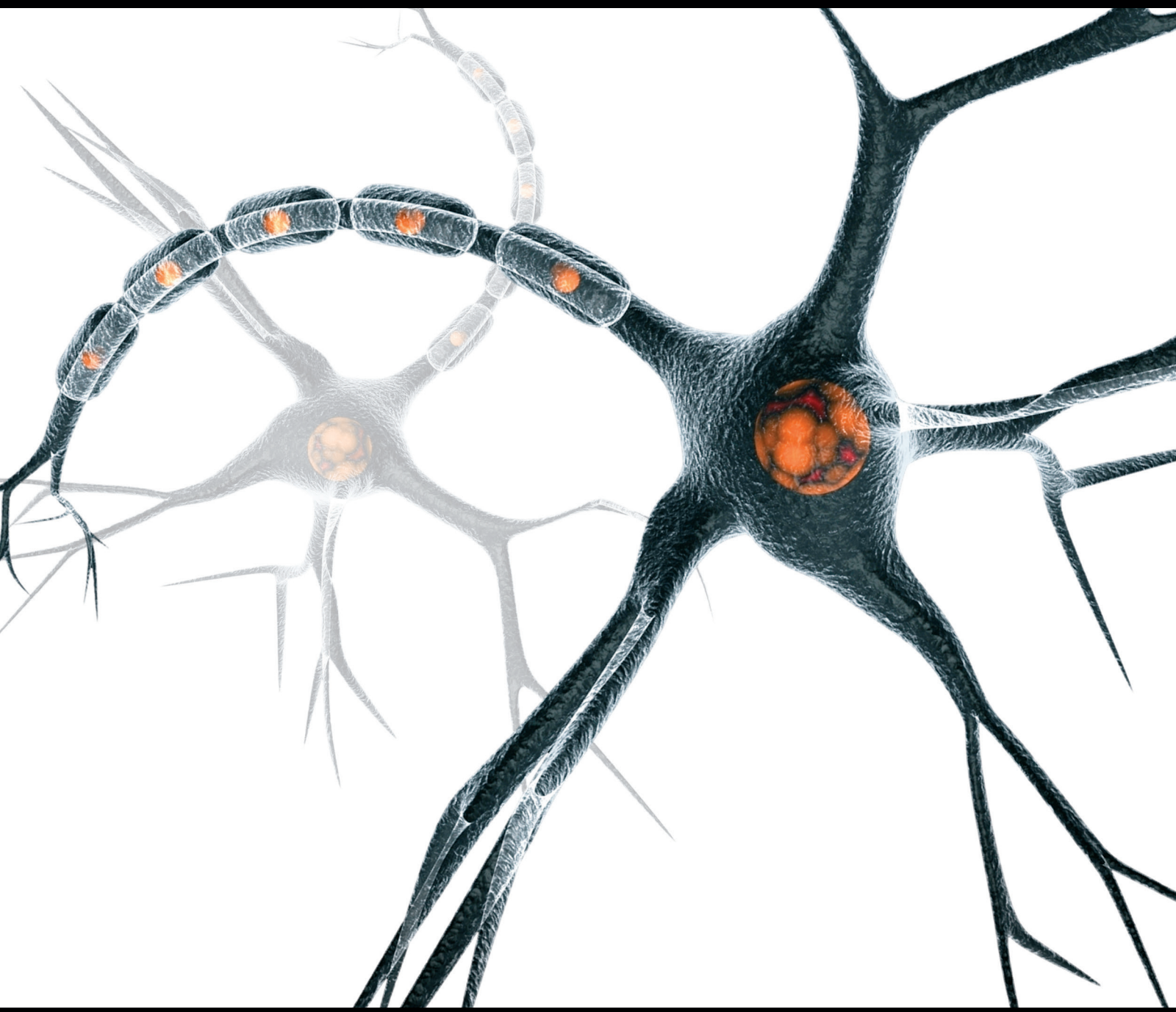
Neural Plasticity

Molecular and Cellular Mechanisms of Synaptopathies

Guest Editors: Alvaro O. Ardiles, Andreas M. Grabrucker, Francisco G. Scholl, Gabby Rudenko, and Tiziana Borsello



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Editorial Board

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Editorial
Molecular and Cellular Mechanisms of Synaptopathies

