism for regulating its translatability," *Science*, vol. 343, no. 6169, pp. 419–422, 2014.

[8] S. Farris, G. Lewandowski, C. D. Cox, and O. Steward, "Selective localization of Arc mRNA in dendrites involves activity- and translation-dependent mRNA degradation," *The Journal of Neuroscience*, vol. 34, no. 13, pp. 4481–4493, 2014.

[9] V. M. Ho, L. O. Dallalzadeh, N. Karathanasis et al., "GluA2 mRNA distribution and regulation by miR-124 in hippocampal neurons," *Molecular and Cellular Neuroscience*, vol. 61, pp. 1–12, 2014.

[10] B. A. Jordan, B. D. Fernholz, M. Boussac et al., "Identification and verification of novel rodent postsynaptic density proteins," *Molecular and Cellular Proteomics*, vol. 3, no. 9, pp. 857–871, 2004.

[11] G. Zhang, T. A. Neubert, and B. A. Jordan, "RNA binding proteins accumulate at the postsynaptic density with synaptic activity," *The Journal of Neuroscience*, vol. 32, no. 2, pp. 599–609, 2012.

[12] M. Kato, T. W. Han, S. Xie et al., "Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels," *Cell*, vol. 149, no. 4, pp. 753–767, 2012.

[13] A. M. Krichevsky and K. S. Kosik, "Neuronal RNA granules: a link between RNA localization and stimulation-dependent translation," *Neuron*, vol. 32, no. 4, pp. 683–696, 2001.

[14] D. St. Johnston, "Moving messages: the intracellular localization of mRNAs," *Nature Reviews Molecular Cell Biology*, vol. 6, no. 5, pp. 363–375, 2005.

[15] W. S. Sossin and L. DesGroseillers, "Intracellular trafficking of RNA in neurons," *Traffic*, vol. 7, no. 12, pp. 1581–1589, 2006.

[16] J. J. An, K. Gharami, G.-Y. Liao et al., "Distinct role of long 3′ UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons," *Cell*, vol. 134, no. 1, pp. 175–187, 2008.

[17] A. Blichenberg, B. Schwanke, M. Rehbein, C. C. Garner, D. Richter, and S. Kindler, "Identification of a cis-acting dendritic targeting element in MAP2 mRNAs," *The Journal of Neuroscience*, vol. 19, no. 20, pp. 8818–8829, 1999.

[18] T. M. Böckers, M. Segger-Junius, P. Iglauer et al., "Differential expression and dendritic transcript localization of Shank family members: identification of a dendritic targeting element in the 3′ untranslated region of Shank1 mRNA," *Molecular and Cellular Neuroscience*, vol. 26, no. 1, pp. 182–190, 2004.

[19] T. Eom, L. N. Antar, R. H. Singer, and G. J. Bassell, "Localization of a beta-actin messenger ribonucleoprotein complex with zipcode-binding protein modulates the density of dendritic filopodia and filopodial synapses," *The Journal of Neuroscience*, vol. 23, no. 32, pp. 10433–10444, 2003.

[20] I. Epstein, G. Tushev, T. J. Will, I. Vlatkovic, I. J. Cajigas, and E. M. Schuman, "Alternative polyadenylation and differential expression of Shank mRNAs in the synaptic neuropil," *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 369, no. 1633, 2014.

[21] H. Kobayashi, S. Yamamoto, T. Maruo, and F. Murakami, "Identification of a cis-acting element required for dendritic targeting of activity-regulated cytoskeleton-associated protein mRNA," *European Journal of Neuroscience*, vol. 22, no. 12, pp. 2977–2984, 2005.

[22] M. Mayford, D. Baranes, K. Podsypanina, and E. R. Kandel, "The 3′-untranslated region of CaMKIIα is a cis-acting signal for the localization and translation of mRNA in dendrites," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 23, pp. 13250–13255, 1996.

[23] E. J. Meer, D. O. Wang, S. Kim, I. Barr, F. Guo, and K. C. Martin, "Identification of a cis-acting element that localizes mRNA to synapses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 12, pp. 4639–4644, 2012.

[24] D. O. Wang, S. M. Kim, Y. Zhao et al., "Synapse- and stimulus-specific local translation during long-term neuronal plasticity," *Science*, vol. 324, no. 5934, pp. 1536–1540, 2009.

[25] A. Blichenberg, M. Rehbein, R. Muller, C. C. Garner, D. Richter, and S. Kindler, "Identification of a cis-acting dendritic targeting element in the mRNA encoding the alpha subunit of Ca^{2+}/calmodulin-dependent protein kinase II," *The European Journal of Neuroscience*, vol. 13, no. 10, pp. 1881–1888, 2001.

[26] M. Itoh, I. Haga, Q.-H. Li, and J.-I. Fujisawa, "Identification of cellular mRNA targets for RNA-binding protein Sam68," *Nucleic Acids Research*, vol. 30, no. 24, pp. 5452–5464, 2002.

[27] M. E. Klein, T. J. Younts, P. E. Castillo, and B. A. Jordan, "RNA-binding protein Sam68 controls synapse number and local β-actin mRNA metabolism in dendrites," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 8, pp. 3125–3130, 2013.

[28] T. J. Bell, K. Y. Miyashiro, J.-Y. Sul et al., "Cytoplasmic BK(Ca) channel intron-containing mRNAs contribute to the intrinsic excitability of hippocampal neurons," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 6, pp. 1901–1906, 2008.

[29] P. T. Buckley, M. T. Lee, J.-Y. Sul et al., "Cytoplasmic intron sequence-retaining transcripts can be dendritically targeted via ID element retrotransposons," *Neuron*, vol. 69, no. 5, pp. 877–884, 2011.

[30] M. Khaladkar, P. T. Buckley, M. T. Lee et al., "Subcellular RNA sequencing reveals broad presence of cytoplasmic intron-sequence retaining transcripts in mouse and rat neurons," *PLoS ONE*, vol. 8, no. 10, Article ID e76194, 2013.

[31] J. Glanzer, K. Y. Miyashiro, J.-Y. Sul et al., "RNA splicing capability of live neuronal dendrites," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 46, pp. 16859–16864, 2005.

[32] P. T. Buckley, M. Khaladkar, J. Kim, and J. Eberwine, "Cytoplasmic intron retention, function, splicing, and the sentinel RNA hypothesis," *Wiley Interdisciplinary Reviews: RNA*, vol. 5, no. 2, pp. 223–230, 2014.

[33] B. A. Jordan and E. Ziff, Eds., *To the Nucleus with Proteomics*, Springer, 2007.

[34] N. Ben Fredj, J. Grange, R. Sadoul, S. Richard, Y. Goldberg, and V. Boyer, "Depolarization-induced translocation of the RNA-binding protein Sam68 to the dendrites of hippocampal neurons," *Journal of Cell Science*, vol. 117, no. 7, pp. 1079–1090, 2004.

[35] G. Chawla, C.-H. Lin, A. Han, L. Shiue, M. Ares Jr., and D. L. Black, "Sam68 regulates a set of alternatively spliced exons during neurogenesis," *Molecular and Cellular Biology*, vol. 29, no. 1, pp. 201–213, 2009.

[36] J. Grange, A. Belly, S. Dupas, A. Trembleau, R. Sadoul, and Y. Goldberg, "Specific interaction between Sam68 and neuronal mRNAs: implication for the activity-dependent biosynthesis of elongation factor eEF1A," *Journal of Neuroscience Research*, vol. 87, no. 1, pp. 12–25, 2009.

[37] J. Grange, V. Boyer, R. Fabian-Fine, N. B. Fredj, R. Sadoul, and Y. Goldberg, "Somatodendritic localization and mRNA association of the splicing regulatory protein Sam68 in the hippocampus and cortex," *Journal of Neuroscience Research*, vol. 75, no. 5, pp. 654–666, 2004.

[38] T. Iijima, Y. Iijima, H. Witte, and P. Scheiffele, "Neuronal cell type-specific alternative splicing is regulated by the KH domain protein SLM1," *The Journal of Cell Biology*, vol. 204, no. 3, pp. 331–342, 2014.

[39] I. J. Cajigas, G. Tushev, T. J. Will, S. Tom Dieck, N. Fuerst, and E. M. Schuman, "The local transcriptome in the synaptic neuropil

revealed by deep sequencing and high-resolution imaging," *Neuron*, vol. 74, no. 3, pp. 453–466, 2012.

[40] J. Eberwine, K. Miyashiro, J. Estee Kacharmina, and C. Job, "Local translation of classes of mRNAs that are targeted to neuronal dendrites," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 13, pp. 7080–7085, 2001.

[41] J. C. Darnell, K. B. Jensen, P. Jin, V. Brown, S. T. Warren, and R. B. Darnell, "Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function," *Cell*, vol. 107, no. 4, pp. 489–499, 2001.

[42] H. Siomi, M. C. Siomi, R. L. Nussbaum, and G. Dreyfuss, "The protein product of the fragile X gene, FMR1, has characteristics of an RNA-binding protein," *Cell*, vol. 74, no. 2, pp. 291–298, 1993.

[43] C. Verhelj, C. E. Bakker, E. de Graaff et al., "Characterization and localization of the FMR-1 gene product associated with fragile X syndrome," *Nature*, vol. 363, no. 6431, pp. 722–724, 1993.

[44] J. C. Darnell, S. J. Van Driesche, C. Zhang et al., "FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism," *Cell*, vol. 146, no. 2, pp. 247–261, 2011.

[45] M. Ascano Jr., N. Mukherjee, P. Bandaru et al., "FMRP targets distinct mRNA sequence elements to regulate protein expression," *Nature*, vol. 492, no. 7429, pp. 382–386, 2012.

[46] M. Arnold, D. C. Ellwanger, M. L. Hartsperger, A. Pfeufer, and V. Stümpflen, "Cis-acting polymorphisms affect complex traits through modifications of MicroRNA regulation pathways," *PLoS ONE*, vol. 7, no. 5, Article ID e36694, 2012.

[47] M. Halvorsen, J. S. Martin, S. Broadaway, and A. Laederach, "Disease-associated mutations that alter the RNA structural ensemble," *PLoS Genetics*, vol. 6, no. 8, Article ID 1001074, 2010.

[48] O. Steward and W. B. Levy, "Preferential localization of polyribosomes under the base of dendritic spines in granule cells of the dentate gyrus," *The Journal of Neuroscience*, vol. 2, no. 3, pp. 284–291, 1982.

[49] M. A. Sutton and E. M. Schuman, "Dendritic protein synthesis, synaptic plasticity, and memory," *Cell*, vol. 127, no. 1, pp. 49–58, 2006.

[50] K. C. Martin and R. S. Zukin, "RNA trafficking and local protein synthesis in dendrites: an overview," *The Journal of Neuroscience*, vol. 26, no. 27, pp. 7131–7134, 2006.

[51] E. M. Schuman, J. L. Dynes, and O. Steward, "Synaptic regulation of translation of dendritic mRNAs," *The Journal of Neuroscience*, vol. 26, no. 27, pp. 7143–7146, 2006.

[52] G. Aakalu, W. B. Smith, N. Nguyen, C. Jiang, and E. M. Schuman, "Dynamic visualization of local protein synthesis in hippocampal neurons," *Neuron*, vol. 30, no. 2, pp. 489–502, 2001.

[53] H. Kang and E. M. Schuman, "A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity," *Science*, vol. 273, no. 5280, pp. 1402–1406, 1996.

[54] K. G. Reymann and J. U. Frey, "The late maintenance of hippocampal LTP: requirements, phases, 'synaptic tagging', 'late-associativity' and implications," *Neuropharmacology*, vol. 52, no. 1, pp. 24–40, 2007.

[55] S. Hüttelmaier, D. Zenklusen, M. Lederer et al., "Spatial regulation of β-actin translation by Src-dependent phosphorylation of ZBP1," *Nature*, vol. 438, no. 7067, pp. 512–515, 2005.

[56] M. E. Klein, P. E. Castillo, and B. A. Jordan, "Coordination between translation and degradation regulates inducibility of mGluR-LTD," *Cell Reports*, vol. 10, no. 9, pp. 1459–1466, 2015.

[57] K. E. Lukong and S. Richard, "Motor coordination defects in mice deficient for the Sam68 RNA-binding protein," *Behavioural Brain Research*, vol. 189, no. 2, pp. 357–363, 2008.

[58] M. P. Paronetto, V. Messina, E. Bianchi et al., "Sam68 regulates translation of target mRNAs in male germ cells, necessary for mouse spermatogenesis," *The Journal of Cell Biology*, vol. 185, no. 2, pp. 235–249, 2009.

[59] S. J. Taylor and D. Shalloway, "An RNA-binding protein associated with Src through its SH2 and SH3 domains in mitosis," *Nature*, vol. 368, no. 6474, pp. 867–871, 1994.

[60] T. Chen, B. B. Damaj, C. Herrera, P. Lasko, and S. Richard, "Self-association of the single-KH-domain family members Sam68, GRP33, GLD-1, and Qk1: role of the KH domain," *Molecular and Cellular Biology*, vol. 17, no. 10, pp. 5707–5718, 1997.

[61] J. J. Derry, S. Richard, H. V. Carvajal et al., "Sik (BRK) phosphorylates sam68 in the nucleus and negatively regulates its RNA binding ability," *Molecular and Cellular Biology*, vol. 20, no. 16, pp. 6114–6126, 2000.

[62] Q. Lin, S. J. Taylor, and D. Shalloway, "Specificity and determinants of Sam68 RNA binding. Implications for the biological function of K homology domains," *The Journal of Biological Chemistry*, vol. 272, no. 43, pp. 27274–27280, 1997.

[63] M. Costa-Mattioli, W. S. Sossin, E. Klann, and N. Sonenberg, "Translational control of long-lasting synaptic plasticity and memory," *Neuron*, vol. 61, no. 1, pp. 10–26, 2009.

[64] K. M. Huber, M. S. Kayser, and M. F. Bear, "Role for rapid dendritic protein synthesis in hippocampal mGluR-dependent long-term depression," *Science*, vol. 288, no. 5469, pp. 1254–1257, 2000.

[65] C. Lüscher and K. M. Huber, "Group 1 mGluR-dependent synaptic long-term depression: mechanisms and implications for circuitry and disease," *Neuron*, vol. 65, no. 4, pp. 445–459, 2010.

[66] B. D. Auerbach, E. K. Osterweil, and M. F. Bear, "Mutations causing syndromic autism define an axis of synaptic pathophysiology," *Nature*, vol. 480, no. 7375, pp. 63–68, 2011.

[67] S. A. Buffington, W. Huang, and M. Costa-Mattioli, "Translational control in synaptic plasticity and cognitive dysfunction," *Annual Review of Neuroscience*, vol. 37, pp. 17–38, 2014.

[68] C. G. Gkogkas, A. Khoutorsky, I. Ran et al., "Autism-related deficits via dysregulated eIF4E-dependent translational control," *Nature*, vol. 493, no. 7432, pp. 371–377, 2013.

[69] E. Santini, T. N. Huynh, A. F. MacAskill et al., "Exaggerated translation causes synaptic and behavioural aberrations associated with autism," *Nature*, vol. 493, no. 7432, pp. 411–415, 2013.

[70] H. Y. Zoghbi and M. F. Bear, "Synaptic dysfunction in neurodevelopmental disorders associated with autism and intellectual disabilities," *Cold Spring Harbor Perspectives in Biology*, vol. 4, 2012.

[71] M. Qin, J. Kang, T. V. Burlin, C. Jiang, and C. B. Smith, "Postadolescent changes in regional cerebral protein synthesis: an *in vivo* study in the *Fmr1* null mouse," *The Journal of Neuroscience*, vol. 25, no. 20, pp. 5087–5095, 2005.

[72] J. C. Darnell and E. Klann, "The translation of translational control by FMRP: therapeutic targets for FXS," *Nature Neuroscience*, vol. 16, no. 11, pp. 1530–1536, 2013.

[73] M. F. Bear, K. M. Huber, and S. T. Warren, "The mGluR theory of fragile X mental retardation," *Trends in Neurosciences*, vol. 27, no. 7, pp. 370–377, 2004.

[74] P.-Y. Deng, Z. Rotman, J. A. Blundon et al., "FMRP regulates neurotransmitter release and synaptic information transmission by modulating action potential duration via BK channels," *Neuron*, vol. 77, no. 4, pp. 696–711, 2013.

[75] L. Ferron, M. Nieto-Rostro, J. S. Cassidy, and A. C. Dolphin, "Fragile X mental retardation protein controls synaptic vesicle exocytosis by modulating N-type calcium channel density," *Nature Communications*, vol. 5, article 3628, 2014.

[76] Y. Zhang, M. R. Brown, C. Hyland et al., "Regulation of neuronal excitability by interaction of fragile X mental retardation protein with slack potassium channels," *The Journal of Neuroscience*, vol. 32, no. 44, pp. 15318–15327, 2012.

[77] N.-P. Tsai, J. R. Wilkerson, W. Guo et al., "Multiple autism-linked genes mediate synapse elimination via proteasomal degradation of a synaptic scaffold PSD-95," *Cell*, vol. 151, no. 7, pp. 1581–1594, 2012.

[78] W. Zhao, S.-C. Chuang, S. R. Young, R. Bianchi, and R. K. S. Wong, "Extracellular glutamate exposure facilitates group I mGluR-mediated epileptogenesis in the hippocampus," *The Journal of Neuroscience*, vol. 35, no. 1, pp. 308–315, 2015.

[79] A. de Bartolomeis, G. Latte, C. Tomasetti, and F. Iasevoli, "Glutamatergic postsynaptic density protein dysfunctions in synaptic plasticity and dendritic spines morphology: relevance to schizophrenia and other behavioral disorders pathophysiology, and implications for novel therapeutic approaches," *Molecular Neurobiology*, vol. 49, no. 1, pp. 484–511, 2014.

[80] D. Tsuboi, K. Kuroda, M. Tanaka et al., "Disrupted-in-schizophrenia 1 regulates transport of ITPR1 mRNA for synaptic plasticity," *Nature Neuroscience*, vol. 18, no. 5, pp. 698–707, 2015.

[81] A. S. L. Ng, R. Rademakers, and B. L. Miller, "Frontotemporal dementia: a bridge between dementia and neuromuscular disease," *Annals of the New York Academy of Sciences*, vol. 1338, pp. 71–93, 2015.

[82] R. Gulino, S. Forte, R. Parenti, and M. Gulisano, "TDP-43 as a modulator of synaptic plasticity in a mouse model of spinal motoneuron degeneration," *CNS & Neurological Disorders—Drug Targets*, vol. 14, no. 1, pp. 55–60, 2015.

[83] R. Gulino, R. Parenti, and M. Gulisano, "Novel mechanisms of spinal cord plasticity in a mouse model of motoneuron disease," *BioMed Research International*, vol. 2015, Article ID 654637, 10 pages, 2015.

[84] B. Bingol and M. Sheng, "Deconstruction for reconstruction: the role of proteolysis in neural plasticity and disease," *Neuron*, vol. 69, no. 1, pp. 22–32, 2011.

[85] N. P. Dantuma and L. C. Bott, "The ubiquitin-proteasome system in neurodegenerative diseases: precipitating factor, yet part of the solution," *Frontiers in Molecular Neuroscience*, vol. 7, article 70, 2014.

[86] H.-C. Tai and E. M. Schuman, "Ubiquitin, the proteasome and protein degradation in neuronal function and dysfunction," *Nature Reviews Neuroscience*, vol. 9, no. 11, pp. 826–838, 2008.

[87] A. C. Belin and M. Westerlund, "Parkinson's disease: a genetic perspective," *FEBS Journal*, vol. 275, no. 7, pp. 1377–1383, 2008.

[88] A. M. Dueñas, R. Goold, and P. Giunti, "Molecular pathogenesis of spinocerebellar ataxias," *Brain*, vol. 129, no. 6, pp. 1357–1370, 2006.

[89] M. Field, P. S. Tarpey, R. Smith et al., "Mutations in the BRWD3 gene cause X-linked mental retardation associated with macrocephaly," *The American Journal of Human Genetics*, vol. 81, no. 2, pp. 367–374, 2007.

[90] G. Froyen, M. Corbett, J. Vandewalle et al., "Submicroscopic duplications of the hydroxysteroid dehydrogenase HSD17B10 and the E3 ubiquitin ligase HUWE1 are associated with mental retardation," *The American Journal of Human Genetics*, vol. 82, no. 2, pp. 432–443, 2008.

[91] M. Lalande and M. A. Calciano, "Molecular epigenetics of Angelman syndrome," *Cellular and Molecular Life Sciences*, vol. 64, no. 7-8, pp. 947–960, 2007.

[92] P. S. Tarpey, F. L. Raymond, S. O'Meara et al., "Mutations in *CUL4B*, which encodes a ubiquitin E3 ligase subunit, cause an X-linked mental retardation syndrome associated with aggressive outbursts, seizures, relative macrocephaly, central obesity, hypogonadism, pes cavus, and tremor," *American Journal of Human Genetics*, vol. 80, no. 2, pp. 345–352, 2007.

[93] B. Bingol and E. M. Schuman, "Activity-dependent dynamics and sequestration of proteasomes in dendritic spines," *Nature*, vol. 441, no. 7097, pp. 1144–1148, 2006.

[94] M. D. Ehlers, "Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system," *Nature Neuroscience*, vol. 6, no. 3, pp. 231–242, 2003.

[95] J. J. Yi and M. D. Ehlers, "Ubiquitin and protein turnover in synapse function," *Neuron*, vol. 47, no. 5, pp. 629–632, 2005.

[96] D. J. Moore, "Parkin: a multifaceted ubiquitin ligase," *Biochemical Society Transactions*, vol. 34, no. 5, pp. 749–753, 2006.

[97] A. W. Lin and H.-Y. Man, "Ubiquitination of neurotransmitter receptors and postsynaptic scaffolding proteins," *Neural Plasticity*, vol. 2013, Article ID 432057, 10 pages, 2013.

[98] L. A. Schwarz and G. N. Patrick, "Ubiquitin-dependent endocytosis, trafficking and turnover of neuronal membrane proteins," *Molecular and Cellular Neuroscience*, vol. 49, no. 3, pp. 387–393, 2012.

[99] E. Pavlopoulos, P. Trifilieff, V. Chevaleyre et al., "Neuralized1 activates CPEB3: a function for nonproteolytic ubiquitin in synaptic plasticity and memory storage," *Cell*, vol. 147, no. 6, pp. 1369–1383, 2011.

[100] M. J. Bianchetta, T. T. Lam, S. N. Jones, and M. A. Morabito, "Cyclin-dependent kinase 5 regulates PSD-95 ubiquitination in neurons," *The Journal of Neuroscience*, vol. 31, no. 33, pp. 12029–12035, 2011.

[101] S. H. Lee, L. Liu, Y. T. Wang, and M. Sheng, "Clathrin adaptor AP2 and NSF interact with overlapping sites of GluR2 and play distinct roles in AMPA receptor trafficking and hippocampal LTD," *Neuron*, vol. 36, no. 4, pp. 661–674, 2002.

[102] M. Joch, A. R. Ase, C. X.-Q. Chen et al., "Parkin-mediated monoubiquitination of the PDZ protein PICK1 regulates the activity of acid-sensing ion channels," *Molecular Biology of the Cell*, vol. 18, no. 8, pp. 3105–3118, 2007.

[103] A. Citri, G. Soler-Llavina, S. Bhattacharyya, and R. C. Malenka, "*N*-methyl-D-aspartate receptor- and metabotropic glutamate receptor-dependent long-term depression are differentially regulated by the ubiquitin-proteasome system," *The European Journal of Neuroscience*, vol. 30, no. 8, pp. 1443–1450, 2009.

[104] R. Fonseca, R. M. Vabulas, F. U. Hartl, T. Bonhoeffer, and U. V. Nägerl, "A balance of protein synthesis and proteasome-dependent degradation determines the maintenance of LTP," *Neuron*, vol. 52, no. 2, pp. 239–245, 2006.

[105] L. Hou, M. D. Antion, D. Hu, C. M. Spencer, R. Paylor, and E. Klann, "Dynamic translational and proteasomal regulation of fragile X mental retardation protein controls mGluR-dependent long-term depression," *Neuron*, vol. 51, no. 4, pp. 441–454, 2006.

[106] A. Karpova, M. Mikhaylova, U. Thomas, T. Knöpfel, and T. Behnisch, "Involvement of protein synthesis and degradation in long-term potentiation of Schaffer collateral CA1 synapses," *The Journal of Neuroscience*, vol. 26, no. 18, pp. 4949–4955, 2006.

[107] V. C. Nalavadi, R. S. Muddashetty, C. Gross, and G. J. Bassell, "Dephosphorylation-induced ubiquitination and degradation of FMRP in dendrites: a role in immediate early mGluR-stimulated translation," *The Journal of Neuroscience*, vol. 32, no. 8, pp. 2582–2587, 2012.

[108] J. Huang, Y. Ikeuchi, M. Malumbres, and A. Bonni, "A Cdh1-APC/FMRP ubiquitin signaling link drives mGluR-dependent synaptic plasticity in the mammalian brain," *Neuron*, vol. 86, no. 3, pp. 726–739, 2015.

[109] M. Li, Y.-H. Shin, L. Hou et al., "The adaptor protein of the anaphase promoting complex Cdh1 is essential in maintaining replicative lifespan and in learning and memory," *Nature Cell Biology*, vol. 10, no. 9, pp. 1083–1089, 2008.

[110] D. Garcia-Arocena and P. J. Hagerman, "Advances in understanding the molecular basis of FXTAS," *Human Molecular Genetics*, vol. 19, no. 1, pp. R83–R89, 2010.

[111] C. Sellier, F. Rau, Y. Liu et al., "Sam68 sequestration and partial loss of function are associated with splicing alterations in FXTAS patients," *The EMBO Journal*, vol. 29, no. 7, pp. 1248–1261, 2010.

[112] T. Iijima, K. Wu, H. Witte et al., "SAM68 regulates neuronal activity-dependent alternative splicing of neurexin-1," *Cell*, vol. 147, no. 7, pp. 1601–1614, 2011.

[113] R. F. Berman, R. A. Buijsen, K. Usdin et al., "Mouse models of the fragile X premutation and fragile X-associated tremor/ataxia syndrome," *Journal of Neurodevelopmental Disorders*, vol. 6, article 25, 2014.

[114] V. Beaumont, N. Zhong, R. Fletcher, R. C. Froemke, and R. S. Zucker, "Phosphorylation and local presynaptic protein synthesis in calcium- and calcineurin-dependent induction of crayfish long-term facilitation," *Neuron*, vol. 32, no. 3, pp. 489–501, 2001.

[115] A. Casadio, K. C. Martin, M. Giustetto et al., "A transient, neuron-wide form of CREB-mediated long-term facilitation can be stabilized at specific synapses by local protein synthesis," *Cell*, vol. 99, no. 2, pp. 221–237, 1999.

[116] H. H. Yin, M. I. Davis, J. A. Ronesi, and D. M. Lovinger, "The role of protein synthesis in striatal long-term depression," *The Journal of Neuroscience*, vol. 26, no. 46, pp. 11811–11820, 2006.

[117] A. M. Taylor, N. C. Berchtold, V. M. Perreau, C. H. Tu, N. Li Jeon, and C. W. Cotman, "Axonal mRNA in uninjured and regenerating cortical mammalian axons," *The Journal of Neuroscience*, vol. 29, no. 15, pp. 4697–4707, 2009.

[118] S. B. Christie, M. R. Akins, J. E. Schwob, and J. R. Fallon, "The FXG: a presynaptic Fragile X granule expressed in a subset of developing brain circuits," *The Journal of Neuroscience*, vol. 29, no. 5, pp. 1514–1524, 2009.

[119] M. Kundel, K. J. Jones, C. Y. Shin, and D. G. Wells, "Cytoplasmic polyadenylation element-binding protein regulates neurotrophin-3-dependent β-catenin mRNA translation in developing hippocampal neurons," *The Journal of Neuroscience*, vol. 29, no. 43, pp. 13630–13639, 2009.

[120] Y. Sasaki, K. Welshhans, Z. Wen et al., "Phosphorylation of zipcode binding protein 1 is required for brain-derived neurotrophic factor signaling of local β-actin synthesis and growth cone turning," *The Journal of Neuroscience*, vol. 30, no. 28, pp. 9349–9358, 2010.

[121] H. L. Zhang, T. Eom, Y. Oleynikov et al., "Neurotrophin-induced transport of a beta-actin mRNP complex increases beta-actin levels and stimulates growth cone motility," *Neuron*, vol. 31, no. 2, pp. 261–275, 2001.

[122] M. Spillane, A. Ketschek, T. T. Merianda, J. L. Twiss, and G. Gallo, "Mitochondria coordinate sites of axon branching through localized intra-axonal protein synthesis," *Cell Reports*, vol. 5, no. 6, pp. 1564–1575, 2013.

[123] J. Baleriola, C. A. Walker, Y. Y. Jean et al., "Axonally synthesized ATF4 transmits a neurodegenerative signal across brain regions," *Cell*, vol. 158, no. 5, pp. 1159–1172, 2014.

[124] J. Blumenthal and I. Ginzburg, "Zinc as a translation regulator in neurons: implications for P-body aggregation," *Journal of Cell Science*, vol. 121, no. 19, pp. 3253–3260, 2008.

[125] J. Tcherkezian, P. A. Brittis, F. Thomas, P. P. Roux, and J. G. Flanagan, "Transmembrane receptor DCC associates with protein synthesis machinery and regulates translation," *Cell*, vol. 141, no. 4, pp. 632–644, 2010.

[126] E. Koenig, R. Martin, M. Titmus, and J. R. Sotelo-Silveira, "Cryptic peripheral ribosomal domains distributed intermittently along mammalian myelinated axons," *The Journal of Neuroscience*, vol. 20, no. 22, pp. 8390–8400, 2000.

[127] S. Viswanathan, M. E. Williams, E. B. Bloss et al., "High-performance probes for light and electron microscopy," *Nature Methods*, vol. 12, no. 6, pp. 568–576, 2015.

[128] M. E. Tanenbaum, L. A. Gilbert, L. S. Qi, J. S. Weissman, and R. D. Vale, "A protein-tagging system for signal amplification in gene expression and fluorescence imaging," *Cell*, vol. 159, no. 3, pp. 635–646, 2014.

[129] O. Steward and P. M. Falk, "Polyribosomes under developing spine synapses: growth specializations of dendrites at sites of synaptogenesis," *Journal of Neuroscience Research*, vol. 13, no. 1-2, pp. 75–88, 1985.

[130] O. Steward and P. M. Falk, "Protein-synthetic machinery at postsynaptic sites during synaptogenesis: a quantitative study of the association between polyribosomes and developing synapses," *The Journal of Neuroscience*, vol. 6, no. 2, pp. 412–423, 1986.

[131] O. Steward and T. M. Reeves, "Protein-synthetic machinery beneath postsynaptic sites on CNS neurons: association between polyribosomes and other organelles at the synaptic site," *The Journal of Neuroscience*, vol. 8, no. 1, pp. 176–184, 1988.

[132] N. Maggio and A. Vlachos, "Synaptic plasticity at the interface of health and disease: new insights on the role of endoplasmic reticulum intracellular calcium stores," *Neuroscience*, vol. 281, pp. 135–146, 2014.

[133] M. S. Rook, M. Lu, and K. S. Kosik, "CaMKIIα 3′ untranslated region-directed mRNA translocation in living neurons: visualization by GFP linkage," *The Journal of Neuroscience*, vol. 20, no. 17, pp. 6385–6393, 2000.

[134] A. R. Buxbaum, Y. J. Yoon, R. H. Singer, and H. Y. Park, "Single-molecule insights into mRNA dynamics in neurons," *Trends in Cell Biology*, vol. 25, no. 8, pp. 468–475, 2015.

[135] L. E. Ostroff, J. C. Fiala, B. Allwardt, and K. M. Harris, "Polyribosomes redistribute from dendritic shafts into spines with enlarged synapses during LTP in developing rat hippocampal slices," *Neuron*, vol. 35, no. 3, pp. 535–545, 2002.

[136] N. M. Sosanya, L. P. Cacheaux, E. R. Workman, F. Niere, N. I. Perrone-Bizzozero, and K. F. Raab-Graham, "Mammalian target of rapamycin (mTOR) tagging promotes dendritic branch variability through the capture of Ca^{2+}/calmodulin-dependent

protein kinase II α (CaMKIIα) mRNAs by the RNA-binding protein HuD," *The Journal of Biological Chemistry*, vol. 290, no. 26, pp. 16357–16371, 2015.

[137] A. Govindarajan, I. Israely, S.-Y. Huang, and S. Tonegawa, "The dendritic branch is the preferred integrative unit for protein synthesis-dependent LTP," *Neuron*, vol. 69, no. 1, pp. 132–146, 2011.

[138] H. Makino and R. Malinow, "Compartmentalized versus global synaptic plasticity on dendrites controlled by experience," *Neuron*, vol. 72, no. 6, pp. 1001–1011, 2011.

[139] S. H. Chang, H. S. Chung, N. K. Fontaine et al., "Mode division multiplexed optical transmission enabled by all-fiber mode multiplexer," *Optics Express*, vol. 22, no. 12, pp. 14229–14236, 2014.

[140] J. H. Lee, E. R. Daugharthy, J. Scheiman et al., "Highly multiplexed subcellular RNA sequencing in situ," *Science*, vol. 343, no. 6177, pp. 1360–1363, 2014.

[141] L. G. Tilney and M. S. Tilney, "The actin filament content of hair cells of the bird cochlea is nearly constant even though the length, width, and number of stereocilia vary depending on the hair cell location.," *The Journal of Cell Biology*, vol. 107, no. 6, pp. 2563–2574, 1988.

[142] J. F. Ross and M. Orlowski, "Growth-rate-dependent adjustment of ribosome function in chemostat-grown cells of the fungus *Mucor racemosus*," *Journal of Bacteriology*, vol. 149, no. 2, pp. 650–653, 1982.

[143] Z. B. Katz, A. L. Wells, H. Y. Park, B. Wu, S. M. Shenoy, and R. H. Singer, "β-actin mRNA compartmentalization enhances focal adhesion stability and directs cell migration," *Genes and Development*, vol. 26, no. 17, pp. 1885–1890, 2012.

[144] S. Saha, M. M. Mundia, F. Zhang et al., "Arginylation regulates intracellular actin polymer level by modulating actin properties and binding of capping and severing proteins," *Molecular Biology of the Cell*, vol. 21, no. 8, pp. 1350–1361, 2010.

[145] F. Zhang, S. Saha, S. A. Shabalina, and A. Kashina, "Differential arginylation of actin isoforms is regulated by coding sequence-dependent degradation," *Science*, vol. 329, no. 5998, pp. 1534–1537, 2010.

[146] L. L. Orefice, E. G. Waterhouse, J. G. Partridge, R. R. Lalchandani, S. Vicini, and B. Xu, "Distinct roles for somatically and dendritically synthesized brain-derived neurotrophic factor in morphogenesis of dendritic spines," *The Journal of Neuroscience*, vol. 33, no. 28, pp. 11618–11632, 2013.

[147] D. C. Dieterich, J. J. L. Hodas, G. Gouzer et al., "In situ visualization and dynamics of newly synthesized proteins in rat hippocampal neurons," *Nature Neuroscience*, vol. 13, no. 7, pp. 897–905, 2010.

[148] K. M. Huber, S. M. Gallagher, S. T. Warren, and M. F. Bear, "Altered synaptic plasticity in a mouse model of fragile X mental retardation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 11, pp. 7746–7750, 2002.

[149] J. M. Halstead, T. Lionnet, J. H. Wilbertz et al., "An RNA biosensor for imaging the first round of translation from single cells to living animals," *Science*, vol. 347, no. 6228, pp. 1367–1671, 2015.

Hindawi Publishing Corporation
Neural Plasticity
Volume 2016, Article ID 8056370, 26 pages
http://dx.doi.org/10.1155/2016/8056370

Review Article

Dendritic Spines in Depression: What We Learned from Animal Models

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Received 7 July 2015; Accepted 26 November 2015

Academic Editor: Maurizio Popoli

Depression, a severe psychiatric disorder, has been studied for decades, but the underlying mechanisms still remain largely unknown. Depression is closely associated with alterations in dendritic spine morphology and spine density. Therefore, understanding dendritic spines is vital for uncovering the mechanisms underlying depression. Several chronic stress models, including chronic restraint stress (CRS), chronic unpredictable mild stress (CUMS), and chronic social defeat stress (CSDS), have been used to recapitulate depression-like behaviors in rodents and study the underlying mechanisms. In comparison with CRS, CUMS overcomes the stress habituation and has been widely used to model depression-like behaviors. CSDS is one of the most frequently used models for depression, but it is limited to the study of male mice. Generally, chronic stress causes dendritic atrophy and spine loss in the neurons of the hippocampus and prefrontal cortex. Meanwhile, neurons of the amygdala and nucleus accumbens exhibit an increase in spine density. These alterations induced by chronic stress are often accompanied by depression-like behaviors. However, the underlying mechanisms are poorly understood. This review summarizes our current understanding of the chronic stress-induced remodeling of dendritic spines in the hippocampus, prefrontal cortex, orbitofrontal cortex, amygdala, and nucleus accumbens and also discusses the putative underlying mechanisms.

1. Introduction

Depression, a severe psychiatric disorder [1, 2], affects up to 20% of the population in the US within their lifetime and is more prevalent in women than men [3–6]. Although depression has been studied for decades, its cellular and molecular mechanisms still remain largely unknown [7]. As many as 30–40% of patients with major depressive disorder have treatment-resistant depression which does not respond to currently available antidepressant therapies [8]. It is therefore important to identify the mechanisms underlying depression in order to develop effective therapeutic strategies.

Chronic stress, especially psychosocial stressors in humans, is one well-known risk factor for the development of depression [6, 9–13]. Enhancement of neuronal plasticity is essential for adaptive intracellular changes during the normal stress response, which promotes dendritic growth, new synapse formation, and facilitates neuronal protein synthesis in the face of an acute challenge. In addition, a successful stress response requires continuity of the response to ensure normal brain function and promote survival [9, 14, 15]. On the one hand, brief or moderate stressors actually enhance neural function in most cases, while severe or chronic stressors are detrimental and can disrupt the ability of the brain to maintain its normal stress response, eventually leading to depression [15–18]. Furthermore, it has been shown that significant but brief stressful events (acute stress) result in the differentiation of stem cells into new nerve cells that improve the mental performance of rats [19]. On the other hand, chronic stress increases the levels of the stress hormone glucocorticoid and suppresses the production of new neurons in the hippocampus. This response results in decreased dendritic spine density and synapse number and impaired memory [17, 20–24]. The relationship between stress and psychiatric diseases has been well established for 20 years in the clinic [25, 26]. Chronic stress paradigms in rodents, the classical animal model of depression, recapitulate many of the core behavioral features

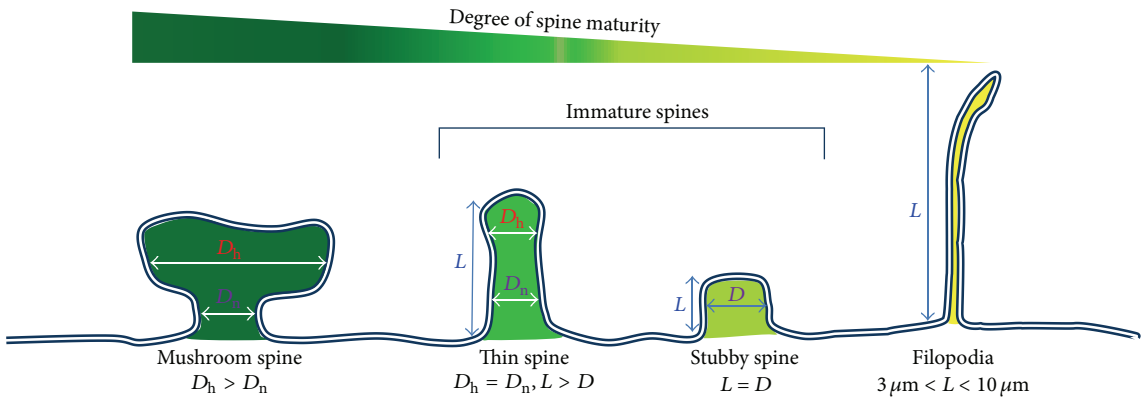

FIGURE 1: Diagram of dendritic spines. Dendritic spines are categorized into mushroom, thin, and stubby spines. Length of spine (L), diameter of spine head (D_h), and diameter of spine neck (D_n). Filopodia are the precursor of dendritic spine.

of depression and respond to antidepressant treatments [10, 23, 27]. However, the precise nature of relationships among the effects of chronic stress, the dysregulation of spine/synapse plasticity, and the molecular mechanisms of depression remain poorly understood [9]. This minireview summarizes our current understanding, obtained from animal models of chronic stress, of remodeling of dendritic spines in five regions of the brain during depression.

2. The Plasticity of Dendritic Spines

Dendritic spines are tiny membranous protrusions from the dendritic shaft of various types of neurons. They typically receive excitatory input from axons, although sometimes both inhibitory and excitatory connections are present on the same spine. Over 90% of all excitatory synapses that occur in the CNS are localized to dendritic spines [60], which are cellular substrates of brain connectivity and the major sites of information processing in the brain [61, 62]. Billions of neurons contact and communicate with each other via synapses. It is widely accepted that the regulation of dendritic spine number, size, and shape is of importance to the plasticity of synapses, as well as learning and memory [63, 64]. The morphology of spines is highly variable and commonly categorized into three types: thin, mushroom, and stubby (Figure 1) [65, 66]. Large mushroom spines are memory spines carrying more biochemical signals [67, 68] and a number of human disease states are associated with alterations with spine morphology and/or spine density [69]. Spines are thin if the length is greater than the neck diameter and the diameters of the head and neck are similar (Figure 1). Spines are classified as mushrooms if the diameter of the head is greater than the diameter of the neck. Spines are considered stubby if the length and width are equal. Spines are defined when they are no longer than three μm [70]. The length of dendritic filopodia is normally >3 μm and <10 μm. The normal dendritic spine density ranges from 0.2 to 3.5 spines per 1 μm of dendrite depending on the neuron type, age, and position along the dendrite as well as the method of counting [71]. Thin and stubby spines, as well as dendritic filopodia, are prevalent during development. Thin and stubby spines are considered to be immature, plastic spines. Dendritic filopodia are precursors of dendritic spines [60, 72]. The spine neck is an important structure for a mushroom spine to perform its normal function because the spine neck prevents Ca^{2+} exchange between the spine head and dendrite shaft. This is important for the regulation of synaptic transmission and may be neuroprotective, preventing excitotoxicity to the dendrite and neuron by restricting excessive influxes of Ca^{2+} within the synaptic region [69, 73]. Different spine types may serve different functions and changes in the ratio of these spines may have a greater effect on neuronal excitability and function [74]. It is generally accepted that thin spines are learning spines, whereas large, mature, and less motile mushroom spines harboring larger and stronger synapses are memory spines that are responsible for the maintenance of neuronal networks and long-term memory [75]. Large mushroom spines with large heads are stable and are likely to contain smooth endoplasmic reticulum, a spine apparatus, polyribosomes, and endosomal compartments in which post-translational modification of proteins, local protein synthesis, local recycling of receptors, and membrane management occur, respectively [64]. Large mushroom spines that contain abundant AMPA receptors are not restricted to pairing with presynaptic axonal terminals containing more synaptic vesicles. They can also associate with presynaptic astroglial terminals, which enhance synapse formation, stabilization, and synapse elimination [64]. Mushroom spines with small heads are motile and unstable and contribute to weak or silent synaptic connections [68].

Dendritic spine pathology is associated with many psychiatric diseases [71, 76–78]. The formation, growth, and elimination of the dendritic spines are precisely controlled, which requires the reorganization of the neural network in response to acute stress or learning processes. These processes are commonly dysregulated or disrupted in chronically stressed animals [46, 79]. Therefore, understanding dendritic spines is fundamental in uncovering the mechanisms underlying depression. It is well established that depression is closely associated with selective structural changes, altered cellular resilience, and neuronal atrophy. Moreover, depression is associated with reduction in astrocytes and reduced/or

increased volume of some brain regions that affect mood and cognition, which involve structural and molecular remodeling of dendritic spines in the hippocampus, prefrontal cortex, amygdala, and nucleus accumbens [7, 23, 49, 62, 80–83]. Antidepressants have reversed some of these structural changes observed in animal models of depression [13, 83, 84]. These studies have generated the hypothesis that alterations of the dendritic spines and the plasticity at excitatory synapses contribute to symptoms of depression [5, 85–88].

3. Chronic Stress and Animal Models of Depression

Animal models are essential tools for studying and understanding specific symptoms of human psychiatric disorders, though none of the current models fully recapitulate stress-related psychiatric disorders described in humans. Most of the current knowledge about the mechanism underlying depression has come from animal models. Several animal models of depression have been used to understand the mechanisms underlying depression [149]. We only discuss the model of chronic stress in this review. Several chronic stress models have been used to model depression-like behaviors in rodents such as chronic restraint stress (CRS), chronic unpredictable stress (CUS), and chronic social defeat stress (CSDS). Behavioral tests of anhedonia (sucrose preference) or despair (forced swim test and tail suspension test) have been widely used to determine depression-like behaviors induced by these three models [150]. Depression-like behaviors induced by these models can often be reversed by chronic antidepressant treatments [27, 86]. It is, however, worth noting that there are some rats or mice that do not respond to traditional antidepressants, which is similar to treatment-resistant depression in human subjects [151]. Here, we briefly summarize our current understanding about these three animal models.

3.1. Chronic Restraint Stress (CRS). CRS has been used widely to study the morphological, hormonal, and behavioral alteration in several brain regions in rodents, such as the hippocampus, prefrontal cortex, amygdala, and nucleus accumbens because it is inexpensive and relatively easy to implement [152] (Tables 1–4). To study dendritic morphology and spine formation, this method typically involves restraining an animal for 1–6 h each day in a restraint device (bag or tube) for a period of 14–21 days or more. A disadvantage of the CRS model is the habituation of rats or mice to repeated exposure to homotypic restraint stressors; the response of plasma corticosterone, the major glucocorticoids in rodents, to the final stressor is diminished in animals that had been stressed for 14 days [153–156]. The pattern of hypothalamic corticotrophin-releasing hormone (CRH) heteronuclear RNA and mRNA responses to CRS is similar to the response of corticosterone, decreasing with increasing frequency of exposure to the repeated restraint stressor [153]. Animals habituate over time and finally show no increase in hypothalamic-pituitary-adrenal (HPA) axis activation and no increase in expression of hypothalamic CRH [30, 153, 156]. The duration of CRS may differentially affect learning/memory and CA3 dendritic atrophy with shorter periods of CRS (7–13 days) serving an adaptive function to enhance learning and memory [157]. On the other hand, longer CRS duration (21 days or more) causes maladaptive changes such as dendrite atrophy, spine loss, and impaired memory [15, 157, 158]. CRS-induced habitation of HPA axis contrasts with the hyperactivity of the HPA axis accompanied by increased CRH levels [43, 159] and the hypersecretion of cortisol [160, 161] in depressed patients, showing that activation of HPA axis is a hallmark of major depression [162, 163]. Depending on duration and intensity of chronic stress, some studies report that exposure of animals to CRS induces depression-like behaviors such as anhedonia (decreased sucrose preference) [164–169], which is a core symptom of human depression [10, 27]. A conflicting report shows CRS could not induce anhedonic-like behavior [170]. The duration and intensity of CRS as well as animal strains may determine whether CRS can be used as a valid animal model of depression to produce anhedonic-like behavior.

3.2. Chronic Unpredictable Mild Stress (CUMS). CUMS is a well-established animal model for depression. The original, three-week chronic unpredictable severe stress (CUS) model with diverse severe and unpredictable stressors (electric shocks, immobilization, cold swimming, isolation housing, and other strong stimuli) was developed by Katz and coworkers [171, 172]. In order to accurately recapitulate the human condition, Willner and colleagues replaced severe stressors in Katz's model with mild stressors. Additionally, Willner and colleagues augmented the CUMS model with a variety of mild and unpredictable stressors (e.g., overnight illumination; presence of novel objects; periods of food and/or water deprivation; cage tilt; change of cage mate) [173]. In Willner's model, exposure of animals to 7–13 mild stressors up to 3 months produced a longer lasting depression-like behavior, anhedonia [173–175]. The CUS model used in Duman's group was modified from Willner's model. In Duman's model, animals were exposed to 10 [108, 176] or 12 [106] unpredictable stressors, 2 times per day, for up to 35 days, which produced depression-like behaviors. The duration of CUS is 21 days for the experiments using CUS alone or 35 days for the experiment using CUS together with antidepressant treatments [106, 108, 176]. It is worth noting that CUS model used by Duman's group is different from the CUMS protocol, not only in the duration and number of stressors/day, but also at the level of stressor intensity (rotation on a shaker 1 hour, cold 4°C 1 hour, lights off for 3 hours, lights on overnight, strobe light overnight, aversive odor overnight, 45° tilted cages overnight, food and water deprivation overnight, crowded housing overnight, and isolation housing overnight) [108, 176]. The modified CUMS model used in our laboratory consists of daily exposure of animals to 8 chronic unpredictable mild stressors, one stressor per day, for 21 days. The same stressor is not applied in two consecutive days [24, 177]. The different abbreviations of chronic unpredictable mild stress (CUS, CMS, or CUMS) were used in several modified versions by different laboratories. We use CUMS as a common denotation in this review. In comparison with the CRS model, CUMS overcomes stress habituation of the

Table 1: The effects of chronic stress on dendritic spines in hippocampus.

#	Stress	Paradigms	Animals	CA1	CA3	References
1	CRS	6 h/day for 21 days	Male SD rats	nd	Apical, not basal dendritic atrophy	[28]
2	CRS	6 h/day for 21 days	Male SD rats	nd	↑ spine density in apical, basal dendrites	[29]
3	CRS or multiple stress (CMS): 3 different stressors	CRS, 6 h/day for 21 days CMS: 3 stressors/day for 21 days	Male SD rats	nd	Apical dendritic atrophy; CORT habituates to 21-day CRS but not 21-day CMS	[30]
4	CRS	6 h/day for 21 days	Male SD rats	nd	Apical dendritic atrophy is blocked by cyanoketone or CGP43487	[31]
5	CRS	6 h/day for 21 days	Male SD rats	nd	↑ synaptic vesicle density in MFT	[32]
6	CRS	6 h/day for 21 days	Male SD rats	nd	Apical dendritic atrophy, recovery after 10 days ↓ spine density	[33]
7	CRS	6 h/day for 21 days	Adult male Wister rats	nd	↑ excitatory MF-CA3 synapses, recovery after maze learning	[34]
8	Acute restraint plus intermittent tail shock	30 shocks: 1 mA, 1 s, 1/min	Adult male and female SD rats	↑ spine density in male and ↓ in female apical dendrites, both 100% blocked by CPP	nd	[35]
9	CRS	6 h/day for 21 days	Male Wistar rats	nd	↓ PSD number; ↓ spine density in apical dendrites Retraction of dendritic TE with ↓ in their volume	[36]
10	CRS	6 h/day for 21 days	Adult SD adult female rats	↔ dendritic atrophy ↑ spine density ↑ spine size	Apical dendritic atrophy Spine density, nd	[37, 38]
11	CRS	6 h/day for 21 days	Male Wistar rats	↑ PSD surface and ↑ PSD volume; ↔ excitatory synapses in stratum	nd	[39]
12	CRS	6 h/day for 21 days	C57/BL6 male Wt mice	↓ spine density in apical dendrite ↓ NR1, NR2B, NR2A, and GAP43	These decreases are tPA and plasminogen dependent	[40]
13	CRS	6 h/day for 21 days	C57/BL6 male Wt mice	↔ dendritic atrophy; ↓ total spine density, ↔ stubby spines ↓ thin and mushroom spine density	Apical, not basal dendritic atrophy ↔ total spine density, ↑ stubby spines, ↓ thin and mushroom spines	[41]
14	CRS	6 h/day for 21 days	Adult SD female rats	↔ dendritic atrophy ↑ spine density ↑ mushroom spine	Apical dendritic atrophy ↓ spine density	[42]
15	CRS	2.5 h/day for 14 days	Male rats	↓ spine density in apical dendrites	nd	[43]
16	CRS	6 h/day for 21 days	Adult SD male rats	↑ spine density	Apical dendritic atrophy, ↓ spine density, and ↑ spinophilin and Homer1	[44]
17	CRS	6 h/day for 21 days	Female mice	↓ spine density in apical dendrites	nd	[45]
18	CRS	2.5 h/day for 14 days	Adult male SD rats	↓ spine density, ↓ cadherin, and ↔ LIMK/cofilin and p-LIMK/p-cofilin	nd	[46]

Table 1: Continued.

#	Stress	Paradigms	Animals	CA1	CA3	References
19	CRS	6 h/day for 25 days	Female, male Long-Evans rats	↓ spine density in basilar dendrites; ↑ apical dendritic arbors in female, not male ventral CA1	Deficits in spatial memory in female but not male	[47]
20	CRS	6 h/day for 21 days	Adult male mice	↓ spine density; ↓ p-Akt, ↓ p-GSK-3β, and ↓ p-Erk1/2	nd	[48]
21	CUMS	1 stressor/day for 30 days	Male Wister rats	↔ apical dendrite	Apical dendritic atrophy; ↓ MF-CA3 synapses	[49]
22	CUMS	2 stressors/day for 10 days	Male Wister rats	nd	↔ CA3 dendrites	[50]
23	CUMS	1 stressor/day for 21 days	Male SD rats	↓ CA1 spine density	↓ CA3 spine density, ↓ Kalirin-7 protein in hippocampus	[24]
24	CUMS	1 stressor/day for 14 days	Male mice	nd	↑ CA3 spine density	[51]
25	CUMS	1 stressor/day for 8 weeks	Male SD rat	↓ PSD thickness in CA1 ↓ PSD95 protein	↓ PSD93, ↓ PSD95, ↓ SYN, ↓ spinophilin, and ↓ synapsin 1	[52]
26	CUMS	2-3 stressors/day for 21–35 days	Adult SD rats	Impaired AMPAR-synaptic excitation at TA-CA1 synapses ↓ GluR1 and PSD95	Induces depression-like behaviors	[53]
27	CUMS	2 stressors/day for 28 days	Male C57/b mice	↓ mGlu2 receptors in susceptible, not resilient mice	mGLu2 deletion in mice results in a more severe susceptibility to stress	[54]
28	Multimodal stress	Adult male C57BL/6J mice	5 h	↓ synapse numbers in dorsal apical dendrites ↓ PSD-95-ir puncta	↓ synapse numbers in dorsal CA3 apical ↓ PSD-95-ir puncta	[55]
29	Psychosocial stress	1 h/day for 28 days	Male tree shrews	nd	Apical, not basal dendritic atrophy ↔ spine density	[56]
30	Psychosocial stress	1 h/day for 28 days	Male rats	nd	Apical dendritic atrophy	[57]
31	Chronic CORT exposure	3-4 weeks	Male SD rats	Impaired AMPAR-synaptic excitation at TA-CA1 synapses ↓ GluR1 protein	Induces depression-like behaviors	[58]
32	CORT exposure	35 days	C57/BL6 male mice	↓ CA1 thin and stubby spine density, but not mushroom spines	↔ CA3 spine density	[59]

CRS: chronic restraint stress. CUMS: chronic unpredictable mild stress. TA: temporoammonic. CORT: corticosterone. MFT: mossy fiber terminals. TE: thorny excrescences in the stratum lucidum of CA3. ↔: no change. ↓: decrease. ↑: increase. nd: not done.

HPA axis occurring during stress, in which the response of plasma corticosterone to the final stressor is still sustained in animals which had been stressed for 15 to 35 days [27, 30, 106, 155]. Depression-like behaviors and deficits in synaptic plasticity are gradually developed during CUMS [24, 173]. The CUMS model recapitulates many of the core behavioral characteristics of human depression that are reversible by chronic treatments with traditional antidepressant agents [10, 27] and is more relevant to human disease. Therefore, the CUMS model has been widely used as an animal (specifically rat) model of depression. Our results show that, during CUMS, rats require three weeks to develop depression-like behaviors accompanied by both functional changes in CA3-CA1 synapses and decreased spine density in the dendrites of CA1 and CA3 pyramidal neurons [24, 177]. This is in line with Willner's CUMS paradigm [173], in which animals were exposed to initial unpredictable stress for three weeks to develop depression-like behaviors prior to the onset of antidepressant treatments. Because of its advantage of the gradual development of depression-like behaviors during CUMS [24, 175], this model is useful in studying depression-like behaviors such as anhedonia [27, 86, 174, 178]. In addition, this CUMS model is useful for inducing depression-like behaviors in female mice because chronic social defeat

Table 2: The effects of chronic stress on dendritic spines in the prefrontal cortex (PFC).

#	Stress	Paradigms	Animals	PFC	Proteins	References
1	CRS	6 h/day for 21 days	Male SD rats	↓ apical dendrite of layers II and III mPFC		[76]
2	CRS	3 h/day for 21 days	Male SD rats	Apical dendrite atrophy ↔ basal dendrites in PL mPFC		[89]
3	CRS	6 h/day for 21 days, 21 day recovery	Male SD rats	↓ apical dendrite length, reversible after 21 d in mPFC		[90]
4	CRS	6 h/day for 21 days	Male SD rats	↓ 20% apical dendritic length, ↓ spine density in PL mPFC		[91]
5	CRS	6 h/day for 21 days, 21-day recovery	Male SD rats	↓ 20% apical dendritic arbors in mPFC		[92]
6	CRS	6 h/day for 21 days	Male SD rats	↓ mushroom spine density ↑ thin spine number in PL mPFC		[93]
7	CRS	1 h/day for 7 days	Male SD rats	↓ spine density in PL mPFC		[94]
8	CRS	6 h/day for 21 days	Male SD rats	↓ apical spine density in apical dendrites Inhibition of PKC prevents spine loss		[95]
9	CRS	6 h/day for 21 days/with 21-day recovery	Male SD rats	↓ apical dendrite arbors, ↓ spine density; partial recovery of dendrites and spine loss in IL mPFC		[96]
10	CRS	3 h/day for 7 days	Male and female SD rats	↓ apical dendrite arbors in male, ↑ apical dendrite arbors in female, which is estradiol dependent in mPFC		[97]
11	CRS	6 h/day for 21 days	Male SD young and aged rats	↓ apical dendrite arbors in young, but not aged, rats are reversible; ↓ spine density in young, but not aged, rats		[98]
12	CRS	6 h/day for 21 days	Male SD rats young, middle-aged, and aged	↓ spine density (↓ thin and stubby spines, ↔ mushroom spines) in young but not middle-aged and aged rats in PL mPFC		[99]
13	CRS	6 h/day for 21 days	Male SD rats	↑ mRNA levels of VAMP2, VAMP1, syntaxin 1A, synapsin, synaptotagmins I and III, and synapsins I and II ↓ SNAP-25 mRNA level	↑ protein levels of VAMP2, syntaxin 1A, and SNAP-25	[100]
14	CRS	2 h/day for 7 days	Adult male WT mice	↓ spine density in mPFC; ↓ apical dendrites	↓ BDNF	[101]
15	CRS	1 h/day for 21 days	Male GIN mice	↔ spine density in mPFC	↑ NCAM, SYN	[102]
16	CRS	6 h/day for 21 days	Male SD rats	↓ spine density in PL mPFC	Alpha-2A-adrenoceptor	[103]
17	CRS	3 h/day for 21 days	Male SD rats PL mPFC	↓ dendritic retraction is prevented by D1R antagonist SCH23390 that causes dendritic retraction in unstressed rats		[104]
18	CRS	2 h/day for 7 days	Male SD rats	↓ glutamatergic transmission in PFC pyramidal neurons		[105]
19	CUMS	15 days or 35 days	Male SD rats	35% ↓ cell proliferation in neocortex		[106]
20	CUMS	3 stressors/day for 21 days	Male Wistar rats	↓ volume of layer I/II of PL and IL ↓ neuronal density of layer II of PL and IL Apical dendritic atrophy in PL and IL ↔ spine density tends to decrease in PL and IL		[107]

Table 2: Continued.

#	Stress	Paradigms	Animals	PFC	Proteins	References
21	CUMS	2 stressors/day for 21 days	Male SD rats	↓ spine density in mPFC; ↓ synapsin I, GluR1, and PSD95		[108]
22	CUMS	1 stressors/day for 21 days	Male SD rats	↓ synaptic length of the active zone in CG1 mPFC ↓ PSD thickness in PL; ↓ PSD93, ↓ PSD95, ↓ spinophilin in CG1 and PL	↓ spinophilin and synapsin 1 in CG1	[52]
23	CIS	2 h/day for 10 days	Male SD rats	↔ apical dendrites in IL-BLA projecting neurons in IL mPFC Apical dendritic atrophy in random selected neurons in IL mPFC ↔ spine density in IL mPFC		[109]
24	Depressed patients		Postmortem dorsolateral PFC	↓ synapse number in dorsolateral PFC, ↓ synaptic-function-related genes	GATA1 ↑ Rab4b ↓	[110]
25	CORT, vehicle	daily injection for 21 days	Male SD rats	↑ spine density proximal to the soma		[111]
26	Forced swim	10 min/day for 3 days	Adult male C57BL/6J mice	↓ apical dendrites in IL mPFC ↔ basal dendrites in IL mPFC; ↔ apical and basal dendrites in PL mPFC		[112]
27	Early-life stress	3 h/day on postnatal days 1–14	Male Wistar rats	↓ spine density in apical and basal dendrites in mPFC	GluR1, GluR2, αCaMKII, and PSD95 ↑	[113]

CRS: chronic restraint stress. CUMS: chronic unpredictable mild stress. CIS: chronic immobilization stress. PL: prelimbic region of the mPFC. IL: infralimbic region of the mPFC. CG1: area 1 of cingulate region of mPFC. CORT: corticosterone. ↔: no change. ↓: decrease. ↑: increase.

stress protocol cannot successfully induce depression-like behaviors in C57BL/6J female mice [179]. A recent report shows that C57BL/6 mice, one of the most widely used mouse strains, are resistant to the commonly used CUMS protocol due to the variety of genetically modified lines. A recently revised, eight-week CUMS protocol has been developed and used to induce depression-like behaviors in C57BL/6 mice [180]. Interestingly, male and female rodents are differentially affected by CUMS, depending on the behavioral and neurobiological markers that are being measured [181].

3.3. Chronic Social Defeat Stress (CSDS). CSDS is one of the most frequently used rodent models for depression and has been used to induce depression-like behaviors in mice such as social avoidance and anhedonia [86, 144, 182–185]. During each defeat period, an intruder, a male C57BL/6J mouse, is allowed to interact for 10 minutes with an aggressive and large CD1 mouse during which the intruder is rapidly investigated, attacked, and defeated by the resident CD-1 mouse. The experimental C57BL/6J mice are exposed to a different resident aggressor for 10 minutes each day for 10 consecutive days [183, 184, 186–188]. On the one hand, after completing the social defeats, 30% of animals do not show depression-like behaviors known as "resilient," a positive adaptation in the face of stress, threat, or severe adversity [189, 190]. On the other hand, a majority of animals (70%) develop depression-like behaviors known as "susceptible." A disadvantage of this model is that it is limited in studying only male mice because female C57BL/6J mice are not easily defeated by CD-1 mice [86]. This model has been widely used to induce depression-like behaviors and study the molecular mechanisms underlying depression [139, 141, 142, 146, 149, 191, 192]. This model is also used to induce depression-like behaviors in rats [192, 193].

4. The Effects of Chronic Stress on Dendritic Spines in Different Brain Regions

4.1. Hippocampus (Table 1). The hippocampus plays an important role in learning and memory and is particularly sensitive to stress and glucocorticoids [194, 195]. Rodent hippocampus contains high levels of glucocorticoid receptors (GRs) and mineralocorticoid-like receptors (MRs). The affinity of MR for corticosterone is 6- to 10-fold higher than that of GR, but it is GR that is activated after stress and is involved in its feedback action on stress-induced neural plasticity [196]. Chronic stress decreases GR expression or its numbers and finally alters the balance of GR/MR in the male hippocampus [197, 198], which is thought to be a protective mechanism against the damaging effects of chronic stress. Chronic exposure of male rats to glucocorticoids induces depression-like behaviors and causes the synaptic deficits in the hippocampus [58]. A recent report shows that GRs, acting via MR, decrease resilience to stress via downregulation of mGlu2 receptors in mice during CUMS [54]. Chronic stress and glucocorticoids impair hippocampal function, which in turn contributes to the HPA axis dysregulation [195, 198]. The blunting of the feedback mechanism is believed to underlie

Table 3: The effects of chronic stress on dendritic spines in the amygdala.

#	Stress	Paradigms	Animals	Amygdala	Function	Proteins	References
1	CRS	6 h/day for 21 days	Wt C57/BL/6 mice	↓ spine density in WT medium spiny stellate neurons MeA, ↑ spine density in Wt BLA	↔ spinogenesis in BLA OF tPA−/− mice	tPA−/− mice reverse stress-induced reduction of spine density in MeA	[114]
2	CRS	6 h/day for 28 days	Male young, Wistar rats	↔ spine density in MePD			[115]
3	CRS	1 h/day for 10 days	Male ICR mice	↓ eIPSC, ↑ LTD GABAergic synapse in BLA	MAGL inhibition prevents depression-like behavior	2-AG ↑, MAGL ↓	[116, 117]
4	CRS	2 h/d for 10 days	Male Wt mice	↑ BLA dendritic branching ↑ spine density in BLA apical and basal dendrites ↑ spine length ↑ anxiety behavior	Fmr1 KO mice fail to show anxiety	In Fmr1 KO mice ↔ spine length in BLA ↓ spine density in BLA	[118]
5	CRS	1 h/day for 21 days	Male GIN mice	↔ spine density, ↓ dendritic arborization in interneurons in LA and BLA		GAD67, synaptophysin and PSA-NCAM ↓	[119]
6	CRS	2 h/day for 10 days	Male ICR mice	↑ dendritic length and branch points in BLA, which are blocked by tianeptine	Depression-like behaviors are blocked by tianeptine	Tianeptine is an antidepressant	[120]
7	CRS	1 h/day for 14 days	Male SD rats	Impaired LTP in the NAc 30 days after stress termination	CB1/2R agonist prevents the stress-impaired LTP	↓ GRs in amygdala and NAc	[121]
8	CRS	20 min/day 7 out of 9 days	Male SD rats	↑ dendritic length in BLA, ↑ spine density in LA and BA, but proximal increase in LA, nonproximal increases in BA	↑ frequency of sEPSC *in vivo*		[122, 123]
9	CRS	6 h/day for 21 days	C57/Bl6 mice	↑ dendritic arborization ↑ spine density in BLA ↑ anxiety-like behaviors	CRS-induced changes in structure and behaviors are abolished in FAAH KO mice		[124]
10	Acute restraint stress CRS	Single 1 h Single 6 h 6 h/day for 28 days	Male young adult	↓ Spine density in the posterodorsal MePD ↔ spine density in MePD			[115]
11	CIS CUMS	2 h/d for 10 days 10 days	Male Wistar rats	↑ dendritic arborization in BLA pyramidal and stellate neurons Dendritic atrophy in BLA bipolar neurons			[50]
12	CIS	2 h/d for 10 days	Male Wistar rats	↔ dendrites in CeA ↑ dendrites in BNST			[125]

Table 3: Continued.

#	Stress	Paradigms	Animals	Amygdala	Function	Proteins	References
13	CIS	2 h/d for 10 days	Male Wistar rats	↑ dendritic length in BLA			[126]
14	CIS	2 h/d for 10 days	Male Wistar rats	↑ spine density in the BLA			[127]
15	CIS	2 h/d for 21 days	Male Wistar rats	↑ dendritic arborization BLA, ↑ spine density ↑ synaptic connectivity	↑ anxiety-like behavior		[128]
16	CIS	2 h/day for 10 days	Male Wistar rats	↑ spine density in BLA LTP ↑ (thalamic-LA)	sIPSC frequency ↓		[129]
17	AIS	2 h	Male Wistar rats	↔ spine density or dendritic arborization 1 d later, ↑ spine density 10 d later in BLA			[127]
18	CUMS	8 weeks	Adult male SD rats	↑ synaptic length of the active zone in BLA ↑ PSD thickness in BLA	↑ synaptic proteins are correlated with depression-like behaviors	↓ PSD93, ↔ PSD95, and ↔ spinophilin ↔ synapsin ↔ synaptophysin	[52]
19	CUMS	14 days	Male Swiss albino mice	↑ spine density in BLA ↑ dendritic length in BLA	Associated with depression-like behaviors		[51]
20	Chronic CORT	20 days	C57BL/6 mice	↑ spine density in BLA, recovery to normal level with a washout period			[130]
21	CORT drinking water	50 μg/mL for 14 days	Adult male SD rats		↑ GluR1 and synaptophysin in the LA	↑ IEGs Arc/Arg3.1 and Egr-1 in the LA	[131]
22	Single prolonged stress	2 h restraint, 20 min forced swimming	Adult male SD rats	↑ dendritic arborization in BLA ↔ in CeA neurons		↑ NPY ↔ CaMKII and MR/GR expression in the BLA	[132]
23	Single elevated platform acute stress	30 min, single	Male SD rats	↑ total spine density ↑ mushroom spine density in BLA; ↓ number and the length of branches in BLA			[133]
24	Chronic social instability stress	1 h/day for 35 days	Adolescent 28-day-old SD rat Adult, 56-day-old SD rat	↓ spine density in BLA ↑ spine density in BLA		↑ truncated TrkB, ↓ full-length TrkB and SNAP-25 ↑ full-length and truncated TrkB	[134]

CRS: chronic restraint stress. CUMS: chronic unpredictable mild stress. CIS: chronic immobilization stress. AIS: acute immobilization stress. BA: the basal nucleus of the amygdala. BLA: the basolateral amygdala. LA: the lateral nucleus of the amygdala. MePD: posterodorsal medial amygdala. eCB: endocannabinoid. 2-AG: eCB 2-arachidonoylglycerol. MAGL: monoacylglycerol lipase, an enzyme for degrading 2-AG. CORT: corticosterone. Tianeptine: an antidepressant. ↔: no change. ↓: decrease. ↑: increase.

Table 4: The effects of chronic stress on dendritic spines on nucleus accumbens (NAc).

#	Stress	Paradigms	Animals	NAc	Function	Proteins or mRNA	References
1	CRS	10 days	Male D1R and D2R mice	↓ AMPAR/NMDAR ratio in D1R-MSNs via MC4R; induces LTD in D1R-MSN	MC4R activation and LTD in NAc are required for stress-induced anhedonia	MC4Rs, **α-MSH**	[135]
2	CRS	1 h/day for 14 days	Male SD rats	Chronic cannabinoid exposure prevents impaired memory via CB1	CB1/2 receptor agonist prevents CRS-induced-impairment LTP in NAc and in the spatial task	↓ glucocorticoid receptors in the Amg, NAc, PFC, and hippocampus	[121]
3	CUMS	3 stressors/day for 21 days	Male Wistar rats	↑ neuron density in DMS; ↓ neuron density in DLS; ↑ dendritic length in DLS; ↔ spine density in DS			[107]
4	CSDS	10 min/day for 10 days	Male C57/BL6J and CD1		↑ ΔFosB induced by CSDS is required for resilience	↑ ΔFosB in resilience mice	[136]
5	CSDS	10 min/day for 10 days	Male C57/BL6 and CD1		↓ fEPSP in NAc; disrupted NMDAR-dependent LTD in cortico-NAc	↓ NR2B surface and PSD95 in NAc; ↔ NR2A, Syn and NR1	[137]
6	CSDS	5 min/each total 3 times	C57BL/6J and CD1 mice	↑ IκK activity ↑ thin spine density in MSNs	IκK enhances social avoidance behavior	↑ inhibitor of κB kinase (IκK)	[138]
7	CSDS	10 min/day for 10 days	C57BL6/J and CD1 mice	↑ stubby spine density in MSNs in susceptible mice	↑ frequency of mEPSCs in NAc of susceptible mice	↑ IκB kinase (IκK) in NAc in susceptible mice	[139]
8	CSDS	10 min/day for 10 days	C57BL6/J and CD1 mice	↑ Dnmt3a levels in NAc ↑ spine density in NAc	Dnmt3a regulates depression-like behaviors	↑ DNA methyltransferases (Dnmt3a)	[140]
9	CSDS	10 min/day for 10 days	Male C57/BL6 and CD1	↑ stubby spine density in MSNs in susceptible mice in a Rac-1-dependent manner	↑ cofilin puncta colocalization with stubby spines	↓ Rac-1 mRNA levels in NAc of susceptible mice ↓ Rac-1 mRNA levels in NAc of depressed patients	[141]
10	CSDS	10 min/day for 10 days	Male C57/BL6 and CD1	Excitatory transmission at ILT-NAc MSN synapses controls susceptibility to CSDS	↑ AMPAR/NMDAR ratio only at ILT inputs to MSNs of susceptible mice	↑ Vglut22, but not Vglut1 in MSN of susceptible mice	[142]
11	CSDS	10 min/day for 10 days	Male C57/BL6 and CD1	↑ uEPSC amplitude in D1R ↓ uEPSC amplitude in D2R mushroom, not thin spines in NAC MSNs in resilient, but not susceptible mice	CSDS does affect uEPSC amplitude mushroom or thin spines of D1-MSNs or D2-MSNs in susceptible mice		[143]
12	CSDS	10 min/day for 10 days	C57BL6/J and CD1 mice	DNA microarrays; some genes specific to susceptibility in VTA and NAC are identified in susceptible mice	↑ firing in VTA DA neurons in susceptible mice	↑ BDNF, Akt, GSK-3β, and ERK1/2 in NAc of susceptible mice	[144]
13	CSDS	10 min/day for 3 days	Male C57/bl6 and CD1	↓ sIPSC frequency in NAc in control, not stressed mice	↑ sensitivity of striatal GABA synapses to the stimulation of cannabinoid CB1R	CB1R ↓	[145]

Table 4: Continued.

#	Stress	Paradigms	Animals	NAc	Function	Proteins or mRNA	References
14	CSDS	10 min/day for 10 days	Male C57/bl6 and CD1	↑ vHIP-NAc synaptic transmission is prosusceptible ↓ LTD of vHIP-NAc synaptic transmission is proresilient	vHIP afferents to NAc uniquely regulate susceptibility to CSDS ↓ activity in vHIP in mice resilient to CSDS		[146]
15	Emotional (ES) and physical stress (PS)	10 min/day for 10 days	Male C57/bl6 adolescent (P35) or adult (P56) and CD1	↑ spine density in NAc in adolescents by ES and PS ↑ spine density in NAc in adult only by PS		ES and PS ↓ p-ERK2 in adolescents but ↑ p-ERK2 in adult	[147]
16	Prenatal stress		Male and female rats	↑ spine density in NAc			[148]

Syn: synaptophysin. CRS: chronic restraint stress. CUMS: chronic unpredictable mild stress. CIS: chronic immobilization stress. CSDS: chronic social defeat stress. MC4R: melanocortin 4 receptor. DMS: dorsal medium striatum. DLM: dorsal lateral striatum. DS: dorsal striatum. ILT: intralaminar thalamus. MSNs: medium spiny neurons. NAc: nucleus accumbens. vHIP: ventral hippocampus. uEPSCs: unitary excitatory postsynaptic currents. ↔: no change. ↓: decrease. ↑: increase.

sustained high levels of glucocorticoids in some depressed patients [199]. People with depression have a significantly smaller hippocampus than healthy individuals [200–205], which may result from a decrease in dendritic arbors and spine density in hippocampal neurons. Hippocampal atrophy in depressed patients is associated with depression severity [206].

CA1 and CA3 Dendrites. Many structural and functional studies show that dendritic retraction or atrophy, characterized by both reduction in total dendritic length and a simplification of dendritic arbors, is found in the dendrites of CA3 pyramidal neurons but not the dendrites of CA1 pyramidal neurons in response to CUMS [49] or CRS [37, 38, 41, 42] (Table 1). Therefore, CA3 dendrites are more sensitive to chronic stress than CA1 dendrites. The different sensitivity of CA1 and CA3 to chronic stress may result from the differences between these two regions in afferents/efferents, the levels of GRs, NMDA receptors, 5-HT receptors, and GABA inhibitory tones [207–211]. GR levels are higher in the CA1 region than the CA3 region, where the receptors are activated by stress hormone corticosteroids [209, 212]. In addition, it has been repeatedly shown that apical dendrites of CA3 pyramidal neurons are more susceptible to the effects of sustained CRS than CA3 basal dendrites. Dendritic retraction in apical but not basal dendrites of CA3 pyramidal neurons is found after CUMS [49], chronic psychosocial stress [56, 57], and CRS [28, 30, 31, 33, 37, 38, 41, 42, 44, 49, 56, 213, 214]. CRS-induced depression-like behaviors and CA3 dendritic atrophy are not permanent but recovered to control levels after certain stress-free period following the end of CRS procedure [33, 49, 158, 213, 215]. Importantly, CA3 dendritic retraction induced by CRS requires corticosterone secretion and intact NMDAR function. Treatments of chronically stressed rats with either the steroid synthesis blocker cyanoketone or competitive NMDA receptor antagonist (CGP 43487) blocked CRS-induced dendritic retraction [31]. Similar to CUMS, rats usually require three weeks to develop depression-like behaviors and CA3 apical dendritic atrophy because only 21 days, but not 7 to 13 days of CRS, induces reversible impairments of spatial memory performance and CA3 apical dendritic atrophy [157, 158]. In addition, atrophy of apical dendrites, but not basal dendrites of CA3 pyramidal neurons, is found after chronic exposure to elevated glucocorticoid levels, which mimics chronic stress [216]. Chronic stress-induced hippocampal CA3 dendritic retraction and elevated glucocorticoid release contribute to impaired spatial memory [217].

CA3 Dendritic Spines. Chronic stress-induced alterations of spine density in CA3 pyramidal neurons depend on stressor types, animal species, sex, and the duration of stress. CRS causes either a decrease [30, 33, 36, 42, 44], an increase [29, 34], or no change [56] in the spine density in the dendrites of male rat CA3 pyramidal neurons. CRS-induced loss of synapses in male rat CA3 apical dendrites can be recovered following water maze training [34, 36]. One report shows that CRS causes a decrease in dendritic spine density, especially in thin and mushroom spines in mouse CA1 pyramidal neurons, but does not affect total spine density in mouse CA3 pyramidal neurons, due to increased stubby spine density and decreased thin and mushroom spine density [41]. The degree of stress-induced spine loss in CA3 pyramidal neurons correlates significantly with the memory defects and loss of LTP in mice [79]. In comparison with CRS, both 21-day CUMS and 30-day CUMS decrease spine density in male rat CA3 pyramidal neurons [24, 49], whereas 14-day CUMS increases spine density in male mouse CA3 pyramidal neurons [51], which is consistent with our report that two-week CUMS enhances LTP induction in CA3-CA1 synapses in male rat hippocampus [24]. Psychosocial stress (1 h/day for 28 days) does not affect spine density in CA3 pyramidal neurons of male tree shrews [56].

CA1 Dendritic Spines. CA1 is a hippocampal region crucial for long-term memory [218]. In comparison with CA3 pyramidal neurons, chronic stress-induced changes in spine density in CA1 pyramidal neurons are less characterized. Stress affects spine density in CA1 pyramidal neurons in a sex-dependent manner. Acute stress (30, 1 sec, 1 mA, 60 Hz shocks to the tail) increases spine density in the apical dendrites of male hippocampal CA1 pyramidal neurons but decreases it in the same area of female hippocampus [219]. These increases and decreases in spine density are dependent on NMDA receptor activation [35]. Similar to acute stress, the same CRS regimen causes a decrease in spine density in the apical dendrites of hippocampal CA1 pyramidal neurons in male rat and male mouse [40, 43, 46, 48] but causes an increase in spine density in the same region in female rats [37, 38, 42]. One recent study shows that CRS decreases spine density in basal dendrites, while it increases apical dendritic arbors in the CA1 pyramidal neurons of the ventral hippocampus in female but not in male rats [47]. In contrast to female rats, female mice show a decrease in spine density in CA1 pyramidal after exposure to same 21-day CRS [45]. Additionally, an ultrastructural study of CA1 synapses shows that 21-day CRS causes an increase in the size of the postsynaptic density in male rat CA1 [39]. Similar to CRS, CUMS also causes a decrease in spine density in the dendrites of CA1 pyramidal neurons in male rat [24]. Stress-induced increase in spine density in the apical dendrites of CA1 pyramidal neurons in female rat and same stress-induced decrease in spine density in the same area in male rat are completely prevented by NMDA receptor antagonist CPP [35, 219], but exposure of NMDA receptor antagonist CPP to the stress procedure does not affect corticosterone levels or the corticosterone response to stress, suggesting a key role of NMDA receptor activation in stress-induced increases or decreases in spine density [35]. Similar to sex-dependent alterations of dendritic spines induced by both acute stress and CRS in hippocampal CA1 pyramidal neurons, there is a sex difference in CRS-induced changes in hippocampal-dependent spatial learning and memory. CRS impairs spatial learning and memory in males but not in females [38, 197]. Furthermore, recent studies suggest that CUMS-induced glutamatergic dysfunction in excitatory temporoammonic- (TA-) CA1 synapses of the hippocampus serves as an underlying cause of depression

[53, 220]. This suggests that restoring spine loss or excitatory synaptic dysfunction in the hippocampus could be a novel therapeutic target for depression. Similar to CUMS, chronic exposure of male rats to corticosterone for 3-4 weeks induces depression-like behaviors and causes a decrease in AMPAR-mediated excitation at temporoammonic-CA1 synapses accompanied by decreased expression of GluR1 protein. Blocking CUMS-induced increase of corticosterone during CUMS with the corticosterone synthesis inhibitor metyrapone prevents stress-induced depression-like behaviors [58]. Similar to male rats, exposure of male mice to 35-day corticosterone treatments shows anxiety/depression-like behaviors, accompanied by a reduction in spine density, mainly in thin and stubby spines but not in mushroom spines in CA1 pyramidal neurons [59]. Mushroom spines are more stable and resistant to corticosterone or CRS [46]. Chronic corticosterone-induced decreases in spine density in the hippocampal CA1 pyramidal neurons and depression-like behaviors recover to normal levels concomitantly after 25-day treatment with fluoxetine [59]. These studies suggest that corticosterone secreted during stress plays a key role in chronic stress-induced depression-like behaviors, dysfunction of excitatory synapses, and alteration of dendritic spines in the hippocampus; rescuing chronic stress-induced loss of dendritic spines and/or synaptic dysfunction may rescue depression-like behaviors.

4.2. Prefrontal Cortex (PFC) (Table 2). The medial PFC (mPFC), an information processing center, is often divided into the anterior cingulate, prelimbic (PL), and infralimbic (IL) subregions. These subregions are different in structure and function [221]. The mPFC plays a critical role in the integration of cognitive and emotionally relevant information, modulation of subcortical systems, and attention [222–225]. The mPFC expressing high levels of glucocorticoid receptors [226] is a target site for glucocorticoids and plays an important role in the regulation of the response of HPA axis to stress and antidepressant response [225, 227, 228]. It is widely reported that the mPFC volume is decreased in a subset of depressed patients [201, 205, 229–233]. However, a recent report shows that the decreased volume of the mPFC is found in male but not in female depressed patients [234]. The decreased volume of the mPFC in depressed patients [201, 205, 229, 230] is in line with decreased expression of synaptic-function-related genes and loss of synapses in the mPFC of subjects with major depression disorder [110]. In addition, glial cell loss, reductions in the density and size of neurons in the postmortem mPFC of subjects with major depression, may contribute to pathology of depression [235, 236]. Animal studies show that the retraction of apical dendrites of pyramidal neurons in the mPFC induced by chronic stress is accompanied by alterations in fear conditioning and extinction [112]. CRS-induced dendritic retraction and spine loss in the hippocampal and mPFC neurons are accompanied by cognitive impairments, which are mediated by each respective structural alteration [92, 109, 217].

It is well documented that CRS results in a retraction of the distal part of apical dendritic arbors of layers II/III pyramidal cells [76, 89, 90, 92] and a decrease in spine density on those neurons [91, 93, 96, 237] in the mPFC of male rats, which is similar to that found in hippocampal CA3 region [41, 42, 44]. The pattern of CRS-induced dendritic reorganization is similar to that seen after daily corticosterone injections [238, 239]. CRS also alters spine morphology with an overall decrease in mean dendritic spine volume and surface area, a reduction in large mushroom spine density, and an increase in small thin spine density in the mPFC of male rats. These findings suggest failure of the spines to mature and stabilize following CRS [93]. One conflicting study, however, reports that CRS-induced decrease in spine density in the male rat mPFC is characterized by a decrease in thin and stubby spine density without affecting mushroom spine density [99].

CRS causes a reduction of length and branch number in the apical dendrites of the neurons in the mPFC of young (3 months) and aged (20 months) male rats. Surprisingly, CRS-induced retraction of apical dendrites, however, is reversed with recovery in young (3 months) but not aged (20 months) animals [98]. In young rats, CRS results in dendritic spine loss and alters the patterns of spine morphology. In contrast, CRS does not affect spine density and spine shape in aged animals, showing that dendritic spines become progressively less plastic in the aging brain [99]. Interestingly, chronic immobilization stress does not affect spine density in a subpopulation of IL neurons in the mPFC that project to the basolateral amygdala (BLA) in male rats, suggesting this pathway may be particularly resilient against the effects of stress [109]. Randomly selected neurons in the IL of the mPFC, however, show dendritic retraction after CRS. Since most layer II/III neurons project intracortically, the majority of randomly selected pyramidal neurons may be local cortical neurons with no projections to the BLA [109]. An independent study reports that IL neurons, but not PL neurons, in the mPFC are highly sensitive to a brief exposure to stress and the same form of stress impairs fear extinction in mice [112]. However, these IL neurons are putative local cortical neurons without projections to the BLA. A conflicting report shows that CRS causes dendritic retraction in PL neurons of rat mPFC, while this dendritic retraction is prevented by the D1R antagonist SCH23390, and the same D1R antagonist causes dendritic retraction in the PL neurons of the mPFC in unstressed rats. However, the effects of CRS on dendrites in the IL neurons of mPFC are not studied in this report [104]. These results show that dopaminergic transmission in the PL neurons of the mPFC during stress may contribute directly to the CRS-induced retraction of apical dendrites, while dopamine transmission in the absence of stress is important in maintaining normal dendritic morphology [104]. Recent reports show that acute foot-shock stress not only produces an increase in the number of excitatory synapses and docked vesicles [240] in the mPFC, but also induces rapid and sustained increases in spine density accompanied by atrophy of apical dendrites in the PL neurons of the mPFC [241]. Importantly, these synaptic changes induced by acute stress are prevented by chronic antidepressant desipramine treatments [240, 241]. Optogenetic activation of the mPFC exerts potent antidepressant-like effects, showing that the activity of the mPFC may play a key role in the development of depression-like behaviors

and antidepressant responses [242]. Similar to hippocampus, alteration of stress-mediated dendritic arbors in the mPFC is sex dependent. CRS causes retraction of apical dendrite arbors in the mPFC in male, while it increases apical dendrite arbors in the female mPFC in which CRS-induced dendritic plasticity is estrogen dependent [97]. Rat mPFC is sexually dimorphic, which is characterized by a bigger and more complex apical dendritic tree in the PL neurons of the mPFC in healthy male rats than that in healthy female rats [243, 244].

4.3. Orbitofrontal Cortex (OFC). The OFC, a part of the PFC in the frontal lobes in the brain, is involved in cognitive functions, decision-making, and emotional processing [245]. The studies from neuroimaging and neuropathology show that the OFC is involved in pathophysiology of major depression [246]. Decreases in cortical thickness, neuronal size, neuronal density, and glia densities in the II–IV cortical layers of the OFC are found in subjects with major depression [236]. The decrease in neuronal sizes in layer 3 of the OFC from depressed subjects is confirmed by another postmortem study [247]. Neuroimaging and functional studies also show that patients with major depression have reduced OFC volume [248] and reduced density of pyramidal neurons in layers V and III of the OFC [249]. In contrast, animal studies show that 3-week CUMS increases both the volume of layers II/III in the lateral orbital subregion and the volume of layer II in the ventral orbital subregion of the OFC, which is accompanied by an increase in the length of apical dendrites in the ventral orbital subregion of the OFC [107]. Interestingly, CRS causes a 43% increase in the dendritic arbors in the OFC neurons, an effect opposite to what is observed in the mPFC neurons where the same CRS causes 20% retraction of apical dendritic arbors in layer II/III pyramidal neurons of the mPFC [92]. The mechanisms through which CRS increases dendritic arbors of the OFC are not known. Further studies are needed to explore the discrepancy between the data from imaging analysis or postmortem studies and the findings from animal models. Our recent study showed that 3-week CUMS caused a decrease in spine density in the OFC pyramidal neurons, which was accompanied by both depression-like behaviors and decreased expression of Kalirin-7 and PSD95 in the OFC (Chang Xu, Shu-Chen An, and Xin-Ming Ma, unpublished). Kalirin-7 plays an essential role in maintaining dendritic spine density, size, and synaptic functions [250, 251]. Expression of Kalirin-7 in the hippocampus is decreased by 3-week CUMS [24]. Similar to CUMS, chronic exposure of male mice to corticosterone for 20 days that recapitulates blood corticosterone levels found after CRS exposure in mice also decreases spine density in the OFC neurons, which fails to recover after one week of washout period [130]. This suggests that chronic stress-induced decrease in spine density is not reversible in the OFC neurons. Additional study is required to address this question.

4.4. Amygdala (Table 3). The amygdala, a structure within the subcortical limbic system, is involved in the processing of emotion and motivation such as fear and anger. The amygdala is also responsible for determining what memories are stored and where they are stored. There are conflicting reports on amygdala volume in major depression [252]. Imaging studies show an increase [253–255] or decrease [256, 257] or no change [258] in amygdala volume or increased activity of amygdala [201, 259, 260] in patients with major depression. A conflicting MRI study reports a trend towards smaller left amygdala volumes in depressed patients compared with healthy controls [203]. A postmortem study shows that depressed subjects have a larger lateral nucleus and a greater number of total BLA neurovascular cells than controls. There are no differences in the number or density of neurons or glia between depressed and control subjects [252]. To our knowledge, it is not clear whether cell size in BLA is altered in depressed patients.

Animal studies show that chronic stress generally results in an increase in spine density and enhanced dendritic arborization in the amygdala (Table 3). This is in contrast to the hippocampus and PFC (Tables 1 and 2). Acute immobilization also causes an increase in spine density without any effects on dendritic arbors in BLA spiny neurons [127], showing that these neurons are very sensitive to stress. Amygdala-dependent fear learning is enhanced by CRS in rats [33]. Chronic stress causes an increase in dendritic arborization and spine density in the BLA spiny neurons of male rats [122, 123, 125–129] and male mice [114, 118, 120, 124]. These neurons are thought to be glutamatergic neurons [261]. In contrast, CRS causes a decrease in spine density in spiny neurons in the medial amygdale, which are GABAergic neurons [114]. CRS-induced increase in dendritic arbors and spine density in the BLA pyramidal neurons and CRS-induced depression-like behavior in wild-type mice are absent in fatty acid amide hydrolase (FAAH) deficient mice [124] suggesting a key role of FAAH in maintaining normal amygdala function in the face of chronic stress. Chronic immobilization stress-induced dendritic hypertrophy in the BLA spiny neurons is not reversible [126]. This is distinct from hippocampal CA3 and mPFC atrophy, which is reversible within the same period of stress-free recovery [33]. A single dose of corticosterone induces dendritic hypertrophy in the BLA accompanied by enhanced anxiety [262]. Chronic exposure of mice to corticosterone for 20 days mimicking chronic stress increases dendritic length and spine density in the BLA [130]. Chronic exposure of rats to corticosterone for 2 weeks causes an increase in the levels of memory-related genes including Arc/Arg3.1 and Egr-1 and enhances the consolidation of fear memory processes in the lateral amygdala [131]. In addition, tianeptine, an antidepressant, exerts the opposite roles in chronic stress-induced synaptic changes in the amygdala and hippocampus [120].

4.5. Nucleus Accumbens (NAc) (Table 4). Animal studies indicate that the neuronal circuitry of the PFC-NAc-ventral tegmental area (VTA) underlies drug reward responses and contributes to relapse to cocaine seeking [263, 264]. Excitatory axonal terminals from glutamatergic neurons of the PFC form the synapse onto NAc medium spiny neurons (MSNs), which also receive dopaminergic (DA) inputs from the VTA. The VTA receives GABAergic inputs from the NAc and glutamatergic inputs from the PFC [265, 266]. In

addition, The NAc also receives glutamatergic inputs from ventral hippocampus and basolateral amygdala [146]. The NAc serves as a hub of the brain's reward pathways [267] and plays a central role in mood and emotion regulation [268]. Depressive symptoms, such as anhedonia, and depression severity are correlated with reduced NAc volume and reduced NAc responses to rewards in depressed patients [205, 269]. An optogenetic study shows that inhibition of the VTA-NAc projection induces resilience, whereas inhibition of the VTA-mPFC projection enhances susceptibility [270], highlighting a key role of PFC-NAc-VTA circuitry in the development of depression. Therefore, dysregulation of PFC-NAc-VTA reward circuitry may contribute to the pathophysiology of depression [13, 146, 271]. Similar to the effect of cocaine abuse, chronic stress may alter dendritic spines and synaptic plasticity in the PFC-NAc-VTA circuitry. A recent study, however, reports that chronic social defeat stress- (CSDS-) mediated increase in glutamatergic transmission at the intralaminar thalamus- (ILT-) NAc but not PFC-NAc circuitry mediates stress-induced postsynaptic plasticity on the MSNs and depression-like behaviors in susceptible mice [142].

The MSNs of dorsal striatum receive not only glutamatergic inputs from the cerebral cortex and the thalamus, but also DA innervation from the midbrain [272]. These MSNs account for >95% of the neurons in the striatum [273, 274]. The dorsal striatum and the NAc are not distinguishable in their populations and expression of DA receptors (DRs, D1R and D2R). Approximately half of the striatal MSNs express the D1R [274, 275]; other half MSNs express the D2R [276, 277]. The degree of D1R/D2R colocalization remains controversial, ranging from 10% to 30% [275, 278, 279]. D1R signaling enhances dendritic excitability and glutamatergic signaling in striatonigral MSNs, while D2R signaling exerts the opposite effect in striatopallidal MSNs (indirect pathway) [280–282]. CRS causes a decrease in AMPAR/NMDAR ratio in the D1R-MSN of the NAc compared to nonstressed control, while it does not affect AMPAR/NMDAR ratios in D2R-MSNs of the NAc. This CRS-induced decrease in the ratio of AMPAR/NMDAR in the D1R-MSN is accompanied by depression-like behaviors, showing a role of NAc D1R-MSNs, at least in part, in the development of depression [135]. This is further supported by two recent reports [143, 283]. One report shows that enhanced activity in D1R-MSNs causes resilient behaviors, while inhibition of these D1R-MSNs induces depression-like behaviors after CSDS [283]. Another report shows that CSDS specifically results in an increase in synaptic strength represented by the increased amplitude of uEPSCs (unitary excitatory postsynaptic currents) in large mushroom spines on D1R-MSNs but decreases synaptic strength on D2R-MSNS mushroom spines in the NAc of resilient mice. CSDS does not affect the uEPSC amplitude in small thin spines on both D1R- and D2R-NAc MSNs in resilient mice [143]. CSDS, however, does not alter synaptic strength in mushroom and thin spines on D1R- or D2R-MSNs in the NAc in susceptible mice [143]. These data show that the NAc D1R-MSN of susceptible mice may be resistant to adaptation and play a critical role in the development of chronic stress-induced depression-like behaviors. In addition, the inhibitor of kappaB kinase (IκK) in the NAc is also a critical regulator of depression-like behavior, and the IκK-nuclear factor kappaB (NFκB) plays a key role in the regulation of synaptic signaling and neuronal morphology *in vitro* and *in vivo* [138]. Overexpression of IκK increases thin spine density in the NAc MSNs. CSDS-induced increase in IκK activity in the NAc enhances social avoidance behavior and promotes the formation of thin spines. Inhibition of IκK signaling results in a reversal of CSDS-induced social avoidance behaviors, suggesting that CSDS-induced depression-like behaviors are associated with IκK-mediated increase in thin spine density in the NAc [138]. Interestingly, CSDS-induced increases in stubby spine density and the frequency of mEPSCs in the NAc in susceptible mice are accompanied by an increase in the levels of IκK in the NAc [139]. These results show that CSDS-induced increases in stubby spine density and IκK expression in the NAc are correlated with depression-like behaviors. CSDS-mediated downregulation of Rac1 through an epigenetic mechanism contributes to depression-like behaviors and enhanced formation of stubby spines in the NAc MSNs of susceptible mice [141]. Furthermore, DeltaFosB, a transcription factor, plays an essential role in the mechanism of resilience in mice, supported by evidence that CSDS-mediated induction of DeltaFosB in the NAc is not only necessary and sufficient for resilience in mice, but also required for the antidepressant fluoxetine to reverse depression-like behaviors induced by CSDS [136]. NR2B in the NAc plays a key role in the modulation of CSDS-induced depression-like behaviors and synaptic plasticity. CSDS-induced reduction in NR2B surface expression in the mouse NAc neurons is restored by fluoxetine treatment. Behaviorally, restoration of NR2B loss prevents the behavioral sensitization of mice to chronic stress [137]. Overexpression of DNA methyltransferase (Dnmt3a) increases dendritic spine density in the NAc MSNs. CSDS-induced depression-like behaviors are accompanied by an increase in the Dnmt3a levels in the NAc, suggesting that CSDS-induced depression-like behaviors are positively correlated with increased spine density in the NAc neurons [140]. These studies highlight an important role of the NAc in chronic stress-induced depression-like behaviors. It is possible that stress may differently affect dendritic spines in the D1R-MSNs and D2R-MSNs of the NAc. More studies are required for a better understanding of the roles of D1R-MSNs and D2R-MSNs in chronic stress-induced depression-like behaviors and the underlying mechanisms.

Reduced NAc volume in depressed patients [205, 269] is not in line with the findings from animal models in which stress generally results in an increase in spine density in the NAc MSNs. CSDS causes an increase in spine density and the frequency of mEPSCs in the mouse NAc MSNs [86]. In addition, the shell of the NAc is thought to be a part of the extended amygdala [284]. Chronic stress increases spine density in the neurons of the BLA and the shell of NAc even though these two neuron types are naturally different. The downstream mechanisms of chronic stress-induced spine formation in these two distinct neuron types are not clear.

Taken together, these data show that altered spine density and synaptic plasticity in the NAc MSNs are correlated with depression-like behaviors induced by chronic stress, which

may be a target for developing the novel treatment strategies for depression.