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Synapses, contact points between neurons, are essential elements supporting the ability of neurons to communicate and to transmit relevant information to each other. They play an integral role in brain development and wiring neurons into neural circuits, for example, those related to our behavior. Therefore, alterations affecting the integrity and/or functionality of synapses can lead to synaptic pathologies or synaptopathies. For instance, many neurological disorders including Alzheimer's disease, Down syndrome, epilepsy, and Parkinson's disease and neurodevelopmental disorders such as autism spectrum disorders, intellectual disability, and fragile X syndrome have consistently been reported to exhibit abnormalities in synaptic composition, morphology, and function. This special issue discusses various aspects of the molecular interactions that underlie synaptic protein networks and the complex signaling pathways that are activated by them, knowledge that is crucial to understand the cellular and molecular mechanisms involved in different synaptopathies. Synapses comprise a presynaptic compartment, consisting of the axon terminal and their protein machinery implicated in the release of neurotransmitters. Upon exocytosis of presynaptic vesicles, neurotransmitters spill out into the extracellular space called the "synaptic cleft" and diffuse to reach a postsynaptic compartment, composed of the protein machinery that receives and transduces the neurotransmitter-induced signals [1]. Most synaptopathies directly or indirectly affect the molecular repertoire of synaptic proteins.

V. I. Torres et al. provide a comprehensive review describing specific pre- and postsynaptic proteins that are involved in the physiopathology of various synaptopathies and how deficits in these molecules contribute to different synaptopathic mechanisms. V. I. Torres et al. catalogue the different presynaptic and postsynaptic proteins that to date have been implicated in neuropsychiatric, neurodevelopmental, and neurodegenerative disorders. A more specific review by G. Rudenko focusses on synaptic adhesion molecules (SAMs), many of which are now implicated in neuropsychiatric, neurodevelopmental, and neurodegenerative diseases as well. SAMs tether to the pre- or postsynaptic membranes extending their extracellular domains into the synaptic cleft where they coordinate protein interaction networks. A much larger diversity of SAMs and their protein interactions exists than has previously been appreciated. In

addition, SAMs drive more complex functions than purely the adhesion of presynaptic and postsynaptic membranes. Furthermore, SAMs are under dynamic control through a variety of mechanisms, enabling them to play a key role in plasticity at synapses. Thus, considering the crucial role that SAMs play in synapse development, they may yield novel therapeutic targets which can be exploited to ameliorate certain synaptopathies.

Several articles in this special issue focus on specific synaptic proteins in detail. S. Biggi et al. present evidence that c-Jun N-terminal kinase (JNK), acting presynaptically, may have an important functional role in synapses. JNK is part of a signaling pathway strongly activated by NMDA stimulation and involved in synaptic plasticity. It is noteworthy that until now, most studies have been focused on the postsynaptic mechanism of JNK action, and less is known about JNK presynaptic localization and its physiological role at this site. S. Biggi et al. demonstrate that activation of presynaptic NMDA receptors leads to the activation of JNK which modulates neurotransmitter release through a direct interaction with SNARE proteins. These findings are relevant considering that JNK activity had been associated with not only neurodegenerative disorders like Alzheimer's, Huntington's, and Parkinson's disease but also psychiatric disorders and intellectual disabilities. F. Longhena et al. review recent evidence indicating that α-synuclein possesses a novel "prion-like" behavior, whereby the protein can spread transsynaptically to trigger the aggregation of α-synuclein in neighboring neurons, a phenomenon observed in Parkinson's disease animal models and patients [2–5]. Monomeric, oligomeric, and fibrillary α-synuclein forms accumulate at the synaptic terminal due to their high affinity for vesicular membranes and can be released in a process that is thought to be mediated mainly by exosomal vesicles [6, 7]. Once released, α-synuclein could affect endogenous α-synuclein on recipient neurons, thereby affecting their function, and/or could interact with membrane lipids leading to both pre- and postsynaptic alterations and hence synaptic failure. The elucidation of this novel mechanism of α-synuclein transmission can shed lights on the contribution of α-synuclein spreading to Parkinson's disease related synaptopathy. E. Pérez-Palma et al. introduce new evidence relating to Wnt/β-catenin signaling and the expression of novel Wnt/β-catenin target genes. In addition to genes involved in neural precursors, forebrain development, and stem cell differentiation, E. Pérez-Palma et al. also identified a significant number of genes with transcription factor activity. The genes modulated by Wnt/β-catenin signaling are involved in biological processes such as neuronal structure and activity, and that are affected in synaptopathies. B. K. Unda et al. provide evidence that the secreted neurotrophic factor neuregulin-1 (NRG1) and its receptor ErbB4 signal through the multifunctional scaffold protein, disrupted in schizophrenia 1 (DISC1). Together, they regulate the development of cortical inhibitory interneurons and thus also impact the proper balance between excitatory and inhibitory transmissions. Since the genes encoding these proteins have been identified independently as risk factors for schizophrenia, a complete understanding of how these proteins interact to regulate the development of cortical inhibitory neuron morphology and synapse formation may provide insights into how these processes progress into synaptopathies.