5. The Mechanisms of Chronic Stress-Induced Alterations in Dendritic Spines

The molecular mechanisms underlying spine loss and dendritic retraction induced by chronic stress in the hippocampus and PFC as well as enhanced spine formation found in the amygdala and NAc in chronically stressed animals are not well understood. Expression of several synapse-related genes is decreased in the postmortem PFC of subjects with major depressive disorder [110]. One of these genes is GATA1 (GATA-binding factor 1), a transcriptional repressor that plays a key role in the formation of dendritic spines and dendrite arbor maintenance [110]. Furthermore, a nuclear pore complex protein, nucleoporin p62 (NUP62), and tyrosine phosphorylation of NUP62 play a critical role in CRS-induced dendritic retraction of hippocampal CA3 pyramidal neurons [285]. Many synaptic proteins including Kalirin-7, spinophilin, Homer1, cofilin, Rac-1, cadherin, p-Akt, p-GSK-3β, p-Erk1/2, PKC, NCAM, PSA-NCAM, SNAP-25, SNAP-29, VAMP1/2, syntaxin 1A, synaptophysin, synapsin 1, Vglut2, GluR1, GluR2, NR1, NR2A, NR2B, PSD95, αCaMKII, melanocortin 4 receptors, CRH receptor 1, and P190RhoGAP play an important role in the regulation of the spine formation and/or synaptic plasticity; expression of these synaptic proteins in the brain is altered by chronic stress, and these proteins may play a key role in chronic stress-induced both depression-like behaviors and spine alterations (Table 1–4) [24, 40, 44, 46, 53, 100, 102, 113, 130, 141, 142, 144, 286–292]. In addition, chronic stress-induced alterations of several signal transduction pathways including cAMP-PKA-CREB, cAMP-ERK1/2-CREB, cAMP-PKA, Ras-ERK, PI3K-Akt, TNFα-Nfκb, GSK-3β, mTOR, and CREB may be also associated with chronic stress-induced spine loss or increase in certain brain areas [7, 22, 293]. A recent report shows that the Homer1/mGluR5 complex is involved in the development of CSDS-induced depression-like behaviors [294], suggesting a role of this complex in chronic stress-mediated spine plasticity. Presynaptic mGlu2 receptors play a key role in CUMS-induced depression-like behaviors in male susceptible mice [54]. The rapid antidepressant-like properties of ketamine, an NMDA receptor antagonist, result from increased synaptic signaling proteins and increased number and function of new spine synapses via activating the mammalian target of rapamycin (mTOR) pathway in the rat mPFC and hippocampus [295–298]. S6K1, a key mediator of activity-dependent synaptic protein synthesis, is the downstream of mTORC1 and plays a critical role in CUMS-induced depression-like behaviors [299]. Postmortem studies show that the levels of NR2A, NR2B, mGLuR5, PSD-95, and mTOR as well as the levels of S6K, eIF4B, and p-eIF4B, the core downstream signaling targets of mTOR, are decreased in the PFC of depressed patients [300]. These studies suggest that mTOR signaling is a promising target for the development of novel antidepressant drugs [297, 301, 302].

Taken together, understanding chronic stress- and/or depression-induced alterations in dendritic spines, synapse plasticity, synaptic proteins, and their upstream/downstream signaling pathways may pave the path for developing efficiency therapeutic strategies for depression. The search for the mechanisms through which chronic stress alters dendritic spines or synapse numbers in different brain regions should be a major future direction.

Conflict of Interests

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

Acknowledgments

This work was supported by National Science Foundation of China (81371512) and Connecticut Innovation Fund (14SCBUCHC11). Thanks are due to Boyu Ma and Dr. Mason Yeh for their reading of the paper.

References

[1] V. Patel, M. Abas, J. Broadhead, C. Todd, and A. Reeler, "Depression in developing countries: lessons from Zimbabwe," *The British Medical Journal*, vol. 322, no. 7284, pp. 482–484, 2001.

[2] K. S. Kendler, M. Gatz, C. O. Gardner, and N. L. Pedersen, "A Swedish national twin study of lifetime major depression," *The American Journal of Psychiatry*, vol. 163, no. 1, pp. 109–114, 2006.

[3] M. R. Levinstein and B. A. Samuels, "Mechanisms underlying the antidepressant response and treatment resistance," *Frontiers in Behavioral Neuroscience*, vol. 8, article 208, 2014.

[4] R. C. Kessler, T. C. Wai, O. Demler, and E. E. Walters, "Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication," *Archives of General Psychiatry*, vol. 62, no. 6, pp. 617–627, 2005.

[5] S. M. Thompson, A. J. Kallarackal, M. D. Kvarta, A. M. Van Dyke, T. A. LeGates, and X. Cai, "An excitatory synapse hypothesis of depression," *Trends in Neurosciences*, vol. 38, no. 5, pp. 279–294, 2015.

[6] D. A. Bangasser and R. J. Valentino, "Sex differences in stress-related psychiatric disorders: neurobiological perspectives," *Frontiers in Neuroendocrinology*, vol. 35, no. 3, pp. 303–319, 2014.

[7] W. N. Marsden, "Synaptic plasticity in depression: molecular, cellular and functional correlates," *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, vol. 43, pp. 168–184, 2013.

[8] X. Zhou, K. D. Michael, Y. Liu et al., "Systematic review of management for treatment-resistant depression in adolescents," *BMC Psychiatry*, vol. 14, article 340, 2014.

[9] P. W. Gold, "The organization of the stress system and its dysregulation in depressive illness," *Molecular Psychiatry*, vol. 20, no. 1, pp. 32–47, 2015.

[10] M. N. Hill, K. G. C. Hellemans, P. Verma, B. B. Gorzalka, and J. Weinberg, "Neurobiology of chronic mild stress: parallels to major depression," *Neuroscience and Biobehavioral Reviews*, vol. 36, no. 9, pp. 2085–2117, 2012.

[11] R. C. Kessler, "The effects of stressful life events on depression," *Annual Review of Psychology*, vol. 48, pp. 191–214, 1997.

[12] C. Kiyohara and K. Yoshimasu, "Molecular epidemiology of major depressive disorder," *Environmental Health and Preventive Medicine*, vol. 14, no. 2, pp. 71–87, 2009.

[13] C. Pittenger and R. S. Duman, "Stress, depression, and neuroplasticity: a convergence of mechanisms," *Neuropsychopharmacology*, vol. 33, no. 1, pp. 88–109, 2008.

[14] J. Herbert, I. M. Goodyer, A. B. Grossman et al., "Do corticosteroids damage the brain?" *Journal of Neuroendocrinology*, vol. 18, no. 6, pp. 393–411, 2006.

[15] J. Radley, D. Morilak, V. Viau, and S. Campeau, "Chronic stress and brain plasticity: mechanisms underlying adaptive and maladaptive changes and implications for stress-related CNS disorders," *Neuroscience & Biobehavioral Reviews*, 2015.

[16] E. R. de Kloet, M. S. Oitzl, and M. Joëls, "Stress and cognition: are corticosteroids good or bad guys?" *Trends in Neurosciences*, vol. 22, no. 10, pp. 422–426, 1999.

[17] B. S. McEwen, "Glucocorticoids, depression, and mood disorders: structural remodeling in the brain," *Metabolism: Clinical and Experimental*, vol. 54, no. 5, pp. 20–23, 2005.

[18] S. J. Lupien and B. S. McEwen, "The acute effects of corticosteroids on cognition: integration of animal and human model studies," *Brain Research Reviews*, vol. 24, no. 1, pp. 1–27, 1997.

[19] E. D. Kirby, S. E. Muroy, W. G. Sun et al., "Acute stress enhances adult rat hippocampal neurogenesis and activation of newborn neurons via secreted astrocytic FGF2," *eLife*, vol. 2013, no. 2, Article ID e00362, 2013.

[20] C. O. Bondi, G. Rodriguez, G. G. Gould, A. Frazer, and D. A. Morilak, "Chronic unpredictable stress induces a cognitive deficit and anxiety-like behavior in rats that is prevented by chronic antidepressant drug treatment," *Neuropsychopharmacology*, vol. 33, no. 2, pp. 320–331, 2008.

[21] Y. Xu, J. Pan, J. Sun et al., "Inhibition of phosphodiesterase 2 reverses impaired cognition and neuronal remodeling caused by chronic stress," *Neurobiology of Aging*, vol. 36, no. 2, pp. 955–970, 2015.

[22] Z. Z. Wang, W. X. Yang, Y. Zhang et al., "Phosphodiesterase-4D knock-down in the prefrontal cortex alleviates chronic unpredictable stress-induced depressive-like behaviors and memory deficits in mice," *Scientific Reports*, vol. 5, Article ID 11332, 2015.

[23] C. H. Duman and R. S. Duman, "Spine synapse remodeling in the pathophysiology and treatment of depression," *Neuroscience Letters*, vol. 601, pp. 20–29, 2015.

[24] H. Qiao, S. C. An, W. Ren, and X. M. Ma, "Progressive alterations of hippocampal CA3-CA1 synapses in an animal model of depression," *Behavioural Brain Research*, vol. 275, pp. 191–200, 2014.

[25] C. Heim, M. J. Owens, P. M. Plotsky, and C. B. Nemeroff, "The role of early adverse life events in the etiology of depression and posttraumatic stress disorder," *Annals of the New York Academy of Sciences*, vol. 821, pp. 194–207, 1997.

[26] R. M. Sapolsky, "Why stress is bad for your brain," *Science*, vol. 273, no. 5276, pp. 749–750, 1996.

[27] P. Willner, "Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS," *Neuropsychobiology*, vol. 52, no. 2, pp. 90–110, 2005.

[28] Y. Watanabe, E. Gould, and B. S. McEwen, "Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons," *Brain Research*, vol. 588, no. 2, pp. 341–345, 1992.

[29] Sunanda, M. S. Rao, and T. R. Raju, "Effect of chronic restraint stress on dendritic spines and excrescences of hippocampal CA3 pyramidal neurons—a quantitative study," *Brain Research*, vol. 694, no. 1-2, pp. 312–317, 1995.

[30] A. M. Magariños and B. S. McEwen, "Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: comparison of stressors," *Neuroscience*, vol. 69, no. 1, pp. 83–88, 1995.

[31] A. M. Magariños and B. S. McEwen, "Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: involvement of glucocorticoid secretion and excitatory amino acid receptors," *Neuroscience*, vol. 69, no. 1, pp. 89–98, 1995.

[32] A. M. Magarinos, J. M. Verdugo, and B. S. McEwen, "Chronic stress alters synaptic terminal structure in hippocampus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 25, pp. 14002–14008, 1997.

[33] C. D. Conrad, J. E. LeDoux, A. M. Magariños, and B. S. McEwen, "Repeated restraint stress facilitates fear conditioning independently of causing hippocampal CA3 dendritic atrophy," *Behavioral Neuroscience*, vol. 113, no. 5, pp. 902–913, 1999.

[34] C. Sandi, H. A. Davies, M. I. Cordero, J. J. Rodriguez, V. I. Popov, and M. G. Stewart, "Rapid reversal of stress induced loss of synapses in CA3 of rat hippocampus following water maze training," *European Journal of Neuroscience*, vol. 17, no. 11, pp. 2447–2456, 2003.

[35] T. J. Shors, J. Falduto, and B. Leuner, "The opposite effects of stress on dendritic spines in male vs. female rats are NMDA receptor-dependent," *European Journal of Neuroscience*, vol. 19, no. 1, pp. 145–150, 2004.

[36] M. G. Stewart, H. A. Davies, C. Sandi et al., "Stress suppresses and learning induces plasticity in CA3 of rat hippocampus: a three-dimensional ultrastructural study of thorny excrescences and their postsynaptic densities," *Neuroscience*, vol. 131, no. 1, pp. 43–54, 2005.

[37] K. J. McLaughlin, S. E. Baran, R. L. Wright, and C. D. Conrad, "Chronic stress enhances spatial memory in ovariectomized female rats despite CA3 dendritic retraction: possible involvement of CA1 neurons," *Neuroscience*, vol. 135, no. 4, pp. 1045–1054, 2005.

[38] K. J. McLaughlin, J. O. Wilson, J. Harman et al., "Chronic 17beta-estradiol or cholesterol prevents stress-induced hippocampal CA3 dendritic retraction in ovariectomized female rats: possible correspondence between CA1 spine properties and spatial acquisition," *Hippocampus*, vol. 20, no. 6, pp. 768–786, 2010.

[39] H. S. Donohue, P. L. A. Gabbott, H. A. Davies et al., "Chronic restraint stress induces changes in synapse morphology in stratum lacunosum-moleculare CA1 rat hippocampus: a stereological and three-dimensional ultrastructural study," *Neuroscience*, vol. 140, no. 2, pp. 597–606, 2006.

[40] R. Pawlak, B. S. S. Rao, J. P. Melchor, S. Chattarji, B. McEwen, and S. Strickland, "Tissue plasminogen activator and plasminogen mediate stress-induced decline of neuronal and cognitive functions in the mouse hippocampus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 50, pp. 18201–18206, 2005.

[41] A. M. Magariños, C. J. Li, J. G. Toth et al., "Effect of brain-derived neurotrophic factor haploinsufficiency on stress-induced remodeling of hippocampal neurons," *Hippocampus*, vol. 21, no. 3, pp. 253–264, 2011.

[42] C. D. Conrad, K. J. McLaughlin, T. N. Huynh, M. El-Ashmawy, and M. Sparks, "Chronic stress and a cyclic regimen of estradiol administration separately facilitate spatial memory: relationship with hippocampal CA1 spine density and dendritic complexity," *Behavioral Neuroscience*, vol. 126, no. 1, pp. 142–156, 2012.

[43] A. Fernández-Guasti, J. L. Fiedler, L. Herrera, and R. J. Handa, "Sex, stress, and mood disorders: at the intersection of adrenal

and gonadal hormones," *Hormone and Metabolic Research*, vol. 44, no. 8, pp. 607–618, 2012.

[44] D. Orlowski, B. Elfving, H. K. Müller, G. Wegener, and C. R. Bjarkam, "Wistar rats subjected to chronic restraint stress display increased hippocampal spine density paralleled by increased expression levels of synaptic scaffolding proteins," *Stress*, vol. 15, no. 5, pp. 514–523, 2012.

[45] M. S. Kassem, J. Lagopoulos, T. Stait-Gardner et al., "Stress-induced grey matter loss determined by MRI is primarily due to loss of dendrites and their synapses," *Molecular Neurobiology*, vol. 47, no. 2, pp. 645–661, 2013.

[46] P. Castañeda, M. Muñoz, G. García-Rojo et al., "Association of N-cadherin levels and downstream effectors of Rho GTPases with dendritic spine loss induced by chronic stress in rat hippocampal neurons," *Journal of Neuroscience Research*, vol. 93, no. 10, p. Spc1, 2015.

[47] A. M. Rico, A. L. Mendoza, D. A. Durán, H. de la Luz Torres, G. A. Mendoza, and A. B. Silva Gómez, "The effects of chronic restraint on the morphology of ventral CA1 neurons in female Long Evans rats," *Stress*, vol. 18, no. 1, pp. 67–75, 2015.

[48] P. Huang, C. Li, T. Fu et al., "Flupirtine attenuates chronic restraint stress-induced cognitive deficits and hippocampal apoptosis in male mice," *Behavioural Brain Research*, vol. 288, pp. 1–10, 2015.

[49] N. Sousa, N. V. Lukoyanov, M. D. Madeira, O. F. X. Almeida, and M. M. Paula-Barbosa, "Reorganization of the morphology of hippocampal neurites and synapses after stress-induced damage correlates with behavioral improvement," *Neuroscience*, vol. 97, no. 2, pp. 253–266, 2000.

[50] A. Vyas, R. Mitra, B. S. Shankaranarayana Rao, and S. Chattarji, "Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons," *Journal of Neuroscience*, vol. 22, no. 15, pp. 6810–6818, 2002.

[51] H. R. Sharma and M. K. Thakur, "Correlation of ERα/ERβ expression with dendritic and behavioural changes in CUMS mice," *Physiology & Behavior*, vol. 145, pp. 71–83, 2015.

[52] X. L. Li, Y. G. Yuan, H. Xu et al., "Changed synaptic plasticity in neural circuits of depressive-like and escitalopram-treated rats," *International Journal of Neuropsychopharmacology*, vol. 18, no. 10, 2015.

[53] A. J. Kallarackal, M. D. Kvarta, E. Cammarata et al., "Chronic stress induces a selective decrease in AMPA receptor-mediated synaptic excitation at hippocampal temporoammonic-CA1 synapses," *The Journal of Neuroscience*, vol. 33, no. 40, pp. 15669–15674, 2013.

[54] C. Nasca, B. Bigio, D. Zelli, F. Nicoletti, and B. S. McEwen, "Mind the gap: glucocorticoids modulate hippocampal glutamate tone underlying individual differences in stress susceptibility," *Molecular Psychiatry*, vol. 20, pp. 755–763, 2015.

[55] P. M. Maras, J. Molet, Y. Chen et al., "Preferential loss of dorsal-hippocampus synapses underlies memory impairments provoked by short, multimodal stress," *Molecular Psychiatry*, vol. 19, no. 7, pp. 811–822, 2014.

[56] A. M. Magariños, B. S. McEwen, G. Flügge, and E. Fuchs, "Chronic psychosocial stress causes apical dendritic atrophy of hippocampal CA3 pyramidal neurons in subordinate tree shrews," *Journal of Neuroscience*, vol. 16, no. 10, pp. 3534–3540, 1996.

[57] C. R. McKittrick, A. M. Magariños, D. C. Blanchard, R. J. Blanchard, B. S. McEwen, and R. R. Sakai, "Chronic social stress reduces dendritic arbors in CA3 of hippocampus and decreases binding to serotonin transporter sites," *Synapse*, vol. 36, no. 2, pp. 85–94, 2000.

[58] M. D. Kvarta, K. E. Bradbrook, H. M. Dantrassy, A. M. Bailey, and S. M. Thompson, "Corticosterone mediates the synaptic and behavioral effects of chronic stress at rat hippocampal temporoammonic synapses," *Journal of Neurophysiology*, vol. 114, no. 3, pp. 1713–1724, 2015.

[59] G. Wang, Y. Cheng, M. Gong et al., "Systematic correlation between spine plasticity and the anxiety/depression-like phenotype induced by corticosterone in mice," *NeuroReport*, vol. 24, no. 12, pp. 682–687, 2013.

[60] K. M. Harris and S. B. Kater, "Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function," *Annual Review of Neuroscience*, vol. 17, pp. 341–371, 1994.

[61] D. A. Fortin, T. Srivastava, and T. R. Soderling, "Structural modulation of dendritic spines during synaptic plasticity," *The Neuroscientist*, vol. 18, no. 4, pp. 326–341, 2012.

[62] M. Banasr, J. M. Dwyer, and R. S. Duman, "Cell atrophy and loss in depression: reversal by antidepressant treatment," *Current Opinion in Cell Biology*, vol. 23, no. 6, pp. 730–737, 2011.

[63] K.-O. Lai and N. Y. Ip, "Structural plasticity of dendritic spines: the underlying mechanisms and its dysregulation in brain disorders," *Biochimica et Biophysica Acta—Molecular Basis of Disease*, vol. 1832, no. 12, pp. 2257–2263, 2013.

[64] C. H. Bailey, E. R. Kandel, and K. M. Harris, "Structural components of synaptic plasticity and memory consolidation," *Cold Spring Harbor Perspectives in Biology*, vol. 7, no. 7, Article ID a021758, 2015.

[65] K. M. Harris, F. E. Jensen, and B. Tsao, "Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation," *The Journal of Neuroscience*, vol. 12, no. 7, pp. 2685–2705, 1992.

[66] A. Tashiro and R. Yuste, "Structure and molecular organization of dendritic spines," *Histology and Histopathology*, vol. 18, no. 2, pp. 617–634, 2003.

[67] J. Bourne and K. M. Harris, "Do thin spines learn to be mushroom spines that remember?" *Current Opinion in Neurobiology*, vol. 17, no. 3, pp. 381–386, 2007.

[68] H. Kasai, M. Matsuzaki, J. Noguchi, N. Yasumatsu, and H. Nakahara, "Structure-stability-function relationships of dendritic spines," *Trends in Neurosciences*, vol. 26, no. 7, pp. 360–368, 2003.

[69] E. A. Nimchinsky, B. L. Sabatini, and K. Svoboda, "Structure and function of dendritic spines," *Annual Review of Physiology*, vol. 64, pp. 313–353, 2002.

[70] X.-M. Ma, J. Huang, Y. Wang, B. A. Eipper, and R. E. Mains, "Kalirin, a multifunctional Rho guanine nucleotide exchange factor, is necessary for maintenance of hippocampal pyramidal neuron dendrites and dendritic spines," *Journal of Neuroscience*, vol. 23, no. 33, pp. 10593–10603, 2003.

[71] C. Sala and M. Segal, "Dendritic spines: the locus of structural and functional plasticity," *Physiological Reviews*, vol. 94, no. 1, pp. 141–188, 2014.

[72] L. J. Petrak, K. M. Harris, and S. A. Kirov, "Synaptogenesis on mature hippocampal dendrites occurs via filopodia and immature spines during blocked synaptic transmission," *Journal of Comparative Neurology*, vol. 484, no. 2, pp. 183–190, 2005.

[73] M. Segal, "Dendritic spines for neuroprotection: a hypothesis," *Trends in Neurosciences*, vol. 18, no. 11, pp. 468–471, 1995.

[74] J. Noguchi, M. Matsuzaki, G. C. R. Ellis-Davies, and H. Kasai, "Spine-neck geometry determines NMDA receptor-dependent Ca^{2+} signaling in dendrites," *Neuron*, vol. 46, no. 4, pp. 609–622, 2005.

[75] J. N. Bourne and K. M. Harris, "Balancing structure and function at hippocampal dendritic spines," *Annual Review of Neuroscience*, vol. 31, pp. 47–67, 2008.

[76] J. J. Radley, H. M. Sisti, J. Hao et al., "Chronic behavioral stress induces apical dendritic reorganization in pyramidal neurons of the medial prefrontal cortex," *Neuroscience*, vol. 125, no. 1, pp. 1–6, 2004.

[77] K. Fénelon, B. Xu, C. S. Lai et al., "The pattern of cortical dysfunction in a mouse model of a schizophrenia-related microdeletion," *The Journal of Neuroscience*, vol. 33, no. 37, pp. 14825–14839, 2013.

[78] P. Penzes, M. E. Cahill, K. A. Jones, J.-E. VanLeeuwen, and K. M. Woolfrey, "Dendritic spine pathology in neuropsychiatric disorders," *Nature Neuroscience*, vol. 14, no. 3, pp. 285–293, 2011.

[79] Y. Chen, E. A. Kramár, L. Y. Chen et al., "Impairment of synaptic plasticity by the stress mediator CRH involves selective destruction of thin dendritic spines via RhoA signaling," *Molecular Psychiatry*, vol. 18, no. 4, pp. 485–496, 2013.

[80] O. von Bohlen und Halbach, "Structure and function of dendritic spines within the hippocampus," *Annals of Anatomy*, vol. 191, no. 6, pp. 518–531, 2009.

[81] R. S. Duman and G. K. Aghajanian, "Synaptic dysfunction in depression: potential therapeutic targets," *Science*, vol. 338, no. 6103, pp. 68–72, 2012.

[82] G. Rajkowska and C. A. Stockmeier, "Astrocyte pathology in major depressive disorder: insights from human postmortem brain tissue," *Current Drug Targets*, vol. 14, no. 11, pp. 1225–1236, 2013.

[83] B. Czéh, M. Simon, B. Schmelting, C. Hiemke, and E. Fuchs, "Astroglial plasticity in the hippocampus is affected by chronic psychosocial stress and concomitant fluoxetine treatment," *Neuropsychopharmacology*, vol. 31, no. 8, pp. 1616–1626, 2006.

[84] C. Rocher, M. Spedding, C. Munoz, and T. M. Jay, "Acute stress-induced changes in hippocampal/prefrontal circuits in rats: effects of antidepressants," *Cerebral Cortex*, vol. 14, no. 2, pp. 224–229, 2004.

[85] E. Castreń, "Neuronal network plasticity and recovery from depression," *JAMA Psychiatry*, vol. 70, no. 9, pp. 983–989, 2013.

[86] D. J. Christoffel, S. A. Golden, and S. J. Russo, "Structural and synaptic plasticity in stress-related disorders," *Reviews in the Neurosciences*, vol. 22, no. 5, pp. 535–549, 2011.

[87] R. S. Duman, "Pathophysiology of depression and innovative treatments: remodeling glutamatergic synaptic connections," *Dialogues in Clinical Neuroscience*, vol. 16, no. 1, pp. 11–27, 2014.

[88] M. Popoli, Z. Yan, B. S. McEwen, and G. Sanacora, "The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission," *Nature Reviews Neuroscience*, vol. 13, no. 1, pp. 22–37, 2012.

[89] S. C. Cook and C. L. Wellman, "Chronic stress alters dendritic morphology in rat medial prefrontal cortex," *Journal of Neurobiology*, vol. 60, no. 2, pp. 236–248, 2004.

[90] J. J. Radley, A. B. Rocher, W. G. M. Janssen, P. R. Hof, B. S. McEwen, and J. H. Morrison, "Reversibility of apical dendritic retraction in the rat medial prefrontal cortex following repeated stress," *Experimental Neurology*, vol. 196, no. 1, pp. 199–203, 2005.

[91] J. J. Radley, A. B. Rocher, M. Miller et al., "Repeated stress induces dendritic spine loss in the rat medial prefrontal cortex," *Cerebral Cortex*, vol. 16, no. 3, pp. 313–320, 2006.

[92] C. Liston, M. M. Miller, D. S. Goldwater et al., "Stress-induced alterations in prefrontal cortical dendritic morphology predict selective impairments in perceptual attentional set-shifting," *Journal of Neuroscience*, vol. 26, no. 30, pp. 7870–7874, 2006.

[93] J. J. Radley, A. B. Rocher, A. Rodriguez et al., "Repeated stress alters dendritic spine morphology in the rat medial prefrontal cortex," *Journal of Comparative Neurology*, vol. 507, no. 1, pp. 1141–1150, 2008.

[94] C. Perez-Cruz, M. Simon, B. Czéh, G. Flügge, and E. Fuchs, "Hemispheric differences in basilar dendrites and spines of pyramidal neurons in the rat prelimbic cortex: activity- and stress-induced changes," *European Journal of Neuroscience*, vol. 29, no. 4, pp. 738–747, 2009.

[95] A. B. Hains, M. A. T. Vu, P. K. Maciejewski, C. H. Van Dyck, M. Gottron, and A. F. T. Arnsten, "Inhibition of protein kinase C signaling protects prefrontal cortex dendritic spines and cognition from the effects of chronic stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 42, pp. 17957–17962, 2009.

[96] D. S. Goldwater, C. Pavlides, R. G. Hunter et al., "Structural and functional alterations to rat medial prefrontal cortex following chronic restraint stress and recovery," *Neuroscience*, vol. 164, no. 2, pp. 798–808, 2009.

[97] J. E. Garrett and C. L. Wellman, "Chronic stress effects on dendritic morphology in medial prefrontal cortex: sex differences and estrogen dependence," *Neuroscience*, vol. 162, no. 1, pp. 195–207, 2009.

[98] E. B. Bloss, W. G. Janssen, B. S. McEwen, and J. H. Morrison, "Interactive effects of stress and aging on structural plasticity in the prefrontal cortex," *The Journal of Neuroscience*, vol. 30, no. 19, pp. 6726–6731, 2010.

[99] E. B. Bloss, W. G. Janssen, D. T. Ohm et al., "Evidence for reduced experience-dependent dendritic spine plasticity in the aging prefrontal cortex," *The Journal of Neuroscience*, vol. 31, no. 21, pp. 7831–7839, 2011.

[100] H. K. Müller, G. Wegener, M. Popoli, and B. Elfving, "Differential expression of synaptic proteins after chronic restraint stress in rat prefrontal cortex and hippocampus," *Brain Research*, vol. 1385, pp. 26–37, 2011.

[101] H. Yu, D.-D. Wang, Y. Wang, T. Liu, F. S. Lee, and Z.-Y. Chen, "Variant brain-derived neurotrophic factor Val66met polymorphism alters vulnerability to stress and response to antidepressants," *Journal of Neuroscience*, vol. 32, no. 12, pp. 4092–4101, 2012.

[102] J. Gilabert-Juan, E. Castillo-Gomez, R. Guirado, M. D. Moltó, and J. Nacher, "Chronic stress alters inhibitory networks in the medial prefrontal cortex of adult mice," *Brain Structure and Function*, vol. 218, no. 6, pp. 1591–1605, 2013.

[103] A. B. Hains, Y. Yabe, and A. F. Arnsten, "Chronic stimulation of alpha-2A-adrenoceptors with guanfacine protects rodent prefrontal cortex dendritic spines and cognition from the effects of chronic stress," *Neurobiology of Stress*, vol. 2, pp. 1–9, 2015.

[104] G. L. Lin, C. B. Borders, L. J. Lundewall, and C. L. Wellman, "D1 receptors regulate dendritic morphology in normal and stressed prelimbic cortex," *Psychoneuroendocrinology*, vol. 51, pp. 101–111, 2015.

[105] E. Y. Yuen, J. Wei, W. Liu, P. Zhong, X. Li, and Z. Yan, "Repeated stress causes cognitive impairment by suppressing glutamate

receptor expression and function in prefrontal cortex," *Neuron*, vol. 73, no. 5, pp. 962–977, 2012.

[106] M. Banasr, G. W. Valentine, X.-Y. Li, S. L. Gourley, J. R. Taylor, and R. S. Duman, "Chronic unpredictable stress decreases cell proliferation in the cerebral cortex of the adult rat," *Biological Psychiatry*, vol. 62, no. 5, pp. 496–504, 2007.

[107] E. Dias-Ferreira, J. C. Sousa, I. Melo et al., "Chronic stress causes frontostriatal reorganization and affects decision-making," *Science*, vol. 325, no. 5940, pp. 621–625, 2009.

[108] N. Li, R.-J. Liu, J. M. Dwyer et al., "Glutamate *N*-methyl-D-aspartate receptor antagonists rapidly reverse behavioral and synaptic deficits caused by chronic stress exposure," *Biological Psychiatry*, vol. 69, no. 8, pp. 754–761, 2011.

[109] R. M. Shansky, C. Hamo, P. R. Hof, B. S. McEwen, and J. H. Morrison, "Stress-induced dendritic remodeling in the prefrontal cortex is circuit specific," *Cerebral Cortex*, vol. 19, no. 10, pp. 2479–2484, 2009.

[110] H. J. Kang, B. Voleti, T. Hajszan et al., "Decreased expression of synapse-related genes and loss of synapses in major depressive disorder," *Nature Medicine*, vol. 18, no. 9, pp. 1413–1417, 2012.

[111] L. M. Seib and C. L. Wellman, "Daily injections alter spine density in rat medial prefrontal cortex," *Neuroscience Letters*, vol. 337, no. 1, pp. 29–32, 2003.

[112] A. Izquierdo, C. L. Wellman, and A. Holmes, "Brief uncontrollable stress causes dendritic retraction in infralimbic cortex and resistance to fear extinction in mice," *The Journal of Neuroscience*, vol. 26, no. 21, pp. 5733–5738, 2006.

[113] A. Chocyk, B. Bobula, D. Dudys et al., "Early-life stress affects the structural and functional plasticity of the medial prefrontal cortex in adolescent rats," *European Journal of Neuroscience*, vol. 38, no. 1, pp. 2089–2107, 2013.

[114] S. Bennur, B. S. Shankaranarayana Rao, R. Pawlak, S. Strickland, B. S. McEwen, and S. Chattarji, "Stress-induced spine loss in the medial amygdala is mediated by tissue-plasminogen activator," *Neuroscience*, vol. 144, no. 1, pp. 8–16, 2007.

[115] S. Marcuzzo, A. Dall'Oglio, M. F. M. Ribeiro, M. Achaval, and A. A. Rasia-Filho, "Dendritic spines in the posterodorsal medial amygdala after restraint stress and ageing in rats," *Neuroscience Letters*, vol. 424, no. 1, pp. 16–21, 2007.

[116] J. J. Sumislawski, T. S. Ramikie, and S. Patel, "Reversible gating of endocannabinoid plasticity in the amygdala by chronic stress: a potential role for monoacylglycerol lipase inhibition in the prevention of stress-induced behavioral adaptation," *Neuropsychopharmacology*, vol. 36, no. 13, pp. 2750–2761, 2011.

[117] S. Patel, P. J. Kingsley, K. MacKie, L. J. Marnett, and D. G. Winder, "Repeated homotypic stress elevates 2-arachidonoylglycerol levels and enhances short-term endocannabinoid signaling at inhibitory synapses in basolateral amygdala," *Neuropsychopharmacology*, vol. 34, no. 13, pp. 2699–2709, 2009.

[118] M. Qin, Z. Xia, T. Huang, and C. B. Smith, "Effects of chronic immobilization stress on anxiety-like behavior and basolateral amygdala morphology in Fmr1 knockout mice," *Neuroscience*, vol. 194, pp. 282–290, 2011.

[119] J. Gilabert-Juan, E. Castillo-Gomez, M. Pérez-Rando, M. D. Moltó, and J. Nacher, "Chronic stress induces changes in the structure of interneurons and in the expression of molecules related to neuronal structural plasticity and inhibitory neurotransmission in the amygdala of adult mice," *Experimental Neurology*, vol. 232, no. 1, pp. 33–40, 2011.

[120] A. G. Pillai, S. Anilkumar, and S. Chattarji, "The same antidepressant elicits contrasting patterns of synaptic changes in the amygdala vs hippocampus," *Neuropsychopharmacology*, vol. 37, no. 12, pp. 2702–2711, 2012.

[121] H. Abush and I. Akirav, "Cannabinoids ameliorate impairments induced by chronic stress to synaptic plasticity and short-term memory," *Neuropsychopharmacology*, vol. 38, no. 8, pp. 1521–1534, 2013.

[122] M. A. Padival, S. R. Blume, and J. A. Rosenkranz, "Repeated restraint stress exerts different impact on structure of neurons in the lateral and basal nuclei of the amygdala," *Neuroscience*, vol. 246, pp. 230–242, 2013.

[123] M. Padival, D. Quinette, and J. A. Rosenkranz, "Effects of repeated stress on excitatory drive of basal amygdala neurons in vivo," *Neuropsychopharmacology*, vol. 38, no. 9, pp. 1748–1762, 2013.

[124] M. N. Hill, S. A. Kumar, S. B. Filipski et al., "Disruption of fatty acid amide hydrolase activity prevents the effects of chronic stress on anxiety and amygdalar microstructure," *Molecular Psychiatry*, vol. 18, no. 10, pp. 1125–1135, 2013.

[125] A. Vyas, S. Bernal, and S. Chattarji, "Effects of chronic stress on dendritic arborization in the central and extended amygdala," *Brain Research*, vol. 965, no. 1-2, pp. 290–294, 2003.

[126] A. Vyas, A. G. Pillai, and S. Chattarji, "Recovery after chronic stress fails to reverse amygdaloid neuronal hypertrophy and enhanced anxiety-like behavior," *Neuroscience*, vol. 128, no. 4, pp. 667–673, 2004.

[127] R. Mitra, S. Jadhav, B. S. McEwen, A. Vyas, and S. Chattarji, "Stress duration modulates the spatiotemporal patterns of spine formation in the basolateral amygdala," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 26, pp. 9371–9376, 2005.

[128] A. Vyas, S. Jadhav, and S. Chattarji, "Prolonged behavioral stress enhances synaptic connectivity in the basolateral amygdala," *Neuroscience*, vol. 143, no. 2, pp. 387–393, 2006.

[129] A. Suvrathan, S. Bennur, S. Ghosh, A. Tomar, S. Anilkumar, and S. Chattarji, "Stress enhances fear by forming new synapses with greater capacity for long-term potentiation in the amygdala," *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 369, no. 1633, Article ID 20130151, 2014.

[130] S. L. Gourley, A. M. Swanson, and A. J. Koleske, "Corticosteroid-induced neural remodeling predicts behavioral vulnerability and resilience," *Journal of Neuroscience*, vol. 33, no. 7, pp. 3107–3112, 2013.

[131] M. S. Monsey, L. M. Boyle, M. L. Zhang et al., "Chronic corticosterone exposure persistently elevates the expression of memory-related genes in the lateral amygdala and enhances the consolidation of a Pavlovian fear memory," *PLoS ONE*, vol. 9, no. 3, Article ID e91530, 2014.

[132] H. Cui, H. Sakamoto, S. Higashi, and M. Kawata, "Effects of single-prolonged stress on neurons and their afferent inputs in the amygdala," *Neuroscience*, vol. 152, no. 3, pp. 703–712, 2008.

[133] M. Maroun, P. J. Ioannides, K. L. Bergman, A. Kavushansky, A. Holmes, and C. L. Wellman, "Fear extinction deficits following acute stress associate with increased spine density and dendritic retraction in basolateral amygdala neurons," *European Journal of Neuroscience*, vol. 38, no. 4, pp. 2611–2620, 2013.

[134] S.-F. Tsai, T.-Y. Huang, C.-Y. Chang et al., "Social instability stress differentially affects amygdalar neuron adaptations and memory performance in adolescent and adult rats," *Frontiers in Behavioral Neuroscience*, vol. 8, article 27, 2014.

[135] B. K. Lim, K. W. Huang, B. A. Grueter, P. E. Rothwell, and R. C. Malenka, "Anhedonia requires MC4R-mediated synaptic

adaptations in nucleus accumbens," *Nature*, vol. 487, pp. 183–189, 2012.

[136] V. Vialou, A. J. Robison, Q. C. Laplant et al., "ΔFosB in brain reward circuits mediates resilience to stress and antidepressant responses," *Nature Neuroscience*, vol. 13, no. 6, pp. 745–752, 2010.

[137] B. Jiang, W. Wang, F. Wang et al., "The stability of NR2B in the nucleus accumbens controls behavioral and synaptic adaptations to chronic stress," *Biological Psychiatry*, vol. 74, no. 2, pp. 145–155, 2013.

[138] D. J. Christoffel, S. A. Golden, M. Heshmati et al., "Effects of inhibitor of κB kinase activity in the nucleus accumbens on emotional behavior," *Neuropsychopharmacology*, vol. 37, no. 12, pp. 2615–2623, 2012.

[139] D. J. Christoffel, S. A. Golden, D. Dumitriu et al., "IκB kinase regulates social defeat stress-induced synaptic and behavioral plasticity," *The Journal of Neuroscience*, vol. 31, no. 1, pp. 314–321, 2011.

[140] Q. LaPlant, V. Vialou, H. E. Covington III et al., "Dnmt3a regulates emotional behavior and spine plasticity in the nucleus accumbens," *Nature Neuroscience*, vol. 13, no. 9, pp. 1137–1143, 2010.

[141] S. A. Golden, D. J. Christoffel, M. Heshmati et al., "Epigenetic regulation of RAC1 induces synaptic remodeling in stress disorders and depression," *Nature Medicine*, vol. 19, no. 3, pp. 337–344, 2013.

[142] D. J. Christoffel, S. A. Golden, J. J. Walsh et al., "Excitatory transmission at thalamo-striatal synapses mediates susceptibility to social stress," *Nature Neuroscience*, vol. 18, no. 7, pp. 962–964, 2015.

[143] L. A. Khibnik, M. Beaumont, M. Doyle et al., "Stress and cocaine trigger divergent and cell type-specific regulation of synaptic transmission at single spines in nucleus accumbens," *Biological Psychiatry*, 2015.

[144] V. Krishnan, M.-H. Han, D. L. Graham et al., "Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions," *Cell*, vol. 131, no. 2, pp. 391–404, 2007.

[145] S. Rossi, V. De Chiara, A. Musella et al., "Chronic psychoemotional stress impairs cannabinoid-receptor-mediated control of GABA transmission in the striatum," *The Journal of Neuroscience*, vol. 28, no. 29, pp. 7284–7292, 2008.

[146] R. C. Bagot, E. M. Parise, C. J. Pena et al., "Ventral hippocampal afferents to the nucleus accumbens regulate susceptibility to depression," *Nature Communications*, vol. 6, article 7062, 2015.

[147] B. L. Warren, O. K. Sial, L. F. Alcantara et al., "Altered gene expression and spine density in nucleus accumbens of adolescent and adult male mice exposed to emotional and physical stress," *Developmental Neuroscience*, vol. 36, no. 3-4, pp. 250–260, 2014.

[148] A. Muhammad and B. Kolb, "Mild prenatal stress-modulated behavior and neuronal spine density without affecting amphetamine sensitization," *Developmental Neuroscience*, vol. 33, no. 2, pp. 85–98, 2011.

[149] V. Krishnan and E. J. Nestler, "Animal models of depression: molecular perspectives," *Current Topics in Behavioral Neurosciences*, vol. 7, no. 1, pp. 121–147, 2011.

[150] E. J. Nestler and S. E. Hyman, "Animal models of neuropsychiatric disorders," *Nature Neuroscience*, vol. 13, no. 10, pp. 1161–1169, 2010.

[151] B. J. Caldarone, V. Zachariou, and S. L. King, "Rodent models of treatment-resistant depression," *European Journal of Pharmacology*, vol. 753, pp. 51–65, 2015.

[152] T. Buynitsky and D. I. Mostofsky, "Restraint stress in biobehavioral research: recent developments," *Neuroscience and Biobehavioral Reviews*, vol. 33, no. 7, pp. 1089–1098, 2009.

[153] X.-M. Ma and S. L. Lightman, "The arginine vasopressin and corticotrophin-releasing hormone gene transcription responses to varied frequencies of repeated stress in rats," *Journal of Physiology*, vol. 510, no. 2, pp. 605–614, 1998.

[154] O. Martí and A. Armario, "Anterior pituitary response to stress: time-related changes and adaptation," *International Journal of Developmental Neuroscience*, vol. 16, no. 3-4, pp. 241–260, 1998.

[155] J. P. Herman, "Neural control of chronic stress adaptation," *Frontiers in Behavioral Neuroscience*, vol. 7, article 61, 2013.

[156] N. Grissom and S. Bhatnagar, "Habituation to repeated stress: get used to it," *Neurobiology of Learning and Memory*, vol. 92, no. 2, pp. 215–224, 2009.

[157] V. Luine, C. Martinez, M. Villegas, A. M. Magariños, and B. S. Mcewen, "Restraint stress reversibly enhances spatial memory performance," *Physiology and Behavior*, vol. 59, no. 1, pp. 27–32, 1996.

[158] V. Luine, M. Villegas, C. Martinez, and B. S. McEwen, "Repeated stress causes reversible impairments of spatial memory performance," *Brain Research*, vol. 639, no. 1, pp. 167–170, 1994.

[159] F. C. Raadsheer, W. J. G. Hoogendijk, F. C. Stam, F. J. H. Tilders, and D. F. Swaab, "Increased numbers of corticotropin-releasing hormone expressing neurons in the hypothalamic paraventricular nucleus of depressed patients," *Neuroendocrinology*, vol. 60, no. 4, pp. 436–444, 1994.

[160] K. J. Parker, A. F. Schatzberg, and D. M. Lyons, "Neuroendocrine aspects of hypercortisolism in major depression," *Hormones and Behavior*, vol. 43, no. 1, pp. 60–66, 2003.

[161] F. Holsboer, "The corticosteroid receptor hypothesis of depression," *Neuropsychopharmacology*, vol. 23, no. 5, pp. 477–501, 2000.

[162] F. P. Varghese and E. S. Brown, "The hypothalamic-pituitary-adrenal axis in major depressive disorder: a brief primer for primary care physicians," *Primary Care Companion to the Journal of Clinical Psychiatry*, vol. 3, no. 4, pp. 151–155, 2001.

[163] M. Belvederi Murri, C. Pariante, V. Mondelli et al., "HPA axis and aging in depression: systematic review and meta-analysis," *Psychoneuroendocrinology*, vol. 41, pp. 46–62, 2014.

[164] S. Aboul-Fotouh, "Chronic treatment with coenzyme Q10 reverses restraint stress-induced anhedonia and enhances brain mitochondrial respiratory chain and creatine kinase activities in rats," *Behavioural Pharmacology*, vol. 24, no. 7, pp. 552–560, 2013.

[165] S. Chiba, T. Numakawa, M. Ninomiya, M. C. Richards, C. Wakabayashi, and H. Kunugi, "Chronic restraint stress causes anxiety- and depression-like behaviors, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 39, no. 1, pp. 112–119, 2012.

[166] A. Zafir, A. Ara, and N. Banu, "In vivo antioxidant status: a putative target of antidepressant action," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 33, no. 2, pp. 220–228, 2009.

[167] J. A. Bravo, G. Díaz-Veliz, S. Mora et al., "Desipramine prevents stress-induced changes in depressive-like behavior and hippocampal markers of neuroprotection," *Behavioural Pharmacology*, vol. 20, no. 3, pp. 273–285, 2009.

[168] Y.-W. Luo, Y. Xu, W.-Y. Cao et al., "Insulin-like growth factor 2 mitigates depressive behavior in a rat model of chronic stress," *Neuropharmacology*, vol. 89, pp. 318–324, 2015.

[169] G. Naert, G. Ixart, T. Maurice, L. Tapia-Arancibia, and L. Givalois, "Brain-derived neurotrophic factor and hypothalamic-pituitary-adrenal axis adaptation processes in a depressive-like state induced by chronic restraint stress," *Molecular and Cellular Neuroscience*, vol. 46, no. 1, pp. 55–66, 2011.

[170] S. Zhu, R. Shi, J. Wang, J.-F. Wang, and X.-M. Li, "Unpredictable chronic mild stress not chronic restraint stress induces depressive behaviours in mice," *NeuroReport*, vol. 25, no. 14, pp. 1151–1155, 2014.

[171] R. J. Katz, K. A. Roth, and B. J. Carroll, "Acute and chronic stress effects on open field activity in the rat: implications for a model of depression," *Neuroscience and Biobehavioral Reviews*, vol. 5, no. 2, pp. 247–251, 1981.

[172] R. J. Katz, "Animal model of depression: pharmacological sensitivity of a hedonic deficit," *Pharmacology Biochemistry and Behavior*, vol. 16, no. 6, pp. 965–968, 1982.

[173] P. Willner, A. Towell, D. Sampson, S. Sophokleous, and R. Muscat, "Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant," *Psychopharmacology*, vol. 93, no. 3, pp. 358–364, 1987.

[174] M. Papp, P. Willner, and R. Muscat, "An animal model of anhedonia: attenuation of sucrose consumption and place preference conditioning by chronic unpredictable mild stress," *Psychopharmacology*, vol. 104, no. 2, pp. 255–259, 1991.

[175] P. Willner, "Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation," *Psychopharmacology*, vol. 134, no. 4, pp. 319–329, 1997.

[176] M. Banasr and R. S. Duman, "Glial loss in the prefrontal cortex is sufficient to induce depressive-like behaviors," *Biological Psychiatry*, vol. 64, no. 10, pp. 863–870, 2008.

[177] D. D. Luo, S. C. An, and X. Zhang, "Involvement of hippocampal serotonin and neuropeptide Y in depression induced by chronic unpredicted mild stress," *Brain Research Bulletin*, vol. 77, no. 1, pp. 8–12, 2008.

[178] P. Willner, R. Muscat, and M. Papp, "Chronic mild stress-induced anhedonia: a realistic animal model of depression," *Neuroscience and Biobehavioral Reviews*, vol. 16, no. 4, pp. 525–534, 1992.

[179] Q. LaPlant, S. Chakravarty, V. Vialou et al., "Role of nuclear factor kappaB in ovarian hormone-mediated stress hypersensitivity in female mice," *Biological Psychiatry*, vol. 65, no. 10, pp. 874–880, 2009.

[180] S. Monteiro, S. Roque, D. de Sá-Calçada, N. Sousa, M. Correia-Neves, and J. J. Cerqueira, "An efficient chronic unpredictable stress protocol to induce stress-related responses in C57BL/6 mice," *Frontiers in Psychiatry*, vol. 6, article 6, 2015.

[181] A. Franceschelli, S. Herchick, C. Thelen, Z. Papadopoulou-Daifoti, and P. M. Pitychoutis, "Sex differences in the chronic mild stress model of depression," *Behavioural Pharmacology*, vol. 25, no. 5-6, pp. 372–383, 2014.

[182] H.-C. Yan, X. Cao, M. Das, X.-H. Zhu, and T.-M. Gao, "Behavioral animal models of depression," *Neuroscience Bulletin*, vol. 26, no. 4, pp. 327–337, 2010.

[183] O. Berton, C. A. McClung, R. J. Dileone et al., "Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress," *Science*, vol. 311, no. 5762, pp. 864–868, 2006.

[184] D. Chou, C.-C. Huang, and K.-S. Hsu, "Brain-derived neurotrophic factor in the amygdala mediates susceptibility to fear conditioning," *Experimental Neurology*, vol. 255, pp. 19–29, 2014.

[185] R. J. Donahue, J. W. Muschamp, S. J. Russo, E. J. Nestler, and W. A. Carlezon, "Effects of striatal ΔFosB over expression and ketamine on social defeat stress-induced anhedonia in mice," *Biological Psychiatry*, vol. 76, no. 7, pp. 550–558, 2014.

[186] S. A. Golden, H. E. Covington III, O. Berton, and S. J. Russo, "A standardized protocol for repeated social defeat stress in mice," *Nature Protocols*, vol. 6, no. 8, pp. 1183–1191, 2011.

[187] N. M. Tsankova, O. Berton, W. Renthal, A. Kumar, R. L. Neve, and E. J. Nestler, "Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action," *Nature Neuroscience*, vol. 9, no. 4, pp. 519–525, 2006.

[188] V. Krishnan, "Defeating the fear: new insights into the neurobiology of stress susceptibility," *Experimental Neurology*, vol. 261, pp. 412–416, 2014.

[189] B. S. McEwen, J. D. Gray, and C. Nasca, "Recognizing resilience: learning from the effects of stress on the brain," *Neurobiology of Stress*, vol. 1, no. 1, pp. 1–11, 2015.

[190] D. Cicchetti, "Resilience under conditions of extreme stress: a multilevel perspective," *World Psychiatry*, vol. 9, no. 3, pp. 145–154, 2010.

[191] V. Krishnan and E. J. Nestler, "The molecular neurobiology of depression," *Nature*, vol. 455, no. 7215, pp. 894–902, 2008.

[192] R. Rygula, N. Abumaria, U. Havemann-Reinecke et al., "Pharmacological validation of a chronic social stress model of depression in rats: effects of reboxetine, haloperidol and diazepam," *Behavioural Pharmacology*, vol. 19, no. 3, pp. 183–196, 2008.

[193] O. Wiborg, "Chronic mild stress for modeling anhedonia," *Cell and Tissue Research*, vol. 354, no. 1, pp. 155–169, 2013.

[194] M. Joëls, N. Pasricha, and H. Karst, "The interplay between rapid and slow corticosteroid actions in brain," *European Journal of Pharmacology*, vol. 719, no. 1–3, pp. 44–52, 2013.

[195] C. D. Conrad, "Chronic stress-induced hippocampal vulnerability: the glucocorticoid vulnerability hypothesis," *Reviews in the Neurosciences*, vol. 19, no. 6, pp. 395–411, 2008.

[196] J. M. H. M. Reul and E. R. de Kloet, "Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation," *Endocrinology*, vol. 117, no. 6, pp. 2505–2511, 1985.

[197] E. Kitraki, O. Kremmyda, D. Youlatos, M. N. Alexis, and C. Kittas, "Gender-dependent alterations in corticosteroid receptor status and spatial performance following 21 days of restraint stress," *Neuroscience*, vol. 125, no. 1, pp. 47–55, 2004.

[198] E. R. De Kloet, E. Vreugdenhil, M. S. Oitzl, and M. Joëls, "Brain corticosteroid receptor balance in health and disease," *Endocrine Reviews*, vol. 19, no. 3, pp. 269–301, 1998.

[199] C. Anacker, P. A. Zunszain, L. A. Carvalho, and C. M. Pariante, "The glucocorticoid receptor: pivot of depression and of antidepressant treatment?" *Psychoneuroendocrinology*, vol. 36, no. 3, pp. 415–425, 2011.

[200] P. Videbech and B. Ravnkilde, "Hippocampal volume and depression: a meta-analysis of MRI studies," *The American Journal of Psychiatry*, vol. 161, no. 11, pp. 1957–1966, 2004.

[201] W. C. Drevets, J. L. Price, and M. L. Furey, "Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression," *Brain Structure and Function*, vol. 213, no. 1-2, pp. 93–118, 2008.

[202] J. D. Bremner, M. Narayan, E. R. Anderson, L. H. Staib, H. L. Miller, and D. S. Charney, "Hippocampal volume reduction in major depression," *The American Journal of Psychiatry*, vol. 157, no. 1, pp. 115–118, 2000.

[203] S. C. Caetano, J. P. Hatch, P. Brambilla et al., "Anatomical MRI study of hippocampus and amygdala in patients with current and remitted major depression," *Psychiatry Research—Neuroimaging*, vol. 132, no. 2, pp. 141–147, 2004.

[204] K. Sawyer, E. Corsentino, N. Sachs-Ericsson, and D. C. Steffens, "Depression, hippocampal volume changes, and cognitive decline in a clinical sample of older depressed outpatients and non-depressed controls," *Aging and Mental Health*, vol. 16, no. 6, pp. 753–762, 2012.

[205] V. Lorenzetti, N. B. Allen, A. Fornito, and M. Yücel, "Structural brain abnormalities in major depressive disorder: a selective review of recent MRI studies," *Journal of Affective Disorders*, vol. 117, no. 1-2, pp. 1–17, 2009.

[206] W. D. Taylor, D. R. McQuoid, M. E. Payne, A. S. Zannas, J. R. MacFall, and D. C. Steffens, "Hippocampus atrophy and the longitudinal course of late-life depression," *The American Journal of Geriatric Psychiatry*, vol. 22, no. 12, pp. 1504–15012, 2014.

[207] S. J. Coultrap, K. M. Nixon, R. M. Alvestad, C. F. Valenzuela, and M. D. Browning, "Differential expression of NMDA receptor subunits and splice variants among the CA1, CA3 and dentate gyrus of the adult rat," *Molecular Brain Research*, vol. 135, no. 1-2, pp. 104–111, 2005.

[208] L. C. Berumen, A. Rodriguez, R. Miledi, and G. Garcia-Alcocer, "Serotonin receptors in hippocampus," *The Scientific World Journal*, vol. 2012, Article ID 823493, 15 pages, 2012.

[209] J. P. Herman, P. D. Patel, H. Akil, and S. J. Watson, "Localization and regulation of glucocorticoid and mineralocorticoid receptor messenger RNAs in the hippocampal formation of the rat," *Molecular Endocrinology*, vol. 3, no. 11, pp. 1886–1894, 1989.

[210] G. Cellot and E. Cherubini, "Functional role of ambient GABA in refining neuronal circuits early in postnatal development," *Frontiers in Neural Circuits*, vol. 7, article 136, 2013.

[211] N. M. van Strien, N. L. M. Cappaert, and M. P. Witter, "The anatomy of memory: an interactive overview of the parahippocampal-hippocampal network," *Nature Reviews Neuroscience*, vol. 10, no. 4, pp. 272–282, 2009.

[212] J. A. M. Van Eekelen, W. Jiang, E. R. De Kloet, and M. C. Bohn, "Distribution of the mineralocorticoid and the glucocorticoid receptor mRNAs in the rat hippocampus," *Journal of Neuroscience Research*, vol. 21, no. 1, pp. 88–94, 1988.

[213] A. N. Hoffman, A. Krigbaum, J. B. Ortiz et al., "Recovery after chronic stress within spatial reference and working memory domains: correspondence with hippocampal morphology," *European Journal of Neuroscience*, vol. 34, no. 6, pp. 1023–1030, 2011.

[214] L. A. M. Galea, B. S. McEwen, P. Tanapat, T. Deak, R. L. Spencer, and F. S. Dhabhar, "Sex differences in dendritic atrophy of CA3 pyramidal neurons in response to chronic restraint stress," *Neuroscience*, vol. 81, no. 3, pp. 689–697, 1997.

[215] J. B. Ortiz, C. M. Mathewson, A. N. Hoffman, P. D. Hanavan, E. F. Terwilliger, and C. D. Conrad, "Hippocampal brain-derived neurotrophic factor mediates recovery from chronic stress-induced spatial reference memory deficits," *European Journal of Neuroscience*, 2014.

[216] C. S. Woolley, E. Gould, and B. S. McEwen, "Exposure to excess glucocorticoids alters dendritic morphology of adult hippocampal pyramidal neurons," *Brain Research*, vol. 531, no. 1-2, pp. 225–231, 1990.

[217] C. D. Conrad, "What is the functional significance of chronic stress-induced CA3 dendritic retraction within the hippocampus?" *Behavioral and Cognitive Neuroscience Reviews*, vol. 5, no. 1, pp. 41–60, 2006.

[218] S. C. McQuown, R. M. Barrett, D. P. Matheos et al., "HDAC3 is a critical negative regulator of long-term memory formation," *Journal of Neuroscience*, vol. 31, no. 2, pp. 764–774, 2011.

[219] T. J. Shors, C. Chua, and J. Falduto, "Sex differences and opposite effects of stress on dendritic spine density in the male versus female hippocampus," *The Journal of Neuroscience*, vol. 21, no. 16, pp. 6292–6297, 2001.

[220] X. Cai, A. J. Kallarackal, M. D. Kvarta et al., "Local potentiation of excitatory synapses by serotonin and its alteration in rodent models of depression," *Nature Neuroscience*, vol. 16, no. 4, pp. 464–472, 2013.

[221] K. M. Moench and C. L. Wellman, "Stress-induced alterations in prefrontal dendritic spines: implications for post-traumatic stress disorder," *Neuroscience Letters*, vol. 601, pp. 41–45, 2015.

[222] G. Bush, P. J. Whalen, B. R. Rosen, M. A. Jenike, S. C. McInerney, and S. L. Rauch, "The counting Stroop: an interference task specialized for functional neuroimaging—validation study with functional MRI," *Human Brain Mapping*, vol. 6, no. 4, pp. 270–282, 1998.

[223] A. W. MacDonald III, J. D. Cohen, V. A. Stenger, and C. S. Carter, "Dissociating the role of the dorsolateral prefrontal and anterior cingulate cortex in cognitive control," *Science*, vol. 288, no. 5472, pp. 1835–1838, 2000.

[224] H. J. Groenewegen and H. B. M. Uylings, "The prefrontal cortex and the integration of sensory, limbic and autonomic information," *Progress in Brain Research*, vol. 126, pp. 3–28, 2000.

[225] D. Diorio, V. Viau, and M. J. Meaney, "The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress," *The Journal of Neuroscience*, vol. 13, no. 9, pp. 3839–3847, 1993.

[226] R. S. Ahima and R. E. Harlan, "Differential corticosteroid regulation of type II glucocorticoid receptor-like immunoreactivity in the rat central nervous system: topography and implications," *Endocrinology*, vol. 129, no. 1, pp. 226–236, 1991.

[227] J. M. McKlveen, B. Myers, J. N. Flak et al., "Role of prefrontal cortex glucocorticoid receptors in stress and emotion," *Biological Psychiatry*, vol. 74, no. 9, pp. 672–679, 2013.

[228] R. J. Hussain and L. Jacobson, "Increased antidepressant sensitivity after prefrontal cortex glucocorticoid receptor gene deletion in mice," *Physiology and Behavior*, vol. 138, pp. 113–117, 2015.

[229] W. C. Drevets, J. L. Price, J. R. Simpson Jr. et al., "Subgenual prefrontal cortex abnormalities in mood disorders," *Nature*, vol. 386, no. 6627, pp. 824–827, 1997.

[230] P. C. M. P. Koolschijn, N. E. M. van Haren, G. J. L. M. Lensvelt-Mulders, H. E. Hulshoff Pol, and R. S. Kahn, "Brain volume abnormalities in major depressive disorder: a meta-analysis of magnetic resonance imaging studies," *Human Brain Mapping*, vol. 30, no. 11, pp. 3719–3735, 2009.

[231] M. J. Kempton, Z. Salvador, M. R. Munafò et al., "Structural neuroimaging studies in major depressive disorder. Meta-analysis and comparison with bipolar disorder," *Archives of General Psychiatry*, vol. 68, no. 7, pp. 675–690, 2011.

[232] E. Bora, B. J. Harrison, C. G. Davey, M. Yücel, and C. Pantelis, "Meta-analysis of volumetric abnormalities in cortico-striatal-pallidal-thalamic circuits in major depressive disorder," *Psychological Medicine*, vol. 42, no. 4, pp. 671–681, 2012.

[233] M. T. Treadway, M. L. Waskom, D. G. Dillon et al., "Illness progression, recent stress, and morphometry of hippocampal subfields and medial prefrontal cortex in major depression," *Biological Psychiatry*, vol. 77, pp. 285–294, 2015.

[234] J. M. Carlson, E. Depetro, J. Maxwell, E. Harmon-Jones, and G. Hajcak, "Gender moderates the association between dorsal medial prefrontal cortex volume and depressive symptoms in a subclinical sample," *Psychiatry Research: Neuroimaging*, vol. 233, no. 2, pp. 285–288, 2015.

[235] D. Cotter, D. Mackay, G. Chana, C. Beasley, S. Landau, and I. P. Everall, "Reduced neuronal size and glial cell density in area 9 of the dorsolateral prefrontal cortex in subjects with major depressive disorder," *Cerebral Cortex*, vol. 12, no. 4, pp. 386–394, 2002.

[236] G. Rajkowska, J. J. Miguel-Hidalgo, J. Wei et al., "Morphometric evidence for neuronal and glial prefrontal cell pathology in major depression," *Biological Psychiatry*, vol. 45, no. 9, pp. 1085–1098, 1999.

[237] J. J. Radley, A. B. Rocher, M. Miller et al., "Repeated stress induces dendritic spine loss in the rat medial prefrontal cortex," *Cerebral Cortex*, vol. 16, no. 3, pp. 313–320, 2005.

[238] C. L. Wellman, "Dendritic reorganization in pyramidal neurons in medial prefrontal cortex after chronic corticosterone administration," *Journal of Neurobiology*, vol. 49, no. 3, pp. 245–253, 2001.

[239] S. M. Brown, S. Henning, and C. L. Wellman, "Mild, short-term stress alters dendritic morphology in rat medial prefrontal cortex," *Cerebral Cortex*, vol. 15, no. 11, pp. 1714–1722, 2005.

[240] N. Nava, G. Treccani, N. Liebenberg et al., "Chronic desipramine prevents acute stress-induced reorganization of medial prefrontal cortex architecture by blocking glutamate vesicle accumulation and excitatory synapse increase," *International Journal of Neuropsychopharmacology*, vol. 18, no. 3, 2015.

[241] N. Nava, G. Treccani, A. Alabsi et al., "Temporal dynamics of acute stress-induced dendritic remodeling in medial prefrontal cortex and the protective effect of desipramine," *Cerebral Cortex*, 2015.

[242] H. E. Covington III, M. K. Lobo, I. Maze et al., "Antidepressant effect of optogenetic stimulation of the medial prefrontal cortex," *The Journal of Neuroscience*, vol. 30, no. 48, pp. 16082–16090, 2010.

[243] M. R. Farrell, D. R. Sengelaub, and C. L. Wellman, "Sex differences and chronic stress effects on the neural circuitry underlying fear conditioning and extinction," *Physiology and Behavior*, vol. 122, pp. 208–215, 2013.

[244] B. Kolb and J. Stewart, "Sex-related differences in dendritic branching of cells in the prefrontal cortex of rats," *Journal of Neuroendocrinology*, vol. 3, no. 1, pp. 95–99, 1991.

[245] A. Bechara, H. Damasio, and A. R. Damasio, "Emotion, decision making and the orbitofrontal cortex," *Cerebral Cortex*, vol. 10, no. 3, pp. 295–307, 2000.

[246] W. C. Drevets, "Orbitofrontal cortex function and structure in depression," *Annals of the New York Academy of Sciences*, vol. 1121, pp. 499–527, 2007.

[247] D. Cotter, L. Hudson, and S. Landau, "Evidence for orbitofrontal pathology in bipolar disorder and major depression, but not in schizophrenia," *Bipolar Disorders*, vol. 7, no. 4, pp. 358–369, 2005.

[248] J. Scheuerecker, E. M. Meisenzahl, N. Koutsouleris et al., "Orbitofrontal volume reductions during emotion recognition in patients with major depression," *Journal of Psychiatry & Neuroscience*, vol. 35, pp. 311–320, 2010.

[249] G. Rajkowska, J. J. Miguel-Hidalgo, P. Dubey, C. A. Stockmeier, and K. R. R. Krishnan, "Prominent reduction in pyramidal neurons density in the orbitofrontal cortex of elderly depressed patients," *Biological Psychiatry*, vol. 58, no. 4, pp. 297–306, 2005.

[250] P. Mandela and X. M. Ma, "Kalirin, a key player in synapse formation, is implicated in human diseases," *Neural Plasticity*, vol. 2012, Article ID 728161, 9 pages, 2012.

[251] X.-M. Ma, D. D. Kiraly, E. D. Gaier et al., "Kalirin-7 is required for synaptic structure and function," *The Journal of Neuroscience*, vol. 28, no. 47, pp. 12368–12382, 2008.

[252] M. J. Rubinow, G. Mahajan, W. May et al., "Basolateral amygdala volume and cell numbers in major depressive disorder: a postmortem stereological study," *Brain Structure and Function*, pp. 1–4, 2014.

[253] C. Lange and E. Irle, "Enlarged amygdala volume and reduced hippocampal volume in young women with major depression," *Psychological Medicine*, vol. 34, no. 6, pp. 1059–1064, 2004.

[254] T. Frodl, E. Meisenzahl, T. Zetzsche et al., "Enlargement of the amygdala in patients with a first episode of major depression," *Biological Psychiatry*, vol. 51, no. 9, pp. 708–714, 2002.

[255] K. Vassilopoulou, M. Papathanasiou, I. Michopoulos et al., "A magnetic resonance imaging study of hippocampal, amygdala and subgenual prefrontal cortex volumes in major depression subtypes: melancholic versus psychotic depression," *Journal of Affective Disorders*, vol. 146, no. 2, pp. 197–204, 2013.

[256] G. Kronenberg, L. Tebartz van Elst, F. Regen, M. Deuschle, I. Heuser, and M. Colla, "Reduced amygdala volume in newly admitted psychiatric in-patients with unipolar major depression," *Journal of Psychiatric Research*, vol. 43, no. 13, pp. 1112–1117, 2009.

[257] Y. Tang, F. Wang, G. Xie et al., "Reduced ventral anterior cingulate and amygdala volumes in medication-naïve females with major depressive disorder: a voxel-based morphometric magnetic resonance imaging study," *Psychiatry Research: Neuroimaging*, vol. 156, no. 1, pp. 83–86, 2007.

[258] S. H. Joshi, R. T. Espinoza, T. Pirnia et al., "Structural plasticity of the hippocampus and amygdala induced by electroconvulsive therapy in major depression," *Biological Psychiatry*, 2015.

[259] P. W. Gold, R. Machado-Vieira, and M. G. Pavlatou, "Clinical and biochemical manifestations of depression: relation to the neurobiology of stres," *Neural Plasticity*, vol. 2015, Article ID 581976, 11 pages, 2015.

[260] W. C. Drevets, T. O. Videen, J. L. Price, S. H. Preskorn, S. T. Carmichael, and M. E. Raichle, "A functional anatomical study of unipolar depression," *Journal of Neuroscience*, vol. 12, no. 9, pp. 3628–3641, 1992.

[261] P. Sah, E. S. L. Faber, M. L. De Armentia, and J. Power, "The amygdaloid complex: anatomy and physiology," *Physiological Reviews*, vol. 83, no. 3, pp. 803–834, 2003.

[262] R. Mitra and R. M. Sapolsky, "Acute corticosterone treatment is sufficient to induce anxiety and amygdaloid dendritic hypertrophy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 14, pp. 5573–5578, 2008.

[263] P. W. Kalivas and K. McFarland, "Brain circuitry and the reinstatement of cocaine-seeking behavior," *Psychopharmacology*, vol. 168, no. 1-2, pp. 44–56, 2003.

[264] S. E. Hyman, R. C. Malenka, and E. J. Nestler, "Neural mechanisms of addiction: the role of reward-related learning and memory," *Annual Review of Neuroscience*, vol. 29, pp. 565–598, 2006.

[265] P. W. Kalivas and C. O'Brien, "Drug addiction as a pathology of staged neuroplasticity," *Neuropsychopharmacology*, vol. 33, no. 1, pp. 166–180, 2008.

[266] D. M. Dietz, K. C. Dietz, E. J. Nestler, and S. J. Russo, "Molecular mechanisms of psychostimulant-induced structural plasticity," *Pharmacopsychiatry*, vol. 42, supplement 1, pp. S69–S78, 2009.

[267] A. E. Kelley, "Ventral striatal control of appetitive motivation: role in ingestive behavior and reward-related learning," *Neuroscience and Biobehavioral Reviews*, vol. 27, no. 8, pp. 765–776, 2004.

[268] C. Nauczyciel, S. Robic, T. Dondaine et al., "The nucleus accumbens: a target for deep brain stimulation in resistant major depressive disorder," *Journal of Molecular Psychiatry*, vol. 1, article 17, 2013.

[269] D. A. Pizzagalli, A. J. Holmes, D. G. Dillon et al., "Reduced caudate and nucleus accumbens response to rewards in unmedicated individuals with major depressive disorder," *The American Journal of Psychiatry*, vol. 166, no. 6, pp. 702–710, 2009.

[270] D. Chaudhury, J. J. Walsh, A. K. Friedman et al., "Rapid regulation of depression-related behaviours by control of midbrain dopamine neurons," *Nature*, vol. 493, no. 7433, pp. 532–536, 2013.

[271] S. J. Russo and E. J. Nestler, "The brain reward circuitry in mood disorders," *Nature Reviews Neuroscience*, vol. 14, no. 9, pp. 609–625, 2013.

[272] K. Gurney, T. J. Prescott, J. R. Wickens, and P. Redgrave, "Computational models of the basal ganglia: from robots to membranes," *Trends in Neurosciences*, vol. 27, no. 8, pp. 453–459, 2004.

[273] P. Svenningsson, A. C. Nairn, and P. Greengard, "DARPP-32 mediates the actions of multiple drugs of abuse," *The AAPS Journal*, vol. 7, no. 2, pp. E353–E360, 2005.

[274] M. Matamales, J. Bertran-Gonzalez, L. Salomon et al., "Striatal medium-sized spiny neurons: identification by nuclear staining and study of neuronal subpopulations in BAC transgenic mice," *PLoS ONE*, vol. 4, no. 3, Article ID e4770, 2009.

[275] J. Bertran-Gonzalez, C. Bosch, M. Maroteaux et al., "Opposing patterns of signaling activation in dopamine D1 and D2 receptor-expressing striatal neurons in response to cocaine and haloperidol," *Journal of Neuroscience*, vol. 28, no. 22, pp. 5671–5685, 2008.

[276] J. W. Kebabian and D. B. Calne, "Multiple receptors for dopamine," *Nature*, vol. 277, no. 5692, pp. 93–96, 1979.

[277] D. R. Sibley, F. J. Monsma Jr., L. D. McVittie, C. R. Gerfen, R. M. Burch, and L. C. Mahan, "Molecular neurobiology of dopamine receptor subtypes," *Neurochemistry International*, vol. 20, supplement, pp. 17S–22S, 1992.

[278] O. Aizman, H. Brismar, P. Uhlén et al., "Anatomical and physiological evidence for D1 and D2 dopamine receptor colocalization in neostriatal neurons," *Nature Neuroscience*, vol. 3, no. 3, pp. 226–230, 2000.

[279] X. Tian, L. Kai, P. E. Hockberger, D. L. Wokosin, and D. J. Surmeier, "MEF-2 regulates activity-dependent spine loss in striatopallidal medium spiny neurons," *Molecular and Cellular Neuroscience*, vol. 44, no. 1, pp. 94–108, 2010.

[280] J. C. Stoof and J. W. Kebabian, "Opposing roles for D-1 and D-2 dopamine receptors in efflux of cyclic AMP from rat neostriatum," *Nature*, vol. 294, no. 5839, pp. 366–368, 1981.

[281] D. J. Surmeier, J. Ding, M. Day, Z. Wang, and W. Shen, "D1 and D2 dopamine-receptor modulation of striatal glutamatergic signaling in striatal medium spiny neurons," *Trends in Neurosciences*, vol. 30, no. 5, pp. 228–235, 2007.

[282] R. J. Smith, M. K. Lobo, S. Spencer, and P. W. Kalivas, "Cocaine-induced adaptations in D1 and D2 accumbens projection neurons (a dichotomy not necessarily synonymous with direct and indirect pathways)," *Current Opinion in Neurobiology*, vol. 23, no. 4, pp. 546–552, 2013.

[283] T. C. Francis, R. Chandra, D. M. Friend et al., "Nucleus accumbens medium spiny neuron subtypes mediate depression-related outcomes to social defeat stress," *Biological Psychiatry*, vol. 77, no. 3, pp. 212–222, 2015.

[284] D. S. Zahm, "An integrative neuroanatomical perspective on some subcortical substrates of adaptive responding with emphasis on the nucleus accumbens," *Neuroscience and Biobehavioral Reviews*, vol. 24, no. 1, pp. 85–105, 2000.

[285] Y. Kinoshita, R. G. Hunter, J. D. Gray et al., "Role for NUP62 depletion and PYK2 redistribution in dendritic retraction resulting from chronic stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 45, pp. 16130–16135, 2014.

[286] K. Wedzony, A. Chocyk, M. Maćkowiak, K. Fijał, and A. Czyrak, "Cortical localization of dopamine D4 receptors in the rat brain—immunocytochemical study," *Journal of Physiology and Pharmacology*, vol. 51, no. 2, pp. 205–221, 2000.

[287] P. Sántha, M. Pákáski, E. K. Fodor et al., "Cytoskeletal protein translation and expression in the rat brain are stressor-dependent and region-specific," *PLoS ONE*, vol. 8, no. 10, Article ID e73504, 2013.

[288] D. Rei, X. Mason, J. Seo et al., "Basolateral amygdala bidirectionally modulates stress-induced hippocampal learning and memory deficits through a p25/Cdk5-dependent pathway," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 23, pp. 7291–7296, 2015.

[289] Y.-C. Lin and A. J. Koleske, "Mechanisms of synapse and dendrite maintenance and their disruption in psychiatric and neurodegenerative disorders," *Annual Review of Neuroscience*, vol. 33, pp. 349–378, 2010.

[290] A. J. Koleske, "Molecular mechanisms of dendrite stability," *Nature Reviews Neuroscience*, vol. 14, no. 8, pp. 536–550, 2013.

[291] S. R. Wainwright and L. A. M. Galea, "The neural plasticity theory of depression: assessing the roles of adult neurogenesis and PSA-NCAM within the hippocampus," *Neural Plasticity*, vol. 2013, Article ID 805497, 14 pages, 2013.

[292] Y. Chen, A. L. Andres, M. Frotscher, and T. Z. Baram, "Tuning synaptic transmission in the hippocampus by stress: the CRH system," *Frontiers in Cellular Neuroscience*, vol. 6, article 13, 2012.

[293] J. D. Gray, T. G. Rubin, R. G. Hunter, and B. S. McEwen, "Hippocampal gene expression changes underlying stress sensitization and recovery," *Molecular Psychiatry*, vol. 19, no. 11, pp. 1171–1178, 2014.

[294] K. V. Wagner, J. Hartmann, C. Labermaier et al., "Homer1/mGluR5 activity moderates vulnerability to chronic social stress," *Neuropsychopharmacology*, vol. 40, pp. 1222–1233, 2015.

[295] N. Li, B. Lee, R.-J. Liu et al., "mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists," *Science*, vol. 329, no. 5994, pp. 959–964, 2010.

[296] J. Tang, W. Xue, B. Xia et al., "Involvement of normalized NMDA receptor and mTOR-related signaling in rapid antidepressant effects of Yueju and ketamine on chronically stressed mice," *Scientific Reports*, vol. 5, Article ID 13573, 2015.

[297] W. Zhou, N. Wang, C. Yang, X.-M. Li, Z.-Q. Zhou, and J.-J. Yang, "Ketamine-induced antidepressant effects are associated with

AMPA receptors-mediated upregulation of mTOR and BDNF in rat hippocampus and prefrontal cortex," *European Psychiatry*, vol. 29, no. 7, pp. 419–423, 2014.

[298] J.-J. Yu, Y. Zhang, Y. Wang et al., "Inhibition of calcineurin in the prefrontal cortex induced depressive-like behavior through mTOR signaling pathway," *Psychopharmacology*, vol. 225, no. 2, pp. 361–372, 2013.

[299] J. M. Dwyer, J. G. Maldonado-Avilés, A. E. Lepack, R. J. DiLeone, and R. S. Duman, "Ribosomal protein S6 kinase 1 signaling in prefrontal cortex controls depressive behavior," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 19, pp. 6188–6193, 2015.

[300] C. S. Jernigan, D. B. Goswami, M. C. Austin et al., "The mTOR signaling pathway in the prefrontal cortex is compromised in major depressive disorder," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 35, no. 7, pp. 1774–1779, 2011.

[301] S. W. Park, J. G. Lee, M. K. Seo et al., "Differential effects of antidepressant drugs on mTOR signalling in rat hippocampal neurons," *International Journal of Neuropsychopharmacology*, vol. 17, pp. 1831–1846, 2014.

[302] X. Liu, L. Luo, R. Mu et al., "Fluoxetine regulates mTOR signalling in a region-dependent manner in depression-like mice," *Scientific Reports*, vol. 5, Article ID 16024, 2015.

Hindawi Publishing Corporation
Neural Plasticity
Volume 2016, Article ID 2819107, 13 pages
http://dx.doi.org/10.1155/2016/2819107

Research Article

Hippocampal Dendritic Spines Are Segregated Depending on Their Actin Polymerization

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Received 3 July 2015; Revised 21 September 2015; Accepted 1 October 2015

Academic Editor: Kimberly Tolias

Dendritic spines are mushroom-shaped protrusions of the postsynaptic membrane. Spines receive the majority of glutamatergic synaptic inputs. Their morphology, dynamics, and density have been related to synaptic plasticity and learning. The main determinant of spine shape is filamentous actin. Using FRAP, we have reexamined the actin dynamics of individual spines from pyramidal hippocampal neurons, both in cultures and in hippocampal organotypic slices. Our results indicate that, in cultures, the actin mobile fraction is independently regulated at the individual spine level, and mobile fraction values do not correlate with either age or distance from the soma. The most significant factor regulating actin mobile fraction was the presence of astrocytes in the culture substrate. Spines from neurons growing in the virtual absence of astrocytes have a more stable actin cytoskeleton, while spines from neurons growing in close contact with astrocytes show a more dynamic cytoskeleton. According to their recovery time, spines were distributed into two populations with slower and faster recovery times, while spines from slice cultures were grouped into one population. Finally, employing fast lineal acquisition protocols, we confirmed the existence of loci with high polymerization rates within the spine.

1. Introduction

Dendritic spines are specializations of glutamatergic synapses. They have been the object of theoretical and experimental studies for more than a century. First identified by Ramón y Cajal [1], their role in synaptic transmission is still under study. Morphologically, spines are clearly identified as tiny protrusions, about one micron long, with a mushroom-like shape, although this static description does not reflect their great variability in size and shape. Spines are dynamic structures that undergo morphological changes in a developmental and activity-dependent manner [2, 3]. In this sense, neurons are able to control synaptic efficiency by adjusting the size and density of spines [4], which have been accepted, in turn, as important regulators of synaptic plasticity, learning, and memory formation [5–7]. In addition, abnormal spine shape and density have been associated with different pathologies, such as Alzheimer's disease, epilepsy, Down's syndrome, and fragile X syndrome [8, 9].

A motile actin cytoskeleton provides the required molecular substrate for the dynamic nature of spines [10]. At the ultrastructural level, dendritic spines are enriched with a branched actin network [11–13]. The actin state depends on

the coordinated action of several actin binding proteins [8, 14]. Said equilibrium, that is, the proportion between filamentous and monomeric actin, can be quantified employing FRAP (Fluorescence Recovery After Photobleaching) [15], FRET (Fluorescence Resonance Energy Transfer) [16], or photoactivated actin [17].

Ongoing actin polymerization exerts a direct control over membrane receptor composition and the stability of the postsynaptic density, and it has been suggested that actin might serve as an anchor place in the synapse [18, 19]. Supporting this notion, spines contain discrete locus of polymerization often associated with postsynaptic density and receptor trafficking [17].

Besides neurons, glial cells also play an important role in synapse physiology and development. During synaptogenesis, glial cells release cholesterol and thrombospondins to increase synapse number and functionality [20]. Astrocytes not only regulate the synaptic microenvironment by removing or releasing neurotransmitters into the extracellular space [21]; they can also directly modulate synaptic transmission, synaptic plasticity [22, 23], and neurodegeneration [24]. Astrocyte interaction with synaptic spines requires physical contact, as demonstrated in electron microscopy reconstructions where 57% of the spines in a mature hippocampus are associated with astrocytes [25]. In organotypic hippocampal cultures, astrocytes rapidly extend and retract fine processes that associate and release from dendritic spines. Changes in astrocytic processes are coordinated with the stabilization of larger spines [26]. Furthermore, astrocyte protrusions are essential in the maturation and stabilization of newly forming spines, and thus astrocyte contact enhances both lifetime and morphological maturation of spines [27].

Here, we employed FRAP techniques to study actin spine dynamics in dissociated cultures of rat hippocampal neurons and organotypic slices. Our results reveal an unexpectedly high degree of variability regarding actin dynamics in individual spines. Moreover, the spine population was segregated into two groups, according to their recovery velocity rate. Additionally, we show that the presence of astrocytes in the culture can regulate actin cytoskeleton dynamics, in that, spines growing in the presence of astrocytes present a higher actin dynamics than those growing in the absence of astrocytes. Finally, we described a simple protocol to demonstrate the presence of polymerization hot spots within the spine structure.

2. Material and Methods

2.1. Hippocampal Neuronal Culture and Transfection. Primary cultures of hippocampal neurons were dissociated from postnatal (P0-P1) rat pups as previously described [28]. In brief, hippocampal neurons were grown in culture media consisting of Neurobasal medium supplemented with 0.5 mM glutamine, 50 mg/mL penicillin, 50 units/mL streptomycin, 4% FBS, and 4% B27 supplement (all from Invitrogen). Cells were plated in Mattek chambers with a 12 mm glass coverslip center (Mattek, USA), previously coated with poly-D-lysine (50 μg/mL) and laminin (4 μg/mL). On days 4, 7, 14, and 21 in vitro (DIV), 500 μL (from a total of 2000 μL) of the culture medium was replaced with 520 μL of new, fresh medium. Two types of cultures were used, depending on the density of astrocytes. Under regular conditions, after the astrocytes grew to form a monolayer (usually after four days to a week in culture), a concentration of 4 μM of cytosine-D-arabinofuranoside (SIGMA) was added to prevent glial cell overgrowth (this condition was referred to as "Ast high"). In the second type of cultures, the inhibitor was added after 2 days in vitro, to obtain cultures growing in the near-absence of glial cells (we referred to this type of cultures as "Ast low" condition). All neurons studied grew in Ast high conditions, unless otherwise indicated. Prior to plating, neurons were transfected with a vector plasmid encoding for the YFP/GFP fused to the N-terminus of chicken β-actin gene, under the control of the platelet-derived growth factor enhancer/promoter region (PDGF; vector kindly provided by Drs. Yukiko Goda and José Airas [29]). Transfection was performed by neuronal electroporation, using the electroporation rat hippocampal neuron kit from AMAXA according to the manufacturer's instructions or with a BioRad Cell electroporator system (exponential discharge protocol with the following parameters: 220 V and 950 mF and resistance fixed to infinitum; cells and plasmids were mixed in BioRad electroporation buffer). In both protocols, 10 μg of plasmidic DNA was mixed with 2–4 million cells.

2.2. Hippocampal Slice Preparation, Culture, and Sindbis Virus Expression. Hippocampal slices were prepared from young rats of both sexes (postnatal days 6-7) as previously described [30]. Briefly, after dissection of the hippocampi in ice-cold gassed (5% CO_2/95% O_2) dissection solution (in mM: 10 glucose, 4 KCl, 24 $NaHCO_3$, 234 sucrose, 0.5 $MgCl_2 \cdot 6H_2O$, 0.7 $CaCl_2 \cdot 2H_2O$, and 0.03 phenol red at pH 7.4), 400 μm transverse slices were prepared using a tissue slicer. Slices were transferred to slice culture inserts (Millipore) and cultured in culture medium (Minimum Essential Media (MEM) supplemented with 20% (v/v) horse serum, 1 mM glutamine, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 1 mg/L insulin, 0.0012% (w/v) ascorbic acid, 30 mM HEPES, 13 mM glucose and 5.2 mM $NaHCO_3$ at pH 7.25, and a final osmolarity of 320 mOsm/L). Cultures were kept at 35°C. The recombinant EGFP-actin was delivered into slices using the Sindbis virus, as previously described [31]. The plasmid pSR5-EGFP-actin was prepared as described in [32]. Recombinant protein expression was typically 12–24 h.

2.3. Fluorescence Recovery After Photobleaching (FRAP). Images were taken either with a TCS-SP5 or a TCS-SL laser-scanning confocal spectral microscopes (both from Leica Microsystems Heidelberg, GmbH). The inverted microscopes were equipped with an incubation system featuring temperature and CO_2 control. All experiments were performed at 35°C and 5% CO_2. Live images were acquired using a 63x oil immersion objective lens (NA 1.32), with a pixel size of 58 nm × 58 nm. The confocal pinhole was set at 4.94 Airy units to minimize changes in fluorescence due to GFP/YFP-actin moving away from the focus plane.

FRAP experiments were performed using the following protocol: 10 single prebleach scans were acquired at 225–300 ms intervals, followed by 10 bleach scans at full laser power, over a circular area of 2 μm in diameter. During the postbleach period, 250 scans were acquired at 225–300 ms intervals, followed by 10 images acquired at 1 s time intervals. In order to resolve the initial fast recovery, some experiments were performed using the Leica fly mode acquisition; bleaching was performed during the X fly forward scan at 100% laser power. During the backward scan, fluorescence was read out with laser intensity set to imaging values (185 ms interval). Postbleach images (30–60) were acquired at the same time interval.

To avoid significant photobleaching, the excitation intensity was attenuated to ~5 to 8% of the laser power during image acquisition. Fluorescence was quantified using the Image Processing Leica Confocal Software. Background fluorescence was measured in a random field outside of the dendrite and subtracted from all the measurements. Dendrite fluorescence was determined for each image and compared with the initial dendrite fluorescence to determine the spontaneous signal lost during imaging.

The fluorescence signal measured in the region of interest (ROI) and normalized to the change in dendrite fluorescence was determined to be $I_{\text{rel}} = I_t/I_0 * T_0/T_t$, where I_t is the average intensity in the region of interest at time t; I_0 is the average intensity in the region of interest during prebleach, T_0 is the dendrite intensity during prebleach, and T_t is the dendrite intensity at time t. The introduction of the correction factor (T_0/T_t) accounts for possible small fluctuations in total fluorescence intensity caused by the bleach itself and yields a more accurate estimate of the fluorescence measured in the ROI.

The net fluorescence recovery (mobile fraction, MF) measured in the region of interest was determined as $\text{MF} = (F_{\text{end}} - F_{\text{post}})/(F_{\text{pre}} - F_{\text{post}})$, where F_{end} is the ROI mean intensity at the steady-state, F_{post} represents ROI intensity after photobleaching, and F_{pre} is the mean ROI intensity prebleach.

Each individual spine recovery curve was fitted by a two-component exponential equation, although the initial fast component, driven by diffusion, was negligible in most of the recordings. Therefore, the recovery time constant (tau, τ) was calculated from the fitting to a monoexponential curve.

Ultrafast recordings were performed employing a x, t acquisition mode. This protocol permits linear scans of 200–300 nm width at 1-ms intervals. For these experiments, three consecutive scans or jobs were acquired (each consisted of 2000 lines × 512 pixels in width): an initial prebleach job (2000 lines), a bleach protocol (2000 lines at maximal laser power), and a final 6 x jobs (2000 lines each), to account for a recovery time of 12 seconds. To avoid significant photobleaching, excitation intensity was attenuated to ~5 to 8% of the laser power during image acquisition.

Latrunculin A, Cytochalasin D, and Jasplakinolide were from SIGMA.

2.4. Two-Photon Fluorescence Imaging of Hippocampal Slice Preparations. Organotypic hippocampal slices (3–7 DIV) expressing EGFP-actin were perfused with ACSF at 30°C. Two-photon fluorescence images were obtained with a Zeiss LSM510 laser-scanning microscope using a 63x water immersion objective and a Mai Tai DeepSee (Spectra Physics) 910 nm laser as light source for excitation. Digital images were acquired using Zen software. For FRAP experiments, images were acquired every 200 ms for 2.7 min (810 images). After 3 images, the EGFP-actin signal from dendritic spines was photobleached with one iteration of high laser intensity. Fluorescence values at the spine were normalized to those of the adjacent dendrite to compensate for ongoing bleaching during imaging. Fluorescence values and the spine area were analyzed using Image J.

2.5. Immunocytochemistry. Immunocytochemical analysis was performed as follows: cultures were rinsed in phosphate buffer saline (PBS) and fixed for 15 min in 4% paraformaldehyde in PBS. Coverslips were then washed three times in PBS and incubated for 30 min in blocking solution (2% goat serum, 2% serum albumin, and 0.2% Triton X-100 in PBS). Antibodies were diluted in blocking solution and incubated for 60 min. GFAP and Synapsin antibodies were from Abcam (rabbit polyclonal reference 7260) and Cell Signaling USA (rabbit polyclonal reference 2312), respectively. Samples were subsequently washed three times in PBS and incubated for 30 min in PBS solution containing the appropriate fluorescence-conjugated secondary antibodies (all from Molecular Probes) and were then washed five more times with PBS buffer and mounted using Mowiol.

2.6. Statistical Analysis. Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). A two-way ANOVA with Tukey's multiple comparison test was performed to detect differences in mobile fraction between neurons, cultures, and distance from somas. A one-way ANOVA was employed to study differences among neurons or between individual neurons and the whole population. A Kolmogorov-Smirnov test was performed to compare cumulative frequency distributions for spine head areas between "Ast high" and "Ast low" conditions. A Mann-Whitney test was used to test for differences between MF in both culture conditions ("Ast high" and "Ast low") or between the culture ("Ast high") and slices. The significance level was set at $p < 0.05$.

A better model of tau distribution was determined by comparing a single Gaussian model versus a sum of two, employing an extra sum-of-squares F test in GraphPad Prism.

For all our experiments in "Ast high" conditions, a minimum of 10 neurons from around 5 independent cultures and approximately 212 spines were analyzed. For the "Ast low" condition, we studied a minimum of 30 neurons from 10 independent cultures with more than 100 spines analyzed.

3. Results

3.1. Hippocampal Dendritic Spines Are Enriched in Actin. Culture hippocampal neurons produce dendritic protrusions with distinct stages of morphological progression [33, 34]. Dendritic filopodia could be observed as early as 6 DIV and became abundant around 9 DIV. By DIV 14, the dominant dendritic protrusions were thin spines, characterized by a relatively long neck and a small head. Mature, mushroom-shaped spines became abundant at about 18–21 DIV.

Several studies have reported that transfected neurons accumulate GFP-actin at dendritic spines, making them clearly visible without affecting synaptic transmission [15, 35, 36]. We transfected cultured hippocampal neurons with a plasmid encoding GFP-actin under the control of a neuronal PDGF (platelet-derived growth factor) promoter to avoid overproduction and toxicity of GFP-actin (Figure 1(a)) [28, 29]. In these neurons, growing for more than 18 DIV, staining with rhodamine phalloidin largely colocalizes with GFP-actin positive dendritic spines (90% of colocalization, data not shown). This result is consistent with the reported enrichment of actin filaments in the spines [37, 38].

To characterize actin dynamics at the spine, we have employed FRAP as previously described [39, 40]. Our approximation is based on the work of Star et al. [15] and Koskinen et al. [36]. Basically, we are assuming that (1) actin monomers are free to move in and out of the spine compartment and (2) most of the actins in the spine are in filamentous form, and these are in a dynamic equilibrium, continuously poly- and depolymerizing. Therefore, the net recovery of fluorescence at the steady-state (the so-called mobile fraction, MF) includes the free diffusion of actin monomers, plus the proportion of filaments in dynamic equilibrium. Assuming that actin monomer diffusion is constant [41], a low proportion of stable filaments should render high values of mobile fraction, and, conversely, a high proportion of stable actin filaments would produce lower mobile fraction values (Figure 1(b)). Finally, the fluorescence recovery rate is proportional to the velocity of actin monomer incorporation to the plus ends of filament, making FRAP a suitable technique to measure actin treadmilling (Figure 1(b)) [36].

3.2. The Mobile Fraction Value Is Specific to Each Individual Spine. In agreement with the previously reported studies, the recovery curve has two clearly distinguished components, each adjusted to a single exponential curve. The fast (initial) component showed a mean time constant of 0.61 ± 0.09 s. Similar time constant diffusion was obtained when spines from monomeric GFP-transfected (mGFP) neurons were analyzed (0.53 ± 0.093 s), supporting the idea that this fast component was driven by pure diffusion (Figure 1(b) insert and Figure 3(e)). The first component was only uncovered when a fly mode acquisition was employed and was ignored in most of the experiments because it does not provide any information about actin cytoskeleton dynamics. The second component was mostly driven by actin polymerization; consistently, Cytochalasin D (5 μM) treatment, a barbed-end capping drug, reduced the mobile fraction to 0.30 ± 0.13 and slowed recovery fluorescence time as previously described (Figure 1(b); [15]). Jasplakinolide 1 μM treatment, a membrane permeable actin filament stabilizer, greatly impairs fluorescence recovery (MF: around 5%; statistically nonsignificant), further confirming that the slower component depends of F-actin polymerization.

The population of mobile fraction values follows a continuous distribution (values range from 0.2 to 1.1; recovery values higher than 110% were discarded), with a mean value of 0.78 ± 0.01 (Figure 1(c)). When several spines from the same neuron were analyzed, we observed a large variability of MF values within a single neuron (see Figure 1(d) as an example). Therefore, our first question concerned the origin of this variability. Was the mobile fraction regulated by the age of the culture or by the proximity of the spine to neuronal soma? Thus, spine mobile fractions were analyzed for a period of five days (18, 20, and 22 days in culture) and MF values were averaged and segregated, according to their dendritic origin (primary, secondary, and tertiary dendrite) and age of the culture. The results (Figure 1(e)) indicate that neither age of the culture nor the distance from the cell body affects spine mobile fraction (similar mean values were obtained when spines were segregated in 20 μm intervals, data not shown). Despite variability, no differences were found when average mobile fraction values were compared among neurons or between single neurons and the whole population of MFs (Figure 1(f)). In summary, considering these results as a whole, we assume that mobile fraction variability can be attributed to the individual spines themselves, and not to neurons or culture age.

3.3. Astrocyte Contacts Modulate Spine Actin Dynamics. From a spurious observation, we began to suspect that the presence of astrocytes might modulate mobile fraction. Therefore, to evaluate the role of astrocytes modulating actin dynamics at the spine level, we performed FRAP experiments with two types of cultures: regular cultures growing over an astrocyte monolayer (condition: "Ast high") and in the partial/total absence of astrocytes (condition: "Ast low") (Figures 2(a) and 2(b)). Both types of cultures developed spines after 16 days in vitro, and recordings were made between 18 and 22 days in vitro. Despite the fact that neurons exhibit a normal growth in the absence of astrocytes, we observed a consistent reduction in basal fluorescence levels at the spines. To test whether differences arise from spine size, we analyzed the spine head area in both experimental conditions. No significant differences were found in average head area between the two conditions ("Ast high": 0.73 ± 0.03; "Ast low": 0.78 ± 0.04 μm^2), although the cumulative frequency distribution indicates that small spine head areas were more abundant in "Ast low" conditions (Figure 2(c)). Moreover, the initial fast component of recovery in "Ast low" conditions had a mean time value of 0.65 ± 0.04 s,

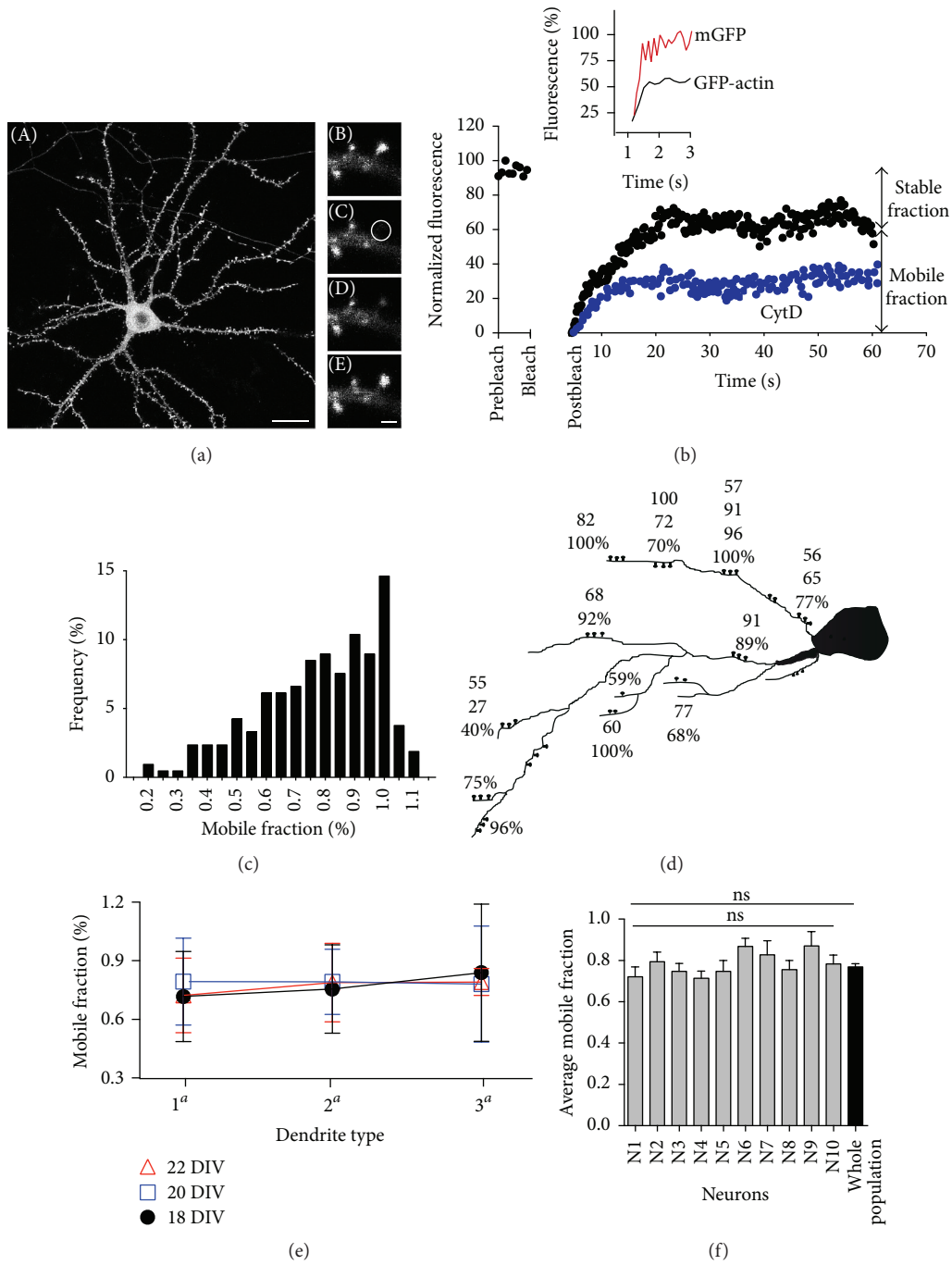

Figure 1: Mobile fraction values do not correlate with culture age or dendrite localization. (a) (A) An example of hippocampal neurons (growing in the presence of astrocytes) transfected with GFP-actin. Scale bar: 20 μm. Pictures on the right (B to E) are higher magnification images showing prebleach (B), bleach (C), postbleach (D), and late phase of recovery (E) (60 s). Scale bar: 1 μm. (b) Normalized fluorescence recovery curve of (a), showing the two fractions of fluorescence recovery: stable and mobile fractions (n = 212 spines, black circles). An example of the recovery in the presence of Cytochalasin D (CytD) (5 μM) in the extracellular solution (n = 4 spines, blue circles). Top insert: comparison of the initial phase of GFP-actin (black line) and monomeric GFP (red line) recovery curves. Note the similarities between both initial phases in the first 1–3 ms. (c) Graph frequency distribution of mobile fractions from neurons 20 DIV growing in the presence of astrocytes. Mean average was 0.78 ± 0.01 (n = 10 neurons, n = 5 independent cultures, and n = 212 spines). (d) Neuronal structure drawing indicating the localization of the recorded spines. Note the variability in MF values along neuronal dendrites. (e) Mobile fraction values were averaged according to their dendrite type (primary, secondary, or tertiary), and the same value was calculated at days 18, 20, and 22 in vitro (n = 10 neurons, n = 5 independent cultures, mean ± SEM). As the graph shows, no differences were found for culture age or dendrite localization (two-way ANOVA with Tukey's multiple comparison test, ns). (f) Average mean of mobile fraction values from ten individual neurons was compared with the overall population of MF mean. No statistical differences were found among neurons or when each neuron was independently compared to the whole population. Only values from 20 DIV were used in this analysis (one-way ANOVA, ns) (n = 10 neurons, n = 5 independent cultures, mean ± SEM).

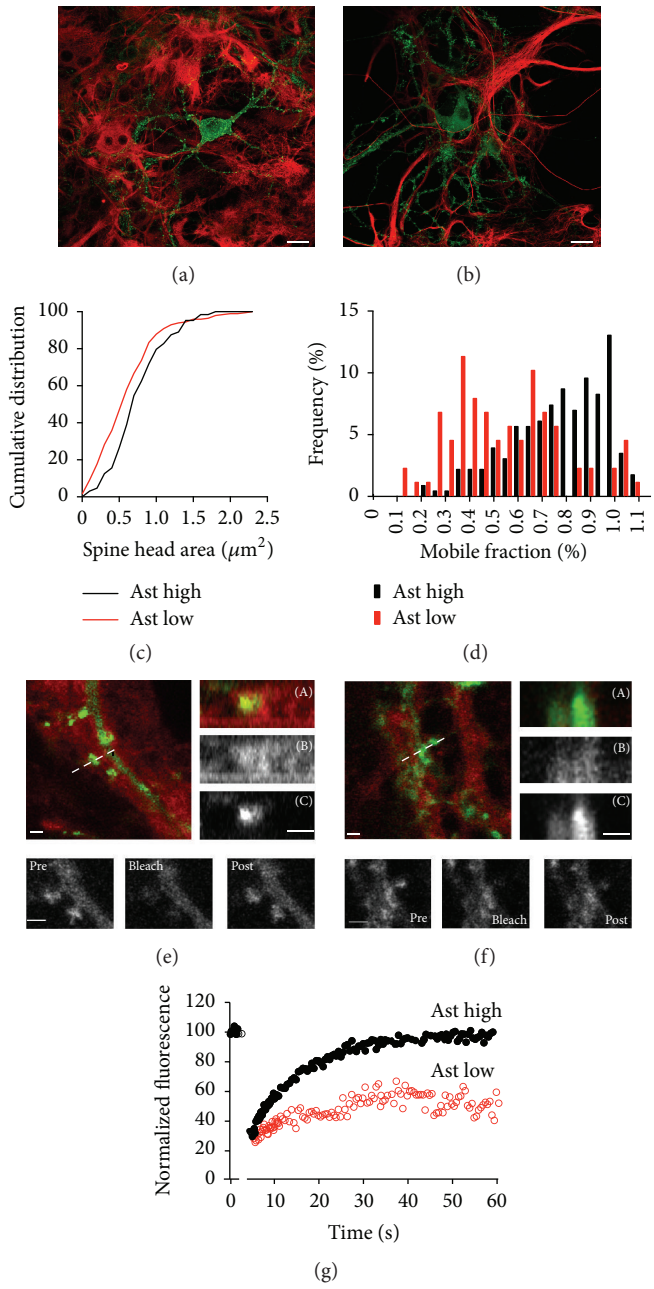

FIGURE 2: Astrocyte contact regulates actin mobile fraction. (a-b) The pictures show two neurons growing over a layer of astrocytes ((a) "Ast high" condition), or in the near-absence/absence of astrocytes ((b) "Ast low" condition). Notice how in the "Ast low" condition a large part of the neuron has no contact with the surrounding astrocytes. Neurons were transfected with GFP-actin (green) and astrocytes were identified by GFAP staining (red). Scale bar: 10 μm, 21 days in vitro. (c) Cumulative distribution of spine head areas comparing "Ast high" (black) versus "Ast low" (red) (n = 198 spines "Ast high" and n = 64 spines "Ast low") (Kolmogorov-Smirnov test, ns). (d) Frequency distribution of mobile fractions in "Ast low" conditions (red bars) (n = 82 spines). Of interest is that the MF was drastically reduced in the "Ast low" condition, as compared to the "Ast high" condition (Mann-Whitney test, $p < 0.0001$). The mobile fraction values distribution of "Ast high" conditions was included for comparison (black bars). (e and f) In vivo confocal images. (e) To evaluate the presence of direct astrocyte contact with the recorded spine, membranes were stained with the lipophilic dye FM4-64 (4 μM, red). The dendrite shown in (e) "Ast high" condition was lying on top of an astrocytic layer. Longitudinal sections at higher magnification were performed to study spine surroundings (approximately marked as a dashed line), pictures (A) to (C) (overlap, FM4-64 and GFP-actin, resp.). Scale bar: 1 μm. Bottom: detailed pictures of selected frames obtained from the FRAP experiment, prebleach, bleach, and postbleach. Scale bar: 1 μm (n = 8). (f) Detailed picture of a dendrite growing in "Ast low" conditions. Scale bar: 1 μm. Notice how FM4-64 staining was only present along the dendrite, but not surrounding the spine. A section of the optical longitudinal acquisition is shown in pictures (A), (B) and (C). Bottom: selected frames obtained from the FRAP experiment, prebleach, bleach, and postbleach. Scale bar: 1 μm (n = 5). (g) Normalized fluorescence recovery curve of the depicted spines growing in "Ast high" (black dots) and "Ast low" (red dots). Notice that spines growing in "Ast high" conditions are characterized by a recovery close to 80%, while spines growing in "Ast low" conditions present a recovery close to 50%.

similar to that obtained in "Ast high" conditions, implying that the diffusion rate is unaffected by the presence/absence of astrocytes in the culture.

In contrast, different results were observed when the mobile fraction was quantified. In "Ast low" conditions, the MF was drastically reduced (Figures 2(d) and 2(g), red open circles). Although some variability is present, the frequency distribution clearly indicates that neurons growing in these conditions have lower mobile fractions, with a range between 0.1 and 0.8 and a mean value of 0.54 ± 0.02 (Figure 2(d)).

To confirm the presence of astrocytes nearby or in close proximity to the spine, in a small number of experiments, FM4-64 (a lipophilic dye) was included in the culture media during the recording conditions. In these conditions, and without stimulated endocytosis, FM4-64 adheres to all extracellular membranes, allowing easy identification of the presence of membranes around the spine. As indicated in Figure 2(e), in "Ast high" conditions, dendrites lie on top of a membrane surface. FM4-64 staining showed a sandwich-like distribution, enfolding dendritic spines (see linear scanning in insert, Figure 2(e)(A, B, and C)), indicative of a membrane around the spine. This spine showed a mobile fraction value close to 80% (Figure 2(g), black closed circles). In clear contrast, the spine from the "Ast low" conditions was almost devoid of red fluorescence around the spine (see insert in Figure 2(f)(A, B and C)). In this case, fluorescence recovery was close to 30% (Figure 2(g), red open circles).

In addition to the physical contact between the dendritic spines and astrocytes, it is possible that glial cells release soluble factors into the medium that affect actin dynamics [42]. In order to test possible contributions of soluble factors, we studied some "Ast low" neurons treated with astrocyte-conditioned medium, but no significant effects were seen in the actin mobile fraction. Due to this absence of significant effects and the fact that the medium composition may depend on many, highly variable factors (age of astrocytes, frequency of medium replacement, degradation, etc.), we chose not to pursue these experiments any further. Nevertheless, we cannot completely rule out that some undetermined soluble factors could affect actin dynamics.

3.4. Spines Can Be Divided into Two Populations, according to Their Recovery Constant. As mentioned earlier, the recovery rate of GFP-actin fluorescence is proportional to actin polymerization velocity. To study the variability of this parameter in our neuronal population, we analyzed the rate of recovery by fitting the second component of the recovery curve to an exponential growth described by a tau value (τ). Recovery time values show a high degree of variability, ranging from 1.1 to 46.8 seconds. The frequency distribution graph suggests the existence of two populations of spines, determined according to their recovery time (Figure 3(a)). The frequency distribution was fitted to a double Gaussian distribution, with two average values of 6.02±2.70 and 14.87± 6.32 seconds, respectively. The kinetics of recovery were also affected by the lack of astrocytes. In these culture conditions, constant times presented a double Gaussian distribution, with two mean values of 13.16 ± 5.84 and 33.22 ± 3.55 (Figure 3(b), red bars).

We then proceeded to evaluate whether there was any relation between recovery times and MF values (in "Ast high" condition). As shown in Figure 3(c), the relation between MF and tau values reinforces the existence of two populations of spines: one characterized by a faster recovery (up to 10 s) and lower MF values and a second population characterized by slower recovery times and higher levels of MF (Figure 3(c)). A similar distribution can be observed when mobile fraction values are plotted versus spine head size. Two populations became apparent in this graph: one with a smaller size and lower mobile fractions and a second one with larger areas and higher mobile fractions (Figure 3(d)).

Therefore, it follows that tau and spine size area are also related, with smaller spines displaying faster recovery times and larger spines being more prone to showing slower recovery times (Figure 3(e), closed dots, left axis). It can be argued that if diffusion is the main driver, the recovery time constant and photobleached area will follow a linear regression that is simply the effect of increasing the bleached area [43]. To test this hypothesis, recovery rates were analyzed for a simple diffusion process employing EGFP transfected neurons, and recovery time values were plotted against spine head areas (Figure 3(e), open circles, right axis). As Figure 3(e) indicates, recovery times are ten times slower when employing GFP-actin, which rules out diffusion as a main driver controlling actin velocity recovery.

To confirm whether this distribution of actin recovery times was a general characteristic of the spines or a peculiarity of the hippocampal cultures, we performed a similar experiment employing hippocampal organotypic slices transfected with GFP-actin (Figure 3(f)). In this condition, the estimated mean MF was 0.84 ± 0.02, which was not statistically different from the MF obtained from the cultures. The differences between these two models emerged when recovery times were analyzed. As Figure 3(f) shows, the frequency distribution of tau values from the organotypic slices indicates the presence of a single population of spines with a mean value of 25.06 ± 1.9 seconds (blue bars). Interestingly, all spines from organotypic cultures have slower recovery times. This result was confirmed when the spine area was plotted against MF. As Figure 3(d) indicates, all values from slices are segregated into a population of spines with larger areas and higher MF values (Figure 3(d), blue circles).

3.5. Spines Contain Polymerization Hot Spots. Previous studies using photoactivated actin in combination with high-resolution techniques suggested the existence of polymerization hot spots along spine head structure [17]. To evaluate this point, we devised a simple experimental protocol employing conventional confocal microscopy. To this end, a line scanning mode (x, t mode) was used to perform FRAP. Employing this acquisition mode, only a narrow longitudinal area was scanned (close to 300 nm wide). This allowed us to reduce time sampling values to 1-2 ms (Figures 4(a)-4(b)). Using this acquisition mode, we were able to differentiate between the recovery rate of the distal part of the spine

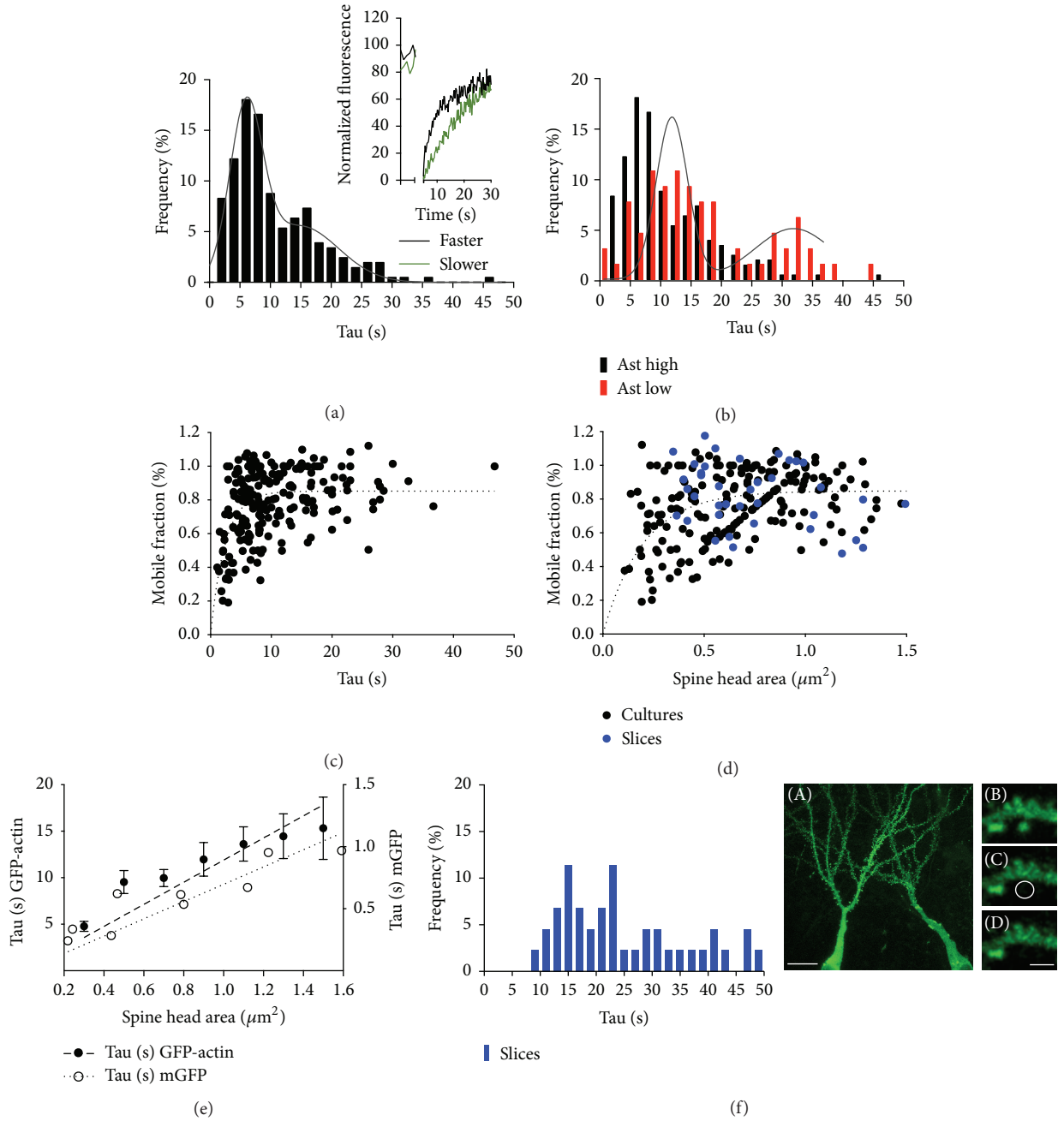

Figure 3: Spines can be divided into two categories, according to their fluorescence recovery time. (a) Frequency distribution of tau values in "Ast high" condition (black bars) (n = 205 spines). Distribution was adjusted to a two-Gaussian distribution, with two peak values of 6.02 ± 2.70 and 14.87 ± 6.32 seconds (F test, $p < 0.0001$) (gray line). Insert depicts two representative recovery curves for each category. Only the initial 30 seconds are displayed. Curves were normalized and scaled to the same initial time. (b) Frequency distribution of tau values in the "Ast low" condition (red bars) (n = 72 spines). Distribution was adjusted to a two-Gaussian distribution, with two estimates peak values of 15.12 ± 2.3 and 35.42 ± 4.22 seconds (F test, $p < 0.0001$) (gray line). Black bars show the frequency distribution of tau values in "Ast high" condition, plotted for comparison. (c) The mobile fraction of each "Ast high" spine was plotted against its recovery time value (dotted line, one phase exponential association). Note that spines with higher mobile fractions present slow recovery times, and vice versa. (d) Mobile fraction values of "Ast high" spines were plotted against their spine head area (black dots) (n = 210 spines). Of interest, spines with a larger head area show higher mobile fractions, and vice versa. Blue dots represent spines from hippocampal culture slices (n = 44 spines). Spines from hippocampal slices show high mobile fractions and larger spine head areas. (e) Left axis: spine head areas were grouped (0.2 μm^2 intervals) and their mean average areas were plotted against their average recovery time (n = 198 spines, mean ± SEM). The value distribution was adjusted to a linear regression (slope: 11.89 ± 0.80). Right axis: same relation employing spines transfected with monomeric GFP (slope: 0.69 ± 0.054) (n = 9 spines). Both graphs show a lineal relation between bleach area and fluorescence recovery constant, although with a tenfold difference in scale. (f) Right picture. (A) Example of two pyramidal hippocampal neurons expressing GFP-actin from an organotypic slice culture. Scale bar: 20 μm. (B to D) Sequential frames of a FRAP experiment. Scale bar: 2 μm. Average mobile fraction was estimated to be 0.84 ± 0.02 (n = 49 spines). Left graph: frequency distribution of tau values obtained from hippocampal slices (n = 42 spines, blue bars). Notice how slice spines fall mostly into a single distribution. Spine MF values from cultures and slices were not statistically different (Mann-Whitney test, ns).

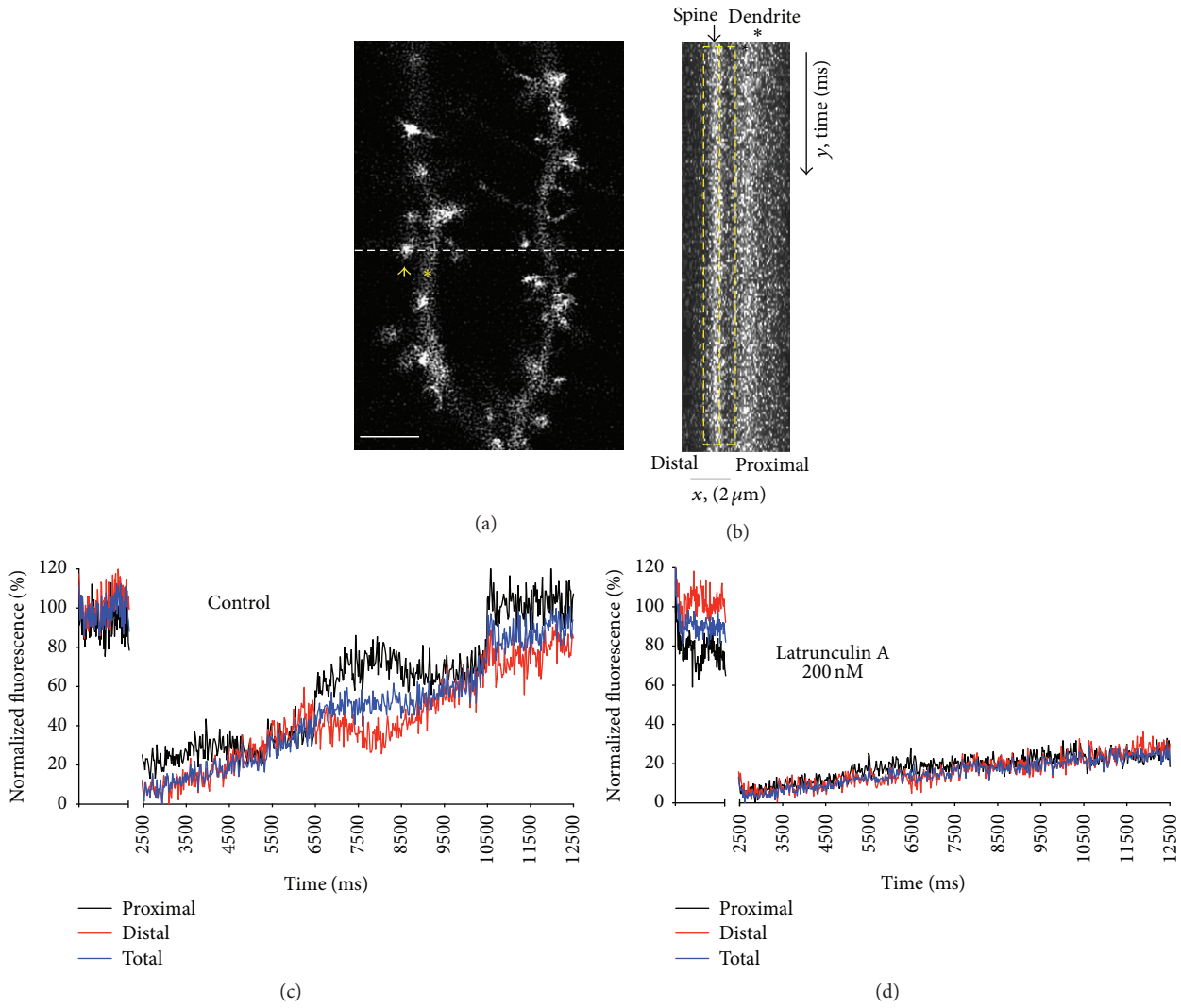

Figure 4: Spine head contains polymerization hot spots. (a) Example of spines from neurons in culture with astrocytes present, transfected with GFP-actin. The white line highlights the area selected for FRAP, employing a line scanning mode. In this mode, the scanned area is limited to the drawn line (around 200–300 nm wide). The spine is labeled with an arrow and the dendrite with an asterisk. Scale bar: 5 μm. (b) Section of a recovery image from the initial postbleach period, obtained with the lineal acquisition mode. The y-axis corresponds to the acquisition time in ms (each line, 1 ms) and the x-axis corresponds to the localization in microns along the line. Spine and dendrite are marked with an arrow and an asterisk, respectively. Spine length was arbitrarily divided into two sections (highlighted by the yellow dashed box), corresponding to the distal and proximal part of the spine (notice how the proximal part includes the neck of the spine and a portion of the head). Scale bar: 2 μm. (c) Example of normalized fluorescence recovery curve of the spine in (b). The distal part of the spine (red) and the proximal area (black) were analyzed and plotted independently. The blue line shows the fluorescence recovery curve of the entire yellow box area (sum of distal and proximal areas). In this particular example, at the times when the distal area recovery rate drops, a proportional increase at the distal area is found. Therefore, the total average fluorescence recovery does not change (blue line). Out of the 25 spines analyzed, 13 (52%) showed differences in the recovery rate between the distal and proximal areas of the spine, as shown in the example. (d) Normalized fluorescence recovery curve after addition to the culture media of Latrunculin A (200 nM), an organic compound that blocks actin polymerization by sequestering actin monomers. Notice the reduction in mobile fraction value and the absence of distributed polymerization.

(cortical head area) and the proximal area (closer to the neck of the spine) (highlighted as a dashed yellow box in Figure 4(b)). When fluorescence recovery curves from each section were independently analyzed, transient changes in the slope of recovery were visible (Figure 4(c)). The changes in one area were accompanied by an equivalent alteration, but in the opposite direction, in the other areas of the spine (Figure 4(c), comparison of recovery between distal and proximal areas). A similar phenomenon was observed in 52% of the spines studied (13 of a total of 25 spines). Oscillations in the slope of recovery were observed in either the distal or proximal areas of the spine in a similar proportion, with no differences between large or small spines. Similar phenomena in the fluorescence profile were also evident during basal

recording, even though the reduction of fluorescence after bleaching facilitated the discrimination (data not shown). To confirm that actin polymerization was the primary cause of these oscillations in fluorescence recovery rate, a series of experiments were performed, adding 200 nM of Latrunculin A (LatA) to the extracellular solution. LatA, an organic compound with a high affinity for monomeric actin, prevents actin polymerization by sequestering actin monomers. Despite a reduction in the recovery rate in the presence of LatA, the recovery profile was similar between the two areas of the spine head (Figure 4(d), $n = 16$). Similar results were obtained when Jasplakinolide 1 μM was added to the culture media (data not shown).

4. Discussion

It has been proposed that a highly dynamic actin cytoskeleton in dendritic spines is necessary to support and regulate spine morphology, as well as synaptic transmission and plasticity. In the present report, we have confirmed the plastic nature of this actin cytoskeleton.

Spine actin mobile fraction values were not homogenous in either slices or cultures. On the contrary, we found a large degree of variability, with values between 20 and 100%, although the majority of spine MF values were concentrated close to 80%. Similar to the results of the pioneering work of Star and colleagues, we found that nearly all spines contained a large amount of dynamic actin [15].

Other authors have previously reported a progressive reduction in mobile fraction associated with culture aging [36]. However, our results show a large and stable MF mean value that is independent of the age of the culture or even the distance to the soma, a result that is in agreement with the lack of changes in hippocampal cultures reported by Star and colleagues [15]. Similar to this work, in our study, large spines that theoretically must bear large postsynaptic densities were associated with large mobile fractions and relatively slow actin recoveries. Confirming these findings, in hippocampal organotypic slices from 7-day-old animals, all spines had a large area and were characterized by highly mobile fraction values. However, it must be recognized that different age and culture conditions, or even FRAP protocols [44], among laboratories would certainly induce different spine actin turnovers that could contribute to explaining the discrepancies in the reported results.

The main finding of our paper is the presence of two spine populations (faster and slower recovery) in culture conditions based on their polymerization rate, and, notably, only one population in organotypic slices (slower). Spine heads typically contain a major dense network of short cross-linked and branched filaments [13]. Since Fluorescence Recovery After Photobleaching quantifies the incorporation of new fluorescent monomers, recovery time constants express, or must be proportional to, the polymerization rate. Attending to the double Gaussian distribution observed, we have classified the spines into slow polymerization (tau values between 10 and 25 seconds) and fast polymerization (between 2 and 10 seconds) groups. Interestingly, analysis of the spine head areas demonstrated that large spines were associated with slower recovery rates, while small spines displayed a faster recovery. Different molecular components at the spine ultrastructural level should easily explain the dynamic differences. A large set of actin binding proteins, such profilin II, gelsolin, debrin, and Arp2/3, have been found to be associated with the spine cytoskeleton (for a review, see Cingolani and Goda [19]). Among them, Cofilin 1/ADF has been recognized as a key regulator controlling F-actin assembly and disassembly [45]. Binding of ADF/Cofilin to actin is controlled via phosphorylation (inactivation) and dephosphorylation (activation) by LIM kinases (LIMK) and slingshot phosphatases, respectively [46], both of which are known to exert powerful control over spine morphology and synaptic plasticity [46]. Overexpression of an inactive form of Cofilin results in more mature spines through an AMPA receptor traffic-dependent mechanism [47]. Inactive Cofilin mutants increase F-actin [48] contents and reduce the actin dynamics measured by FRAP [49]. On the other hand, Cofilin 1 promotes F-actin assembly during LTP [50]; conversely, it is required for F-actin disassembly and spine shrinkage during LTD [51]. Such a dual function of Cofilin 1 thus suggests that it may be responsible, at least in part, for the observed variability among turnovers and actin mobile fractions. However, based on the complexity of the signal cascades that control actin dynamics, it is very likely that additional molecular pathways are also involved. An accurate proportion and compartmentalization of the actin binding proteins inside the spine would be crucial to ensuring proper spine morphology and function. Future experiments quantifying and analyzing the distribution of proteins controlling polymerization within the spine are necessary.

Spine size distribution was different between primary cultures and slices. In primary cultures, a large proportion of spines were smaller than their counterparts found in slices. We must keep in mind that our measurements are relative, based on an estimation of the spine area from a two-dimensional image. Levels of transfection among neurons or even the microscope employed might affect this variable. Nevertheless, a simple explanation might be the different developmental stage of spines in these two systems. Thus, young spines of small size may be more abundant and more easily found in primary cultures, while this category of spines progressively diminishes in slices until its final elimination [52]. Further experiments analyzing spine size distribution, comparing primary cultures and slices, would be needed to clarify this point.

An unexpected result was the role of astrocytes, which participated in the dynamics of the actin cytoskeleton of the spine. The absence of astrocytes shifted the actin mobile fraction distribution to smaller values and slower recoveries. A substantial series of reports have demonstrated that astrocytes play a critical role in regulating synapse formation and activity in the central nervous system [53, 54]. Astrocyte presence increases synapse formation, maturation, and stabilization [20, 27, 55, 56]. Several soluble factors secreted by astrocytes have been already identified, including thrombospondins [57], cholesterol complexes [58], and SPARC

[59], which are known to be involved in synaptic formation and maturation. Moreover, the age of astrocytes in cultures regulates the probability of release and synapse maturity of cocultured neurons [60]. In addition to secreted factors, astrocytes can regulate synaptogenesis through physical interactions, and local contact by astrocytes thus elicited PKC activation by means of integrin receptor activation within the neuron, facilitating glutamatergic synaptogenesis [61]. Ephrin interactions between neurons and astrocytes have been implicated in spine morphology regulation. EphA4, a family of tyrosine kinase receptors, is enriched in dendritic spines and its ligand ephrin-A3 is localized at the astrocytic processes [56]. Acute inhibition of ephrin/EphA4 signaling in hippocampal neuronal cultures produces irregular spines with thinner heads [56]. Consistent with a role in neuron-astrocyte signaling, acute application of EphA4/Fc (which inhibits endogenous interaction of EphA4) decreases the contact lifetime between astrocyte processes and spines and reduces astrocyte-dependent stabilization of newly formed dendritic spines in organotypic hippocampal cultures [27]. Therefore, synaptic maturation and neuronal activity are among the many forms of astrocytic control. At this point, we cannot determine which signaling pathways might mediate the effect of surrounding astrocytes on the actin cytoskeleton within the spine. Nevertheless, we cannot rule out the influence of secreted factors. Further experiments will be needed to address this issue.

Finally, our experiments confirmed the existence of polymerization hot spots along the spine structure, as previously shown by Frost and colleagues [17]. In their work employing a combination of PALM techniques, the authors demonstrate the existence of discrete and separate foci along the spine head and neck, where actin polymerization velocity was elevated. The authors conclude that some of these hot spots can be associated with areas of receptor endocytosis. Our results are based on the use of fast line scans with a low spatial resolution, but a fast acquisition rate (1-2 ms). Spatial resolution is limited under these conditions. Our calculations employing fixed cells established a wide 300-nm range, limiting the measured area and therefore reducing the probabilities of detecting simultaneous hot spots. Interestingly, the presence of a polymerization hot spot was accompanied by a similar area of slower polymerization, suggesting a flux of actin monomers within the spine. This net flux of actin monomers would remain undetected when whole spine fluorescence is measured.

Synapses are inherently plastic and undergo persistent changes in strength and postsynaptic receptor composition [62]. Spine cytoarchitecture has been also associated with synaptic plasticity. Synaptic changes that support long-term plasticity (i.e., LTP) evolve through consecutive stages, and every stage involves a different set of actin functions (for a review, see Rudy [63]). Remarkably, these changes are not coupled with changes in nearby spines [64–67], supporting the functional/biochemical independence of each spine. Interestingly, the development of the two-photon glutamate uncaging technique has allowed the stimulation of a single spine while simultaneously imaging its morphology [68]. With this approach, it has been found that, upon stimulation, a single dendritic spine rapidly changes its morphology, enlarging its head for the first few minutes and eventually experiencing a whole-volume change that lasts for hours [67, 68] (for a review, see Nishiyama and Yasuda [69]).

We have observed a large degree of actin variability among spines, even on the same dendrite. This finding reinforces the notion that, at the biochemical and structural levels, each spine is self-regulated independently of its neighbors. One can speculate about the reasons for the observed variability among the spines, but an independently regulated actin cytoskeleton would indisputably subserve a large degree of systemic plasticity. In other words, every spine would independently adapt its structure to the ongoing synaptic strength, with the actin cytoskeleton being the main element responsible for these changes. As Professor Yuste proposed, the electrical and biochemical independence of each spine supports the brain's ability to form a plastic nonsaturated distributed circuit, where every spine is independently regulated [70].

It goes without saying that we are still far from having a complete understanding of actin dynamic participation in spine morphogenesis and physiology. We believe that future work must be undertaken to understand the different roles of actin binding proteins within the spine and to specifically quantify the participation of actin dynamics in the process of AMPA glutamate receptor endocytosis.

5. Conclusions

The main findings of our report are, first, the confirmation of the dynamic nature of the actin cytoskeleton at the spine head level. This dynamic is individually regulated by each spine, independently of neuron age or distance from the cell body. Second, we have found that the presence of astrocytes is an important regulator of the actin mobile fraction and polymerization rate. Third, according to their polymerization rate, spines can be categorized into two populations in primary cultures, or a single population in organotypic slices. Finally, our results confirm the presence of polymerization hot spots within the spine.

Conflict of Interests

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

Acknowledgments

This paper is supported by Grants from the Spanish Ministry of the Economy and Competitiveness: BFU 2010-1753 (Miguel Morales) and SAF2011-24730 (José Antonio Esteban). The authors would like to thank Anna Bosch from Advanced Optical Microscopy Unit from CCiTUB from University of Barcelona for her support with confocal and image analysis.

References

[1] S. Ramón y Cajal, *La Textura del Sistema Nervioso del Hombre y los Vertebrados*, Springer, Berlin, Germany, 1998.

[2] C.-C. Chen, J. Lu, and Y. Zuo, "Spatiotemporal dynamics of dendritic spines in the living brain," *Frontiers in Neuroanatomy*, vol. 8, article 28, 2014.

[3] H. Hering and M. Sheng, "Dendritic spines: structure, dynamics and regulation," *Nature Reviews in Neuroscience*, vol. 2, no. 12, pp. 880–888, 2001.

[4] R. Araya, T. P. Vogels, and R. Yuste, "Activity-dependent dendritic spine neck changes are correlated with synaptic strength," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 28, pp. E2895–E2904, 2014.

[5] M. Bosch and Y. Hayashi, "Structural plasticity of dendritic spines," *Current Opinion in Neurobiology*, vol. 22, no. 3, pp. 383–388, 2012.

[6] L. Enriquez-Barreto, G. Cuesto, N. Dominguez-Iturza et al., "Learning improvement after PI3K activation correlates with de novo formation of functional small spines," *Frontiers in Molecular Neuroscience*, vol. 6, article 54, 2014.

[7] J. N. Bourne and K. M. Harris, "Balancing structure and function at hippocampal dendritic spines," *Annual Review of Neuroscience*, vol. 31, pp. 47–67, 2008.

[8] P. Penzes, M. E. Cahill, K. A. Jones, J.-E. VanLeeuwen, and K. M. Woolfrey, "Dendritic spine pathology in neuropsychiatric disorders," *Nature Neuroscience*, vol. 14, no. 3, pp. 285–293, 2011.

[9] J. C. Fiala, J. Spacek, and K. M. Harris, "Dendritic spine pathology: cause or consequence of neurological disorders?" *Brain Research Reviews*, vol. 39, no. 1, pp. 29–54, 2002.

[10] N. Honkura, M. Matsuzaki, J. Noguchi, G. C. R. Ellis-Davies, and H. Kasai, "The subspine organization of actin fibers regulates the structure and plasticity of dendritic spines," *Neuron*, vol. 57, no. 5, pp. 719–729, 2008.

[11] C. Dillon and Y. Goda, "The actin cytoskeleton: integrating form and function at the synapse," *Annual Review of Neuroscience*, vol. 28, pp. 25–55, 2005.

[12] B. Calabrese, M. S. Wilson, and S. Halpain, "Development and regulation of dendritic spine synapses," *Physiology*, vol. 21, no. 1, pp. 38–47, 2006.

[13] F. Korobova and T. M. Svitkina, "Molecular architecture of synaptic actin cytoskeleton in hippocampal neurons reveals a mechanism of dendritic spine morphogenesis," *Molecular Biology of the Cell*, vol. 21, no. 1, pp. 165–176, 2010.

[14] P. Hotulainen and C. C. Hoogenraad, "Actin in dendritic spines: connecting dynamics to function," *The Journal of Cell Biology*, vol. 189, no. 4, pp. 619–629, 2010.

[15] E. N. Star, D. J. Kwiatkowski, and V. N. Murthy, "Rapid turnover of actin in dendritic spines and its regulation by activity," *Nature Neuroscience*, vol. 5, no. 3, pp. 239–246, 2002.

[16] K.-I. Okamoto, T. Nagai, A. Miyawaki, and Y. Hayashi, "Rapid and persistent modulation of actin dynamics regulates postsynaptic reorganization underlying bidirectional plasticity," *Nature Neuroscience*, vol. 7, no. 10, pp. 1104–1112, 2004.

[17] N. A. Frost, H. Shroff, H. Kong, E. Betzig, and T. A. Blanpied, "Single-molecule discrimination of discrete perisynaptic and distributed sites of actin filament assembly within dendritic spines," *Neuron*, vol. 67, no. 1, pp. 86–99, 2010.

[18] S. S. Correia, S. Bassani, T. C. Brown et al., "Motor protein-dependent transport of AMPA receptors into spines during long-term potentiation," *Nature Neuroscience*, vol. 11, no. 4, pp. 457–466, 2008.

[19] L. A. Cingolani and Y. Goda, "Actin in action: the interplay between the actin cytoskeleton and synaptic efficacy," *Nature Reviews Neuroscience*, vol. 9, no. 5, pp. 344–356, 2008.

[20] E. M. Ullian, S. K. Sapperstein, K. S. Christopherson, and B. A. Barres, "Control of synapse number by glia," *Science*, vol. 291, no. 5504, pp. 657–661, 2001.

[21] P. G. Haydon and G. Carmignoto, "Astrocyte control of synaptic transmission and neurovascular coupling," *Physiological Reviews*, vol. 86, no. 3, pp. 1009–1031, 2006.

[22] A. Araque and G. Perea, "Glial modulation of synaptic transmission in culture," *Glia*, vol. 47, no. 3, pp. 241–248, 2004.

[23] G. Perea and A. Araque, "Astrocytes potentiate transmitter release at single hippocampal synapses," *Science*, vol. 317, no. 5841, pp. 1083–1086, 2007.

[24] Z. Šišková and M.-È. Tremblay, "Microglia and synapse: interactions in health and neurodegeneration," *Neural Plasticity*, vol. 2013, Article ID 425845, 10 pages, 2013.

[25] R. Ventura and K. M. Harris, "Three-dimensional relationships between hippocampal synapses and astrocytes," *Journal of Neuroscience*, vol. 19, no. 16, pp. 6897–6906, 1999.

[26] M. Haber, L. Zhou, and K. K. Murai, "Cooperative astrocyte and dendritic spine dynamics at hippocampal excitatory synapses," *Journal of Neuroscience*, vol. 26, no. 35, pp. 8881–8891, 2006.

[27] H. Nishida and S. Okabe, "Direct astrocytic contacts regulate local maturation of dendritic spines," *Journal of Neuroscience*, vol. 27, no. 2, pp. 331–340, 2007.

[28] M. Morales, M. A. Colicos, and Y. Goda, "Actin-dependent regulation of neurotransmitter release at central synapses," *Neuron*, vol. 27, no. 3, pp. 539–550, 2000.

[29] M. A. Colicos, B. E. Collins, M. J. Sailor, and Y. Goda, "Remodeling of synaptic actin induced by photoconductive stimulation," *Cell*, vol. 107, no. 5, pp. 605–616, 2001.

[30] B. H. Gähwiler, M. Capogna, D. Debanne, R. A. McKinney, and S. M. Thompson, "Organotypic slice cultures: a technique has come of age," *Trends in Neurosciences*, vol. 20, no. 10, pp. 471–477, 1997.

[31] R. Malinow, Y. Hayashi, M. Maletic-Savatic et al., "Introduction of green fluorescent protein (GFP) into hippocampal neurons through viral infection," *Cold Spring Harbor Protocols*, vol. 2010, no. 4, 2010.

[32] M. Benoist, R. Palenzuela, C. Rozas et al., "MAP1B-dependent Rac activation is required for AMPA receptor endocytosis during long-term depression," *The EMBO Journal*, vol. 32, no. 16, pp. 2287–2299, 2013.

[33] H. Takahashi, Y. Sekino, S. Tanaka, T. Mizui, S. Kishi, and T. Shirao, "Drebrin-dependent actin clustering in dendritic filopodia governs synaptic targeting of postsynaptic density-95 and dendritic spine morphogenesis," *Journal of Neuroscience*, vol. 23, no. 16, pp. 6586–6595, 2003.

[34] K. Goslin, G. Banker, and H. Asmussen, "Rat hippocampal neurons in low density cultures," in *Culturing nerve cells*, pp. 339–370, MIT Press, Cambridge, Mass, USA, 1998.

[35] M. Fischer, S. Kaech, D. Knutti, and A. Matus, "Rapid actin-based plasticity in dendritic spines," *Neuron*, vol. 20, no. 5, pp. 847–854, 1998.

[36] M. Koskinen, E. Bertling, R. Hotulainen, K. Tanhuanpää, and P. Hotulainen, "Myosin IIb controls actin dynamics underlying the dendritic spine maturation," *Molecular and Cellular Neuroscience*, vol. 61, pp. 56–64, 2014.

[37] E. Fifkova and R. J. Delay, "Cytoplasmic actin in neuronal processes as a possible mediator of synaptic plasticity," *The Journal of Cell Biology*, vol. 95, no. 1, pp. 345–350, 1982.

[38] A. Matus, M. Ackermann, G. Pehling, H. R. Byers, and K. Fujiwara, "High actin concentrations in brain dendritic spines and postsynaptic densities," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 79, no. 23 I, pp. 7590–7594, 1982.

[39] M. R. Chacón, A. I. Navarro, G. Cuesto et al., "Focal adhesion kinase regulates actin nucleation and neuronal filopodia formation during axonal growth," *Development*, vol. 139, no. 17, pp. 3200–3210, 2012.

[40] M. Martinez de Lagran, R. Benavides-Piccione, I. Ballesteros-Yañez et al., "Dyrk1A influences neuronal morphogenesis through regulation of cytoskeletal dynamics in mammalian cortical neurons," *Cerebral Cortex*, vol. 22, no. 12, pp. 2867–2877, 2012.

[41] F. Lanni and B. R. Ware, "Detection and characterization of actin monomers, oligomers, and filaments in solution by measurement of fluorescence photobleaching recovery," *Biophysical Journal*, vol. 46, no. 1, pp. 97–110, 1984.

[42] N. J. Allen and B. A. Barres, "Signaling between glia and neurons: focus on synaptic plasticity," *Current Opinion in Neurobiology*, vol. 15, no. 5, pp. 542–548, 2005.

[43] D. Axelrod, D. E. Koppel, J. Schlessinger, E. Elson, and W. W. Webb, "Mobility measurement by analysis of fluorescence photobleaching recovery kinetics," *Biophysical Journal*, vol. 16, no. 9, pp. 1055–1069, 1976.

[44] M. Koskinen and P. Hotulainen, "Measuring F-actin properties in dendritic spines," *Frontiers in Neuroanatomy*, vol. 8, article 74, 2014.

[45] G. Hild, L. Kalmár, R. Kardos, M. Nyitrai, and B. Bugyi, "The other side of the coin: functional and structural versatility of ADF/cofilins," *European Journal of Cell Biology*, vol. 93, no. 5-6, pp. 238–251, 2014.

[46] M. B. Rust, "ADF/cofilin: a crucial regulator of synapse physiology and behavior," *Cellular and Molecular Life Sciences*, vol. 72, no. 18, pp. 3521–3529, 2015.

[47] J. Gu, C. W. Lee, Y. Fan et al., "ADF/cofilin-mediated actin dynamics regulate AMPA receptor trafficking during synaptic plasticity," *Nature Neuroscience*, vol. 13, no. 10, pp. 1208–1215, 2010.

[48] M. B. Rust, C. B. Gurniak, M. Renner et al., "Learning, AMPA receptor mobility and synaptic plasticity depend on n-cofilin-mediated actin dynamics," *The EMBO Journal*, vol. 29, no. 11, pp. 1889–1902, 2010.

[49] P. Hotulainen, O. Llano, S. Smirnov et al., "Defining mechanisms of actin polymerization and depolymerization during dendritic spine morphogenesis," *The Journal of Cell Biology*, vol. 185, no. 2, pp. 323–339, 2009.

[50] M. Bosch, J. Castro, T. Saneyoshi, H. Matsuno, M. Sur, and Y. Hayashi, "Structural and molecular remodeling of dendritic spine substructures during long-term potentiation," *Neuron*, vol. 82, no. 2, pp. 444–459, 2014.

[51] Q. Zhou, K. J. Homma, and M.-M. Poo, "Shrinkage of dendritic spines associated with long-term depression of hippocampal synapses," *Neuron*, vol. 44, no. 5, pp. 749–757, 2004.

[52] K. M. Harris, F. E. Jensen, and B. Tsao, "Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation," *The Journal of Neuroscience*, vol. 12, no. 7, pp. 2685–2705, 1992.

[53] N. J. Allen and B. A. Barres, "Neuroscience: glia—more than just brain glue," *Nature*, vol. 457, no. 7230, pp. 675–677, 2009.

[54] A. Pérez-Alvarez and A. Araque, "Astrocyte-neuron interaction at tripartite synapses," *Current Drug Targets*, vol. 14, no. 11, pp. 1220–1224, 2013.

[55] C. Eroglu and B. A. Barres, "Regulation of synaptic connectivity by glia," *Nature*, vol. 468, no. 7321, pp. 223–231, 2010.

[56] K. K. Murai, L. N. Nguyen, F. Irie, Y. Yamaguchi, and E. B. Pasquale, "Control of hippocampal dendritic spine morphology through ephrin-A3/EphA4 signaling," *Nature Neuroscience*, vol. 6, no. 2, pp. 153–160, 2003.

[57] K. S. Christopherson, E. M. Ullian, C. C. A. Stokes et al., "Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis," *Cell*, vol. 120, no. 3, pp. 421–433, 2005.

[58] D. H. Mauch, K. Nägier, S. Schumacher et al., "CNS synaptogenesis promoted by glia-derived cholesterol," *Science*, vol. 294, no. 5545, pp. 1354–1357, 2001.

[59] E. V. Jones, Y. Bernardinelli, Y. C. Tse, S. Chierzi, T. P. Wong, and K. K. Murai, "Astrocytes control glutamate receptor levels at developing synapses through SPARC-beta-integrin interactions," *Journal of Neuroscience*, vol. 31, no. 11, pp. 4154–4165, 2011.

[60] H. Kawano, S. Katsurabayashi, Y. Kakazu et al., "Long-term culture of astrocytes attenuates the readily releasable pool of synaptic vesicles," *PLoS ONE*, vol. 7, no. 10, Article ID e48034, 2012.

[61] H. Hama, C. Hara, K. Yamaguchi, and A. Miyawaki, "PKC signaling mediates global enhancement of excitatory synaptogenesis in neurons triggered by local contact with astrocytes," *Neuron*, vol. 41, no. 3, pp. 405–415, 2004.

[62] S. Knafo, C. Venero, C. Sánchez-Puelles et al., "Facilitation of AMPA receptor synaptic delivery as a molecular mechanism for cognitive enhancement," *PLoS Biology*, vol. 10, no. 2, Article ID e1001262, 2012.

[63] J. W. Rudy, "Variation in the persistence of memory: an interplay between actin dynamics and AMPA receptors," *Brain Research*, vol. 1621, pp. 29–37, 2015.

[64] R. C. Malenka and M. F. Bear, "LTP and LTD: an embarrassment of riches," *Neuron*, vol. 44, no. 1, pp. 5–21, 2004.

[65] R. Yuste and W. Denk, "Dendritic spines as basic functional units of neuronal integration," *Nature*, vol. 375, no. 6533, pp. 682–684, 1995.

[66] B. L. Sabatini, T. G. Oertner, and K. Svoboda, "The life cycle of Ca^{2+} ions in dendritic spines," *Neuron*, vol. 33, no. 3, pp. 439–452, 2002.

[67] S.-J. R. Lee, Y. Escobedo-Lozoya, E. M. Szatmari, and R. Yasuda, "Activation of CaMKII in single dendritic spines during long-term potentiation," *Nature*, vol. 458, no. 7236, pp. 299–304, 2009.

[68] M. Matsuzaki, G. C. R. Ellis-Davies, T. Nemoto, Y. Miyashita, M. Iino, and H. Kasai, "Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons," *Nature Neuroscience*, vol. 4, no. 11, pp. 1086–1092, 2001.

[69] J. Nishiyama and R. Yasuda, "Biochemical computation for spine structural plasticity," *Neuron*, vol. 87, no. 1, pp. 63–75, 2015.

[70] R. Yuste, "Dendritic spines and distributed circuits," *Neuron*, vol. 71, no. 5, pp. 772–781, 2011.

Hindawi Publishing Corporation
Neural Plasticity
Volume 2016, Article ID 8301737, 9 pages
http://dx.doi.org/10.1155/2016/8301737

Review Article

Emerging Roles of BAI Adhesion-GPCRs in Synapse Development and Plasticity

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Received 14 July 2015; Revised 6 October 2015; Accepted 12 October 2015

Academic Editor: Lin Xu

Synapses mediate communication between neurons and enable the brain to change in response to experience, which is essential for learning and memory. The sites of most excitatory synapses in the brain, dendritic spines, undergo rapid remodeling that is important for neural circuit formation and synaptic plasticity. Abnormalities in synapse and spine formation and plasticity are associated with a broad range of brain disorders, including intellectual disabilities, autism spectrum disorders (ASD), and schizophrenia. Thus, elucidating the mechanisms that regulate these neuronal processes is critical for understanding brain function and disease. The brain-specific angiogenesis inhibitor (BAI) subfamily of adhesion G-protein-coupled receptors (adhesion-GPCRs) has recently emerged as central regulators of synapse development and plasticity. In this review, we will summarize the current knowledge regarding the roles of BAIs at synapses, highlighting their regulation, downstream signaling, and physiological functions, while noting the roles of other adhesion-GPCRs at synapses. We will also discuss the relevance of BAIs in various neurological and psychiatric disorders and consider their potential importance as pharmacological targets in the treatment of these diseases.

1. Introduction

Mental, emotional, and autonomic functions of the brain arise from interactions between the nearly 100 billion neurons that comprise this organ in humans. On average, each neuron forms 1,000 specialized contacts, or synapses, with other neurons. Synapses are asymmetric, complex, and highly dynamic [1, 2]. The plasticity of synapses and dendritic spines, the morphological structures that are the loci of most excitatory synapses in the central nervous system (CNS), are widely believed to underlie learning and memory and are frequently altered in neurodevelopmental and neurodegenerative diseases [3, 4]. Thus, understanding the development, dynamics, and elimination of synapses is crucial for human health. A dizzying array of signals coordinates these processes, and thus receptors are an integral component of the synaptic regulatory machinery [1–4]. Receptors also represent the most accessible point at which to manipulate these processes pharmacologically [5].

2. Adhesion-GPCRs

G-protein coupled receptors (GPCRs) comprise a superfamily of approximately 800 members in humans, including many important drug targets [6]. They exhibit a characteristic seven-transmembrane (7TM) core structure by which GPCRs interact with and activate a variety of heterotrimeric G-proteins, which in turn activate or repress intracellular signaling cascades [7]. Adhesion-GPCRs are a GPCR subfamily with 33 members in humans that are characterized by an extended N-terminal extracellular segment connected to the core GPCR structure by a distinctive GPCR autoproteolysis-inducing (GAIN) domain, which is present in all adhesion-GPCRs except GPR123 [8, 9]. The N-terminal segments of

most adhesion-GPCRs contain multiple domains capable of binding to other cells or the extracellular matrix [8–10]. These include at least 16 different types of domain, with multiple types frequently occurring within the same protein; domains include cadherin-like repeats, thrombospondin-like repeats, rhamnose-binding lectin domains, and calnexin domains. Adhesion-GPCRs can be divided into 9 subfamilies based on phylogenetic analysis of the GPCR moiety; members of the different subfamilies generally also have related complements of N-terminal adhesive domains [9, 10]. GAIN domains mediate autoproteolytic cleavage of adhesion-GPCRs during translation in the ER at a site within the GAIN domain called the GPCR proteolysis site (GPS) [11, 12]. After cleavage, the N- and C-terminal fragments (NTFs, CTFs) of most adhesion-GPCRs remain noncovalently associated [9, 10]. However, this scenario is complicated. Some adhesion-GPCRs do not undergo autoproteolysis, and some that do may even swap NTFs with other adhesion-GPCRs resulting in "hybrid" adhesion-GPCRs [9, 13, 14]. Cell type and ligand binding may affect cleavage and association of the resulting fragments. It has been widely believed that the NTFs may repress the signaling mediated by CTFs, and that ligand binding relieves this inhibition, possibly by causing dissociation of the NTF from the CTF [8, 15]. Recently, a peptide agonist sequence named Stachel was identified on the C-terminal side of the GPS of adhesion-GPCRs. This sequence, which is specific for a given adhesion-GPCR, can activate G-protein dependent signaling through the adhesion-GPCR when it is unmasked by removal of the NTF or conformational changes in the protein (either of which is presumably ligand-induced) [16]. Identification of the GAIN domain and Stachel sequence are both recent findings, illustrating a rapid advance in the knowledge of adhesion-GPCR biology after years lagging behind other GPCRs.

Adhesion-GPCRs function in various tissues throughout organisms [8, 9], but an important driving force of recent rapid advances in adhesion-GPCR biology has been the discovery that adhesion-GPCRs regulate the development and function of many aspects of the nervous system. These include migration of neuronal precursors, axon guidance, myelination of axons, vascularization of the brain, and synapse formation and function [8, 9]. In this brief review, we highlight the roles of the brain-specific angiogenesis inhibitor (BAI) subfamily of adhesion-GPCRs at neuronal synapses. Adhesion-GPCR nomenclature arose over a long period of time and in a nonsystemic manner. Recently, a systemized nomenclature was proposed for this family [9]. Thus, the members of the BAI subfamily, BAI1–3, would now be named ADGRB1–3. This new nomenclature is not yet in standard use, and we will use the traditional names for adhesion-GPCRs, noting the new designations of adhesion-GPCRs we discuss. For general information on adhesion-GPCR function we direct the reader to several excellent recent reviews [8–10, 12].

3. The BAI Subfamily of Adhesion-GPCRs

BAI1, BAI2, and BAI3 (ADGRB1–3) comprise a subfamily of adhesion-GPCRs that are highly expressed in the brain [9, 17]. BAIs are large proteins, approximately 200 kDa in size, with each possessing a long N-terminal region containing multiple adhesive thrombospondin type 1 repeats (TSRs), a hormone-binding domain, and the autoproteolysis-inducing GAIN domain (Figure 1). BAIs also contain an extended intracellular region C-terminal to the conserved 7TM GPCR domain that terminates in a PDZ-binding motif, QTEV (Gln-Thr-Glu-Val) [18]. BAI1 contains an additional TSR (five in total), an integrin-binding RGD (Arg-Gly-Asp) motif, and a C-terminal proline-rich region not present in the other two BAI family members (Figure 1).

BAI1 was initially identified as a target gene of the tumor suppressor p53 [19]. Genes encoding BAI2 and BAI3 were subsequently discovered based on their homology with *BAI1* [20]. BAIs are widely expressed in postnatal and adult brain, with *BAI1* and *BAI2* mRNA levels peaking at postnatal day 10 (P10), while the level of *BAI3* mRNA is highest 1 day after birth [21]. BAI1 protein is present in neurons, glia, and macrophages, with particularly high expression in cortical and hippocampal pyramidal neurons [22–26]. Less is known about the cellular distribution of BAI2 and BAI3 proteins, although BAI3 is abundant in cerebellar Purkinje cells [27–29]. In neurons, BAI1 and BAI3 are both enriched in the postsynaptic density (PSD), suggesting a role for these proteins in synapse development and/or function [25, 30, 31].

Like most adhesion-GPCRs, BAIs possess a GAIN domain, but their ability to undergo autoproteolytic cleavage appears to be cell-type specific and not required for proper surface trafficking [31]. For instance, while BAI1 is cleaved at the GPS site in mouse brain and human malignant glioma cells [11, 32–34], uncleaved full-length BAI1 is also clearly present in hippocampal and cortical neurons [25]. Cleavage of the BAI1 GAIN domain generates a secreted 120 kDa fragment called Vasculostatin-120 (Vstat120), which is capable of inhibiting angiogenesis and tumor formation [32, 33]. BAI1 is also cleaved at a second site N-terminal to the GAIN domain by matrix metalloproteinase 14 (MMP-14) [35]. This cleavage event generates a 40 kDa fragment called Vasculostatin-40 (Vstat40), which also has antiangiogenic activity [35]. The antiangiogenic effects of Vstat120 and Vstat40 are primarily mediated by the TSRs, which bind to the scavenger receptor CD36 and induce proapoptotic signaling [33]. While proteolytic cleavage of BAI proteins is thought to both modulate the function of the full-length receptors and release their NTFs, which can exert their own physiological effects [18], more work needs to be done to understand how cleavage is regulated and what precise consequences it has on BAI function.

Research in the last decade has revealed a number of important roles for BAI family members in diverse cellular processes [17, 36]. As indicated above, BAI proteins can function as potent inhibitors of angiogenesis and tumor progression [36]. BAI1 expressed in macrophages has also been shown to bind to phosphatidylserine (PS) and lipopolysaccharide (LPS) and mediate the engulfment of apoptotic cells and Gram-negative bacteria, respectively [24, 37]. BAI1 promotes engulfment in response to PS or LPS binding by activating the associated ELMO/DOCK180 signaling module, which in turn activates the small GTPase Rac1 and induces

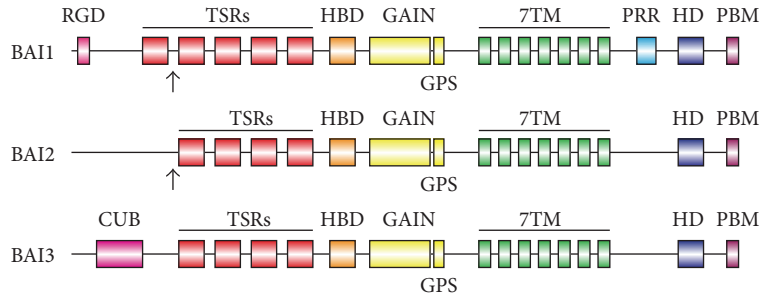

Figure 1: Schematic representation of BAI family members. BAI adhesion-GPCRs have a diverse collection of signaling and structural domains. These include thrombospondin type I repeats (TSRs), a hormone binding domain (HBD), the GAIN domain (GAIN), the GPCR autoproteolysis site (GPS), the characteristic seven-transmembrane domain (7TM), an α-helical RKR motif (HD), and the PDZ-binding motif (PBM), which are shared between all three family members. BAI1 has five TSRs, while BAI2 and BAI3 only have four. BAI1 and BAI2 are cleaved by proteases (BAI1 by matrix metalloprotease-14, BAI2 by Furin), which generates truncated fragments at the indicated locations marked by arrows. BAI1 has an additional integrin-binding RGD motif in the N-terminus and a proline-rich region (PRR) in the C-terminus. BAI1 also has a slightly truncated third intracellular loop compared to the other family members. BAI3 has a unique CUB domain in the N-terminus.

Rac1-dependent actin cytoskeletal remodeling required for internalization of apoptotic cells or bacteria [24, 37]. The ability to BAI1 to bind to PS is also important for myoblast fusion, and loss of BAI1 results in a reduction in myofiber size and impaired muscle regeneration in mice [38]. The TSRs on the N-terminus of BAI family members are essential for their capacity to regulate these diverse cellular processes, and therefore proteolysis of the BAI extracellular domain may dramatically alter BAI function [36].

4. Roles of BAIs at Synapses

Despite the recent advances in our understanding of BAI function, until recently, little was known about the roles of BAI adhesion-GPCRs in neurons. Over the last few years, BAIs have emerged as important regulators of synaptogenesis and synaptic plasticity. Below, we consider the synaptic functions of each of the BAI family members in turn.

4.1. BAI1 Function at Synapses. BAI1 is enriched in, though not exclusively localized to, the PSD in dendritic spines in hippocampal neurons; this has been shown by biochemical fractionation and immunocytochemistry in rat hippocampal neurons and mouse brains [25, 31]. This enrichment indicated that BAI1 might play a role in synaptic formation or function, and this problem was attacked in two different ways. In both cases, synaptic effects were found, though the details vary.

Our approach was to acutely knock down BAI1 both *in vitro* using cultured rat hippocampal neurons and *in vivo* using *in utero* electroporation of shRNAs directed against BAI1 [25]. In both systems, we found that BAI1 plays a key role in dendritic spine formation. Knockdown of BAI1 in cultured primary hippocampal neurons resulted in a loss of spine and synapse density with a shift of remaining spines to an immature elongated morphology [25]. *In vivo* knockdown also resulted in a dramatic loss of spine density and a shift toward less mature spines in the somatosensory and the cingulate cortices [25]. BAI1's prospinogenic and prosynaptogenic activities are mediated through its interactions with the cell polarity complex Tiam1/Par3 through its C-terminal PDZ-binding motif [25] (Figure 2). Tiam1 is an activator of the small GTPase Rac1, which directs the actin cytoskeletal remodeling that drives spine and synapse development [39]. Tiam1 couples Rac1-dependent spine and synapse formation to extracellular signals, including glutamate (via NMDA receptors) [40], ephrin-B (via EphB receptors) [41], and BDNF (via TrkB receptors) [42]. BAI1 anchors the Tiam1/Par3 complex to dendritic spines where localized Rac1 activation promotes the formation of dendritic spines and subsequent excitatory synaptogenesis. Of note, although other Rac1 activators such as ELMO/DOCK180 bind to BAI1 [24], Rac1 activation leading to spinogenesis requires only Tiam1, as BAI1 mutants lacking the Tiam1/Par3-interacting motif cannot rescue the knockdown phenotype, whereas mutants that do not interact with ELMO/DOCK180 can [25].

Consistent with these results, knockout mouse studies recently revealed a requirement for BAI1 in spatial learning and synaptic plasticity [26]. BAI1-null mice have severe deficits in both hippocampus-dependent spatial learning and memory along with enhanced long-term potentiation (LTP) and impaired long-term depression (LTD) [26]. An interesting result arising from this study was the discovery that BAI1 contributes to proper synapse formation through its ability to stabilize the expression of the postsynaptic scaffold protein PSD95. BAI1-null mice show significant decreases in PSD95 at dendritic spines/synapses. It was determined that BAI1 binds to and inhibits the E3 ubiquitin ligase MDM2, thereby preventing the PSD95 degradation that was responsible for the spatial learning and plasticity phenotypes observed in BAI1-null mice [26] (Figure 2).

Although both of these studies agreed that BAI1 plays a role in synapse function, there were important differences in the results. Our results using shRNAs against BAI1 led to stark and obvious loss of spines, while the results with the BAI1-null mice showed no difference in spine density. There are obvious

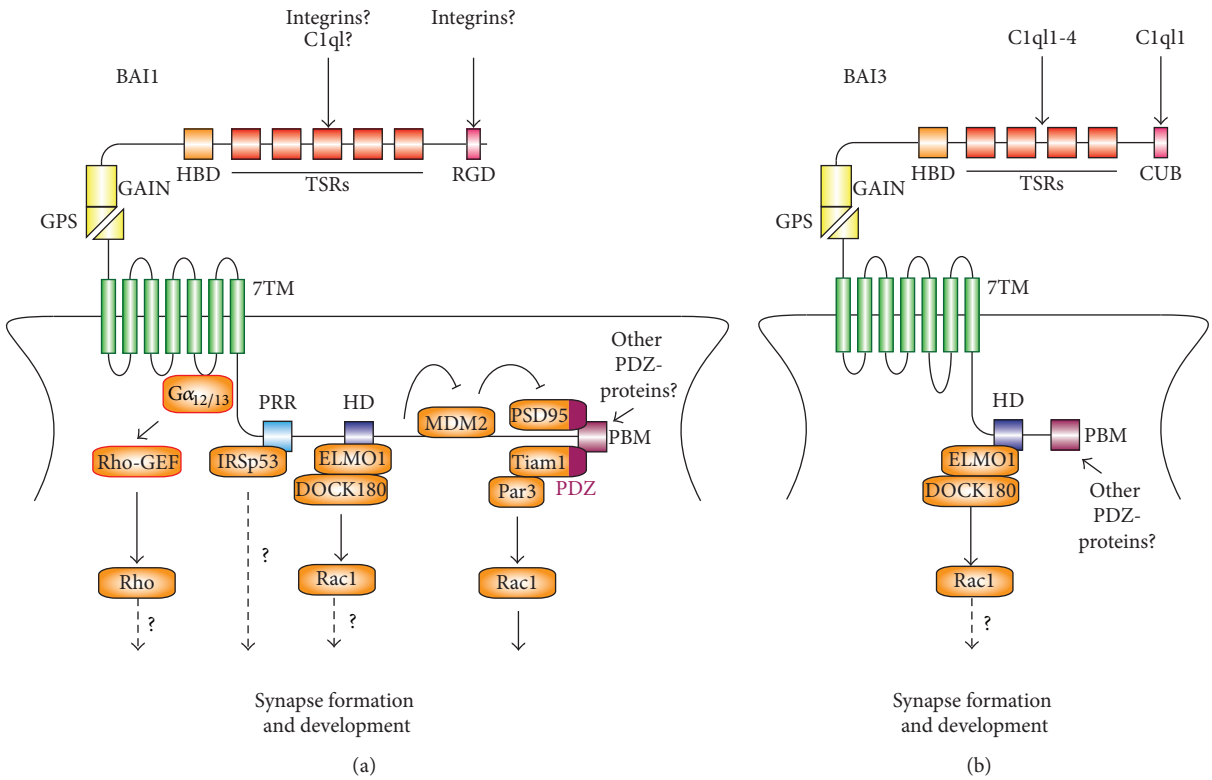

Figure 2: Synaptic binding partners and signaling pathways of BAI adhesion-GPCRs. (a) Synaptic interactions of BAI1. On the N-terminal segment of BAI1, the TSRs and the RGD motif are predicted to bind integrins. The TSRs also putatively bind complement C1ql factors, although the function of this interaction is unclear. BAI1 activates the RhoA pathway by coupling with $G\alpha_{12/13}$, although this has only been shown in cultured HEK293T cells and requires confirmation in neurons (red outline). The C-terminal region of BAI1 binds to IRSp53 via its proline-rich region (PRR), but the function of this interaction needs to be further explored. BAI1 also interacts with the Rac1 activator modules ELMO1/DOCK180 (via the α-helical RKR motif (HD)) and Tiam1/Par3 (via the PDZ-binding motif (PBM)). However, only the Tiam1/Par3 interaction is required for BAI1's effects on dendritic spine formation and excitatory synaptogenesis. In addition, BAI binds to the ubiquitin E3 ligase MDM2 and suppresses its polyubiquitination activity on PSD95, stabilizing PSD95 expression levels. (b) Synaptic interactions of BAI3. The TSRs and the CUB domain of BAI3 have been shown to bind complement C1ql factors C1ql3 and C1ql1, respectively. In cerebellar development, the C1ql1-BAI3 interaction helps establish proper synaptic connectivity in Purkinje cells and maintain a single-winner climbing fiber. The α-helical RKR motif (HD) of BAI3 also interacts with ELMO1/DOCK180 to regulate dendritogenesis, but the role of this interaction in synaptogenesis remains to be determined.

differences in the techniques used that could have given rise to these differences, and we will return to this issue below.

BAI1's C-terminal PDZ-binding motif also interacts with a variety of other synaptic molecules. Proteomic analysis reveals that the C-terminal segment of BAI1 can bind to PDZ-domain-containing proteins such as SAP97 (DLG1), Densin-180, MAGI-1/BAP1, MAGI-2, and MAGI-3 [31]. However, the exact functions of the majority of these interactions are not well understood. One potentially interesting BAI1-binding protein is the insulin receptor substrate 53 (IRSp53), which binds to a proline-rich region in BAI1's intracellular C-terminal segment and is also enriched in the PSD [43, 44]. Since IRSp53 is itself a downstream effector of Rac1 and Cdc42 and a regulator of dendrite spine morphogenesis [45], future studies that explore the effects of IRSp53-BAI1 interactions could elucidate key mechanisms of spinogenesis and synaptogenesis. IRSp53's potential role in autism spectrum disorder (ASD) makes this an even more interesting interaction to investigate [46].

4.2. BAI2 Function at Synapses. Like BAI1, BAI2 is broadly expressed in the brain, primarily in neurons and astrocytes [47]. However, the subcellular localization of BAI2 remains unclear. Roles for BAI2 in neurogenesis and synaptogenesis have been suggested but not well established experimentally. BAI2-deficient mice were found to display increased resistance to social defeat stress and reduced immobility in the tail suspension test, two behavioral assays that assess depressive behavior in rodents [48]. BAI2-deficient mice were also shown to exhibit increased neurogenesis in the dentate gyrus of the hippocampus, where BAI2 is highly expressed [47, 48]. These two observations are likely related since enhanced adult neurogenesis has been shown to positively correlate with resistance to depression [49]. It is also consistent with reports that BAI2 suppresses the expression of vascular endothelial growth factor (VEGF) [50], as VEGF stimulates adult neurogenesis in the dentate gyrus [51]. Loss of BAI2 could therefore increase VEGF levels, resulting in enhanced neurogenesis and increased resistance to stress.

This idea will need to be further investigated. Furthermore, since stress and depression are known to induce synapse loss, while antidepressants promote synaptogenesis [52], in future studies it will be interesting to investigate the possible roles of BAI2 at synapses.

4.3. BAI3 Function at Synapses. Biochemical fractionation studies have revealed that like BAI1, BAI3 localizes to excitatory synapses in the brain [30, 53]. Furthermore, overexpression studies examining the localization of BAI3 in transfected hippocampal neurons have shown that it is highly enriched in spines where it colocalizes with the postsynaptic marker PSD95 [28]. Together these findings suggest that BAI3 may play an important role at excitatory synapses. Indeed, recently BAI3 was shown to regulate excitatory synapse connectivity and formation in the mouse cerebellum [28, 29] (Figure 2). Knockdown of BAI3 using lentivirus-delivered shRNA in P7 pups induced clear deficits in connectivity between cerebellar climbing fibers and their target Purkinje cells *and* between parallel fibers and Purkinje cells by P21 [28]. Dendritic spine density and vGlut1-positive synaptic contacts were both decreased in Purkinje cells with reduced BAI3 levels [28]. Similarly, mice lacking BAI3 specifically in Purkinje cells show a significant decrease in the number of vGlut2-positive puncta in the cerebellum [29].

BAI3's role at climbing fiber synapses is mediated through its interactions with a class of secreted complement proteins known as the C1q-like complement (C1ql) family. C1ql proteins are broadly expressed in the brain with different spatial and temporal expression patterns shown by family members C1ql1–4 [54]. In particular, C1ql1 is highly expressed during the first 2 postnatal weeks in various neuronal populations, particularly in the hippocampus, cerebral cortex, and cerebellum [54]. Transient C1ql1 secretion in the cerebellum promotes Purkinje cell spinogenesis, and the effect of modulating C1ql1 expression on Purkinje cell spinogenesis depends on the expression levels of BAI3 [28]. Critically, the C1ql1-BAI3 interaction promotes developmental synapse refinement and triggers elimination of surplus climbing fiber synapses, helping to select and maintain a single winning climbing fiber [29]. BAI3 expression in Purkinje cells is required for this process, and the climbing fiber is the source of C1ql. Moreover, continued expression of BAI3 is necessary for maintenance of climbing fiber synapses, and adult mice lacking C1ql, which possess excess climbing fiber synapses per Purkinje cell, eliminate these extra synapses when C1ql is introduced into the animals [29].

C1ql1 interacts with BAI3 through the N-terminal CUB domain, which is unique to BAI3 [29]. BAI3 also interacts with another C1ql family member, C1ql3, through its TSRs [55]. Incubating cultured hippocampal neurons with C1ql3 was shown to decrease excitatory synaptic density, and this effect was reversed by adding the isolated TSRs of BAI3 to the culture [55]. This result suggests a role for BAI3/C1ql3 in hippocampal synapse development akin to the BAI3/C1ql1-mediated pruning function in the cerebellum described above. It is not known if BAI3 also plays an earlier role in promoting synapse formation in the hippocampus. Further, since the TSRs in BAI3 are present in all BAIs, it is possible that C1ql3 also interacts with BAI1 and BAI2, but this remains to be investigated.

BAI3's role in synapse elimination during cerebellar development could shed some light on the differences observed in the shRNA-transfected versus BAI1-null mice described above. If proper spine formation requires a competition to sort out the "winning" synapse, expression profiles of relevant proteins in participating neurons might contribute to the resolution of this competition. In the neurons in which BAI1 was removed via shRNA, only the transfected cells had a deficit in BAI1, and they represented a small fraction (<5%) of the total population. If they were in competition with BAI1-expressing neurons for the establishment of synapses, *and* BAI1 promotes winning the competition, then the BAI1 knockdown neurons would be at a decided disadvantage relative to the vast majority of neurons expressing normal levels of BAI1. This state of affairs would hold for both the cultured neurons and the *in vivo* preparations. On the other hand, the neurons examined in the BAI1 null mice existed on a background of BAI1 null neurons. Therefore, the unmarked neurons would not have an advantage in preserving synapses and this may explain why no loss of dendritic spines was observed. Such argument by analogy can only go so far, and compensation by other BAI family members could also be a factor, but this hypothesis warrants further investigation.

5. Other Adhesion-GPCRs Involved in Synapses

In addition to the roles that BAIs play in synaptogenesis and synaptic function, there is evidence that additional adhesion-GPCRs function in these roles. Latrophilins are an adhesion-GPCR subfamily comprised of 3 members latrophilins 1–3 (Lphn1–3 or ADGRL1–3) and ELTD1 (ADGRL4) in humans and represent one of only two subfamilies conserved in invertebrates [9]. Latrophilins were identified as receptors for the black widow spider toxin α-latrotoxin, which causes a massive Ca^{2+}-mediated exocytosis of neurotransmitter-containing vesicles from the presynaptic side of the synapses [56]. Lphn1 and Lphn3 are largely restricted to the brain, while Lphn2 is expressed in many tissues [9]. In addition to their GAIN domains, Lphns contain a hormone receptor motif, an olfactomedin-like domain, and a rhamnose-binding lectin domain in their NTFs [9]. Both Lphn1 and Lphn3 have been implicated in synapse formation. Lphn1 is thought to mediate its effects on synapse formation via interactions with teneurin-2/lasso [57, 58], neurexin-1β/2β [59], and fibronectin leucine-rich transmembrane proteins (FLRTs) [58]. All three of these proteins have been implicated independently in synapse formation. Presynaptic Lphn1 binds to teneurin-2 via its lectin domain with nanomolar affinity in a manner regulated by alternate splicing of Lphn1 [57, 58]. This interaction supports cell adhesion, while homophilic interaction between teneurins does not [58]. The Lphn1/teneurin interaction leads to presynaptic Ca^{2+} increases [57], and disruption of the interaction using the teneurin-binding segment of the Lphn1 NTF decreases both excitatory and inhibitory synapse density in rat hippocampal neurons [58]. Lphn1's interaction with neurexins also has

nanomolar affinity and is regulated by alternate splicing of neurexins but is largely mediated by Lphn1's olfactomedin domain [59]. This interaction is especially intriguing because neurexins and their canonical binding partners, neuroligins, form trans-synaptic complexes and are strongly implicated in ASD [60]. Postsynaptic Lphn1 binds to presynaptic neurexins competitively with neuroligins [59]. It is not yet known what function the Lphn1/neurexin interaction serves at synapses, but given the known roles of both proteins, it is likely to be of high interest. Similarly, the role of the Lphn1/FLRT-3 interaction is not completely understood. Lphn3 has received increased attention of late due to a strong emerging correlation with attention deficit/hyperactivity disorder (ADHD) in humans [61, 62]. Lphn3 binds to FLRT-3 via its olfactomedin domain and to teneurin-1 via its olfactomedin and lectin domains [63, 64]. Presynaptic Lphn3 interacts with postsynaptic FLRT-3 to promote synapse formation in hippocampal neurons and in cortical synapses from layers 2/3 to layer 5 [63, 64]. Interestingly, FLRT-3 and teneurins vary in their distributions throughout the layered structure of the cortex, suggesting that Lphn3 could serve different functions in different regions of the brain by interacting with distinct ligands [64]. In short, Lphns are implicated in both presynaptic function and in directing synapse formation by forming complexes with transmembrane ligands in neuronal membranes.

The Celsr adhesion-GPCR subfamily is characterized by the presence of atypical cadherin repeats, calcium-binding EGF-like domains, laminin G domains, and a hormone receptor motif in their NTFs in addition to the GAIN domain [9, 10]. Like latrophilins, this subfamily is conserved in invertebrates, with Flamingo in *Drosophila melanogaster*, Fmi-1/2 in *Caenorhabditis elegans*, and Celsr1–3 (ADGRC1–3) in humans [9, 10]. Adhesion-GPCRs of the Flamingo/CELSR subfamily function in many aspects of nervous system development, including neural tube closure, axon guidance, and the formation of dendritic arbors [9, 65–68]. These effects are mediated through the now classical interaction of these proteins with the cellular planar cell polarity (PCP) machinery, as well as cAMP- and Ca^{2+}-dependent mechanisms [9, 65, 66, 68]. Synaptic defects are observed when expression of Celsr-subfamily adhesion-GPCRs is altered or repressed, but it is difficult to determine whether these are direct effects on synaptic formation and/or maintenance, or whether they arise secondarily from malformation of axons and dendrites. Loss of Flamingo leads to formation of ectopic neuromuscular junctions, or synapses between axons and muscle, in Drosophila [69]. It also leads to malformed en passant synapses in this system, though these synapses are functional [69]. Further, aging animals lacking Flamingo exhibit a decrease in neuromuscular junctions, though this appears to be an effect of axonal degeneration [69]. Numerous questions remain to be answered in order to determine the specific roles of Celsr subfamily adhesion-GPCRs in synaptic formation and function. Finally, very large GPCR 1 (VLGR1 or ADGRV1) has been implicated in the formation of cochlear synapses, though its specific role remains unclear [70]. Many adhesion-GPCRs have not yet been tested for a role in synapses. Identification of adhesion-GPCRs involved in synaptic formation and function as well as elucidation of the mechanisms and signals that underlie these roles are important challenges for both adhesion-GPCR and synaptic biology.

6. BAIs' Disease Relevance and Potential as Therapeutic Targets

Given the important roles that BAI adhesion-GPCRs play in promoting synapse development and plasticity and inhibiting angiogenesis and tumor formation [18], it is not surprising that they have been implicated in a number of human diseases. For instance, single nucleotide polymorphisms (SNPs) and copy number variations in the human *BAI3* gene have been associated with schizophrenia [71–73], bipolar disorder [74], and drug addiction [75], brain disorders characterized by synapse abnormalities [4]. Furthermore, BAI3 expression is affected by lithium treatment, which is often used to treat patients with bipolar disorder and schizophrenia [74, 76]. The human *BAI1* gene is also located in a hot spot for *de novo* germline mutations in patients with autism [77], and BAI1 expression is upregulated in mouse models of Rett and MeCP2 Duplication Syndromes [78]. Conversely, *BAI1* expression is downregulated in glioblastoma and is inversely correlated with neovascularization in colorectal and lung cancers [36]. The growing evidence that BAIs play critical roles in human disease suggests that they may make good therapeutic targets in the future. GPCRs are generally considered to be the most successful therapeutic targets for a broad spectrum of diseases. Indeed, greater than 50% of the current therapeutic agents on the market target these proteins [79, 80]. Greater insight into the regulation and function of BAIs could therefore facilitate the development of novel therapies for the treatment of brain disorders and cancer.

7. Conclusions

After years of relative obscurity, there have been rapid recent advances in understanding the biology of BAIs and other adhesion-GPCRs. These molecules are intriguing because they tend to have multiple ligand binding domains that suggest that they are signal integrators, recognize large, complex substrates, and/or detect coincidences. The complexities added by NTF swapping, signaling by both GPCR-dependent and -independent modes, splice variants, and potential formation of higher level complexes are only beginning to be understood in a functional context. These complexities lend themselves to neuronal and synaptic function, given the role that these cells and structures play in storing and processing information. BAIs in particular are demonstrating key roles in synaptic function, though they play other roles in and out of the brain as well. A full appreciation of BAI function will require the identification of all BAI ligands, complete elucidation of BAI expression patterns and localization, identification of all binding partners and modes of signaling, and dynamic measurements of these properties. These are exciting challenges that hold great promise for increasing

our understanding of synaptic function, as well as treating synaptic dysfunction.

Conflict of Interests

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

Acknowledgments

This work was supported by the National Institute of Neurological Disorders and Stroke Grant R01NS062829 (Kimberley F. Tolias) and the National Institute of Mental Health Grants R01MH103108 (Kimberley F. Tolias) and K01MH089112 (Joseph G. Duman).

References

[1] C. L. Waites, A. M. Craig, and C. C. Garner, "Mechanisms of vertebrate synaptogenesis," *Annual Review of Neuroscience*, vol. 28, pp. 251–274, 2005.

[2] A. K. McAllister, "Dynamic aspects of CNS synapse formation," *Annual Review of Neuroscience*, vol. 30, pp. 425–450, 2007.

[3] Y. C. Lin and A. J. Koleske, "Mechanisms of synapse and dendrite maintenance and their disruption in psychiatric and neurodegenerative disorders," *Annual Review of Neuroscience*, vol. 33, no. 1, pp. 349–378, 2010.

[4] K.-O. Lai and N. Y. Ip, "Structural plasticity of dendritic spines: the underlying mechanisms and its dysregulation in brain disorders," *Biochimica et Biophysica Acta*, vol. 1832, no. 12, pp. 2257–2263, 2013.

[5] M. D. Thompson, D. E. Cole, V. Capra et al., "Pharmacogenetics of the G protein-coupled receptors," *Methods in Molecular Biology*, vol. 1175, pp. 189–242, 2014.

[6] T. K. Bjarnadóttir, D. E. Gloriam, S. H. Hellstrand, H. Kristiansson, R. Fredriksson, and H. B. Schiöth, "Comprehensive repertoire and phylogenetic analysis of the G protein-coupled receptors in human and mouse," *Genomics*, vol. 88, no. 3, pp. 263–273, 2006.

[7] A. Chollet and G. Turcatti, "Biophysical approaches to G protein-coupled receptors: structure, function and dynamics," *Journal of Computer Aided Molecular Design*, vol. 13, no. 3, pp. 209–219, 1999.

[8] I. Liebscher, T. Schöneberg, and S. Prömel, "Progress in demystification of adhesion G protein-coupled receptors," *Biological Chemistry*, vol. 394, no. 8, pp. 937–950, 2013.

[9] J. Hamann, G. Aust, D. Araç et al., "International Union of Basic and Clinical Pharmacology. XCIV. Adhesion G protein-coupled receptors," *Pharmacological Reviews*, vol. 67, no. 2, pp. 338–367, 2015.

[10] T. Langenhan, G. Aust, and J. Hamann, "Sticky signaling—adhesion class G protein-coupled receptors take the stage," *Science Signaling*, vol. 6, no. 276, article re3, 2013.

[11] D. Araç, A. A. Boucard, M. F. Bolliger et al., "A novel evolutionarily conserved domain of cell-adhesion GPCRs mediates autoproteolysis," *The EMBO Journal*, vol. 31, no. 6, pp. 1364–1378, 2012.

[12] S. Prömel, T. Langenhan, and D. Araç, "Matching structure with function: the GAIN domain of Adhesion-GPCR and PKD1-like proteins," *Trends in Pharmacological Sciences*, vol. 34, no. 8, pp. 470–478, 2013.

[13] Y.-S. Huang, N.-Y. Chiang, C.-H. Hu et al., "Activation of myeloid cell-specific adhesion class G protein-coupled receptor EMR2 via ligation-induced translocation and interaction of receptor subunits in lipid raft microdomains," *Molecular and Cellular Biology*, vol. 32, no. 8, pp. 1408–1420, 2012.

[14] J.-P. Silva, V. Lelianova, C. Hopkins, K. E. Volynski, and Y. Ushkaryov, "Functional cross-interaction of the fragments produced by the cleavage of distinct adhesion G-protein-coupled receptors," *Journal of Biological Chemistry*, vol. 284, no. 10, pp. 6495–6506, 2009.

[15] K. J. Paavola, J. R. Stephenson, S. L. Ritter, S. P. Alter, and R. A. Hall, "The N terminus of the adhesion G protein-coupled receptor GPR56 controls receptor signaling activity," *The Journal of Biological Chemistry*, vol. 286, no. 33, pp. 28914–28921, 2011.

[16] N. Scholz, J. Gehring, C. Guan et al., "The adhesion GPCR latrophilin/CIRL shapes mechanosensation," *Cell Reports*, vol. 11, no. 6, pp. 866–874, 2015.

[17] D. Park and K. S. Ravichandran, "Emerging roles of brain-specific angiogenesis inhibitor 1," *Advances in Experimental Medicine and Biology*, vol. 706, pp. 167–178, 2010.

[18] J. R. Stephenson, R. H. Purcell, and R. A. Hall, "The BAI subfamily of adhesion GPCRs: synaptic regulation and beyond," *Trends in Pharmacological Sciences*, vol. 35, no. 4, pp. 208–215, 2014.

[19] H. Nishimori, T. Shiratsuchi, T. Urano et al., "A novel brain-specific p53-target gene, BAI1, containing thrombospondin type 1 repeats inhibits experimental angiogenesis," *Oncogene*, vol. 15, no. 18, pp. 2145–2150, 1997.

[20] T. Shiratsuchi, H. Nishimori, H. Ichise, Y. Nakamura, and T. Tokino, "Cloning and characterization of BAI2 and BAI3, novel genes homologous to brain-specific angiogenesis inhibitor 1 (BAI1)," *Cytogenetics and Cell Genetics*, vol. 79, no. 1-2, pp. 103–108, 1997.

[21] H. J. Kee, K. Y. Ahn, K. C. Choi et al., "Expression of brain-specific angiogenesis inhibitor 3 (BAI3) in normal brain and implications for BAI3 in ischemia-induced brain angiogenesis and malignant glioma," *FEBS Letters*, vol. 569, no. 1–3, pp. 307–316, 2004.

[22] K. Mori, Y. Kanemura, H. Fujikawa et al., "Brain-specific angiogenesis inhibitor 1 (BAI1) is expressed in human cerebral neuronal cells," *Neuroscience Research*, vol. 43, no. 1, pp. 69–74, 2002.

[23] J. D. Sokolowski, S. L. Nobles, D. S. Heffron, D. Park, K. S. Ravichandran, and J. W. Mandell, "Brain-specific angiogenesis inhibitor-1 expression in astrocytes and neurons: implications for its dual function as an apoptotic engulfment receptor," *Brain, Behavior, and Immunity*, vol. 25, no. 5, pp. 915–921, 2011.

[24] D. Park, A.-C. Tosello-Trampont, M. R. Elliott et al., "BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module," *Nature*, vol. 450, no. 7168, pp. 430–434, 2007.

[25] J. G. Duman, C. P. Tzeng, Y.-K. Tu et al., "The adhesion-GPCR BAI1 regulates synaptogenesis by controlling the recruitment of the Par3/Tiam1 polarity complex to synaptic sites," *The Journal of Neuroscience*, vol. 33, no. 16, pp. 6964–6978, 2013.

[26] D. Zhu, C. Li, A. M. Swanson et al., "BAI1 regulates spatial learning and synaptic plasticity in the hippocampus," *The Journal of Clinical Investigation*, vol. 125, no. 4, pp. 1497–1508, 2015.

[27] V. Lanoue, A. Usardi, S. M. Sigoillot et al., "The adhesion-GPCR *BAI3*, a gene linked to psychiatric disorders, regulates dendrite

morphogenesis in neurons," *Molecular Psychiatry*, vol. 18, no. 8, pp. 943–950, 2013.

[28] S. Sigoillot, K. Iyer, F. Binda et al., "The secreted protein C1QL1 and its receptor BAI3 control the synaptic connectivity of excitatory inputs converging on cerebellar purkinje cells," *Cell Reports*, vol. 10, no. 5, pp. 820–832, 2015.

[29] W. Kakegawa, N. Mitakidis, E. Miura et al., "Anterograde C1ql1 signaling is required in order to determine and maintain a single-winner climbing fiber in the mouse cerebellum," *Neuron*, vol. 85, no. 2, pp. 316–330, 2015.

[30] M. O. Collins, H. Husi, L. Yu et al., "Molecular characterization and comparison of the components and multiprotein complexes in the postsynaptic proteome," *Journal of Neurochemistry*, vol. 97, supplement 1, pp. 16–23, 2006.

[31] J. R. Stephenson, K. J. Paavola, S. A. Schaefer, B. Kaur, E. G. Van Meir, and R. A. Hall, "Brain-specific angiogenesis inhibitor-1 signaling, regulation, and enrichment in the postsynaptic density," *The Journal of Biological Chemistry*, vol. 288, no. 31, pp. 22248–22256, 2013.

[32] B. Kaur, D. J. Brat, N. S. Devi, and E. G. Van Meir, "Vasculostatin, a proteolytic fragment of brain angiogenesis inhibitor 1, is an antiangiogenic and antitumorigenic factor," *Oncogene*, vol. 24, no. 22, pp. 3632–3642, 2005.

[33] B. Kaur, S. M. Cork, E. M. Sandberg et al., "Vasculostatin inhibits intracranial glioma growth and negatively regulates in vivo angiogenesis through a CD36-dependent mechanism," *Cancer Research*, vol. 69, no. 3, pp. 1212–1220, 2009.

[34] D. Okajima, G. Kudo, and H. Yokota, "Brain-specific angiogenesis inhibitor 2 (BAI2) may be activated by proteolytic processing," *Journal of Receptors and Signal Transduction*, vol. 30, no. 3, pp. 143–153, 2010.

[35] S. M. Cork, B. Kaur, N. S. Devi et al., "A proprotein convertase/MMP-14 proteolytic cascade releases a novel 40kDa vasculostatin from tumor suppressor BAI1," *Oncogene*, vol. 31, no. 50, pp. 5144–5152, 2012.

[36] S. M. Cork and E. G. Van Meir, "Emerging roles for the BAI1 protein family in the regulation of phagocytosis, synaptogenesis, neurovasculature, and tumor development," *Journal of Molecular Medicine*, vol. 89, no. 8, pp. 743–752, 2011.

[37] S. Das, K. A. Owen, K. T. Ly et al., "Brain angiogenesis inhibitor 1 (BAI1) is a pattern recognition receptor that mediates macrophage binding and engulfment of Gram-negative bacteria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 5, pp. 2136–2141, 2011.

[38] A. E. Hochreiter-Hufford, C. S. Lee, J. M. Kinchen et al., "Phosphatidylserine receptor BAI1 and apoptotic cells as new promoters of myoblast fusion," *Nature*, vol. 497, no. 7448, pp. 263–267, 2013.

[39] K. F. Tolias, J. G. Duman, and K. Um, "Control of synapse development and plasticity by Rho GTPase regulatory proteins," *Progress in Neurobiology*, vol. 94, no. 2, pp. 133–148, 2011.

[40] K. F. Tolias, J. B. Bikoff, A. Burette et al., "The Rac1-GEF Tiam1 couples the NMDA receptor to the activity-dependent development of dendritic arbors and spines," *Neuron*, vol. 45, no. 4, pp. 525–538, 2005.

[41] K. F. Tolias, J. B. Bikoff, C. G. Kane, C. S. Tolias, L. Hu, and M. E. Greenberg, "The Rac1 guanine nucleotide exchange factor Tiam1 mediates EphB receptor-dependent dendritic spine development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 17, pp. 7265–7270, 2007.

[42] K.-O. Lai, A. S. L. Wong, M.-C. Cheung et al., "TrkB phosphorylation by Cdk5 is required for activity-dependent structural plasticity and spatial memory," *Nature Neuroscience*, vol. 15, no. 11, pp. 1506–1515, 2012.

[43] M.-A. Abbott, D. G. Wells, and J. R. Fallon, "The insulin receptor tyrosine kinase substrate p58/53 and the insulin receptor are components of CNS synapses," *The Journal of Neuroscience*, vol. 19, no. 17, pp. 7300–7308, 1999.

[44] J. Bockmann, M. R. Kreutz, E. D. Gundelfinger, and T. M. Böckers, "ProSAP/Shank postsynaptic density proteins interact with insulin receptor tyrosine kinase substrate IRSp53," *Journal of Neurochemistry*, vol. 83, no. 4, pp. 1013–1017, 2002.

[45] J. Choi, J. Ko, B. Racz et al., "Regulation of dendritic spine morphogenesis by insulin receptor substrate 53, a downstream effector of Rac1 and Cdc42 small GTPases," *The Journal of Neuroscience*, vol. 25, no. 4, pp. 869–879, 2005.

[46] C. Toma, A. Hervás, N. Balmaña et al., "Association study of six candidate genes asymmetrically expressed in the two cerebral hemispheres suggests the involvement of BAIAP2 in autism," *Journal of Psychiatric Research*, vol. 45, no. 2, pp. 280–282, 2011.

[47] H. J. Kee, J. T. Koh, M.-Y. Kim et al., "Expression of brain-specific angiogenesis inhibitor 2 (BAI2) in normal and ischemic brain: involvement of BAI2 in the ischemia-induced brain angiogenesis," *Journal of Cerebral Blood Flow and Metabolism*, vol. 22, no. 9, pp. 1054–1067, 2002.

[48] D. Okajima, G. Kudo, and H. Yokota, "Antidepressant-like behavior in brain-specific angiogenesis inhibitor 2-deficient mice," *Journal of Physiological Sciences*, vol. 61, no. 1, pp. 47–54, 2011.

[49] B. A. Samuels and R. Hen, "Neurogenesis and affective disorders," *European Journal of Neuroscience*, vol. 33, no. 6, pp. 1152–1159, 2011.

[50] B. C. Jeong, M.-Y. Kim, J. H. Lee et al., "Brain-specific angiogenesis inhibitor 2 regulates VEGF through GABP that acts as a transcriptional repressor," *FEBS Letters*, vol. 580, no. 2, pp. 669–676, 2006.

[51] J. L. Warner-Schmidt and R. S. Duman, "VEGF is an essential mediator of the neurogenic and behavioral actions of antidepressants," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 11, pp. 4647–4652, 2007.

[52] R. S. Duman and G. K. Aghajanian, "Synaptic dysfunction in depression: potential therapeutic targets," *Science*, vol. 338, no. 6103, pp. 68–72, 2012.

[53] F. Selimi, I. M. Cristea, E. Heller, B. T. Chait, and N. Heintz, "Proteomic studies of a single CNS synapse type: the parallel fiber/purkinje cell synapse," *PLoS Biology*, vol. 7, no. 4, Article ID e1000083, 2009.

[54] T. Iijima, E. Miura, M. Watanabe, and M. Yuzaki, "Distinct expression of C1q-like family mRNAs in mouse brain and biochemical characterization of their encoded proteins," *European Journal of Neuroscience*, vol. 31, no. 9, pp. 1606–1615, 2010.

[55] M. F. Bolliger, D. C. Martinelli, and T. C. Südhof, "The cell-adhesion G protein-coupled receptor BAI3 is a high-affinity receptor for C1q-like proteins," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 6, pp. 2534–2539, 2011.

[56] M. A. Rahman, A. C. Ashton, F. A. Meunier, B. A. Davletov, J. O. Dolly, and Y. A. Ushkaryov, "Norepinephrine exocytosis stimulated by alpha-latrotoxin requires both external and stored Ca^{2+} and is mediated by latrophilin, G proteins and phospholipase C," *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 354, no. 1381, pp. 379–386, 1999.

[57] J.-P. Silva, V. G. Lelianova, Y. S. Ermolyuk et al., "Latrophilin 1 and its endogenous ligand Lasso/teneurin-2 form a high-affinity transsynaptic receptor pair with signaling capabilities," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 29, pp. 12113–12118, 2011.

[58] A. A. Boucard, S. Maxeiner, and T. C. Südhof, "Latrophilins function as heterophilic cell-adhesion molecules by binding to teneurins: regulation by alternative splicing," *Journal of Biological Chemistry*, vol. 289, no. 1, pp. 387–402, 2014.

[59] A. A. Boucard, J. Ko, and T. C. Südhof, "High affinity neurexin binding to cell adhesion G-protein-coupled receptor CIRL1/latrophilin-1 produces an intercellular adhesion complex," *Journal of Biological Chemistry*, vol. 287, no. 12, pp. 9399–9413, 2012.

[60] A. C. Reichelt, R. J. Rodgers, and S. J. Clapcote, "The role of neurexins in schizophrenia and autistic spectrum disorder," *Neuropharmacology*, vol. 62, no. 3, pp. 1519–1526, 2012.

[61] M. Arcos-Burgos, M. Jain, M. T. Acosta et al., "A common variant of the latrophilin 3 gene, *LPHN3*, confers susceptibility to ADHD and predicts effectiveness of stimulant medication," *Molecular Psychiatry*, vol. 15, no. 11, pp. 1053–1066, 2010.

[62] E. M. Bruxel, A. Salatino-Oliveira, G. C. Akutagava-Martins et al., "LPHN3 and attention-deficit/hyperactivity disorder: a susceptibility and pharmacogenetic study," *Genes, Brain and Behavior*, vol. 14, no. 5, pp. 419–427, 2015.

[63] M. L. O'Sullivan, J. de Wit, J. N. Savas et al., "FLRT proteins are endogenous latrophilin ligands and regulate excitatory synapse development," *Neuron*, vol. 73, no. 5, pp. 903–910, 2012.

[64] M. L. O'Sullivan, F. Martini, S. von Daake, D. Comoletti, and A. Ghosh, "LPHN3, a presynaptic adhesion-GPCR implicated in ADHD, regulates the strength of neocortical layer 2/3 synaptic input to layer 5," *Neural Development*, vol. 9, article 7, 2014.

[65] Y. Shima, S.-Y. Kawaguchi, K. Kosaka et al., "Opposing roles in neurite growth control by two seven-pass transmembrane cadherins," *Nature Neuroscience*, vol. 10, no. 8, pp. 963–969, 2007.

[66] X.-J. Wang, D.-L. Zhang, Z.-G. Xu et al., "Understanding cadherin EGF LAG seven-pass G-type receptors," *Journal of Neurochemistry*, vol. 131, no. 6, pp. 699–711, 2014.

[67] A. Steimel, L. Wong, E. H. Najarro, B. D. Ackley, G. Garriga, and H. Hutter, "The flamingo ortholog FMI-1 controls pioneer-dependent navigation of follower axons in *C. elegans*," *Development*, vol. 137, no. 21, pp. 3663–3673, 2010.

[68] T. Nishimura, H. Honda, and M. Takeichi, "Planar cell polarity links axes of spatial dynamics in neural-tube closure," *Cell*, vol. 149, no. 5, pp. 1084–1097, 2012.

[69] H. Bao, M. L. Berlanga, M. Xue et al., "The atypical cadherin flamingo regulates synaptogenesis and helps prevent axonal and synaptic degeneration in *Drosophila*," *Molecular and Cellular Neuroscience*, vol. 34, no. 4, pp. 662–678, 2007.

[70] M. Zallocchi, D. T. Meehan, D. Delimont et al., "Role for a novel Usher protein complex in hair cell synaptic maturation," *PLoS ONE*, vol. 7, no. 2, Article ID e30573, 2012.

[71] P. DeRosse, T. Lencz, K. E. Burdick, S. G. Siris, J. M. Kane, and A. K. Malhotra, "The genetics of symptom-based phenotypes: toward a molecular classification of schizophrenia," *Schizophrenia Bulletin*, vol. 34, no. 6, pp. 1047–1053, 2008.

[72] H.-M. Liao, Y.-L. Chao, A.-L. Huang et al., "Identification and characterization of three inherited genomic copy number variations associated with familial schizophrenia," *Schizophrenia Research*, vol. 139, no. 1–3, pp. 229–236, 2012.

[73] E. S. Lips, L. N. Cornelisse, R. F. Toonen et al., "Functional gene group analysis identifies synaptic gene groups as risk factor for schizophrenia," *Molecular Psychiatry*, vol. 17, no. 10, pp. 996–1006, 2012.

[74] M. J. McCarthy, C. M. Nievergelt, J. R. Kelsoe, and D. K. Welsh, "A survey of genomic studies supports association of circadian clock genes with bipolar disorder spectrum illnesses and lithium response," *PLoS ONE*, vol. 7, no. 2, Article ID e32091, 2012.

[75] Q.-R. Liu, T. Drgon, C. Johnson, D. Walther, J. Hess, and G. R. Uhl, "Addiction molecular genetics: 639,401 SNP whole genome association identifies many 'cell adhesion' genes," *American Journal of Medical Genetics, Part B: Neuropsychiatric Genetics*, vol. 141, no. 8, pp. 918–925, 2006.

[76] A. McQuillin, M. Rizig, and H. M. D. Gurling, "A microarray gene expression study of the molecular pharmacology of lithium carbonate on mouse brain mRNA to understand the neurobiology of mood stabilization and treatment of bipolar affective disorder," *Pharmacogenetics and Genomics*, vol. 17, no. 8, pp. 605–617, 2007.

[77] J. J. Michaelson, Y. Shi, M. Gujral et al., "Whole-genome sequencing in autism identifies hot spots for de novo germline mutation," *Cell*, vol. 151, no. 7, pp. 1431–1442, 2012.

[78] M. Chahrour, Y. J. Sung, C. Shaw et al., "MeCP2, a key contributor to neurological disease, activates and represses transcription," *Science*, vol. 320, no. 5880, pp. 1224–1229, 2008.

[79] C. Du and X. Xie, "G protein-coupled receptors as therapeutic targets for multiple sclerosis," *Cell Research*, vol. 22, no. 7, pp. 1108–1128, 2012.

[80] R. Lappano and M. Maggiolini, "G protein-coupled receptors: novel targets for drug discovery in cancer," *Nature Reviews Drug Discovery*, vol. 10, no. 1, pp. 47–60, 2011.

Hindawi Publishing Corporation
Neural Plasticity
Volume 2016, Article ID 4258171, 11 pages
http://dx.doi.org/10.1155/2016/4258171

Research Article

The Gα_o Activator Mastoparan-7 Promotes Dendritic Spine Formation in Hippocampal Neurons

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Received 23 May 2015; Revised 26 July 2015; Accepted 27 August 2015

Academic Editor: Xin-Ming Ma

Mastoparan-7 (Mas-7), an analogue of the peptide mastoparan, which is derived from wasp venom, is a direct activator of *Pertussis toxin*- (PTX-) sensitive G proteins. Mas-7 produces several biological effects in different cell types; however, little is known about how Mas-7 influences mature hippocampal neurons. We examined the specific role of Mas-7 in the development of dendritic spines, the sites of excitatory synaptic contact that are crucial for synaptic plasticity. We report here that exposure of hippocampal neurons to a low dose of Mas-7 increases dendritic spine density and spine head width in a time-dependent manner. Additionally, Mas-7 enhances postsynaptic density protein-95 (PSD-95) clustering in neurites and activates Gα_o signaling, increasing the intracellular Ca^{2+} concentration. To define the role of signaling intermediates, we measured the levels of phosphorylated protein kinase C (PKC), c-Jun N-terminal kinase (JNK), and calcium-calmodulin dependent protein kinase IIα (CaMKIIα) after Mas-7 treatment and determined that CaMKII activation is necessary for the Mas-7-dependent increase in dendritic spine density. Our results demonstrate a critical role for Gα_o subunit signaling in the regulation of synapse formation.

1. Introduction

G proteins are highly expressed in the mammalian brain and play a critical role in the regulation and development of synaptic transmission because they act as transducers for the G protein-coupled receptors (GPCRs) [1]. They are composed of a guanine nucleotide-binding α subunit (Gα) and a $\beta\gamma$ complex (G$\beta\gamma$). In mammals, 20 different G proteins have been described, each composed of one of the 19α subunits, one of the 5β subunits, and one of the 12γ subunits [2]. In the resting state, Gα is bound to GDP and associated with G$\beta\gamma$ and a GPCR. This complex is dissociated when Gα binds to GTP, causing the activation of Gα and the G$\beta\gamma$ complex, allowing them to regulate their downstream effectors [3]. Gα subunits are separated into four families based on sequence homology (Gα_s, Gα_q, G$\alpha_{i/o}$, G$\alpha_{12/13}$); each of these proteins activates a different pathway [4].

The majority of excitatory synaptic connections in the central nervous system are located on small dendritic protrusions, which are enriched in signaling molecules and serve to compartmentalize individual postsynaptic structures [5]. In mouse and human brains, the G protein subunits Gα_o, Gα_i, Gα_q, Gα_z, Gα_s, Gα_{12}, Gα_{13}, and Gα_{14} are present in postsynaptic densities (PSDs) [6], suggesting a key role of these signal transducer proteins in the synaptic regulation.

To study the specific role of G proteins in the regulation of dendritic spines, we used Mas-7, a potent analogue of the peptide mastoparan, which is obtained from the venom of *Vespula lewisii* [7]. Mas-7 has a substitution of an alanine for a lysine at position 12 [8] and binds to the plasma membrane to form an α-helix structure that activates G$\alpha_{o/i}$ subunits without requiring the activation of a GPCR [9]. This peptide shows a wide variety of biological effects, including antiviral activity [10], histamine release from mast cells [11],

the induction of potent mitochondrial permeability [12], and tumor cell cytotoxicity [13]. However, the effect of Mas-7 in hippocampal neurons has not been studied.

Here, we show that a low dose of Mas-7 activates $G\alpha_o$, causing the switch from GDP to GTP in hippocampal neurons. Functionally, Mas-7 increases dendritic spine density through a Ca^{2+}-dependent mechanism in hippocampal neurons. Mas-7 also activates a variety of Ca^{2+}-sensitive proteins, including CaMKIIα, which is necessary for the increase in dendritic spine density.

These results suggest that G protein activation, especially the $G\alpha_o$ subunit activation, may contribute to dendritic spine remodeling in neurons.

2. Materials and Methods

2.1. Reagents. Mas-7 was purchased from Sigma-Aldrich (St. Louis, MO); Fura-2AM from Molecular Probes (Eugene, OR); and KN93 from Calbiochem (San Diego, CA).

2.2. Hippocampal Neuronal Culture. Rat hippocampal cultures were prepared from Sprague-Dawley rats of both sexes at embryonic day 18, as previously described [14]. On day two, neurons were treated with 2 μM cytosine arabinoside for 24 h to avoid glial cell growth. Then, the neurons were cultured with Neurobasal medium supplemented with 1% B27 from Invitrogen (Eugene, OR).

2.3. Measurements of Intracellular Ca^{2+} in Hippocampal Neurons. Cytosolic Ca^{2+} signals were determined in cells seeded at 160,000 per 35 mm coverslip; the cells were loaded with 4.5 μM Fura-2-AM for 30 min as previously described [15]. The experiments were performed in an isotonic calcium-free solution (in mM): 140 NaCl, 2.5 KCl, 1.7 $MgCl_2$, 5 glucose, 0.5 EGTA, and 10 HEPES (305 mOsm/L, pH 7.4 with Tris). An Olympus Spinning Disc IX81 microscope was used in live-imaging experiments recording 1 photo every 5 seconds. The increases in cytosolic Ca^{2+} are represented by the normalized ratio of the fluorescence emitted at 510 nm after excitation at 340 (which determine the probe bound to Ca^{2+}) and 380 nm (probe not bound to Ca^{2+}) relative to the ratio measured prior to cell stimulation. The integration of the area under the curve was performed with GraphPad Prism5 software (La Jolla, CA) using the first minute before the stimuli application as a baseline.

2.4. Immunoprecipitation of Activated $G\alpha_o$ Subunit. The $G\alpha_o$ activation assay kit from New East Bioscience (#80901, King of Prussia, PA) was used. The protocol recommended by the manufacturer was employed with modifications. Briefly, neurons at 14 days *in vitro* (DIV) (seeded at 900,000 cells/well) were treated with 1 μM Mas-7 for 5 or 30 min. Then, the cells were lysed with 0.5 mL 1x kit buffer (#30303) and centrifuged at 12,000 ×g 4°C for 10 min. The supernatants were incubated with 1 μL mouse monoclonal antibody specific for $G\alpha_o$ bound to GTP (active form) (#26907) and 20 μL A/G agarose beads (#30301) for 2 h at 4°C with orbital rotation. As a positive control, untreated neurons were lysed and then incubated with 10 mM GTPγS (#30302) and 10 mM $MgCl_2$ for 90 min at RT, and as a negative control, the lysed neurons were incubated with 10 mM GDP (#30304) and 10 mM $MgCl_2$. Later, the lysates were washed 3 times and the beads were suspended in 20 μL Laemmli 2x loading buffer and boiled for 5 min. The total level of $G\alpha_o$ was detected by immunoblotting with a polyclonal anti-$G\alpha_o$ antibody (#21015, 1 : 1000).

2.5. Western Blot. Neurons at 14 DIV were seeded at 400,000 cells/well and treated with 1 μM Mas-7 and were lysed on ice and immediately processed. Immunoblotting was performed as described [16]. The primary antibodies used included mouse anti-CaMKIIα (sc-5306, 1 : 1000), mouse anti-phospho-Tyr286-CaMKIIα (sc-32289, 1 : 1000), rabbit anti-PKCβII (sc-210, 1 : 1000), rabbit anti-β-tubulin (sc-9104, 1 : 1000), mouse anti-GAPDH (sc-32233, 1 : 5000), and rabbit anti-GSK-3β (sc-9166, 1 : 1000) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); rabbit anti-JNK (#9252, 1 : 1000), rabbit anti-phospho-Thr183/Tyr185-JNK (#4668, 1 : 1000), and rabbit anti-phospho-Ser9-GSK-3β (#9336, 1 : 1000) from Cell Signaling Technology (Beverly, MA); rabbit anti-phospho-Ser660-PKCβII (ab75837, 1 : 10000) and rabbit anti-$G\alpha_o$ (ab136535, 1 : 5000) from Abcam (Cambridge, MA); mouse anti-PSD-95 (k28/43, 1 : 1000) from UC Davis/NIH NeuroMab Facility; and mouse anti-β-actin (11978, 1 : 10000) from Sigma (St. Louis, MO). Equal amounts of protein were loaded (20 μg).

2.6. PSD-95 Immunofluorescence and Image Analysis. Neurons at 14 DIV were plated at 35,000 cells/coverslip and treated with 1 μM Mas-7, fixed with a freshly prepared solution of 4% paraformaldehyde plus 4% sucrose in PBS for 20 min at 4°C, permeabilized with 0.2% Triton X-100 for 5 min at room temperature (RT), and then blocked with 1% BSA in PBS (blocking solution) for 30 min at 37°C. This procedure was followed by an overnight incubation at 4°C with anti-PSD-95 (k28/43, 1 : 400) from UC Davis/NIH NeuroMab Facility and Synapsin I (SynI) (sc-20780, 1 : 2000) from Santa Cruz Biotechnology Inc. The neurons were washed with PBS and incubated for 30 min at 37°C with Phalloidin-Alexa-633 and the secondary antibody (Molecular Probes). Images were acquired from 10 microscope fields for each condition with an Olympus Fluoview FV 1000 confocal microscope. To quantify PSD-95 clusters, we used a previously described protocol [17] using NIH ImageJ software (NIH, Baltimore, MD). The synaptic contacts were measured as previously reported [18].

2.7. Transfection and Dendritic Spine Morphology Analysis. Hippocampal neurons plated at 60,000 per poly-D-lysine-coated 12 mm glass coverslip were transfected at 10 DIV with an EGFP plasmid (pEGFP-N1, Clontech, Mountain View, CA) using a NeuroMag kit (KC 30800) from OZ Bioscience (Marseille, France) as described previously [19]. First, the neurons were washed for 30 min with Neurobasal medium. Then, 0.8 μg DNA/1.25 μL magnetobeads per cover were mixed and incubated for 15 min in 100 μL Neurobasal medium at RT. Next, the mix was added by drops to the neurons, and the magnetobeads were allowed to enter the

cells by use of the magnet for 15 min (37°C, 5% CO_2). Subsequently, the magnet was removed for 40 min, and, finally, the transfected medium was replaced with fresh medium. At 14 DIV, the neurons were depleted for 2 h with Neurobasal medium without B7 supplement before being treated with 1 μM Mas-7 or 10 μM KN93 plus Mas-7 at different times. An Olympus Fluoview FV 1000 confocal microscope was used to obtain digital confocal stacks from 15 to 20 serial images with a Z step size of 0.25 μm. Dendritic Z-stacks were reconstructed using the super-pass module of Imaris software. Accurate reconstruction of spine head diameter was achieved using the approximate circle algorithm with a threshold of 0.8. Ten neurons were analyzed for each condition. The mean spines length and spines head width of each neurite were reported. Spine density was calculated by measuring the total number of spines per neurite length (spine density/10 μm).

2.8. Live-Cell Imaging of Dendritic Spine Morphogenesis. Hippocampal neurons cultured in round 35 mm coverslips at a density of 160,000 cells/coverslip were transfected with EGFP at 11 DIV. Then, at 14 DIV the neurons were placed in the imaging chamber in an isotonic solution (in mM: 1.2 $CaCl_2$, 140 NaCl, 2.5 KCl, 0.5 $MgCl_2$, 5 glucose, and 10 HEPES (305 mOsm/L, pH 7.4 with Tris)). The EGFP-positive neurons were imaged with an Olympus Spinning Disc IX81 microscope every 5 min for 45 min after the treatment with 1 μM Mas-7. The images were processed and analyzed using ImageJ software.

2.9. Statistical Analysis. Statistical analysis was performed using Prism 5 software. The values are expressed as the mean ± standard error of the mean. The statistical significance of differences was assessed with one-way ANOVA with Bonferroni's posttest for multiple comparisons and with Student's t-test for comparisons between two conditions ($p < 0.05$ was considered significant). The number of independent experiments is indicated in the corresponding figure legends.

3. Results

3.1. Mas-7 Activates the $G\alpha_o$ Subunit in Hippocampal Neurons. To determine whether the Mas-7 peptide activates G proteins, specifically the $G\alpha_o$ subunit in cultured rat hippocampal neurons, we treated them with Mas-7 and then performed immunoprecipitation assays, using a commercial specific antibody that recognizes $G\alpha_o$ bound to GTP ($G\alpha_o$-GTP), the active form of the $G\alpha_o$ protein, in combination with SDS-PAGE and immunoblotting. Additionally, we immunoprecipitated $G\alpha_o$-GTP from lysates of hippocampal neurons that were incubated with nonhydrolyzable GTP (GTPγS) or with GDP as a positive and negative control, respectively.

Our data show that Mas-7 induced activation of the $G\alpha_o$ subunit in cultured hippocampal neurons after 5 min of exposure (Figure 1(a), *left panel*); the activation then declined at 30 min. By contrast, Mas-7 was unable to increase the total $G\alpha_o$ protein level, even after 2 h of treatment (Figure 1(b)). Furthermore, incubation with GTPγS produced an increase in the activation of $G\alpha_o$, and GDP incubation decreased the activation (Figure 1(a), *right panel*). These findings show that Mas-7 produces rapid activation of $G\alpha_o$ subunit, suggesting that the $G\alpha_o$-dependent signaling cascade is also activated.

3.2. Mas-7 Increases the Intracellular Calcium Concentration in Hippocampal Neurons. In different cell types, mastoparan and Mas-7 produce an increase in intracellular calcium (Ca^{2+}) [20, 21]. For example, in rat cerebellar granule neurons, 15 μM mastoparan produces a robust elevation in intracellular Ca^{2+} [22]. To assess whether Mas-7 generates a similar effect in cultured hippocampal neurons, we treated them with Mas-7 at a lower dose (1–5 μM) to avoid a toxic effect. To quantify the response of individual neurons to Mas-7, we used Fura-2 AM in a Ca^{2+}-free solution and measured the fluorescence emitted by the probe at 510 nm after the 340/380 excitation in a live imaging experiments. In Figure 1(c) (Fura-2 AM 340/380 ratio images), it is observed that Mas-7 increased the Ca^{2+} concentration in the soma and dendrites of the hippocampal neurons. Additionally, Mas-7 induced a large elevation of Ca^{2+} in a concentration-dependent manner (Figure 1(d)). There was a significant difference between the Ca^{2+} elevations induced by 1 and 5 μM (Figure 1(e)). This finding suggests that the activation of $G\alpha_o$ by Mas-7 produces the release of Ca^{2+} from internal cellular stores in hippocampal neurons.

3.3. Mas-7 Activates CamKII, PKC, and JNK in Mature Hippocampal Neurons. Because Mas-7 increased the Ca^{2+} concentration in hippocampal neurons, we evaluated whether Mas-7 could activate Ca^{2+}-dependent kinases, such as CaMKIIα and PKCβII. Previous reports in neuronal and nonneuronal cells have shown that mastoparan can induce a rise in Ca^{2+} via a phospholipase C- (PLC-) dependent mechanism [7, 22]. The ability of Mas-7 to activate these kinases was determined using anti-phospho-Tyr286-CaMKIIα or anti-phospho-Ser-660-PKCβII antibodies in mature hippocampal neurons.

As illustrated in Figure 2, Mas-7 stimulated the activity of CaMKIIα in a time-dependent manner. We found that hippocampal neurons treated with Mas-7 showed a slight but significant increase in the active form of CaMKIIα after 5 min. Then, the phosphorylation of CaMKIIα decreased and remained at a similar level after 1-2 h with respect to the control condition. In addition, Mas-7 treatment increased PKC phosphorylation after 5 min, with a peak at 60 min (Figure 2). The total levels of PKC were not affected by Mas-7 treatment. Moreover, we analyzed the effect of Mas-7 on the phosphorylation state of other kinases, such as JNK and glycogen synthase kinase-3β (GSK-3β). Mas-7 also induced an increase in JNK phosphorylation (p-JNK-Thr183/Tyr185) after 15 min, mainly of the JNK1 isoform, which corresponds to the lower band. However, hippocampal neurons exposed to Mas-7 did not show any change in the phosphorylation of GSK-3β at serine 9 (p-GSK-3β-Ser9), even after 2 h of treatment. These findings indicate that $G\alpha_o$ activation by Mas-7 promotes the activation of CaMKII, PKC, and JNK in hippocampal neurons.

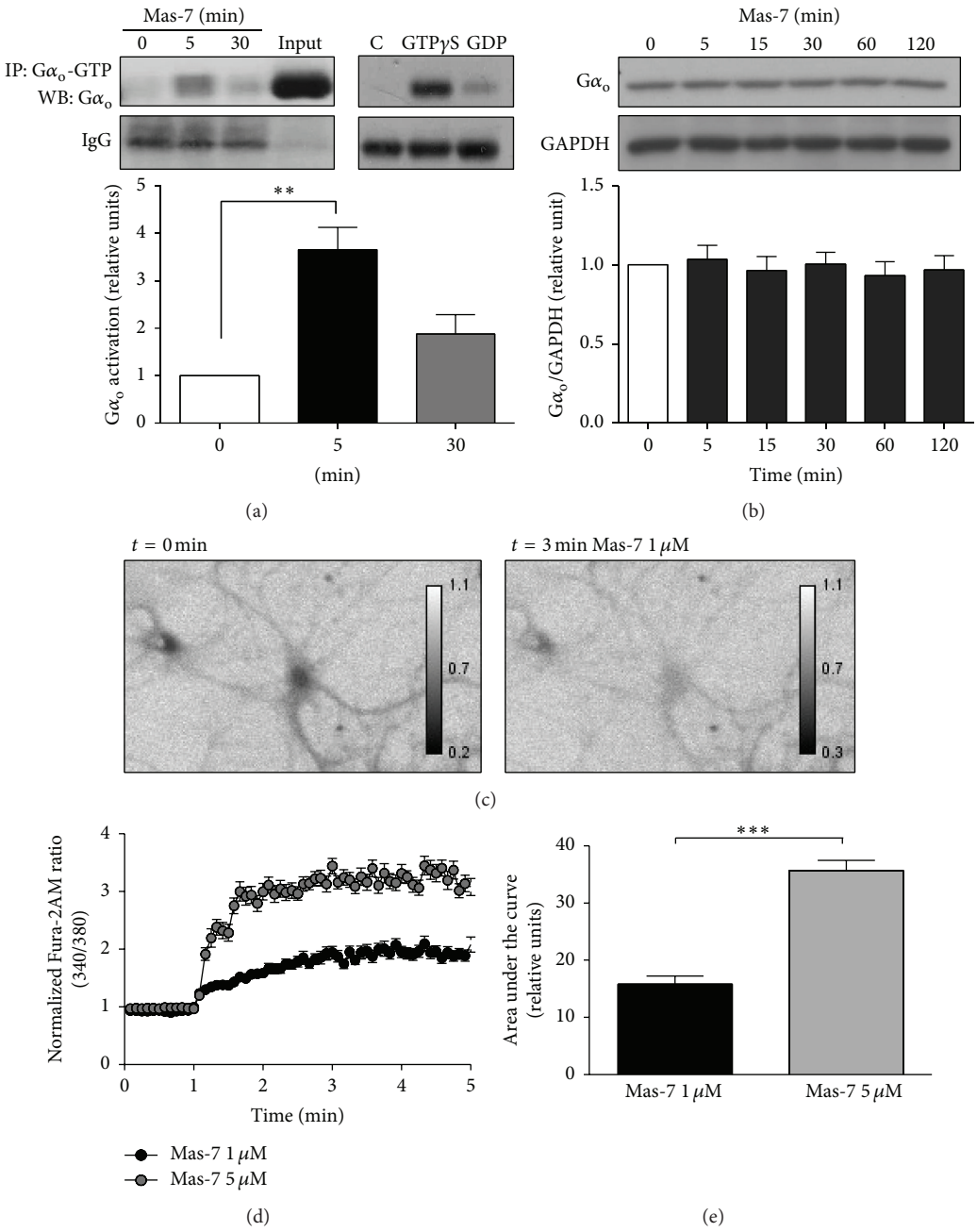

Figure 1: Mas-7 activates the Gα$_o$ subunit and increases the intracellular Ca^{2+} concentration in hippocampal neurons. (a) *Left panel*, 14 DIV hippocampal neurons were stimulated with 1 μM Mas-7 for 0, 5, or 30 min. The neurons were lysed and incubated with anti-Gα$_o$-GTP for 2 h and then analyzed by immunoblotting using an anti-Gα$_o$ antibody ($n = 4$). The input lane corresponds to a lysate sample before the immunoprecipitation. $^{**}p < 0.01$. *Right panel,* lysates from untreated (control) hippocampal neurons were incubated with GTPγS as a positive control or with GDP as a negative control for 90 min at RT. Then, the Gα$_o$-GTP was immunoprecipitated and analyzed by western blotting to determine the total level of Gα$_o$. The IgG band shows that an equal amount of antibody was used for the immunoprecipitation. (b) Representative western blot and quantification of the total level of Gα$_o$ in 14 DIV neurons incubated for different periods of time with 1 μM Mas-7. GAPDH was used as a loading control ($n = 4$). (c) Ratio images (340/380) of the Fura-2AM probe from hippocampal neurons under basal conditions ($t = 0$) or after 3 min of 1 μM Mas-7 treatment. (d) Quantification of measurements of the intracellular Ca^{2+} increase in hippocampal neurons bathed in a Ca^{2+}-free solution with different concentrations of Mas-7 ($n = 3$, 70–79 neurons, each condition). (e) Area under the curve of the Ca^{2+} increase after Mas-7 treatment. $^{***}p < 0.001$.

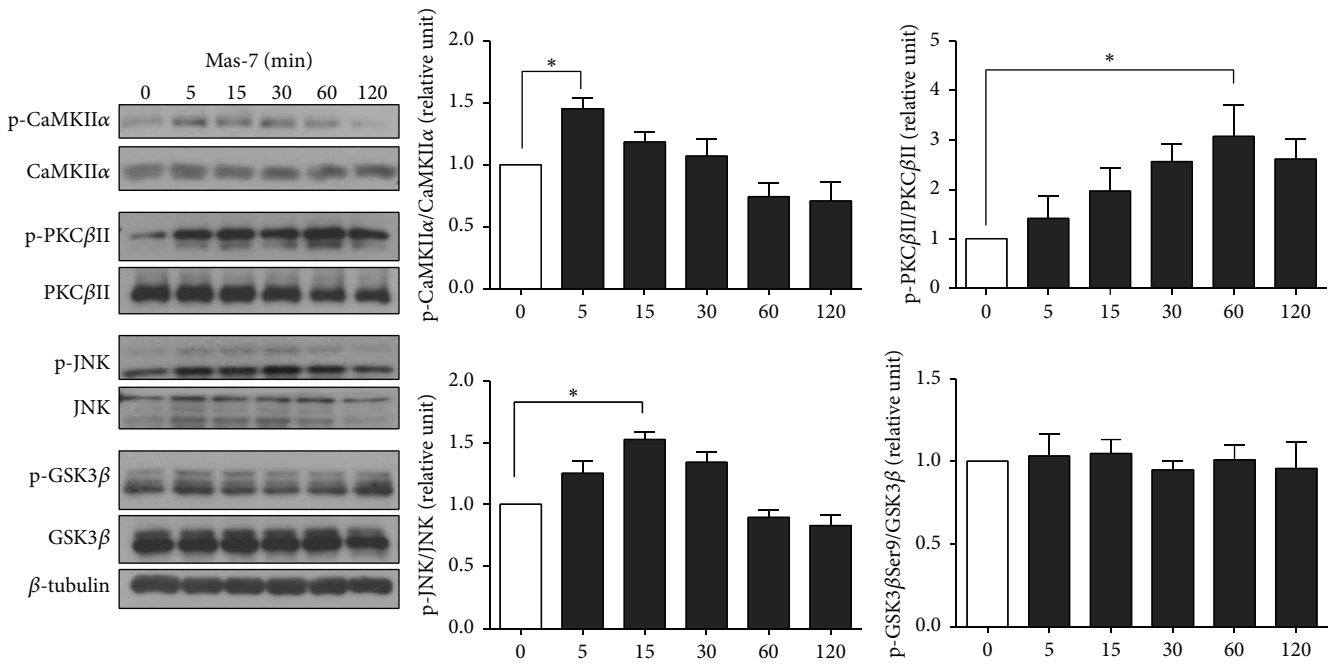

Figure 2: Mas-7 activates CaMKII, PKC, and JNK in hippocampal neurons. Representative western blot and quantification of total and phosphorylated levels of CaMKIIα ($n = 4$), PKCβII ($n = 4$), JNK ($n = 3$), and GSK-3β ($n = 3$) in 14 DIV hippocampal neurons incubated with 1 μM Mas-7 for different times. β-tubulin was used as a loading control. $^{*}p < 0.05$.

3.4. New PSD-95 Clusters Are Induced by Mas-7 Treatment in Hippocampal Neurons. PSD-95 is a scaffold protein that plays a key role in synapse organization, during dendritic spine formation [23]. Here, we examined whether the Mas-7 peptide could regulate the postsynaptic region in mature hippocampal neurons. Specifically, we analyzed whether Mas-7 would induce an increase in PSD-95 clustering in 14 DIV neurons by measuring PSD-95 density and the area of the clusters by immunofluorescence, as described previously [17].

The effect of Mas-7 in the clustering of PSD-95 was evaluated, and a time-dependent increase in the number of PSD-95 clusters was observed. As indicated in Figures 3(a) and 3(b), PSD-95 clustering increased approximately 50% after 60 min of Mas-7 treatment and remained elevated at 2 h in comparison with the control. To evaluate whether the increase in PSD-95 density was due to an increase in the expression of PSD-95, we measured the area of PSD-95 clusters and the levels of PSD-95 protein in total extracts. Mas-7 did not change the area of the PSD-95 clusters (Figure 3(c)) or the PSD-95 total protein level (Figure 3(d)) over the same temporal course for which we observed the increase in the number of PSD-95 clusters, suggesting redistribution of existing PSD-95 proteins, rather than changes in expression.

Thus, our findings suggest that $G\alpha_o$ activation by Mas-7 triggers PSD-95 remodeling that promotes clustering and additional postsynaptic assembly, without changing the expression of the PSD-95 protein.

3.5. Mas-7 Changes the Morphology and Density of Dendritic Spines in Hippocampal Neurons. The presence of PSD-95 clusters in excitatory neurons is well correlated with the number of mature dendritic spines [24]. For this reason, we attempted to determine the role of Mas-7 in dendritic spine formation by transfecting mature hippocampal neurons with EGFP at 10 DIV and then exposed them to 1 μM Mas-7 at different times at 14 DIV. Dendritic spine protrusions below 3 μm in length were analyzed using Imaris software to measure spine length, width, and density. Hippocampal neurons exposed to Mas-7 exhibited increased dendritic spine density after 30 min and 1 h (Figure 4(a)), with a peak at 2 h. Additionally, Mas-7 increased spine head width after 30 min (Figure 4(c)); however, the length of the spines was not significantly affected (Figure 4(d)). An increase in dendritic spine head width has been related to the strength of synaptic transmission [25], which suggests that Mas-7 might also regulate that process.

To provide additional evidence of the effect of Mas-7 in the development of dendritic spine protrusions, live cell time-lapse imaging of the formation of dendritic spines was performed. EGFP-transfected neurons were treated for 45 min with Mas-7 (Figure 4(e)), and we determined that Mas-7 produced *de novo* formation of a dendritic spine. This new protrusion presented a recognizable head and appeared after 30 min of treatment.

Together, these results suggest that the activation of $G\alpha_o$ has a regulatory effect on spine morphogenesis in hippocampal neurons.

3.6. The Activation of CaMKIIα Is Involved in the Dendritic Spine Density Increase Induced by Mas-7. Mas-7 is capable of activating CaMKIIα very rapidly; thus, we sought to

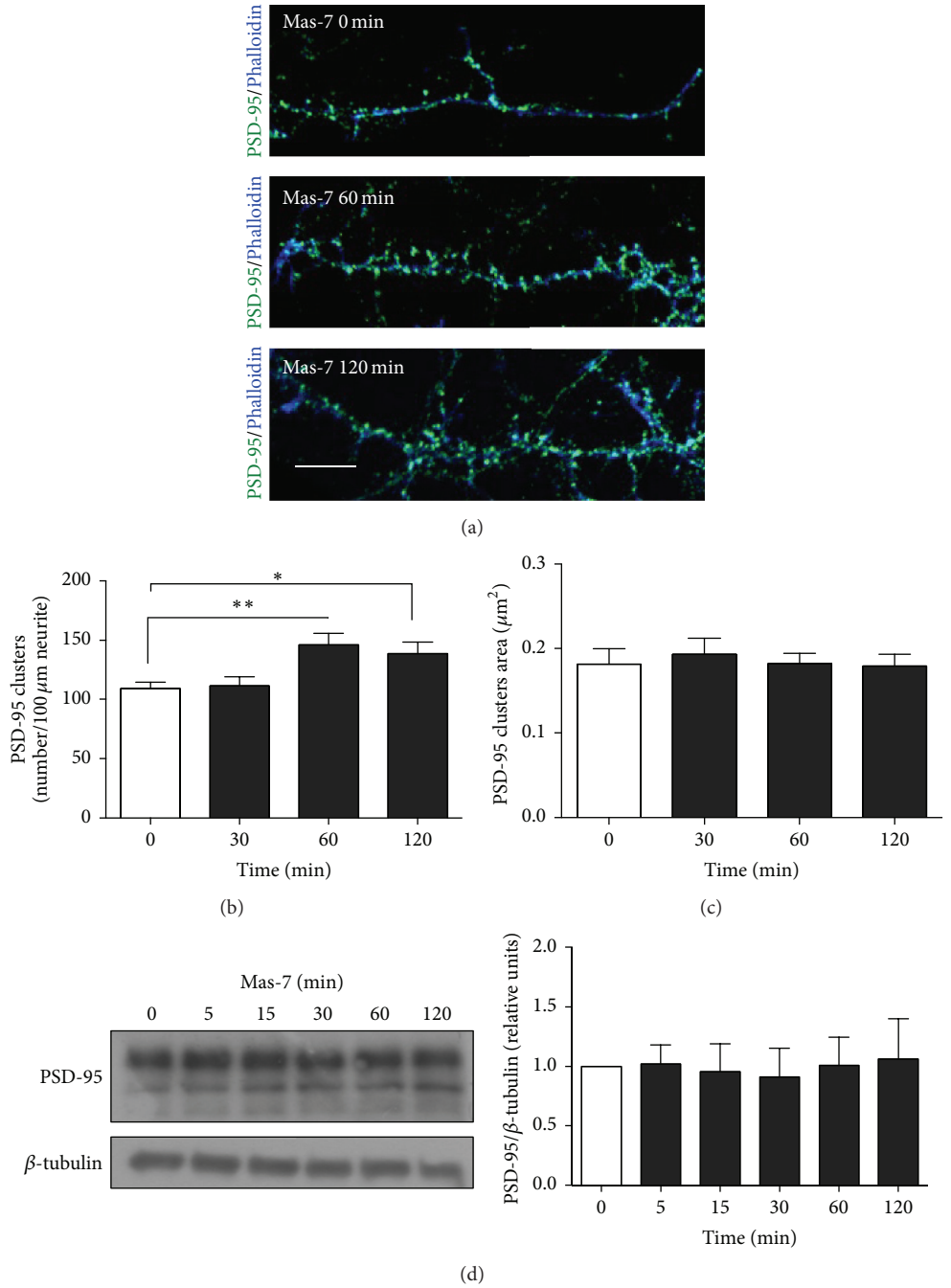

Figure 3: Mas-7 increases the number of PSD-95 clusters in hippocampal neurons. (a) Representative immunofluorescence images for PSD-95 (green) and Phalloidin (blue) in hippocampal neurons exposed to Mas-7 for 0, 60, and 120 min. Scale bar = 6 μm. (b) Quantification of number of PSD-95 clusters/100 μm of neurite (n = 4). (c) Quantification of PSD-95 cluster area (n = 4). (d) Western blot and quantification of total levels of PSD-95 in hippocampal neurons exposed to Mas-7 for different lengths of time (n = 3). $^{**}P < 0.01$ and $^{*}P < 0.05$.

establish whether this activation was required to produce the increase in dendritic spines density to understand the cellular mechanism of Mas-7. Interestingly, CaMKIIα has been related to dendritic spine formation and regulation [26].

We used KN93, a classic inhibitor of CaMKII activation [27]. In hippocampal neurons, KN93 completely blocked the increase of dendritic spine density induced by Mas-7 at 2 h, without affecting density when applied alone (Figures 5(a) and 5(b)). These results indicate that the activation of CAMKIIα by the increase in intracellular Ca^{2+} concentration is required for the regulation of spinogenesis induced by Mas-7.

3.7. Mas-7 Induces Synaptic Contacts in Hippocampal Neurons. Our previous findings of Mas-7 regulation of spine formation as well as of PSD-95 cluster remodelling (Figures 3 and 4)

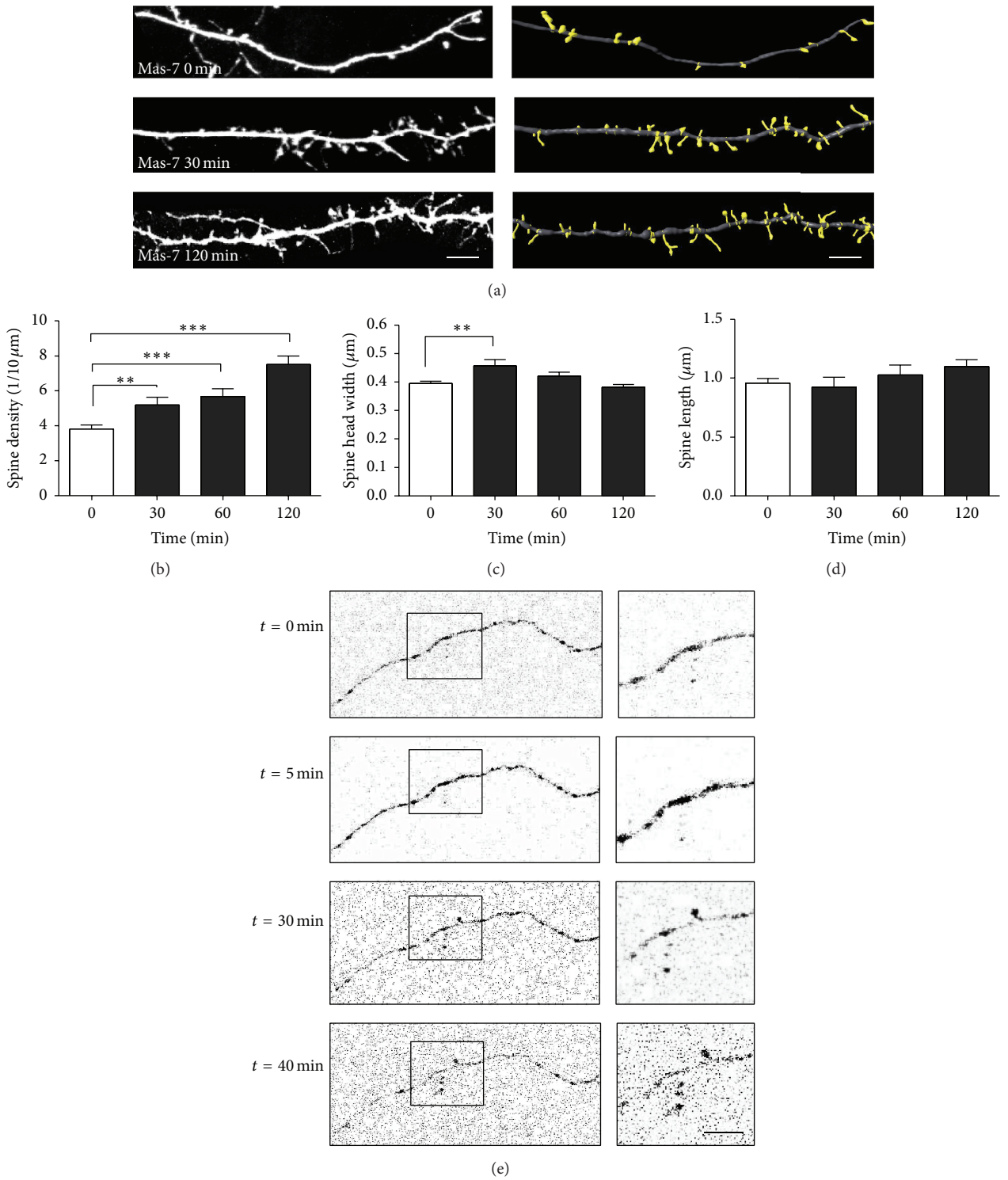

FIGURE 4: $G\alpha_o$ activation by Mas-7 regulates dendritic spine morphogenesis. (a) *Left panel*, representative images of 14 DIV hippocampal neurons treated with Mas-7 for 0, 30, or 120 min. *Right panel*, 3D reconstructions of neurites. Scale bar = 5 μm. (b) Quantification of dendritic spine density. (c) Quantification of spine head width. (d) Quantification of spine length ($n = 3$). $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$. (e) Mas-7 induces *de novo* formation of dendritic protrusions. Live cell time-lapse imaging of the formation of dendritic spines in response to Mas-7 in 14 DIV hippocampal neurons. A dendrite of an EGFP-transfected neuron is shown before and after 5, 30, and 40 min of treatment with Mas-7. Scale bar = 2 μm.

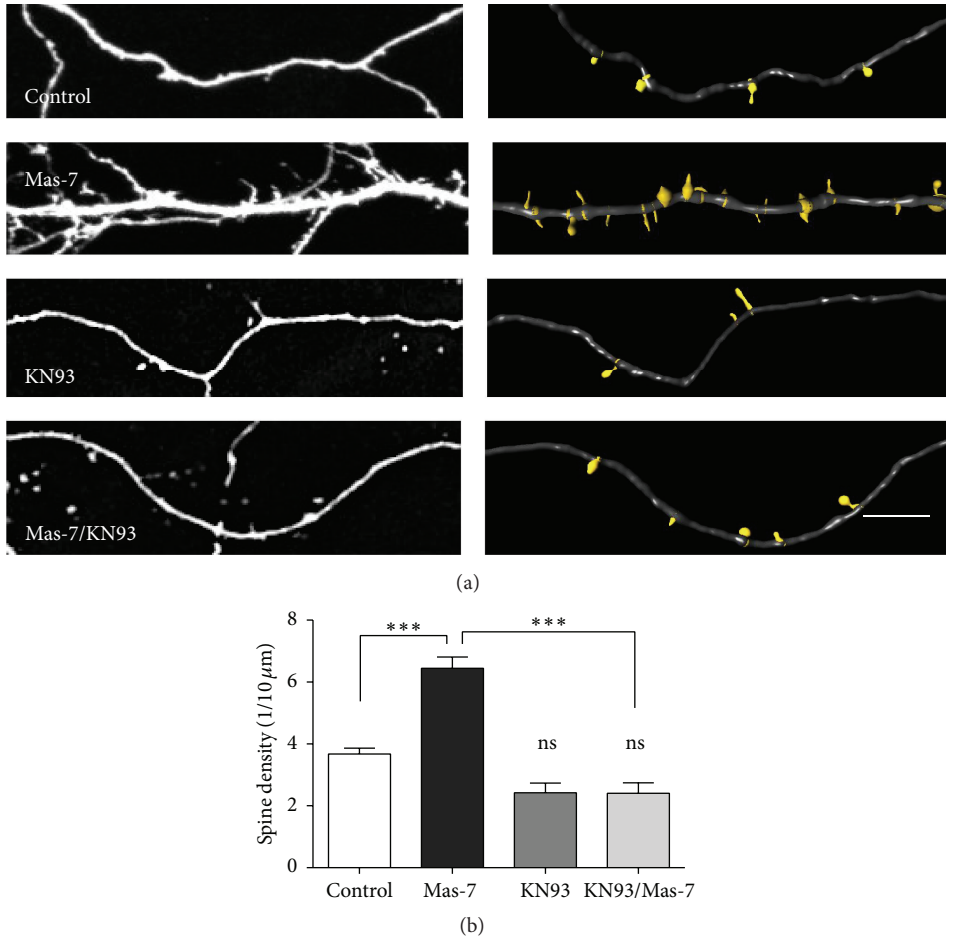

Figure 5: Mas-7 induced spinogenesis through the activation of CaMKII. (a) *Left panel*, representative images of 14 DIV hippocampal neurons untreated (control) or treated with or without Mas-7 for 2 h and coincubated with KN93. *Right panel*, representative 3D reconstructions. (b) Quantification of dendritic spine density for all of the conditions ($n = 4$). Scale bar = 5 μm. $^{***}p < 0.001$. ns = no significant difference.

led us to suggest that Mas-7 is a regulator of the synapse. To address this alternative, hippocampal neurons at 14 DIV were incubated for 1 or 2 h with Mas-7. Treatment with 1 μM Mas-7 significantly increased the number of synaptic contacts after 1-2 h of treatment (Figure 6). The synaptic contacts were observed by the staining for PSD-95 (green) and for the presynaptic protein Syn I (red), where both stains are facing directly. Interestingly, our results suggest that Mas-7 rapidly increases the number of PSD-95 puncta at 1 and 2 h and simultaneously increases the number of synaptic contacts.

4. Discussion

In the present work, we studied the effect of Mas-7, a peptide used to pharmacologically activate G proteins, on synaptic structure.

First, our findings show that the $G\alpha_o$ subunit is activated in hippocampal neurons by Mas-7 treatment, which is consistent with previous data obtained in other cellular contexts, through a mechanism similar to the action of GPCRs [9, 28]. The rapid activation of $G\alpha_o$ occurred after 5 min of Mas-7 exposure in hippocampal neurons, as its analogue mastoparan, because it promotes the dissociation of GDP and enhances the GTP binding [29]. Additionally, mastoparan increases the intrinsic GTPase activity of G proteins, which suggests that the active state of $G\alpha_o$ is transient [30]. We observed that, after 30 min of treatment, the activation of $G\alpha_o$ decayed, which is consistent with an increase in GTPase activity.

Furthermore, it is possible that Mas-7 can activate other G proteins subunits in hippocampal neurons, such as $G\alpha_i$, because mastoparan has been shown to be able to activate both these subunits in a biochemical assay [9].

In hippocampal neurons, Mas-7 presents an immediate effect, increasing the intracellular Ca^{2+} in a concentration-dependent manner, as previously reported in neuroblastoma cells [21] and in neutrophils [20]. This increase remained at least 4-5 min after treatment with Mas-7. It is known that, in addition to being an activator of G proteins, Mas-7 is an inhibitor of ATPase activity from the endoplasmic reticulum [31], which could explain the sustained rise. This elevation in the levels of Ca^{2+} led to the activation of CaMKIIα and PKCβII, two Ca^{2+}-dependent kinases, as well as JNK. The activation of CaMKII was fast, but Mas-7 activated PKC after

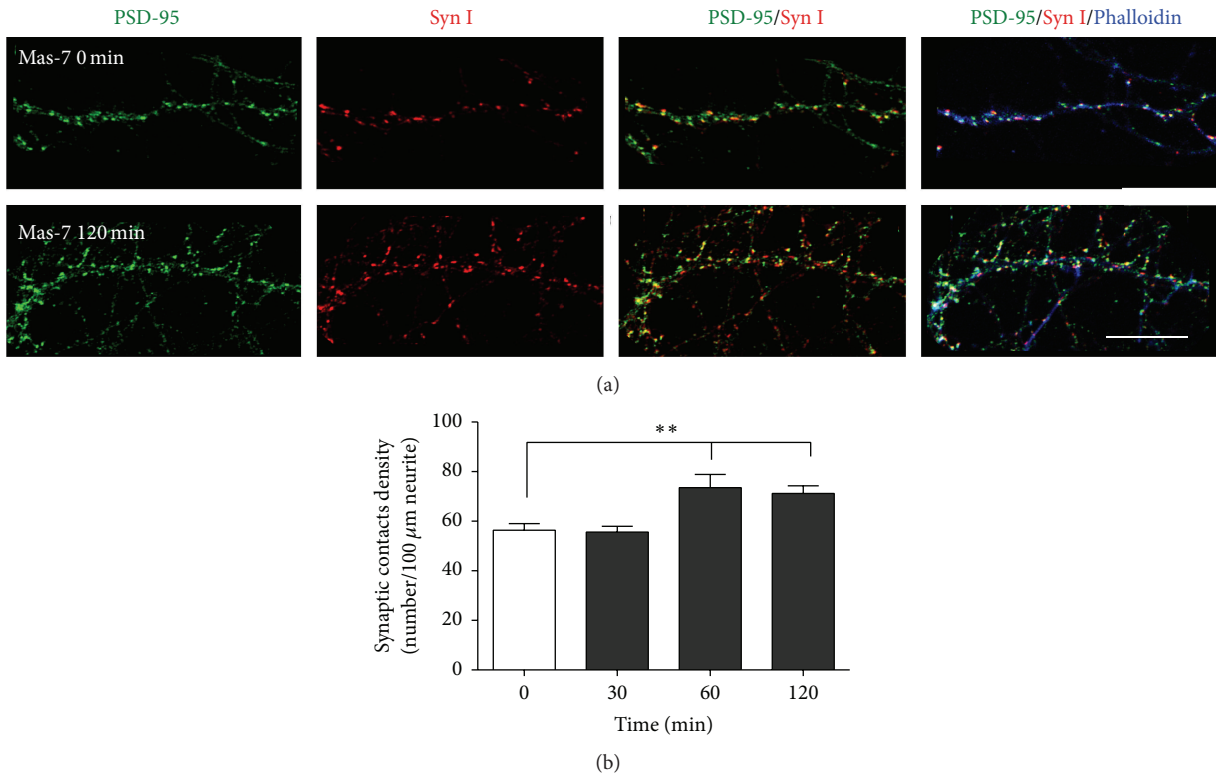

Figure 6: Mas-7 induces the formation of synaptic contacts. (a) Representative images of hippocampal neurons at 14 DIV treated with 1 μM Mas-7 for 0 or 120 min. PSD-95 (green), SynI (red), and Phalloidin (blue) immunofluorescence. Scale bar = 4 μm. (b) Quantification of the density of synaptic contacts (10 neurons were analyzed in each experiment). $^{**}p < 0.01$.

1 h. It would be interesting to study how they regulate the biological effects of Mas-7.

On the other hand, it has been suggested that mastoparan treatment can produce apoptosis in cerebellar granule cells through Ca^{2+} release, probably via activation of a transduction pathway involving PLC and IP_3, using 10–20 μM concentration [22]. In the present study, Mas-7 mimicked the increase in Ca^{2+} from internal stores; however, we used a lower concentration of Mas-7 for a shorter period of time, which did not replicate the apoptotic effect in hippocampal neurons. Higher concentrations of Mas-7 can probably also trigger a similar apoptotic effect in mature hippocampal neurons.

Previous studies have established that $G\alpha_o$ is highly expressed in the central and peripheral nervous systems, where it represents approximately 1% of membrane proteins [32]. Additionally, the $G\alpha_o$ subunit has been linked to cognitive and memory functions in the adult brain. The corresponding knockout mice exhibit neurological impairments, such as reduced motor control, hyperactivity, hyperalgesia, and a shortened lifespan [33]. Moreover, $G\alpha_o$ is required for the formation of associative memory in mushroom body neurons in *D. melanogaster* [34].

Functionally, we observed that Mas-7 was able to modulate the postsynaptic region in the mammalian CNS. In the postsynaptic region, the regulation of dendritic spines is a key process in neuronal plasticity and memory. Dendritic spines undergo structural modifications in response to a diverse range of stimuli [35]. Here, we showed that Mas-7 can regulate dendritic spine formation, increase dendritic spine density and head width, and increase PSD-95 clustering. Although there are several studies that support a model in which PSD-95 is recruited in an activity-dependent manner to new spines, where it contributes to the stabilization of nascent spines [36, 37], we sought to study whether these new spines could form synapses. We found that Mas-7 also increased the number of synaptic contacts, which suggests that $G\alpha_o$ activation is able to generate functional synapses. Further studies are required to demonstrate whether the increase in the dendritic spine density induced by Mas-7 has a positive impact on memory and learning *in vivo*.

Effects of Mas-7 treatment on other aspects of neuronal development have been previously demonstrated, such as a significant increase in axonal growth in hippocampal neurons [38], which suggests that activation of the $G\alpha_o$ subunit generates several structural effects that could produce cytoskeleton remodeling. In particular, we propose that the Ca^{2+} increase produced by Mas-7 and the subsequent activation of the downstream Ca^{2+}-sensitive kinases PKCβII and CaMKIIα as well as JNK can explain the dendritic spine remodeling. These kinases are known regulators of dendritic spine morphology [26, 39, 40], but, particularly, it is known that CaMKIIα is highly expressed in spines and is important for long-term potentiation (LTP) [41]. We found that blocking the

activity of CaMKIIα prevents the Mas-7-dependent increase in dendritic spine density, helping to elucidate the mechanism by which Mas-7 acts. Certainly, understanding the role of PKC and JNK could provide us with a global vision of the involvement of Gα_o signaling in spine remodeling and synapse formation.

All of these findings indicate that Gα_o might be important for the regulation and maintenance of synapses. We suggest a mechanism, in which the activation of this G protein subunit increases the levels of Ca^{2+}, activates CaMKIIα to remodel the postsynaptic region, and ultimately leads to the formation of synaptic contacts.

5. Conclusion

In this work, we demonstrated that the peptide Mas-7 produced several biological effects in mature hippocampal neurons, including activation of Gα_o signaling and of CaMKIIα, JNK, and PKCβII. Functionally, our results suggest that Mas-7 causes dendritic spine remodeling, increases the number of spines, and recruits PSD-95 protein into spines to produce functional synapses.

Conflict of Interests

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

Acknowledgments

This work was supported by grants from FONDECYT (no. 1120156) and the Basal Center of Excellence in Science and Technology (CONICYT-PFB12/2007) to Nibaldo C. Inestrosa, a predoctoral grant to Valerie T. Ramírez, and a postdoctoral fellowship from CONICYT (Fondecyt Postdoctorado No. 3140355) to Eva Ramos-Fernández. The authors thank Gloria Méndez for the neuronal culture preparations.

References

[1] B. K. Atwood, D. M. Lovinger, and B. N. Mathur, "Presynaptic long-term depression mediated by Gi/o-coupled receptors," *Trends in Neurosciences*, vol. 37, no. 11, pp. 663–673, 2014.

[2] M. Hillenbrand, C. Schori, J. Schöppe, and A. Plückthun, "Comprehensive analysis of heterotrimeric G-protein complex diversity and their interactions with GPCRs in solution," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 11, pp. E1181–E1190, 2014.

[3] G. Milligan and E. Kostenis, "Heterotrimeric G-proteins: a short history," *British Journal of Pharmacology*, vol. 147, supplement 1, pp. S46–S55, 2006.

[4] E. J. Neer, "Heterotrimeric C proteins: organizers of transmembrane signals," *Cell*, vol. 80, no. 2, pp. 249–257, 1995.

[5] T. M. Newpher and M. D. Ehlers, "Spine microdomains for postsynaptic signaling and plasticity," *Trends in Cell Biology*, vol. 19, no. 5, pp. 218–227, 2009.

[6] À. Bayés, M. O. Collins, M. D. R. Croning, L. N. van de Lagemaat, J. S. Choudhary, and S. G. N. Grant, "Comparative study of human and mouse postsynaptic proteomes finds high compositional conservation and abundance differences for key synaptic proteins," *PLoS ONE*, vol. 7, no. 10, Article ID e46683, 2012.

[7] B. K. Drobak and P. A. C. Watkins, "Inositol(1,4,5)trisphosphate production in plant cells: stimulation by the venom peptides, melittin and mastoparan," *Biochemical and Biophysical Research Communications*, vol. 205, no. 1, pp. 739–745, 1994.

[8] H. S. Park, S. Y. Lee, Y. H. Kim, J. Y. Kim, S. J. Lee, and M.-U. Choi, "Membrane perturbation by mastoparan 7 elicits a broad alteration in lipid composition of L1210 cells," *Biochimica et Biophysica Acta—Molecular and Cell Biology of Lipids*, vol. 1484, no. 2-3, pp. 151–162, 2000.

[9] T. Higashijima, J. Burnier, and E. M. Ross, "Regulation of Gi and Go by mastoparan, related amphiphilic peptides, and hydrophobic amines: Mechanism and structural determinants of activity," *The Journal of Biological Chemistry*, vol. 265, no. 24, pp. 14176–14186, 1990.

[10] C. J. Sample, K. E. Hudak, B. E. Barefoot et al., "A mastoparan-derived peptide has broad-spectrum antiviral activity against enveloped viruses," *Peptides*, vol. 48, pp. 96–105, 2013.

[11] Y. Ozaki, Y. Matsumoto, Y. Yatomi, M. Higashihara, T. Kariya, and S. Kume, "Mastoparan, a wasp venom, activates platelets via pertussis toxin-sensitive GTP-binding proteins," *Biochemical and Biophysical Research Communications*, vol. 170, no. 2, pp. 779–785, 1990.

[12] T. Yamamoto, M. Ito, K. Kageyama et al., "Mastoparan peptide causes mitochondrial permeability transition not by interacting with specific membrane proteins but by interacting with the phospholipid phase," *FEBS Journal*, vol. 281, no. 17, pp. 3933–3944, 2014.

[13] R. A. de Azevedo, C. R. Figueiredo, A. K. Ferreira et al., "Mastoparan induces apoptosis in B16F10-Nex2 melanoma cells via the intrinsic mitochondrial pathway and displays antitumor activity *in vivo*," *Peptides*, vol. 68, pp. 113–119, 2015.

[14] A. R. Alvarez, J. A. Godoy, K. Mullendorff, G. H. Olivares, M. Bronfman, and N. C. Inestrosa, "Wnt-3a overcomes beta-amyloid toxicity in rat hippocampal neurons," *Experimental Cell Research*, vol. 297, no. 1, pp. 186–196, 2004.

[15] J. Fernandes, I. M. Lorenzo, Y. N. Andrade et al., "IP3 sensitizes TRPV4 channel to the mechano-and osmotransducing messenger 5′-6′-epoxyeicosatrienoic acid," *Journal of Cell Biology*, vol. 181, no. 1, pp. 143–155, 2008.

[16] L. Varela-Nallar, C. P. Grabowski, I. E. Alfaro, A. R. Alvarez, and N. C. Inestrosa, "Role of the Wnt receptor Frizzled-1 in presynaptic differentiation and function," *Neural Development*, vol. 4, article 41, 2009.

[17] G. G. Farías, I. E. Alfaro, W. Cerpa et al., "Wnt-5a/JNK signaling promotes the clustering of PSD-95 in hippocampal neurons," *The Journal of Biological Chemistry*, vol. 284, no. 23, pp. 15857–15866, 2009.

[18] L. Varela-Nallar, J. Parodi, G. G. Farías, and N. C. Inestrosa, "Wnt-5a is a synaptogenic factor with neuroprotective properties against Aβ toxicity," *Neurodegenerative Diseases*, vol. 10, no. 1–4, pp. 23–26, 2012.

[19] S. Di Angelantonio, E. Murana, S. Cocco et al., "A role for intracellular zinc in glioma alteration of neuronal chloride equilibrium," *Cell Death and Disease*, vol. 5, no. 10, Article ID e1501, 2014.

[20] A. Perianin and R. Snyderman, "Mastoparan, a wasp venom peptide, identifies two discrete mechanisms for elevating

cytosolic calcium and inositol trisphosphates in human polymorphonuclear leukocytes," *Journal of Immunology*, vol. 143, no. 5, pp. 1669–1673, 1989.

[21] B.-C. Suh, S.-K. Song, Y.-K. Kim, and K.-T. Kim, "Induction of cytosolic Ca^{2+} elevation mediated by Mas-7 occurs through membrane pore formation," *Journal of Biological Chemistry*, vol. 271, no. 51, pp. 32753–32759, 1996.

[22] S.-Z. Lin, G.-M. Yan, K. E. Koch, S. M. Paul, and R. P. Irwin, "Mastoparan-induced apoptosis of cultured cerebellar granule neurons is initiated by calcium release from intracellular stores," *Brain Research*, vol. 771, no. 2, pp. 184–195, 1997.

[23] P. Steiner, M. J. Higley, W. Xu, B. L. Czervionke, R. C. Malenka, and B. L. Sabatini, "Destabilization of the postsynaptic density by PSD-95 serine 73 phosphorylation inhibits spine growth and synaptic plasticity," *Neuron*, vol. 60, no. 5, pp. 788–802, 2008.

[24] V. A. Alvarez and B. L. Sabatini, "Anatomical and physiological plasticity of dendritic spines," *Annual Review of Neuroscience*, vol. 30, pp. 79–97, 2007.

[25] M. Matsuzaki, N. Honkura, G. C. R. Ellis-Davies, and H. Kasai, "Structural basis of long-term potentiation in single dendritic spines," *Nature*, vol. 429, no. 6993, pp. 761–766, 2004.

[26] S.-J. R. Lee, Y. Escobedo-Lozoya, E. M. Szatmari, and R. Yasuda, "Activation of CaMKII in single dendritic spines during long-term potentiation," *Nature*, vol. 458, no. 7236, pp. 299–304, 2009.

[27] M. Sumi, K. Kiuchi, T. Ishikawa et al., "The newly synthesized selective Ca2+/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells," *Biochemical and Biophysical Research Communications*, vol. 181, no. 3, pp. 968–975, 1991.

[28] T. Higashijima, S. Uzu, T. Nakajima, and E. M. Ross, "Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G proteins)," *The Journal of Biological Chemistry*, vol. 263, no. 14, pp. 6491–6494, 1988.

[29] A. E. Remmers, R. Posner, and R. R. Neubig, "Fluorescent guanine nucleotide analogs and G protein activation," *The Journal of Biological Chemistry*, vol. 269, no. 19, pp. 13771–13778, 1994.

[30] M. Zorko, M. Pooga, K. Saar, K. Rezaei, and Ü. Langel, "Differential regulation of GTPase activity by mastoparan and galparan," *Archives of Biochemistry and Biophysics*, vol. 349, no. 2, pp. 321–328, 1998.

[31] C. L. Longland, M. Mezna, and F. Michelangeli, "The mechanism of inhibition of the Ca^{2+}-ATPase by mastoparan. Mastoparan abolishes cooperative Ca2+ binding," *The Journal of Biological Chemistry*, vol. 274, no. 21, pp. 14799–14805, 1999.

[32] P. F. Worley, J. M. Baraban, and C. Van Dop, "Go, a guanine nucleotide-binding protein: Immunohistochemical localization in rat brain resembles distribution of second messenger systems," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 83, no. 12, pp. 4561–4565, 1986.

[33] M. Jiang, M. S. Gold, G. Boulay et al., "Multiple neurological abnormalities in mice deficient in the G protein G_o," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 6, pp. 3269–3274, 1998.

[34] J. Ferris, H. Ge, L. Liu, and G. Roman, "G(o) signaling is required for *Drosophila* associative learning," *Nature Neuroscience*, vol. 9, no. 8, pp. 1036–1040, 2006.

[35] J. N. Bourne and K. M. Harris, "Balancing structure and function at hippocampal dendritic spines," *Annual Review of Neuroscience*, vol. 31, pp. 47–67, 2008.

[36] O. Prange and T. H. Murphy, "Modular transport of postsynaptic density-95 clusters and association with stable spine precursors during early development of cortical neurons," *The Journal of Neuroscience*, vol. 21, no. 23, pp. 9325–9333, 2001.

[37] I. Ehrlich, M. Klein, S. Rumpel, and R. Malinow, "PSD-95 is required for activity-driven synapse stabilization," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 10, pp. 4176–4181, 2007.

[38] J. C. Nordman, W. S. Phillips, N. Kodama, S. G. Clark, C. A. Del Negro, and N. Kabbani, "Axon targeting of the alpha 7 nicotinic receptor in developing hippocampal neurons by Gprin1 regulates growth," *Journal of Neurochemistry*, vol. 129, no. 4, pp. 649–662, 2014.

[39] M. Goldin and M. Segal, "Protein kinase C and ERK involvement in dendritic spine plasticity in cultured rodent hippocampal neurons," *European Journal of Neuroscience*, vol. 17, no. 12, pp. 2529–2539, 2003.

[40] E. T. Coffey, "Nuclear and cytosolic JNK signalling in neurons," *Nature Reviews Neuroscience*, vol. 15, no. 5, pp. 285–299, 2014.

[41] J. Lisman, R. Yasuda, and S. Raghavachari, "Mechanisms of CaMKII action in long-term potentiation," *Nature Reviews Neuroscience*, vol. 13, no. 3, pp. 169–182, 2012.

Hindawi Publishing Corporation
Neural Plasticity
Volume 2016, Article ID 6170509, 11 pages
http://dx.doi.org/10.1155/2016/6170509

Research Article

Large and Small Dendritic Spines Serve Different Interacting Functions in Hippocampal Synaptic Plasticity and Homeostasis

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Received 7 July 2015; Revised 2 September 2015; Accepted 11 October 2015

Academic Editor: Bryen A. Jordan

The laying down of memory requires strong stimulation resulting in specific changes in synaptic strength and corresponding changes in size of dendritic spines. Strong stimuli can also be pathological, causing a homeostatic response, depressing and shrinking the synapse to prevent damage from too much Ca^{2+} influx. But do all types of dendritic spines serve both of these apparently opposite functions? Using confocal microscopy in organotypic slices from mice expressing green fluorescent protein in hippocampal neurones, the size of individual spines along sections of dendrite has been tracked in response to application of tetraethylammonium. This strong stimulus would be expected to cause both a protective homeostatic response and long-term potentiation. We report separation of these functions, with spines of different sizes reacting differently to the same strong stimulus. The immediate shrinkage of large spines suggests a homeostatic protective response during the period of potential danger. In CA1, long-lasting growth of small spines subsequently occurs consolidating long-term potentiation but only after the large spines return to their original size. In contrast, small spines do not change in dentate gyrus where potentiation does not occur. The separation in time of these changes allows clear functional differentiation of spines of different sizes.

1. Introduction

Dendritic spines form the postsynaptic element of most excitatory synapses in the mammalian cortex and hippocampus and their differing sizes and morphologies are directly related to synaptic strength [1]. The strength of spine synapses is highly plastic which is important for homeostatic protection from excitotoxicity but also for the laying down and retrieval of memory [2–4]. Being directly related to the strength of synapses, it is not surprising that the size of spines also changes with plasticity of synaptic transmission [5–7]. However, it remains controversial whether the diversity of spine morphologies represents a continuum, with size simply reflecting the history of the synapse or rather that spines with different morphological classifications represent different functional entities. To address this question, we investigate how different spines react and interact when they are strongly and simultaneously stimulated across the network. Application of tetraethylammonium chloride (TEA) results in "chemical long-term potentiation" (LTP) at CA3-CA1 synapses [8] and has been shown to cause growth in a subset of small spines when imaged 2 hours after induction [9]. However, such global stimulation would also be expected to cause an immediate protective homeostatic response due to both massive depolarisation and resulting glutamate release. Moreover, possible effects of the osmolarity change caused by adding 25 mM TEA must be considered, a control that has seldom been included in previous studies. Here, we report that, in response to TEA, not only the direction but also the time course of changes in the response of larger and smaller spines can be separated. Moreover, in DG granule cells, where TEA does not cause long-term potentiation [10], the response of spines differs from that of CA1 pyramidal cells confirming the functional link between spine size and synaptic plasticity.

2. Material and Methods

2.1. Animals and Slices. Organotypic slices were prepared using standard methods [11] from 5- to 6-day-old mice of either sex expressing green fluorescent protein (GFP) on the

Thy1 promoter (GFPS mice) [12], resulting in a subset of their glutamatergic neurones being fluorescent. Organotypic slices used for granule cell imaging and electrophysiological recording were made with the standard protocol of parasagittal sections. For imaging of CA1 pyramidal cells, slices were angled as for preparation of acute slices for electrophysiological recording (~15° off parasagittal) as this maintains more CA1 neurones intact and ensures that the preparations for imaging and recording were as similar as possible.

Acute slices were made using standard methods [13] adapted for mouse [14]. Each hemisphere was sectioned (400 μm) in ice-cold dissection artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 2.4 KCl, 26 $NaHCO_3$, 1.4 NaH_2PO_4, 20 D-glucose, 3 $MgCl_2$, and 0.5 $CaCl_2$, pH 7.4, ~315 mOsm/L. The hippocampus with a portion of entorhinal cortex was dissected and placed into a chamber containing bubbled dissection ACSF at room temperature (~20°C). After 5 minutes, the chamber was warmed to 35°C. Slices were then, at 5-minute intervals, consecutively transferred to increasingly physiological Ca^{2+} and Mg^{2+} ion concentration (in mM): (i) 1 Mg^{2+}, 0.5 Ca^{2+}; (ii) 1 Mg^{2+}, 1 Ca^{2+}; (iii) 1 Mg^{2+}, 2 Ca^{2+} (standard ACSF). After 20 minutes at 35°C, slices were allowed to return to room temperature for at least 40 min before recording.

All animal procedures were performed in compliance with the United Kingdom Animals (Scientific Procedures) Act 1986.

2.2. Chemical LTP. TEA (25 mM) dissolved in ACSF was bath perfused (~1 mL/min) for 5 minutes before returning to standard ACSF [8]. For time control experiments TEA was not included but experiments were otherwise identical with and without TEA. In osmolarity control experiments, the protocol was identical but 50 mM sorbitol replaced the TEA.

2.3. Imaging and Analysis. Dendrites chosen at random were scanned (confocal microscope: Olympus Fluoview 300 or Zeiss LSM 510; Olympus 60x water immersion objective, N.A. 0.9) at 6x gain with 0.2 μm steps. The microscope used did not affect the results. For maximum resolution, all imaging experiments were carried out in organotypic hippocampal slices (2-3 weeks *in vitro*) at 30 ± 1°C. After deconvolution (AutoQuant, Media Cybernetics), images were reconstructed in 3D using the Filament Tracer module of Imaris (Bitplane) to estimate spine diameter. Filament Tracer estimates the diameter of a sphere equivalent to the volume estimated from several automatically defined sections of the spine taken through the z-plane (Figure 1(a)). Thus, the "diameters" reported are not a direct measure (which would be beyond the resolution of the image) but rather a back extrapolation from several images estimating the overall 3D head volume. This calculated value, rather than being an accurate absolute measure of the diameter of the active zone, is a high-resolution method of comparing changes in individual spines across time, while avoiding the assumption of where on the spine the synaptic contact would be situated. Moreover, using diameter rather than volume transforms the skewed volume data to a normal distribution, facilitating analysis. All image analysis was carried out blind to treatment and to the time point of the experiment. Data in figures are presented for spines in which size could be reliably estimated at the initial control time point (−10 minutes, to which all other time points were compared) and at least 3 of the 5 postinduction time points (diameter > z-interval, ensuring at least two intersecting planes). Note that this excludes the smallest spines. In some cases, we were also unable to model the largest spines, so they were also excluded. This may relate to irregularities in the shapes of spines being better resolved in these cases and so causing problems with the algorithm used by Imaris (e.g., see Figure 1(a), granule cell dendrite). The time course and direction of change for both small and large spines in both CA1 pyramidal cells and dentate gyrus (DG) granule cells were consistent whether all spines were considered individually or the results were averaged by experiment. Results shown are for individual spines independent of preparation.

2.4. Electrophysiology. Field recording is the method of choice for measuring effects over the network and for avoiding effects of cell dialysis in LTP experiments. However, as the CA1 cell layer tends to spread out in organotypic slices, the interface between the cell body layer and dendrites becomes too diffuse for recording field excitatory postsynaptic potentials (fEPSPs). We have previously demonstrated however that the morphology of dendritic spines in CA1 is very similar in acute and organotypic preparations [15, 16] and so fEPSPs were recorded in acute slices from 4-week-old male mice, being the most similar preparation suited to these recordings. In the case of dentate gyrus, the cell layer is often less affected by the spread of the cell layers over time and so in some preparations it is possible to obtain field recordings. We have thus concentrated on acute slices throughout the field experiments but compared the dentate results to organotypic slices where possible.

Slices were transferred as needed to a heated (30 ± 1°C), submerged chamber, perfused with ACSF, and allowed to recover for 1 h in the recording chamber. A glass stimulating electrode (filled with ACSF, resistance 1–3 MΩ) was positioned in the appropriate projection (stratum radiatum or alternately medial or lateral perforant path). A glass recording electrode (filled with ACSF, resistance 1–3 MΩ) was positioned in stratum radiatum of CA1 or in the molecular layer of the dentate gyrus to record a dendritic field potential. Stimulation intensity was set at ~50% of the intensity required to evoke a population spike and recording continued until a 15-minute stable baseline was achieved. LTP conditioning consisted of either application of TEA (as above) or 3 trains of tetani, each consisting of 20 pulses at 100 Hz, 1.5 s intertrain interval, and recording (0.1 Hz) was then continued for another 60 minutes. Data are displayed as averages of 6 consecutive responses over 1 minute. Recording and analysis were carried out using WinWCP synaptic analysis software (Dr. John Dempster; http://spider.science.strath.ac.uk/sipbs/software.htm).

2.5. Statistics. Statistics were performed using SPSS (Version 23) or Graphpad Prism (Version 6). All data are expressed as

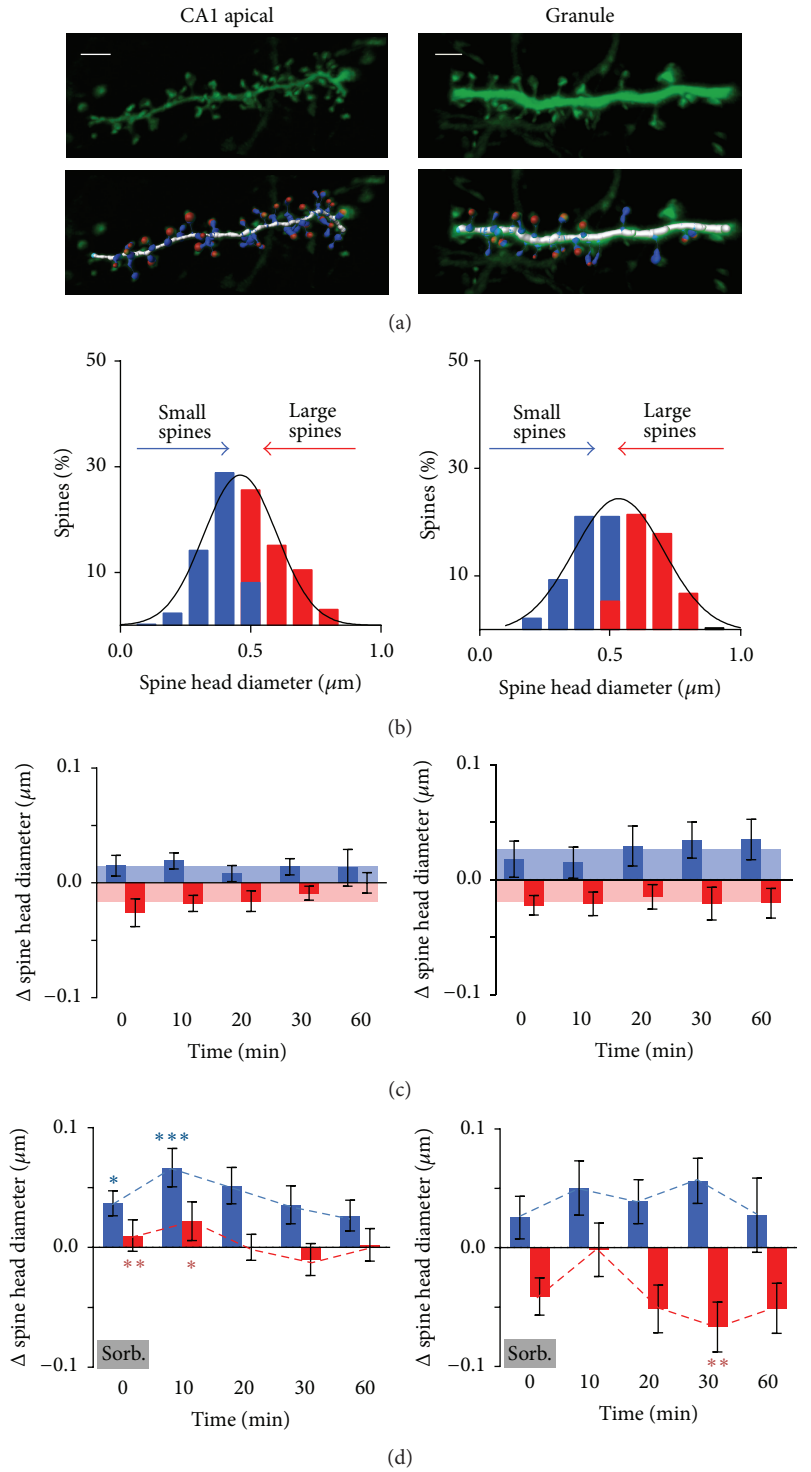

Figure 1: Control data from CA1 pyramidal cells or DG granule cells tend to fluctuate towards the mean but are similar over time (left panels: CA1 pyramidal cells; right panels: DG granule cells). (a) 3D reconstruction of deconvolved confocal images of sections of dendrite of a hippocampal organotypic slice and model of the dendritic spines as superimposed by Imaris. Scale bar: 2 μm. Note one large spine not modelled by Imaris (see Section 2). (b) Before application of TEA, estimated spine head diameters are normally distributed. Small (blue) and large (red) spines are defined as spines with diameters less or greater than the mean diameter, respectively. (c) Change in spine diameter after repeated imaging in control ACSF. Limits of the shaded region represent the mean of all time points for small (blue) and large (red) spines. (d) Change in spine diameter after repeated imaging following transient high osmolarity ACSF perfusion (50 mM sorbitol, 5 min). Dotted line represents mean change of small (blue) and large (red) spines at that time point. Note the data at time 0 are sampled during the sorbitol wash-in. *Post hoc* analysis of control data versus sorbitol (3-way interaction between size, time, and treatment): $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

means ± SEM. For analysis of spine head size, a Generalised Linear Mixed Model was used to compare control versus sorbitol, control versus TEA, and sorbitol versus TEA in each of the two cell types. Repeated measures of the change in size over time (using unstructured covariance) on each spine compared to the pretreatment time point took into account the different preparations for each treatment group. A 3-way comparison was made using time, size, and treatment as fixed effects. Reported probabilities refer to the *post hoc* analysis of the 3-way interactions using sequential Sidak adjustment for multiple comparisons. A robust estimate was used for missing data points. (As outlined above, data were included if reliable estimates could be obtained before treatment and for at least 3 of the subsequent 5 time points.)

All other analyses used paired or unpaired t-tests as appropriate.

3. Results

Using confocal microscopy, stretches of hippocampal dendrites were repeatedly scanned, reconstructed in 3D, and modelled (Figure 1(a)) at 10-minute intervals before (−10 min), during (0 min), and at several time points after (10, 20, 30, and 60 min) exposing the slice to TEA or sorbitol (osmotic control) or at the same time points with no change of solution (time control).

3.1. Controls. Spines were classified in terms of size and location. Estimated spine head diameters on apical dendrites of CA1 pyramidal cells (0.49 ± 0.006 μm, $n = 396$) were significantly lower than for DG granule cells (0.53 ± 0.009 μm, $n = 280$; Student's t-test $p < 0.0001$ versus CA1 apical spines). Spines were thus divided into those smaller or larger than 0.49 μm for CA1 apical and 0.53 μm for DG granule cells and this formed the initial distribution (designated −10 min, Figure 1(b)). Each spine was then compared to its own initial value over time (0, 10, 20, 30, and 60 min; Δ spine head size). (Note that using 0.53 μm versus 0.49 μm as the size threshold for DG granule cells made no qualitative difference to the result.)

Effects of Time and Natural Fluctuation. As would be expected from random fluctuation [17], in control experiments estimated spine diameter fluctuated on average towards the mean, small spines becoming, on average, slightly larger and large spines slightly smaller (Figure 1(c)). There was no significant difference in the fluctuation over the time course of the experiment (2-way ANOVA size versus time, both apical CA1 pyramidal cells and DG granule cells: significant effect of head size $p < 0.0001$, no effect of time, $p \sim 0.6$, and no interaction, $p \sim 0.5$; $n = 176$ spines in 7 preparations for CA1 cells and 83 spines in 4 preparations for granule cells). For the purpose of illustration, the mean change in spine head size of each group was averaged across all time points (outer limit of shaded region, Figures 1(c), 2(b), and 3(b)) although the relevant time point was used for statistical comparison with test data.

Effects of Osmolarity. The eventual aim of the study was to understand the role of different spine types when simultaneously stimulated by an induction protocol that would cause LTP, in this case 25 mM TEA. Addition of 25 mM TEA altered the osmolarity of the solution from 315 mOsm to 365 mOsm. As it is not possible to prevent this increase in osmolarity without changing Na^+ concentration which would alter the excitability of the system and compromise the LTP, parallel experiments were conducted to assess the contribution of osmolarity to both the slope of the field potentials and the change in spine size. To this end, sorbitol (50 mM) was substituted for TEA, increasing osmolarity (by 50 mOsm without causing LTP) in otherwise identical experiments to the TEA experiments below. Sorbitol caused a transient decrease in field potential slope reaching a minimum level of ~50% of baseline values at around 10 minutes in both CA1 and dentate gyrus which then returned to baseline level by 20 minutes in both areas (Figures 2(c) and 3(c)). The sorbitol was tested in acute slices in both CA1 ($n = 5$) and dentate gyrus ($n = 13$) and the time course confirmed in the dentate gyrus in organotypic slices ($n = 2$). This decrease in synaptic strength was not accompanied by a change in paired-pulse ratio (PPR) and hence was probably not due to a presynaptic effect such as osmotically induced depletion of the readily releasable vesicle pool. We hypothesized that the osmolarity-induced depression may have been associated with a decrease in the size of spines, particularly large spines due to loss of H_2O down the osmotic gradient. However, in the CA1 region, the decrease in field potential slope was not associated with any decrease in spine size ($n = 122$ spines in 4 preparations). In fact, both small and large spines transiently showed a significant increase in size when compared to controls, peaking at 10 minutes during washout of sorbitol. Large spines consistently returned to baseline by 20 minutes while small spines returned to control levels at a variable rate but always by 60 minutes (Figure 1(d) and dotted lines in Figures 2(b) and 3(b)). In contrast, in the dentate gyrus, increased osmolarity resulted in delayed shrinkage of the large spines peaking with a significant change from control data at 30 minutes. Osmolarity had no significant effect on small spines compared to controls at any time point in dentate gyrus ($n = 75$ spines in 4 preparations).

Hence, the transiently decreased synaptic response resulting from increased osmolarity was accompanied by a trend towards growth of spines rather than shrinkage, particularly in the CA1 region, opposing the expected effect of osmolarity and breaking the usual association between synaptic strength and spine morphology.

3.2. Effects of TEA. In order to assess the interaction of long-term plasticity and spine size, TEA was applied as a global stimulus to hippocampal slices. TEA blocks potassium channels causing widespread depolarization and glutamate release. The effect of TEA on spines of CA1 pyramidal cells, in which application of TEA consistently causes LTP, was compared to effects in DG granule cells where TEA fails to induce LTP [10]. Effects of TEA differed between the CA1 region and the dentate gyrus in both the electrical and morphological changes observed but in both cases changes in

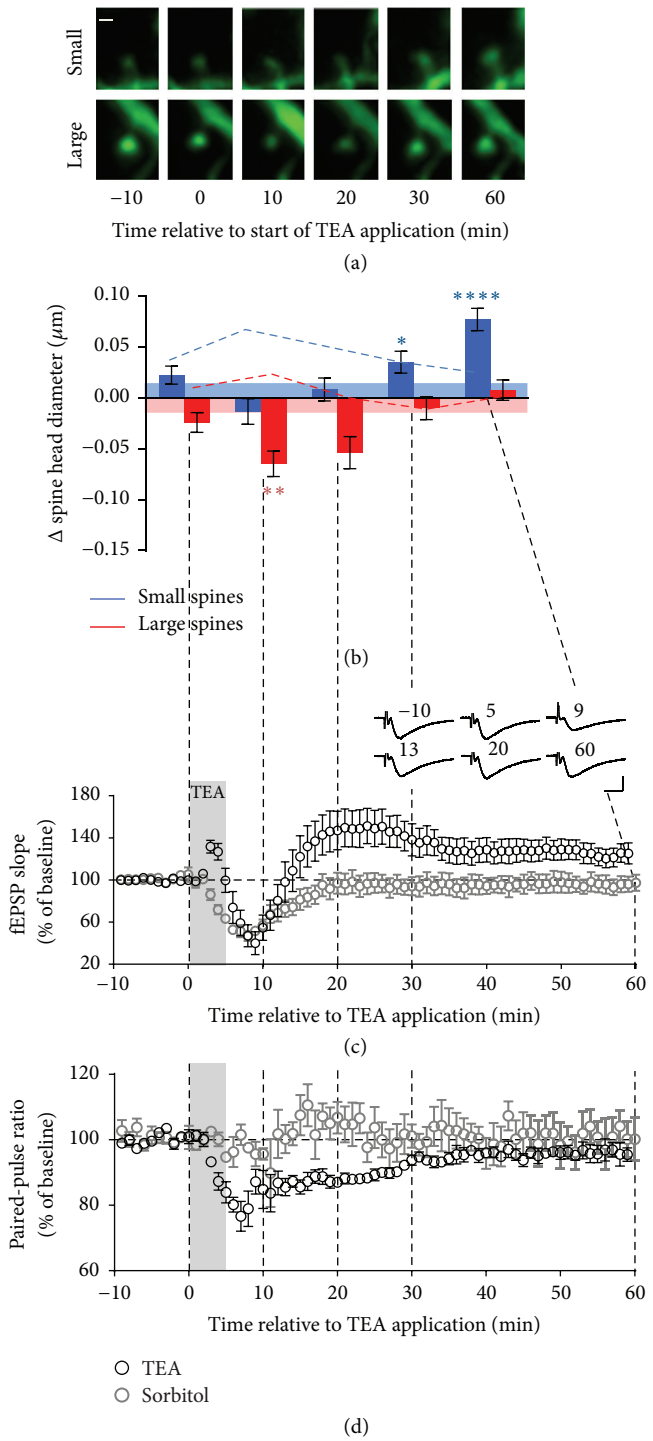

Figure 2: Large and small dendritic spines in CA1 pyramidal cells respond to TEA with different time courses corresponding to different phases of the synaptic response. (a) Typical example of a small and a large spine imaged before (−10), during (0), and after washout (10–60 min) of TEA (25 mM, 5 min). Scale bar: 0.5 μm. (b) Quantification of changes in spine size relative to the pre-TEA measurement. Small spines, blue; large spines, red. Limits of the shading represent the mean change across time in the absence of TEA (time course control experiments). The dotted lines represent the mean change at each time point in response to sorbitol (osmolarity control experiments). *Post hoc* analysis of control data versus TEA (3-way interaction between size, time, and treatment): $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$. (c) $fEPSP_{slope}$ and (d) PPR recorded in the CA1 region of acute hippocampal slices in response to stimulation of the Schaffer collateral before, during, and after application of TEA (black symbols) or sorbitol (grey symbols) as above. Error bars: SEM. Grey shading: TEA perfusion. Inset: averages of fEPSPs recorded from a typical slice over 1 min at 10 s intervals at the time indicated (min). Scale bar: 1 mV, 10 ms.

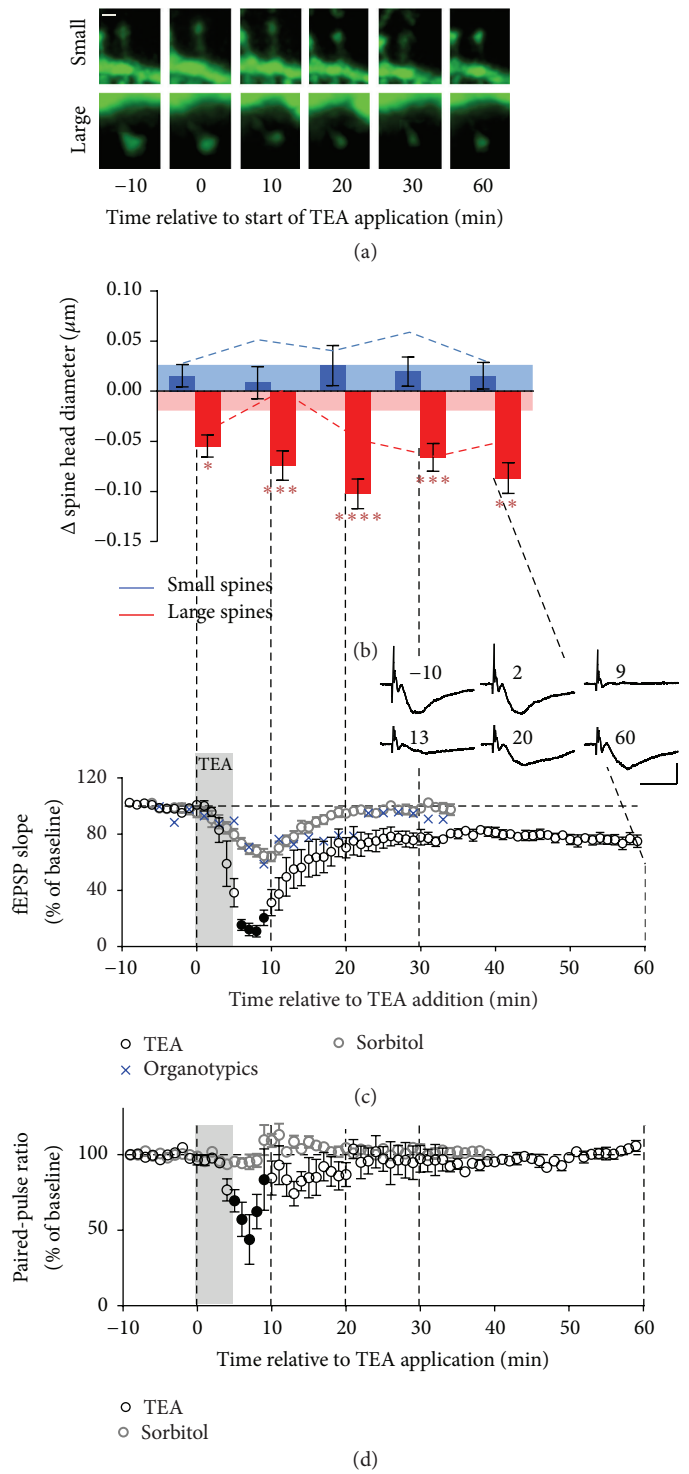

FIGURE 3: Synapses in DG granule cells behave differently from CA1 synapses in response to the same TEA stimulus. (a) Typical example of a small and a large spine imaged before (−10), during (0), and after washout (10–60 min) of TEA (25 mM, 5 min). Scale bar: 0.5 μm. (b) Quantification of changes in spine size. Small spines, blue; large spines, red. Limits of the shading represent the mean change across time in the absence of TEA (time course control experiments). *Post hoc* analysis of control data versus TEA (3-way interaction between size, time, and treatment): $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. ((c), (d)) Slope and PPR of fEPSP recorded in the DG granule cells region in organotypic and acute hippocampal slices in response to stimulation of the perforant path, before and after application of TEA (black), as above, and high osmolarity ACSF (grey), as above. Closed symbols represent points where responses were too small for reliable measurement. Grey shading: TEA perfusion. Error bars: SEM. Inset: averages of fEPSPs recorded from a typical slice over 1 min at 10 s intervals at the time indicated (min). Scale bar: 1 mV, 10 ms.

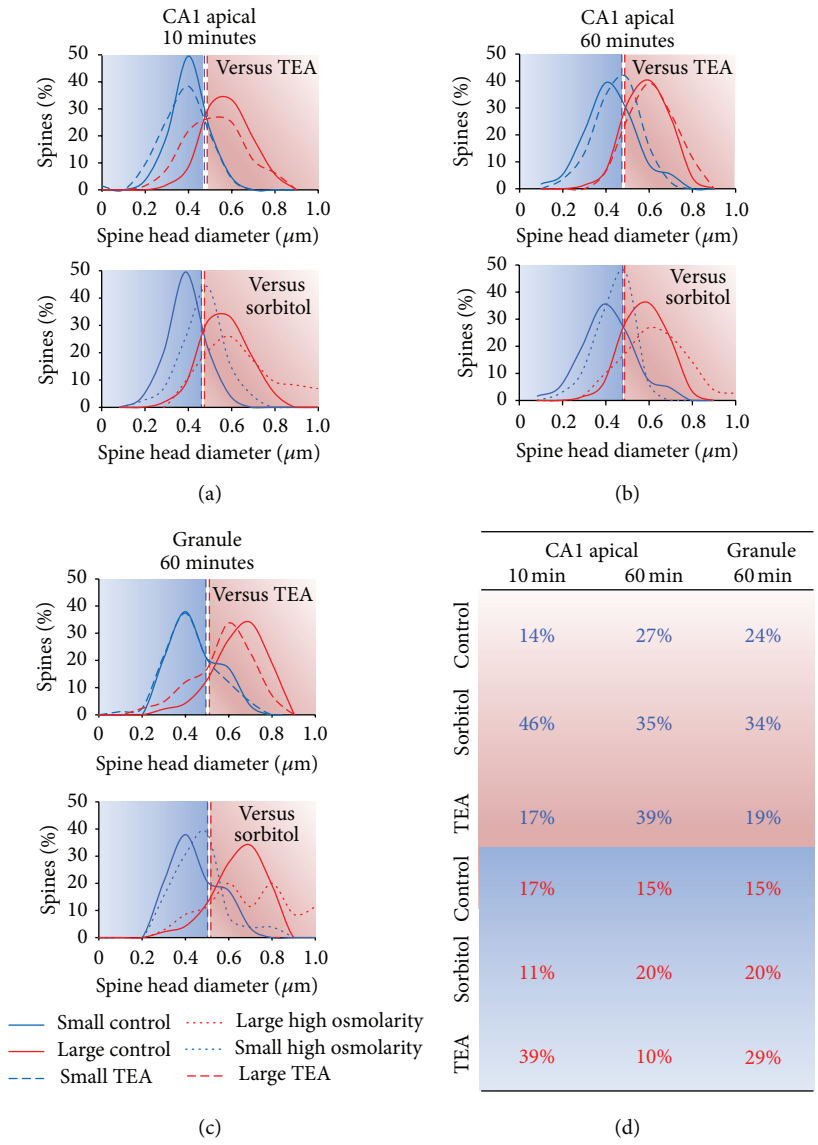

Figure 4: Comparison of small (blue) and large (red) spine head distributions in control versus TEA and control versus sorbitol experiments. ((a), (b)) Distribution of diameters estimated from computed volume measurement of spines in CA1 pyramidal cell apical dendrites (a) 10 and (b) 60 min after application of control (unbroken lines) versus upper panel, TEA (dashed lines), or lower panel, sorbitol (dotted lines), at the same time points. By 60 min, large spines (red) have returned to their original size being no different from control spines, whereas the small spines have grown, indicated by the shift of the distribution (blue) to the right. (c) Dentate granule cell distribution of spine diameters at 60 min. Upper panel: the distribution of small spines (blue) is not greatly affected by TEA (dashed lines) whereas the distribution of large spines (red) shifts to the left showing the persistent decrease in spine head diameter compared to controls (unbroken lines). Lower panel: sorbitol (dotted lines) shows no significant change compared to control (unbroken lines). The blue and red backgrounds represent the diameters defined as small or large, respectively, in the initial category definition at −10 min according to mean diameters (vertical dashed lines). (d) Percentages of spines belonging to each size category (as defined at −10 min) that cross the mean into another category at specified time points after application of TEA or sorbitol. Starting category indicated by font colour (blue text, small; red text, large); final category indicated by background colour (blue, small; red, large). Hence, blue writing on a red background indicates a spine that was initially in the small category but that moved across the threshold to the large category by the time point indicated.

small spines and changes in large spines differed in direction and time course (Figures 2–4). Interestingly, in the presence of TEA, simultaneous changes in the size of large and small spines were never observed. Changes in spine size in the TEA experiments were analysed relative to both control and osmolarity experiments.

3.2.1. CA1 Pyramidal Cells. Changes in spine size were measured in apical dendrites of CA1 pyramidal cells (Figures 2(a) and 2(b), $n = 176$ spines in 7 preparations). Application of TEA immediately caused shrinkage of large spines, reaching a minimum at 10 minutes ($p < 0.01$) but returning to baseline by 30 minutes. Note that, as outlined above, this decrease in

the size of large spines could not be explained by a response to a change in osmolarity because the small change observed when in sorbitol was in the opposite direction to the change caused by TEA ($p < 0.0001$ sorbitol versus TEA, 10 min).

In contrast to the shrinkage of large spines, within the same dendritic segments, small spines showed not only a different direction of change but an entirely different time course. Initially, in the presence of TEA and over the following 20 minutes of washout, TEA caused a similar fluctuation in small spines to control conditions. Hence, addition of TEA opposed the growth of small spines caused by increased osmolarity ($p < 0.0001$, 10 min; $p < 0.05$, 20 min). However, when the effect of osmolarity washed out and the large spines had returned to control levels, small spines began to grow significantly and, by 60 minutes, showed a 4-fold greater increase on average than that shown by small spines either in the control condition ($p < 0.0001$) or in the presence of sorbitol ($p < 0.05$). Effect on the distribution of spine sizes of sorbitol or TEA versus control conditions at 10 and 60 minutes is illustrated in Figure 4.

In order to investigate how changes in spine morphology were related to TEA-induced changes in synaptic strength, fEPSPs were recorded under conditions as close as possible to those of the imaging experiments (Figure 2(c); see Section 2). The addition of TEA to the bath initially resulted in a brief increase in $fEPSP_{slope}$ which was apparently largely presynaptic in origin, as it was paralleled by a drop in PPR, usually indicative of an increase in release probability. This was followed by substantial depression of the measured postsynaptic response with $fEPSP_{slope}$ decreasing over 10 min. This depression can largely be explained by the effect of increasing osmolarity as it is mirrored by the application of sorbitol. A tendency of the presynaptic volley to widen and decrease suggests a loss of excitability of the presynaptic axons; however, considering the apparent increase in release probability, this was unlikely to be the major reason for the depression (Figure 2(c), inset). The decrease in PPR lasted for 30 minutes after TEA application and could be largely attributed to a presynaptic effect of TEA, rather than osmolarity, as this was not seen in sorbitol control experiments. As the effect of osmolarity declined, the size of the postsynaptic response returned towards baseline eventually revealing potentiation of the fEPSP with an ongoing contribution from increased presynaptic release. The PPR returned to baseline level by about 30 minutes at which time the synaptic response settled to a plateau of potentiation ($125 \pm 9\%$, $n = 7$; $p < 0.05$; paired t-test lasts 10 minutes versus baseline) presumably mediated postsynaptically.

It is notable that while the early loss of electrical response could be wholly attributed to the effect of increased osmolarity, the decrease in the size of large spines was entirely TEA dependent as was the change in PPR. Moreover, these two purely TEA-induced effects occur with a similar time course suggesting that the transient shrinkage of large spines could be a short-term homeostatic response to the increased release probability and general spill-over of glutamate resulting from the TEA-induced global stimulation. This would have the protective effect of preventing an excessive postsynaptic response. Note that the decrease in PPR suggests an increase in release probability that would be expected to cause an increase in the postsynaptic response if it were not for opposing postsynaptic factors. Moreover, the final stable potentiation by 30 min after TEA application, once PPR had returned to baseline, was also consistent with consolidation of a postsynaptic change by the delayed growth of small spines.

3.2.2. Dentate Granule Cells. When similar imaging experiments were carried out in DG granule cells in organotypic slices, the pattern of change was different from spines in the apical dendrites of CA1 pyramidal cells (Figure 3, $n = 167$ spines in 10 preparations). TEA had no effect on small spines which behaved similarly across the time course of the experiment, tending to be even more stable than when osmolarity was changed in the absence of TEA ($p < 0.05$, 10 min). In contrast, large spines showed an immediate small but significant decrease in size on application of TEA but, unlike in the CA1 region, the decrease persisted throughout the experiment being statistically significant compared to control at all time points ($p < 0.05$ at 0, peaking at $p < 0.0001$ at 20 min). This result was however difficult to interpret as, with the exception of the 10-minute time point ($p < 0.01$), the effect of TEA on large spines was similar to the effect of sorbitol-induced increased osmolarity.

TEA however clearly affected the small and large spines differently (interaction between treatment and size, $p = 0.01$).

The spine response was again reflected in the field recordings. As suggested by the spine morphology, application of TEA induced LTD in the DG granule cells of organotypic slices (Figure 3(c), $n = 7$). Thus, even after washout and recovery from the extreme depression caused by the presence of TEA, the stable plateau reached by 20–30 min was significantly lower than baseline ($73 \pm 3.4\%$, $n = 9$; $p < 0.0005$). To assess whether the difference in effect of TEA on field response in the dentate gyrus versus the CA1 region was due to the organotypic preparation versus the acute slice, we also compared the effect of TEA in acute slices in dentate gyrus and tetanus-induced LTP in both CA1 and dentate gyrus. In both cases, robust LTP was measured in the CA1 region and no LTP was observed in the dentate gyrus, although responses in acute slices returned to baseline, rather than showing the long-term depression seen in organotypic slices (data not shown). The effects of osmolarity were however similar in both preparations. Hence, in dentate gyrus where TEA failed to cause LTP, the delayed response of small spines was absent whereas large spines still showed a similar homeostatic response to the strong stimulus, albeit not recovering once the stimulus was removed.

4. Discussion

In the present study, application of TEA is used as a tool to stimulate many spines simultaneously in order to investigate how they interact when both protective homeostatic and long-term potentiating responses would be expected; specifically, we aim to tease out whether different types of spines subserve different functions. To this end, we studied spines

both in the CA1 region where TEA causes LTP and in the dentate gyrus where LTP was absent under these conditions.

In earlier studies, spines have frequently been defined into categories such as filopodia, stubby, thin, and mushroom according to head size, head size as a ratio to neck diameter, and length of spine or other criteria [18]. In particular, transitions between thin and mushroom spines have been suggested to play a role in synaptic plasticity (for review, see [19]). These categories have been very useful particularly in relation to electron microscopy studies that allow resolution of the presence of specialized endoplasmic reticulum and other features of the spine (for review, see [20]). However, whether there is a clear distinction or a continuum between spine types is not clear and certainly with confocal microscopy, accurate measurement of spine necks is not possible and the size of spine heads shows a near-Gaussian distribution. Hence, many spines would fall into an area between thin and mushroom relying on subjective judgments for definition. In the present study, we have restricted analysis to spines with clear heads hence excluding stubby spines or filopodia. The division between small and large spines would be roughly equivalent to thin and mushroom spines, respectively, but the clear cut-off at mean diameter allows an entirely objective division that has recently been preferred in light microcopy studies [7]. The analysis here includes all spines that could be well fitted by Imaris. This excludes spines too small for resolution and some very large spines (see Section 2). It is possible that additional changes of interest would be seen if such spines could be included.

It has been previously reported that when stimulation is applied to single spines in the CA1 apical dendrites, using repetitive photolysis of MNI-glutamate, response to glutamate and spine head size increases immediately, independent of the starting size [7]. However, while this growth and the resulting synaptic potentiation can be long-lasting for small spines, it is only transient in large spines. This shows that stimulation of individual synapses affects small and large spines differently but does not clarify the question of different functional entities, as it may reflect a continuum limited by the maximum head size that an individual spine can maintain. Moreover, single spines would rarely be activated in isolation under physiological or indeed pathological conditions and the response to stimulation of a spine may be influenced by the responses of neighboring spines.

Here we report that responses in large and small spines can be functionally differentiated when stimulated simultaneously. Our results in apical dendrites of CA1 pyramidal cells are in agreement with a previous TEA study in CA1 of hippocampal organotypic slices, which also showed that long-term changes are mostly related to small spines [9] as is also true for individual spine stimulation [7]. However, Hosokawa and colleagues [9] only investigated effects 2 hours after TEA application and so the shrinkage of large spines and stability of small spines observed here, in the presence and during washout of TEA, would have been missed. We suggest that the most likely reason for the immediate shrinkage, which reverses during TEA washout, is a homeostatic response to overstimulation. Although increased osmolarity has been used to cause glutamate release in single boutons in culture [21, 22], these studies used 6–10-fold the osmolarity used in the present study and demonstrated that neighbouring spines, which would have been exposed to lower level osmolarity changes, were not affected. The suggestion that changing osmolarity by 50 mOsm is unlikely to have changed glutamate release is supported by the stability of the PPR when sorbitol was added in the osmolarity controls for the present study. In another study, using electron microscopy, Stewart and coworkers were unable to detect changes in spine volume one hour after TEA washout [23]. In this study, the stimulus was more extreme (25 mM TEA applied for 20 min in the presence of 10 mM Ca^{2+} and 5 mM K^{+} and in the absence of Mg^{2+}) and again, only a single time point was observed. Clearly under such conditions recovery from stimulation would be likely to occur later and so, even if similar effects occurred to those observed in the present study, it is possible that the point of sampling happened to coincide with the time at which large spines recovered and small spines had not yet started to grow.

The delay observed here, before small spines grow, is also different from the immediate growth reported when a small spine is stimulated individually [7]. This suggests that the delay is the result of interaction between spines when they are simultaneously stimulated. The delay is particularly remarkable in the light of increased osmolarity apparently causing a transient increase in the size of small spines in the absence of changes in large spines, suggesting that either the TEA-induced change in large spines or other effects of TEA actively prevent this change. Possibly the head size of small spines would also be seen to decrease similarly to the effect seen in large spines if the osmolarity could be kept constant.

Considering the depressive effect of osmolarity on synaptic transmission in both CA1 and dentate gyrus, it is interesting that this is not reflected in spine size. This is an example where the generally close link between spine size and synaptic response becomes dissociated. One possibility is that the change in osmolarity alters the geometry of the synapse such that pre- and postsynaptic sides temporarily lose their close apposition. This would mean that, even with increased release suggested by the changed PPR in the presence of TEA, the response would remain depressed.

The application of TEA causes many effects including broadening of the action potential, which likely influences our electrophysiological measurement of synaptic response, so that the relative contributions of pre- and postsynaptic factors to the depression of the electrophysiological responses in the presence of TEA are hard to assess. However, the TEA-dependent decrease in PPR in both regions over the first 20 minutes after TEA application would be expected to correlate with an increase in the response. Hence, the depression associated with the decrease in the size of large spines is likely to be underestimated. The field recordings do serve to indicate a time course of the maximal acute effects of TEA however and the substantial shrinkage of the large spines strongly suggests a postsynaptic component in organotypic slices under these conditions. It is perhaps surprising that the increased osmolarity does not contribute to this shrinkage, although it clearly is a factor in the transient synaptic depression. Indeed, shrinkage would be the expected

effect of osmolarity as H_2O moves towards the hyperosmotic compartment; however, dendritic spines appear to be able to resist any osmolarity-induced shrinkage within these limits.

The electrophysiological responses confirm the difference in the effect of TEA on CA1 pyramidal cells and DG granule cells. It is interesting to note that DG granule cells show a similar immediate response to TEA but the depression is greater and shows a very different long-term response both electrically and in the changes seen in spine morphology. The question arises whether the initial extreme depression is irreversible in some large spines in the dentate gyrus possibly reflecting lack of recovery of function in a subset of synapses rather than a true long-term depression across the population. The present observations that conditions causing LTP in the CA1 region do not cause potentiation in the dentate gyrus are in agreement with previous electrophysiological studies in acute rat hippocampal slices [10]. Note that if stimulated separately, the synapses of the medial and lateral perforant path have different characteristics in relation to short-term plasticity but behave similarly in terms of tetanus-induced LTP when recorded in the absence of $GABA_A$ receptor antagonists [24]. Moreover, in organotypic slices, these pathways are likely to be less clearly defined and use of chemical LTP will stimulate all pathways equally. Hence, while both pathways were stimulated, the results were pooled in this study.

5. Conclusions

In conclusion, we suggest that, in the CA1 region, a subset of spines has specific functions that do not represent a continuum across the spectrum of spine morphologies. In both CA1 and DG, we propose that it is large spines that are important for immediate short-term homeostatic protection while, at least in the CA1 region, the delayed growth of small spines follows the increase in synaptic response, stabilizing the alteration in AMPA receptors that may underlie learning and memory. Moreover, throughout this study in both CA1 and DG, small and large spines never change simultaneously. Occurrence of LTD and LTP depend strongly on the Ca^{2+} dynamics in individual spines and have previously been reported to be mutually inhibitory via the phosphorylation and dephosphorylation of glycogen synthase kinase-3 [25, 26]. Such a mechanism may be involved in the interactions reported here. Moreover, under normal physiological stimuli onto individual spines, Ca^{2+} transients are large and rapid but restricted to the spine [27], whereas, under a strong stimulus such as that used here, the diffusion of Ca^{2+} between large and small spines may contribute to communication between spines of different sizes [28].

The network-wide stimulation used in this study could be compared to the pathological effects of ischemia or epilepsy rather than the more subtle stimuli required for the specific laying down of memory. These observations could thus be important in the well-established interactions that occur between such pathological processes and memory [29]. Moreover, the separation of these effects could have important implications in relation to the link between acute pathological insults and eventual long-term effects in Alzheimer's disease or other neurodegenerative conditions. We suggest that the strong synaptic depression mediated by shrinkage of large spines during and immediately after the application of TEA and the resulting delay in small spine growth combine to protect the neurone by decreasing the influx of Ca^{2+} and the damage that this could cause, short- and long-term, if uncontrolled (see [30] for review of implications in neurodegeneration). Indeed some of the earliest changes suggested to occur in Alzheimer's disease are related to Ca^{2+} homeostasis [31]. Large spines are known to be anatomically different from small spines, containing considerably more smooth endoplasmic reticulum, often associated with a spine apparatus that is not present in small spines [32]. Although the reason for these differences is not clear, such specialized organelles could be essential if large spines serve specific functions in relation to protecting the neurone from excessive Ca^{2+} influx in pathological situations. It may therefore be possible to target the failure of such specialized spines selectively without changing the memory supporting functions of the small spines.

Disclosure

Peter Haslehurst's present address is Department of Pharmacology, Oxford University, and Joshua D. Jackson's present address is Manchester Pharmacy School, University of Manchester.

Conflict of Interests

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

Acknowledgments

The authors thank Rivka Steinberg, Rima Hussein, and Sam Ranasinghe for technical and microscopy assistance. They also thank Dervis Salih for making and teaching others to make organotypic slices. They thank Lion Shahab for expert assistance with the statistics. And they also thank Zahid Padamsey, Nigel Emptage, and Dervis Salih for helpful comments on the paper. Peter Haslehurst and Zelah Joel were supported by Case Studentship from the Biotechnology and Biological Sciences Research Council UK and Engineering and Physical Sciences Research Council UK, respectively, and GlaxoSmithKline. Damian Cummings was supported by the Medical Research Council UK.

References

[1] M. Matsuzaki, G. C. R. Ellis-Davies, T. Nemoto, Y. Miyashita, M. Iino, and H. Kasai, "Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons," *Nature Neuroscience*, vol. 4, no. 11, pp. 1086–1092, 2001.

[2] M. J. Hasbani, M. L. Schlief, D. A. Fisher, and M. P. Goldberg, "Dendritic spines lost during glutamate receptor activation reemerge at original sites of synaptic contact," *Journal of Neuroscience*, vol. 21, no. 7, pp. 2393–2403, 2001.

[3] S. L. Parsley, S. M. Pilgram, F. Soto, K. P. Giese, and F. A. Edwards, "Enriching the environment of αCaMKIIT286A mutant mice reveals that LTD occurs in memory processing but must be subsequently reversed by LTP," *Learning and Memory*, vol. 14, no. 1, pp. 75–83, 2007.

[4] L. R. Squire, "Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans," *Psychological Review*, vol. 99, no. 2, pp. 195–231, 1992.

[5] K. M. Harris, J. C. Fiala, and L. Ostroff, "Structural changes at dendritic spine synapses during long-term potentiation," *Philosophical Transactions of the Royal Society of London B*, vol. 358, pp. 745–748, 2003.

[6] N. Honkura, M. Matsuzaki, J. Noguchi, G. C. R. Ellis-Davies, and H. Kasai, "The subspine organization of actin fibers regulates the structure and plasticity of dendritic spines," *Neuron*, vol. 57, no. 5, pp. 719–729, 2008.

[7] M. Matsuzaki, N. Honkura, G. C. R. Ellis-Davies, and H. Kasai, "Structural basis of long-term potentiation in single dendritic spines," *Nature*, vol. 429, no. 6993, pp. 761–766, 2004.

[8] L. Anikstejn and Y. Ben-Ari, "NMDA-independent form of long-term potentiation produced by tetraethylammonium in the hippocampal CA1 region," *European Journal of Pharmacology*, vol. 181, no. 1-2, pp. 157–158, 1990.

[9] T. Hosokawa, D. A. Rusakov, T. V. P. Bliss, and A. Fine, "Repeated confocal imaging of individual dendritic spines in the living hippocampal slice: evidence for changes in length and orientation associated with chemically induced LTP," *The Journal of Neuroscience*, vol. 15, no. 8, pp. 5560–5573, 1995.

[10] D. Song, X. Xie, Z. Wang, and T. W. Berger, "Differential effect of TEA on long-term synaptic modification in hippocampal CA1 and dentate gyrus in vitro," *Neurobiology of Learning and Memory*, vol. 76, no. 3, pp. 375–387, 2001.

[11] L. Stoppini, P.-A. Buchs, and D. Muller, "A simple method for organotypic cultures of nervous tissue," *Journal of Neuroscience Methods*, vol. 37, no. 2, pp. 173–182, 1991.

[12] G. Feng, R. H. Mellor, M. Bernstein et al., "Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP," *Neuron*, vol. 28, no. 1, pp. 41–51, 2000.

[13] F. A. Edwards, A. Konnerth, B. Sakmann, and T. Takahashi, "A thin slice preparation for patch clamp recordings from neurones of the mammalian central nervous system," *Pflügers Archiv European Journal of Physiology*, vol. 414, no. 5, pp. 600–612, 1989.

[14] D. M. Cummings, W. Liu, E. Portelius et al., "First effects of rising amyloid-beta in transgenic mouse brain: synaptic transmission and gene expression," *Brain*, vol. 138, no. 7, pp. 1992–2004, 2015.

[15] A. De Simoni, C. B. Griesinger, and F. A. Edwards, "Development of rat CA1 neurones in acute versus organotypic slices: role of experience in synaptic morphology and activity," *Journal of Physiology*, vol. 550, no. 1, pp. 135–147, 2003.

[16] A. De Simoni and F. A. Edwards, "Pathway specificity of dendritic spine morphology in identified synapses onto rat hippocampal CA1 neurons in oranotypic slices," *Hippocampus*, vol. 16, no. 12, pp. 1111–1124, 2006.

[17] N. Yasumatsu, M. Matsuzaki, T. Miyazaki, J. Noguchi, and H. Kasai, "Principles of long-term dynamics of dendritic spines," *The Journal of Neuroscience*, vol. 28, no. 50, pp. 13592–13608, 2008.

[18] K. M. Harris and J. K. Stevens, "Dendritic spines of CA1 pyramidal cells in the rat hippocampus: serial electron microscopy with reference to their biophysical characteristics," *Journal of Neuroscience*, vol. 9, no. 8, pp. 2982–2997, 1989.

[19] F. A. Edwards, "Anatomy and electrophysiology of fast central synapses lead to a structural model for long-term potentiation," *Physiological Reviews*, vol. 75, no. 4, pp. 759–787, 1995.

[20] J. N. Bourne and K. M. Harris, "Balancing structure and function at hippocampal dendritic spines," *Annual Review of Neuroscience*, vol. 31, pp. 47–67, 2008.

[21] C. Collin, C. Vicario-Abejon, M. E. Rubio, R. J. Wenthold, R. D. G. McKay, and M. Segal, "Neurotrophins act at presynaptic terminals to activate synapses among cultured hippocampal neurons," *European Journal of Neuroscience*, vol. 13, no. 7, pp. 1273–1282, 2001.

[22] Y. Sara, M. G. Mozhayeva, X. R. Liu, and E. T. Kavalali, "Fast vesicle recycling supports neurotransmission during sustained stimulation at hippocampal synapses," *Journal of Neuroscience*, vol. 22, no. 5, pp. 1608–1617, 2002.

[23] M. G. Stewart, N. I. Medvedev, V. I. Popov et al., "Chemically induced long-term potentiation increases the number of perforated and complex postsynaptic densities but does not alter dendritic spine volume in CA1 of adult mouse hippocampal slices," *European Journal of Neuroscience*, vol. 21, no. 12, pp. 3368–3378, 2005.

[24] E. Hanse and B. Gustafsson, "Long-term potentiation and field EPSPs in the lateral and medial perforant paths in the dentate gyrus in vitro: a comparison," *European Journal of Neuroscience*, vol. 4, no. 11, pp. 1191–1201, 1992.

[25] S. Peineau, C. Taghibiglou, C. Bradley et al., "LTP inhibits LTD in the hippocampus via regulation of GSK3beta," *Neuron*, vol. 53, no. 5, pp. 703–717, 2007.

[26] L. Shahab, F. Plattner, E. E. Irvine, D. M. Cummings, and F. A. Edwards, "Dynamic range of GSK3α not GSK3β is essential for bidirectional synaptic plasticity at hippocampal CA3-CA1 synapses," *Hippocampus*, vol. 24, no. 12, pp. 1413–1416, 2014.

[27] B. L. Sabatini, T. G. Oertner, and K. Svoboda, "The life cycle of Ca^{2+} ions in dendritic spines," *Neuron*, vol. 33, no. 3, pp. 439–452, 2002.

[28] J. Noguchi, M. Matsuzaki, G. C. R. Ellis-Davies, and H. Kasai, "Spine-neck geometry determines NMDA receptor-dependent Ca^{2+} signaling in dendrites," *Neuron*, vol. 46, no. 4, pp. 609–622, 2005.

[29] J. Chin and H. E. Scharfman, "Shared cognitive and behavioral impairments in epilepsy and Alzheimer's disease and potential underlying mechanisms," *Epilepsy and Behavior*, vol. 26, no. 3, pp. 343–351, 2013.

[30] M. J. Berridge, "Calcium hypothesis of Alzheimer's disease," *Pflugers Archiv European Journal of Physiology*, vol. 459, no. 3, pp. 441–449, 2010.

[31] S. Chakroborty, J. Kim, C. Schneider, C. Jacobson, J. Molgó, and G. E. Stutzmann, "Early presynaptic and postsynaptic calcium signaling abnormalities mask underlying synaptic depression in presymptomatic Alzheimer's disease mice," *The Journal of Neuroscience*, vol. 32, no. 24, pp. 8341–8353, 2012.

[32] J. Spacek and K. M. Harris, "Three-dimensional organization of smooth endoplasmic reticulum in hippocampal CA1 dendrites and dendritic spines of the immature and mature rat," *Journal of Neuroscience*, vol. 17, no. 1, pp. 190–203, 1997.

Hindawi Publishing Corporation
Neural Plasticity
Volume 2016, Article ID 3423267, 31 pages
http://dx.doi.org/10.1155/2016/3423267

Review Article

Emerging Roles of Filopodia and Dendritic Spines in Motoneuron Plasticity during Development and Disease

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Received 19 June 2015; Revised 10 September 2015; Accepted 21 September 2015

Academic Editor: Deepak P. Srivastava

Motoneurons develop extensive dendritic trees for receiving excitatory and inhibitory synaptic inputs to perform a variety of complex motor tasks. At birth, the somatodendritic domains of mouse hypoglossal and lumbar motoneurons have dense filopodia and spines. Consistent with Vaughn's synaptotropic hypothesis, we propose a developmental unified-hybrid model implicating filopodia in motoneuron spinogenesis/synaptogenesis and dendritic growth and branching critical for circuit formation and synaptic plasticity at embryonic/prenatal/neonatal period. Filopodia density decreases and spine density initially increases until postnatal day 15 (P15) and then decreases by P30. Spine distribution shifts towards the distal dendrites, and spines become shorter (stubby), coinciding with decreases in frequency and increases in amplitude of excitatory postsynaptic currents with maturation. In transgenic mice, either overexpressing the mutated human Cu/Zn-superoxide dismutase ($hSOD1^{G93A}$) gene or deficient in GABAergic/glycinergic synaptic transmission (gephyrin, GAD-67, or VGAT gene knockout), hypoglossal motoneurons develop excitatory glutamatergic synaptic hyperactivity. Functional synaptic hyperactivity is associated with increased dendritic growth, branching, and increased spine and filopodia density, involving actin-based cytoskeletal and structural remodelling. Energy-dependent ionic pumps that maintain intracellular sodium/calcium homeostasis are chronically challenged by activity and selectively overwhelmed by hyperactivity which eventually causes sustained membrane depolarization leading to excitotoxicity, activating microglia to phagocytose degenerating neurons under neuropathological conditions.

1. Introduction

It has been well over 100 years since spines on dendrites of cerebellar Purkinje cells of hen were first discovered by Ramon y Cajal in 1888 [1, 2]. Subsequently he identified dendritic spines and filopodia in other neurons including cortical and hippocampal pyramidal cells, cerebellar basket and Golgi cells, and spinal motoneurons from various species including humans, cats, dogs, birds, and rabbits at different developmental stages from embryonic to adulthood [3], using the Golgi method [4]. Cajal noted that spine density was higher in early postnatal development than at later stages. He made the first observation of spine plasticity in pyramidal neurons and proposed that the spines might help to increase and modify synaptic connections. Dendritic spines have fascinated scientists ever since and were assumed to underlie the physical substrate of long-term memory in the brain, after the first electron microscopic analysis of spines in cortical neurons [5]. Despite more than a century of research, a definitive role for dendritic spines remains elusive: a recently emerging view is that they are strategically positioned postsynaptic cellular compartments likely to play key roles in neuronal functions such as information processing and plasticity under normal and neuropathological conditions [6–22].

Dendritic spines are protrusions from the dendritic shaft of neurons (Figure 1) that comprise the receptive postsynaptic compartment at most excitatory synapses in the brain [5, 6, 9, 23]. Time-lapse imaging of dendritic spines in hippocampal slices has revealed an amazingly plastic structure that undergoes continuous changes in shape and size, which are not intuitively related to its assumed role in long-term memory and neuroplasticity [7, 10]. The spine can dynamically form, change its shape, and disappear in response to afferent stimulation, indicating that spine morphology and density are an important vehicle for structuring synaptic interactions and plasticity [7, 9, 10, 12, 17, 20, 22, 24]. Functionally, the spine has

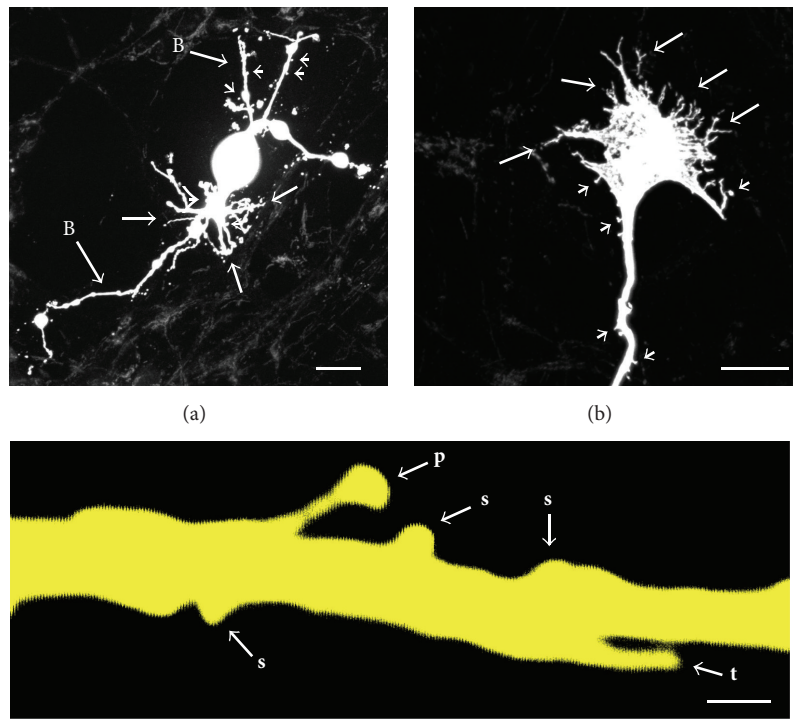

Figure 1: (a and b) show formation of filopodia and spine-like processes (protospines) on neuritis and dendritic growth cones from developing (immature) human cortical neuron-like cells derived from induced pluripotent stem (iPS) cells in culture without synaptic inputs. These cells were dye-filled from their soma with a less invasive semiloose seal Neurobiotin electroporation method, and Neurobiotin was visualized by incubating cells in Streptavidin Cy3 (for methods see Kanjhan and Vaney, 2008 [25]). (a) shows the formation of neurite branches ("B-arrows"), filopodia (long arrows), and spine-like processes or protospines (short arrows) protruding from the soma and neurites in a developing immature neuron-like cell. (b) shows a high-magnification image of a dendritic growth cone with filopodia (thin long processes) and spine-like processes (shorter protrusions; short arrows) protruding from its circumference, in the absence of any synaptic inputs. The same cell had a much longer axonal growth cone extending from soma in opposite direction (not shown). (c) shows the types of spines found in the dendrites of a hypoglossal motoneuron from a 15-postnatal-day-old wild-type C57/Bl6 mouse. In this panel, all types of spines previously reported in other neuronal types are evident. These include (p) pedunculated spines that are thin and longer with prominent necks and heads resembling mushrooms; (s) sessile spines that show stubby or short lacking clear necks; (t) thin spines that are longer filopodia-like spines and lack clear necks and mushroom-like heads. All images were taken with a 100x oil objective (NA 1.35) using 2.5x (b) and 10x (c) optical zoom using an Olympus BX61 (Olympus Fluoview ver. 1.7c) microscope. Each micrograph is a confocal image stack of $10 \times 0.35\,\mu$m (a and b) and $3 \times 0.35\,\mu$m (c) thick optical sections. Scale bar = $10\,\mu$m in (a-b) and $1\,\mu$m in (c).

been shown to be an independent cellular compartment, able to regulate calcium concentration independently of its parent dendrite [8]. While this role is crucial in the developing nervous system, large variations in spine shape and density in the adult brain and under neuropathological conditions indicate that tuning of synaptic inputs and plasticity may be a role of spines throughout the life of a neuron [9, 11–13, 17, 19, 20, 22].

Most of our knowledge today about dendritic spines and filopodia has primarily come from studies on cortical, hippocampal, and cerebellar neurons. Our recent research suggests significant roles for dendritic spines and filopodia in motoneuron function and plasticity, particularly during embryonic-postnatal development and under neuropathological conditions. In the following sections, we will review previous studies of filopodia and spines in motoneurons in the light of these recent advances.

2. Morphological Studies of Filopodia and Spines on Motoneurons: A Historical Perspective

Motoneurons located in the lower brainstem (i.e., cranial motor nuclei III to XII) and through the entire length of the spinal cord play vital roles in the control of motor functions such as respiration, posture, and locomotion. Humans possess more than 500 different skeletal muscles, capable of contracting in a precise temporal and spatial coordination to execute many refined complex motor functions. Motoneurons have developed elaborate dendritic structures to meet these highly complex demands. Somatic and dendritic motoneuron morphology was first revealed in the drawings of Ramon y Cajal using the Golgi method [1, 2]. Growth cones, filopodia, and spines can be seen on dendrites of embryonic chicken and adult cat spinal motoneurons [1–3].

Subsequently a number of studies, primarily using the Golgi method, described the presence of filopodia and spines on motoneurons [28–31]. In their early studies in the adult cat and monkey, M. E. Scheibel and A. B. Scheibel described that the majority of motoneuron dendrites were spine-bearing, but their distribution was not nearly as regular as in cortical neurons [28]. Scheibel and colleagues however in their following studies described spine-like processes as protospines (filopodia-like long thin immature spines with no obvious necks) on the soma and dendrites (proximal and distal), and they argued that these or primitive polymorphic spines were a feature of perinatal motoneuron dendrites, present only during early development with a peak around postnatal day 11 (P11) and then declining in numbers due to resorption onto the dendritic surface or shafts, almost entirely disappearing with formation of dendritic bundles [29, 30, 32]. They suggested that repository spinal programs involved in controlling rhythmic behaviors such as respiration and locomotion were originally loaded via an archaic system of presynaptic fibers terminating on the polymorphic protospines covering most of the dendrites and soma during the prenatal phase and were subsequently lost with rearrangement of dendrites in tightly packed bundles around the fascicles of myelinated axons. The programs were then conceived to function autonomously, as loss of spines coincided with development of bundles, throughout the organism's existence, subject to modification and override by newer systems [29, 30, 32]. These conclusions of M. E. Scheibel and A. B. Scheibel resulted in a loss of interest in investigation of dendritic spines on motoneurons by many laboratories around the world. A significant number of subsequent studies, some of which are mentioned above, using various methods, including Golgi, retrograde tracing, calcium imaging, and dye-filling, did not report or discuss filopodia or spine presence on motoneurons in the adult or during development [33–37]. This will be discussed in detail later in this section (see below).

Despite this setback emanating from M. E. Scheibel and A. B. Scheibel's conclusions, at least some Golgi studies consistently reported the presence of spines or filopodia on motoneurons from various species studied. In the rat, subtle differences were noted in the descriptions derived from neonatal and young adult Golgi preparations [38]. For example, in most of the neonatal materials, profuse spine-like excrescences, the heteromorphic protospines were noted on the somatodendritic domains of motoneurons. At P10- to P14-day-old juvenile rats, the protospines became fewer and were comparable numerically to the spines counted on motoneurons of much older preparations (35 to 65 days postnatally) [38]. Interestingly, this loss of protospines coincides with the refinement of motor neuron circuit, namely, the loss of polyneuronal innervation of muscle [39–41]. Following this developmental period, occasional sessile (stubby and short spines lacking clear necks) and rare pedunculated (thin and longer spines with prominent necks and heads resembling mushrooms) spines and appendages on the soma and dendrites of hypoglossal motor neurons from adult primates have been reported [42].

Development of electron microscopy techniques in the 1950s revealed morphological and synaptic properties of spines at ultrastructural level [5]. In the 1970s and 1980s Vaughn put forward his synaptotropic hypothesis on spine and synapse formation based on his ultrastructural and Golgi studies on embryonic and newborn mouse spinal cord motoneurons, where he reported synaptic contacts occurring on motoneurons as early as embryonic day 11 (E11) [43–46]. Vaughn's synaptotropic hypothesis postulates that dendritic filopodia, arising from growth cones capture axons, establish synaptic contacts and then gradually turn into spines or dendritic shafts and that filopodia also produce motoneuron dendritic branches [3, 12, 45–47]. This filopodial model of spine, synapse, and dendrite formation will be discussed in detail below in Section 4. Other scientists have also studied synaptic density and contacts at ultrastructural level in various motoneuron pools in adult animals, including rat phrenic [48] and hypoglossal [49] and cat lumbosacral motoneurons [50].

In the 1980s, development of neuroanatomical and retrograde tracing of motoneurons from their target muscles with wheat germ agglutinin (WGA) or cholera toxin- (CT-) conjugated horseradish peroxidase (HRP) and biocytin and fluorescent tracers (e.g., Fluorogold) provided a new tool targeting of specific motoneuron populations or pools in different species [31, 51–55]. However, most of these studies like those indicated before barely mentioned dendritic spines or filopodia in motoneurons.

Another major development in the study of motoneurons started in the early 1950s, with intracellular microelectrode recordings from spinal motoneurons allowing a detailed analysis of their electrophysiological properties [56]. Use of sharp electrodes also allowed the development of dye-filling methods of individual motoneurons in the 1960s [57]. The morphology of functionally characterized different types of mammalian spinal and brainstem motoneurons started to emerge from this type of *in vivo* research [58, 59]. Subsequent studies, using dye-filling with HRP, fluorescent dyes (e.g., Lucifer Yellow), and biocytin or Neurobiotin revealed dendritic morphology of functionally characterized motoneurons primarily from the *in vivo* adult cat studies [60–65], followed by studies in the adult rat *in vivo* [66–69] and in developing rat brainstem slices [70, 71].

Dye-filling studies of individual motoneurons reported occasional sessile and rare pedunculated spines and appendages on the soma and dendrites of motoneurons from adult animals including cat hypoglossal [65], phrenic [72], and hind limb [62] motoneurons and rat phrenic motoneurons [31]. A developmental study identified frequent growth cones, filopodia, and lamellipodial and fusiform processes in kitten at birth, most processes disappearing at P45 [62]. Therefore, one possibility is that, in older animals, the spines or spine-like processes (e.g., filopodia or appendages) are significantly reduced compared to newborns; however, another study reported a few spines in developing cat phrenic motoneurons [73], with a similar spine density to that of adult cat phrenic motoneurons [72]. By contrast, another dye-filling study in developing rat (P1 to P30) hypoglossal motoneurons, despite performing detailed morphometric analysis, did not mention anything about dendritic spines or filopodia [70, 71].

In the past 15 years mice have increasingly become the species of choice for studying mammalian motoneurons, primarily due to the availability of transgenic mice. In mice, most of the available information about motoneuron dendritic structure comes from studies that used retrograde tracers and immunochemical or Golgi staining to visualize and draw projections of motoneuron dendrites in single sections without full reconstruction [74–78]. The quantification of motoneuron dendritic spines by Golgi or HRP staining combined with light microscopy is limited due to dense and dark reaction products, as significant number of spines under or above the dendrites are likely to be unaccounted for [79]. Also with these dark reaction products, it would be hard to detect some of the very short and stubby spines (0.1 to 0.4 μm long), as shown in Figure 1(c) at high magnification. Although some of these difficulties have been overcome recently by using fluorescent molecules such as Fluorogold in retrograde labeling studies [80] or using transgenic mice with fluorescently (e.g., Green Fluorescent Protein (GFP) or Yellow Fluorescent Protein (YFP)) expressing motoneurons [81], there are still potential limitations with nonspecific and partial uptake of labels not reaching intensities sufficient to reveal fine structural detail such as dendritic spines in distal dendrites (see below) [55]. Only a handful of studies have carried out dye-filling studies of individual motoneurons in mice using *in vitro* hemisected lumbar spinal cords from P3 to P13 mice [82] and in brainstem spinal cord preparations from P3 to P9 mice [83, 84]. Dendritic elongation and branching abnormalities of lumbar motoneurons were reported in mutated human Cu/Zn-superoxide dismutase ($hSOD1^{G85R}$) overexpressing mice compared to wild-type mice at P3–P9 [83, 84]. It is again surprising that there was no mention of spines and filopodia on neonatal mice lumbar motoneurons dye-filled with biocytin or Neurobiotin [82–84] or Ca^{2+} Green-1 and Texas Red dextrans [37]. Of these earlier studies in the mice, only one study reported and quantified dendritic spines in cervical motoneurons using a rapid Golgi method [74]. This study reported significant reduction in dendritic length, branching, and spine density in presumed alpha motoneurons from the 2–6-month-old *Wobbler* mouse (a model of human infantile spinal muscular atrophy) compared to wild-type mice [74].

Recently, we have developed a highly sensitive and less invasive (i.e., minimal cellular damage) dye electroporation method to fill individual cells using Neurobiotin, a small molecule (molecular weight (MW) = 323) with the advantage of comprehensive intracellular distribution (Figures 1, 2, and 3) [25, 26, 85]. We have been using voltage pulses to electroporate the membrane in giga-seal or semiloose seal modes, instead of direct and variable suction that can damage the plasma membrane and intracellular organelles [25, 26, 85]. Combining our less-invasive and sensitive Neurobiotin electroporation method with the high magnification (100x objective with 2.5x to 10x optical zoom) laser confocal and super resolution microscopy has allowed detection and quantification of significant numbers of dendritic spines and filopodia in developing hypoglossal motoneurons from mouse brainstem slices (Figures 1(c) and 3) [26, 27]. Using this method we have recently studied somatodendritic morphology of over 100 hypoglossal motoneurons in developing mice from E17 to P28 [86]. Our most recent studies on 300–500 μm transverse spinal cord slice preparations from newborn mice also indicate the abundant presence of filopodia and spines on somatodendritic domains of dye-filled lumbar motoneurons (Figures 2(a)–2(c)) that received both excitatory glutamatergic NMDA (*N*-methyl-D-aspartic acid) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and inhibitory gamma-aminobutyric acid (GABA) and glycinergic synaptic currents, based on their activation/inactivation kinetics (Figures 2(d)-2(e)). In motoneurons we find all types of spines previously reported in other neuronal types [6, 12, 23], including pedunculated (thin and longer spines with prominent necks and heads resembling mushrooms), sessile (stubby or short spines lacking clear necks), and thin and longer filopodia-like spines lacking clear necks (Figure 1(c)). We have also occasionally seen branched spines with Y-shaped tips in dendrites of developing motoneurons (see Figure 6(c), asterisk). General understanding is that mushroom (pedunculated) and stubby (sessile) spines represent more mature and stable spines, while thin and branched spines tend to be more plastic and immature [6, 12, 23]. In the following sections, we will show examples and argue that the filopodia and spine-like structures form the structural bases of motoneuron plasticity during development and under neuropathological conditions.

Reasons for lack of detection or reporting of spines and filopodia on motoneuronal somatodendritic domain by many previous morphological studies are not clear. We believe there may be many contributing factors such as significant neuronal injury and tissue damage during recording or dye-filling, sensitivity of detection method and dye used, and sufficient magnification (minimum 100x objective with 2.5x optical zoom) to detect short and stubby spines (0.1 to 0.4 μm long; Figure 1(c)) that are the most common type in the adult. We noted that most previous morphological studies mentioned above, especially prior to use of confocal microscopy, did not image cells at high enough magnification to identify spines and filopodia on motoneurons. From our experience, any major physical damage to the cellular soma or dendrites during whole-cell patch-clamping, electrode pull-out, prolonged recordings with patch or sharp (high impedance intracellular recordings *in vivo*) electrodes under unstable conditions (i.e., movement), or using inappropriate pipette solutions (e.g., osmolarity, ionic composition, and pH), can result in membrane damage and subsequent swelling, vacuolization, blebbing, or beading of cellular membranes or compartments [25, 26, 85]. Under such conditions, either filopodia and spines will become undetectable due to retraction or they will simply integrate with swellings, vacuoles, blebs, or beads and disguise upon losing their structural support. Indeed when we closely look at the most of the previous morphological studies (see above), especially in *in vivo* animal preparations with intracellular filling including our own work [68, 69], beading and blebbing are common in somatodendritic domains of motoneurons. Dendritic spines can also structurally restrict access of dyes, especially larger molecules such as HRP (MW ~ 44 kilo Dalton (kDa))

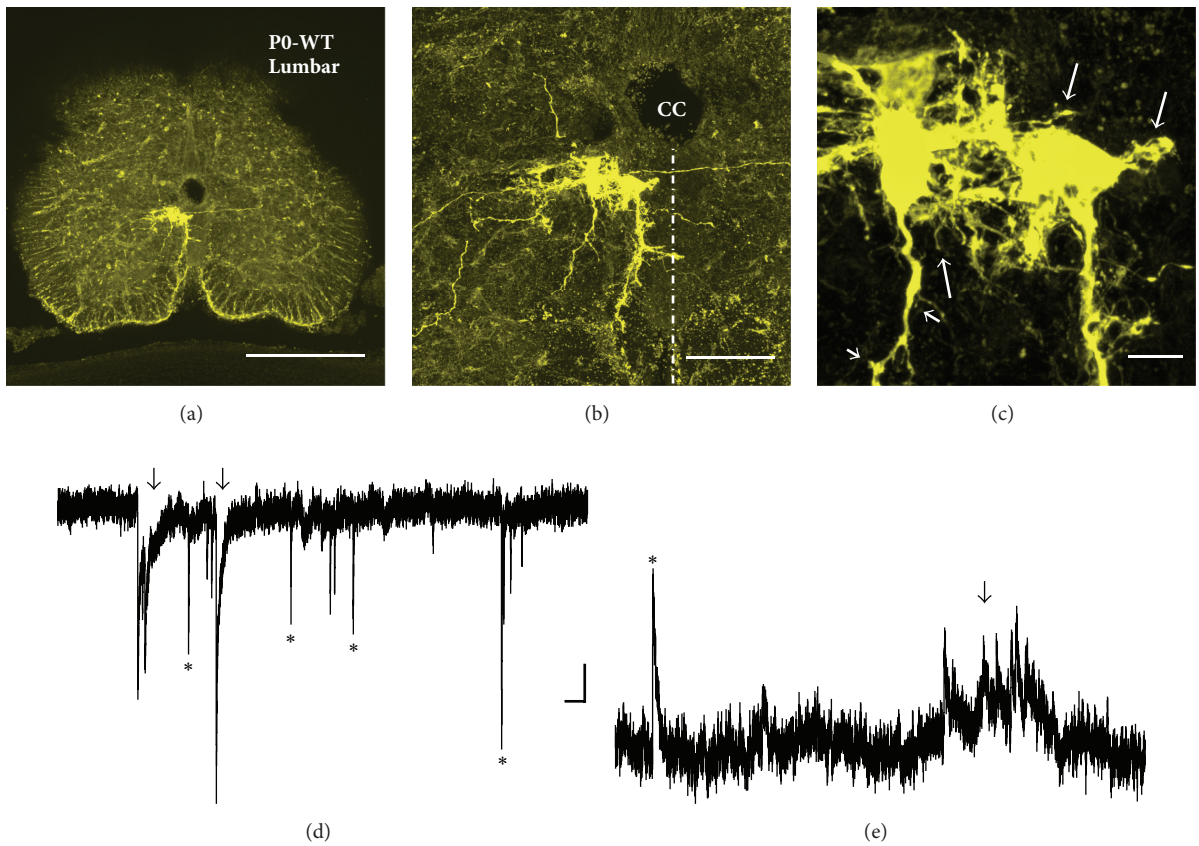

Figure 2: The somatodendritic and synaptic properties of lumbar motoneurons from a newborn wild-type C57/Bl6 (P0-WT) mouse. (a) Low-power image showing a lumbar spinal cord slice in transverse plane and the location of dye-filled motoneurons (yellow cells). (b) Medium-power confocal image showing two dye-coupled motoneurons located ventrolateral to the central canal (CC), with commissural dendrites crossing the midline (dashed line) to the contralateral side of the spinal cord. (c) High-power confocal image of these two motoneurons, which displayed action potential firing upon membrane depolarization (not shown), each displaying extensive filopodia (long arrows) and spine-like processes (short arrows) present in their somatodendritic domains. (d) Excitatory postsynaptic currents (downward deflections) recorded at a holding potential of −60 mV. Fast inactivating excitatory currents may be AMPA receptor-mediated (asterisks), whereas slowly inactivating excitatory currents likely include NMDA currents alone or together with AMPA (short arrows). (e) Inhibitory postsynaptic currents (upward deflections) recorded at a holding potential of 0 mV. Fast inactivating inhibitory currents may be glycine-mediated (asterisk), whereas slowly inactivating currents likely include GABA-mediated currents (short arrows). Scale bar is 200 μm in (a), 50 μm in (b), and 10 μm in (c). Scale bars for (d and e) are 250 ms and 25 pA.

compared to small size Neurobiotin (MW = 323) (Kanjhan, unpublished observations). Furthermore, the intensity of dye is always much higher in dendritic shafts, particularly in distal dendrites, compared to spines protruding from them, even with smaller dyes Neurobiotin, biocytin (MW = 372), Lucifer Yellow (MW = 522), and Alexa Fluor (MW = 570) [25]. Another limitation is the working distance of the objectives and penetration of light through the 300 μm thick brain tissue; the spines and filopodia can reliably be detected and imaged only if they are located on the surface of the tissue (ideally within the top 20 μm). The clarity and resolution of the image are lost in dendrites deeper than 25–30 μm from the surface of the brain slice and the spines become too blurry for any reliable detection and measurement. One way around this problem could be further slicing of the 300 μm thick brain slice to ≤20 μm sections following fixation of the tissue.

3. Molecular Mechanisms Involved in the Development of Motoneuron Dendritic Trees, Filopodia, and Spines: Remodeling under Neuropathological Conditions

During early embryonic development, motoneurons emerge from dividing progenitor cells located in the medial portion of the ventral neural tube [87]. Motoneuron identities are established by patterning cues working in cooperation with intrinsic sets of transcription factors [87–92]. As the embryo develops, motoneurons further differentiate in a stepwise manner to form compact anatomical groups, termed motor pools or motor columns, connecting to a unique muscle target [88, 92–94]. The lateral motor column (LMC), positioned in the brachial and lumbar enlargements of the spinal cord, contains motoneurons that innervate the skeletal

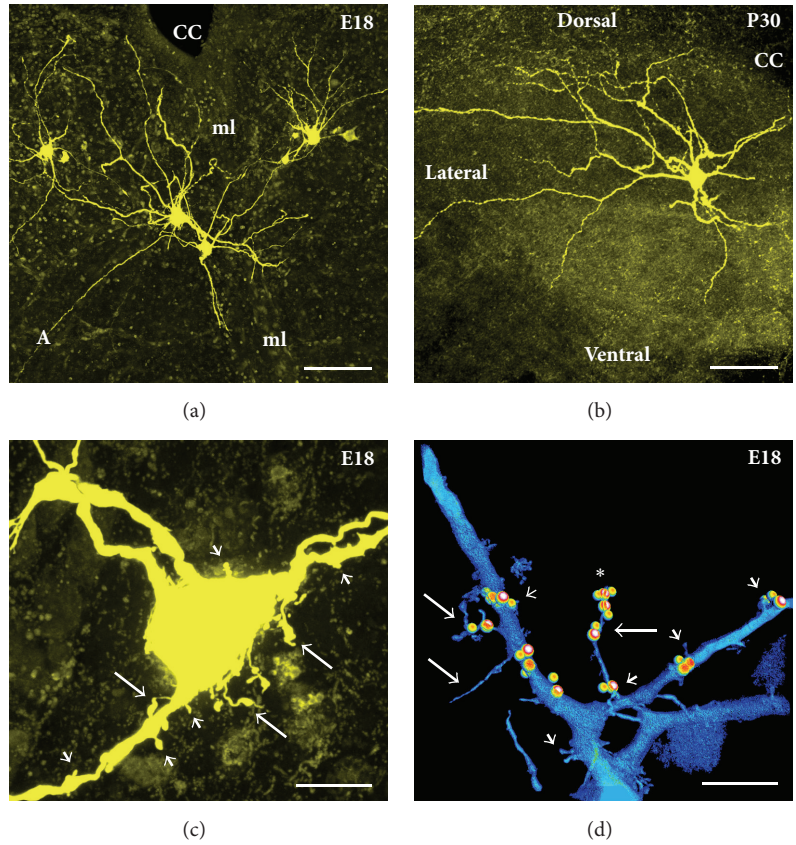

Figure 3: Morphological properties of hypoglossal motoneurons in brainstem slices obtained from embryonic and adult C57/Bl6 wild-type mice (for methodology see Kanjhan and Bellingham, 2013 [26, 27]). (a) Image showing 4 hypoglossal motoneurons filled with Neurobiotin in a 300 μm slice preparation obtained from a mice at embryonic day 18 (E18). Note that the motoneuron on the dorsal right-hand side is dye-coupled to 4 adjacent motoneurons. Two motoneurons on the ventromedial portion of the hypoglossal nucleus have dendrites crossing the midline (ml) to the contralateral side. Axon (A) of one of the motoneurons is clearly visible projecting in the ventrolateral direction to join the hypoglossal nerve outlet. (b) A hypoglossal motoneuron from an adult mouse at postnatal day 30 (P30). Note a significantly larger dendritic tree in the adult mice. (c) A high-power confocal image showing filopodia (long arrows) and spine-like processes (short arrows) at the soma and primary dendrites of a motoneuron from a WT mouse at E18. (d) A rendered 3D reconstruction generated by Imaris software illustrating an overlapping localization of the presynaptic vesicular glutamate transporter-2 (VGLUT-2) terminals (small spheres) and the postsynaptic density protein-95 (PSD-95) (larger spheres) on filopodia (note as many as 4 excitatory synaptic contacts on a single filopodium marked as $*$) and spine-like processes on the primary dendrites of a motoneuron from an E18 WT mouse. CC: central canal. Scale bars = 100 μm in (a and b); 10 μm in (c and d).

muscles of the limbs, while the mediomedial column (MMC), positioned throughout the rostrocaudal extent of the spinal cord, contains motoneurons that for the most part innervate the axial trunk muscles [92–94].

Motoneurons are unique in the vertebrate central nervous system, in the sense that they are arguably the only neurons for which both function and precise target tissue innervation are known. Since the pioneering work of Sir Charles Scott Sherrington, it is widely recognized that motoneurons link the central nervous system to the muscles [95]. Motoneurons are thus the final common effector pathway, where all the peripheral sensory and central premotor and interneuron pathways converge to elicit motor output. A single motoneuron drives a subset of muscle fibers within a muscle, forming a motor unit [95].

Motoneuron pools are not homogeneous and comprise diverse subtypes, according to the muscle fiber type they innervate [41, 89, 96, 97]. Based on their somatodendritic structure, synaptic inputs, axonal projection, and gene expression profiles, every motor column consists of three major motoneuron subtypes: the alpha, beta, and gamma motoneurons; with further subdivision, seven categories of motoneurons have been described based on their innervation pattern [36, 89, 96, 97]. The large multipolar alpha motoneurons innervate the extrafusal muscle fibers in the skeletal muscle and receive input from the proprioceptive sensory afferent neurons. Alpha motoneurons form three types of motor units: (1) fast-twitch fatigable (FF) alpha motoneurons have the biggest soma size and axon diameter, control a large number of type IIB extrafusal muscle fibers, have large neuromuscular synapses, and display phasic/delayed firing patterns; (2) fast-twitch fatigue-resistant (FR) alpha motoneurons are slightly smaller than FF motoneurons, innervate type IIA extrafusal muscle fibers, have large neuromuscular

synapses, and display high-frequency tonic/delayed firing patterns; (3) by contrast, slow-twitch (S) alpha motoneurons are smaller than FF and FR types, innervate fewer type I myosin heavy chain (MHC) muscle fibers, form smaller neuromuscular synapses, and produce tonic/immediate action potential firing [36, 89, 96, 97]. The morphological properties of beta motoneurons resemble alpha motoneurons, but they are skeleton-fusimotor and innervate both the extrafusal fibers in the skeletal muscle and the intrafusal muscle fibers in the muscle spindle. There are two types of beta motoneurons: (1) beta static motoneurons innervate type IIA or IIB extrafusal fibers and the intrafusal nuclear bag2 fiber; (2) beta dynamic motoneurons innervate type I extrafusal muscle fibers and the intrafusal nuclear bag1 fiber [89, 96, 97]. We currently do not know much about specific properties of the beta motoneurons. The gamma motoneurons, which make up for ~30% of all motoneurons, are the smallest of all motoneuron types and innervate exclusively the intrafusal muscle fibers in the muscle spindles, without a direct input from proprioceptive afferents. There are two types of gamma motoneurons: (1) gamma static motoneurons innervate the intrafusal nuclear bag2 fiber and/or the nuclear chain fibers; (2) gamma dynamic motoneurons innervate the intrafusal nuclear bag1 fiber [89, 96, 97]. The gamma motoneurons can also be distinguished from the neuronal nuclear antigen (NeuN) expressing alpha motoneurons, on the basis of expression of transcription factor Err3 (estrogen receptor-related protein 3) or a muscle spindle-derived signaling molecule Wnt-7a (wingless type 7a) [36, 89, 90].

Motoneurons are cholinergic neurons that receive excitatory glutamatergic and inhibitory GABAergic and glycinergic synaptic inputs, as well as many additional modulatory inputs including noradrenergic, serotonergic, dopaminergic, cholinergic, and purinergic inputs [33–35, 97–102]. The intrinsic membrane properties of motoneurons are precisely tuned within each category of motoneurons in order to produce an output that is adapted to the contractile properties of their specific muscle targets [97, 103]. The voltage-dependent delayed rectifier K^+ channel (Kv2.1) contributing to neuronal excitability has been specifically located at the postsynaptic site of large cholinergic C-bouton inputs to somatodendritic domains of alpha motoneurons [104]. There is some evidence that primarily large alpha motoneurons, especially FF type phasic motor units, are selectively lost in aging and motoneuron diseases; their neuromuscular junctions (NMJs) become first denervated (i.e., endplate denervation) and are then partially reinnervated or compensated by the newly grown axon collaterals of adjacent motoneurons [41, 89, 105–107]. Alpha motor axon terminals at NMJs have been shown to undergo bouts of degeneration and regeneration in young asymptomatic $SOD1^{G93A}$ mice, but later in life alpha motor axons selectively degenerate via a process termed "dying back," resulting in the appearance of neurological symptoms due to denervation of muscle fibers and loss of motor neurons [41, 107, 108].

Intercellular communication is essential for the regulation of embryonic development. During early development at the time of cell division, immature neurons start to put out processes known as growth cones that bear filopodia, spine-like structures, and small branches, as shown here with dye-filling by Neurobiotin electroporation of developing human cortical neurons (equivalent of less than 90-day-old fetal neurons) derived from induced pluripotent stem cells (Figures 1(a)-1(b)). Note that isolated developing fetal cells in culture conditions are able to form filopodia and spines from their growth cones in the absence of synaptic inputs (Figures 1(a)-1(b)). Growth cones and filopodia on motoneurons were first described by Ramon y Cajal [1–3]. The growth cones give rise to filopodia, which are composed largely of filamentous- (F-) actin bundles or polymers [47, 109]. Filopodia are long (~2–20 μm) and thin (<0.3 μm in diameter) protrusions or stalks that lack a knobby head (Figures 1(a), 2(c), and 3(c)-3(d)); they are present during development on the soma, dendrites, and axons of neurons and are much more dynamic than dendritic spines [3, 12, 47, 110–112]. It is rare to see filopodia on mature neurons, and therefore their function may be primarily developmental, except under pathological conditions when they may reappear as a regenerative response to injury [62, 111–114]. However, a number of manipulations have also been shown to induce filopodia growth including high-frequency focal synaptic stimulation by activation of glutamatergic NMDA receptors, overexpression of glutamatergic AMPA receptor subtype 2 (GluR2) and the transmembrane agrin (TM-agrin), proteolytic cleavage of agrin by neurotrypsin, or activation of the small conductance Ca^{2+}-activated K^+ channel subtype 3 (SK3) [3, 12, 47, 110].

Activation of the central regulators of actin dynamics Ras (rat sarcoma) and Ras homolog (Rho) family of small GTPases (guanosine triphosphatases), including most studied members RhoA, Rac1 (Ras-related C3 botulinum toxin substrate 1), and cdc42 (cell division cycle 42), and their downstream intermediates results in the polymerization of actin fibers by enabled vasodilator-stimulated phosphoprotein (Ena/Vasp) homology proteins [112, 115]. Growth factors bind to receptor tyrosine kinases resulting in the polymerization of actin filaments, which, when cross-linked, make up the supporting cytoskeletal elements of filopodia [112, 115–118]. Rho activity also results in activation by phosphorylation of ezrin-radixin-moesin (ERM) family proteins that link actin filaments to the filopodia membrane [115]. Myosin-X (Myo10) is a MyTH4-FERM (myosin tail homology 4-for protein 4.1, ezrin, radixin, and moesin) myosin that is a molecular motor localized to the tips of filopodia and functions in filopodia formation by acting downstream of small GTPase cdc42 [119]. Filopodia are much more dynamic than dendritic spines, and issues that are not understood or clear include the role of Ca^{2+} influx in filopodia dynamics [112].

Rats and mice are born with relatively immature forebrains, and spinogenesis in cortical pyramidal neurons starts postnatally; at this stage these neurons are primarily depolarized by GABA instead of glutamate, as excitatory glutamatergic synapses are not formed yet [120]. By contrast, motoneurons and especially hypoglossal motoneurons, which need to be functional at birth for the newborn to breath and suckle, reach maturity at late embryonic stages (Figures 3 and 4). Our results show abundant filopodia and spine-like

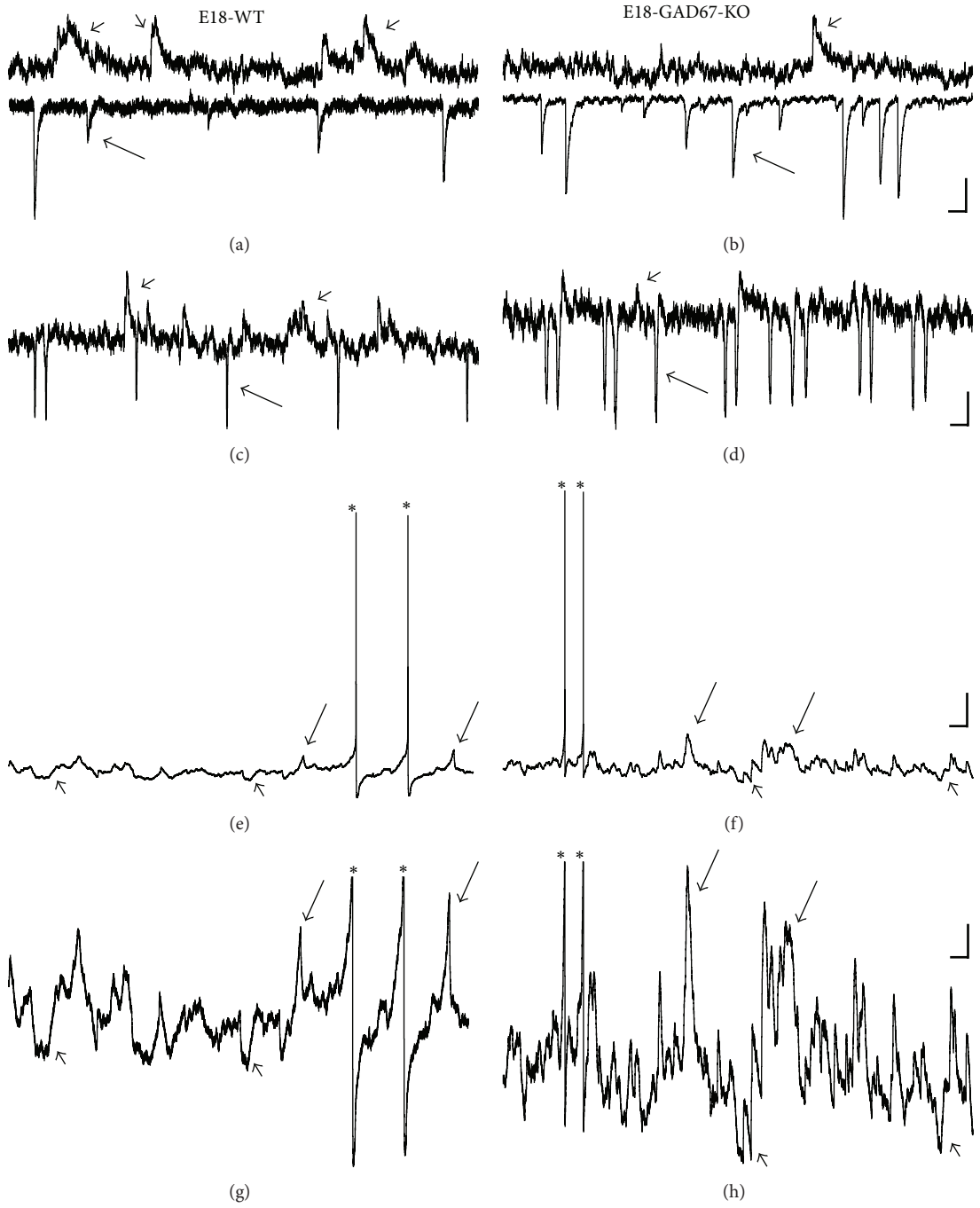

Figure 4: Electrophysiological recordings of hypoglossal motoneurons from C57/Bl6 WT mice compared to GAD67-KO mice at E18 (for methodology see Kanjhan and Bellingham, 2013 [26]). (a-b) Somatic recordings of excitatory (downward deflections, long arrows, EPSCs) and inhibitory (upward deflections, short arrows, IPSCs) postsynaptic currents using low resistance electrodes (3-4 mΩ) at −60 mV and 0 mV holding potentials, respectively. (c-d) Dendritic recording of EPSCs (downward deflections, long arrows) and IPSCs (upward deflections, short arrows) at 0 mV holding potential using higher impedance electrodes (10–15 mΩ). (e-f) Current-clamp recording of membrane potential at action potential (∗) firing threshold, showing subthreshold excitatory (long arrows, EPSPs) and inhibitory (short arrows, IPSPs) postsynaptic potentials. (g-h) Magnified baseline traces of (e)-(f), respectively. Scale bars = (a-b) = 50 pA, 0.1 s; (c-d) = 25 pA, 0.1 s; (e-f) = 20 mV, 1 s; and (g-h) = 3 mV, 1 s.

processes at E17-E18 motoneurons, some of which contain both pre- and postsynaptic components of glutamatergic synapses: vesicular glutamate transporter 2 (VGLUT2) and postsynaptic density 95 (PSD-95), respectively (Figures 3(c)-3(d)). Some of the long filopodia had more than one VGLUT2 and PSD-95 appositions (Figure 3(d)), suggesting multiple potential synaptic sites. These observations are in agreement with Vaughn's ultrastructural findings that synaptic contacts form in mice spinal motoneurons primarily on filopodia as early as E11-E12 [44]. The spinal cord starts to convey first synaptic activity recorded from motoneurons at E12.5, that is, GABAergic [102]. GABAergic and cholinergic signaling together generate earliest spontaneous spinal motor activity [102]. GABAergic synaptic transmission to motoneurons is soon supported by glycinergic synaptic transmission. Glutamatergic synaptic transmission to motoneurons likely activates at around E14.5 [102]. Functionality of excitatory and inhibitory synapses at E18 has been confirmed by our electrophysiological recordings from motoneurons, which were subsequently filled by Neurobiotin electroporation for morphological analysis (for methodological details see Kanjhan and Vaney, 2008, and Kanjhan and Bellingham, 2013 [25, 26]). Somatic patch-clamp recordings show that at E18 wild-type (WT) mice hypoglossal motoneurons receive both glutamatergic excitatory postsynaptic currents (EPSCs) and GABAergic/glycinergic inhibitory postsynaptic currents (IPSCs) under voltage clamp (Figure 4(a)). EPSCs and IPSCs are translated into excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs) under current-clamp conditions, respectively (Figures 4(e) and 4(g)). In lumbar motoneurons, the earliest age we have tested was P0, and at that age group we have seen well-developed excitatory and inhibitory synaptic currents (Figures 2(d)-2(e)). Activation and inactivation kinetics of these currents suggest presence of fast-inactivating AMPA and slow-inactivating NMDA receptor type-mediated excitatory synaptic currents and fast inactivating glycinergic and slow inactivating GABA-mediated inhibitory synaptic currents (Figures 2(d)-2(e)). We have also studied excitatory and inhibitory synaptic currents in transgenic mice deficient in GABAergic inhibitory synaptic transmission, due to elimination of 67 kDa glutamic acid decarboxylase (GAD-67) enzyme, which is the major isoform catalyzing the decarboxylation of glutamate to GABA in prenatal and neonatal brains. Inhibitory postsynaptic currents are mostly lost in mice that lack GAD-67, while there is an increase in the frequency of excitatory synaptic inputs (Figure 4(b)). Patch-clamp recordings from the proximal dendrites of motoneurons in WT and GAD-67 knockout (KO) mice confirmed these results (Figures 4(c)-4(d)). In addition, dendritic recordings show that at least some of the inhibitory outward currents are generated in the dendrites. While somatic and perisomatic inhibition of motoneurons has been studied in detail [33–35, 121] and immunolabelling studies suggest the presence of dendritic inhibition [122], there is no functional data showing dendritic inhibition in motoneurons [123]. We have recently shown that presynaptic vesicular inhibitory amino acid transporter (VGAT) terminals and postsynaptic $GABA_A$ receptor alpha-1 subunits form close appositions on dendrites of hypoglossal motoneurons [27]. Our functional and morphological evidences suggest that inhibitory synaptic modulation may be involved in dendritic integration of synaptic inputs and localized regulation of neuronal Ca^{2+} signaling [124] in motoneuronal information processing [33, 100, 101], as shown in detail for other neuronal types such as retinal ganglion cells [125] and hippocampal neurons [126]. In current-clamp recordings, IPSPs were significantly reduced in amplitude and frequency in hypoglossal motoneurons from the mice deficient in inhibitory synaptic transmission; instead an increase in the frequency and amplitude of EPSPs was observed, which increased the action potential firing probability and reduced the interspike interval (Figures 4(f) and 4(h)). GABA and glycine can activate depolarizing chloride currents in some neurons, such as hippocampal pyramidal cells, during postnatal development due to high intracellular chloride concentrations [102, 120]. Therefore, the presence of IPSCs and IPSPs seen at E18 hypoglossal motoneurons confirms the maturity of hypoglossal motoneurons, as suggested by previous studies [34, 127]. However, the maturation of GABAergic/glycinergic responses from depolarizing to hyperpolarizing may differ among various motoneurons pools [102, 127].

We see filopodia and spine-like processes on the soma and dendrites of developing lumbar (Figures 2(b)-2(c)) and hypoglossal motoneurons from mice (Figures 3(c)-3(d), 5(a), 5(c), 5(d), 6(a)–6(c), and 6(e)) and rats (Figures 6(g)-6(h)). These findings show that the localization of filopodia and spines on motoneurons is not unique to a species or to a specific motoneuron pool. Filopodia-like long processes were also frequently observed on developing motoneuron axons projecting ventrolaterally (not shown). We often saw a variation in density, size, and shape of filopodia and spine-like structures among neighboring motoneurons and even between the dendrites originating from the same motoneuron. In some motoneurons, dorsolaterally projecting dendrites, that likely receive various sensory afferent inputs, had more filopodia and spine-like processes compared to the ventral dendrites. Such specific spine distribution may be important in sensory experience-dependent plasticity of large F type alpha motoneurons that receive sensory inputs. This may have a potential involvement in the selective degeneration of F-type motoneurons in amyotrophic lateral sclerosis (ALS) or motoneuron disease, as well as in aging [41, 89, 105–107]. In the spinal cord, nerve endings from Ia/II proprioceptive sensory neurons, directly contacting alpha motoneurons, are preferentially affected in ALS and degenerate much earlier than those from Ib sensory neurons in $SOD1^{G93A}$ and transactivation response element (TAR) deoxyribonucleic acid- (DNA-) binding protein-43 ($TDP\text{-}43^{A315T}$) mutant mice [107]. It is possible that the sensory input changes to motoneurons, such as increases in excitatory synaptic inputs, may be at least partly involved in increases in frequency of excitatory synaptic inputs shown in Figure 7. On motoneuron dendrites we observed both terminal type (at the tips of distal dendrites; Figures 6(a)-6(b)) and collateral type (emerging from dendritic shafts; Figures 3(d), 6(a)–6(c), and 6(h)) filopodia. While the terminal

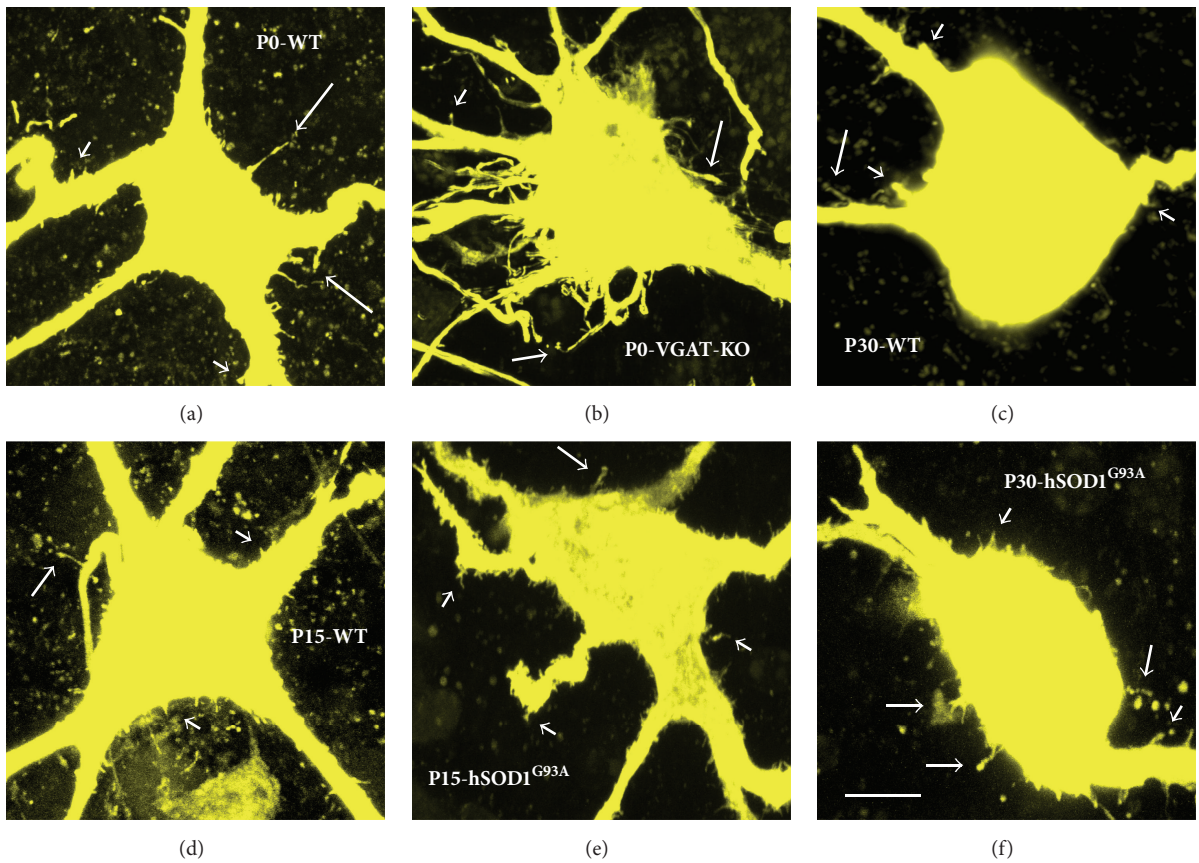

Figure 5: Somatodendritic morphologies of hypoglossal motoneurons during postnatal development and under neuropathological conditions. (a) Filopodia (long arrows) and spine-like processes (short arrows) in the somatodendritic domain of a motoneuron from a P0 WT mouse. (b) Increased density and size of filopodia and spine-like processes from a P0 mice lacking vesicular inhibitory amino acid transporter (VGAT-KO). (c-d) Filopodia and spine-like processes in the soma decrease in density and size during postnatal development from WT mice at P15 (d) and P30 (c). (e-f) Density and size of filopodia and spine-like processes are higher in motoneuronal somatodendritic domain in mice overexpressing the mutated human Cu/Zn-superoxide dismutase ($hSOD1^{G93A}$) gene at P15 (e) and P30 (f). All micrographs are assembled from confocal image stacks of 20 to 40 optical images collected at 0.35 μm steps using an Olympus BX61 (Olympus Fluoview ver. 1.7c) microscope. Scale bar in (f) = 10 μm (applies to all panels).

filopodia may be involved in dendritic growth and branching, the collateral filopodia may be involved in spinogenesis or synaptogenesis, as suggested previously [47, 62, 128–130]. Spines on Purkinje and pyramidal cells show morphological variability and have traditionally been classified based on their appearances as stubby (sessile), thin, and mushroom (pedunculated) types [12, 131]. In motoneuronal somatodendritic domain, the mushroom-shaped pedunculated spines were less frequent than the stubby (sessile) and thin and filopodia-like spines (Figure 1(c)), the sessile (stubby) spines being most common at P30.

In consistency with previous studies on other parts of the brain, such as the pyramidal cells from the rat visual cortex where transiently appearing filopodia mostly disappear after P12 [111, 132], there was a gradual developmental decrease in the filopodia density on the soma and dendrites of motoneurons at P15 (Figures 5(d) and 6(c)) and P30 (Figures 5(c) and 6(e)) compared to E17/P0 WT mice (Figures 3(c)-3(d), 5(a), and 6(a)-6(b)). This may coincide with the developmental downregulation of the GluR1 subunit of AMPA receptors, which has previously been shown to promote filopodia numbers in spinal motoneurons during postnatal development [133]. Spine density on the other hand increased, especially on the distal dendrites at P15 (Figures 5(d) and 6(c)), but then showed a decline at P30 WT mice (Figures 5(c) and 6(e)), especially on soma and proximal dendrites (Figure 6(e)). With maturation, the proportion of shorter and stubby spines on the soma and dendrites increased. One possibility is that some of the taller spine-like processes during early development may actually be shorter filopodia. However, longer filopodia-like processes were still present on distal dendrites, but not as common at P30 (Figure 6(e)). Other studies have also shown that higher levels of spine formation and loss occur in cortical and pyramidal cells from adolescent mice versus adults [13, 22, 111, 134]. During postnatal development, reduction in filopodia numbers in motoneurons may be subsequently followed by a reduction in spine numbers. The reduction in filopodia may limit the formation of new dendritic segments and synapses, promoting stabilized synaptic connectivity during transition to adulthood.

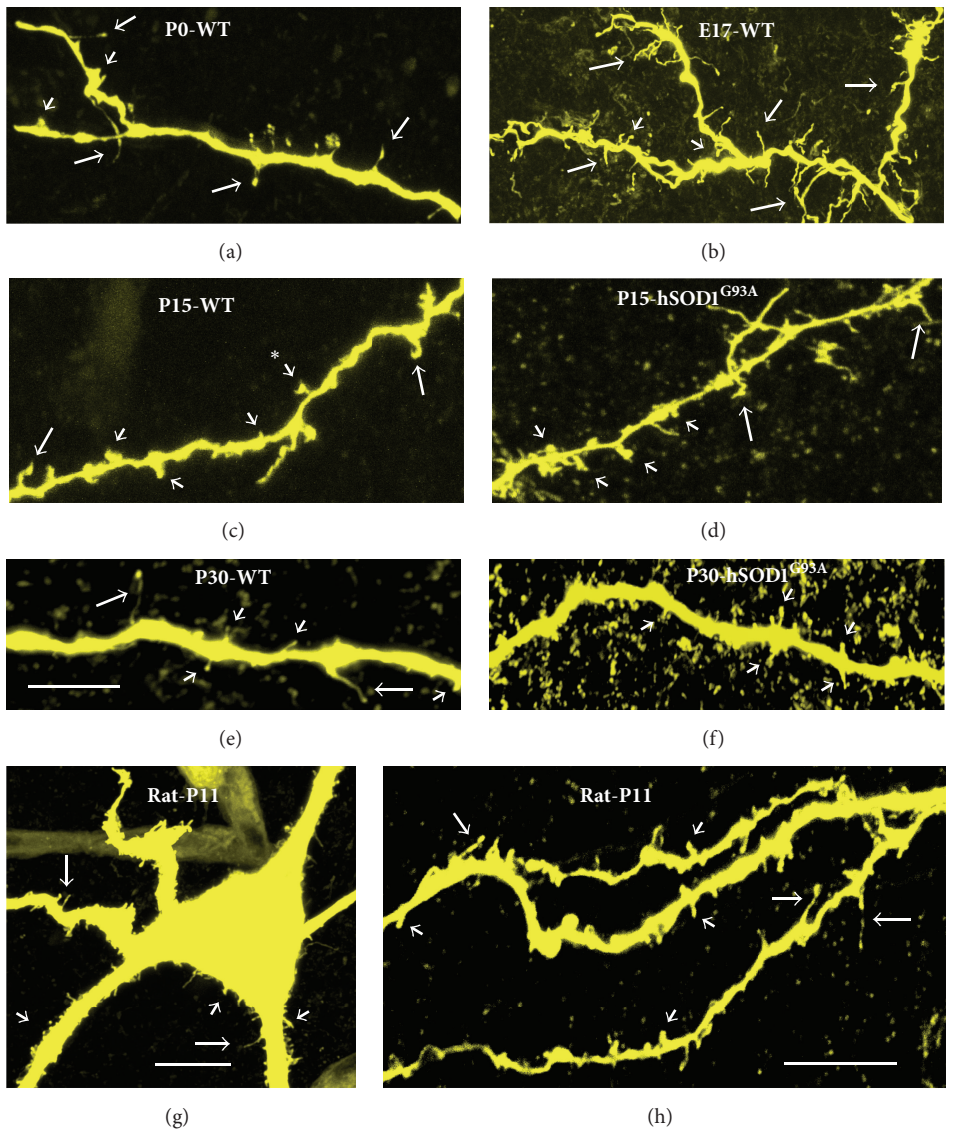

Figure 6: Developmental changes seen in filopodia (long arrows) and spine-like processes (short arrows) in distal dendrites of hypoglossal motoneurons from C57/Bl6 WT mice, Wistar rat (P11; WT), and hSOD1^{G93A} mutant mice. (a-b) The filopodia were long and common and spine-like processes started to form along the distal dendrites at E17-P0. Long and very dense filopodia were seen in some distal dendrites at late embryonic and newborn mice (b). (c, e) Filopodia density compared to E17/P0 gradually reduced with postnatal maturation at P15 (c) and further reduced by P30 (e) WT mice. Spine-like processes decreased in length becoming gradually stubby shaped with postnatal maturation; spine density increased by P15 (c) but decreased at P30 (e). A rare Y-shaped branched spine is marked with an asterisk $*$ (c). (d–f) Increased density of spine-like processes in mice overexpressing the mutated human SOD1^{G93A} gene at P15 (d) and P30 (f). (g-h) Similar filopodia and spine distribution were seen in the P11 rat hypoglossal motoneuronal soma, primary (g) and distal dendrites (h). All micrographs are assembled from confocal image stacks of 20 to 40 optical images collected at 0.35 μm steps. Scale bars in (e), (g), (h) = 10 μm. Scale bar in (e) applies to panels (a) to (f).

In mutant mice strains with impaired inhibitory synaptic transmission (lacking VGAT, GAD-67, or gephyrin), we saw an increase in the density and length of filopodia and spine-like structures on the soma and primary dendrites (Figure 5(b)) compared to WT littermates at E18-P0 (Figure 5(a)). Increases in density and length of filopodia and spine density may be a compensatory reaction to form functional GABAergic and glycinergic synapses. This also implies that the filopodia may also be involved in formation of GABAergic and glycinergic inhibitory synapses, not just excitatory glutamatergic synapses. There is evidence for GABA and glutamate corelease in the brain, and GABAergic synapses can be formed not only on dendritic shafts but also on dendritic spines of pyramidal neurons, where GABA may play a key role in the localized regulation of neuronal Ca^{2+} signaling [124]. It has been proposed that the active excitatory inputs may specifically attract (or repel) an inhibitory bouton; for example, GABAergic inputs may be recruited by the presence of specific glutamatergic afferents, as spines receiving a GABAergic synapse seem to be targeted

by excitatory terminals expressing synaptic marker VGLUT2 [124]. We have shown that VGLUT2 is common in presynaptic terminals to motoneurons (Figure 3(d)), raising the possibility that similar mechanisms may also be implicated during motoneuron development and synaptogenesis. By increasing the length and density of filopodial and spine-like processes, and dendritic branching, motoneurons may be trying to increase their chances of finding GABAergic and glycinergic terminals. Given that the majority of synaptic contacts are found on filopodia of spinal motoneurons during early development [43, 135] (Figure 3(d)), it is possible that, after a long period of nascent inhibitory synaptogenesis, these filopodial processes are ending up with extra excitatory synapse formations. This is consistent with patch-clamp recordings showing significant decrease in the frequency and amplitude of inhibitory postsynaptic currents and potentials and a significant increase in the frequency of excitatory postsynaptic currents and potentials in mutant mice compared to their WT littermates (Figure 4). These findings fit with significant increases in hypoglossal nerve output in brainstem-spinal cord preparations of gephyrin knockout mice, compared to WT littermates [127]. Unfortunately, mice deficient in inhibitory synaptic transmission (GAD-67-KO, VGAT-KO, and gephyrin-KO) die soon after birth [127], preventing the acquisition of data past P0 from any of these mutants. Interestingly, mice overexpressing the mutated hSOD1^{G93A} gene, widely used as a mouse model of inherited ALS, also show increased density of filopodia and spine-like structures in their soma (Figures 5(e)-5(f)) and dendrites (Figures 6(d) and 6(f)) at P15–P30 of presymptomatic age, compared to WT littermates at P15 (Figures 5(c) and 5(d)) and P30 (Figures 6(c) and 6(d)). As for mice deficient in inhibitory synaptic transmission, these morphological changes correlate with increases in the frequency of EPSCs in motoneurons from mice overexpressing the hSOD1^{G93A} gene (Figure 7).

We have found that increased filopodia and spine-like processes were usually associated with increased dendritic length and branching in hSOD1^{G93A} mutants and inhibitory synaptic transmission deficient strains of mice including gephyrin, GAD67, and VGAT knockout mice compared to WT littermates. In WT mice, dendritic growth and branching and increases in spine density continued until about P15, when the filopodia density was high; after loss of filopodia, the growth of the motoneuronal dendritic length and branching, as well as increases in dendritic spine density, were reduced. Taken together these findings suggest that at least some of the filopodia may be involved in dendritic lengthening and branching and increased spine density during normal motoneuron development and that exaggerated filopodial formation leads to increased structural responses in hSOD1^{G93A}, gephyrin, GAD67, and VGAT mutants compared to WT mice. The remaining fewer filopodia in WT adult motoneurons may reflect a residual regeneration capacity of motoneurons, while increases in filopodial number following injury or under neuropathological conditions may reflect reactivated capacity of motoneurons to regenerate.

The mechanisms driving increases in filopodial and spine density and shape (i.e., shortening and thickening), as well as dendritic branching and length, during normal development and under neuropathological conditions or injury are not clear. It is likely that multiple factors are involved: including innate genetic factors, neurotrophic and growth factors, hormones (e.g., androgens and estrogens), guidance cues, neuronal cell adhesion molecules (NCAMs), extracellular matrix, and neuronal activity regulated by the excitatory and inhibitory synaptic inputs and intrinsic membrane properties. Each of these factors will be discussed in detail below.

Brain-derived neurotrophic factor (BDNF) is a strong neuroplasticity candidate that can transform functional activity into morphological changes and dendritic complexity and stability, either during development or as a consequence of changed neuronal activity [20, 136–138]. BDNF can be released from neuronal dendrites or axons in response to neuronal activity or activation of glutamate receptors [116, 136, 138, 139]. BDNF acts upon tyrosine kinase B (or tropomyosin-related kinase B; TrkB) receptors or other signaling pathways such as serum inducible kinase (SKN) to regulate dendritic complexity, filopodial and spine density, and stability of these structures [20, 116–118, 137, 138, 140]. TrkB receptor signaling pathways are well characterized and involve the activation of rat sarcoma/extracellular signal-regulated kinases (Ras/ERK), phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B), mitogen-activated protein kinase (MAPK), and phospholipase C- (PLC-) gamma pathways [117, 137]. MAPK and PI3K play crucial roles in translation and/or trafficking of proteins induced by synaptic activity, whereas PLC-gamma regulates intracellular Ca^{2+} that can drive transcription via cyclic adenosine monophosphate (cAMP) and protein kinase C (PKC) [117, 137]. In the neuromuscular system, androgens regulate BDNF and TrkB expression levels in spinal motoneurons and BDNF levels in target muscles [140, 141]. Androgen-BDNF interactions have important implications in motoneuron dendritic morphology [140, 141], possibly involving cytoskeletal proteins such as actin and tubulin. Castration results in decreased BDNF-TrkB and subsequent regression in dendritic morphology [140, 141]. Estrogens can also have significant effect on motoneuron dendritic growth during early postnatal development [142]. Estrogens are also known to interact with BDNF and to play important roles in brain neuroplasticity [143]; however details of this interaction have not yet been explored in regard to motoneuron plasticity [142]. Activation of BDNF-TrkB complex has been implicated in motoneuron vulnerability to SOD1^{G37R} mutations and toxicity [144]. After spinal cord injury, treadmill training induced lumbar motoneuron dendritic plasticity and functional recovery have been related to an increase in BDNF expression [145]. It has been reported that glutamatergic and GABAergic synapses react differently to postsynaptic BDNF: glutamatergic synaptic inputs increase, whereas GABAergic inputs decrease in a TrkB receptor-dependent manner [146].

NMDA receptors were originally considered to be the sole source of glutamate-mediated Ca^{2+} influx. However, AMPA receptors lacking developmentally regulated GluR2 subunit also allow a significant influx of Ca^{2+} ions

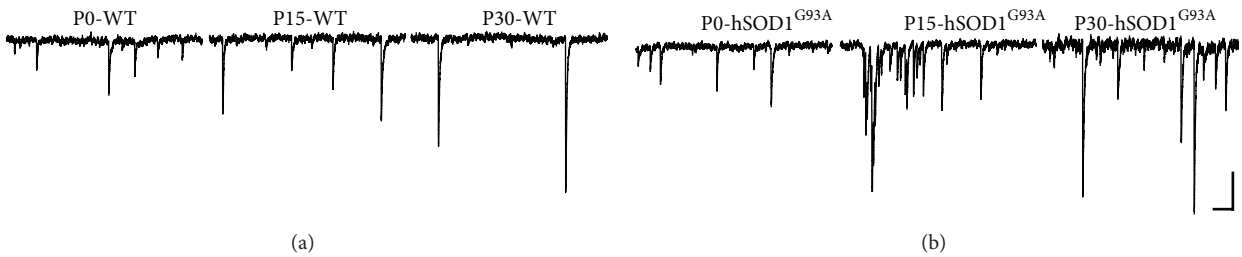

Figure 7: Developmental changes in the amplitude and frequency of spontaneous excitatory postsynaptic currents (EPSCs; downward deflections) in hypoglossal motoneurons recorded from the C57/Bl6 WT mice (a), compared to the mice overexpressing the mutated human $SOD1^{G93A}$ gene (b) at P0, P15, and P30. The amplitude of EPSCs increased by ~3-fold, while the frequency of EPSCs reduced by ~50% with postnatal maturation (from P0 to P30) in WT mice. In the mutated $hSOD1^{G93A}$ mice the frequency of EPSCs was increased, without a clear change in EPSC amplitude, compared to age-matched WT littermates. Scale bars = 100 pA, 0.1 s.

[124, 147]. AMPA receptors also contribute to Ca^{2+} signaling by depolarizing the membrane, which activates voltage-gated calcium channels and relieves Mg^{2+} block from NMDA receptors [124]. Calcium influx activated by glutamate acting via AMPA/kainate receptors has been shown to have distinct and specific effects on the growth and development of motoneuron dendrites in E15 rat embryos [147]. This study suggested a potential physiological role for excitatory neurotransmission in dendrite growth and morphology during development when synaptic contacts are forming between afferent neurons and spinal motoneurons [147]. Increased glutamatergic synaptogenesis in other neuronal networks is also thought to occur through alterations in Ca^{2+} dynamics and/or glutamate-dependent synaptic plasticity [8, 12, 148]. For example, Ca^{2+} influx mediated by AMPA and NMDA glutamate receptors promotes or restricts spine growth in a concentration-dependent manner [9, 149], while overactivation of neurons can elicit increases in spine number and structure [7]. The complexity of dendritic arbor and branching of spinal motoneurons are refined in an activity-dependent manner that is sensitive to blockade of NMDA receptors during postnatal development, but not during late-adult postnatal life [75, 150]. However, NR3B subunit NMDA receptor expression is upregulated in adult motoneurons, and overexpression of NR3B increases dendritic complexity and branching and filopodia numbers [151]. During early postnatal life, synaptic activity promotes dendrite elaboration and growth in spinal motoneurons utilizing GluR1-containing AMPA receptors [152]. Overexpression of the AMPA receptor GluR1 subunit also resulted in an increase in filopodia numbers and in dendritic length and arbor complexity [133]. In contrast, AMPA receptor GluR2 overexpression did not alter dendritic complexity but was associated with increased arbor length and decreased filopodia numbers [133]. These authors concluded that downregulation of GluR1 during postnatal development might limit the formation of new dendrite segments and synapses, promoting stabilized synaptic connectivity with maturation [133]. Other studies have suggested a role for activity-regulated cytoskeletal associated protein involvement in NR2A subtype NMDA, GluR1 and GluR2 type AMPA receptor-mediated chronic spontaneous functional recovery of the paralyzed diaphragm muscle following cervical spinal cord hemisection [153].

Using genetic manipulations of plasma membrane K^+ channel expression in *Drosophila*, it has previously been shown that increased intrinsic neuronal excitability can cause increased dendritic branch formation, whereas decreased intrinsic excitability can cause increased dendrite branch elongation of motoneurons [154]. Therefore, changes in dendritic complexity and plasticity can also be driven by alteration of intrinsic membrane excitability, independent of excitatory glutamatergic synaptic inputs. Since increases in excitatory synaptic inputs were common to motoneurons in all mutant mice used in our studies (Figures 4 and 7), we think it is likely that increases in dendritic branching, filopodia, and spine numbers reported here in mice overexpressing $hSOD1^{G93A}$ and in mice with impaired inhibitory synaptic transmission (VGAT, GAD-67, and gephyrin knockouts) may be driven by increases in glutamatergic synaptic inputs onto motoneurons. Therefore, glutamate- and activity-dependent pathways are likely to be major players in structural remodeling of dendrites, spines, and filopodia under these neuropathological conditions.

However, the signal transduction mechanisms linking glutamate receptor activation to intracellular effectors that accomplish structural and functional plasticity are not well understood. Ultrastructural studies have revealed that the postsynaptic density (PSD) is a highly organized structure that scaffolds the receptors (such as NMDA and AMPA), enzymes, and signaling molecules required for synaptic transduction [16, 20, 155]. Spines are enriched with a complex network of actin, termed the actin spinoskeleton, which supports and determines the physical structure and shape of the dendritic spine [20, 155]. The actin spinoskeleton also organizes the postsynaptic signaling machinery, drives changes in spine structure, and maintains spine stability [16, 20, 156]. The highly dynamic actin cytoskeleton is regulated by an abundance of actin-binding proteins and upstream signaling pathways that modulate actin polymerization and depolymerization [109]. A long list of actin-binding proteins includes actin depolymerizing factor (ADF)/cofilin, actin-related proteins 2/3 (Arp2/3) complex, brevin, calpactin, cortactin, calcium/calmodulin-dependent protein kinase II (CaMKII), calponin, cofilin, drebrin, dystrophin, epidermal growth factor (EGF) receptor, ERM proteins, gelsolin, G-proteins, myosins, myelin basic protein, neurexins, plastin,

phalloidin, PKC, rapsyn, suppressor of ras^{Val14} (srv2), synaptopodin, spectrin, tau, tropomyosin, and Wiskott-Aldrich syndrome protein (WASP) [109]. Glutamate receptor activation is one of the most characterized regulators of actin-based dendritic spine plasticity [16, 20, 157, 158]. CaMKII-dependent phosphorylation of kalirin-7 and subsequent activation of the small GTPase Rac1 are required for NMDA receptor-dependent rapid changes in spine morphology (i.e., enlargement of existing spines) and GluR1 AMPA receptor insertion into synapses of pyramidal neurons [159]. Inactive CaMKII can bind F-actin, thereby limiting access of actin-regulating proteins to F-actin and stabilizing spine structure [156]. Activation of CaMKII dissociates CaMKII from F-actin and permits F-actin remodeling by regulatory proteins followed by reassociation and restabilization [156]. Thus CaMKII kinase can regulate a transient interplay between the kinase and structural functions during the induction of synaptic plasticity.

Recent studies have also identified a neuronal cell adhesion molecule, Dscam1 (Down's syndrome cell adhesion molecule 1), to be critical in regulating developmental dendritic arbor growth, spine and synapse formation, and circuit wiring in mice cerebral cortical and hippocampal pyramidal neurons [160, 161] and in dendritic arbor growth and branching of *Drosophila* motoneurons [162, 163]. Significant loss of Dscam1 function in *Drosophila* prevented stable dendrites from being formed and mutant motoneurons were devoid of mature dendritic branches, instead of displaying a dense meshwork of filopodia-like and lamellipodia-like processes, normally seen during early pupal development [162]. Therefore, Dscam1 may be required for the transformation of actin-rich highly dynamic filopodia into stable dendrites, although the underlying mechanism remains to be elucidated [162]. It is possible that Dscam1 affects intracellular signaling pathways, such as small Rho GTPase, which regulate dendritic growth cone dynamics and spine formation and stability. The intracellular domains of *Drosophila* and human Dscam interact with P21-activated kinase (PAK1), which, in turn, provides a possible mechanistic link to Rho GTPases and actin polymerization [162]. Dendritic translation of Dscam1 is regulated by NMDA receptor activation, and impairment of NMDA-mediated regulation of Dscam1 has been implicated in alterations in dendritic morphology and synaptic plasticity in Down's syndrome [160]. Surprisingly, Dscam1 deficient *Drosophila* flight motoneurons that lack 90% of their dendrites but have normal axonal structure and membrane currents can still satisfactorily perform the vast majority of basic motor functions [163]. Motoneurons with significant dendritic defects can still be contacted by appropriate synaptic partners and can produce qualitatively normal firing patterns and wing movements during flight and courtship song behaviors [163]. However, a normal complex 3D dendritic architecture is essential for intricate regulation and fine tuning of behavior and particularly challenging tasks, such as the integration of optomotor input for adequate control of flight power output or the temporal accuracy of switching between different song elements during courtship to ensure mating success [163].

In vertebrates, cadherins and catenins are the major cell adhesion molecules involved in regulation of dendritic branching and synaptic morphogenesis [164, 165]. The cadherins are glycosylated transmembrane proteins associated with a group of cytosolic proteins, the catenins, and they form cell adhesion complexes in various tissues [165]. A recent study showed that the spine pruning and maturation in the mouse somatosensory cortex are coordinated via the cadherin/catenin cell adhesion complex and bidirectionally regulated by sensory experience [166]. This study concluded that activity-induced interspine competition for beta-catenin provides specificity for concurrent spine maturation and elimination and thus is critical for the molecular control of spine pruning during neural circuit refinement as well as under neuropathological conditions such as autism [166]. The cadherin family is composed of more than 100 members and classified into several subfamilies, including classical cadherins and protocadherins. Protocadherins constitute the largest cadherin family, with 68 members in humans and 70 in murine [165]. Inhibition of cadherin function in cultured hippocampal neurons using a dominant negative approach resulted in abnormal morphogenesis of spines, including filopodia-like elongation and spine head bifurcation, along with disruptions at postsynaptic and presynaptic proteins and synaptic vesicle recycling [164]. In the spinal cord, early studies showed that protocadherin gamma proteins were required for survival of spinal interneurons, synaptic development, and maturation of spinal neurons [167]. Mice lacking all 22 genes of the protocadherin gamma cluster have decreased numbers of spinal cord synapses, are nearly immobile, and die shortly after birth [167]. More recent study showed that protocadherins were involved in mediating dendritic self-avoidance process, in which branches arising from a single neuron repel each other, in the mammalian retinal starburst amacrine interneurons and cerebellar Purkinje cells, mirroring those reported for Dscam1 function in *Drosophila* mentioned above [168]. The clustered protocadherins regulate neuronal survival, as well as dendritic self-avoidance. Nonclustered protocadherins promote cell motility rather than the stabilization of cell adhesion, unlike the classic cadherins, and mediate dynamic cellular processes, such as growth cone migration [165, 169]. Cadherin superfamily members are implicated in several neuronal disorders including Alzheimer's disease, schizophrenia, autism, mental retardation, and epilepsy [165, 169].

Cell adhesion molecules also connect to both the presynaptic partner and the extracellular matrix (ECM), which is composed of glycoproteins (e.g., laminins, tenascins, and thrombospondins) and proteoglycans that form a complex interactive meshwork in and around the synaptic cleft [170]. The neurons, glial cells, and the space adjacent to and between synapses are surrounded by ECM providing a "glue or gel" to attach cells and processes to each other [170]. At the synaptic cleft, pre- and postsynaptic cell adhesion molecules associate with one another and with the ECM to initiate and maintain synaptic contact [170]. These transmembrane cell adhesion proteins connect to the intracellular dendritic spine actin network and influence the activities of actin

regulatory molecules, thereby controlling spine shape. The ECM has therefore been implicated in spine and synapse stability, remodeling, and plasticity during development and adulthood [170]. These are important functions as loss of spine stability has been implicated in a number of neurodegenerative diseases [170].

ECM also attaches signals with special domains docking to cell surface receptors and presents soluble molecules such as basic fibroblast growth factors (bFGFs) or wingless/Int-1 (Wnt)—proteins critical for neuronal survival and identity determination [92]. The availability of these molecules depends on the matrix composition and influences the transcription factor code of each cell. Recent research has also provided strong evidence that depletion of single matrix molecules like Tenascin C (TnC) can lead to developmental changes within the motoneuron progenitor pools [92]. Modulation of pathways involving potently inhibitory ECM may be critical in recovery from spinal cord injuries [171]. Following spinal injury, digestion with chondroitinase ABC of the upregulated chondroitin sulphate proteoglycans, that restrict functional plasticity and stabilize spines, is beneficial in motoneuronal plasticity and functional recovery of paralyzed diaphragm [171].

Upon neuronal activation or stimulation, the actin spinoskeleton is uniquely regulated within microdomains to modulate spine morphology, PSD structure, and membrane trafficking that involves the dynamic processes of exocytosis, endocytosis, internalization, endosomal recycling, and localization of molecules such as AMPA receptors important in synaptic transmission and neuroplasticity [172]. Actin dynamics generate forces that manipulate membranes in the process of vesicle biogenesis and also for propelling vesicles through the cytoplasm to reach their destination [172]. In addition, trafficking mechanisms exploit more stable aspects of the actin cytoskeleton by using actin-based motor proteins such as myosins to traffic vesicular cargo along actin filaments [172, 173]. Myosins are a large family of actin-based cytoskeletal motors that use energy derived from adenosine triphosphate (ATP) hydrolysis to generate movement and force for functions such as regulation of actin cytoskeleton dynamics in dendritic spines and powering of synaptic cargo transport [173]. In summary, mounting evidence indicates that the actin molecule with rapid dynamics is at the centre stage of structural regulation, maintenance, and remodeling of synaptic plasticity [16, 20, 155, 156].

Recent studies have found that alterations or disturbances in the cytoskeletal actin pathway in motoneurons and redox alterations in the inflammatory compartment contribute to ALS pathogenesis and disease outcome [174–178]. Profilin 1 (PFN-1) is crucial for the conversation of monomeric (globular) G-actin to polymer microfilament (filamentous) F-actin in response to extracellular signals [109], and mutations in PFN-1 gene are shown to cause familial ALS [175, 177]. Primary motoneurons expressing mutant PFN-1 display smaller growth cones with a reduced F/G-actin ratio [175]. Actin-binding protein plastin 3 (PLS-3) levels are reduced in spinal muscular atrophy (SMA), and transgenic reintroduction of PLS-3 rescues functional defects in SMA [174, 178]. SMA is due to gene mutations or deletions in the survival motor neuron 1 (*smn1*) gene, decreasing the availability of SMN protein, which in turn leads to an early degeneration of lower motor neurons in children [178]. SMN protein regulates actin dynamics, and SMN overexpression in cultured neuronal cells promotes neurite outgrowth [174, 176, 178]. Small GTPase Rac1 dysregulation or alterations in structure and function have also been implicated in the pathogenesis of ALS and SMA [176]. Rac1 plays a key regulatory function of both actin and microtubule cytoskeletal dynamics and thus it is central to axonal and dendritic growth and stability, as well as dendritic and spine structural plasticity during development and under neuropathological conditions. Rac1 is also a crucial regulator of nicotinamide adenine dinucleotide phosphate-oxidase- (NADPH-) dependent membrane oxidase (NOX), a prominent source of reactive oxygen species (ROS), thus having a central role in the inflammatory response and neurotoxicity mediated by microglia [176]. SOD1 directly binds to Rac1 in a redox-sensitive manner: in reducing conditions SOD1 binds to Rac1 and stimulates its activity; conversely, in oxidizing conditions SOD1 dissociates from Rac1 and inhibits its activity [176].

Figure 7 illustrates how these developmental and neuropathological changes (in the case of $hSOD1^{G93A}$-mutated mice) in dendritic, filopodial, and spine morphology are reflected in functional properties of motoneurons, as recorded in the form of excitatory postsynaptic currents (EPSCs) received by the motoneurons. At all age groups, in particular at P15 and P30, the frequency of EPSCs is significantly higher in large-sized hypoglossal motoneurons from $hSOD1^{G93A}$ transgenic mice (Figure 7(b)), compared to age-matched WT mice (Figure 7(a)). Amplitude of EPSCs was slightly larger in $hSOD1^{G93A}$ overexpressing motoneurons than WT motoneurons, but not as dramatic as changes in EPSC frequency (Figure 7). This suggests that changes at the synaptic level are primarily due to increases in the total number of glutamatergic excitatory synapses on to $hSOD1^{G93A}$ overexpressing hypoglossal motoneurons. This is consistent with denser filopodia and spine numbers in motoneurons from $hSOD1^{G93A}$ mice (Figures 5(e), 5(f), 6(d), and 6(f)) compared to WT (Figures 5(a), 5(c)-5(d), 6(a)–6(c), and 6(e)).

In WT motoneurons, there is an increase in motoneuron EPSC amplitudes associated with a decrease in frequency during motoneuron development (Figure 7(a)), coinciding with decreases in dendritic spine numbers and transition to shorter and stubby spines (Figures 5(c)-5(d), and 6(e)). Indeed, these size and shape changes (i.e., shortening and increase in thickness) in spines and filopodia during postnatal development may reflect the strengthening of synapses in an activity-dependent manner, as shown for the pyramidal neurons [20, 111, 179]. In particular, stubby spines seem to be much more stable and persistent than longer and thinner spines [179], and filopodial processes that are much more dynamic than dendritic spines may also be less stable [111, 179]. These developmental changes in the size and density of spines and increases in the amplitude of EPSCs reported

here suggest an increase in synaptic strength and efficacy with maturation of motoneurons. Spine size correlates with synaptic strength and larger spines commonly contain larger PSDs with more AMPA-type glutamate receptors and appose axon terminals with larger readily releasable pools of neurotransmitter [6, 170, 179]. Therefore, the presence of stubby and large diameter spines is more likely to produce strong excitatory postsynaptic currents and has greater influence on neuronal firing and network signaling. While small amplitude excitatory synaptic inputs will need to summate to cause firing, a large amplitude excitatory synaptic input from a single presynaptic fiber can trigger action potentials in the postsynaptic neuron [180, 181]. As excitatory synaptic strength increases, the motoneuron may receive fewer but larger amplitude (stronger) excitatory synaptic inputs to bring the membrane potential at the axon initial segment to firing threshold by activating voltage-gated sodium channels. Riluzole-sensitive persistent sodium current present in motoneurons would be expected to further assist these large synaptic depolarizations [26, 182]. This makes sense as motoneurons, once activated, often have an intrinsic tendency to fire tonically in rhythmic bursts of relatively low frequencies (0.5–2 Hz burst frequency), interrupted by inhibitory inputs and after hyperpolarizations, such as during walking and breathing [33, 68, 69, 98, 183–185].

In pyramidal and Purkinje cells, spines do not only appear and disappear, but their basic morphology also seems to change continuously [9, 157, 186], often in an activity- or experience-dependent manner [10, 14, 17, 22, 187–191]. Although glutamatergic pyramidal cells and GABAergic Purkinje cells are very different in their morphology and function from cholinergic motoneurons, similar molecular mechanisms of plasticity, such as dynamic actin-based cytoskeletal remodeling of dendritic spine structure, size, and shape, can also take place in motoneurons in response to experience and activity or under neuropathological conditions. Such dynamic plasticity of sensory- and activity-dependent development of the neuromotor system and the modifications that take place after disease or injury have been the focus of many studies [18, 76, 127, 145, 178, 192–194].

4. Spinogenesis/Synaptogenesis and Plasticity during Motoneuron Development

As most spines are believed to be postsynaptic compartments receiving excitatory synaptic inputs [8], spinogenesis is linked to synaptogenesis [12]. An initial period of spine proliferation during early development is probably intrinsic to the neuron, as spines can emerge in the absence of axon terminals; the activity of the synapse and the neuron regulates a later decline in spinogenesis [12, 190]. A large proportion of spinogenesis and synaptogenesis in the primary visual cortex occurs before eye opening in mice, and the only morphological event that seems to correlate with eye opening is the elongation of the spine neck [132]. We also regularly observe filopodia and spines on growth cones and dendritic processes of developing human neurons derived from induced pluripotent stem (iPS) cells, in culture prior to formation of synaptic inputs (Figures 1(a)-1(b)). Therefore it is likely that the sensory input or sensory evoked activity and axonal input are not essential for ontogenetic spinogenesis/synaptogenesis in neurons, such as visual cortical neurons, during embryonic/prenatal or even neonatal period [12, 13, 132, 190]. Instead the robust spontaneous activity of the developing brain and spinal network *in utero* is potentially important for normal spinogenesis [12, 13, 132, 190]. However, the spine stability and modifications to spine shape and size can be strongly modulated by sensory manipulations and activity [13, 20, 22, 24, 189–191, 195, 196]. Previous studies have shown spine [7] and filopodia [197] generation after induction of synaptic potentiation using two-photon laser microscopy. Using similar methods in mice *in vivo*, others have shown that spines are remarkably stable throughout life in cerebral cortical neurons [14, 15, 111, 134, 179, 189, 191, 198], including primary visual cortical layer 5 pyramidal neurons in the primary visual cortex of living transgenic mice expressing yellow fluorescent protein [111, 134]. The turnover of spines is higher during the critical period of postnatal development than in adult life [13, 22, 111, 134]. These *in vivo* studies also found that spines undergo considerable changes in shape and size during development and in the adult mice, as previously shown in *in vitro* preparations [10, 134, 157, 158].

Numerous cell surface receptors, scaffold proteins, and actin binding proteins present in spines engaged in spine morphogenesis [156, 199–204]. Molecular studies indicate that multiple signaling pathways, particularly those involving Rho and Ras families of small GTPases, such as Racl, RhoA, and cdc42, play important roles in morphogenesis of dendritic spines, spinogenesis, spine loss or retraction, and synaptic plasticity, by converging on the actin cytoskeleton to bidirectionally regulate spine morphology and dynamics [199–204]. Overexpression of these small GTPase proteins results in the creation of new spines *in vivo* and *in vitro* in Purkinje cells and pyramidal neurons [199, 200, 205]. Spine density, size, and length are controlled by different members of the Rho and Ras families in developing and mature Purkinje cells and pyramidal neurons [201–205]. Therefore even mature neurons have the entire molecular complement required for spinogenesis in response to injury and neuropathological conditions.

Spine stability is most likely due to binding of inactive CaMKII to F-actin, thereby limiting access of actin-regulating proteins to F-actin [156], as well as contribution from cell adhesion molecules and the extracellular matrix [170]. Activation of CaMKII by sensory stimulation or synaptic activity may be important in altering the structural stability of actin and spines [156]. This role is critical as most brain disorders and neurodegenerative diseases including Alzheimer's, Parkinson's, and Huntington's diseases and schizophrenia involve loss of dendritic spine stability in adulthood [170].

Despite our increasing understanding of the molecular players in spinogenesis, the mechanism of spine formation is still widely debated. Three major models of spinogenesis in the nervous system have been put forward by previous studies [3, 12]: these are proposed by Miller/Peters [132], by Sotelo [206, 207], and by the filopodia model derived from

Vaughn's synaptotropic hypothesis [43–46] (Figure 8). The Miller/Peters model is based on Golgi-electron microscopic data from developing rat visual cortex pyramidal cells; in this model, protrusions with a stubby appearance are first formed on dendritic shafts; then an apposed presynaptic region of the axon shows a swelling as synaptic vesicles accumulate, inducing the dendritic shaft to initiate elongated spine formation; in the final stage (at around P21 in mice), many spines are thin or mushroom shaped [132]. However, more recent studies have found that dendritic spines can grow directly from a dendritic shaft in contact with an axon which induces the clustering of Rac1, a small GTPase from Rho family involved in dynamic regulation of actin and the microtubule cytoskeleton, leading to spinogenesis [208], so that spine formation precedes synapse formation [190, 209]. The Sotelo model, based on data from cerebellar Purkinje cells of normal and mutant mice, contradicts the Miller/Peters model; as Purkinje cells from mutant mice lacking parallel fibers form abundant spine, the Sotelo model proposes that the presynaptic axonal terminal has only a minor role in spinogenesis [206, 207].

The filopodia model is derived from Vaughn's synaptotropic hypothesis, based on ultrastructural and Golgi studies on embryonic and neonatal mice spinal cord motoneurons [43–46]. The filopodial model posits that spines arise by transformation of existing filopodial precursors or protospines, as has been shown by live confocal time-lapse imaging studies [47, 129]. Studies of hippocampal and cortical pyramidal neurons have provided supporting evidence for this model by confirming that existing filopodia can produce both sessile and pedunculated spines [47, 129, 130].

A unifying model for synaptogenesis during different developmental stages has recently been put forward by García-López et al. [3], which incorporates elements of these previous models and proposes that synaptogenesis may happen in three different modes, corresponding to prenatal (Sotelo model), neonatal (filopodial model), and mature (Miller/Peters model) age groups, respectively (Figure 8). Our data spans this developmental spectrum, and our observations fit most closely with the filopodial model during embryonic/prenatal/neonatal periods; although Sotelo model may also contribute particularly in neonatal/juvenile period, Miller/Peters model is likely involved primarily in adults. The unified model also overlaps with our results obtained from developing motoneurons. However our motoneuron-based model also differs from the unified model [3] in the sequence of events, with filopodial model, instead of Sotelo model, being dominant during embryonic/prenatal/neonatal periods; then it is followed by involvement of Sotelo model at neonatal/juvenile periods, and the Miller/Peters model may get involved much later at juvenile/adult stages (Figure 8). Our data from embryonic/neonatal/juvenile motoneurons is least consistent with the Miller/Peters model based on pyramidal cells, as this model may be more applicable to spinogenesis/synaptogenesis in adult motoneurons (Figure 8). Spinogenesis in motoneurons therefore seems to have a uniquely sequenced unified-hybrid model differing in sequence of events from previous models based on pyramidal and Purkinje neurons, as summarized in Figure 8. This may be due to major developmental differences between the pyramidal/Purkinje cells and hypoglossal/lumbar motoneurons. These include the following. (1) Motoneurons are cholinergic, receive excitatory glutamatergic synaptic inputs before birth, and are inhibited by GABA and glycine prior to birth (Figure 4); by contrast, pyramidal neurons do not have excitatory glutamatergic synapses until after birth and are excited by GABA during prenatal and neonatal periods. (2) Filopodia and long thin spine-like processes appear on embryonic motoneurons forming synapses (Figures 3(c)-3(d), 5(a), and 6(a)-6(b)), as previously shown [43, 44]. We have seen filopodia on motoneuron soma and dendrites at the earliest age that we have examined (E16), and filopodia likely appear earlier than that, as others have shown synapses forming on dendritic filopodia as early as E11 in mice spinal motoneurons [44, 46]. Indeed, around 70% of synaptic contacts are found on filopodia in the developing mice [43] and chicken [135] spinal cord motoneurons. By contrast, in pyramidal cells, filopodia of the collateral type are transient and appear later postnatally at P3–P12 and rarely form synapses [132]. Synapses on dendritic shafts predominate in pyramidal cells in early development, with spine formation beginning in the first week after birth [132, 210]. (3) In motoneurons, filopodia and spine-like processes are thin and longer at late embryonic and early postnatal ages, becoming shorter and stubby with postnatal development, and mushroom spines are rare (Figures 5(c)-5(d), 6(c)–6(e)). In cerebral pyramidal neurons during early postnatal development, short and stubby spines are common, while in the adult, thin and mushroom spines are more common, although many stubby spines are still present [132, 211]. (4) Pyramidal and Purkinje neurons are innervated by regular parallel fibers, by contrast to developing presynaptic axon terminations within the hypoglossal nucleus following convoluted routes with many three-dimensional twists and turns in mouse and rat (Kanjhan, unpublished observations), as shown for the adult rat [69]. This suggests that formation of synapses on filopodia during development follows contact with searching axon terminations (Figure 3(d)) [43, 44, 135], before being transformed into dendritic spines or incorporated into the dendritic shaft by filopodial retraction. Filopodia contacting several axons can distinguish between distinct inputs and choose the most active ones [3].

It is possible that cholinergic motoneurons, which extend long axonal projections to muscles in the periphery and which receive descending premotor and local interneuron inputs, use different spinogenesis strategies than inhibitory (GABAergic) Purkinje cells and excitatory (glutamatergic) pyramidal neurons. One feature that supports this is that spine shapes in different cell types (e.g., pyramidal, Purkinje, and motoneurons) fall into different dominant categories. In the somatodendritic domain of mature motoneurons (P30), stubby and short type spines are seen more frequently than the relatively sparse longer mushroom-shaped spines, which are the dominant spine shape in adult pyramidal and Purkinje cells [12, 131, 132, 211]. While motoneuron spines get shorter (stubby) with maturation, in contrast the pyramidal and Purkinje neuron spines seem to be getting longer.

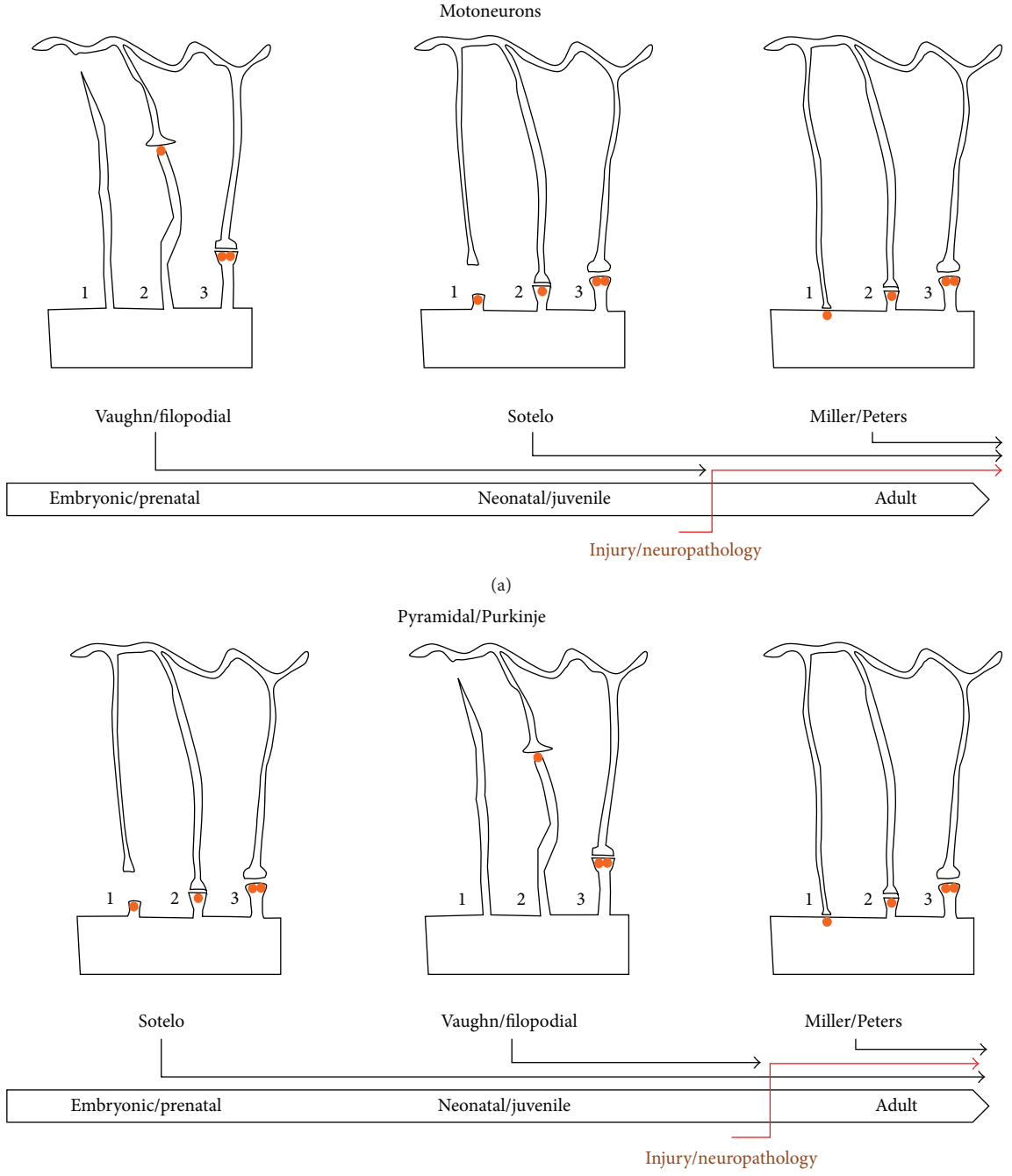

Figure 8: A schematic presentation of a unified-hybrid model of spinogenesis/synaptogenesis in motoneurons compared to pyramidal/Purkinje neurons at different developmental stages involving previously described models. (a) In motoneurons, the Vaughn/filopodial model (left) predominates during embryonic, prenatal, and neonatal development but becomes less common during juvenile development, where the Sotelo model (middle) becomes more frequent. The Miller/Peters model (right) may play a role in adult plasticity. (b) The sequence of spinogenesis/synaptogenesis in motoneurons seems to significantly differ from that described for pyramidal and Purkinje cells. The Sotelo model predominates in spinogenesis in pyramidal/Purkinje cells during embryonic/prenatal period. The filopodial model is less involved in all cell types in the adult but is likely reactivated as part of the regenerative/remodelling processes following injury/neuropathological conditions (red lines). 1, 2, and 3 indicate the sequence of pre- and postsynaptic development; filled circles represent postsynaptic receptor clusters.

Unfortunately there are no specific markers, which can distinguish different spine types or differentiate spines from filopodia. The filopodial model of spinogenesis, which is relatively less active in juvenile/adult period, may become reactivated following neuronal injury/neuropathological events as part of the structural regenerative remodelling of the neuronal morphology (Figure 8).

We have noted differences in filopodia and spine density between motoneurons from the same slice preparation, as well as between the dendrites of the same motoneuron. Morphological differences, such as spine density and shape (short and stubby spines) and dendritic branching, among categories of motoneurons may also be important in providing structural bases for synaptic hyperexcitability leading to neuropathological conditions, such as selective degeneration of certain types of motoneurons (e.g., vulnerable F type phasic large alpha motoneurons) in ALS [89, 106, 107, 194, 212–214]. The potential neuropathological roles of morphological changes will be discussed below.

5. Excitatory Hypersynaptogenesis and Intrinsic Membrane Hyperexcitability Are Linked to Insufficiencies in Cellular Energy Metabolism and Selective Motoneuron Degeneration

Amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) are neurodegenerative disorders characterized by selective loss of motoneurons, most likely due to cellular excitotoxicity and oxidative stress caused by accumulation or deregulation of intracellular Ca^{2+} levels resulting primarily from glutamate receptor activation [107, 178, 193, 194, 212–218]. Elevated intracellular Ca^{2+} can activate cytoplasmic apoptotic proteins such as calcineurin and calpain, deregulate of Ca^{2+} in the endoplasmic reticulum, and overload the mitochondria with Ca^{2+}, resulting in mitochondrial dysfunction and oxidative stress ultimately promoting neuronal death [216, 218, 219]. Most cases of ALS are sporadic with no known genetic linkage, while approximately 10% are associated with familial forms, presenting mutations in over 20 genes encoding for distinct proteins with varied functions, including SOD1, fused in sarcoma (FUS), TDP-43, chromosome 9 open reading frame 72 (C9ORF72), PFN-1, vacuolar protein sorting-associated protein 9- (VPS9-) ankyrin repeat protein (VARP), alsin, ataxin-2, and matrin-3 [194, 218]. Mutations in these proteins may increase the susceptibility for the dysregulated intracellular Ca^{2+}-mediated degenerative processes to occur, suggesting existence of a common pathogenic pathway centered around intracellular Ca^{2+} and its handling [194, 216, 218]. For example, misfolded and aggregated SOD1 mutants localized within the mitochondrial membrane of spinal cord motoneurons cause dysfunction in oxidative phosphorylation and lead to endoplasmic reticulum stress [194, 216, 218, 219].

Consistent with glutamate-mediated excitotoxicity hypothesis, our studies of motoneurons from $hSOD1^{G93A}$ mutant mice show that increases in dendritic spine density compared to age-matched littermates are associated with increased frequency of EPSCs, as well as an enhanced developmental increase in EPSC amplitudes (Figure 7(b)). Increased frequency of excitatory and inhibitory synaptic inputs to hypoglossal motoneurons, together with increased intrinsic persistent sodium currents resulting in increased action potential firing rates, was previously reported in $hSOD1^{G93A}$ mutant mice as early as P4 to P10 [192]. These modifications in incoming excitatory synaptic inputs interact with changes in the intrinsic membrane properties of the motoneurons. For example, increases in persistent sodium and calcium currents may result in longer lasting depolarizations following excitatory synaptic inputs [97, 215, 217, 220], leading to excessive sodium and calcium loading of the cytoplasm and specific compartments such as spine heads. Such longer depolarizations may in turn reduce the excitability and firing properties of motoneurons, by depolarization block or partial inactivation of the voltage-gated sodium channels [217]. This may be the mechanism of hypoexcitability of lumbar motoneurons reported in adult $hSOD1^{G93A}$ mutant mice [221]. Accumulation of intracellular calcium will also result in disturbances in calcium homeostasis and protein folding, endoplasmic reticulum stress [194, 212, 215, 217, 219, 222], and perturbations in the function and motility of the actin-based cytoskeleton and spinoskeleton [10, 16, 20, 155, 158, 174, 175, 177, 178, 203]. However, recent studies have questioned the role of hyperexcitability in motoneuron degeneration [36, 37, 221]. The excitability of large F-type motoneurons was unchanged in the $mSOD1^{G93A}$ mutant neonatal mice, but, surprisingly, the small S-type motoneurons displayed intrinsic hyperexcitability [36]. Another study using two-photon imaging found that calcium transients in motoneuron dendrites of $hSOD1^{G93A}$ mutants are smaller, compared to WT mice at P4–P11 [37]. These findings may not be conclusive, given the wide variability in their Ca^{2+} responses and the inability to measure the total Ca^{2+} entry into the cell. However, data presented here and overwhelming evidence from various labs around the world, including recent studies from human motoneurons derived from ALS patient induced pluripotent stem cells (iPS cells, see below), support a role for hyperexcitability in the development of motoneuron degeneration [91, 223].

A reduction or depletion of intracellular ATP will have further consequences on neuronal activity, by cyclical activation ATP-sensitive potassium (K^{ATP}) channels that set burst frequency and duration in motoneurons [184, 215, 217]. Motoneuronal bursting under neuropathological conditions, such as inhibition of glutamate uptake by astrocytes, may involve persistent glutamatergic activation of NMDA, AMPA (GluR2 lacking Ca^{2+} permeable), and metabotropic glutamate receptor type-1 (mGluR1) receptors to cyclical activation of K^{ATP} conductances, linking electrical discharge properties to the cellular energy metabolism in motoneurons [215, 217]. Firing properties of motoneurons may be further boosted by activity-dependent increases in extracellular K^+ levels (~6 mM) and decreases in Ca^{2+} levels (~0.9 mM) consequent to increased locomotion or hyperactivity [224].

Another intrinsic factor that is critical in motoneuron excitability is the relative contribution from various K^+ channels including the delayed-rectifier K^+ current (Kv7 or M-current) [225, 226] and TASK-1 two-pore domain leak K^+ channels that are regulated by many extracellular and intracellular factors including several neurotransmitters such as glutamate, serotonin, and noradrenaline [33, 227, 228]. A reduction in K^+ currents would increase the input resistance and the intrinsic excitability of motoneurons, potentially increasing the effects of excitatory synaptic inputs. Similar mechanisms have been implicated in a mouse model of neuronal atrophy in spinocerebellar ataxia type 1, involving abnormal membrane depolarizations due to a reduction in K^+ channels, including TASK-1-like background K^+ currents [229]. Postnatal increases in expression of TASK-1 channels likely dampen the excitability of motoneurons, perhaps serving to increase precision in muscle control and to reduce involuntary muscle contractions, as well as serving as a neuroprotector by letting K^+ out of the cell in a voltage-independent manner [228]. Activation of glutamatergic metabotropic mGluR1 receptors inhibits TASK-1 like background K^+ channels, subsequently increasing input resistance, motoneuronal excitability, and bursting activity [217]. Motoneurons may become more reliant on these TASK-1 leak K^+ channels with aging [228], and changes such as increased inhibition of these channels via glutamatergic mGluR1 or peroxide-mediated oxidative stress may have undesired effects [217]. The role of K^+ channels may become more critical when the extracellular K^+ levels are raised to ~6 mM by increased locomotion in an activity-dependent manner [224]. Another important role is also played by astrocytes surrounding motoneurons, as they can clear extracellular K^+ and glutamate in an activity-dependent manner using their ionic pumps (e.g., Na^+-K^+ ATPase, Na^+-K^+-$2Cl^-$), K^+ channels (e.g., inwardly rectifying K^+ channels, especially Kir4.1), excitatory amino acid transporters EAAT1 (GLAST) and EAAT2 (GLT-1), and gap junctions made of connexins, Cx43 and Cx30 [230, 231].

Thus, reduced intracellular ATP availability increases the metabolic cost of a single action potential and disrupts K^+ and Na^+ homeostasis, resulting in a chronic depolarization and mitochondrial stress and dysfunction, which subsequently leads a cascade of events to selective degeneration of motoneurons [232]. Distinct subsets of motoneurons may also have variable bioenergetics needs. Motoneurons are large neurons with extensive dendritic trees and longest axonal projections requiring continuous and metabolically demanding transport of various molecules and mitochondria to the terminals [232]. Motoneurons are extremely active, continuously firing action potentials to maintain tonic posture or to generate the complex firing patterns needed for muscle contraction during specific movements, adding to the metabolic burden that must be met by ATP, produced both via oxidative phosphorylation and glycolysis [232]. Increased synaptic hyperactivity and longer lasting depolarizations will increase this metabolic demand, putting stress on mitochondria, as cellular Na^+ and Ca^{2+} overloading increase energy use by many homeostatic exchangers, such as plasma membrane Na^+-K^+ ATPase (sodium pump), plasma membrane and sarcoplasmic Ca^{2+}-ATPase (calcium pumps), and plasma membrane and mitochondrial Na^+-Ca^{2+} exchangers to maintain the ionic homeostasis critical for neuronal survival [232]. Interestingly, the $\alpha 1$ isoform of Na^+-K^+ ATPase is differentially expressed in large alpha motoneurons, compared to small gamma motoneurons, which express the $\alpha 3$ isoform [233]. The $\alpha 1$ isoform of Na^+-K^+ ATPase extrudes intracellular Na^+ at a slower rate than $\alpha 3$ [234], and this may well be a factor relevant to the selective degeneration of larger F-type alpha motoneurons in ALS patients and $hSOD1^{G93A}$ mutant mice [89, 106, 107, 194, 214]. Na^+-K^+ ATPase is vulnerable to aberrant SOD1 activity, and global loss of Na^+-K^+ ATPase and its nitric oxide-mediated regulation occur in mice overexpressing $hSOD1^{G93A}$ [235].

Defects in mitochondrial transport are implicated in the pathogenesis of several major neurological disorders [236]. Recent studies have identified mitochondrial Rho1 (Miro1) GTPases, a mitochondrial calcium sensor for glutamate receptor-dependent localization of mitochondria at synapses, as a key determinant of how energy supply is matched to energy usage in neurons [236, 237]. Trafficking of mitochondria to dendritic and axonal locations in neurons, where there are large Na^+ and Ca^{2+} fluxes requiring active function of pumps, is essential for maintaining neuronal function and health. In fact, mitochondrial trafficking is regulated by Ca^{2+} flux through ionotropic glutamate receptors [237]. Miro1 links mitochondria to kinesin-1 family 5 (KIF5) motor proteins in a Ca^{2+}-dependent manner (i.e., inhibited by micromolar Ca^{2+} binding to Miro1), allowing mitochondria to move along microtubules (anterograde or retrograde) until mitochondrial stopping induced by glutamate or neuronal activity [236, 237]. For example, activation of NMDA receptors leads to Miro1 positioning mitochondria at the postsynaptic side of synapses [237]. Miro1 is essential for development of cranial motor nuclei and maintenance of upper motor neurons, and neuron-specific loss of Miro1 causes depletion of mitochondria from corticospinal tract axons and progressive neurological deficits [238]. Defects in Miro1-mediated mitochondrial motility and distribution are sufficient to cause neurological disease such as upper motoneuron disease [238]. A significant reduction in Miro1 levels in the spinal cord tissue of ALS patients and transgenic models of ALS ($SOD1^{G93A}$, $TDP\text{-}43^{M337V}$) was recently shown by immunoblot analysis [239]. The same study also showed that excessive glutamate challenge leads to a significant reduction in Miro1 expression in spinal motoneurons of mice, suggesting that glutamate excitotoxicity may cause Miro1 deficiency leading to motoneuron degeneration [239].

These studies together suggest that excessive glutamatergic synaptic activity and changes in intrinsic membrane properties leading to sustained membrane depolarization will increase the energy demand of a motoneuron. If the supply of energy falls behind the consumption of energy required to maintain physiological levels of cytoplasmic Ca^{2+} and Na^+, the rise in the intracellular levels of these ions will activate process that will lead to motoneuron degeneration.

Recent developments in stem cell technologies have allowed generation of human motoneurons from somatic or skin cells of normal and patients with ALS, paving the way for opportunities to develop patient-specific treatments [91, 223]. Motoneurons derived from induced pluripotent stem cells (iPS cells) from ALS patients, harboring SOD1, C9orf72, and fused-in-sarcoma (FUS) mutations, have been reported to display reduced delayed-rectifier K^+ current (Kv7 or M-current) amplitudes relative to control-derived motor neurons [91]. The M-current activator retigabine both blocks the hyperexcitability and improves motor neuron survival *in vitro* when tested in SOD1 mutant ALS patient iPS cell-derived motoneurons in culture [91]. A more recent study also reported initial hyperexcitability followed by progressive loss of action potential output and synaptic activity, due to a progressive decrease in voltage-activated Na^+ and K^+ currents, in patient iPS cell-derived motoneurons, harboring transactivation response element (TAR) DNA-binding protein (TARDBP) or C9ORF72 ALS-causing mutations [223]. These studies from human iPS cell-derived motoneurons are consistent with our results, discussion, and conclusions primarily based on mice models of ALS.

6. Potential Roles of Microglia in Motoneuron Plasticity and Neurodegeneration

It is increasingly accepted that ALS is a complex neurodegenerative syndrome that involves not only motoneurons but also a wide range of different tissues and cell types, including interneurons, muscle cells, astrocytes, oligodendrocytes, and microglia [178, 193, 194, 214, 230, 240, 241]. Although ALS primarily affects motoneurons, astrocyte and microglia activation and skeletal muscle atrophy (sarcopenia) are also typical hallmarks of the disease. However, the functional relationship between motoneurons, astrocytes, microglia, and skeletal muscle in the pathogenic process remains unclear. Neuroinflammation is evident in rodent models of inherited ALS overexpressing mutant SOD1 and in ALS human patients [176, 240–246]. A consistent neuropathologic feature of ALS is the extensive inflammation around motor neurons evidenced by the accumulation of reactive astrocytes and activated microglia [240–244].

Microglia are the resident macrophages in the nervous system where they form a nonoverlapping mosaic or microglial network, which monitors and controls the environment and activity of neurons (Figure 9(a)) [247–249]. Microglia are considered the most susceptible sensors of neuronal environment and brain pathology and have additional roles in providing cytokines, growth factors, and neurotransmitters during development and neuronal plasticity [247–249]. Microglia are located in close proximity to synapses; with their highly dynamic and motile processes containing actin-based cytoskeleton, they can scan their territorial domains and display transient interactions with the synapses [247–249]. Microglial surveillance and synaptic pruning have been shown to be important in normal brain development and synaptic maturation [248, 250, 251]. Therefore deficits or changes in microglial function may contribute to synaptic abnormalities seen in neurodegenerative and neurodevelopmental diseases [246, 248, 250–252]. Signs of nervous tissue damage, lesion, or dysfunction result in a complex and multistage activation process that converts resident microglial cells to their activated form [247, 249]. Once activated, microglia can migrate to the injured or dysfunctional site, proliferate, and form new processes; and then they are able to destroy neurons either by direct phagocytosis or by indirectly secreting neurotoxic substances [245, 247, 249]. Selective changes in motoneuronal activity, such as synaptic hyperactivity in $hSOD1^{G93A}$ mutants (Figure 7(b)), or pharmacologic block of the inhibitory synaptic transmission leading to disinhibited motoneuron bursting [184, 217] will increase metabolic and energy demand by depleting intracellular ATP (see above) and this will subsequently activate microglia to attack and phagocytose the motoneuronal soma and dendrites (Figure 9). Microglial attack and phagocytosis were seen in presymptomatic $hSOD1^{G93A}$ mutant mice in a minority (~5%) of cells dye-filled with Neurobiotin (Figures 9(a)-9(b)). By contrast, microglial attack and phagocytosis were never observed in WT hypoglossal motoneurons under normal conditions. However, pharmacological blocking of inhibitory synaptic transmission activated microglia acutely (within minutes) and the microglial response was intense and seen in all the cells tested ($n = 6$) from the $hSOD1^{G93A}$ mutant or WT mice (Figures 9(c)-9(d)). Therefore it is likely that the intensity of microglial response correlates with the level of motoneuronal activity and metabolic demand due to intracellular ATP depletion. Although our example shown here is acute, as blocking inhibitory synaptic transmission occurs within minutes (Figures 9(c)-9(d)), it is possible that lower levels of chronically increased activity may also cause significant damage over time [240, 242–244]. Once microglia are activated, we see swelling and vacuolization in soma, dendrites, filopodia, and spines of motoneurons (Figure 9), subsequently resulting in rapid disintegration of affected motoneurons. In support of this idea, a recent study has shown that the modulation of microglial activation by Fasudil, a Rho kinase inhibitor drug, prolongs survival and improves motor function in $hSOD1^{G93A}$ mice [253].

7. Conclusions

Previous studies and our observations suggest that filopodia and dendritic spines are central structural elements of motoneuronal development and plasticity under both normal and neuropathological conditions. Both lumbar and hypoglossal motoneurons display dense filopodia and spine-like structures in their somatodendritic domains at late embryonic (prenatal) and newborn stages. During normal postnatal maturation of motoneurons, the density of filopodia reduced whilst spine-like stubby processes increased until around P15 and then decreased by P30. Spine distribution shifted towards the distal dendrites, and spine density decreased and spines became shorter and thick (stubby; 0.1 to 0.4 μm long) with postnatal maturation. This coincided with a decreased frequency and increased amplitude of excitatory postsynaptic currents in motoneurons by ~2- to 3-fold at

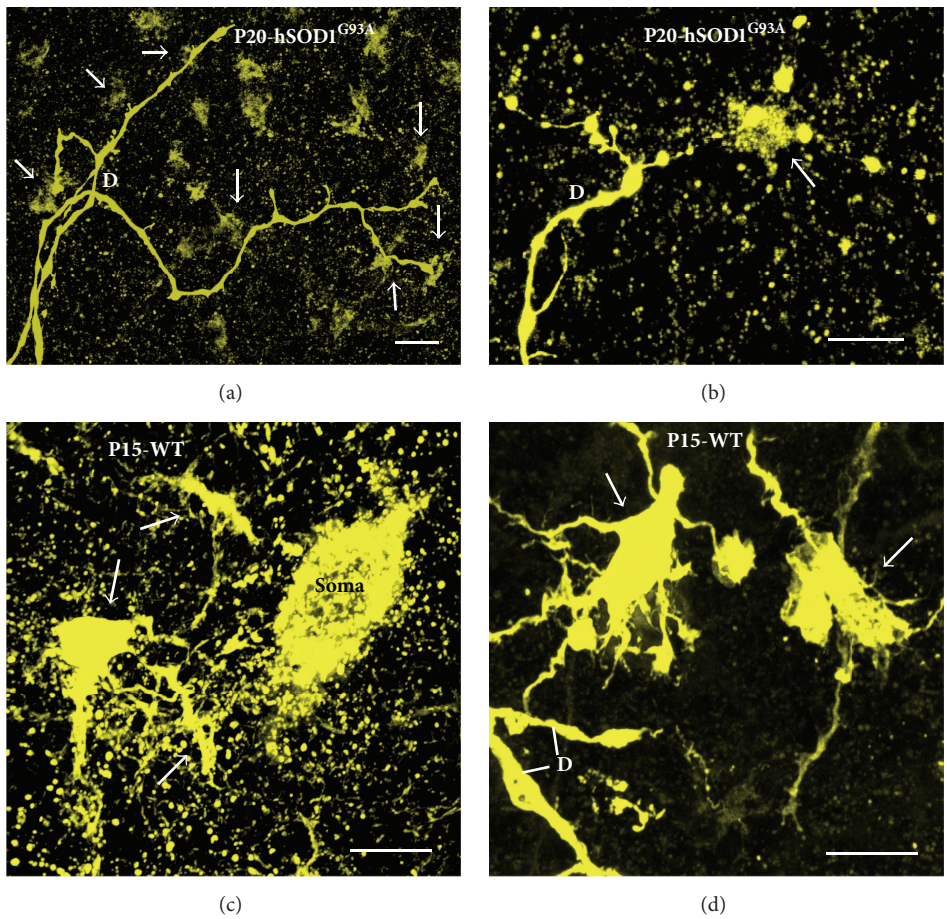

Figure 9: Microglial control of motoneuronal territory. Microglia were indirectly labeled by Neurobiotin, which they engulfed by phagocytosis from Neurobiotin-filled motoneurons, evident from detection of Neurobiotin both in dye-filled motoneuron and microglia. Neurobiotin was visualized by incubating brain slices in Streptavidin Cy3, as usual (standard) in all our preparations presented in this study (for details see Kanjhan and Vaney, 2008 [25], and Kanjhan and Bellingham, 2013 [26]). (a) A mosaic of activated microglia (arrows; with amoeboid appearance and loss of stellate processes) seen in close contact to the Neurobiotin-filled distal dendrites of a hypoglossal motoneuron in a brain slice from a P20 mice overexpressing the mutated human $SOD1^{G93A}$ gene. (b) An example of resident microglia (arrow) engulfing distal dendrites (D) of a dye-filled hypoglossal motoneuron from $hSOD1^{G93A}$ mutant mouse, as indicated by the blebbing of the dendrite and that both dendrite and microglia contain Neurobiotin. (c-d) Resident microglia (arrows), activated by blocking inhibitory synaptic transmission by addition of 5 μM bicuculline and 2 μM strychnine for >15 minutes, engulfing the soma (c) and dendrites (d) of a dye-filled hypoglossal motoneuron in brainstem slices from P15 WT mice. Bright appearance of microglia is due to Neurobiotin taken by phagocytosis from the soma and dendrites of motoneuron. Note swelling, beading, blebbing, and vacuolization in soma (c), dendrites (b, d), and spines (b). Scale bars = 10 μm.

P30. Our findings, consistent with Vaughn's synaptotropic hypothesis, suggest that filopodia may be involved in spinogenesis and synaptogenesis, as well as dendritic growth and branching critical for circuit formation and synaptic plasticity during embryonic/prenatal and neonatal development. The sequences of spinogenesis/synaptogenesis in motoneurons differ from pyramidal and Purkinje cells and fit with a unique unified-hybrid model (Figure 8). Dendritic length and branching and filopodia and spine density, shape, and length are all dynamic and regulated by development (e.g., genetics) and by neuronal activity determined by synaptic or intrinsic properties. The soma and dendritic trees of motoneurons receive highly orchestrated excitatory and inhibitory synaptic inputs, allowing motoneurons to control and coordinate highly complex and refined motor tasks. Any significant and prolonged changes to the balance of excitatory-inhibitory synaptic inputs can result in synaptic hyperactivity and changes in intrinsic membrane properties (i.e., hyperexcitability), with associated changes in neuronal dendritic tree, filopodia, and spine morphology. Remodeling of synaptic, intrinsic membrane, and morphological properties of motoneurons can ultimately lead to excitotoxicity and subsequent motoneuronal damage. Microglial synaptic pruning and phagocytosis may shape this remodeling process. Future studies need to address the molecular mechanisms driving the changes in microglia and motoneurons during normal development, in the genesis of synaptic hyperactivity, and in subsequent motoneuron loss in neurodegenerative

and neurodevelopmental diseases. Finally, we are hoping that this contribution will make an impact and stimulate new research on dendritic spine structure and function during development and disease, particularly in the motoneuron field.

Abbreviations

ADF/cofilin: Actin depolymerizing factor/cofilin
Akt: Protein kinase B
ALS: Amyotrophic lateral sclerosis
AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Arp2/3: Actin-related proteins 2/3
ATP: Adenosine triphosphate
BDNF: Brain-derived neurotrophic factor
bFGFs: Basic fibroblast growth factors
C9ORF72: Chromosome 9 open reading frame 72
CaMKII: Calcium/calmodulin-dependent protein kinase II
cAMP: Cyclic adenosine monophosphate
cdc42: Cell division cycle 42
CT: Cholera toxin
Dscam1: Down's syndrome cell adhesion molecule 1
DNA: Deoxyribonucleic acid
E: Embryonic day
ECM: Extracellular matrix
EGF: Epithelial growth factor
EPSCs: Excitatory postsynaptic currents
EPSPs: Excitatory postsynaptic potentials
Ena/Vasp: Enabled vasodilator-stimulated phosphoprotein
ERM: Ezrin-radixin-moesin
Err3: Estrogen receptor-related protein 3
GABA: Gamma-aminobutyric acid
F-actin: Filamentous actin
FF: Fast-twitch fatigable
FR: Fast-twitch fatigue resistant
Fus: Fused in sarcoma
HRP: Horse radish peroxidase
hSOD1: Human Cu/Zn-superoxide dismutase
G-actin: Globular actin
GAD-67: 67 kDa glutamic acid decarboxylase
GFP: Green fluorescent protein
GluR1: AMPA receptor type 1
GluR2: AMPA receptor type 2
GTPases: Guanosine triphosphatases
iPS cells: Induced pluripotent stem cells
IPSCs: Inhibitory postsynaptic currents
IPSPs: Inhibitory postsynaptic potentials
Kif5: Kinesin-1 family 5
KO: Knockout
Kv2.1: Voltage-dependent delayed rectifier K^+ channel 2.1
LMC: Lateral motor column
MAPK: Mitogen-activated protein kinase
mGluR: Metabotropic glutamate receptor
Miro1: Mitochondrial Rho1
MMC: Medial motor column
MW: Molecular weight
Myo10: Myosin-X
MyTH4-FERM: Myosin tail homology 4-for protein 4.1, ezrin, radixin, and moesin
NADPH: Nicotinamide adenine dinucleotide phosphate-oxidase
NCAM: Neuronal cell adhesion molecules
NeuN: Neuronal nuclear antigen
NMDA: *N*-Methyl-D-aspartic acid
NMJ: Neuromuscular junction
NOX: NADPH-dependent membrane oxidase
NR3B: NMDA receptor type 3B
P: Postnatal day
PAK1: P21-activated kinase 1
PFN-1: Profilin 1
PI3K: Phosphatidylinositol 3-kinase
PKC: Protein kinase C
PLC: Phospholipase C
PLS-3: Plastin 3
PSD-95: Postsynaptic density 95
Rac1: Ras-related C3 botulinum toxin substrate 1
Ras: Rat sarcoma
Ras/ERK: Rat sarcoma/extracellular signal regulated kinases
Rho: Ras homolog
ROS: Reactive oxygen species
S: Slow-twitch
SK3: Small conductance Ca^{2+}-activated K^+ channel subtype 3
SMA: Spinal muscular atrophy
smn 1: Survival motor neuron 1 gene
SKN: Serum inducible kinase
srv2: Suppressor of ras^{Val14}
TARDBP: Transactivation response element (TAR) DNA-binding protein
TDP-43: Transactivation response element (TAR) DNA-binding protein-43
TM-agrin: Transmembrane agrin
TnC: Tenascin C
TrkB: Tyrosine kinase B (also called, tropomyosin-related kinase B)
VPS9: Vacuolar protein sorting-associated protein 9
VARP: VPS9-ankyrin repeat protein
VGAT: Vesicular inhibitory amino acid transporter
VGLUT2: Vesicular glutamate transporter 2
WASP: Wiskott-Aldrich syndrome protein
WGA: Wheat-germ agglutinin
Wnt: Wingless/Int-1
WT: Wild type
YFP: Yellow fluorescent protein.

Conflict of Interests

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

Authors' Contribution

Peter G. Noakes and Mark C. Bellingham contributed equally to this work.

Acknowledgments

Mr. Patrick Fortuna and Professor Ernst Wolvetang (AIBN, University of Queensland) are thanked for generously supplying the iPS-derived cells used in Figures 1(a)-1(b). Professor Yuchio Yanagawa (Gunma University, Japan) is thanked for his kind and generous supply of VGAT and GAD-67 transgenic mice (used in Figures 4(b), 4(d), 4(e), 4(h), and 5(b)). Ms. Holly Brand and Ms. Rose Norton are thanked for their help with lumbar spinal cord preparations (used in Figure 2) during their undergraduate Winter Research Scholarship Program, supported by the UQ Advantage Program. The study was supported by grants to Peter G. Noakes and Mark C. Bellingham (National Health and Medical Research Council Project Grants 1065884 and 401579; Motor Neuron Disease Research Institute of Australia).

References

[1] S. Ramon y Cajal, "The structure and connexions of neurons," in *Nobel Lecture*, 1906.

[2] S. Ramon y Cajal, *Histologie du Systeme Nerveux de l'Homme et des Vertebres*, vol. 1, Maloine, Paris, France, 1909.

[3] P. García-López, V. García-Marín, and M. Freire, "Dendritic spines and development: towards a unifying model of spinogenesis—a present day review of Cajal's histological slides and drawings," *Neural Plasticity*, vol. 2010, Article ID 769207, 29 pages, 2010.

[4] C. Golgi, "The neuron doctrine—theory and facts," in *Nobel Prize Lecture*, 1906.

[5] E. G. Gray, "Electron microscopy of synaptic contacts on dendrite spines of the cerebral cortex," *Nature*, vol. 183, no. 4675, pp. 1592–1593, 1959.

[6] K. M. Harris and S. B. Kater, "Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function," *Annual Review of Neuroscience*, vol. 17, pp. 341–371, 1994.

[7] F. Engert and T. Bonhoeffer, "Dendritic spine changes associated with hippocampal long-term synaptic plasticity," *Nature*, vol. 399, no. 6731, pp. 66–70, 1999.

[8] R. Yuste, A. Majewska, and K. Holthoff, "From form to function: calcium compartmentalization in dendritic spines," *Nature Neuroscience*, vol. 3, no. 7, pp. 653–659, 2000.

[9] H. Hering and M. Sheng, "Dendritic spines: structure, dynamics and regulation," *Nature Reviews Neuroscience*, vol. 2, no. 12, pp. 880–888, 2001.

[10] T. Bonhoeffer and R. Yuste, "Spine motility. Phenomenology, mechanisms, and function," *Neuron*, vol. 35, no. 6, pp. 1019–1027, 2002.

[11] M. Segal, "Changing views of Cajal's neuron: the case of the dendritic spine," *Progress in Brain Research*, vol. 136, pp. 101–107, 2002.

[12] R. Yuste and T. Bonhoeffer, "Genesis of dendritic spines: insights from ultrastructural and imaging studies," *Nature Reviews Neuroscience*, vol. 5, no. 1, pp. 24–34, 2004.

[13] M. Segal, "Dendritic spines and long-term plasticity," *Nature Reviews Neuroscience*, vol. 6, no. 4, pp. 277–284, 2005.

[14] Y. Zuo, A. Lin, P. Chang, and W.-B. Gan, "Development of long-term dendritic spine stability in diverse regions of cerebral cortex," *Neuron*, vol. 46, no. 2, pp. 181–189, 2005.

[15] J. Grutzendler and W.-B. Gan, "Two-photon imaging of synaptic plasticity and pathology in the living mouse brain," *NeuroRx*, vol. 3, no. 4, pp. 489–496, 2006.

[16] M. Segal, A. Vlachos, and E. Korkotian, "The spine apparatus, synaptopodin, and dendritic spine plasticity," *Neuroscientist*, vol. 16, no. 2, pp. 125–131, 2010.

[17] M. Fu and Y. Zuo, "Experience-dependent structural plasticity in the cortex," *Trends in Neurosciences*, vol. 34, no. 4, pp. 177–187, 2011.

[18] A. M. Tan and S. G. Waxman, "Spinal cord injury, dendritic spine remodeling, and spinal memory mechanisms," *Experimental Neurology*, vol. 235, no. 1, pp. 142–151, 2012.

[19] W. Yu and B. Lu, "Synapses and dendritic spines as pathogenic targets in Alzheimer's disease," *Neural Plasticity*, vol. 2012, Article ID 247150, 8 pages, 2012.

[20] A. J. Koleske, "Molecular mechanisms of dendrite stability," *Nature Reviews Neuroscience*, vol. 14, no. 8, pp. 536–550, 2013.

[21] R. Yuste, "Electrical compartmentalization in dendritic spines," *Annual Review of Neuroscience*, vol. 36, pp. 429–449, 2013.

[22] C. Sala and M. Segal, "Dendritic spines: the locus of structural and functional plasticity," *Physiological Reviews*, vol. 94, no. 1, pp. 141–188, 2014.

[23] K. M. Harris, F. E. Jensen, and B. Tsao, "Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation," *The Journal of Neuroscience*, vol. 12, no. 7, pp. 2685–2705, 1992.

[24] Y. Zuo, G. Yang, E. Kwon, and W.-B. Gan, "Long-term sensory deprivation prevents dendritic spine loss in primary somatosensory cortex," *Nature*, vol. 436, no. 7048, pp. 261–265, 2005.

[25] R. Kanjhan and D. I. Vaney, "Semi-loose seal neurobiotin electroporation for combined structural and functional analysis of neurons," *Pflugers Archiv*, vol. 457, no. 2, pp. 561–568, 2008.

[26] R. Kanjhan and M. C. Bellingham, "Neurobiotin electroporation for combined structural and functional analysis of neurons in developing mouse brain slices," in *Stimulation and Inhibition of Neurons*, P. M. Pilowsky, M. M. J. Farnham, and A. Y. Fong, Eds., vol. 78 of *Neuromethods*, pp. 151–165, Humana Press, New York, NY, USA, 2013.

[27] M. J. Fogarty, L. A. Hammond, R. Kanjhan, M. C. Bellingham, and P. G. Noakes, "A method for the three-dimensional reconstruction of Neurobiotin-filled neurons and the location of their synaptic inputs," *Frontiers in Neural Circuits*, vol. 7, article 153, 2013.

[28] M. E. Scheibel and A. B. Scheibel, "Organization of spinal motoneuron dendrites in bundles," *Experimental Neurology*, vol. 28, no. 1, pp. 106–112, 1970.

[29] M. E. Scheibel, T. L. Davies, and A. B. Scheibel, "Maturation of reticular dendrites: loss of spines and development of bundles," *Experimental Neurology*, vol. 38, no. 2, pp. 301–310, 1973.

[30] M. E. Scheibel and A. B. Scheibel, "Dendrite bundles as sites for central programs: an hypothesis," *International Journal of Neuroscience*, vol. 6, no. 4, pp. 195–202, 1973.

[31] H. G. Goshgarian and J. A. Rafols, "The phrenic nucleus of the albino rat: a correlative HRP and Golgi study," *Journal of Comparative Neurology*, vol. 201, no. 3, pp. 441–456, 1981.

[32] R. P. Hammer Jr., R. D. Lindsay, and A. B. Scheibel, "Development of the brain stem reticular core: an assessment of dendritic state and configuration in the perinatal rat," *Brain Research*, vol. 227, no. 2, pp. 179–190, 1981.

[33] J. C. Rekling, G. D. Funk, D. A. Bayliss, X.-W. Dong, and J. L. Feldman, "Synaptic control of motoneuronal excitability," *Physiological Reviews*, vol. 80, no. 2, pp. 767–852, 2000.

[34] J. J. Greer and G. D. Funk, "Perinatal development of respiratory motoneurons," *Respiratory Physiology and Neurobiology*, vol. 149, no. 1–3, pp. 43–61, 2005.

[35] A. J. Berger, "Development of synaptic transmission to respiratory motoneurons," *Respiratory Physiology and Neurobiology*, vol. 179, no. 1, pp. 34–42, 2011.

[36] F. Leroy, B. Lamotte d'Incamps, R. D. Imhoff-Manuel, and D. Zytnicki, "Early intrinsic hyperexcitability does not contribute to motoneuron degeneration in amyotrophic lateral sclerosis," *eLife*, vol. 3, 2014.

[37] K. A. Quinlan, J. B. Lamano, J. Samuels, and C. J. Heckman, "Comparison of dendritic calcium transients in juvenile wild type and SOD1(G93A) mouse lumbar motoneurons," *Frontiers in Cellular Neuroscience*, vol. 9, article 139, 2015.

[38] A. B. Odutola, "Cell grouping and Golgi architecture of the hypoglossal nucleus of the rat," *Experimental Neurology*, vol. 52, no. 3, pp. 356–371, 1976.

[39] M. C. Brown, J. K. S. Jansen, and D. Van Essen, "Polyneuronal innervation of skeletal muscle in new-born rats and its elimination during maturation," *Journal of Physiology*, vol. 261, no. 2, pp. 387–422, 1976.

[40] M. R. Bennett, "Development of neuromuscular synapses," *Physiological Reviews*, vol. 63, no. 3, pp. 915–1048, 1983.

[41] D. Frey, C. Schneider, L. Xu, J. Borg, W. Spooren, and P. Caroni, "Early and selective loss of neuromuscular synapse subtypes with low sprouting competence in motoneuron diseases," *Journal of Neuroscience*, vol. 20, no. 7, pp. 2534–2542, 2000.

[42] M. H. Cooper, "The hypoglossal nucleus of the primate: a Golgi study," *Neuroscience Letters*, vol. 21, no. 3, pp. 249–254, 1981.

[43] J. E. Vaughn, C. K. Hendrikson, and J. A. Grieshaber, "A quantitative study of synapses on motor neuron dendritic growth cones in developing mouse spinal cord," *The Journal of Cell Biology*, vol. 60, no. 3, pp. 664–672, 1974.

[44] J. E. Vaughn, T. Sims, and M. Nakashima, "A comparison of the early development of axodendritic and axosomatic synapses upon embryonic mouse spinal motor neurons," *Journal of Comparative Neurology*, vol. 175, no. 1, pp. 79–100, 1977.

[45] J. E. Vaughn, R. P. Barber, and T. J. Sims, "Dendritic development and preferential growth into synaptogenic fields: a quantitative study of Golgi-impregnated spinal motor neurons," *Synapse*, vol. 2, no. 1, pp. 69–78, 1988.

[46] J. E. Vaughn, "Fine structure of synaptogenesis in the vertebrate central nervous system," *Synapse*, vol. 3, no. 3, pp. 255–285, 1989.

[47] M. E. Dailey and S. J. Smith, "The dynamics of dendritic structure in developing hippocampal slices," *Journal of Neuroscience*, vol. 16, no. 9, pp. 2983–2994, 1996.

[48] H. G. Goshgarian and J. A. Rafols, "The ultrastructure and synaptic architecture of phrenic motor neurons in the spinal cord of the adult rat," *Journal of Neurocytology*, vol. 13, no. 1, pp. 85–109, 1984.

[49] J. R. O'Kusky, "Postnatal changes in the numerical density and total number of asymmetric and symmetric synapses in the hypoglossal nucleus of the rat," *Developmental Brain Research*, vol. 108, no. 1-2, pp. 179–191, 1998.

[50] B. Ulfhake, U. Arvidsson, S. Cullheim et al., "an ultrastructural study of 5-hydroxytryptamine-, thyrotropin-releasing hormone- and substance P-immunoreactive axonal boutons in the motor nucleus of spinal cord segments L7-S1 in the adult cat," *Neuroscience*, vol. 23, no. 3, pp. 917–929, 1987.

[51] X. S. T. Wan, J. Q. Trojanowski, J. O. Gonatas, and C. N. Liu, "Cytoarchitecture of the extranuclear and commissural dendrites of hypoglossal nucleus neurons as revealed by conjugates of horseradish peroxidase with cholera toxin," *Experimental Neurology*, vol. 78, no. 1, pp. 167–175, 1982.

[52] N. G. Forger and S. M. Breedlove, "Seasonal variation in mammalian striated muscle mass and motoneuron morphology," *Journal of Neurobiology*, vol. 18, no. 2, pp. 155–165, 1987.

[53] S. M. Altschuler, X. Bao, and R. R. Miselis, "Dendritic architecture of hypoglossal motoneurons projecting to extrinsic tongue musculature in the rat," *Journal of Comparative Neurology*, vol. 342, no. 4, pp. 538–550, 1994.

[54] L. D. Aldes, "Subcompartmental organization of the ventral (protrusor) compartment in the hypoglossal nucleus of the rat," *Journal of Comparative Neurology*, vol. 353, no. 1, pp. 89–108, 1995.

[55] C. B. Mantilla, W.-Z. Zhan, and G. C. Sieck, "Retrograde labeling of phrenic motoneurons by intrapleural injection," *Journal of Neuroscience Methods*, vol. 182, no. 2, pp. 244–249, 2009.

[56] L. G. Brock, J. S. Coombs, and J. C. Eccles, "The recording of potentials from motoneurones with an intracellular electrode," *The Journal of Physiology*, vol. 117, no. 4, pp. 431–460, 1952.

[57] R. C. Thomas and V. J. Wilson, "Marking single neurons by staining with intracellular recording microelectrodes," *Science*, vol. 151, no. 3717, pp. 1538–1539, 1966.

[58] J. N. Barrett and K. Graubard, "Fluorescent staining of cat motoneurons in vivo with beveled micropipettes," *Brain Research*, vol. 18, no. 3, pp. 565–568, 1970.

[59] J.-O. Kellerth, "Intracellular staining of cat spinal motoneurons with procion yellow for ultrastructural studies," *Brain Research*, vol. 50, no. 2, pp. 415–418, 1973.

[60] M. D. Egger, N. C. Freeman, and E. Proshansky, "Morphology of spinal motoneurones mediating a cutaneous spinal reflex in the cat," *The Journal of Physiology*, vol. 306, no. 1, pp. 349–363, 1980.

[61] P. K. Rose and F. J. R. Richmond, "White-matter dendrites in the upper cervical spinal cord of the adult cat: a light and electron microscopic study," *Journal of Comparative Neurology*, vol. 199, no. 2, pp. 191–203, 1981.

[62] B. Ulfhake and S. Cullheim, "Postnatal development of cat hind limb motoneurons. II: in vivo morphology of dendritic growth cones and the maturation of dendrite morphology," *Journal of Comparative Neurology*, vol. 278, no. 1, pp. 88–102, 1988.

[63] M. D. David Egger and L. Daniel Egger, "Quantitative morphological analysis of spinal motoneurons," *Brain Research*, vol. 253, no. 1-2, pp. 19–30, 1982.

[64] A. Yoshida, K. Tsuru, Y. Mitsuhiro, K. Otani, and Y. Shigenaga, "Morphology of masticatory motoneurons stained intracellularly with horseradish peroxidase," *Brain Research*, vol. 416, no. 2, pp. 393–401, 1987.

[65] Y. Fukunishi, Y. Nagase, A. Yoshida et al., "Quantitative analysis of the dendritic architectures of cat hypoglossal motoneurons stained intracellularly with horseradish peroxidase," *Journal of Comparative Neurology*, vol. 405, no. 3, pp. 345–358, 1999.

[66] D. L. Becker, J. Dekkers, R. Navarrete, C. R. Green, and J. E. Cook, "Enhancing the laser scanning confocal microscopic visualization of Lucifer yellow filled cells in whole-mounted tissue," *Scanning Microscopy*, vol. 5, no. 3, pp. 619–624, 1991.

[67] J. Dekkers, D. L. Becker, J. E. Cook, and R. Navarrete, "Early postnatal changes in the somatodendritic morphology of ankle flexor motoneurons in the rat," *European Journal of Neuroscience*, vol. 6, no. 1, pp. 87–97, 1994.

[68] B. Kruszewska, J. Lipski, and R. Kanjhan, "An electrophysiological and morphological study of esophageal motoneurons in rats," *The American Journal of Physiology*, vol. 266, no. 2, part 2, pp. R622–R632, 1994.

[69] J. Lipski, X. Zhang, B. Kruszewska, and R. Kanjhan, "Morphological study of long axonal projections of ventral medullary inspiratory neurons in the rat," *Brain Research*, vol. 640, no. 1-2, pp. 171–184, 1994.

[70] P. A. Nunez-Abades, F. He, G. Barrionuevo, and W. E. Cameron, "Morphology of developing rat genioglossal motoneurons studied in vitro: changes in length, branching pattern, and spatial distribution of dendrites," *Journal of Comparative Neurology*, vol. 339, no. 3, pp. 401–420, 1994.

[71] P. A. Nunez-Abades and W. E. Cameron, "Morphology of developing rat genioglossal motoneurons studied in vitro: relative changes in diameter and surface area of somata and dendrites," *Journal of Comparative Neurology*, vol. 353, no. 1, pp. 129–142, 1995.

[72] W. E. Cameron, D. B. Averill, and A. J. Berger, "Morphology of cat phrenic motoneurons as revealed by intracellular injection of horseradish peroxidase," *Journal of Comparative Neurology*, vol. 219, no. 1, pp. 70–80, 1983.

[73] W. E. Cameron, F. He, P. Kalipatnapu, J. S. Jodkowski, and R. D. Guthrie, "Morphometric analysis of phrenic motoneurons in the cat during postnatal development," *Journal of Comparative Neurology*, vol. 314, no. 4, pp. 763–776, 1991.

[74] W. Y. Ma and L. L. Vacca-Galloway, "Reduced branching and length of dendrites detected in cervical spinal cord motoneurons of Wobbler mouse, a model for inherited motoneuron disease," *Journal of Comparative Neurology*, vol. 311, no. 2, pp. 210–222, 1991.

[75] F. M. Inglis, F. Furia, K. E. Zuckerman, S. M. Strittmatter, and R. G. Kalb, "The role of nitric oxide and NMDA receptors in the development of motor neuron dendrites," *Journal of Neuroscience*, vol. 18, no. 24, pp. 10493–10501, 1998.

[76] F. M. Inglis, K. E. Zuckerman, and R. G. Kalb, "Experience-dependent development of spinal motor neurons," *Neuron*, vol. 26, no. 2, pp. 299–305, 2000.

[77] F. M. Inglis, R. Crockett, S. Korada, W. C. Abraham, M. Hollmann, and R. G. Kalb, "The AMPA receptor subunit GluR1 regulates dendritic architecture of motor neurons," *Journal of Neuroscience*, vol. 22, no. 18, pp. 8042–8051, 2002.

[78] Z. Zhang, D. M. Casey, J.-P. Julien, and Z. Xu, "Normal dendritic arborization in spinal motoneurons requires neurofilament subunit L," *Journal of Comparative Neurology*, vol. 450, no. 2, pp. 144–152, 2002.

[79] M. L. Feldman and A. Peters, "A technique for estimating total spine numbers on Golgi-impregnated dendrites," *Journal of Comparative Neurology*, vol. 188, no. 4, pp. 527–542, 1979.

[80] D. C. Gordon and F. J. R. Richmond, "Topography in the phrenic motoneuron nucleus demonstrated by retrograde multiple-labelling techniques," *Journal of Comparative Neurology*, vol. 292, no. 3, pp. 424–434, 1990.

[81] G. Feng, R. H. Mellor, M. Bernstein et al., "Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP," *Neuron*, vol. 28, no. 1, pp. 41–51, 2000.

[82] Y. Li, D. Brewer, R. E. Burke, and G. A. Ascoli, "Developmental changes in spinal motoneuron dendrites in neonatal mice," *Journal of Comparative Neurology*, vol. 483, no. 3, pp. 304–317, 2005.

[83] J. Amendola and J. Durand, "Morphological differences between wild-type and transgenic superoxide dismutase 1 lumbar motoneurons in postnatal mice," *Journal of Comparative Neurology*, vol. 511, no. 3, pp. 329–341, 2008.

[84] A. A. Filipchuk and J. Durand, "Postnatal dendritic development in lumbar motoneurons in mutant superoxide dismutase 1 mouse model of amyotrophic lateral sclerosis," *Neuroscience*, vol. 209, pp. 144–154, 2012.

[85] R. Kanjhan and B. Sivyer, "Two types of ON direction-selective ganglion cells in rabbit retina," *Neuroscience Letters*, vol. 483, no. 2, pp. 105–109, 2010.

[86] R. Kanjhan, M. J. Fogarty, P. G. Noakes, and M. C. Bellingham, "Developmental changes in the morphology of mouse hypoglossal motor neurons," *Brain Structure and Function*, In press.

[87] N. Stifani, "Motor neurons and the generation of spinal motor neuron diversity," *Frontiers in Cellular Neuroscience*, vol. 8, article 293, 2014.

[88] T. M. Jessell, "Neuronal specification in the spinal cord: inductive signals and transcriptional codes," *Nature Reviews Genetics*, vol. 1, no. 1, pp. 20–29, 2000.

[89] K. C. Kanning, A. Kaplan, and C. E. Henderson, "Motor neuron diversity in development and disease," *Annual Review of Neuroscience*, vol. 33, pp. 409–440, 2010.

[90] S. Ashrafi, M. Lalancette-Hébert, A. Friese et al., "Wnt7A identifies embryonic gamma-motor neurons and reveals early postnatal dependence of gamma-motor neurons on a muscle spindle-derived signal," *The Journal of Neuroscience*, vol. 32, no. 25, pp. 8725–8731, 2012.

[91] B. J. Wainger, E. Kiskinis, C. Mellin et al., "Intrinsic membrane hyperexcitability of amyotrophic lateral sclerosis patient-derived motor neurons," *Cell Reports*, vol. 7, no. 1, pp. 1–11, 2014.

[92] S. Wiese and A. Faissner, "The role of extracellular matrix in spinal cord development," *Experimental Neurology*, 2015.

[93] V. Luria and E. Laufer, "Lateral motor column axons execute a ternary trajectory choice between limb and body tissues," *Neural Development*, vol. 2, article 13, 2007.

[94] D. Bonanomi and S. L. Pfaff, "Motor axon pathfinding," *Cold Spring Harbor Perspectives in Biology*, vol. 2, no. 3, Article ID a001735, 2010.

[95] E. G. T. Liddel and C. S. Sherrington, "Recruitment and some other factors of reflex inhibition," *Proceedings of the Royal Society of London B*, vol. 97, no. 686, pp. 488–518, 1925.

[96] R. E. Burke, "Revisiting the notion of 'motor unit types'," *Progress in Brain Research*, vol. 123, pp. 167–175, 1999.

[97] M. Manuel and D. Zytnicki, "Alpha, beta and gamma motoneurons: functional diversity in the motor systems final pathway," *Journal of Integrative Neuroscience*, vol. 10, no. 3, pp. 243–276, 2011.

[98] G. D. Funk, R. Kanjhan, C. Walsh et al., "P2 receptor excitation of rodent hypoglossal motoneuron activity in vitro and in vivo: a molecular physiological analysis," *Journal of Neuroscience*, vol. 17, no. 16, pp. 6325–6337, 1997.

[99] M. C. Bellingham and G. D. Funk, "Cholinergic modulation of respiratory brain-stem neurons and its function in sleep-wake state determination," *Clinical and Experimental Pharmacology and Physiology*, vol. 27, no. 1-2, pp. 132–137, 2000.

[100] C. J. Heckman, J. J. Kuo, and M. D. Johnson, "Synaptic integration in motoneurons with hyper-excitable dendrites," *Canadian Journal of Physiology and Pharmacology*, vol. 82, no. 8-9, pp. 549–555, 2004.

[101] A. S. Hyngstrom, M. D. Johnson, and C. J. Heckman, "Summation of excitatory and inhibitory synaptic inputs by motoneurons with highly active dendrites," *Journal of Neurophysiology*, vol. 99, no. 4, pp. 1643–1652, 2008.

[102] A.-E. Allain, H. Le Corronc, A. Delpy et al., "Maturation of the GABAergic transmission in normal and pathologic motoneurons," *Neural Plasticity*, vol. 2011, Article ID 905624, 13 pages, 2011.

[103] A. J. Berger, D. A. Bayliss, M. C. Bellingham, M. Umemiya, and F. Viana, "Postnatal development of hypoglossal motoneuron intrinsic properties," *Advances in Experimental Medicine and Biology*, vol. 381, pp. 63–71, 1996.

[104] E. A. L. Muennich and R. E. W. Fyffe, "Focal aggregation of voltage-gated, Kv2.1 subunit-containing, potassium channels at synaptic sites in rat spinal motoneurones," *The Journal of Physiology*, vol. 554, no. 3, pp. 673–685, 2004.

[105] L. Larsson and T. Ansved, "Effects of ageing on the motor unit," *Progress in Neurobiology*, vol. 45, no. 5, pp. 397–458, 1995.

[106] M. V. Yasvoina, B. Genç, J. H. Jara et al., "eGFP expression under UCHL1 promoter genetically labels corticospinal motor neurons and a subpopulation of degeneration-resistant spinal motor neurons in an ALS mouse model," *Journal of Neuroscience*, vol. 33, no. 18, pp. 7890–7904, 2013.

[107] S. K. Vaughan, Z. Kemp, T. Hatzipetros, F. Vieira, and G. Valdez, "Degeneration of proprioceptive sensory nerve endings in mice harboring amyotrophic lateral sclerosis-causing mutations," *Journal of Comparative Neurology*, 2015.

[108] M. Dadon-Nachum, E. Melamed, and D. Offen, "The "dying-back" phenomenon of motor neurons in ALS," *Journal of Molecular Neuroscience*, vol. 43, no. 3, pp. 470–477, 2011.

[109] C. G. dos Remedios, D. Chhabra, M. Kekic et al., "Actin binding proteins: regulation of cytoskeletal microfilaments," *Physiological Reviews*, vol. 83, no. 2, pp. 433–473, 2003.

[110] J. C. Fiala, M. Feinberg, V. Popov, and K. M. Harris, "Synaptogenesis via dendritic filopodia in developing hippocampal area CA1," *Journal of Neuroscience*, vol. 18, no. 21, pp. 8900–8911, 1998.

[111] J. Grutzendler, N. Kasthuri, and W.-B. Gan, "Long-term dendritic spine stability in the adult cortex," *Nature*, vol. 420, no. 6917, pp. 812–816, 2002.

[112] C. A. Heckman and H. K. Plummer III, "Filopodia as sensors," *Cellular Signalling*, vol. 25, no. 11, pp. 2298–2311, 2013.

[113] D. P. Purpura, "Dendritic spine 'dysgenesis' and mental retardation," *Science*, vol. 186, no. 4169, pp. 1126–1128, 1974.

[114] P. K. Rose, V. MacDermid, M. Joshi, and M. Neuber-Hess, "Emergence of axons from distal dendrites of adult mammalian neurons following a permanent axotomy," *European Journal of Neuroscience*, vol. 13, no. 6, pp. 1166–1176, 2001.

[115] Y. Ohta, N. Suzuki, S. Nakamura, J. H. Hartwig, and T. P. Stossel, "The small GTPase RalA targets filamin to induce filopodia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 5, pp. 2122–2128, 1999.

[116] B. W. Luikart, W. Zhang, G. A. Wayman, C.-H. Kwon, G. L. Westbrook, and L. F. Parada, "Neurotrophin-dependent dendritic filopodial motility: a convergence on PI3K signaling," *Journal of Neuroscience*, vol. 28, no. 27, pp. 7006–7012, 2008.

[117] A. Yoshii and M. Constantine-Paton, "Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease," *Developmental Neurobiology*, vol. 70, no. 5, pp. 304–322, 2010.

[118] S.-L. Guo, G.-H. Tan, S. Li et al., "Serum inducible kinase is a positive regulator of cortical dendrite development and is required for BDNF-promoted dendritic arborization," *Cell Research*, vol. 22, no. 2, pp. 387–398, 2012.

[119] A. B. Bohil, B. W. Robertson, and R. E. Cheney, "Myosin-X is a molecular motor that functions in filopodia formation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 33, pp. 12411–12416, 2006.

[120] E. Cherubini, J. L. Gaiarsa, and Y. Ben-Ari, "GABA: an excitatory transmitter in early postnatal life," *Trends in Neurosciences*, vol. 14, no. 12, pp. 515–519, 1991.

[121] C. Marchetti, S. Pagnotta, R. Donato, and A. Nistri, "Inhibition of spinal or hypoglossal motoneurons of the newborn rat by glycine or GABA," *European Journal of Neuroscience*, vol. 15, no. 6, pp. 975–983, 2002.

[122] G. Örnung, O. P. Ottersen, S. Cullheim, and B. Ulfhake, "Distribution of glutamate-, glycine- and GABA-immunoreactive nerve terminals on dendrites in the cat spinal motor nucleus," *Experimental Brain Research*, vol. 118, no. 4, pp. 517–532, 1998.

[123] M. E. Larkum, T. Launey, A. Dityatev, and H.-R. Lüscher, "Integration of excitatory postsynaptic potentials in dendrites of motoneurons of rat spinal cord slice cultures," *Journal of Neurophysiology*, vol. 80, no. 2, pp. 924–935, 1998.

[124] M. J. Higley, "Localized GABAergic inhibition of dendritic Ca^{2+} signalling," *Nature Reviews Neuroscience*, vol. 15, no. 9, pp. 567–572, 2014.

[125] B. Sivyer and S. R. Williams, "Direction selectivity is computed by active dendritic integration in retinal ganglion cells," *Nature Neuroscience*, vol. 16, no. 12, pp. 1848–1856, 2013.

[126] F. E. Müllner, C. J. Wierenga, and T. Bonhoeffer, "Precision of inhibition: dendritic inhibition by individual gabaergic synapses on hippocampal pyramidal cells is confined in space and time," *Neuron*, vol. 87, no. 3, pp. 576–589, 2015.

[127] G. B. Banks, R. Kanjhan, S. Wiese et al., "Glycinergic and GABAergic synaptic activity differentially regulate motoneuron survival and skeletal muscle innervation," *Journal of Neuroscience*, vol. 25, no. 5, pp. 1249–1259, 2005.

[128] M. Berry and P. Bradley, "The growth of the dendritic trees of Purkinje cells in the cerebellum of the rat," *Brain Research*, vol. 112, no. 1, pp. 1–35, 1976.

[129] N. E. Ziv and S. J. Smith, "Evidence for a role of dendritic filopodia in synaptogenesis and spine formation," *Neuron*, vol. 17, no. 1, pp. 91–102, 1996.

[130] Z. Parnass, A. Tashiro, and R. Yuste, "Analysis of spine morphological plasticity in developing hippocampal pyramidal neurons," *Hippocampus*, vol. 10, no. 5, pp. 561–568, 2000.

[131] A. Peters and I. R. Kaiserman-Abramof, "The small pyramidal neuron of the rat cerebral cortex. The perikaryon, dendrites and spines," *American Journal of Anatomy*, vol. 127, no. 4, pp. 321–355, 1970.

[132] M. Miller and A. Peters, "Maturation of rat visual cortex. II. A combined Golgi-electron microscope study of pyramidal neurons," *Journal of Comparative Neurology*, vol. 203, no. 4, pp. 555–573, 1981.

[133] R. Prithviraj, K. M. Kelly, R. Espinoza-Lewis, T. Hexom, A. B. Clark, and F. M. Inglis, "Differential regulation of dendrite

complexity by AMPA receptor subunits GluR1 and GluR2 in motor neurons," *Developmental Neurobiology*, vol. 68, no. 2, pp. 247–264, 2008.

[134] J. Grutzendler, G. Yang, F. Pan, C. N. Parkhurst, and W. B. Gan, "Transcranial two-photon imaging of the living mouse brain," *Cold Spring Harbor Protocols*, vol. 2011, no. 9, 2011.

[135] R. P. Skoff and V. Hamburger, "Fine stucure of dendritic and axonal growth cones in embryonic chick spinal cord," *Journal of Comparative Neurology*, vol. 153, no. 2, pp. 107–147, 1974.

[136] X. Tao, A. E. West, W. G. Chen, G. Corfas, and M. E. Greenberg, "A calcium-responsive transcription factor, CaRF, that regulates neuronal activity-dependent expression of BDNF," *Neuron*, vol. 33, no. 3, pp. 383–395, 2002.

[137] M. R. Bennett and J. Lagopoulos, "Stress and trauma: BDNF control of dendritic-spine formation and regression," *Progress in Neurobiology*, vol. 112, pp. 80–99, 2014.

[138] M. Zagrebelsky and M. Korte, "Form follows function: BDNF and its involvement in sculpting the function and structure of synapses," *Neuropharmacology*, vol. 76, pp. 628–638, 2014.

[139] M. Hartmann, R. Heumann, and V. Lessmann, "Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses," *EMBO Journal*, vol. 20, no. 21, pp. 5887–5897, 2001.

[140] T. Verhovshek, L. M. Rudolph, and D. R. Sengelaub, "Brain-derived neurotrophic factor and androgen interactions in spinal neuromuscular systems," *Neuroscience*, vol. 239, pp. 103–114, 2013.

[141] E. N. Ottem, D. J. Bailey, C. L. Jordan, and S. Marc Breedlove, "With a little help from my friends: androgens tap BDNF signaling pathways to alter neural circuits," *Neuroscience*, vol. 239, pp. 124–138, 2013.

[142] L. M. Rudolph and D. R. Sengelaub, "Critical period for estrogen-dependent motoneuron dendrite growth is coincident with ERalpha expression in target musculature," *Developmental Neurobiology*, vol. 73, no. 1, pp. 72–84, 2013.

[143] D. P. Srivastava, K. M. Woolfrey, and P. Penzes, "Insights into rapid modulation of neuroplasticity by brain estrogens," *Pharmacological Reviews*, vol. 65, no. 4, pp. 1318–1350, 2013.

[144] G.-B. Jeong, J. Mojsilovic-Petrovic, M. Boccitto, and R. Kalb, "Signaling events in axons and/or dendrites render motor neurons vulnerable to mutant superoxide dismutase toxicity," *Journal of Neuroscience*, vol. 31, no. 1, pp. 295–299, 2011.

[145] H. Wang, N. K. Liu, Y. P. Zhang et al., "Treadmill training induced lumbar motoneuron dendritic plasticity and behavior recovery in adult rats after a thoracic contusive spinal cord injury," *Experimental Neurology*, vol. 271, pp. 368–378, 2015.

[146] B. Singh, C. Henneberger, D. Betances et al., "Altered balance of glutamatergic/GABAergic synaptic input and associated changes in dendrite morphology after BDNF expression in BDNF-deficient hippocampal neurons," *The Journal of Neuroscience*, vol. 26, no. 27, pp. 7189–7200, 2006.

[147] F. Metzger, S. Wiese, and M. Sendtner, "Effect of glutamate on dendritic growth in embryonic rat motoneurons," *The Journal of Neuroscience*, vol. 18, no. 5, pp. 1735–1742, 1998.

[148] R. Yuste and T. Bonhoeffer, "Morphological changes in dendritic spines associated with long-term synaptic plasticity," *Annual Review of Neuroscience*, vol. 24, pp. 1071–1089, 2001.

[149] M. Segal and P. Andersen, "Dendritic spines shaped by synaptic activity," *Current Opinion in Neurobiology*, vol. 10, no. 5, pp. 582–586, 2000.

[150] R. G. Kalb, "Regulation of motor neuron dendrite growth by NMDA receptor activation," *Development*, vol. 120, no. 11, pp. 3063–3071, 1994.

[151] R. Prithviraj and F. M. Inglis, "Expression of the N-methyl-d-aspartate receptor subunit NR3B regulates dendrite morphogenesis in spinal motor neurons," *Neuroscience*, vol. 155, no. 1, pp. 145–153, 2008.

[152] L. Zhang, J. Schessl, M. Werner et al., "Role of GluR1 in activity-dependent motor system development," *Journal of Neuroscience*, vol. 28, no. 40, pp. 9953–9968, 2008.

[153] W. J. Alilain and H. G. Goshgarian, "Glutamate receptor plasticity and activity-regulated cytoskeletal associated protein regulation in the phrenic motor nucleus may mediate spontaneous recovery of the hemidiaphragm following chronic cervical spinal cord injury," *Experimental Neurology*, vol. 212, no. 2, pp. 348–357, 2008.

[154] C. Duch, F. Vonhoff, and S. Ryglewski, "Dendrite elongation and dendritic branching are affected separately by different forms of intrinsic motoneuron excitability," *Journal of Neurophysiology*, vol. 100, no. 5, pp. 2525–2536, 2008.

[155] L. A. Colgan and R. Yasuda, "Plasticity of dendritic spines: subcompartmentalization of signaling," *Annual Review of Physiology*, vol. 76, pp. 365–385, 2014.

[156] K. Kim, G. Lakhanpal, H. Lu et al., "A Temporary gating of actin remodeling during synaptic plasticity consists of the interplay between the kinase and structural functions of CaMKII," *Neuron*, vol. 87, no. 4, pp. 813–826, 2015.

[157] A. Dunaevsky, A. Tashiro, A. Majewska, C. Mason, and R. Yuste, "Developmental regulation of spine motility in the mammalian central nervous system," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 23, pp. 13438–13443, 1999.

[158] M. Fischer, S. Kaech, U. Wagner, H. Brinkhaus, and A. Matus, "Glutamate receptors regulate actin-based plasticity in dendritic spines," *Nature Neuroscience*, vol. 3, no. 9, pp. 887–894, 2000.

[159] Z. Xie, D. P. Srivastava, H. Photowala et al., "Kalirin-7 controls activity-dependent structural and functional plasticity of dendritic spines," *Neuron*, vol. 56, no. 4, pp. 640–656, 2007.

[160] A. Alves-Sampaio, J. A. Troca-Marín, and M. L. Montesinos, "NMDA-Mediated regulation of DSCAM dendritic local translation is lost in a mouse model of Down's syndrome," *Journal of Neuroscience*, vol. 30, no. 40, pp. 13537–13548, 2010.

[161] K. R. Maynard and E. Stein, "DSCAM contributes to dendrite arborization and spine formation in the developing cerebral cortex," *Journal of Neuroscience*, vol. 32, no. 47, pp. 16637–16650, 2012.

[162] K. M. Hutchinson, F. Vonhoff, and C. Duch, "Dscam1 is required for normal dendrite growth and branching but not for dendritic spacing in *Drosophila motoneurons*," *The Journal of Neuroscience*, vol. 34, no. 5, pp. 1924–1931, 2014.

[163] S. Ryglewski, D. Kadas, K. Hutchinson, N. Schuetzler, F. Vonhoff, and C. Duch, "Dendrites are dispensable for basic motoneuron function but essential for fine tuning of behavior," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 50, pp. 18049–18054, 2014.

[164] H. Togashi, K. Abe, A. Mizoguchi, K. Takaoka, O. Chisaka, and M. Takeichi, "Cadherin regulates dendritic spine morphogenesis," *Neuron*, vol. 35, no. 1, pp. 77–89, 2002.

[165] E. Seong, L. Yuan, and J. Arikkath, "Cadherins and catenins in dendrite and synapse morphogenesis," *Cell Adhesion & Migration*, vol. 9, no. 3, pp. 202–213, 2014.

[166] W. J. Bian, W. Miao, S. He, Z. Qiu, and X. Yu, "Coordinated spine pruning and maturation mediated by inter-spine competition

for cadherin/catenin complexes," *Cell*, vol. 162, no. 4, pp. 808–822, 2015.

[167] J. A. Weiner, X. Wang, J. C. Tapia, and J. R. Sanes, "Gamma protocadherins are required for synaptic development in the spinal cord," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 1, pp. 8–14, 2005.

[168] J. L. Lefebvre, D. Kostadinov, W. V. Chen, T. Maniatis, and J. R. Sanes, "Protocadherins mediate dendritic self-avoidance in the mammalian nervous system," *Nature*, vol. 488, no. 7412, pp. 517–521, 2012.

[169] S. Hayashi and M. Takeichi, "Emerging roles of protocadherins: from self-avoidance to enhancement of motility," *Journal of Cell Science*, vol. 128, no. 8, pp. 1455–1464, 2015.

[170] A. D. Levy, M. H. Omar, and A. J. Koleske, "Extracellular matrix control of dendritic spine and synapse structure and plasticity in adulthood," *Frontiers in Neuroanatomy*, vol. 8, article 116, 2014.

[171] W. J. Alilain, K. P. Horn, H. Hu, T. E. Dick, and J. Silver, "Functional regeneration of respiratory pathways after spinal cord injury," *Nature*, vol. 475, no. 7355, pp. 196–200, 2011.

[172] J. G. Hanley, "Actin-dependent mechanisms in AMPA receptor trafficking," *Frontiers in Cellular Neuroscience*, vol. 8, article 381, 2014.

[173] M. Kneussel and W. Wagner, "Myosin motors at neuronal synapses: drivers of membrane transport and actin dynamics," *Nature Reviews Neuroscience*, vol. 14, no. 4, pp. 233–247, 2013.

[174] L. T. Hao, M. Wolman, M. Granato, and C. E. Beattie, "Survival motor neuron affects plastin 3 protein levels leading to motor defects," *Journal of Neuroscience*, vol. 32, no. 15, pp. 5074–5084, 2012.

[175] C.-H. Wu, C. Fallini, N. Ticozzi et al., "Mutations in the profilin 1 gene cause familial amyotrophic lateral sclerosis," *Nature*, vol. 488, no. 7412, pp. 499–503, 2012.

[176] N. D'Ambrosi, S. Rossi, V. Gerbino, and M. Cozzolino, "Rac1 at the crossroad of actin dynamics and neuroinflammation in Amyotrophic Lateral Sclerosis," *Frontiers in Cellular Neuroscience*, vol. 8, article 279, 2014.

[177] A. E. Renton, A. Chiò, and B. J. Traynor, "State of play in amyotrophic lateral sclerosis genetics," *Nature Neuroscience*, vol. 17, no. 1, pp. 17–23, 2014.

[178] S. Tisdale and L. Pellizzoni, "Disease mechanisms and therapeutic approaches in spinal muscular atrophy," *Journal of Neuroscience*, vol. 35, no. 23, pp. 8691–8700, 2015.

[179] J. T. Trachtenberg, B. E. Chen, G. W. Knott et al., "Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex," *Nature*, vol. 420, no. 6917, pp. 788–794, 2002.

[180] H. Markram, J. Lübke, M. Frotscher, A. Roth, and B. Sakmann, "Physiology and anatomy of synaptic connections between thick tufted pyramidal neurones in the developing rat neocortex," *The Journal of Physiology*, vol. 500, no. 2, pp. 409–440, 1997.

[181] H. Markram, J. Lübke, M. Frotscher, and B. Sakmann, "Regulation of synaptic efficacy by coincidence of postsynaptic APs and EPSPs," *Science*, vol. 275, no. 5297, pp. 213–215, 1997.

[182] M. C. Bellingham, "Pre- and postsynaptic mechanisms underlying inhibition of hypoglossal motor neuron excitability by riluzole," *Journal of Neurophysiology*, vol. 110, no. 5, pp. 1047–1061, 2013.

[183] R. Kanjhan, J. Lipski, B. Kruszewska, and W. Rong, "A comparative study of pre-sympathetic and Bötzinger neurons in the rostral ventrolateral medulla (RVLM) of the rat," *Brain Research*, vol. 699, no. 1, pp. 19–32, 1995.

[184] A. Nistri, K. Ostroumov, E. Sharifullina, and G. Taccola, "Tuning and playing a motor rhythm: how metabotropic glutamate receptors orchestrate generation of motor patterns in the mammalian central nervous system," *Journal of Physiology*, vol. 572, part 2, pp. 323–334, 2006.

[185] S. Grillner and T. M. Jessell, "Measured motion: searching for simplicity in spinal locomotor networks," *Current Opinion in Neurobiology*, vol. 19, no. 6, pp. 572–586, 2009.

[186] M. Fischer, S. Kaech, D. Knutti, and A. Matus, "Rapid actin-based plasticity in dendritic spines," *Neuron*, vol. 20, no. 5, pp. 847–854, 1998.

[187] A. Dunaevsky and C. A. Mason, "Spine motility: a means towards an end?" *Trends in Neurosciences*, vol. 26, no. 3, pp. 155–160, 2003.

[188] S. Oray, A. Majewska, and M. Sur, "Effects of synaptic activity on dendritic spine motility of developing cortical layer V pyramidal neurons," *Cerebral Cortex*, vol. 16, no. 5, pp. 730–741, 2006.

[189] A. Holtmaat, L. Wilbrecht, G. W. Knott, E. Welker, and K. Svoboda, "Experience-dependent and cell-type-specific spine growth in the neocortex," *Nature*, vol. 441, no. 7096, pp. 979–983, 2006.

[190] U. V. Nägerl, G. Köstinger, J. C. Anderson, K. A. C. Martin, and T. Bonhoeffer, "Protracted synaptogenesis after activity-dependent spinogenesis in hippocampal neurons," *Journal of Neuroscience*, vol. 27, no. 30, pp. 8149–8156, 2007.

[191] A. Holtmaat and K. Svoboda, "Experience-dependent structural synaptic plasticity in the mammalian brain," *Nature Reviews Neuroscience*, vol. 10, no. 9, pp. 647–658, 2009.

[192] B. van Zundert, M. H. Peuscher, M. Hynynen et al., "Neonatal neuronal circuitry shows hyperexcitable disturbance in a mouse model of the adult-onset neurodegenerative disease amyotrophic lateral sclerosis," *Journal of Neuroscience*, vol. 28, no. 43, pp. 10864–10874, 2008.

[193] M. Sendtner, "Motoneuron disease," in *Neurotrophic Factors*, vol. 220 of *Handbook of Experimental Pharmacology*, pp. 411–441, Springer, Berlin, Germany, 2014.

[194] S. Vucic, J. D. Rothstein, and M. C. Kiernan, "Advances in treating amyotrophic lateral sclerosis: insights from pathophysiological studies," *Trends in Neurosciences*, vol. 37, no. 8, pp. 433–442, 2014.

[195] B. Lendvai, E. A. Stern, B. Chen, and K. Svoboda, "Experience-dependent plasticity of dendritic spines in the developing rat barrel cortex *in vivo*," *Nature*, vol. 404, no. 6780, pp. 876–881, 2000.

[196] D. Meyer, T. Bonhoeffer, and V. Scheuss, "Balance and stability of synaptic structures during synaptic plasticity," *Neuron*, vol. 82, no. 2, pp. 430–443, 2014.

[197] M. Maletic-Savatic, R. Malinow, and K. Svoboda, "Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity," *Science*, vol. 283, no. 5409, pp. 1923–1927, 1999.

[198] A. J. G. D. Holtmaat, J. T. Trachtenberg, L. Wilbrecht et al., "Transient and persistent dendritic spines in the neocortex in vivo," *Neuron*, vol. 45, no. 2, pp. 279–291, 2005.

[199] L. Luo, T. K. Hensch, L. Ackerman, S. Barbel, L. Y. Jan, and Y. N. Jan, "Differential effects of the Rac GTPase on Purkinje cell axons and dendritic trunks and spines," *Nature*, vol. 379, no. 6568, pp. 837–840, 1996.

[200] A. Y. Nakayama, M. B. Harms, and L. Luo, "Small GTPases Rac and Rho in the maintenance of dendritic spines and branches in

hippocampal pyramidal neurons," *Journal of Neuroscience*, vol. 20, no. 14, pp. 5329–5338, 2000.

[201] A. Tashiro, A. Minden, and R. Yuste, "Regulation of dendritic spine morphology by the Rho family of small GTPases: antagonistic roles of Rac and Rho," *Cerebral Cortex*, vol. 10, no. 10, pp. 927–938, 2000.

[202] L. Luo, "Rho GTPases in neuronal morphogenesis," *Nature Reviews Neuroscience*, vol. 1, no. 3, pp. 173–180, 2000.

[203] H. Murakoshi, H. Wang, and R. Yasuda, "Local, persistent activation of Rho GTPases during plasticity of single dendritic spines," *Nature*, vol. 472, no. 7341, pp. 100–104, 2011.

[204] T. R. Stankiewicz and D. A. Linseman, "Rho family GTPases: key players in neuronal development, neuronal survival, and neurodegeneration," *Frontiers in Cellular Neuroscience*, vol. 8, article 314, 2014.

[205] A. Tashiro and R. Yuste, "Role of Rho GTPases in the morphogenesis and motility of dendritic spines," *Methods in Enzymology*, vol. 439, pp. 285–302, 2008.

[206] C. Sotelo, "Purkinje cell ontogeny: formation and maintenance of spines," *Progress in Brain Research*, vol. 48, pp. 149–170, 1978.

[207] C. Sotelo, "Cerebellar synaptogenesis: what we can learn from mutant mice," *Journal of Experimental Biology*, vol. 153, pp. 225–249, 1990.

[208] K. M. Wiens, H. Lin, and D. Liao, "Rac1 induces the clustering of AMPA receptors during spinogenesis," *Journal of Neuroscience*, vol. 25, no. 46, pp. 10627–10636, 2005.

[209] G. W. Knott, A. Holtmaat, L. Wilbrecht, E. Welker, and K. Svoboda, "Spine growth precedes synapse formation in the adult neocortex in vivo," *Nature Neuroscience*, vol. 9, no. 9, pp. 1117–1124, 2006.

[210] P. Rakic, J.-P. Bourgeois, M. F. Eckenhoff, N. Zecevic, and P. S. Goldman-Rakic, "Concurrent overproduction of synapses in diverse regions of the primate cerebral cortex," *Science*, vol. 232, no. 4747, pp. 232–235, 1986.

[211] R. Benavides-Piccione, I. Ballesteros-Yáñez, J. DeFelipe, and R. Yuste, "Cortical area and species differences in dendritic spine morphology," *Journal of Neurocytology*, vol. 31, no. 3–5, pp. 337–346, 2002.

[212] D. W. Cleveland and J. D. Rothstein, "From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS," *Nature Reviews Neuroscience*, vol. 2, no. 11, pp. 806–819, 2001.

[213] M. Jiang, J. E. Schuster, R. Fu, T. Siddique, and C. J. Heckman, "Progressive changes in synaptic inputs to motoneurons in adult sacral spinal cord of a mouse model of amyotrophic lateral sclerosis," *Journal of Neuroscience*, vol. 29, no. 48, pp. 15031–15038, 2009.

[214] S. Vinsant, C. Mansfield, R. Jimenez-Moreno et al., "Characterization of early pathogenesis in the SOD1G93A mouse model of ALS: part II, results and discussion," *Brain and Behavior*, vol. 3, no. 4, pp. 431–457, 2013.

[215] E. Sharifullina and A. Nistri, "Glutamate uptake block triggers deadly rhythmic bursting of neonatal rat hypoglossal motoneurons," *Journal of Physiology*, vol. 572, no. 2, pp. 407–423, 2006.

[216] J. Grosskreutz, L. Van Den Bosch, and B. U. Keller, "Calcium dysregulation in amyotrophic lateral sclerosis," *Cell Calcium*, vol. 47, no. 2, pp. 165–174, 2010.

[217] A. Cifra, F. Nani, and A. Nistri, "Respiratory motoneurons and pathological conditions: lessons from hypoglossal motoneurons challenged by excitotoxic or oxidative stress," *Respiratory Physiology and Neurobiology*, vol. 179, no. 1, pp. 89–96, 2011.

[218] S. S. Leal and C. M. Gomes, "Calcium dysregulation links ALS defective proteins and motor neuron selective vulnerability," *Frontiers in Cellular Neuroscience*, vol. 9, article 225, 2015.

[219] S. Saxena, E. Cabuy, and P. Caroni, "A role for motoneuron subtype-selective ER stress in disease manifestations of FALS mice," *Nature Neuroscience*, vol. 12, no. 5, pp. 627–636, 2009.

[220] M. Manuel, Y. Li, S. M. ElBasiouny et al., "NMDA induces persistent inward and outward currents that cause rhythmic bursting in adult rodent motoneurons," *Journal of Neurophysiology*, vol. 108, no. 11, pp. 2991–2998, 2012.

[221] N. Delestrée, M. Manuel, C. Iglesias, S. M. Elbasiouny, C. J. Heckman, and D. Zytnicki, "Adult spinal motoneurones are not hyperexcitable in a mouse model of inherited amyotrophic lateral sclerosis," *The Journal of Physiology*, vol. 592, no. 7, pp. 1687–1703, 2014.

[222] T. Prell, J. Lautenschläger, and J. Grosskreutz, "Calcium-dependent protein folding in amyotrophic lateral sclerosis," *Cell Calcium*, vol. 54, no. 2, pp. 132–143, 2013.

[223] A.-C. Devlin, K. Burr, S. Borooah et al., "Human iPSC-derived motoneurons harbouring TARDBP or C9ORF72 ALS mutations are dysfunctional despite maintaining viability," *Nature Communications*, vol. 6, article 5999, 2015.

[224] F. Brocard, N. A. Shevtsova, M. Bouhadfane et al., "Activity-dependent changes in extracellular Ca^{2+} and K^+ reveal pacemakers in the spinal locomotor-related network," *Neuron*, vol. 77, no. 6, pp. 1047–1054, 2013.

[225] M. F. Ireland, G. D. Funk, and M. C. Bellingham, "Muscarinic acetylcholine receptors enhance neonatal mouse hypoglossal motoneuron excitability in vitro," *Journal of Applied Physiology*, vol. 113, no. 7, pp. 1024–1039, 2012.

[226] G. B. Miles, Y. Dai, and R. M. Brownstone, "Mechanisms underlying the early phase of spike frequency adaptation in mouse spinal motoneurons," *Journal of Physiology*, vol. 566, no. 2, pp. 519–532, 2005.

[227] E. M. Talley, Q. Lei, J. E. Sirois, and D. A. Bayliss, "TASK-1, a two-pore domain K^+ channel, is modulated by multiple neurotransmitters in motoneurons," *Neuron*, vol. 25, no. 2, pp. 399–410, 2000.

[228] R. Kanjhan, A. M. Anselme, P. G. Noakes, and M. C. Bellingham, "Postnatal changes in TASK-1 and TREK-1 expression in rat brain stem and cerebellum," *Neuroreport*, vol. 15, no. 8, pp. 1321–1324, 2004.

[229] J. M. Dell'Orco, A. H. Wasserman, R. Chopra et al., "Neuronal atrophy early in degenerative ataxia is a compensatory mechanism to regulate membrane excitability," *Journal of Neuroscience*, vol. 35, no. 32, pp. 11292–11307, 2015.

[230] K. Yamanaka, S. J. Chun, S. Boillee et al., "Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis," *Nature Neuroscience*, vol. 11, no. 3, pp. 251–253, 2008.

[231] G. Cheung, J. Sibille, J. Zapata, and N. Rouach, "Activity-dependent plasticity of astroglial potassium and glutamate clearance," *Neural Plasticity*, vol. 2015, Article ID 109106, 16 pages, 2015.

[232] G. Le Masson, S. Przedborski, and L. F. Abbott, "A computational model of motor neuron degeneration," *Neuron*, vol. 83, no. 4, pp. 975–988, 2014.

[233] I. J. Edwards, G. Bruce, C. Lawrenson et al., "NA^+/K^+ ATPase alpha3 isoforms are differentially expressed in alpha- and gamma-motoneurons," *The Journal of Neuroscience*, vol. 33, no. 24, pp. 9913–9919, 2013.

[234] G. Azarias, M. Kruusmägi, S. Connor et al., "A specific and essential role for Na,K-ATPase $\alpha 3$ in neurons co-expressing $\alpha 1$ and $\alpha 3$," *The Journal of Biological Chemistry*, vol. 288, no. 4, pp. 2734–2743, 2013.

[235] D. Z. Ellis, J. Rabe, and K. J. Sweadner, "Global loss of Na,K-ATPase and its nitric oxide-mediated regulation in a transgenic mouse model of amyotrophic lateral sclerosis," *Journal of Neuroscience*, vol. 23, no. 1, pp. 43–51, 2003.

[236] Z.-H. Sheng and Q. Cai, "Mitochondrial transport in neurons: impact on synaptic homeostasis and neurodegeneration," *Nature Reviews Neuroscience*, vol. 13, no. 2, pp. 77–93, 2012.

[237] A. F. MacAskill, J. E. Rinholm, A. E. Twelvetrees et al., "Miro1 is a calcium sensor for glutamate receptor-dependent localization of mitochondria at synapses," *Neuron*, vol. 61, no. 4, pp. 541–555, 2009.

[238] T. T. Nguyen, S. S. Oh, D. Weaver et al., "Loss of Miro1-directed mitochondrial movement results in a novel murine model for neuron disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 35, pp. E3631–E3640, 2014.

[239] F. Zhang, W. Wang, S. L. Siedlak et al., "Miro1 deficiency in amyotrophic lateral sclerosis," *Frontiers in Aging Neuroscience*, vol. 7, article 100, 2015.

[240] S. Boillée, K. Yamanaka, C. S. Lobsiger et al., "Onset and progression in inherited ALS determined by motor neurons and microglia," *Science*, vol. 312, no. 5778, pp. 1389–1392, 2006.

[241] J. D. Lee, N. A. Kamaruzaman, J. N. T. Fung et al., "Dysregulation of the complement cascade in the hSOD1G93A transgenic mouse model of amyotrophic lateral sclerosis," *Journal of Neuroinflammation*, vol. 10, article 119, 2013.

[242] D. J. Graber, W. F. Hickey, and B. T. Harris, "Progressive changes in microglia and macrophages in spinal cord and peripheral nerve in the transgenic rat model of amyotrophic lateral sclerosis," *Journal of Neuroinflammation*, vol. 7, article 8, 2010.

[243] T. Sanagi, S. Yuasa, Y. Nakamura et al., "Appearance of phagocytic microglia adjacent to motoneurons in spinal cord tissue from a presymptomatic transgenic rat model of amyotrophic lateral sclerosis," *Journal of Neuroscience Research*, vol. 88, no. 12, pp. 2736–2746, 2010.

[244] T. Sanagi, Y. Nakamura, E. Suzuki et al., "Involvement of activated microglia in increased vulnerability of motoneurons after facial nerve avulsion in presymptomatic amyotrophic lateral sclerosis model rats," *GLIA*, vol. 60, no. 5, pp. 782–793, 2012.

[245] D. Brites and A. R. Vaz, "Microglia centered pathogenesis in ALS: insights in cell interconnectivity," *Frontiers in Cellular Neuroscience*, vol. 8, article 117, 2014.

[246] F. L. Heppner, R. M. Ransohoff, and B. Becher, "Immune attack: the role of inflammation in Alzheimer disease," *Nature Reviews Neuroscience*, vol. 16, no. 6, pp. 358–372, 2015.

[247] K. Helmut, U.-K. Hanisch, M. Noda, and A. Verkhratsky, "Physiology of microglia," *Physiological Reviews*, vol. 91, no. 2, pp. 461–553, 2011.

[248] R. M. Ransohoff and B. Stevens, "Neuroscience. How many cell types does it take to wire a brain?" *Science*, vol. 333, no. 6048, pp. 1391–1392, 2011.

[249] H. Kettenmann, F. Kirchhoff, and A. Verkhratsky, "Microglia: new roles for the synaptic stripper," *Neuron*, vol. 77, no. 1, pp. 10–18, 2013.

[250] R. C. Paolicelli, G. Bolasco, F. Pagani et al., "Synaptic pruning by microglia is necessary for normal brain development," *Science*, vol. 333, no. 6048, pp. 1456–1458, 2011.

[251] D. P. Schafer, E. K. Lehrman, A. G. Kautzman et al., "Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner," *Neuron*, vol. 74, no. 4, pp. 691–705, 2012.

[252] Y. Zhan, R. C. Paolicelli, F. Sforazzini et al., "Deficient neuron-microglia signaling results in impaired functional brain connectivity and social behavior," *Nature Neuroscience*, vol. 17, no. 3, pp. 400–406, 2014.

[253] L. Tönges, R. Günther, M. Suhr et al., "Rho kinase inhibition modulates microglia activation and improves survival in a model of amyotrophic lateral sclerosis," *GLIA*, vol. 62, no. 2, pp. 217–232, 2014.

Hindawi Publishing Corporation
Neural Plasticity
Volume 2016, Article ID 3145019, 10 pages
http://dx.doi.org/10.1155/2016/3145019

Review Article

Polarity Determinants in Dendritic Spine Development and Plasticity

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Received 18 June 2015; Revised 16 October 2015; Accepted 1 November 2015

Academic Editor: Kwok-On Lai

The asymmetric distribution of various proteins and RNAs is essential for all stages of animal development, and establishment and maintenance of this cellular polarity are regulated by a group of conserved polarity determinants. Studies over the last 10 years highlight important functions for polarity proteins, including apical-basal polarity and planar cell polarity regulators, in dendritic spine development and plasticity. Remarkably, many of the conserved polarity machineries function in similar manners in the context of spine development as they do in epithelial morphogenesis. Interestingly, some polarity proteins also utilize neuronal-specific mechanisms. Although many questions remain unanswered in our understanding of how polarity proteins regulate spine development and plasticity, current and future research will undoubtedly shed more light on how this conserved group of proteins orchestrates different pathways to shape the neuronal circuitry.

1. Introduction

Neurons are probably the most polarized/compartmentalized cell type in the human body. Their polarity establishment starts with the specification of dendrites and axons. Further compartmentalization occurs during the formation of dendritic spines, which receive most of the excitatory synaptic inputs in the brain. Thus, the formation and maintenance of dendritic spines can be seen as a localized form of polarity establishment, where separation and maintenance of different membrane and cytoplasmic domains are needed. This makes proteins regulating cellular polarity ideally suited to function in dendritic spine development. Indeed, recent studies from a number of laboratories highlight key roles for different classes of polarity proteins in dendritic spine development and plasticity. In this review, I will summarize recent advances in studying the role of cell polarity regulators, including apical-basal polarity and planar polarity determinants, in dendritic spine development and plasticity, and discuss possible future avenues of investigation.

2. The Spine Cytoskeleton

The actin and microtubule cytoskeleton provides the structural basis for cell polarity in most cell types. For example, asymmetric actin polymerization allows a migrating cell to polarize and extend lamellipodia in the direction of movement. In addition, polarized vesicular trafficking along microtubules is essential for the establishment and maintenance of apical versus basolateral domains in epithelial cells [1]. Similarly, dendritic spines depend on the unique organization of the cytoskeleton to maintain their polarized morphology. Dendritic spines are highly actin-rich structures that extend from the microtubule-rich dendritic shaft. Spines typically consist of an enlarged spine head containing a dense network of short branched actin filaments. The spine head is connected to the main dendritic shaft through the spine neck, which contains both long linear and short branched actin filaments [2–4]. Although actin constitutes the main cytoskeletal element of dendritic spines, dynamic microtubules do enter spines, a process that is regulated by

neuronal activity [5, 6]. This activity-dependent microtubule invasion is important for synaptic plasticity [7–9]. Thus, the dynamic actin and microtubule cytoskeleton is important for the morphogenesis and plasticity of dendritic spines. Not surprisingly, many of the upstream polarity regulators target the cytoskeleton to regulate spine growth, maturation, and function, as will be discussed in the following sections.

3. Partitioning-Defective (Par) Proteins

The partitioning-defective (Par) proteins play an essential role in various contexts of polarity establishment, including embryogenesis, directional motility, epithelial morphogenesis, and axon specification [10]. These proteins were initially discovered in the *C. elegans* zygote, where mutations in the *par* genes cause defects in partitioning of the zygote into asymmetric daughter cells [11]. The Par proteins (except for Par2) are conserved from worms to mammals. Par1 and Par4 are Ser/Thr kinases. Par3 and Par6 are PDZ domain-containing scaffolding/adaptor proteins. Par5 is a member of the 14-3-3 family of proteins, which binds to phosphorylated Ser/Thr residues [12]. Par proteins can have distinct distribution patterns. For example, in the developing zygote, Par3 and Par6, which form a complex with atypical PKC (aPKC), are localized to the anterior pole while Par1 is localized to the posterior pole. In epithelial cells, the Par3/Par6/aPKC complex is localized apically, while Par1 is localized basolaterally. This polarized distribution is partially achieved by the two complexes mutually excluding each other from their respective domains [13]. Par1 is phosphorylated by aPKC, which leads to the binding of Par1 with Par5. This interaction will lead to the inhibition of Par1 membrane binding and kinase activity. In this way, Par1 is excluded from the membrane domain occupied by the Par3/Par6/aPKC complex [14, 15]. Conversely, Par1 can phosphorylate Par3, which leads to Par5/14-3-3 binding and triggers the release of Par3 from the cell membrane [16], thus preventing the Par3/6 complex from localizing to the lateral membrane (Figure 1). This mutual exclusion mechanism helps cells establish and maintain polarity by compartmentalizing signaling processes in a spatially specific manner.

The highly compartmentalized nature of neurons and their dendritic spines makes Par proteins ideal candidates to function in spine morphogenesis and plasticity. Indeed, the Par3/Par6/aPKC complex was found to play an important role in dendritic spine morphogenesis in hippocampal neurons. Depletion of Par3 results in immature spines that are filopodial- and lamellipodial-like [17]. This phenotype is mediated by the guanine nucleotide exchange factor TIAM1, which activates the small GTPase Rac1. TIAM1 interacts directly with the C-terminus of Par3 [18, 19]. Further experiments show that Par3 functions by spatially restricting Rac activation to dendritic spines through targeting TIAM1. Since Rac is a key regulator of actin dynamics, it was proposed that Par3 and TIAM1 locally modulate the actin cytoskeleton, which is important for proper spine development. In the absence of Par3, TIAM1 becomes mislocalized causing aberrant activation of Rac, which disrupts normal spine morphogenesis [17]. Recently, the adhesion G-protein coupled receptor (GPCR) brain-specific angiogenesis inhibitor 1 (BAI1) was found to be the upstream regulator of the Par3/TIAM1 complex [20]. BAI1 interacts with the Par3/TIAM1 complex and targets it to dendritic spines. In the absence of BAI1, the Par3/TIAM1 complex is mislocalized, and Rac activation is lost in dendritic spines. These recent results elegantly demonstrate for the first time a cell surface receptor that targets and regulates the Par polarity complex at the postsynapse. It also positions the Par3/TIAM1 complex in a key position to link a synaptic adhesion receptor to local modulation of actin dynamics.

While Par3 functions through TIAM1 and Rac in spine morphogenesis [17], the Par6/aPKC complex was also found to play a distinct role in spine development. Overexpression of Par6 or enzymatic activation of aPKC promotes spine development, while depletion of Par6 or inhibiting aPKC disrupts spine morphogenesis. Unexpectedly, the Par6/aPKC complex was found to function through p190 RhoGAP and the small GTPase RhoA. Overexpression of Par6 inhibits RhoA activation while knockdown of Par6 elevates RhoA activity [21]. Since prolonged activation of RhoA negatively regulates spine development [22, 23], the Par6/aPKC complex promotes spine development by keeping RhoA activity low in dendritic spines. It is interesting to note that in *Drosophila* the Par3/Par6/aPKC complex regulates glutamatergic synapse formation at the neuromuscular junction (NMJ) by modulating actin and microtubule dynamics [24, 25]. Moreover, the localization of Par3 and Par6 to the NMJ is dependent on aPKC kinase activity [24, 25], and the retention of Par3 at the NMJ depends on its dephosphorylation by the lipid and protein phosphatase PTEN [24]. Whether similar mechanisms are involved in the mammalian dendritic spines remains to be determined.

As mentioned above, in developing zygotes and epithelia, the Par3/Par6/aPKC complex antagonizes the function of another polarity protein, Ser/Thr kinase Par1, also known as the Microtubule Affinity Regulating Kinase (MARK). The mammalian Par1/MARK was originally discovered as a family of kinases that phosphorylates microtubule-associated proteins (MAPs), such as MAP2 and tau, leading to the disassembly of microtubules [26]. There are four members of the mammalian Par1/MARK family, including Par1c/MARK1, Par1b/MARK2, Par1a/MARK3, and Par1d/MARK4. A number of other substrates have since been identified, including doublecortin [27, 28], histone deacetylase 7 (HDAC7) [29], plakophilin 2 [30], Cdc25 [31], and Par3 [16, 32]. In rat hippocampal neurons, depletion of Par1b/MARK2 inhibits dendritic spine maturation, resulting in elongated filopodia-like protrusions. Live imaging studies revealed that in Par1b depleted neurons microtubule growth is reduced. Further, it was found that the microtubule plus end binding protein p140Cap showed reduced accumulation in dendritic spines when Par1b was depleted [33]. Together these studies suggest that Par1 promotes dendritic spine development through modulating microtubule dynamics. Interestingly, in *Drosophila*, the Par1/MARK homolog dPar1 phosphorylates discs large (Dlg) and regulates neuromuscular junction formation [34]. This phosphorylation mechanism is conserved as the mammalian Par1/MARK phosphorylates

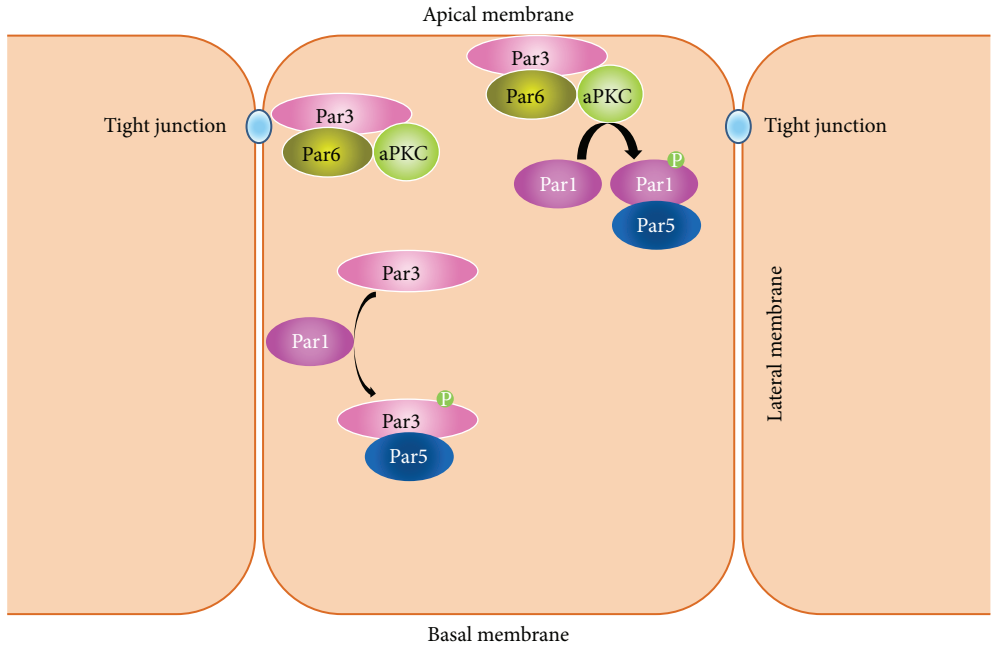

Figure 1: Par polarity proteins maintain their polarized distribution through a mutual exclusion mechanism. In epithelial cells, the Par3/Par6/aPKC complex is localized to the apical membrane while Par1 is localized to the basolateral membrane. Par1 is phosphorylated by aPKC, which leads to the binding of Par1 with Par5, a 14-3-3 protein. This interaction will lead to the inhibition of Par1 membrane binding and kinase activity. In this way, Par1 is excluded from the membrane domain occupied by the Par3/Par6/aPKC complex. Conversely, Par1 can phosphorylate Par3, which leads to Par5/14-3-3 binding and triggers the release of Par3 from the cell membrane, thus preventing the Par3/6 complex from localizing to the lateral membrane.

the Dlg homolog PSD-95 on the conserved Ser561 site. Phosphorylation of this site is important for the function of Par1 in dendritic spine morphogenesis, as a phosphomimetic mutant of PSD-95 can rescue the spine formation defects in hippocampal neurons expressing kinase-dead Par1 [35]. In addition, Par1/MARK was found to function downstream of NMDA receptors through a mechanism that depends on PKA and another member of the Par proteins, Par4, also known as LKB1 [36]. Together, these studies show that Par1 is important for spine development through regulating both microtubule dynamics and the synaptic scaffolding protein PSD-95. It will be interesting to examine whether Par1 participates in NMDA receptor-dependent synaptic plasticity and whether the known antagonistic effects of the Par4/Par1 and Par3/Par6 complexes play any role in spine development (Figure 2).

4. The Septin GTPases

Septins are cytoskeletal proteins that regulate cell polarity by forming filamentous structures underneath the plasma membrane to function as diffusion barriers. They belong to the GTPase family that binds to and hydrolyzes GTP into GDP. There are 13 mammalian septin genes, many of which exist in multiple isoforms [37]. Different septins interact with each other to form heterooligomeric complexes. These oligomers then assemble end-to-end to form filamentous structures. Septin filaments can be straight, curved, or circular and function as scaffolds and/or diffusion barriers [38]. For example, in the budding yeast *Saccharomyces cerevisiae*, where these proteins were initially discovered over 40 years ago, septins form a ring around the neck between mother and bud [37, 38]. More recent studies show that this septin diffusion barrier is important for the asymmetric segregation of age during yeast budding. Aging factor such as circular DNA is retained in the mother cell by a septin-dependent lateral diffusion barrier. This ensures that age is reset in the newborn bud so species propagation can be achieved [39].

Given the geometrical similarities between a yeast bud and a dendritic spine, different groups hypothesized that septins may form a ring around the spine neck to limit diffusion in and out of dendritic spines, thus biochemically compartmentalizing the spine (Figure 3). Indeed it was known that a fraction of dendritic spines are diffusionally isolated [40]; however the molecular identity of this barrier was not clear at the time. In 2007, two groups discovered that septins are indeed present at the spine neck and play an important role in dendritic spine morphogenesis. Both groups independently found that septin 7 (Sept7) is localized to the base of dendritic filopodia, branch points, and the base of dendritic spines. Overexpression of Sept7 increases dendritic branching and protrusion density [41], while depletion of Sept7 results in reduced dendritic arborization and immature, elongated spines [41, 42], suggesting that Sept7 is important for spine maturation.

While the localization of Sept7 to spine neck indicates a role in barrier function, this was not experimentally demonstrated until a recent study by the Choquet group [43]. They measured diffusion of the GluA2 receptor, bulk

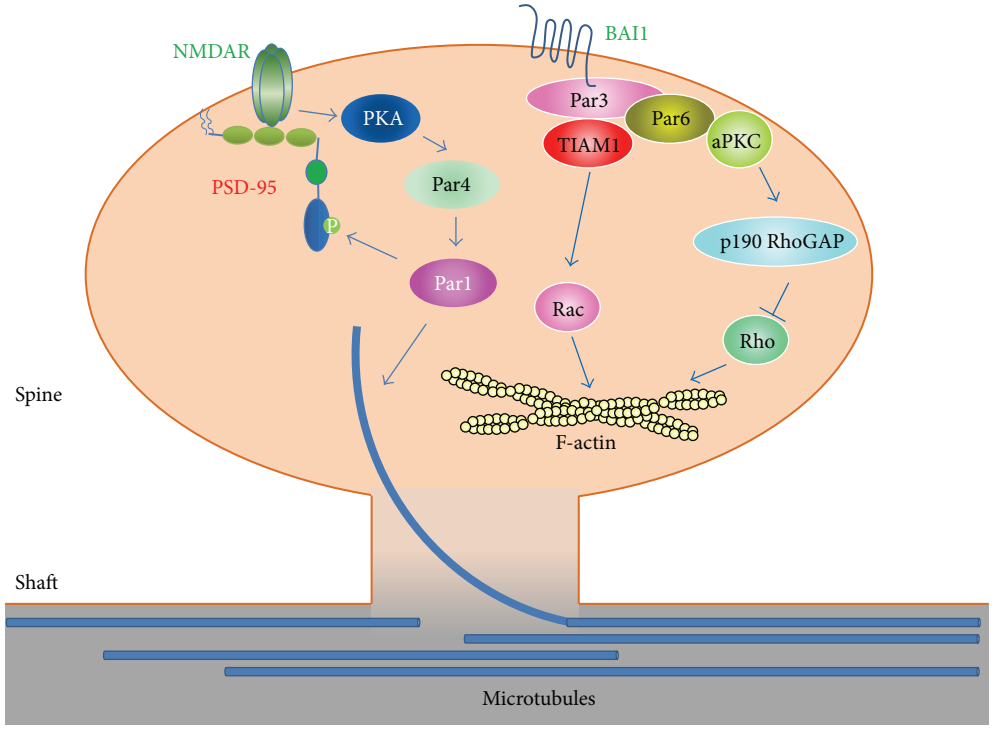

Figure 2: Par polarity proteins in dendritic spines. Members of the partitioning-defective (Par) polarity proteins regulate dendritic spine development through different pathways. Par1 functions downstream of NMDA receptors (NMDAR) to regulate dynamic microtubules and to phosphorylate PSD-95. Par3 functions downstream of the BAI1 receptor and targets TIAM1 to modulate Rac activity. Par6 and aPKC function through p190 RhoGAP to inhibit RhoA. Both Rac and Rho are central regulators of actin dynamics.

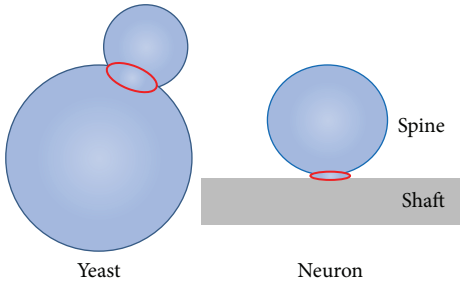

Figure 3: Septin diffusion barriers in yeast and spines. Septins form filamentous structures that constitute diffusion barriers in the yeast bud neck. Similarly, septin diffusion barriers have been found in the spine neck.

membrane, and cytoplasmic proteins across the spine neck, using fluorescence recovery after photobleaching (FRAP) imaging. Diffusion of GluA2 and membrane-bound mRFP is slower in spines containing the septin barrier, while diffusion of cytoplasmic mRFP is not affected [43]. This suggests that Sept7 regulates the lateral diffusion of membrane proteins in and out of spines, which is in line with known septin functions in other organisms. It is intriguing to speculate that septins contribute to the heterogeneity of dendritic spines by forming a barrier on certain spine necks but not others. Further research is needed to elucidate how septin-containing spines and septin-free spines differ in their physiological functions.

5. Planar Cell Polarity Proteins

Planar cell polarity (PCP) is a phenomenon in which coordinated orientation of cells and their appendages, such as stereocilia or hair, occurs within the plane of the epithelial sheet. Thus in the case of PCP, asymmetry is established at the tissue level rather than the cellular level. Genetic studies in *Drosophila* have revealed conserved PCP proteins such as Frizzled (Fz), Dishevelled (Dvl), and Van Gogh (Vang). Studies in the mammalian cochlea have identified additional PCP factors including Vangl2 (a mammalian homologue of the *Drosophila* Vang) and Scrb1 (mammalian homologue of the *Drosophila* Scribble) [44]. From a basic cell biological perspective, the core function of PCP proteins is similar to other polarity proteins, which is to compartmentalize the membrane, except that the compartmentalization occurs on the anterior-posterior body axis instead of the apical-basal axis. Thus, it is perhaps not surprising that several of the PCP proteins are also found to be important for dendritic spine morphogenesis.

5.1. Scribble. Scribble (Scrib) is a large scaffolding protein containing 16 leucine-rich repeats (LRR) on the N-terminus followed by four PDZ domains. It was originally identified

in *Drosophila* as a determinant of apical-basolateral polarity [45] and a tumor suppressor [46]. Scrib localizes to the basolateral domain of epithelial cells and promotes basolateral membrane identity together with its binding partners lethal giant larvae (Lgl) and discs large (Dlg). Depletion of Scrib disrupts E-cadherin mediated adhesion in Madin-Darby Canine Kidney epithelial cells [47, 48]. In mammalian cochlear hair cells, a mutation in the Scrib gene causes defects in PCP as reflected by disrupted orientation of stereociliary bundles of hair cells [49]. Furthermore, Scrib genetically and physically interacts with the PCP core protein Vang and functions as its effector during PCP establishment in *Drosophila* [50]. Thus Scrib is a determinant of both apical-basal polarity and planar polarity.

In *Drosophila*, Scrib regulates the architecture of the presynaptic terminal. Scrib mutant flies show fewer synaptic vesicles in the active zone and more in the reserve pool, resulting in defects in short-term synaptic plasticity [51]. In mammals, this presynaptic effect of Scrib is believed to be downstream of β-catenin [52]. On the postsynaptic side, Scrib recruits the neuronal nitric oxide synthase 1 adaptor protein (NOS1AP) to the G-protein coupled receptor interacting protein 1 (GIT1)/β-p21-activated kinase- (PAK-) interacting exchange factor (β-PIX)/PAK complex to regulate dendritic spine morphogenesis. As the GIT1/β-PIX complex functions to regulate PAK activity through Rac [53, 54], the Scrib-NOS1AP complex also regulates spine morphogenesis through influencing Rac activity [55]. Indeed Scrib mutant mice show increased Rac activation [56]. Furthermore, these mutant mice show impaired synaptic transmission and plasticity in the hippocampus. Overall dendritic spine density is reduced in Scrib mutant mice; however individual spines are enlarged [56]. Together these studies suggest that Scrib functions through Rac to regulate dendritic spine development and plasticity.

5.2. The Wnt/Fz/Dvl Pathway and Vangl. Wnts are a family of secreted proteins that are important for many aspects of tissue development. Wnt proteins function through the seven-transmembrane Frizzled receptor (Fz) and the cytoplasmic adaptor protein Disheveled (Dvl). There are two main branches of the Wnt signaling pathway. The canonical Wnt pathway involves downstream phosphorylation of β-catenin and regulation of gene transcription. The noncanonical Wnt PCP pathway involves regulation of RhoA and actomyosin contractility [57]. During animal development, the Wnt PCP pathway regulates key processes such as convergent extension and neural tube closure [58]. The Wnt pathway is also crucial for multiple cellular processes during brain development, including proliferation and differentiation of neuronal precursors [59], neuronal migration [60], and axon guidance [61]. More recent studies show that Wnt signaling promotes dendritic spine formation in hippocampal neurons [62]. Several different Wnts, including Wnt2, Wnt5a, and Wnt7a, have been shown to increase dendritic spine density [63–65]. Wnt5a increases synaptic transmission [64] and clustering of PSD-95 [66], and Wnt7a increases excitatory, but not inhibitory, synaptic transmission through Dvl1 and the calcium-calmodulin dependent kinase II (CaMKII) [65]. The specific receptors mediating these effects include Fz5, which may act both pre- and postsynaptically [67]. Other Fz receptors involved may include Fz1 and Fz3, both of which are highly localized to synaptic sites [68]. It will be interesting to examine the involvement of other Wnt receptors, including the receptor tyrosine kinase Ryk and receptor tyrosine kinase-like orphan receptor 2 (ROR2). Indeed a recent study shows that depletion of ROR2 inhibits dendritic spine maturation [69].

The *Drosophila* Vang and its mammalian homologue Vangl are tetramembrane spanning proteins that function as core components of the PCP pathway. In the *Drosophila* wing epithelia, Fz and Vang segregate into distinct domains [70]. Fz concentrates on the distal edges of cells while Vang localizes to the proximal edges. How this spatial segregation is achieved is unclear and several different models have been proposed [71]; however the direct transcellular interaction between Fz and Vang is likely involved [72, 73] (Figure 4). In vertebrates, there are two Vangl genes, Vangl1 and Vangl2. Vangl2 is highly expressed in neuronal tissues and regulates various aspects of brain development including neurulation [74, 75], neuronal migration [76, 77], and growth cone guidance [78]. Recent studies show that Vangl2 is also important for dendritic spine development. Vangl2 forms a direct interaction through its C-terminal PDZ-binding motif with PSD-95 on the third PDZ domain [79]. In addition, Vangl2 directly interacts with N-cadherin and enhances its internalization [80]. In hippocampal neurons depleted of Vangl2, both dendritic branching and spine density are reduced [81]. Formation of synapses is also reduced as shown by the decreased clustering of pre- and postsynaptic markers [80]. These studies show that Vangl2 is important for dendritic spine development. It will be interesting to determine how interactions between different PCP proteins contribute to spine development and plasticity (Figure 5).

6. Crosstalk between Polarity Proteins

The interplay within and between different groups of polarity proteins has been most extensively examined in epithelial cells of *Drosophila* and mammals. As described above, the reciprocal exclusions of the Par1-Par3/Par6/aPKC complexes and the Fz-Vang complexes are important for the establishment of apical-basal and planar cell polarity, respectively. However how interactions within different groups of polarity proteins contribute to dendritic spine development and function is largely unknown. Since the interplay between polarity proteins is important for establishing different cellular domains in nonneuronal cells, it is intriguing to speculate that these reciprocal interactions are involved in establishing different spine domains or subdomains. Recent studies using superresolution microscopy have revealed interesting microdomain organizations within dendritic spines [82]. It will be interesting to see whether the organization of these microdomains depends on the balancing acts of the polarity complexes.

Crosstalk between different groups of polarity proteins also occurs. As described above, Scribble interacts with both apical-basal polarity determinants like Lgl and PCP

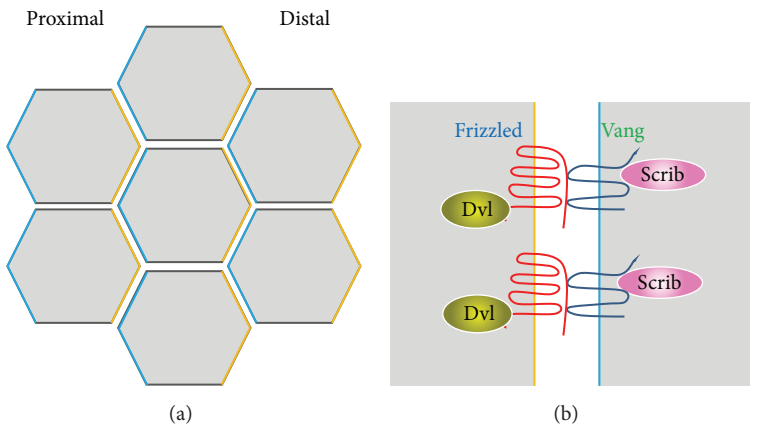

Figure 4: Asymmetric localization of core planar cell polarity (PCP) proteins in the fly wing. (a) Cells can polarize within the plane of the epithelial sheet, a phenomenon called planar cell polarity. (b) The core PCP proteins Frizzled and Vang form transcellular interactions and are distributed asymmetrically along the epithelial plane. Vang is concentrated in the proximal membrane while Frizzled is localized to the distal membrane.

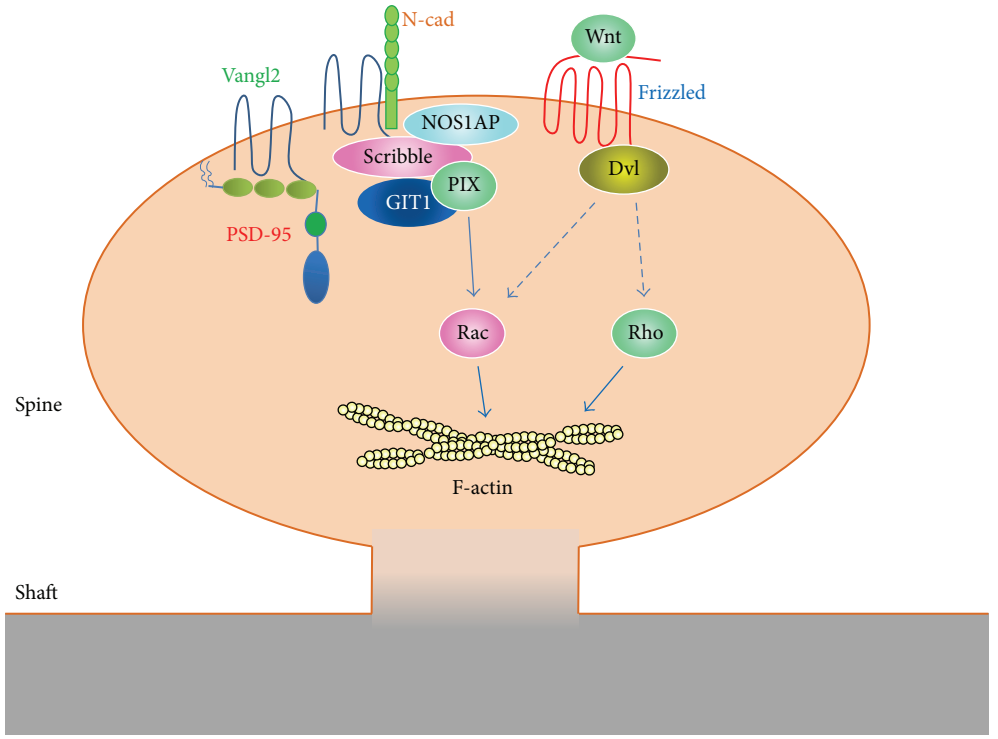

Figure 5: Planar cell polarity proteins in dendritic spines. Planar cell polarity (PCP) proteins play important roles in dendritic spine development. Wnt signals via the Frizzled receptor and Disheveled (Dvl) to regulate spine morphogenesis, possibly through the Rho GTPases. The PCP protein Vangl2 interacts directly with PSD-95 and N-cadherin and regulates N-cadherin endocytosis. Vangl2 also interacts with Scribble, which forms a complex with NOS1AP, GIT1, and PIX to regulate Rac activity in dendritic spines. Dashed arrows represent pathways that have been established in other cell types but have not been directly demonstrated in dendritic spines.

determinants like Vang. Interestingly, recent studies show that the apical-basal polarity determinants Par3/Par6/aPKC can become planar polarized [83, 84], which leads to different fates of the daughter cells [84]. This indicates crosstalk between the Par complex and the PCP machinery. Indeed the Wnt/Dvl pathway has been shown to regulate the Par complex through the interaction between Dvl and aPKC [85]. Finally, Par4/LKB1 and Par1/MARK can regulate the basolateral localization of Scribble [86]. How these crosstalks are involved in dendritic spine development and function remains to be determined.

Interestingly, many of these polarity determinants target the actin and microtubule cytoskeleton to regulate spine development and plasticity. For example, the Par complex,

Scribble, and the Wnt/Dvl complex all target the Rho family GTPases, which are core regulators of the actin cytoskeleton. Rho GTPases have also been shown to modulate microtubule dynamics [87]. Moreover, Par1 and Wnt/Dvl are known regulators of microtubule dynamics [88, 89]. Further studies will shed light on how signals from diverse groups of polarity determinants converge on the cytoskeleton to modulate dendritic spine development and function.

7. Conclusions

The establishment of cell polarity is essential at all stages of animal development, as segregation of different cellular domains is key to the physiological functions of all cell types. Studies from traditional model systems, such as *S. cerevisiae*, *C. elegans*, and *D. melanogaster*, have provided significant insight into the mechanisms by which a conserved group of polarity proteins, including apical-basal polarity proteins and planer polarity proteins, functions in different contexts of polarity establishment. Recent studies in mammalian neurons have highlighted the remarkable diversity of functions for this conserved group of cell polarity proteins. Evolution has bestowed novel roles upon these polarity regulators in the development of dendritic spines, which is a more complex level of neuronal compartmentalization that occurs primarily in vertebrates. While great progress has been made in understanding the function of this important group of proteins in spine development, many questions remain. For example, dendritic spines are heterogeneous in both their morphology and function. Do polarity proteins regulate this heterogeneity? Some polarity proteins show segregated distribution in epithelial cells. Do they distribute to different spine subdomains in neurons? If so, how does this contribute to synaptic functions? Recent advances in imaging techniques, including superresolution imaging, will help address some of these questions. Future research will pave the way to understanding of how these conserved polarity proteins help shape the synaptic connections and how they contribute to cognitive functions of the brain.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by National Institute of Health Grants NS065183 and NS089578 and startup fund from the Rutgers Robert Wood Johnson Medical School to Huaye Zhang.

References

[1] E. Rodriguez-Boulan and I. G. Macara, "Organization and execution of the epithelial polarity programme," *Nature Reviews Molecular Cell Biology*, vol. 15, no. 4, pp. 225–242, 2014.

[2] D. M. D. Landis and T. S. Reese, "Cytoplasmic organization in cerebellar dendritic spines," *Journal of Cell Biology*, vol. 97, no. 4, pp. 1169–1178, 1983.

[3] P. Hotulainen and C. C. Hoogenraad, "Actin in dendritic spines: connecting dynamics to function," *The Journal of Cell Biology*, vol. 189, no. 4, pp. 619–629, 2010.

[4] F. Korobova and T. M. Svitkina, "Molecular architecture of synaptic actin cytoskeleton in hippocampal neurons reveals a mechanism of dendritic spine morphogenesis," *Molecular Biology of the Cell*, vol. 21, no. 1, pp. 165–176, 2010.

[5] J. Gu, B. L. Firestein, and J. Q. Zheng, "Microtubules in dendritic spine development," *Journal of Neuroscience*, vol. 28, no. 46, pp. 12120–12124, 2008.

[6] X. Hu, C. Viesselmann, S. Nam, E. Merriam, and E. W. Dent, "Activity-dependent dynamic microtubule invasion of dendritic spines," *The Journal of Neuroscience*, vol. 28, no. 49, pp. 13094–13105, 2008.

[7] J. Jaworski, L. C. Kapitein, S. M. Gouveia et al., "Dynamic microtubules regulate dendritic spine morphology and synaptic plasticity," *Neuron*, vol. 61, no. 1, pp. 85–100, 2009.

[8] E. B. Merriam, D. C. Lumbard, C. Viesselmann et al., "Dynamic microtubules promote synaptic NMDA receptor-dependent spine enlargement," *PLoS ONE*, vol. 6, no. 11, Article ID e27688, 2011.

[9] X. Hu, L. Ballo, L. Pietila et al., "Bdnf-induced increase of PSD-95 in dendritic spines requires dynamic microtubule invasions," *Journal of Neuroscience*, vol. 31, no. 43, pp. 15597–15603, 2011.

[10] I. G. Macara, "Parsing the polarity code," *Nature Reviews Molecular Cell Biology*, vol. 5, no. 3, pp. 220–231, 2004.

[11] K. J. Kemphues, J. R. Priess, D. G. Morton, and N. Cheng, "Identification of genes required for cytoplasmic localization in early *C. elegans* embryos," *Cell*, vol. 52, no. 3, pp. 311–320, 1988.

[12] D. G. Morton, D. C. Shakes, S. Nugent et al., "The *Caenorhabditis elegans par-5* Gene encodes a 14-3-3 protein required for cellular asymmetry in the early embryo," *Developmental Biology*, vol. 241, no. 1, pp. 47–58, 2002.

[13] B. Goldstein and I. G. Macara, "The PAR proteins: fundamental players in animal cell polarization," *Developmental Cell*, vol. 13, no. 5, pp. 609–622, 2007.

[14] J. B. Hurov, J. L. Watkins, and H. Piwnica-Worms, "Atypical PKC phosphorylates PAR-1 kinases to regulate localization and activity," *Current Biology*, vol. 14, no. 8, pp. 736–741, 2004.

[15] A. Suzuki, M. Hirata, K. Kamimura et al., "aPKC acts upstream of PAR-1b in both the establishment and maintenance of mammalian epithelial polarity," *Current Biology*, vol. 14, no. 16, pp. 1425–1435, 2004.

[16] R. Benton and D. St Johnston, "*Drosophila* PAR-1 and 14-3-3 inhibit Bazooka/PAR-3 to establish complementary cortical domains in polarized cells," *Cell*, vol. 115, no. 6, pp. 691–704, 2003.

[17] H. Zhang and I. G. Macara, "The polarity protein PAR-3 and TIAM1 cooperate in dendritic spine morphogenesis," *Nature Cell Biology*, vol. 8, no. 3, pp. 227–237, 2006.

[18] X. Chen and I. G. Macara, "Par-3 controls tight junction assembly through the Rac exchange factor Tiam1," *Nature Cell Biology*, vol. 7, no. 3, pp. 262–269, 2005.

[19] T. Nishimura, T. Yamaguchi, K. Kato et al., "PAR-6-PAR-3 mediates Cdc42-induced Rac activation through the Rac GEFs STEF/Tiam1," *Nature Cell Biology*, vol. 7, no. 3, pp. 270–277, 2005.

[20] J. G. Duman, C. P. Tzeng, Y.-K. Tu et al., "The adhesion-GPCR BAI1 regulates synaptogenesis by controlling the recruitment of the Par3/Tiam1 polarity complex to synaptic sites," *The Journal of Neuroscience*, vol. 33, no. 16, pp. 6964–6978, 2013.

[21] H. Zhang and I. G. Macara, "The PAR-6 polarity protein regulates dendritic spine morphogenesis through p190 RhoGAP and the Rho GTPase," *Developmental Cell*, vol. 14, no. 2, pp. 216–226, 2008.

[22] A. Tashiro, A. Minden, and R. Yuste, "Regulation of dendritic spine morphology by the Rho family of small GTPases: antagonistic roles of Rac and Rho," *Cerebral Cortex*, vol. 10, no. 10, pp. 927–938, 2000.

[23] E.-E. Govek, S. E. Newey, C. J. Akerman, J. R. Cross, L. Van der Veken, and L. Van Aelst, "The X-linked mental retardation protein oligophrenin-1 is required for dendritic spine morphogenesis," *Nature Neuroscience*, vol. 7, no. 4, pp. 364–372, 2004.

[24] P. Ramachandran, R. Barria, J. Ashley, and V. Budnik, "A critical step for postsynaptic F-actin organization: regulation of Baz/Par-3 localization by aPKC and PTEN," *Developmental Neurobiology*, vol. 69, no. 9, pp. 583–602, 2009.

[25] C. Ruiz-Canada, J. Ashley, S. Moeckel-Cole, E. Drier, J. Yin, and V. Budnik, "New synaptic bouton formation is disrupted by misregulation of microtubule stability in aPKC mutants," *Neuron*, vol. 42, no. 4, pp. 567–580, 2004.

[26] G. Drewes, A. Ebneth, U. Preuss, E.-M. Mandelkow, and E. Mandelkow, "MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption," *Cell*, vol. 89, no. 2, pp. 297–308, 1997.

[27] B. T. Schaar, K. Kinoshita, and S. K. McConnell, "Doublecortin microtubule affinity is regulated by a balance of kinase and phosphatase activity at the leading edge of migrating neurons," *Neuron*, vol. 41, no. 2, pp. 203–213, 2004.

[28] T. Sapir, A. Shmueli, T. Levy et al., "Antagonistic effects of doublecortin and MARK2/Par-1 in the developing cerebral cortex," *The Journal of Neuroscience*, vol. 28, no. 48, pp. 13008–13013, 2008.

[29] F. Dequiedt, M. Martin, J. Von Blume et al., "New role for hPar-1 kinases EMK and C-TAK1 in regulating localization and activity of class IIa histone deacetylases," *Molecular and Cellular Biology*, vol. 26, no. 19, pp. 7086–7102, 2006.

[30] J. Müller, D. A. Ritt, T. D. Copeland, and D. K. Morrison, "Functional analysis of C-TAK1 substrate binding and identification of PKP2 as a new C-TAK1 substrate," *The EMBO Journal*, vol. 22, no. 17, pp. 4431–4442, 2003.

[31] C.-Y. Peng, P. R. Graves, S. Ogg et al., "C-TAK1 protein kinase phosphorylates human Cdc25C on serine 216 and promotes 14-3-3 protein binding," *Cell Growth and Differentiation*, vol. 9, no. 3, pp. 197–208, 1998.

[32] T. W. Hurd, S. Fan, C.-J. Liu, H. K. Kweon, K. Hakansson, and B. Margolis, "Phosphorylation-dependent binding of 14-3-3 to the polarity protein Par3 regulates cell polarity in mammalian epithelia," *Current Biology*, vol. 13, no. 23, pp. 2082–2090, 2003.

[33] K. Hayashi, A. Suzuki, S.-I. Hirai, Y. Kurihara, C. C. Hoogenraad, and S. Ohno, "Maintenance of dendritic spine morphology by partitioning-defective 1b through regulation of microtubule growth," *The Journal of Neuroscience*, vol. 31, no. 34, pp. 12094–12103, 2011.

[34] Y. Zhang, H. Guo, H. Kwan, J.-W. Wang, J. Kosek, and B. Lu, "PAR-1 kinase phosphorylates Dlg and regulates its postsynaptic targeting at the *Drosophila neuromuscular* junction," *Neuron*, vol. 53, no. 2, pp. 201–215, 2007.

[35] Q. Wu, V. L. DiBona, L. P. Bernard, and H. Zhang, "The polarity protein partitioning-defective 1 (PAR-1) regulates dendritic spine morphogenesis through phosphorylating postsynaptic density protein 95 (PSD-95)," *The Journal of Biological Chemistry*, vol. 287, no. 36, pp. 30781–30788, 2012.

[36] L. P. Bernard and H. Zhang, "MARK/Par1 kinase is activated downstream of NMDA receptors through a PKA-dependent mechanism," *PLoS ONE*, vol. 10, no. 5, Article ID e0124816, 2015.

[37] Y. Oh and E. Bi, "Septin structure and function in yeast and beyond," *Trends in Cell Biology*, vol. 21, no. 3, pp. 141–148, 2011.

[38] S. Mostowy and P. Cossart, "Septins: the fourth component of the cytoskeleton," *Nature Reviews Molecular Cell Biology*, vol. 13, no. 3, pp. 183–194, 2012.

[39] Z. Shcheprova, S. Baldi, S. B. Frei, G. Gonnet, and Y. Barral, "A mechanism for asymmetric segregation of age during yeast budding," *Nature*, vol. 454, no. 7205, pp. 728–734, 2008.

[40] B. L. Bloodgood and B. L. Sabatini, "Neuronal activity regulates diffusion across the neck of dendritic spines," *Science*, vol. 310, no. 5749, pp. 866–869, 2005.

[41] T. Tada, A. Simonetta, M. Batterton, M. Kinoshita, D. Edbauer, and M. Sheng, "Role of Septin cytoskeleton in spine morphogenesis and dendrite development in neurons," *Current Biology*, vol. 17, no. 20, pp. 1752–1758, 2007.

[42] Y. Xie, J. P. Vessey, A. Konecna, R. Dahm, P. Macchi, and M. A. Kiebler, "The GTP-binding protein Septin 7 is critical for dendrite branching and dendritic-spine morphology," *Current Biology*, vol. 17, no. 20, pp. 1746–1751, 2007.

[43] H. Ewers, T. Tada, J. D. Petersen, B. Racz, M. Sheng, and D. Choquet, "A septin-dependent diffusion barrier at dendritic spine necks," *PLoS ONE*, vol. 9, no. 12, Article ID e113916, 2014.

[44] M. Simons and M. Mlodzik, "Planar cell polarity signaling: from fly development to human disease," *Annual Review of Genetics*, vol. 42, pp. 517–540, 2008.

[45] D. Bilder and N. Perrimon, "Localization of apical epithelial determinants by the basolateral PDZ protein Scribble," *Nature*, vol. 403, no. 6770, pp. 676–680, 2000.

[46] D. Bilder, M. Li, and N. Perrimon, "Cooperative regulation of cell polarity and growth by *Drosophila* tumor suppressors," *Science*, vol. 289, no. 5476, pp. 113–116, 2000.

[47] M. Lohia, Y. Qin, and I. G. Macara, "The Scribble polarity protein stabilizes E-cadherin/p120-catenin binding and blocks retrieval of E-cadherin to the Golgi," *PLoS ONE*, vol. 7, no. 11, Article ID e51130, 2012.

[48] Y. Qin, C. Capaldo, B. M. Gumbiner, and I. G. Macara, "The mammalian Scribble polarity protein regulates epithelial cell adhesion and migration through E-cadherin," *The Journal of Cell Biology*, vol. 171, no. 6, pp. 1061–1071, 2005.

[49] M. Montcouquiol, R. A. Rachel, P. J. Lanford, N. G. Copeland, N. A. Jenkins, and M. W. Kelley, "Identification of Vangl2 and Scrb1 as planar polarity genes in mammals," *Nature*, vol. 423, no. 6936, pp. 173–177, 2003.

[50] J.-R. Courbard, A. Djiane, J. Wu, and M. Mlodzik, "The apical/basal-polarity determinant Scribble cooperates with the PCP core factor Stbm/Vang and functions as one of its effectors," *Developmental Biology*, vol. 333, no. 1, pp. 67–77, 2009.

[51] J. P. Roche, M. C. Packard, S. Moeckel-Cole, and V. Budnik, "Regulation of synaptic plasticity and synaptic vesicle dynamics by the PDZ protein scribble," *The Journal of Neuroscience*, vol. 22, no. 15, pp. 6471–6479, 2002.

[52] Y. Sun, M. Aiga, E. Yoshida, P. O. Humbert, and S. X. Bamji, "Scribble interacts with β-catenin to localize synaptic vesicles to synapses," *Molecular Biology of the Cell*, vol. 20, no. 14, pp. 3390–3400, 2009.

[53] H. Zhang, D. J. Webb, H. Asmussen, and A. F. Horwitz, "Synapse formation is regulated by the signaling adaptor GIT1," *Journal of Cell Biology*, vol. 161, no. 1, pp. 131–142, 2003.

[54] H. Zhang, D. J. Webb, H. Asmussen, S. Niu, and A. F. Horwitz, "A GIT1/PIX/Rac/PAK signaling module regulates spine morphogenesis and synapse formation through MLC," *The Journal of Neuroscience*, vol. 25, no. 13, pp. 3379–3388, 2005.

[55] L. Richier, K. Williton, L. Clattenburg et al., "NOS1AP associates with scribble and regulates dendritic spine development," *Journal of Neuroscience*, vol. 30, no. 13, pp. 4796–4805, 2010.

[56] M. M. Moreau, N. Piguel, T. Papouin et al., "The planar polarity protein scribble1 is essential for neuronal plasticity and brain function," *Journal of Neuroscience*, vol. 30, no. 29, pp. 9738–9752, 2010.

[57] B. Gao, "Wnt regulation of planar cell polarity (PCP)," *Current Topics in Developmental Biology*, vol. 101, pp. 263–295, 2012.

[58] S. Y. Sokol, "Spatial and temporal aspects of Wnt signaling and planar cell polarity during vertebrate embryonic development," *Seminars in Cell & Developmental Biology*, vol. 42, pp. 78–85, 2015.

[59] H. Bielen and C. Houart, "The Wnt cries many: Wnt regulation of neurogenesis through tissue patterning, proliferation, and asymmetric cell division," *Developmental Neurobiology*, vol. 74, no. 8, pp. 772–780, 2014.

[60] H. Wada and H. Okamoto, "Roles of noncanonical Wnt/PCP pathway genes in neuronal migration and neurulation in zebrafish," *Zebrafish*, vol. 6, no. 1, pp. 3–8, 2009.

[61] K. Onishi, E. Hollis, and Y. Zou, "Axon guidance and injury-lessons from Wnts and Wnt signaling," *Current Opinion in Neurobiology*, vol. 27, pp. 232–240, 2014.

[62] V. Budnik and P. C. Salinas, "Wnt signaling during synaptic development and plasticity," *Current Opinion in Neurobiology*, vol. 21, no. 1, pp. 151–159, 2011.

[63] B. G. Hiester, D. F. Galati, P. C. Salinas, and K. R. Jones, "Neurotrophin and Wnt signaling cooperatively regulate dendritic spine formation," *Molecular and Cellular Neuroscience*, vol. 56, pp. 115–127, 2013.

[64] L. Varela-Nallar, I. E. Alfaro, F. G. Serrano, J. Parodi, and N. C. Inestrosa, "Wingless-type family member 5A (Wnt-5a) stimulates synaptic differentiation and function of glutamatergic synapses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 49, pp. 21164–21169, 2010.

[65] L. Ciani, K. A. Boyle, E. Dickins et al., "Wnt7a signaling promotes dendritic spine growth and synaptic strength through Ca^{2+}/Calmodulin-dependent protein kinase II," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 26, pp. 10732–10737, 2011.

[66] G. G. Farías, I. E. Alfaro, W. Cerpa et al., "Wnt-5a/JNK signaling promotes the clustering of PSD-95 in hippocampal neurons," *The Journal of Biological Chemistry*, vol. 284, no. 23, pp. 15857–15866, 2009.

[67] M. Sahores, A. Gibb, and P. C. Salinas, "Frizzled-5, a receptor for the synaptic organizer Wnt7a, regulates activity-mediated synaptogenesis," *Development*, vol. 137, no. 13, pp. 2215–2225, 2010.

[68] L. Varela-Nallar, V. T. Ramirez, C. Gonzalez-Billault, and N. C. Inestrosa, "Frizzled receptors in neurons: from growth cones to the synapse," *Cytoskeleton*, vol. 69, no. 7, pp. 528–534, 2012.

[69] I. E. Alfaro, L. Varela-Nallar, M. Varas-Godoy, and N. C. Inestrosa, "The ROR2 tyrosine kinase receptor regulates dendritic spine morphogenesis in hippocampal neurons," *Molecular and Cellular Neuroscience*, vol. 67, pp. 22–30, 2015.

[70] J. R. K. Seifert and M. Mlodzik, "Frizzled/PCP signalling: a conserved mechanism regulating cell polarity and directed motility," *Nature Reviews Genetics*, vol. 8, no. 2, pp. 126–138, 2007.

[71] J. Wu and M. Mlodzik, "A quest for the mechanism regulating global planar cell polarity of tissues," *Trends in Cell Biology*, vol. 19, no. 7, pp. 295–305, 2009.

[72] J. Wu and M. Mlodzik, "The frizzled extracellular domain is a ligand for Van Gogh/Stbm during nonautonomous planar cell polarity signaling," *Developmental Cell*, vol. 15, no. 3, pp. 462–469, 2008.

[73] J. M. Carvajal-Gonzalez and M. Mlodzik, "Mechanisms of planar cell polarity establishment in *Drosophila*," *F1000Prime Reports*, vol. 6, article 98, 2014.

[74] B. Ciruna, A. Jenny, D. Lee, M. Mlodzik, and A. F. Schier, "Planar cell polarity signalling couples cell division and morphogenesis during neurulation," *Nature*, vol. 439, no. 7073, pp. 220–224, 2006.

[75] M. Williams, W. Yen, X. Lu, and A. Sutherland, "Distinct apical and basolateral mechanisms drive planar cell polarity-dependent convergent extension of the mouse neural plate," *Developmental Cell*, vol. 29, no. 1, pp. 34–46, 2014.

[76] D. M. Glasco, V. Sittaramane, W. Bryant et al., "The mouse Wnt/PCP protein Vangl2 is necessary for migration of facial branchiomotor neurons, and functions independently of Dishevelled," *Developmental Biology*, vol. 369, no. 2, pp. 211–222, 2012.

[77] V. Sittaramane, X. Pan, D. M. Glasco et al., "The PCP protein Vangl2 regulates migration of hindbrain motor neurons by acting in floor plate cells, and independently of cilia function," *Developmental Biology*, vol. 382, no. 2, pp. 400–412, 2013.

[78] B. Shafer, K. Onishi, C. Lo, G. Colakoglu, and Y. Zou, "Vangl2 promotes Wnt/planar cell polarity-like signaling by antagonizing Dvl1-mediated feedback inhibition in growth cone guidance," *Developmental Cell*, vol. 20, no. 2, pp. 177–191, 2011.

[79] T. Yoshioka, A. Hagiwara, Y. Hida, and T. Ohtsuka, "Vangl2, the planner cell polarity protein, is complexed with postsynaptic density protein PSD-95," *FEBS Letters*, vol. 587, no. 10, pp. 1453–1459, 2013.

[80] T. Nagaoka, R. Ohashi, A. Inutsuka et al., "The Wnt/planar cell polarity pathway component Vangl2 induces synapse formation through direct control of N-cadherin," *Cell Reports*, vol. 6, no. 5, pp. 916–927, 2014.

[81] A. Hagiwara, M. Yasumura, Y. Hida, E. Inoue, and T. Ohtsuka, "The planar cell polarity protein Vangl2 bidirectionally regulates dendritic branching in cultured hippocampal neurons," *Molecular Brain*, vol. 7, article 79, 2014.

[82] H. MacGillavry, Y. Song, S. Raghavachari, and T. Blanpied, "Nanoscale scaffolding domains within the postsynaptic density concentrate synaptic ampa receptors," *Neuron*, vol. 78, no. 4, pp. 615–622, 2013.

[83] S. D. M. Simões, J. T. Blankenship, O. Weitz et al., "Rho-kinase directs bazooka/Par-3 planar polarity during drosophila axis elongation," *Developmental Cell*, vol. 19, no. 3, pp. 377–388, 2010.

[84] C. Besson, F. Bernard, F. Corson et al., "Planar cell polarity breaks the symmetry of PAR protein distribution prior to mitosis in *Drosophila* sensory organ precursor cells," *Current Biology*, vol. 25, no. 8, pp. 1104–1110, 2015.

[85] X. Zhang, J. Zhu, G.-Y. Yang et al., "Dishevelled promotes axon differentiation by regulating atypical protein kinase C," *Nature Cell Biology*, vol. 9, no. 7, pp. 743–754, 2007.

[86] M. Mohseni, J. Sun, A. Lau et al., "A genetic screen identifies an LKB1-MARK signalling axis controlling the Hippo-YAP pathway," *Nature Cell Biology*, vol. 16, no. 1, pp. 108–117, 2014.

[87] A. Hall, "Rho family GTPases," *Biochemical Society Transactions*, vol. 40, no. 6, pp. 1378–1382, 2012.

[88] P. C. Salinas, "Retrograde signalling at the synapse: a role for Wnt proteins," *Biochemical Society Transactions*, vol. 33, no. 6, pp. 1295–1298, 2005.

[89] D. Matenia and E.-M. Mandelkow, "The tau of MARK: a polarized view of the cytoskeleton," *Trends in Biochemical Sciences*, vol. 34, no. 7, pp. 332–342, 2009.

Hindawi Publishing Corporation
Neural Plasticity
Volume 2016, Article ID 6790743, 7 pages
http://dx.doi.org/10.1155/2016/6790743

Research Article

Phosphorylation of CRMP2 by Cdk5 Regulates Dendritic Spine Development of Cortical Neuron in the Mouse Hippocampus

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Received 7 June 2015; Revised 11 July 2015; Accepted 21 July 2015

Academic Editor: Kwok-On Lai

Proper density and morphology of dendritic spines are important for higher brain functions such as learning and memory. However, our knowledge about molecular mechanisms that regulate the development and maintenance of dendritic spines is limited. We recently reported that cyclin-dependent kinase 5 (Cdk5) is required for the development and maintenance of dendritic spines of cortical neurons in the mouse brain. Previous *in vitro* studies have suggested the involvement of Cdk5 substrates in the formation of dendritic spines; however, their role in spine development has not been tested *in vivo*. Here, we demonstrate that Cdk5 phosphorylates collapsin response mediator protein 2 (CRMP2) in the dendritic spines of cultured hippocampal neurons and *in vivo* in the mouse brain. When we eliminated CRMP2 phosphorylation in $CRMP2^{KI/KI}$ mice, the densities of dendritic spines significantly decreased in hippocampal CA1 pyramidal neurons in the mouse brain. These results indicate that phosphorylation of CRMP2 by Cdk5 is important for dendritic spine development in cortical neurons in the mouse hippocampus.

1. Introduction

For the development of functional neural circuitry, the formation of synapses between appropriate partners is a critical step. The majority of excitatory synapses of postsynaptic neurons are localized in specialized cellular structures called dendritic spines. The formation, maturation, and maintenance of dendritic spines are tightly regulated by different extracellular signals including semaphorin 3A (Sema3A). Collapsin response mediator proteins (CRMPs), initially identified as a signaling molecule of Sema3A [1], are composed of five homologous cytosolic phosphoproteins (CRMP1–5) and are highly expressed in developing and adult nervous systems [2–5]. CRMPs bind with tubulin heterodimers, whereas the sequential phosphorylation of CRMPs lowers their binding affinity to tubulin [6]. CRMP2 also colocalizes with the actin cytoskeleton [7] and coimmunoprecipitates with actin [8, 9]. Phosphorylation of CRMP1 and CRMP2 by Cdk5 and sequential phosphorylation of CRMP2 by GSK-3β are crucial for Sema3A-induced growth cone collapse response in dorsal root ganglia (DRG) neurons [10, 11].

Recently, we demonstrated that Cdk5/p35 is necessary for dendritic spine development and maintenance [12]. Additionally, we previously showed that Sema3A-induced spine development is mediated through phosphorylation of CRMP1 by Cdk5 [13] and that CRMP1 and CRMP2 have functional redundancy in neuronal development [14]. Therefore, we hypothesized that phosphorylation of CRMP2 by Cdk5 is also important for the development of dendritic spines *in vivo*. To test this, we first analyzed the localization of phosphorylated forms of CRMP2 in the synapses of cultured hippocampal neurons and *in vivo* in the mouse hippocampus. We observed phosphorylation of CRMP2 by Cdk5 in the dendritic spines of hippocampal neurons *in vitro*

and *in vivo*. We then analyzed spine densities of hippocampal CA1 pyramidal neurons in CRMP2$^{KI/KI}$ mice in which the Cdk5 phosphorylation site of CRMP2 at amino acid 522 was changed from Ser to Ala [14]. We found reduced dendritic spine densities in hippocampal neurons in CRMP2$^{KI/KI}$ mice. These results indicate that CRMP2 phosphorylation by Cdk5 is important for the development of dendritic spines in hippocampal neurons *in vivo*.

2. Materials and Methods

2.1. Mice. The mice used in our experiments were housed in accordance with protocols approved by the Institutional Animal Care and Use Committee at Waseda University. CRMP2$^{KI/KI}$ mice were generated and genotyped as described previously [14]. GFP-M mice, a gift from J. Sanes [15], were crossed with these mutant mice for the present study.

2.2. Neuronal Culture and Immunocytochemistry. Primary cultures of hippocampal neurons were prepared from E18 Wistar rats as described previously [16], with the following modifications: cells were plated at a density of 5.0×10^4 cells/well on coverslips coated with 200 μg/mL poly-L-lysine (Sigma Japan, Tokyo) in 24-well plates. Neurobasal-A (Life Technologies Japan, Tokyo), B27-supplement (Miltenyi Biotec, Tokyo), 2 mM L-glutamine (Life Technologies Japan), and penicillin/streptomycin (Nacalai Tesque, Kyoto) were used as culture medium. Immunocytochemistry was performed as previously described [17]. Briefly, after washing with phosphate-buffered saline (PBS), cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature (RT). After washing with PBS, cells were incubated with primary antibodies, which were diluted in PBS/0.01% Triton X-100, at 4°C overnight. They were then washed 3 times with PBS and incubated with Alexa-Fluor 488 (1 : 1000) or Alexa-Fluor 568 (1 : 1000) secondary antibodies for 1 h. After 3 further washes with PBS, the sections were embedded in Vectashield mounting media (Vector Labs, Burlingame, CA). Images were obtained using a laser scanning confocal microscope based on an FV1000 scanning unit (Olympus, Japan). Primary antibodies used in this study are anti-PSD95 (mouse monoclonal, Millipore), anti-synaptophysin (mouse monoclonal, Millipore), and pCRMP2(S522), which recognizes phospho-CRMP2 at Ser522 (rabbit polyclonal, EMC Biosciences).

2.3. Histological Analysis

2.3.1. Immunohistochemistry. Mice were anesthetized using diethyl ether and then perfused transcardially with 4% PFA in PBS. Brain samples were fixed in 4% PFA in PBS overnight at 4°C. GFP-M mice used in this study were 4–6 weeks of age. After dehydration in 20% sucrose in PBS, samples were embedded in OCT compound (Sakura Finetek, Japan). Cryosections were cut at 14 μm thickness. For immunostaining, sections were incubated with anti-pCRMP2(S522) antibody at 4°C overnight. After washing with PBS, the secondary antibody, AlexaFluor, was applied, and sections were mounted with Vectashield. All immunostaining images were captured with a confocal microscope (FV1000).

2.3.2. Rapid Golgi Staining. Male CRMP2$^{KI/KI}$ and CRMP2$^{+/+}$ mice at P18 and at 5 weeks of age ($n = 3$ for each genotype and age) were used in this study. For modified Golgi-Cox staining, an FD Rapid GolgiStain kit was used (FD NeuroTechnologies, MD). Stained slices were sectioned at a thickness of 200 μm. Pyramidal hippocampal CA1 neurons in each mouse were selected for the analysis as described in our previous work [12, 13]. Dendritic spines of CA1 pyramidal neurons were counted in 50 μm segments of proximal branches of apical dendrites under a BX50 microscope (Olympus) with a UPlanSApo 40x (NA = 0.95) objective. In a typical experiment, more than 2000 spines were counted on more than 50 dendritic segments in 25 neurons. Average spine densities per 50 μm dendritic segments were then calculated for each genotype group. Groups of spines were compared using Student's t-test.

3. Results

3.1. CRMP2 Is Phosphorylated in Dendritic Spines of Cultured Hippocampal Neurons. We tested the possible function of CRMP2 phosphorylation in synapses. We first examined the subcellular localization of phospho-CRMP2 (pCRMP2) in cultured hippocampal neurons. We used anti-synaptophysin or anti-PSD-95 antibodies as presynaptic and postsynaptic markers, respectively. Double staining with anti-pCRMP2 and anti-synaptophysin or anti-PSD-95 antibodies showed that pCRMP2 colocalized with both synaptophysin and PSD-95 in dendritic protrusions (Figure 1). These results demonstrate that CRMP2 is phosphorylated by Cdk5 in the presynapse and dendritic spines in cultured hippocampal neurons, suggesting the possible involvement of CRMP2 phosphorylation in the development of dendritic spines in hippocampal neurons.

3.2. Reduced Spine Densities of Hippocampal CA1 Pyramidal Neurons in Juvenile CRMP2$^{KI/KI}$ Mice. We examined dendritic spine density by Golgi staining in P18 CRMP2$^{KI/KI}$ mice. Golgi staining of forebrain slices showed a reduction in the number of spines in hippocampal CA1 pyramidal neurons in CRMP2$^{KI/KI}$ mice compared with those in CRMP2$^{+/+}$ mice (Figure 2). These results indicate that phosphorylation of CRMP2 by Cdk5 is required for proper formation of dendritic spines in the mouse brain.

3.3. CRMP2 Is Phosphorylated in Dendritic Spines of Hippocampal CA1 Pyramidal Neurons in Mouse Brains. CRMP2 is expressed in hippocampal neurons in adult mice [2]. Thus, we examined its phosphorylation in dendritic spines in hippocampal CA1 pyramidal neurons. For this purpose, we performed immunostaining of hippocampal sections from GFP-M mice at 4–6 weeks of age with anti-pCRMP2 antibody. In GFP-M mice, some hippocampal CA1 pyramidal neurons express GFP [15]. As shown in Figure 3, we detected pCRMP2 immunoreactivity in dendritic spines in hippocampal CA1 pyramidal neurons of GFP-M mice. In

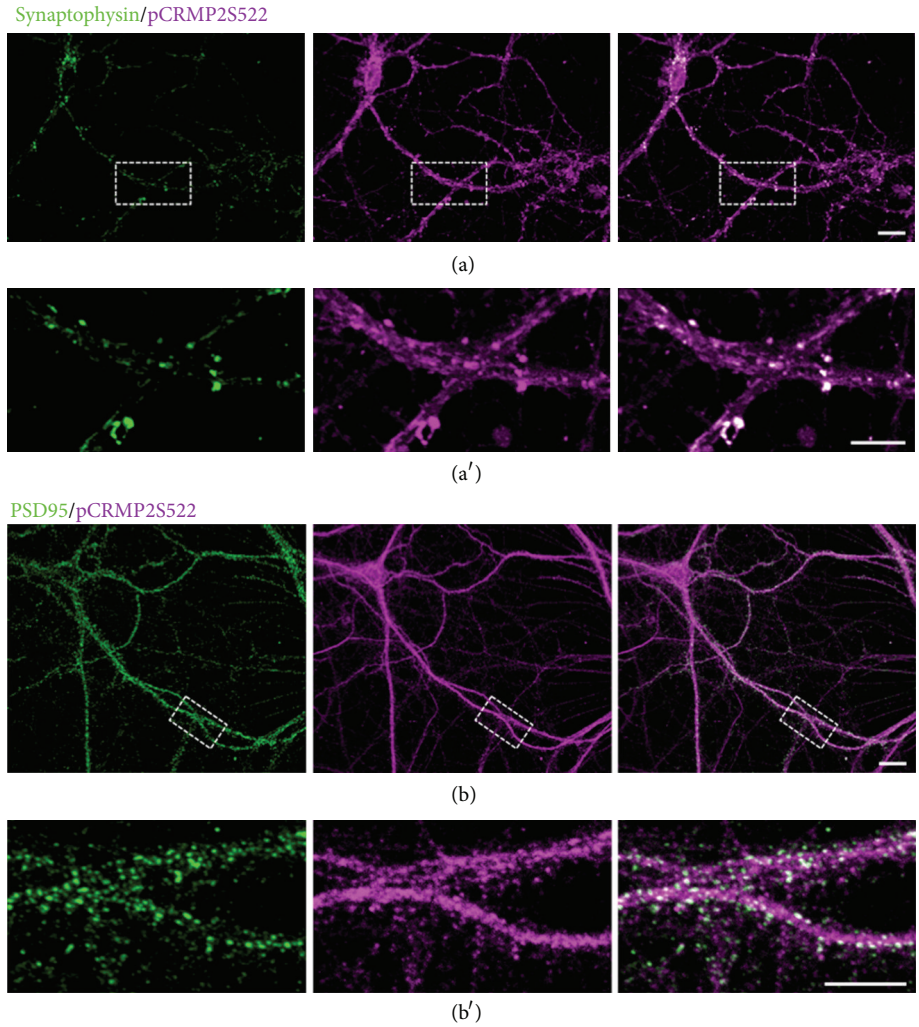

Figure 1: Subcellular localization of phospho-CRMP2 in cultured hippocampal neurons. (a) Immunocytochemistry with anti-phospho-CRMP2 and synaptophysin antibodies. Higher magnification is shown in (a′). (b) Immunocytochemistry with anti-phospho-CRMP2 and PSD95 antibodies in cultured rat hippocampal neurons 28 days *in vitro* (DIV). Merged images are shown. Higher magnification shown in (b′). Scale bar, 20 μm.

contrast its immunoreactivity was very low in those of GFP-M, CRMP2$^{KI/KI}$ double mutant, which is attributable to a cross reactivity of this antibody to pCRMP1 [14]. These results suggest that Cdk5 phosphorylates CRMP2 in dendritic spines of hippocampal CA1 pyramidal neurons in the mouse brain.

3.4. Reduced Spine Densities of Hippocampal CA1 Pyramidal Neurons in 5-Week-Old CRMP2$^{KI/KI}$ Mice. We examined dendritic spine density by Golgi staining in 5-week-old CRMP2$^{KI/KI}$ mice. Golgi staining of forebrain slices showed a reduction in the numbers of spines in hippocampal CA1 pyramidal neurons in CRMP2$^{KI/KI}$ mice compared with those in CRMP2$^{+/+}$ mice (Figure 4). These results indicate that phosphorylation of CRMP2 by Cdk5 is required for the development of proper dendritic spine density in the adult mouse brain.

4. Discussion

Recent studies have demonstrated that Cdk5 substrates are involved in the regulation of spine formation. Synaptic proteins phosphorylated by Cdk5 including ephexin1 [18], WAVE1 [19], CRMP1 [13], TrkB [20], PSD95 [21], drebrin [22], and p70 ribosomal S6 kinase (S6K) [23] have been shown to play a role in spine formation [13, 19, 21, 23] and maintenance [18, 20]. However, their functions differ such that some of them are crucial for spine formation [13] and some for spine retraction [18]. We recently reported reductions of dendritic spine densities in hippocampal CA1 pyramidal neurons of inducible-*p35* cKO, *p39* KO mice and CA1-*p35* cKO, *p39* KO mice with a p35 deletion in the CA1 region of the hippocampus after P17 [12]. We also reported reduction of spine densities in cerebral layer V neurons and hippocampal CA1 pyramidal neurons in inducible-*p35* cKO,

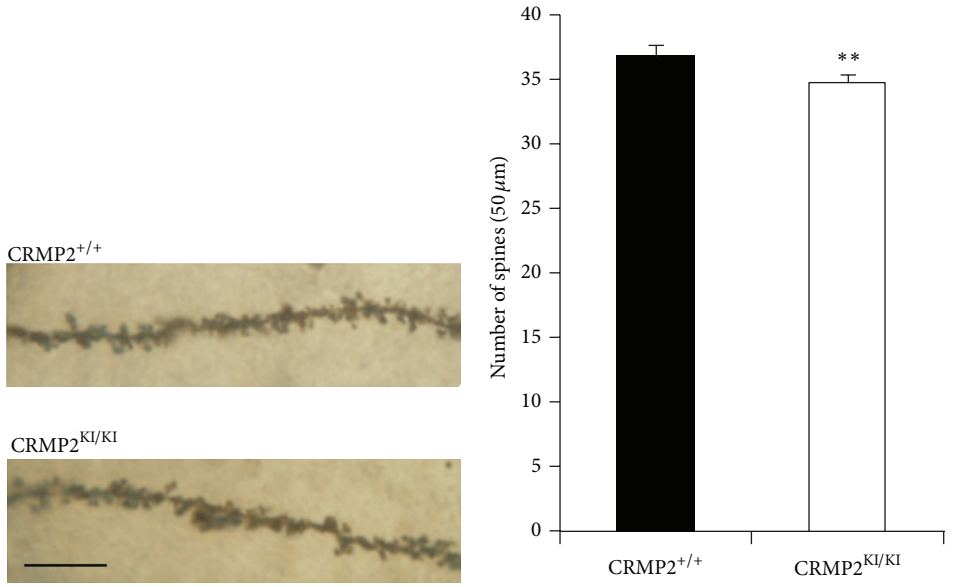

FIGURE 2: Reduction of dendritic spine density in hippocampal CA1 pyramidal neurons in CRMP2$^{KI/KI}$ mice at P18. (a) Representative photographs of dendritic segments of hippocampal CA1 pyramidal neurons at P18. Scale bar, 10 μm. (b) Reduced dendritic spine density was observed in hippocampal CA1 pyramidal neurons of CRMP2$^{KI/KI}$ mice compared to those of control mice. 50 neurons in each area from three mice in each genotype were analyzed. $^{*}P < 0.05$.

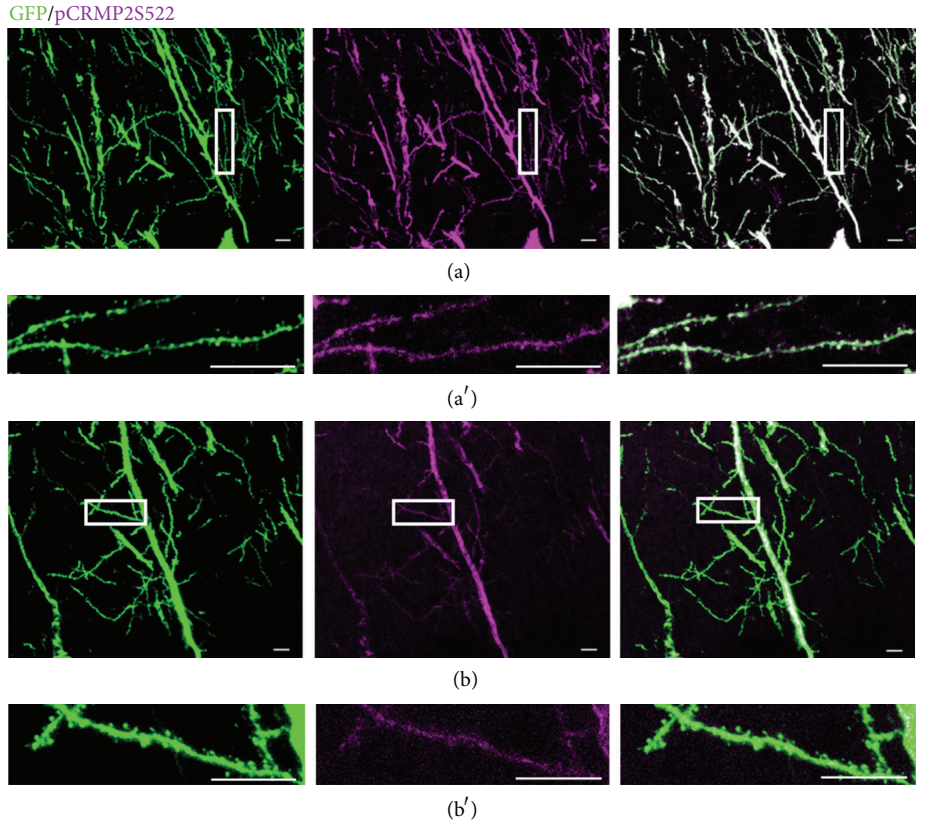

FIGURE 3: Localization of phospho-CRMP2 at dendritic spines of hippocampal CA1 pyramidal neurons. (a) Representative images of immunostaining of apical dendrites and their branches with phospho-CRMP2(S522) (pCRMP2S522) antibody in hippocampal CA1 pyramidal neurons from GFP-M mice. Magnified images of the areas indicated in (a) are shown in (a′). Scale bar, 10 μm. (b) Representative images of immunostaining of apical dendrites and their branches with pCRMP2S522 antibody of hippocampal CA1 pyramidal neurons in GFP-M, CRMP2$^{KI/KI}$ mice. Magnified images of the areas indicated in (b) are shown in (b′). Scale bar, 10 μm.

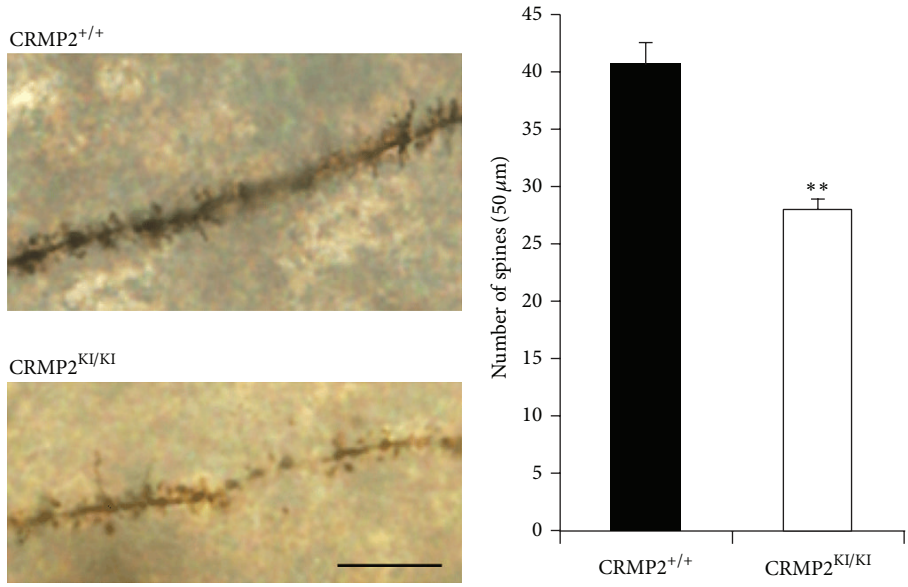

Figure 4: Reduction of dendritic spine density in hippocampal CA1 pyramidal neurons in 5 week-old CRMP2$^{KI/KI}$ mice. (a) Representative photographs of dendritic segments of hippocampal CA1 pyramidal neurons at 5 weeks old. Scale bar, 10 μm. (b) Reduced dendritic spine density was observed in hippocampal CA1 pyramidal neurons of CRMP2$^{KI/KI}$ mice compared to those of control mice. 50 neurons in each area from three mice in each genotype were analyzed. $^{**}P < 0.01$.

p39 KO mice when we deleted the p35 gene in 4-month-old animals [12]. These findings indicate that spine formation and maintenance are dependent on Cdk5 kinase activity in the mouse brain.

Our previous study showed that Sema3A-induced spine development is mediated by phosphorylation of CRMP1 by Cdk5 [13]. Because CRMP1 and CRMP2 have functional similarities in brain development [14], we examined whether phosphorylation of CRMP2 by Cdk5 is also important for the development and maintenance of dendritic spines *in vivo*. Cdk5 specifically phosphorylates Ser residue of CRMP2 at 522 [10]. We previously generated CRMP2$^{KI/KI}$ mice to study the function of Cdk5-mediated CRMP2 phosphorylation by replacing Ser at 522 to Ala [14]. Our present analysis of dendritic spine densities in hippocampal CA1 pyramidal neurons in CRMP2$^{KI/KI}$ mice at P18 showed reduced spine densities in these neurons compared to those of controls (Figure 2). Along with our previous study on CRMP1KO mice [13], these results indicate that CRMPs are important substrates of Cdk5 for spine formation. The results obtained in the present study will provide a new insight into the regulatory mechanisms underlying the effect of Cdk5 on dendritic spine density.

Our analysis of 5-week-old CRMP2$^{KI/KI}$ mice showed further reduction of spine densities in hippocampal CA1 pyramidal neurons (Figure 4). These results exclude the possibility that reduced spine densities of hippocampal neurons in CRMP2$^{KI/KI}$ mice at P18 (Figure 2) are due to the delay of brain development. In the cerebral cortex of macaque monkeys and humans, the number of dendritic spines rapidly increases after birth and peaks in an early phase of the infantile period [24]. Spine density then decreases during the later infantile period and adolescence period to reach the adult level [25]. Decrease of dendritic spine density during the transition from puberty to adulthood has also been reported in the mouse hippocampus [26]. These studies indicate ontogenetic similarity between rodent, primate, and human in spine formation and pruning. This overshoot-type time course of spine formation and pruning is attractive for researchers because it is possibly involved in developmental and psychiatric disorders [27]. Further studies are also required for the analysis of the involvement of CRMP2 phosphorylation in spine pruning and maintenance.

Cdk5 and its activator p35 play multiple roles in brain development, especially in neuronal migration [28]. Emerging evidence suggests that Cdk5/p35 is also involved in synaptic plasticity [29]. Cdk5/p35 is localized at neuronal synapses and phosphorylates many synaptic proteins [21, 30–33]. Furthermore, the induction of synaptic plasticity and spatial learning are impaired in Cdk5/p35 mutant mice [34–36]. The role of Cdk5 in synaptic plasticity and learning was initially studied using Cdk5 inhibitors, which showed inhibition of hippocampal LTP induction and context-dependent fear conditioning [30, 37]. We have previously reported the impairment of long-term depression (LTD) induction and spatial learning and memory in p35 KO mice [36]. Our recent study of p35 conditional KO (cKO) mice, which lack histological abnormalities in the brain, also showed impairment of spatial learning and memory and LTD induction [38]. Importantly, electrophysiological analysis of hippocampal slices from p35 cKO mice revealed reduced synaptic

transmission in hippocampal CA1 pyramidal neurons [38]. Since we observed reduced spine densities of hippocampal CA1 pyramidal neurons in CRMP2$^{KI/KI}$ mice, further electrophysiological studies of hippocampal synaptic plasticity and behavioral analysis in CRMP2$^{KI/KI}$ mice will provide further knowledge of the significance of Cdk5-mediated CRMP2 phosphorylation in synaptic plasticity and in learning and memory.

5. Conclusions

CRMP2 is phosphorylated in dendritic spines of rodent hippocampal neurons *in vitro* and *in vivo*. When we eliminated Cdk5-mediated phosphorylation of CRMP2 at S522 in the mouse brain, the densities of dendritic spines of hippocampal neurons were reduced in the mouse brain. These results suggest the regulation of spine density of hippocampal neurons by CRMP2 phosphorylation.

Conflict of Interests

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

Authors' Contribution

Xiaohua Jin and Kodai Sasamoto contributed equally to this work.

Acknowledgments

The authors thank Dr. Joshua R. Sanes for GFP-M transgenic mice. This work was supported by grants from Grant-in-Aid for JSPS KAKENHI Grant no. 26430043 (Toshio Ohshima).

References

[1] Y. Goshima, F. Nakamura, P. Strittmatter, and S. M. Strittmatter, "Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33," *Nature*, vol. 376, no. 6540, pp. 509–514, 1995.

[2] L.-H. Wang and S. M. Strittmatter, "A family of rat CRMP genes is differentially expressed in the nervous system," *The Journal of Neuroscience*, vol. 16, no. 19, pp. 6197–6207, 1996.

[3] M. Fukada, I. Watakabe, J. Yuasa-Kawada et al., "Molecular characterization of CRMP5, a novel member of the collapsin response mediator protein family," *The Journal of Biological Chemistry*, vol. 275, no. 48, pp. 37957–37965, 2000.

[4] R. Inatome, T. Tsujimura, T. Hitomi et al., "Identification of CRAM, a novel unc-33 gene family protein that associates with CRMP3 and protein-tyrosine kinase(s) in the developing rat brain," *The Journal of Biological Chemistry*, vol. 275, no. 35, pp. 27291–27302, 2000.

[5] J. Yuasa-Kawada, R. Suzuki, F. Kano, T. Ohkawara, M. Murata, and M. Noda, "Axonal morphogenesis controlled by antagonistic roles of two CRMP subtypes in microtubule organization," *European Journal of Neuroscience*, vol. 17, no. 11, pp. 2329–2343, 2003.

[6] Y. Fukata, T. J. Itoh, T. Kimura et al., "CRMP-2 binds to tubulin heterodimers to promote microtubule assembly," *Nature Cell Biology*, vol. 4, no. 8, pp. 583–591, 2002.

[7] N. Arimura, C. Ménager, Y. Kawano et al., "Phosphorylation by Rho kinase regulates CRMP-2 activity in growth cones," *Molecular and Cellular Biology*, vol. 25, no. 22, pp. 9973–9984, 2005.

[8] M. Varrin-Doyer, A. Nicolle, R. Marignier et al., "Human T lymphotropic virus type 1 increases T lymphocyte migration by recruiting the cytoskeleton organizer CRMP2," *Journal of Immunology*, vol. 188, no. 3, pp. 1222–1233, 2012.

[9] M. Tan, C. Cha, Y. Ye et al., "CRMP4 and CRMP2 interact to coordinate cytoskeleton dynamics, regulating growth cone development and axon elongation," *Neural Plasticity*, vol. 2015, Article ID 947423, 13 pages, 2015.

[10] Y. Uchida, T. Ohshima, Y. Sasaki et al., "Semaphorin3A signalling is mediated via sequential Cdk5 and GSK3beta phosphorylation of CRMP2: implication of common phosphorylating mechanism underlying axon guidance and Alzheimer's disease," *Genes to Cells*, vol. 10, no. 2, pp. 165–179, 2005.

[11] T. Yoshimura, Y. Kawano, N. Arimura, S. Kawabata, A. Kikuchi, and K. Kaibuchi, "GSK-3β regulates phosphorylation of CRMP-2 and neuronal polarity," *Cell*, vol. 120, no. 1, pp. 137–149, 2005.

[12] N. Mita, X. He, K. Sasamoto, T. Mishiba, and T. Ohshima, "Cyclin-dependen kinase 5 regulates dendritic spine formation and maintenance of cortical neuron in the mouse brain," *Cerebral Cortex*, 2014.

[13] N. Yamashita, A. Morita, Y. Uchida et al., "Regulation of spine development by semaphorin3A through cyclin-dependent kinase 5 phosphorylation of collapsin response mediator protein 1," *The Journal of Neuroscience*, vol. 27, no. 46, pp. 12546–12554, 2007.

[14] N. Yamashita, T. Ohshima, F. Nakamura et al., "Phosphorylation of CRMP2 (collapsin response mediator protein 2) is involved in proper dendritic field organization," *The Journal of Neuroscience*, vol. 32, no. 4, pp. 1360–1365, 2012.

[15] G. Feng, R. H. Mellor, M. Bernstein et al., "Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP," *Neuron*, vol. 28, no. 1, pp. 41–51, 2000.

[16] K. Goslin, H. Asmussen, and G. Banker, "Rat hippocampal neurons in low-density culture," in *Culturing Nerve Cells*, G. Banker and K. Goslin, Eds., pp. 339–370, MIT Press, Cambridge, UK, 1998.

[17] E. Niisato, J. Nagai, N. Yamashita et al., "CRMP4 suppresses apical dendrite bifurcation of CA1 pyramidal neurons in the mouse hippocampus," *Developmental Neurobiology*, vol. 72, no. 11, pp. 1447–1457, 2012.

[18] W.-Y. Fu, Y. Chen, M. Sahin et al., "Cdk5 regulates EphA4-mediated dendritic spine retraction through an ephexin1-dependent mechanism," *Nature Neuroscience*, vol. 10, no. 1, pp. 67–76, 2007.

[19] Y. Kim, J. Y. Sung, I. Ceglia et al., "Phosphorylation of WAVE1 regulates actin polymerization and dendritic spine morphology," *Nature*, vol. 442, no. 7104, pp. 814–817, 2006.

[20] K.-O. Lai, A. S. L. Wong, M.-C. Cheung et al., "TrkB phosphorylation by Cdk5 is required for activity-dependent structural plasticity and spatial memory," *Nature Neuroscience*, vol. 15, no. 11, pp. 1506–1515, 2012.

[21] M. A. Morabito, M. Sheng, and L.-H. Tsai, "Cyclin-dependent kinase 5 phosphorylates the N-terminal domain of the postsynaptic density protein PSD-95 in neurons," *Journal of Neuroscience*, vol. 24, no. 4, pp. 865–876, 2004.

[22] K. Tanabe, H. Yamazaki, Y. Inaguma et al., "Phosphorylation of drebrin by cyclin-dependent kinase 5 and its role in neuronal migration," *PLoS ONE*, vol. 9, no. 3, Article ID e92291, 2014.

[23] K. O. Lai, Z. Liang, E. Fei, H. Huang, and N. Y. Ip, "Cdk5-dependent phosphorylation of p70 ribosomal S6 kinase (S6K) is required for dendritic spine morphogenesis," *The Journal of Biological Chemistry*, vol. 290, no. 23, pp. 14637–14646, 2015.

[24] P. R. Huttenlocher, "Morphometric study of human cerebral cortex development," *Neuropsychologia*, vol. 28, no. 6, pp. 517–527, 1990.

[25] M. Missler, A. Wolff, H.-J. Merker, and J. R. Wolff, "Pre- and postnatal development of the primary visual cortex of the common marmoset. II. Formation, remodelling, and elimination of synapses as overlapping processes," *Journal of Comparative Neurology*, vol. 333, no. 1, pp. 53–67, 1993.

[26] G. Meyer, R. Ferres-Torres, and M. Mas, "The effects of puberty and castration on hippocampal dendritic spines of mice. A Golgi study," *Brain Research*, vol. 155, no. 1, pp. 108–112, 1978.

[27] P. Penzes, M. E. Cahill, K. A. Jones, J.-E. Vanleeuwen, and K. M. Woolfrey, "Dendritic spine pathology in neuropsychiatric disorders," *Nature Neuroscience*, vol. 14, no. 3, pp. 285–293, 2011.

[28] T. Ohshima, J. M. Ward, C.-G. Huh et al., "Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 20, pp. 11173–11178, 1996.

[29] K.-O. Lai and N. Y. Ip, "Recent advances in understanding the roles of Cdk5 in synaptic plasticity," *Biochimica et Biophysica Acta*, vol. 1792, no. 8, pp. 741–745, 2009.

[30] B.-S. Li, M.-K. Sun, L. Zhang et al., "Regulation of NMDA receptors by cyclin-dependent kinase-5," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 22, pp. 12742–12747, 2001.

[31] M. Matsubara, M. Kusubata, K. Ishiguro, T. Uchida, K. Titani, and H. Taniguchi, "Site-specific phosphorylation of synapsin I by mitogen-activated protein kinase and Cdk5 and its effects on physiological functions," *Journal of Biological Chemistry*, vol. 271, no. 35, pp. 21108–21113, 1996.

[32] S. C. Su, J. Seo, J. Q. Pan et al., "Regulation of N-type voltage-gated calcium channels and presynaptic function by cyclin-dependent kinase 5," *Neuron*, vol. 75, no. 4, pp. 675–687, 2012.

[33] K. Tomizawa, S. Sunada, Y.-F. Lu et al., "Cophosphorylation of amphiphysin I and dynamin I by Cdk5 regulates clathrin-mediated endocytosis of synaptic vesicles," *Journal of Cell Biology*, vol. 163, no. 4, pp. 813–824, 2003.

[34] J.-S. Guan, S. C. Su, J. Gao et al., "Cdk5 is required for memory function and hippocampal plasticity via the cAMP signaling pathway," *PLoS ONE*, vol. 6, no. 9, Article ID e25735, 2011.

[35] A. H. Hawasli, D. R. Benavides, C. Nguyen et al., "Cyclin-dependent kinase 5 governs learning and synaptic plasticity via control of NMDAR degradation," *Nature Neuroscience*, vol. 10, no. 7, pp. 880–886, 2007.

[36] T. Ohshima, H. Ogura, K. Tomizawa et al., "Impairment of hippocampal long-term depression and defective spatial learning and memory in $p35^{-/-}$ mice," *Journal of Neurochemistry*, vol. 94, no. 4, pp. 917–925, 2005.

[37] A. Fischer, F. Sananbenesi, C. Schrick, J. Spiess, and J. Radulovic, "Cyclin-dependent kinase 5 is required for associative learning," *Journal of Neuroscience*, vol. 22, no. 9, pp. 3700–3707, 2002.

[38] T. Mishiba, M. Tanaka, N. Mita et al., "Cdk5/p35 functions as a crucial regulator of spatial learning and memory," *Molecular Brain*, vol. 7, article 82, 2014.

Hindawi Publishing Corporation
Neural Plasticity
Volume 2016, Article ID 5136286, 10 pages
http://dx.doi.org/10.1155/2016/5136286

Review Article

The Involvement of Neuron-Specific Factors in Dendritic Spinogenesis: Molecular Regulation and Association with Neurological Disorders

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Received 15 June 2015; Accepted 26 July 2015

Academic Editor: Deepak P. Srivastava

Dendritic spines are the location of excitatory synapses in the mammalian nervous system and are neuron-specific subcellular structures essential for neural circuitry and function. Dendritic spine morphology is determined by the F-actin cytoskeleton. F-actin remodeling must coordinate with different stages of dendritic spinogenesis, starting from dendritic filopodia formation to the filopodia-spines transition and dendritic spine maturation and maintenance. Hundreds of genes, including F-actin cytoskeleton regulators, membrane proteins, adaptor proteins, and signaling molecules, are known to be involved in regulating synapse formation. Many of these genes are not neuron-specific, but how they specifically control dendritic spine formation in neurons is an intriguing question. Here, we summarize how ubiquitously expressed genes, including syndecan-2, NF1 (encoding neurofibromin protein), VCP, and CASK, and the neuron-specific gene CTTNBP2 coordinate with neurotransmission, transsynaptic signaling, and cytoskeleton rearrangement to control dendritic filopodia formation, filopodia-spines transition, and dendritic spine maturation and maintenance. The aforementioned genes have been associated with neurological disorders, such as autism spectrum disorders (ASDs), mental retardation, learning difficulty, and frontotemporal dementia. We also summarize the corresponding disorders in this report.

1. Introduction

The tiny protrusions emerging from dendrites known as dendritic spines are the primary subcellular locations of excitatory synapses in the mammalian central nervous system [1]. Dendritic spines are typically ~1-2 μm in length and 0.5–1 μm in width of the spine head, with diverse morphologies, such as mushroomlike, stubby, and thin spines. These structures are mainly supported by the F-actin cytoskeleton. Thus, F-actin cytoskeletal proteins and regulators are important factors for generating dendritic spines. Many membrane proteins and adaptor and signaling molecules are also involved in controlling dendritic spine formation and maintenance [2]. Several mechanisms have been described to form dendritic spines [3]. The most popular mechanism is that dendritic filopodia serve as precursors for dendritic spine formation. Interestingly, filopodia are ubiquitously found on various cell types. In contrast, dendritic spines are neuron-specific structures. Thus, the transition from filopodia to spines should be controlled by neuron-specific factors.

Neuron-specific factors controlling dendritic spinogenesis fall into two categories. The first group is proteins specifically expressed in neurons. The second group is neuron-specific cellular responses or processes. These proteins or responses directly or indirectly regulate F-actin rearrangement and dynamics to promote dendritic spine formation. Studies of cytoskeleton-associated cortactin-binding protein 2 (CTTNBP2) and heparan sulfate transmembrane proteoglycan (HSPG) syndecan-2 serve as examples for these two categories, respectively. CTTNBP2 is a neuron-specific cytoskeleton-associated protein and that is enriched at dendritic spines of mature neurons. Although syndecan-2 is widely expressed in many cell types, it is highly concentrated at

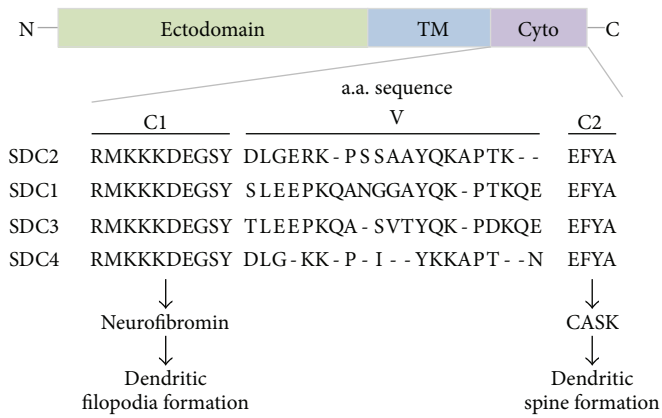

Figure 1: Schematic structure and amino acid sequences of syndecans. C1, conserved domain 1; C2, conserved domain 2; Cyto, cytoplasmic domain; SDC2, syndecan-2; SDC1, syndecan-1; SDC3, syndecan-3; SDC4, syndecan-4; TM, transmembrane; V, variable region.

synapses in neurons. Syndecan-2 cooperates with other proteins to trigger neurotransmission through a neuron-specific signal to induce dendritic spine formation. Genomic analyses of patients with autism spectrum disorders (ASDs) indicated that both CTTNBP2 and syndecan-2 were associated with ASDs [4, 5]. Additionally, neurofibromin, CASK, and VCP coordinate with syndecan-2 to control dendritic spinogenesis and were also associated with neurological disorders. These findings suggest that these genes are critical for neuronal function, likely through their regulation of dendritic spine formation. In this review, we will summarize the functions of these proteins in dendritic spinogenesis and use these proteins as examples to discuss how neuron-specific molecules coordinate with ubiquitously expressed proteins to generate neuron-specific signals for dendritic spine formation.

2. The HSPG Syndecan-2 Triggers Dendritic Spine Formation

2.1. Syndecan-2 Is Enriched at Dendritic Spines and Is Required for Dendritic Spine Formation. Syndecan-2 is a type I membrane protein with a heparan sulfate modification at its ectodomain (Figure 1) [6]. In mammals, the syndecan protein family contains four members, syndecan-1, syndecan-2, syndecan-3, and syndecan-4 [7]. In rodent brains, syndecan-2 and syndecan-3 are the two major syndecans expressed in neurons with differential distribution; syndecan-2 is highly concentrated at synapses, while syndecan-3 is distributed along the axonal shaft [8]. Syndecan-2 is involved in cell-cell and cell-matrix interactions through its heparan sulfate modification. It can also bind growth factors, such as fibroblast growth factor (FGF) and epidermal cell growth factor, and it acts as a coreceptor for these growth factors [7]. Syndecan-2 is broadly and dynamically expressed in several tissues and cell types [7, 8]. During neural development, its expression gradually increases concurrent with synapse formation [8, 9]. In mature neurons, such as cultured rat hippocampal neurons at 18 days after plating *in vitro* (DIV) or later, syndecan-2 is highly enriched at dendritic spines [9, 10]. More importantly, overexpression of syndecan-2 in immature rat hippocampal cultured neurons, such as 1-2 DIV, when endogenous syndecan-2 is not yet expressed, dendritic filopodia are massively induced at 4-5 DIV and dendritic filopodia are then transformed to dendritic spines at 8-9 DIV [9, 11]. Those dendritic spines are expected to be functional, as they are adjacent to the presynaptic marker synaptophysin based on confocal microscopy [11, 12]. Syndecan-2-induced dendritic spinogenesis serves as a model to explore the mechanisms underlying the initiation of dendritic spinogenesis (namely, dendritic filopodia formation), the transition from filopodia to spines, and dendritic spine maturation and maintenance.

2.2. The C1 and C2 Motifs of Syndecan-2 Work Sequentially to Promote Dendritic Spinogenesis. The ectodomain of syndecan-2 heparan sulfate modification is involved in cell-cell and cell-matrix interactions [7]. Its transmembrane domain is required for homodimerization or oligomerization [13], which is critical for the protein-protein interactions of syndecan-2 [14]. The cytoplasmic domain of syndecan-2 contains only 32 amino acid residues (Figure 1). Although it is short, it is divided into three motifs, conserved domain 1 (C1), the variable region (V), and conserved domain 2 (C2). The C1 and C2 motifs are conserved among different syndecans, while the sequences of the V regions vary (Figure 1). The C1 motif is essential for syndecan-2-induced dendritic filopodia formation of rat hippocampal cultured neurons, as the syndecan-2ΔC1 mutant completely loses the ability to promote filopodia formation and spine formation at 5 as well as 9 DIV [11, 15]. The C2 is required for the dendritic filopodia-spines transition and dendritic spine maintenance [15, 16]. Expression of the C2 deletion mutant syndecan-2ΔC2 at 2 DIV promotes dendritic filopodia formation at 5 DIV. However, those filopodia are unable to transform into dendritic spine at 9 DIV [11, 15, 16]. These analyses indicate that the function of syndecan-2 in dendritic spinogenesis can be separated into two sequential steps, namely, filopodia and spine formation, which are controlled by two distinct motifs in syndecan-2.

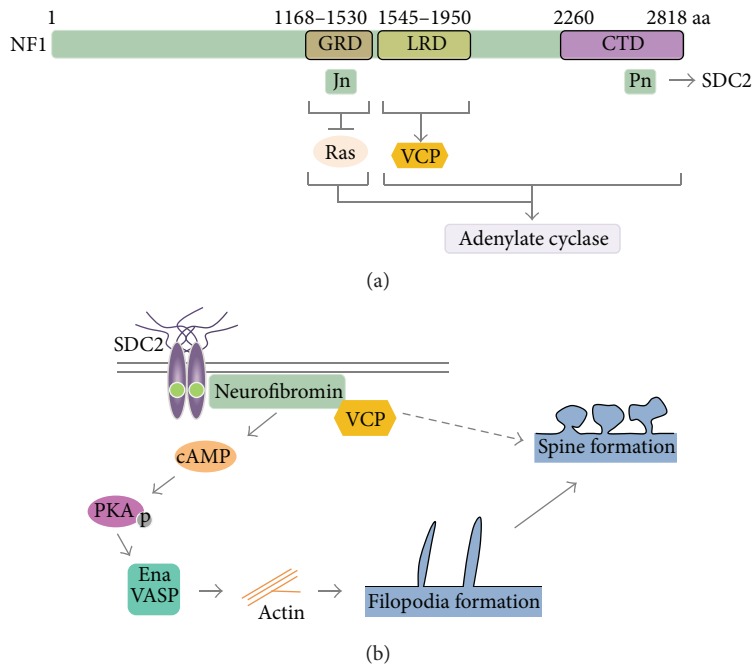

Figure 2: Function of neurofibromin in neurons. (a) Neurofibromin-interacting proteins. The Jn and Pn fragments interact with syndecan-2. The leucine-rich domain (LRD) binds VCP. The GAP-related domain (GRD) downregulates the Ras pathway. Both GRD and the C-terminal half of neurofibromin are involved in adenylate cyclase activity regulation. CTD, C-terminal region. (b) Neurofibromin controls dendritic filopodia and spine formation through the PKA-Ena/VASP and VCP pathways, respectively.

Because both C1 and C2 motifs are short and lack recognizable enzymatic domains, syndecan-2 binding partners have been identified to determine its molecular mechanism underlying dendritic spine formation. Several direct binding partners (summarized in Table 1) have been identified for the C1 domains of syndecan-2, including neurofibromin [17] and ezrin [18]. The C2 motif directly interacts with syntenin [19], CASK [10], and synbindin [20]. Among these, the interactions between syndecan-2 and neurofibromin and CASK have been shown to be relevant in dendritic spine formation. Because the cytoplasmic tail of syndecan-2 is very short, it is unlikely that a single syndecan-2 molecule can simultaneously interact with all of its binding partners. Because the C1 and C2 motifs are involved in two sequential processes, it is likely that neurofibromin and CASK sequentially interact with syndecan-2. Alternatively, it is possible that because syndecan-2 forms at least a dimer through its transmembrane domain, different syndecan-2 molecules in dimers or oligomers separately interact with neurofibromin and CASK. This would suggest that syndecan-2, neurofibromin, and CASK form a single large complex. Further investigation, including coimmunoprecipitation experiments, is required to address this question.

Table 1: SDC2 interacting proteins.

	Binding site in SDC2	Binding site for SDC2	Function
NF1	C1	LRD	Filopodia formation
Ezrin	C1	N-ter.	Links to actin cytoskeleton
Syntenin	C2	PDZ	Cell adhesion and migration
CASK	C2	PDZ	Dendritic spine formation
Synbindin	C2	PDZ-like	Vesicle transport

2.3. Neurofibromin Interacts with the C1 Motif of Syndecan-2 and Promotes Syndecan-2-Induced Dendritic Filopodia Formation. Neurofibromin encoded by the neurofibromatosis type I (*NF1*) gene is characterized by its RasGAP- (Ras GTPase activating protein-) related domain (GRD) (Figure 2(a)) [21–24]. Similar to syndecan-2, neurofibromin is widely expressed in different cell types, though its expression level is much higher in the nervous system [25]. NF1 is one of the most common human inherited disorders featured by changes in skin pigmentation, benign tumor growth, and learning difficulty [26, 27]. Neurofibromin suppresses tumor growth through its ability to downregulate the RAS pathway [28]. In addition to its RAS activity, neurofibromin can increase cAMP concentration by activating adenylate cyclase [29]. Although the molecular mechanisms are less clear, the GRD and C-terminal region of neurofibromin are required for cAMP pathway activation (Figure 2(a)) [30]. Both Gs-dependent and Gs-independent pathways are involved in neurofibromin-regulated adenylate cyclase activation [30]. The cAMP pathway has been shown to be involved in learning

and memory in *Drosophila* [31] and dendritic spine formation in the mammalian nervous system [11].

In a yeast two-hybrid screen using different fragments of neurofibromin as baits, syndecan-2 was identified as a neurofibromin binding partner [17]. Notably, neurofibromin has two independent interacting domains for the C1 motif of syndecan-2. One is the Jn fragment corresponding to amino acid residues 1357–1473 in the GRD of human neurofibromin; the other is the Pn fragment containing amino acid residues 2619–2719 (Figure 2(a)) [17]. The Jn and Pn compete for binding to the C1 motif of syndecan-2. In addition to biochemical studies demonstrating the direct interaction between syndecan-2 and neurofibromin, fluorescence immunostaining further demonstrated the colocalization of syndecan-2 and neurofibromin at synapses in cultured hippocampal neurons [17]. Moreover, both Nf1 knockdown and haploinsufficiency reduce the density of dendritic spines in both rat hippocampal and mouse cortical cultured neurons and in brains [11, 32], consistent with a function of neurofibromin in regulating dendritic spine formation.

The next question is how the syndecan-2-neurofibromin complex regulates dendritic spine formation. One study examined syndecan-2 downstream signaling for triggering filopodia formation. Using a panel of inhibitors to suppress various kinase activities, protein kinase A (PKA) was identified to be required for syndecan-2-induced filopodia formation [11]. Combined with the analysis using different motif deletion mutants of syndecan-2, we found that the C1 motif of syndecan-2 is essential for PKA-dependent filopodia formation [11]. Because neurofibromin interacts with the C1 motif and also activates the cAMP pathway, cultured hippocampal neurons were then used to investigate whether neurofibromin mediates syndecan-2-induced filopodia formation. Both Nf1 knockdown and Jn fragment expression, which acts as a dominant-negative to disrupt the interaction between endogenous neurofibromin and syndecan-2, suppress syndecan-2-induced dendritic filopodia formation of rat hippocampal cultured neurons at 5 DIV [11]. Thus, neurofibromin mediates the signal from syndecan-2 to the cAMP pathway to initiate dendritic spinogenesis.

Because filopodia are supported by F-actin bundles, the syndecan-2-neurofibromin-cAMP pathway has to induce F-actin polymerization and bundle formation to promote dendritic filopodia formation. The Ena (Enabled)/VASP (Vasodilator-Stimulated Phosphoprotein) protein family is a group of F-actin regulators that initiate actin polymerization and bundling [33]. PKA phosphorylation promotes Ena/VASP protein activity to regulate the F-actin cytoskeleton [34]. Upon syndecan-2 overexpression, Ena/VASP phosphorylation increases, consistent with cAMP pathway activation. Moreover, disruption of Ena/VASP activity impairs syndecan-2-induced dendritic filopodia formation [11]. In summary, these studies indicate that syndecan-2 overexpression enhances the ability of neurofibromin to activate the PKA pathway, which then induces the Ena/VASP activity to promote F-actin bundling and filopodia formation.

Although the PKA pathway is required for dendritic filopodia formation, increased intracellular cAMP concentrations alone cannot induce dendritic filopodia formation [11], suggesting that other factor(s) are involved. From an immunoprecipitation-mass spectrometry study, valosin-containing protein (VCP, also known as P97) was identified as a neurofibromin-binding protein [32]. The entire D1 and D2 ATPase domains of VCP are required for the interaction with the leucine-rich domain (LRD) of neurofibromin [32]. VCP is a causative gene of inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia (IBMPFD) [35]. IBMPFD patients frequently suffer from dementia. In addition, VCP mutations are associated with ASDs and amyotrophic lateral sclerosis [36, 37]. These evidences suggest that VCP mutations impair brain function. A combination of human genetic studies, mouse genetic models, and cultured hippocampal and cortical neurons have indicated that neurofibromin interacts with VCP and guides VCP to promote dendritic spinogenesis [32]. The roles of VCP and neurofibromin in dendritic spine formation may account for the neural phenotypes in patients with mutations in the NF1 and VCP genes. However, it is still unclear how VCP regulates dendritic spine formation. To fully address the molecular regulation of neurofibromin and VCP in dendritic spinogenesis, further studies are required.

The function of the syndecan-2-neurofibromin interaction in dendritic spine formation is summarized in Figure 2(b).

2.4. CASK and Syndecan-2 Interactions Regulate Dendritic Spine Maturation. CASK is a ubiquitously expressed gene and is critical for brain development and function [38]. Mutations in the human CASK gene result in X-linked mental retardation and microcephaly with pontine and cerebellar hypoplasia [39–43]. CASK belongs to the membrane-associated guanylate kinase (MAGUK) family and functions as a scaffold protein to interact with more than two dozen cellular proteins [44]. It is widely distributed in neurons, including synapses, dendrites, axons, and soma [10]. At synapses, it localizes to both pre- and postsynaptic sites [10]. In mouse pontine explants and rat hippocampal cultured neurons, CASK knockdown impairs synapse formation at the pre- and postsynapse, respectively [16, 45]. At presynaptic sites, it binds the membrane protein neurexin and other scaffold proteins, such as Mint1, mLin7, and liprin, to control presynaptic button formation [45–48]. CASK uses its PDZ domain at the postsynaptic site to interact with the C2 motif of syndecan-2 [10]. In cultured hippocampal neurons, expression of the PDZ alone of CASK or the C-terminal tail of syndecan-2 that disrupts the interaction between endogenous CASK and syndecan-2 reduces dendritic spine density, narrows spine heads, and shortens spine length at 18 DIV, suggesting that the CASK-syndecan-2 interaction is critical for dendritic spine formation [16].

To investigate whether CASK is involved in dendritic spinogenesis initiation or dendritic spine stabilization, a time course study using a knockdown approach in cultured hippocampal neurons has been performed [16]. The time window of 15–18 DIV covering the initiation toward maturation of dendritic spinogenesis was used for analysis. At 15 DIV,

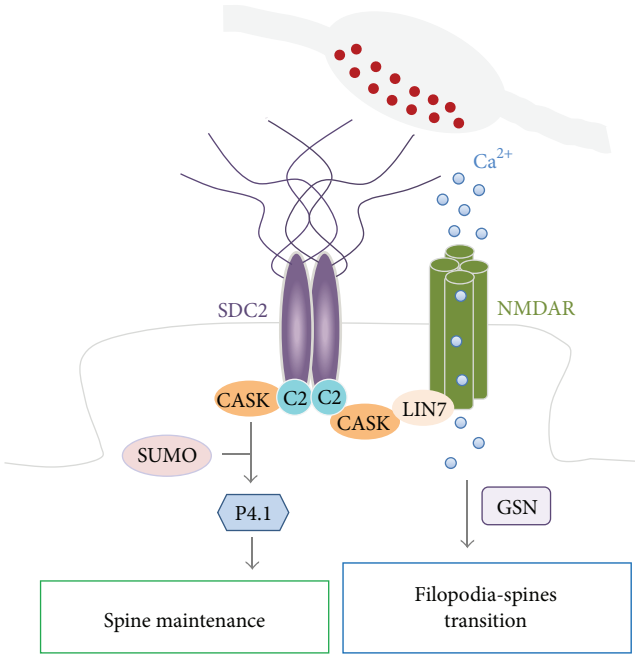

Figure 3: Syndecan-2 coordinates with calcium influx to control dendritic spine formation and maturation. Syndecan-2 links the CASK-mLIN7-NMDAR complex through its C2 motif and directs this complex to target to filopodial tips. It increases the accessibility of postsynaptic filopodia to presynaptic stimulation, which is critical for calcium influx to induce the filopodia-spines transition. In addition to linking mLIN7 and NMDAR, CASK interacts with the protein 4.1-F-actin cytoskeleton. This interaction provides a physical link between the membrane and cytoskeleton to stabilize the dendritic spine structure. GSN, gelsolin.

wild-type dendritic spines are immature, long, and thin, and they are present at a low density. As they mature at 18 DIV, dendritic spine density increases, spine length decreases, and spine width increases. Compared to control neurons, CASK knockdown does not affect spine density, length, or size at 15 DIV, suggesting that CASK is not critical for dendritic spinogenesis initiation. At 18 DIV, CASK knockdown induces dendritic spines withdraw and the spine heads fail to enlarge. The spine density is decreased compared to control neurons [16]. The data indicate that CASK is important for dendritic spine maturation, likely by linking the membrane protein syndecan-2 to the F-actin cytoskeleton via protein 4.1 to stabilize dendritic spines (Figure 3) [16].

2.5. Neurotransmission-Induced Calcium Influx Is Critical for the Syndecan-2-Induced Filopodia-Spines Transition. In human embryonic kidney HEK293 cells, syndecan-2 overexpression induced numerous filopodia on the cell surface [11]. However, these filopodia cannot mature into spines. Because neurofibromin and CASK are also expressed in HEK293 cells, the aforementioned studies cannot explain why syndecan-2-induced dendritic spines are only present in neurons. A neuron-specific factor must be present to control dendritic spine formation. Because neurotransmission is a neuron-specific event and because dendritic filopodia are able to receive neurotransmission signals from presynaptic buttons [49], neurotransmission seems a likely factor that triggers the filopodia-spines transformation in a neuron-specific manner. Indeed, EGTA treatment to chelate extracellular calcium or AP5 treatment to block NMDAR activity, a major neurotransmitter gated calcium channel, impairs the endogenous filopodia-spines transition at 15–17 DIV and syndecan-2-induced filopodia-spines transition at 5–9 DIV [15]. In syndecan-2-overexpressing neurons, intracellular calcium concentration is increased compared to control neurons at 5 DIV. This increase is due to NMDAR-regulated calcium influx because AP5 treatment effectively reduced the intracellular calcium concentration induced by syndecan-2 [15]. The C2 motif of syndecan-2 is required for syndecan-2 overexpression-induced calcium influx [15], suggesting that the interaction with CASK is involved in calcium influx. Previous studies have shown that CASK interacts with mLIN7 via the L27 domains in both proteins [50–52] and that mLIN7 interacts with the C-terminal tail of NMDAR subunit 2b (NMDAR2b) through its PDZ domain [53]. Thus, the CASK-mLIN7 complex links NMDAR to syndecan-2. The interaction between syndecan-2, CASK, mLIN7, and NMDAR2b facilitates NMDAR localization to the tips of dendritic filopodia, where NMDAR may be activated by presynaptic stimulation, namely, glutamate, and induce calcium influx. Disruption of the syndecan-2, CASK, mLIN7, and NMDAR complex by overexpressing the interacting domains impairs NMDAR filopodial distribution, calcium influx, and the filopodia-spines transition [15], suggesting that syndecan-2 triggers calcium influx via the CASK-mLIN7-NMDAR complex and induces the filopodia-spines transition (Figure 3).

The morphological feature of the filopodia-spines transition is dendritic spine head enlargement and spine length shortening. The F-actin cytoskeleton must be rearranged to allow for this morphological change. Calcium is known to regulate F-actin dynamics in dendritic spines [54–56], and gelsolin is a calcium-activated F-actin regulator. It acts as an F-actin severing and capping protein [57–59]. Gelsolin deficiency impairs filopodial retraction of developing neurons [60] and inhibits activity-dependent F-actin remodeling in mature dendritic spines [61]. It is also critical for the filopodia-spines transition induced by the syndecan-2-CASK-mLIN7-NMDAR complex, as gelsolin knockdown maintains syndecan-2-induced protrusions at the filopodial stage [15]. It is possible that other calcium regulated F-actin regulators also act downstream of syndecan-2 to control the filopodia-spines transition. More investigations are required to further elucidate the regulation.

2.6. Conclusion of the Role of Syndecan-2 Signaling in Dendritic Spine Formation. Through its interactions with intracellular binding partners, the ubiquitously expressing protein syndecan-2 modulates the F-actin cytoskeleton, triggers neurotransmission, and promotes neuron-specific synapse formation. From dendritic filopodia formation, filopodia-spines transition to dendritic spine maturation, syndecan-2 interacts with different binding partners to control F-actin behaviors. Syndecan-2 first activates the PKA pathway via neurofibromin to promote F-actin polymerization and bundling for

dendritic filopodia formation [11]. It recruits NMDAR to filopodial tips through its interaction with the CASK-mLIN7 complex and increases the postsynaptic responsiveness to presynaptic stimulation [15]. Calcium influx induces F-actin cytoskeleton rearrangement to allow for the morphological change from filopodia to spines [15]. To further promote dendritic spine maturation and maintenance, syndecan-2 binds to the protein 4.1 through interactions with CASK [16]. Throughout the entire process, neuron specificity falls within NMDAR-mediated calcium influx, which induces F-actin cytoskeleton remodeling to result in morphological changes to the dendritic spine. These studies provide a comprehensive example of how a neuron-specific ion channel coordinates with other adhesion molecules and synaptic proteins to control dendritic spine formation.

3. The Neuron-Specific Cytoskeleton Regulator CTTNBP2 Is Highly Associated with Autism Spectrum Disorders

To identify a neuron-specific F-actin regulator involved in dendritic spinogenesis, we searched the database and literature and focused on cortactin-binding protein 2 (CTTNBP2). CTTNBP2 gene encodes a brain-specific protein that interacts with the SH3 domain of cortactin through its proline-rich domain [62]. Cortactin promotes and stabilizes F-actin branching [63, 64] and thus plays a critical role for dendritic spine morphological maintenance [65]. Because cortactin is a ubiquitously expressed protein, its function in controlling dendritic spinogenesis must be regulated by a neuron-specific factor. The specific expression of CTTNBP2 in the brain makes it a good candidate to control cortactin in dendritic spinogenesis. Furthermore, *de novo* mutations in the CTTNBP2 gene have been repeatedly identified in ASD patients [5, 37, 66]. In a genomic analysis covering 3871 ASD patients, results indicated that CTTNBP2 is a high-confidence risk factor for ASDs with a false discovery rate less than 0.05% [5]. These genetic data support a critical role for CTTNBP2 in brain development and function.

3.1. CTTNBP2 Variant Transcripts and ASD Mutations. In the expression tag sequence (EST) database (http://www.ncbi.nlm.nih.gov/), three variants have been identified as CTTNBP2 transcripts, namely, CTTNBP2-Short (CTTNBP2-S), CTTNBP2-Intron (CTTNBP2-I), and CTTNBP2-Long (CTTNBP2-L). Based on the nucleotide sequence, the first 625 predicted amino acid residues are shared among all variants [67]. Using an antibody against the common region of the CTTNBP variants, immunoblotting revealed that the Short form of CTTNBP2 is the predominant protein product in brains. The protein products of the Intron and Long forms are undetectable in adult brains [67]. Thus, the following studies of CTTNBP2 in neurons focused on CTTNBP2-S. It is still unclear whether the CTTNBP2-I and CTTNBP2-L variants play any role in neurons. Therefore, mutation analysis of ASD patients is meaningful when the mutation was located within the CTTNBP2-S variant sequences. Seven *de novo* ASD mutations in the CTTNBP2 gene have been identified in the exons encoding CTTNBP2-S [5]. To further explore the association of CTTNBP2 with ASD, these mutations should be investigated in detail to determine their effects on CTTNBP2 molecular function and neuronal morphogenesis.

Analysis of the amino acid sequence of CTTNBP2-S predicts a coiled-coil domain at the N-terminal region and proline-rich domain at the C-terminus. The middle region does not contain any recognizable protein structure [67]. The N-terminal coiled-coil domain mediates CTTNBP2-S homooligomerization and heterooligomerization of CTTNBP2-S and the striatin family [68, 69]. The C-terminal proline-rich domain interacts with cortactin [62]. The middle region is required for the protein's association with the microtubule cytoskeleton [69]. The functions of these interactions are discussed below (Figure 4).

3.2. CTTNBP2-S Controls Cortactin Mobility and Regulates Dendritic Spine Formation and Maintenance. CTTNBP2-S localizes to dendritic spines to control the cortactin-F-actin cytoskeleton. Both endogenous CTTNBP2-S and overexpressed Myc-tagged CTTNBP2-S were found to be highly concentrated at dendritic spines in mature cultured hippocampal neurons. Immunofluorescence analysis of adult brains also indicated that CTTNBP2-S colocalized with F-actin puncta *in vivo*, presumably to dendritic spines [67]. CTTNBP2-S is critical for dendritic spine formation, as CTTNBP2 knockdown right before dendritic spinogenesis at 12 DIV reduces spine density and spine head width measured at 18 DIV. Consistent with the morphological changes, the frequency of mEPSC (miniature excitatory postsynaptic synaptic current) is lower in CTTNBP2 knockdown neurons at 18 DIV [67]. In addition to dendritic spine formation, CTTNBP2-S is involved in dendritic spine maintenance, as CTTNBP2-S knockdown in mature neurons, such as 20 DIV, still reduces dendritic spine density at 26 DIV [67]. Cortactin is required for CTTNBP2-S's regulation of dendritic spinogenesis, as a CTTNBP2-S mutant that cannot interact with cortactin cannot rescue CTTNBP2 knockdown-induced spine deficiency [67]. Moreover, fluorescence recovery after photobleaching (FRAP) analysis indicates that CTTNBP2-S regulates cortactin mobility in mature dendritic spines. In the presence of CTTNBP2-S, cortactin more stably localizes to dendritic spines. The data suggest that CTTNBP2-S retains cortactin in dendritic spines and controls dendritic spine formation and maintenance [67].

CTTNBP2-S also controls distribution of striatin family proteins in dendritic spines [68]. The striatin protein family contains three mammalian members, namely, striatin, zinedin, and SG2NA. They function as B-type regulatory subunits of protein phosphatase 2A (PP2A) to control PP2A subcellular location and substrate specificity [70, 71]. All three striatin family members are highly enriched in dendritic spines [72]. Striatin protein distribution to synapses is mediated by its interactions with CTTNBP2-S through the N-terminal coiled-coil domains of both CTTNBP2-S and striatin family members. Similar to cortactin, CTTNBP2

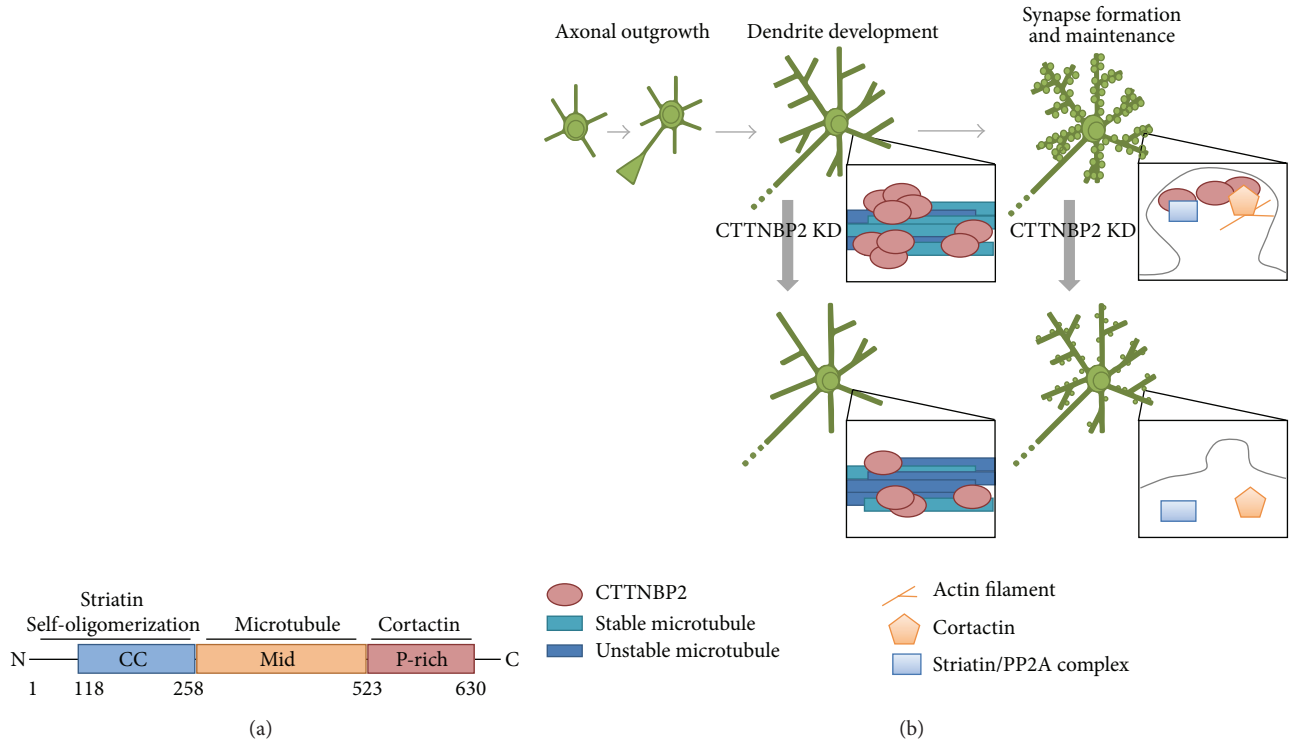

Figure 4: CTTNBP2 and neuronal differentiation. (a) Schematic domain structure and CTTNBP2-S-interacting proteins. CC, coiled-coil domain; Mid, middle region; P-rich, proline-rich domain. (b) The function of CTTNBP2-S in neuronal morphogenesis. CTTNBP2-S controls microtubule stability in the dendritic shafts and cortactin mobility in dendritic spines. Upon CTTNBP2 knockdown during dendritic extension, dendritic complexity decreases. During synaptogenesis, CTTNBP2-S helps maintain cortactin in dendritic spines and promotes dendritic spine formation and maintenance.

knockdown impairs dendritic spine targeting of striatins [68]. In conclusion, CTTNBP2-S regulates F-actin dynamics and PP2A signaling at dendritic spines.

3.3. CTTNBP2-S Modulates Microtubule Stability and Regulates Dendritic Arborization. In COS cells, exogenous CTTNBP2-S was unexpectedly associated with the microtubule cytoskeleton in addition to the cortactin-F-actin cytoskeleton [69]. Cell-matrix interactions influence the cytoskeleton association of CTTNBP2-S. In COS cells, CTTNBP2-S preferentially associates with the F-actin cytoskeleton within one hour after plating. CTTNBP2-S gradually shifts its preference to the microtubule cytoskeleton when establishing cell-matrix interactions [69]. CTTNBP2-S cyto skeletal associations also change in neurons. CTTNBP2-S is highly concentrated at dendritic spines in mature neurons where CTTNBP2-S interacts with F-actin cytoskeletons. In the premature stages when dendritic spines have not yet formed, CTTNBP2-S is already expressed and forms puncta attached on microtubule bundles along the dendritic shaft [69]. The association of CTTNBP2-S with microtubules increases microtubule stability by bundling the microtubules. Two CTTNBP2-S domains are required for microtubule bundling. The Mid domain is required for the association of microtubule, and the N-terminal coiled-coil domain is involved in CTTNBP2-S oligomerization. Oligomerization allows the CTTNBP2-S oligomer to contain multiple microtubule binding sites to induce microtubule bundling [69]. During the dendritic extension stage, CTTNBP2-S knockdown or disruption of microtubule bundling by overexpression of the N-terminal coiled-coil domain or Mid domain impairs dendritic arborization [69]. The studies suggest that, in addition to controlling the F-actin cytoskeleton, CTTNBP2-S regulates microtubule stability to influence dendrite morphology.

3.4. Outstanding Questions about CTTNBP2. The dual roles of CTTNBP2-S in controlling F-actin and the microtubule cytoskeletons require further investigation. As a neuron-specific morphology regulator and a high-confidence risk factor for ASDs, CTTNBP2 deserves further study. Several questions remain to be addressed. For instance, what is the molecular mechanism regulating the association between CTTNBP2-S and F-actin and microtubules? Are the associations of CTTNBP2-S with F-actin and microtubules mutually exclusive? Alternatively, can CTTNBP2-S act as a bridge to link F-actin and microtubules? Only cultured hippocampal neurons have been examined in functional studies of CTTNBP2-S. In the future, *in vivo* studies should be considered. Particularly, to address the association of CTTNBP2 with ASDs, a mouse genetic model is required. The impact of CTTNBP2 ASD mutations on the molecular function

of CTTNBP2-S, brain development, and cognition must be studied to further understand the biological significance of CTTNBP2.

4. Conclusions

Although hundreds of genes are involved in dendritic spine formation, they should be either neuron-specific or directly or indirectly controlled by or linked to neuron-specific signaling or proteins to specifically regulate dendritic spine formation in neurons. In this review, syndecan-2-induced dendritic spine formation and the role of CTTNBP2-S in controlling neuronal morphology provide two distinct examples of how neuronal morphology can be regulated in a neuron-specific manner. The regulation of neuronal morphology is critical for normal brain function. Understanding these regulations is crucial for basic research and for understanding neurological disorder etiology, which could contribute to potential therapeutic treatments of the diseases.

Conflict of Interests

The authors declare no competing financial interests.

Acknowledgments

This work was supported by grants from Academia Sinica (AS-103-TP-B05) and the Ministry of Science and Technology (MOST 103-2321-B-001-002, 103-2321-B-001-018, and 104-2321-B-001-050) to Yi-Ping Hsueh. Hsiao-Tang Hu is supported by the Postdoctoral Fellowship of Academia Sinica.

References

[1] K. M. Harris and J. K. Stevens, "Dendritic spines of CA1 pyramidal cells in the rat hippocampus: serial electron microscopy with reference to their biophysical characteristics," *Journal of Neuroscience*, vol. 9, no. 8, pp. 2982–2997, 1989.

[2] C. Sala and M. Segal, "Dendritic spines: the locus of structural and functional plasticity," *Physiological Reviews*, vol. 94, no. 1, pp. 141–188, 2014.

[3] I. M. Ethell and E. B. Pasquale, "Molecular mechanisms of dendritic spine development and remodeling," *Progress in Neurobiology*, vol. 75, no. 3, pp. 161–205, 2005.

[4] Y. Ishikawa-Brush, J. F. Powell, P. Bolton et al., "Autism and multiple exostoses associated with an X;8 translocation occurring within the GRPR gene and 3′ to the SDC2 gene," *Human Molecular Genetics*, vol. 6, no. 8, pp. 1241–1250, 1997.

[5] S. De Rubeis, X. He, A. P. Goldberg et al., "Synaptic, transcriptional and chromatin genes disrupted in autism," *Nature*, vol. 515, no. 7526, pp. 209–215, 2014.

[6] V. Lories, J.-J. Cassiman, H. D. Van Berghe, and G. David, "Differential expression of cell surface heparan sulfate proteoglycans in human mammary epithelial cells and lung fibroblasts," *The Journal of Biological Chemistry*, vol. 267, no. 2, pp. 1116–1122, 1992.

[7] M. Bernfield, M. Götte, P. W. Park et al., "Functions of cell surface heparan sulfate proteoglycans," *Annual Review of Biochemistry*, vol. 68, pp. 729–777, 1999.

[8] Y.-P. Hsueh and M. Sheng, "Regulated expression and subcellular localization of syndecan heparan sulfate proteoglycans and the syndecan-binding protein CASK/LIN-2 during rat brain development," *The Journal of Neuroscience*, vol. 19, no. 17, pp. 7415–7425, 1999.

[9] I. M. Ethell and Y. Yamaguchi, "Cell surface heparan sulfate proteoglycan syndecan-2 induces the maturation of dendritic spines in rat hippocampal neurons," *The Journal of Cell Biology*, vol. 144, no. 3, pp. 575–586, 1999.

[10] Y.-P. Hsueh, F.-C. Yang, V. Kharazia et al., "Direct interaction of CASK/LIN-2 and syndecan heparan sulfate proteoglycan and their overlapping distribution in neuronal synapses," *The Journal of Cell Biology*, vol. 142, no. 1, pp. 139–151, 1998.

[11] Y.-L. Lin, Y.-T. Lei, C.-J. Hong, and Y.-P. Hsueh, "Syndecan-2 induces filopodia and dendritic spine formation via the neurofibromin-PKA-Ena/VASP pathway," *The Journal of Cell Biology*, vol. 177, no. 5, pp. 829–841, 2007.

[12] H. T. Hu, H. Umemori, and Y. P. Hsueh, "Postsynaptic syndecan-2 induces transsynaptic signaling via fibroblast growth factor-22 for bidirectional synaptic differentiation," Under Review.

[13] S. Choi, E. Lee, S. Kwon et al., "Transmembrane domain-induced oligomerization is crucial for the functions of syndecan-2 and syndecan-4," *The Journal of Biological Chemistry*, vol. 280, no. 52, pp. 42573–42579, 2005.

[14] M. Volta, S. Calza, A. M. Roberts, and R. G. Roberts, "Characterisation of the interaction between syndecan-2, neurofibromin and CASK: dependence of interaction on syndecan dimerization," *Biochemical and Biophysical Research Communications*, vol. 391, no. 2, pp. 1216–1221, 2010.

[15] H.-T. Hu and Y.-P. Hsueh, "Calcium influx and postsynaptic proteins coordinate the dendritic filopodium-spine transition," *Developmental Neurobiology*, vol. 74, no. 10, pp. 1011–1029, 2014.

[16] H.-W. Chao, C.-J. Hong, T.-N. Huang, Y.-G. Lin, and Y.-P. Hsueh, "SUMOylation of the MAGUK protein CASK regulates dendritic spinogenesis," *The Journal of Cell Biology*, vol. 182, no. 1, pp. 141–155, 2008.

[17] Y.-P. Hsueh, A. M. Roberts, M. Volta, M. Sheng, and R. G. Roberts, "Bipartite interaction between neurofibromatosis type I protein (neurofibromin) and syndecan transmembrane heparan sulfate proteoglycans," *Journal of Neuroscience*, vol. 21, no. 11, pp. 3764–3770, 2001.

[18] F. Granés, C. Berndt, C. Roy, P. Mangeat, M. Reina, and S. Vilaró, "Identification of a novel Ezrin-binding site in syndecan-2 cytoplasmic domain," *FEBS Letters*, vol. 547, no. 1–3, pp. 212–216, 2003.

[19] J. J. Grootjans, P. Zimmermann, G. Reekmans et al., "Syntenin, a PDZ protein that binds syndecan cytoplasmic domains," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 25, pp. 13683–13688, 1997.

[20] I. M. Ethell, K. Hagihara, Y. Miura, F. Irie, and Y. Yamaguchi, "Synbindin, a novel syndecan-2-binding protein in neuronal dendritic spines," *The Journal of Cell Biology*, vol. 151, no. 1, pp. 53–67, 2000.

[21] G. F. Xu, B. Lin, K. Tanaka et al., "The catalytic domain of the neurofibromatosis type 1 gene product stimulates ras GTPase and complements ira mutants of *S. cerevisiae*," *Cell*, vol. 63, no. 4, pp. 835–841, 1990.

[22] G. Xu, P. O'Connell, D. Viskochil et al., "The neurofibromatosis type 1 gene encodes a protein related to GAP," *Cell*, vol. 62, no. 3, pp. 599–608, 1990.

[23] R. M. Cawthon, R. Weiss, G. Xu et al., "A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations," *Cell*, vol. 62, no. 1, pp. 193–201, 1990.

[24] R. Ballester, D. Marchuk, M. Boguski et al., "The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins," *Cell*, vol. 63, no. 4, pp. 851–859, 1990.

[25] M. M. Daston and N. Ratner, "Neurofibromin, a predominantly neuronal GTPase activating protein in the adult, is ubiquitously expressed during development," *Developmental Dynamics*, vol. 195, no. 3, pp. 216–226, 1993.

[26] D. H. Gutmann and F. S. Collins, "Recent progress toward understanding the molecular biology of von Recklinghausen neurofibromatosis," *Annals of Neurology*, vol. 31, no. 5, pp. 555–561, 1992.

[27] B. A. J. Ponder, "Neurofibromatosis: from gene to phenotype," *Seminars in Cancer Biology*, vol. 3, no. 3, pp. 115–120, 1992.

[28] T. Jacks, T. S. Shih, E. M. Schmitt, R. T. Bronson, A. Bernards, and R. A. Weinberg, "Tumour predisposition in mice heterozygous for a targeted mutation in Nf1," *Nature Genetics*, vol. 7, no. 3, pp. 353–361, 1994.

[29] H.-F. Guo, I. The, F. Hannan, A. Bernards, and Y. Zhong, "Requirement of *Drosophila* NF1 for activation of adenylyl cyclase by PACAP38-like neuropeptides," *Science*, vol. 276, no. 5313, pp. 795–798, 1997.

[30] J. Tong, F. Hannan, Y. Zhu, A. Bernards, and Y. Zhong, "Neurofibromin regulates G protein-stimulated adenylyl cyclase activity," *Nature Neuroscience*, vol. 5, no. 2, pp. 95–96, 2002.

[31] H.-F. Guo, J. Tong, F. Hannan, L. Luo, and Y. Zhong, "A neurofibromatosis-1-regulated pathway is required for learning in *Drosophila*," *Nature*, vol. 403, no. 6772, pp. 895–898, 2000.

[32] H.-F. Wang, Y.-T. Shih, C.-Y. Chen, H.-W. Chao, M.-J. Lee, and Y.-P. Hsueh, "Valosin-containing protein and neurofibromin interact to regulate dendritic spine density," *The Journal of Clinical Investigation*, vol. 121, no. 12, pp. 4820–4837, 2011.

[33] M. Krause, E. W. Dent, J. E. Bear, J. J. Loureiro, and F. B. Gertler, "Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration," *Annual Review of Cell and Developmental Biology*, vol. 19, pp. 541–564, 2003.

[34] C. Lebrand, E. W. Dent, G. A. Strasser et al., "Critical role of Ena/VASP proteins for filopodia formation in neurons and in function downstream of netrin-1," *Neuron*, vol. 42, no. 1, pp. 37–49, 2004.

[35] G. D. J. Watts, J. Wymer, M. J. Kovach et al., "Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein," *Nature Genetics*, vol. 36, no. 4, pp. 377–381, 2004.

[36] J. O. Johnson, J. Mandrioli, M. Benatar et al., "Exome sequencing reveals VCP mutations as a cause of familial ALS," *Neuron*, vol. 68, no. 5, pp. 857–864, 2010.

[37] I. Iossifov, M. Ronemus, D. Levy et al., "De novo gene disruptions in children on the autistic spectrum," *Neuron*, vol. 74, no. 2, pp. 285–299, 2012.

[38] Y.-P. Hsueh, "Calcium/calmodulin-dependent serine protein kinase and mental retardation," *Annals of Neurology*, vol. 66, no. 4, pp. 438–443, 2009.

[39] J. Najm, D. Horn, I. Wimplinger et al., "Mutations of CASK cause an X-linked brain malformation phenotype with microcephaly and hypoplasia of the brainstem and cerebellum," *Nature Genetics*, vol. 40, no. 9, pp. 1065–1067, 2008.

[40] G. Piluso, M. Carella, M. D'Avanzo et al., "Genetic heterogeneity of FG syndrome: a fourth locus (FGS4) maps to Xp11.4-p11.3 in an Italian family," *Human Genetics*, vol. 112, no. 2, pp. 124–130, 2003.

[41] P. S. Tarpey, R. Smith, E. Pleasance et al., "A systematic, large-scale resequencing screen of X-chromosome coding exons in mental retardation," *Nature Genetics*, vol. 41, no. 5, pp. 535–543, 2009.

[42] M. J. Lyons, J. M. Graham Jr., G. Neri et al., "Clinical experience in the evaluation of 30 patients with a prior diagnosis of FG syndrome," *Journal of Medical Genetics*, vol. 46, no. 1, pp. 9–13, 2009.

[43] S. Hayashi, N. Okamoto, Y. Chinen et al., "Novel intragenic duplications and mutations of CASK in patients with mental retardation and microcephaly with pontine and cerebellar hypoplasia (MICPCH)," *Human Genetics*, vol. 131, no. 1, pp. 99–110, 2012.

[44] Y.-P. Hsueh, "The role of the MAGUK protein CASK in neural development and synaptic function," *Current Medicinal Chemistry*, vol. 13, no. 16, pp. 1915–1927, 2006.

[45] B. A. Samuels, Y.-P. Hsueh, T. Shu et al., "Cdk5 promotes synaptogenesis by regulating the subcellular distribution of the MAGUK family member CASK," *Neuron*, vol. 56, no. 5, pp. 823–837, 2007.

[46] Y. Hata, S. Butz, and T. C. Südhof, "CASK: a novel dlg/PSD95 homolog with an N-terminal calmodulin-dependent protein kinase domain identified by interaction with neurexins," *Journal of Neuroscience*, vol. 16, no. 8, pp. 2488–2494, 1996.

[47] O. Olsen, K. A. Moore, M. Fukata et al., "Neurotransmitter release regulated by a MALS-liprin-alpha presynaptic complex," *Journal of Cell Biology*, vol. 170, no. 7, pp. 1127–1134, 2005.

[48] S. A. Spangler, S. K. Schmitz, J. T. Kevenaar et al., "Liprin-alpha2 promotes the presynaptic recruitment and turnover of RIM1/CASK to facilitate synaptic transmission," *The Journal of Cell Biology*, vol. 201, no. 6, pp. 915–928, 2013.

[49] C. Lohmann and T. Bonhoeffer, "A role for local calcium signaling in rapid synaptic partner selection by dendritic filopodia," *Neuron*, vol. 59, no. 2, pp. 253–260, 2008.

[50] J.-P. Borg, S. W. Straight, S. M. Kaech et al., "Identification of an evolutionarily conserved heterotrimeric protein complex involved in protein targeting," *Journal of Biological Chemistry*, vol. 273, no. 48, pp. 31633–31636, 1999.

[51] S. Butz, M. Okamoto, and T. C. Südhof, "A tripartite protein complex with the potential to couple synaptic vesicle exocytosis to cell adhesion in brain," *Cell*, vol. 94, no. 6, pp. 773–782, 1998.

[52] S. M. Kaech, C. W. Whitfield, and S. K. Kim, "The LIN-2/LIN-7/LIN-10 complex mediates basolateral membrane localization of the *C. elegans* EGF receptor LET-23 in vulval epithelial cells," *Cell*, vol. 94, no. 6, pp. 761–771, 1998.

[53] K. Jo, R. Derin, M. Li, and D. S. Bredt, "Characterization of MALS/Velis-1, -2, and -3: a family of mammalian LIN-7 homologs enriched at brain synapses in association with the postsynaptic density-95/NMDA receptor postsynaptic complex," *The Journal of Neuroscience*, vol. 19, no. 11, pp. 4189–4199, 1999.

[54] P. Hotulainen and C. C. Hoogenraad, "Actin in dendritic spines: connecting dynamics to function," *The Journal of Cell Biology*, vol. 189, no. 4, pp. 619–629, 2010.

[55] K. F. Tolias, J. G. Duman, and K. Um, "Control of synapse development and plasticity by Rho GTPase regulatory proteins," *Progress in Neurobiology*, vol. 94, no. 2, pp. 133–148, 2011.

[56] T. Saneyoshi and Y. Hayashi, "The Ca^{2+} and rho gtpase signaling pathways underlying activity-dependent actin remodeling at dendritic spines," *Cytoskeleton*, vol. 69, no. 8, pp. 545–554, 2012.

[57] H. Q. Sun, M. Yamamoto, M. Mejillano, and H. L. Yin, "Gelsolin, a multifunctional actin regulatory protein," *The Journal of Biological Chemistry*, vol. 274, no. 47, pp. 33179–33182, 1999.

[58] A. M. McGough, C. J. Staiger, J.-K. Min, and K. D. Simonetti, "The gelsolin family of actin regulatory proteins: modular structures, versatile functions," *FEBS Letters*, vol. 552, no. 2-3, pp. 75–81, 2003.

[59] P. Silacci, L. Mazzolai, C. Gauci, N. Stergiopulos, H. L. Yin, and D. Hayoz, "Gelsolin superfamily proteins: key regulators of cellular functions," *Cellular and Molecular Life Sciences*, vol. 61, no. 19-20, pp. 2614–2623, 2004.

[60] M. Lu, W. Witke, D. J. Kwiatkowski, and K. S. Kosik, "Delayed retraction of filopodia in gelsolin null mice," *The Journal of Cell Biology*, vol. 138, no. 6, pp. 1279–1287, 1997.

[61] E. N. Star, D. J. Kwiatkowski, and V. N. Murthy, "Rapid turnover of actin in dendritic spines and its regulation by activity," *Nature Neuroscience*, vol. 5, no. 3, pp. 239–246, 2002.

[62] Y. Ohoka and Y. Takai, "Isolation and characterization of cortactin isoforms and a novel cortactin-binding protein, CBP90," *Genes to Cells*, vol. 3, no. 9, pp. 603–612, 1998.

[63] T. Uruno, J. Liu, P. Zhang et al., "Activation of Arp2/3 complex-mediated actin polymerization by cortactin," *Nature Cell Biology*, vol. 3, no. 3, pp. 259–266, 2001.

[64] A. M. Weaver, A. V. Karginov, A. W. Kinley et al., "Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation," *Current Biology*, vol. 11, no. 5, pp. 370–374, 2001.

[65] H. Hering and M. Sheng, "Activity-dependent redistribution and essential role of cortactin in dendritic spine morphogenesis," *The Journal of Neuroscience*, vol. 23, no. 37, pp. 11759–11769, 2003.

[66] J. Cheung, E. Petek, K. Nakabayashi, L.-C. Tsui, J. B. Vincent, and S. W. Scherer, "Identification of the human cortactin-binding protein-2 gene from the autism candidate region at 7q31," *Genomics*, vol. 78, no. 1-2, pp. 7–11, 2001.

[67] Y.-K. Chen and Y.-P. Hsueh, "Cortactin-binding protein 2 modulates the mobility of cortactin and regulates dendritic spine formation and maintenance," *The Journal of Neuroscience*, vol. 32, no. 3, pp. 1043–1055, 2012.

[68] Y.-K. Chen, C.-Y. Chen, H.-T. Hu, and Y.-P. Hsueh, "CTTNBP2, but not CTTNBP2NL, regulates dendritic spinogenesis and synaptic distribution of the striatin-PP2A complex," *Molecular Biology of the Cell*, vol. 23, no. 22, pp. 4383–4392, 2012.

[69] P.-Y. Shih, S.-P. Lee, Y.-K. Chen, and Y.-P. Hsueh, "Cortactin-binding protein 2 increases microtubule stability and regulates dendritic arborization," *Journal of Cell Science*, vol. 127, no. 16, pp. 3521–3534, 2014.

[70] J. D. Arroyo and W. C. Hahn, "Involvement of PP2A in viral and cellular transformation," *Oncogene*, vol. 24, no. 52, pp. 7746–7755, 2005.

[71] M. Benoist, S. Gaillard, and F. Castets, "The striatin family: a new signaling platform in dendritic spines," *Journal of Physiology Paris*, vol. 99, no. 2-3, pp. 146–153, 2006.

[72] S. Gaillard, Y. Bailly, M. Benoist et al., "Targeting of proteins of the striatin family to dendritic spines: role of the coiled-coil domain," *Traffic*, vol. 7, no. 1, pp. 74–84, 2006.

Hindawi Publishing Corporation
Neural Plasticity
Volume 2016, Article ID 9828517, 11 pages
http://dx.doi.org/10.1155/2016/9828517

Research Article

Effect of Associative Learning on Memory Spine Formation in Mouse Barrel Cortex

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Received 19 June 2015; Revised 31 August 2015; Accepted 14 September 2015

Academic Editor: Bryen A. Jordan

Associative fear learning, in which stimulation of whiskers is paired with mild electric shock to the tail, modifies the barrel cortex, the functional representation of sensory receptors involved in the conditioning, by inducing formation of new inhibitory synapses on single-synapse spines of the cognate barrel hollows and thus producing double-synapse spines. In the barrel cortex of conditioned, pseudoconditioned, and untreated mice, we analyzed the number and morphological features of dendritic spines at various maturation and stability levels: sER-free spines, spines containing smooth endoplasmic reticulum (sER), and spines containing spine apparatus. Using stereological analysis of serial sections examined by transmission electron microscopy, we found that the density of double-synapse spines containing spine apparatus was significantly increased in the conditioned mice. Learning also induced enhancement of the postsynaptic density area of inhibitory synapses as well as increase in the number of polyribosomes in such spines. In single-synapse spines, the effects of conditioning were less pronounced and included increase in the number of polyribosomes in sER-free spines. The results suggest that fear learning differentially affects single- and double-synapse spines in the barrel cortex: it promotes maturation and stabilization of double-synapse spines, which might possibly contribute to permanent memory formation, and upregulates protein synthesis in single-synapse spines.

1. Introduction

It is now widely accepted that behavioral experience altering the neuronal activity induces changes in the density of synapses and dendritic spines [1–3]. Synaptic plasticity has also been shown to alter synaptic efficiency by remodeling of the existing synapses [4–7].

The barrel cortex of rodents as sensory representation of whiskers as well as its afferent pathway is a useful model for studying associative learning-dependent neuronal plasticity. Classical conditioning, in which stimulation of a row of whiskers (conditioned stimulus) is paired with mild electric shock to the tail (unconditioned stimulus), changes the motor behavior of the animals and modifies the cortical representation of sensory receptors involved in the conditioning [8].

Mapping of brain activation pattern with [14C]2-deoxyglucose autoradiography showed learning-dependent expansion of functional cortical representation of the whisker row stimulated during conditioning [8]. This plasticity is associated with changes in both excitatory and inhibitory neurotransmission.

The plasticity of excitatory circuits was manifested by an increase in expression of NR2A (subunit of NMDA receptor specific for excitatory synapses) mRNA and protein [9]. In spite of that, the density of excitatory synapses or single-synapse spines did not change after conditioning [3]. However,

we observed an upregulation of the number of polyribosomes associated with both excitatory and inhibitory synapses accompanied by an increase in postsynaptic density (PSD) area that suggested synaptic potentiation [7].

Conditioning also affected inhibitory transmission, inducing upregulation of GAD 67 mRNA and protein, a marker of inhibitory synapses, within the affected barrels [10], accompanied by an increase in the density of GABAergic neurons [11]. Our previous electron microscopic studies demonstrated that conditioning caused the formation of new inhibitory synapses, producing double-synapse spines in the cognate barrel hollows [3], and remodeled the morphology of double-synapse spines towards mushroom-shaped spines with shorter but thicker necks [12].

The stepwise morphological transformation of dendritic spines during their plastic remodeling that leads to formation of stable spines includes shape and size change [13, 14], acquisition of smooth ER (sER) to the spine, and formation of spine apparatus (SA) [15]. The spines containing SA are the largest [16] and it has been established that the largest spines have the longest half-life *in vivo* [17–19]. SA is a smooth ER-related membrane structure [16, 20] containing synaptopodin, a SA-specific actin-binding protein [21]. It is believed that SA is associated with the regulation of calcium storage and release [22–24] and that together with polyribosomes it can participate in the local protein synthesis [25–27]. SA is also postulated to play a role in the potentiation of synapses located on dendritic spines and in the formation of stable spines involved in memory storage and therefore called "memory spines" [23, 27, 28].

Although their function is still unknown, it seems probable that spines containing SA are involved in the synaptic plasticity [4, 29]. Inactivation of synaptopodin gene leading to the total absence of SA limited induction of long-term potentiation (LTP) and caused deficits in spatial learning [27, 30]. It was observed that fear conditioning increased the number of such spines and the number of SA-associated polyribosomes in the lateral amygdala [4].

Since the data concerning involvement of dendritic spines and spine apparatus in conditioning-induced plasticity of the somatosensory cortex are scarce, the aim of this study was to investigate the effect of short-lasting fear learning on the number and morphological features of dendritic spines in the barrel cortex by using the whisker-to-barrel pathway model and serial section transmission electron microscopy-based stereology. The barrel cortex contains two types of spines: single-synapse spines with single excitatory synapses which account for about 90% of all spines in this region and double-synapse spines with two different synapses: one excitatory and one inhibitory [12]. In each type, we separately analyzed three categories of spines, presumably representing successive levels of spine maturity: sER-free, containing sER, and containing SA.

2. Materials and Methods

2.1. Animals. The experiments were performed on Swiss Webster female mice aged 6-7 weeks, kept in standard conditions. All experiments were compliant with the Council Directive 2010/63EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes and approved by the Animal Care and Use Committees of the Polish Academy of Sciences and the Jagiellonian University.

2.2. Behavior Training. The mice ($n = 15$) were divided into conditioned group ($n = 5$), pseudoconditioned group ($n = 5$), and control, untreated group ($n = 5$). Before the onset of the conditioning procedure, all animals were habituated in a homemade restrainer which holds the mouse neck stationary, leaving the rest of the body, including the head, free. During the habituation period, mice spent 10 min per day for 3 weeks in the restrainer.

After habituation, mice were conditioned using a classical conditioning paradigm. Manual stimulation of the selected whiskers (B row; conditioned stimulus, CS) on the left side of the snout was paired with a mild electric shock to the tail (unconditioned stimulus, UCS) [8]. The pairing procedure included three sweeps back and forth along the entire whisker row with a small paintbrush lasting 3 s each, repeated at a frequency of four times per minute for 10 min, applied for 3 consecutive days. The UCS was a weak, 0.5 mA electric current applied to the tail for 0.5 s at the end of the last sweep in the series. In pseudoconditioned animals (random pairing of CS and UCS), the number and frequency of stimuli applied were the same.

2.3. Transmission Electron Microscopy. Twenty-four hours after completion of the conditioning, the mice were anesthetized with Vetbutal (100 mg/kg body weight; Biowet, Puławy) and perfused through the heart with 20 mL of rinse buffer (0.2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) followed by 100–150 mL of fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). The brains were removed immediately after perfusion and left in the same fixative for 24 h at 4°C.

The next day, after washing in 0.1 M phosphate buffer (pH 7.4), 60 μm tangential vibratome sections were cut from the right barrel cortex. Sections were examined under a stereomicroscope (Nikon Optiphot, Japan) and those containing the barrel field cortex were collected for further processing. The sections were washed in 0.1 M cacodylate buffer (pH 7.4), postfixed twice with 1% osmium tetroxide in 0.1 M cacodylate buffer (the first time with 1.5% potassium ferrocyanide), washed in 70% ethanol containing 1% uranyl acetate, and, after dehydration in graded series of ethanol, embedded in Epon resin (Polysciences Inc., USA) between two silicone-coated glass slides.

The region of B2 and B3 barrels was trimmed for ultrathin sectioning. Series of 30 to 50 successive sections (65–70 nm thick) were cut from each sample. The sections were collected on formvar-coated copper-palladium slots and contrasted with 1% lead citrate. The central regions of the B2 barrel, layer 4, in which cell bodies are sparse and the vast majority of structures observed under TEM are dendrites, axons, and synapses were photographed at 7 K using JEOL 100SX transmission electron microscope (JEOL, Japan). We

examined the collection of ultrathin sections used in our previous study [3].

Ten to twelve serial electron micrographs were taken from successive sections for 3D reconstruction of dendritic spines. The micrographs were initially aligned in Adobe Photoshop CS software, in which stacks of serial images were taken at the final magnification of 30 K.

2.4. Quantitative Analysis of Dendritic Spines. Quantitative analysis of dendritic spines was carried out using NIH Image J Cell Counter software (http://rsb.info.nih.gov/ij/) by placing a grid of two-dimensional sampling frame over the stack of serial sections. The dendritic spines were counted per volume unit (μm^3). Each spine was counted only once in the stack and only spines located fully within the frame or intersecting the left and the upper borderlines of the frame were included. Synapses and spines were defined according to Knott et al. [2]. The density of single- and double-synapse spines containing smooth endoplasmic reticulum (sER) and spine apparatus (SA) and sER-free spines was calculated according to the stereological formula $N_A = \Sigma Q^- / a$, where ΣQ^- is the number of dendritic spines found in the volume a [31]. The counting was done blind: the observer did not know whether the micrographs were taken from conditioned, pseudoconditioned, or control animal.

2.5. Morphological Analysis of Spines. Serial images of 180 spines (90 single-synapse spines and 90 double-synapse spines) from control group, conditioned group, and pseudoconditioned group were selected. The selection criteria included (1) complete series of successive sections (micrographs) allowing 3D reconstruction of the spine, (2) well visible synapses, and (3) the content: SA, sER only, and none. Twelve dendritic spines meeting the above criteria, two in each group (sER-free, sER, and SA), in case of both single-synapse and double-synapse spines, were randomly selected from each animal to yield 10 single-synapse spines and 10 double-synapse spines of each category (sER-free, sER, and SA) per each experimental group (control, conditioned, and pseudoconditioned).

In every spine, length of the spine and diameter of the spine head and neck as well as excitatory and inhibitory (only in double-synapse spines) PSD areas were measured. PSD area was calculated according to Ostroff et al. [32]. Length of the spine was measured after 3D reconstruction. Spine head diameter was measured at the widest part of the head, parallel to the PSD [6]. Three measurements of the neck width at different levels were made and the mean value was calculated as neck diameter. Volume of SA was calculated by summing the values of area of SA multiplied by section thickness of all serial sections in which it appeared.

Three measurements of all parameters from every micrograph containing profiles of the selected spines were made using NIH Image J software. 3D reconstructions of the spines were performed using 3D Studio Max software (Discreet Logic, Montreal, Canada) and the location of spine apparatus (head, head/neck, or neck of the spine) as well as the number of polyribosomes in the dendritic spine was estimated.

The shapes of spines were defined according to Harris et al. [15]. Spines were divided into three shape categories on the basis of their length (l), diameter of the spine head (dh), and diameter of the neck (dn). Very long spines ($l \geq 3 \times$ dn) with similar diameters of the head and neck (dh $\approx$ dn) were termed thin spines. Spine with large heads and narrow necks (dh $\geq 2.5 \times$ dn) were termed mushroom spines. Very short spines with the length close to diameter of the neck ($l \approx$ dn) were termed stubby spines. Spines with more than one head were not observed.

2.6. Statistical Analysis. All data were analysed using GraphPad Prism 5.01 software (GraphPad Software Inc., USA). Differences in the densities of dendritic spines containing SA, containing sER, and sER-free as well as the SA volume across the experimental groups were analysed by Kolmogorov-Smirnov normality test and homogeneity Bartlett's test for equal variances, followed by one-way ANOVA test with *post hoc* Tukey's test. To compare the combined effect of training and spine content on the morphological measurements across the experimental groups, two-way ANOVA with *post hoc* Bonferroni test was used. Differences in shapes of spines and in the location of SA in dendritic spines between control, pseudoconditioned, and conditioned groups and cooccurrence of SA and polyribosomes were assessed by chi square test. In the text of results and in graphs, data are presented as means ± SEM.

3. Results

3.1. Sampling Areas. Dendritic spines were counted in the following total tissue volumes: control group, $484.51 \pm 10.92\ \mu m^3$ (mean volume per animal $96.90 \pm 7.13\ \mu m^3$); conditioned group, $457.92 \pm 12.78\ \mu m^3$ (mean volume per animal $91.58 \pm 7.26\ \mu m^3$); pseudoconditioned group, $479.93 \pm 15.16\ \mu m^3$ (mean volume per animal $95.99 \pm 8.94\ \mu m^3$). The sampling volumes were not significantly different across the groups ($F_{(1,14)} = 0.13$, $P = 0.88$).

3.2. Density of Dendritic Spines. Dendritic spines were classified into three categories according to their content: sER-free spines (Figures 1(a) and 1(d)), spines containing sER only (Figures 1(b) and 1(e)), and spines containing SA (Figures 1(c) and 1(f)). The sER was visible as membranous cisternae inside the spines (Figure 1(b)). The SA was identified as an array of membranous cisternae interleaved with electron-dense plates (Figure 1(c)), as described by Ostroff et al. [4].

3.2.1. Density of Single-Synapse Spines. The density of sER-free single-synapse spines increased approximately twofold after pseudoconditioning (pseudoconditioned group: $0.96 \pm 0.08/\mu m^3$; control group: $0.54 \pm 0.04/\mu m^3$; $F_{(1,44)} = 29.12$, $P < 0.0001$) but did not show any significant change after conditioning ($0.44 \pm 0.01/\mu m^3$; Figure 2(a)). In the conditioned and pseudoconditioned animals, the mean densities of single-synapse spines containing sER and SA did not significantly change (sER: $0.21 \pm 0.02/\mu m^3$ and $0.47 \pm 0.09/\mu m^3$, SA: $0.18 \pm 0.03/\mu m^3$ and $0.26 \pm 0.04/\mu m^3$, resp.) compared to

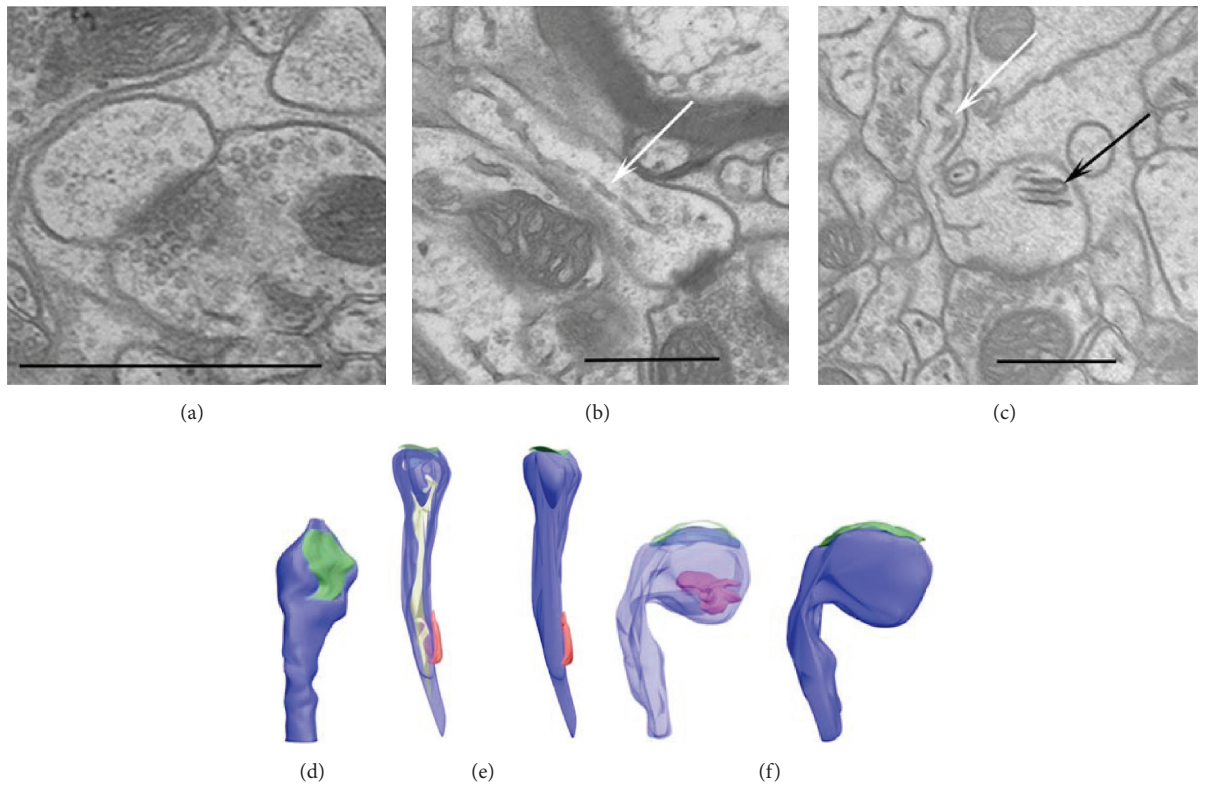

Figure 1: 3D serial section EM reconstruction of three spine types from B2 barrel hollow, also shown in single electron micrographs: sER-free spine (a), spine containing sER (b), and spine containing spine apparatus (c). White arrows indicate sER ((b) and (c)) and black arrow indicates spine apparatus (c). (d)–(f) show reconstruction of dendritic spines (blue): excitatory synapses (green), inhibitory synapse (red; only (e)), smooth endoplasmic reticulum (yellow; (e)), and spine apparatus (red; (f)). Scale bars: 0.5 μm.

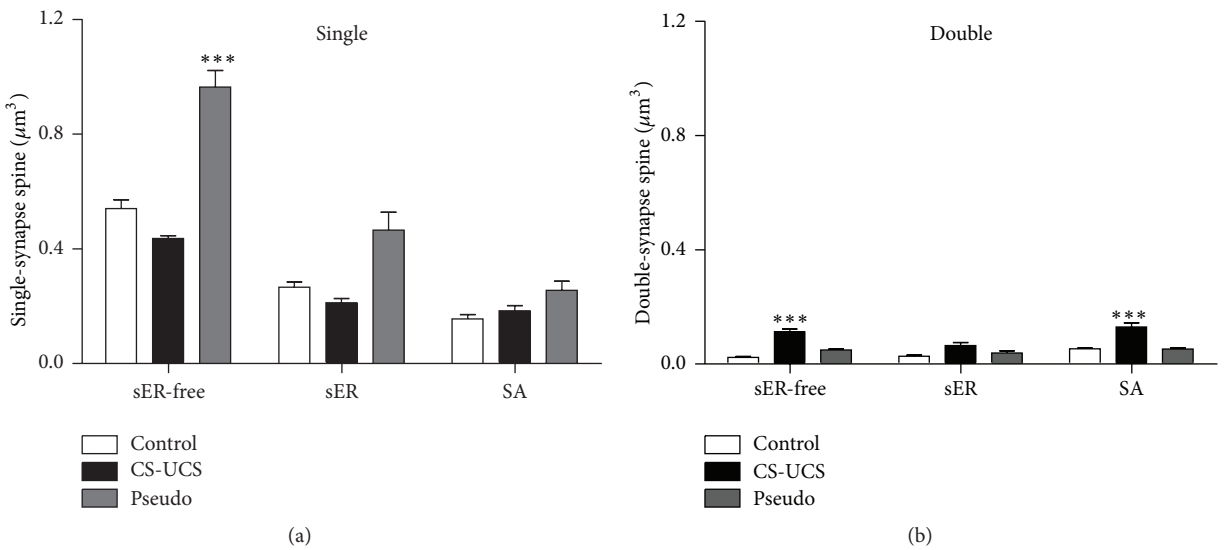

Figure 2: Density of single- (a) and double-synapse spines (b): sER-free, containing sER, and containing spine apparatus (SA). The graphs show means ± SEM (one-way ANOVA with *post hoc* Tukey's test, $^{***}P < 0.001$).

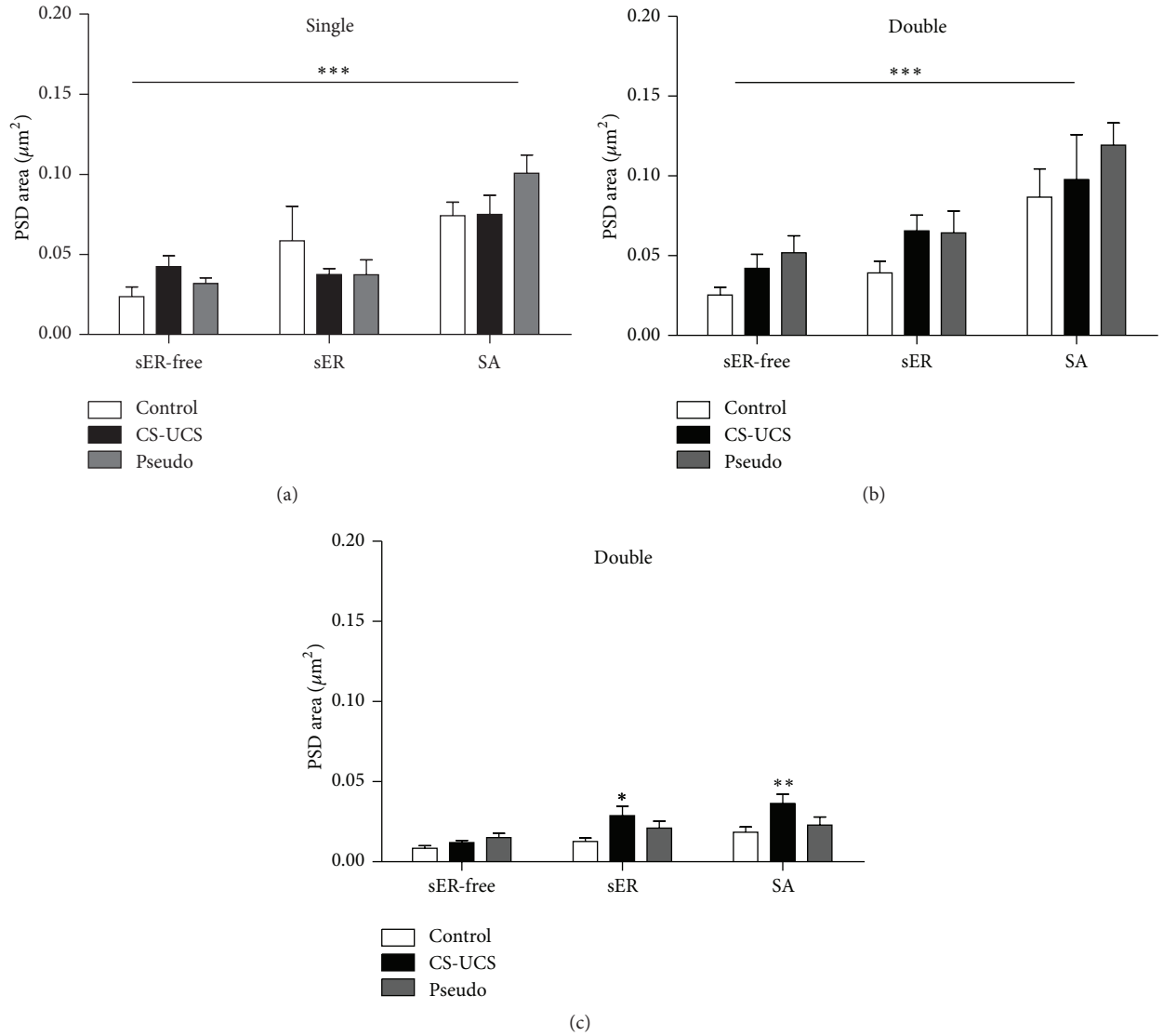

Figure 3: PSD area of excitatory ((a) and (b)) and inhibitory (c) synapses of single- and double-synapse spines: sER-free, containing sER, and containing SA. The graphs show means ± SEM (two-way ANOVA with *post hoc* Bonferroni test; $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$).

control group (sER: 0.27 ± 0.03/μm^3, SA: 0.16 ± 0.02/μm^3; Figure 2(a)).

3.2.2. Density of Double-Synapse Spines. A twofold increase and a fourfold increase in the density of double-synapse spines containing SA and sER-free spines, respectively, were found in the conditioned animals (SA: 0.13 ± 0.02/μm^3, sER-free: 0.11 ± 0.01/μm^3) when compared with control animals (SA: 0.05 ± 0.004/μm^3, sER-free: 0.02 ± 0.005/μm^3; $F_{(1,44)} = 11.26$, $P < 0.0001$), whereas statistically significant differences were not observed between the pseudoconditioned (SA: 0.05 ± 0.006/μm^3, sER-free: 0.05 ± 0.005/μm^3) and control mice (Figure 2(b)). The density of spines containing sER was the highest in the conditioned group, but differences between the groups failed to reach significance (control: 0.03 ± 0.005/μm^3; conditioned: 0.06 ± 0.01/μm^3; pseudoconditioned: 0.04 ± 0.01/μm^3; Figure 2(b)).

3.3. Morphological Analysis of Spines. Results of the measurements are presented in supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/9828517.

3.3.1. Area of Postsynaptic Density (PSD)

PSD of Excitatory Synapses. In all groups, single- and double-synapse spines containing SA had larger PSD area of excitatory synapses as compared to sER-free spines and spines containing sER in the same group of animals (Figures 3(a) and 3(b)). sER-free spines had similar PSD area of excitatory synapses in the control, conditioned, and pseudoconditioned

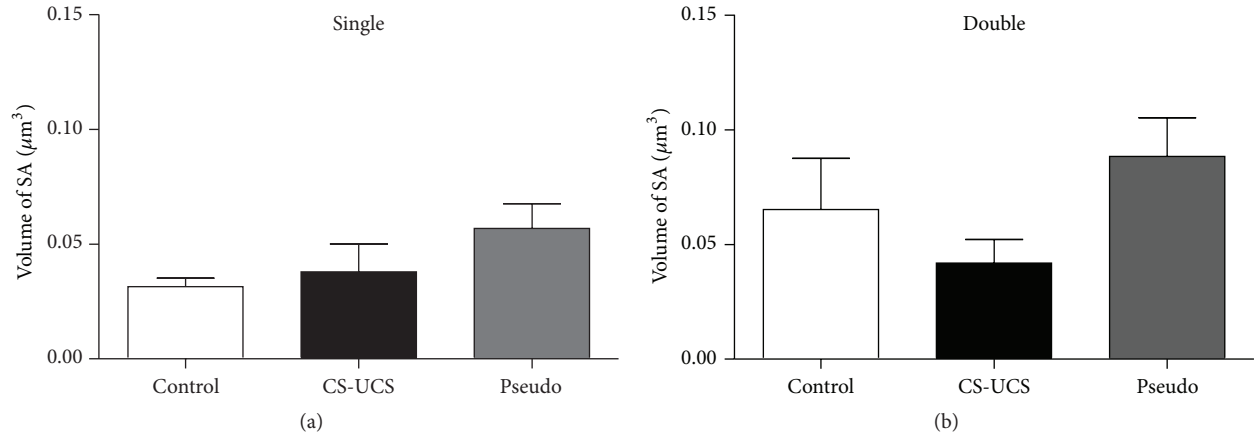

Figure 4: Volume of spine apparatus in single- (a) and double-synapse spines (b). The graphs show means ± SEM (one-way ANOVA with *post hoc* Tukey's test).

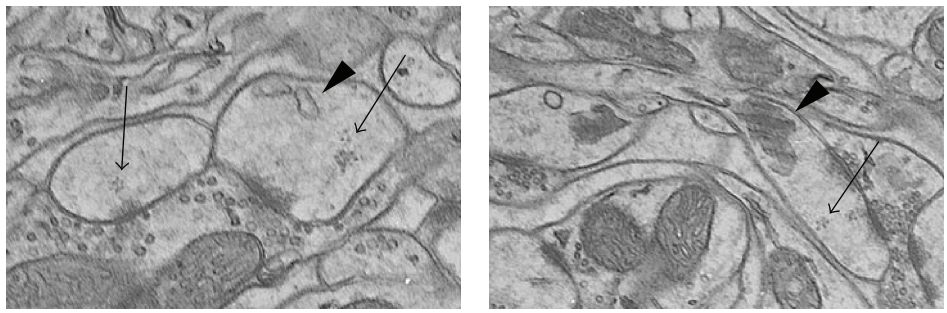

Figure 5: Electron micrographs showing polyribosomes in sER-free, sER-containing (a), and SA-containing (b) spines. Arrows: polyribosomes; arrowhead: sER (a) and spine apparatus (b).

animals. Similarly, spines containing sER had the same PSD area of excitatory synapses in control, conditioned, and pseudoconditioned animals.

PSD of Inhibitory Synapses. Conditioning induced a significant increase in the PSD area of inhibitory synapses located on double-synapse spines containing sER and SA (Figure 3(c)). The PSD area in sER-free spines did not significantly change in conditioned and pseudoconditioned animals as compared with the control group.

3.3.2. Volume and Location of Spine Apparatus in Dendritic Spines. The SA usually included 2–6 cisternae and conditioning did not influence that number. No differences were also observed in the volume of SA between the groups of animals (Figures 4(a) and 4(b)).

The vast majority of SA (single: 56.67%; double: 63.33%) was located on the border between spine head and neck and this location did not change after conditioning or pseudoconditioning.

3.3.3. Occurrence of Polyribosomes in Dendritic Spines. Polyribosomes (Figure 5) were identified as described previously [7] and the mean number of polyribosomes per spine was assessed. Almost all single- and double-synapse spines (96.67%) containing spine apparatus also contained polyribosomes in all experimental groups.

Polyribosomes located in the single-synapse spines were more frequent in the sER-free spines of conditioned and pseudoconditioned animals, and, conversely, the density of polyribosomes located in single-synapse spines containing sER decreased after training. There were no changes in the number of polyribosomes located in single-synapse spines containing SA (Figure 6(a)). Conditioning increased the number of polyribosomes located in double-synapse spines containing SA, while pseudoconditioning increased the number of polyribosomes located in the sER-free spines. There were no changes in the number of polyribosomes located in double-synapse spines containing sER (Figure 6(b)).

3.4. Shape of Spines. Among 60 spines selected for reconstruction (Figure 7), in the control group, the rarest were stubby spines (Figure 7(a)), constituting 23.33% of single-synapse spines and only 3.33% of double-synapse spines. The proportions of other spine shapes were also dependent on type of spine. Single-synapse spines showed predominance of thin spines (Figure 7(b)) that constituted almost half of all such spines (46.67%), while among double-synapse spines about

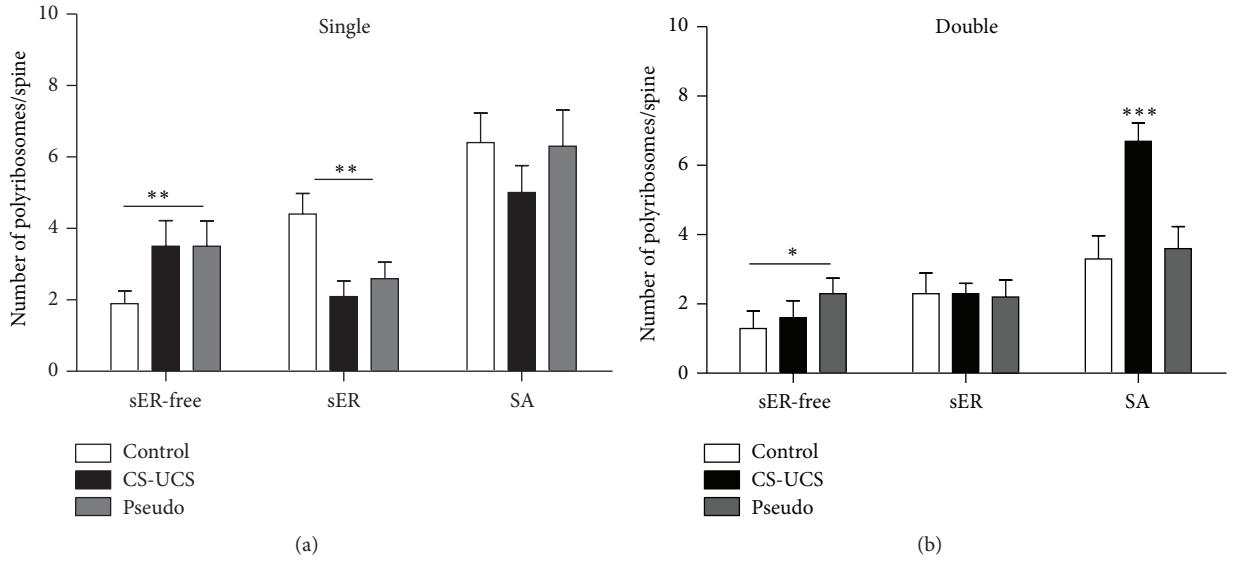

FIGURE 6: Number of polyribosomes in the single- (a) and double-synapse spines (b): sER-free, containing sER, and containing SA. The graphs show means ± SEM (chi square test and one-way ANOVA with *post hoc* Tukey's test: $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$).

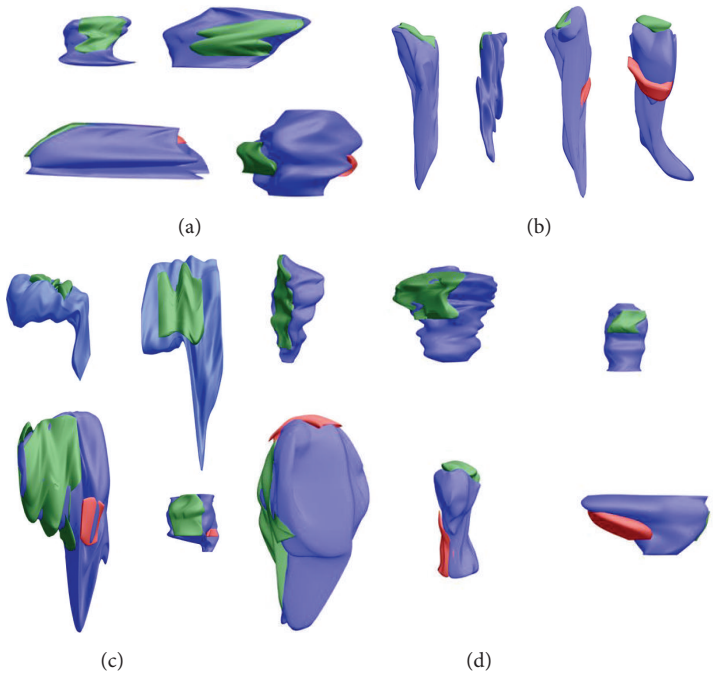

FIGURE 7: 3D reconstruction of single- and double-synapse spines from serial micrographs showing different shapes of spines: stubby (a), thin (b), mushroom (c), and intermediate (d). Color areas as in Figure 1.

one-third were thin spines (36.67%). Mushroom-shaped spines (Figure 7(c)) accounted for 30% of single-synapse spines and for 60% of double-synapse spines (Figure 8).

The majority of stubby spines were sER-free (single: 57.14%; double: 100%). Thin spines mostly contained sER (single: 42.86%; double: 54.55), while mushroom spines predominantly contained SA (single: 66.67%; double: 44.44%).

Conditioning and pseudoconditioning induced an increase in the proportion of mushroom single-synapse spines at the expense of stubby and thin (only conditioning) single-synapse spines ($\chi^2_{(4)} = 10.31$, $P = 0.0356$; Figure 8(a)). There were no experience-dependent changes in shapes of double-synapse spines ($\chi^2_{(4)} = 3.716$, $P = 0.4458$).

We also observed intermediate shapes of spines [33]: short thin spines (the length of spine being 2-3 times longer than the diameter of neck and similar diameters of head and neck) or stubby-mushroom spines (the diameter of spine head being 1.5–2.5 times bigger than the diameter of neck and

Table 1: Statistically significant effects of conditioning and pseudoconditioning on dendritic spines in the barrel cortex.

	Single-synapse spines	Double-synapse spines
Effects after conditioning	Decrease in the number of thin spines	Increase in the number of sER-free and SA-containing spines Increase in inhibitory PSD area in sER- and SA-containing spines Increase in the number of polyribosomes in SA-containing spines
Effects after conditioning and pseudoconditioning	Increase in the number of polyribosomes in sER-free spines Decrease in the number of polyribosomes in sER-containing spines Increase in the number of mushroom spines Decrease in the number of stubby spines	
Effects after pseudoconditioning only	Increase in the number of sER-free spines	Increase in the number of polyribosomes in sER-free spines

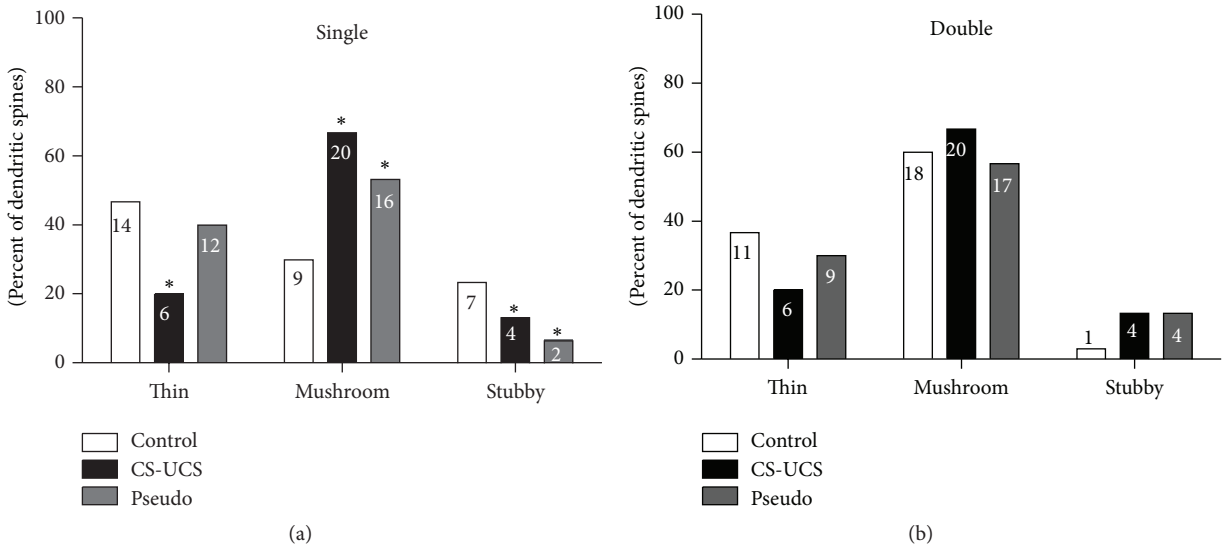

Figure 8: Shapes of single- (a) and double-synapse spines (b): thin, mushroom, and stubby. The graphs show percentages of spine types and their numbers inside the bars (chi square test: $^{*}P < 0.05$).

the length of spine being about 1.5–2.5 times longer than the diameter of neck) (Figure 7(d)). Intermediate spines were rare (3.89% of all spines).

Results of the study are summarized in Table 1.

4. Discussion

This study presents for the first time the effect of fear conditioning on a broad range of morphological parameters of dendritic spines in the somatosensory cortex. Some of its results obtained in control animals confirm findings reported from other areas of the central nervous system, demonstrating that the spines containing spine apparatus (SA) are mostly mushroom-shaped [4, 15, 34]—in the barrel cortex about 80% of double-synapse spines and 77% of single—synapse spines—and that they almost always contain polyribosomes [4]. However, proportions of spine shapes found in the somatosensory cortex differ from that observed in other brain regions; for example, in the hippocampus of adult rat, about 65% of spines were thin, 25% were mushroom, and only about 10% were stubby spines [15]. These apparent differences might reflect differences in spine types in different brain regions.

The percentage of sER-free spines, spines containing sER, and spines containing SA is similar in all analyzed groups of animals. In the lateral amygdala, about 20% spines contained SA, and approximately 10% spines contained sER but not SA [4], while in the hippocampus, depending on different location, from 14% to 37% spines expressed synaptopodin, a marker of SA [27]. The above results are the most consistent with our findings in somatosensory cortex, where about 20% of single-synapse spines and almost a half of double-synapse spines constituting only about 10% of all spines [2, 12] contained SA. Our 3D reconstruction analysis of spine shapes showed that mushroom spines mostly contained spine apparatus and thin spines mostly contained sER, while stubby spines were predominantly sER-free.

We have found that associative fear learning differentially regulates the density of single- and double-synapse spines and exerts a more profound effect on the latter spines. An increase in density was observed in case of sER-free and SA-containing double-synapse spines in somatosensory cortex always bearing an excitatory and an inhibitory synapse [2, 12, 35]. There were no changes, however, in the density of single-synapse spines, associated exclusively with excitatory synapses. Other effects of learning on double-synapse spines included an increase in PSD area of inhibitory synapses in spines containing sER and SA as well as an increase in the number of polyribosomes in SA-containing spines. Single-synapse spines responded to conditioning by a decrease in the number of thin spines.

These findings are consistent with the results of our previous studies demonstrating conditioning-induced formation of inhibitory synapses on double-synapse spines in the barrel cortex [3] and increase in the density of polyribosomes associated with both excitatory and inhibitory synapses located on dendritic spines [7]. The present study shows that the learning-associated changes of double-synapse spines mainly involve sER-free and SA-containing spines. The sER-free spines are the smallest and are considered to be the most unstable, transient spines with the highest motility and very short half-life *in vivo* [17, 23, 28], whereas the spines containing SA are the largest spines; they form stable synaptic connections [23]. These spines have larger PSD areas, as also observed in the present study, and more numerous AMPA glutamate receptors, which could enhance the strength of their synapses [28, 36, 37], and they contain more frequent polyribosomes, suggesting local protein synthesis [4, 26]. Hence, in case of double-synapse spines, associative learning seems to act bidirectionally: it temporarily enhances learning capacity by adding sER-free transient spines and promotes transformation of preexisting spines into the most stable SA-containing spines to stabilize connectivity.

Ostroff et al. [4] proposed that SA may be required for the induction of local translation or for posttranslational protein changes. Memory formation seems to involve strengthening and stabilization of synapses requiring newly produced proteins. In the barrel cortex, conditioning upregulates the local protein synthesis in both single-synapse spines and double-synapse spines but this effect seems to be more effective in double-synapse spines containing SA, as it is accompanied by increase in PSD area. Hence, double-synapse spines are the preferential candidates for participation in learning-associated memory pathways in the barrel cortex.

Learning-induced increase in the number of polyribosomes in single-synapse spines is accompanied by enhanced frequency of mushroom-shaped spines but not of spines containing SA. On the other hand, in double-synapse spines, the response to conditioning includes increased incidence of SA but not increase in the number of mushroom spines. In a previous study we suggested that during conditioning inhibitory inputs could be added to preexisting single-synapse spines [3]. Now, we propose that only those single-synapse spines, which undergo special "preparation" during the learning process, including increase in the density of polyribosomes, increased PSD areas of excitatory synapses, and the spine shapes changing toward mushroom spines, are ready for the acquisition of new inhibitory synapses and formation of SA, which might complete their transformation into "memory spines."

These observations suggest that differential regulation of single- and double-synapse spines by associative learning might involve local synthesis of proteins participating in shape remodeling of single-synapse spines and involved in formation of SA in double-synapse spines.

Other morphological parameters of dendritic spines did not seem to be influenced by learning, neither total spine area (results not shown) nor location of SA and its volume.

We also used the pseudoconditioned group to test whether the observed changes were directly associated with the influence of learning or resulted only from the random application of two kinds of sensory stimuli. Pseudoconditioning is believed to induce a general sensitization of the animal to the conditioned stimulus [38]. Some effects observed in this study (increase in the number of polyribosomes in sER-free spines, decrease in the number of polyribosomes in sER-containing spines, increase in the number of mushroom spines, and decrease in the number of stubby spines) were observed after both, conditioning and pseudoconditioning, so they should be attributed to such sensitization. However, pseudoconditioning alone brought about a significant increase in the density of sER-free single-synapse spines. Such effects of pseudoconditioning alone were only occasionally reported. Cybulska-Klosowicz and Kossut [38] observed that pseudoconditioning activated the contralateral and ipsilateral barrel field, in contrast to a decrease in bilateral activation seen in the conditioned groups. In our previous studies on the barrel cortex we found pseudoconditioning-induced increase in the density of single-synapse spines [3] and a decrease in the density of polyribosomes in dendritic shafts not associated with synapses [7]. The explanation of a sole effect of pseudoconditioning can only be speculative: in contrast to the situation in which an animal learns a sequence of events, a random application of an unpleasant stimulus seems to induce some kind of stress, influencing brain plasticity in a different manner. The present finding suggests that this effect mainly concerns the smallest and most transient spines.

5. Conclusions

Results of the present study demonstrate that associative fear learning produces different effects on single- and double-synapse spines in the barrel cortex: it promotes maturation and stabilization of double-synapse spines, which might possibly contribute to permanent memory formation, and upregulates protein synthesis in single-synapse spines, which might prepare them to accept new inhibitory synapses and transform into double-synapse spines.

Conflict of Interests

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

Acknowledgments

The research was supported by statutory funds (K/ZDS/005680) from the Jagiellonian University Medical College to Ewa Jasek and the Iuventus Plus grant (IP2011 019971) from the Polish Ministry of Science and Higher Education to Malgorzata Jasinska.

References

[1] K. D. Micheva and C. Beaulieu, "An anatomical substrate for experience-dependent plasticity of the rat barrel field cortex," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 25, pp. 11834–11838, 1995.

[2] G. W. Knott, C. Quairiaux, C. Genoud, and E. Welker, "Formation of dendritic spines with GABAergic synapses induced by whisker stimulation in adult mice," *Neuron*, vol. 34, no. 2, pp. 265–273, 2002.

[3] M. Jasinska, E. Siucinska, A. Cybulska-Klosowicz et al., "Rapid, learning-induced inhibitory synaptogenesis in murine barrel field," *Journal of Neuroscience*, vol. 30, no. 3, pp. 1176–1184, 2010.

[4] L. E. Ostroff, C. K. Cain, J. Bedont, M. H. Monfils, and J. E. LeDoux, "Fear and safety learning differentially affect synapse size and dendritic translation in the lateral amygdala," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 20, pp. 9418–9423, 2010.

[5] I. Lushnikova, G. Skibo, D. Muller, and I. Nikonenko, "Excitatory synaptic activity is associated with a rapid structural plasticity of inhibitory synapses on hippocampal CA1 pyramidal cells," *Neuropharmacology*, vol. 60, no. 5, pp. 757–764, 2011.

[6] J. N. Bourne and K. M. Harris, "Coordination of size and number of excitatory and inhibitory synapses results in a balanced structural plasticity along mature hippocampal CA1 dendrites during LTP," *Hippocampus*, vol. 21, no. 4, pp. 354–373, 2011.

[7] M. Jasinska, E. Siucinska, E. Jasek, J. A. Litwin, E. Pyza, and M. Kossut, "Fear learning increases the number of polyribosomes associated with excitatory and inhibitory synapses in the barrel cortex," *PLoS ONE*, vol. 8, no. 2, Article ID e54301, 2013.

[8] E. Siucinska and M. Kossut, "Short-lasting classical conditioning induces reversible changes of representational maps of vibrissae in mouse SI cortex—a 2DG study," *Cerebral Cortex*, vol. 6, no. 3, pp. 506–513, 1996.

[9] A. Skibinska, M. Lech, and M. Kossut, "Differential regulation of cortical NMDA receptor subunits by sensory learning," *Brain Research*, vol. 1065, no. 1-2, pp. 26–36, 2005.

[10] M. Gierdalski, B. Jablonska, E. Siucinska, M. Lech, A. Skibinska, and M. Kossut, "Rapid regulation of GAD67 mRNA and protein level in cortical neurons after sensory learning," *Cerebral Cortex*, vol. 11, no. 9, pp. 806–815, 2001.

[11] E. Siucinska, M. Kossut, and M. G. Stewart, "GABA immunoreactivity in mouse barrel field after aversive and appetitive classical conditioning training involving facial vibrissae," *Brain Research*, vol. 843, no. 1-2, pp. 62–70, 1999.

[12] M. Jasińska, E. Siucińska, S. Głazewski, E. Pyza, and M. Kossut, "Characterization and plasticity of the double synapse spines in the barrel cortex of the mouse," *Acta Neurobiologiae Experimentalis*, vol. 66, no. 2, pp. 99–104, 2006.

[13] V. I. Popov, H. A. Davies, V. V. Rogachevsky et al., "Remodelling of synaptic morphology but unchanged synaptic density during late phase long-term potentiation (LTP): a serial section electron micrograph study in the dentate gyrus in the anaesthetised rat," *Neuroscience*, vol. 128, no. 2, pp. 251–262, 2004.

[14] K. M. Harris, J. C. Fiala, and L. Ostroff, "Structural changes at dendritic spine synapses during long-term potentiation," *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 358, no. 1432, pp. 745–748, 2003.

[15] K. M. Harris, F. E. Jensen, and B. Tsao, "Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation," *Journal of Neuroscience*, vol. 12, no. 7, pp. 2685–2705, 1992.

[16] J. Spacek and K. M. Harris, "Three-dimensional organization of smooth endoplasmic reticulum in hippocampal CA1 dendrites and dendritic spines of the immature and mature rat," *Journal of Neuroscience*, vol. 17, no. 1, pp. 190–203, 1997.

[17] H. Kasai, M. Matsuzaki, J. Noguchi, N. Yasumatsu, and H. Nakahara, "Structure-stability-function relationships of dendritic spines," *Trends in Neurosciences*, vol. 26, no. 7, pp. 360–368, 2003.

[18] A. J. G. D. Holtmaat, J. T. Trachtenberg, L. Wilbrecht et al., "Transient and persistent dendritic spines in the neocortex *in vivo*," *Neuron*, vol. 45, no. 2, pp. 279–291, 2005.

[19] J. T. Trachtenberg, B. E. Chen, G. W. Knott et al., "Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex," *Nature*, vol. 420, no. 6917, pp. 788–794, 2002.

[20] E. G. Gray, "Electron microscopy of synaptic contacts on dendrite spines of the cerebral cortex," *Nature*, vol. 183, no. 4675, pp. 1592–1593, 1959.

[21] T. Deller, T. Merten, S. U. Roth, P. Mundel, and M. Frotscher, "Actin-associated protein synaptopodin in the rat hippocampal formation: localization in the spine neck and close association with the spine apparatus of principal neurons," *Journal of Comparative Neurology*, vol. 418, no. 2, pp. 164–181, 2000.

[22] E. Fifková, J. A. Markham, and R. J. Delay, "Calcium in the spine apparatus of dendritic spines in the dentate molecular layer," *Brain Research*, vol. 266, no. 1, pp. 163–168, 1983.

[23] A. Vlachos, E. Korkotian, E. Schonfeld, E. Copanaki, T. Deller, and M. Segal, "Synaptopodin regulates plasticity of dendritic spines in hippocampal neurons," *The Journal of Neuroscience*, vol. 29, no. 4, pp. 1017–1033, 2009.

[24] E. Korkotian, M. Frotscher, and M. Segal, "Synaptopodin regulates spine plasticity: mediation by calcium stores," *Journal of Neuroscience*, vol. 34, no. 35, pp. 11641–11651, 2014.

[25] O. Steward and T. M. Reeves, "Protein-synthetic machinery beneath postsynaptic sites on CNS neurons: association between polyribosomes and other organelles at the synaptic site," *The Journal of Neuroscience*, vol. 8, no. 1, pp. 176–184, 1988.

[26] J. P. Pierce, T. Mayer, and J. B. McCarthy, "Evidence for a satellite secretory pathway in neuronal dendritic spines," *Current Biology*, vol. 11, no. 5, pp. 351–355, 2001.

[27] T. Deller, C. B. Orth, D. Del Turco et al., "A role for synaptopodin and the spine apparatus in hippocampal synaptic plasticity," *Annals of Anatomy*, vol. 189, no. 1, pp. 5–16, 2007.

[28] J. Bourne and K. M. Harris, "Do thin spines learn to be mushroom spines that remember?" *Current Opinion in Neurobiology*, vol. 17, no. 3, pp. 381–386, 2007.

[29] P. Jedlicka, A. Vlachos, S. W. Schwarzacher, and T. Deller, "A role for the spine apparatus in LTP and spatial learning," *Behavioural Brain Research*, vol. 192, no. 1, pp. 12–19, 2008.

[30] T. Deller, M. Korte, S. Chabanis et al., "Synaptopodin-deficient mice lack a spine apparatus and show deficits in synaptic plasticity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 18, pp. 10494–10499, 2003.

[31] J. C. Fiala and K. M. Harris, "Extending unbiased stereology of brain ultrastructure to three-dimensional volumes," *Journal of the American Medical Informatics Association*, vol. 8, no. 1, pp. 1–16, 2001.

[32] L. E. Ostroff, J. C. Fiala, B. Allwardt, and K. M. Harris, "Polyribosomes redistribute from dendritic shafts into spines with enlarged synapses during LTP in developing rat hippocampal slices," *Neuron*, vol. 35, no. 3, pp. 535–545, 2002.

[33] J. I. Arellano, R. Benavides-Piccione, J. Defelipe, and R. Yuste, "Ultrastructure of dendritic spines: correlation between synaptic and spine morphologies," *Frontiers in Neuroscience*, vol. 1, no. 1, pp. 131–143, 2007.

[34] M. Kuwajima, J. Spacek, and K. M. Harris, "Beyond counts and shapes: Studying pathology of dendritic spines in the context of the surrounding neuropil through serial section electron microscopy," *Neuroscience*, vol. 251, pp. 75–89, 2013.

[35] E. G. Jones and T. P. Powell, "Morphological variations in the dendritic spines of the neocortex," *Journal of Cell Science*, vol. 5, no. 2, pp. 509–529, 1969.

[36] R. C. Malenka and R. A. Nicoll, "Long-term potentiation—a decade of progress?" *Science*, vol. 285, no. 5435, pp. 1870–1874, 1999.

[37] M. Matsuzaki, G. C. R. Ellis-Davies, T. Nemoto, Y. Miyashita, M. Iino, and H. Kasai, "Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons," *Nature Neuroscience*, vol. 4, no. 11, pp. 1086–1092, 2001.

[38] A. Cybulska-Klosowicz and M. Kossut, "Early-phase of learning enhances communication between brain hemispheres," *European Journal of Neuroscience*, vol. 24, no. 5, pp. 1470–1476, 2006.