Further, in this special issue, two papers focus on the use of animal models to yield a wealth of information about the underlying mechanisms of synaptopathies. Epilepsy is a neuropsychiatric condition characterized by an abnormal and excessive neural activity leading to a predisposition to recurrent unprovoked seizures affecting both excitatory and inhibitory synapses, synaptic plasticity, and behavior [8]. M. Lenz et al. use a pilocarpine-induced status epilepticus animal model to study synaptopodin, an actin-binding protein expressed in cortical neurons that is involved in the modulation of synaptic plasticity and spine remodeling [9, 10]. They propose that synaptopodin is a valuable diagnostic marker to detect alterations in the ability of neurons to undergo synaptic plasticity such as can be observed in seizure-induced synaptopathies; M. Lenz et al. provide further support for the role of synaptopodin in the ability of hippocampal neurons to exhibit synaptic plasticity, demonstrating that intraperitoneal pilocarpine injection reduces synaptopodin levels and affects the induction of long-term potentiation (LTP). M. I. Herrera et al. review the literature linking perinatal asphyxia and synaptic dysfunction obtained from an experimental model of perinatal asphyxia. Perinatal asphyxia is an obstetric complication resulting to abnormal brain development in term and preterm neonates; if affected babies survive, they develop neurological disorders such as epilepsy, cerebral palsy, mental retardation, attention-deficit disorder, and schizophrenia. M. I. Herrera et al. present evidence that perinatal asphyxia induces a series of modifications in synaptic composition, structure, and function leading to alterations in synapses and their function.

Lastly, focusing on the role of gene-environment interaction in mental disorders, S. Pfaender et al. provide evidence that zinc signaling plays a role in proliferation and neuronal differentiation of stem cells. They show that zinc deficiency during brain development impairs neurogenesis and modulates the expression of synaptic proteins, primary mechanisms influencing brain function.

In summary, articles published in this special issue highlight the very complex nature of synaptopathies, the participation of a very diverse portfolio of synaptic proteins linked to synaptopathies, and emphasize the importance of understanding these proteins to identify potential novel targets which might benefit the development of therapies for neuropsychiatric, neurodevelopment, and neurodegenerative diseases.

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Research Article

Evidence of Presynaptic Localization and Function of the c-Jun N-Terminal Kinase

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The c-Jun N-terminal kinase (JNK) is part of a stress signalling pathway strongly activated by NMDA-stimulation and involved in synaptic plasticity. Many studies have been focused on the post-synaptic mechanism of JNK action, and less is known about JNK presynaptic localization and its physiological role at this site. Here we examined whether JNK is present at the presynaptic site and its activity after presynaptic NMDA receptors stimulation. By using N-SIM Structured Super Resolution Microscopy as well as biochemical approaches, we demonstrated that presynaptic fractions contained significant amount of JNK protein and its activated form. By means of modelling design, we found that JNK, via the JBD domain, acts as a physiological effector on T-SNARE proteins; then using biochemical approaches we demonstrated the interaction between Syntaxin-1-JNK, Syntaxin-2-JNK, and Snap25-JNK. In addition, taking advance of the specific JNK inhibitor peptide, D-JNKI1, we defined JNK action on the SNARE complex formation. Finally, electrophysiological recordings confirmed the role of JNK in the presynaptic modulation of vesicle release. These data suggest that JNK-dependent phosphorylation of T-SNARE proteins may have an important functional role in synaptic plasticity.

1. Introduction

The c-Jun NH_2-terminal protein kinase (JNKs) is part of the mitogen-activated protein kinase (MAPK) family [1, 2] and is represented in mammals by three genes coding for three isoforms: JNK1, JNK2, and JNK3. JNK1 and JNK2 expression is essentially ubiquitous, while JNK3 presence is restricted to brain and testis [3]. JNKs activation is led by several types of extracellular stress stimuli such as heat shock, UV irradiation, inflammatory cytokines, Aβ oligomers-induced toxicity, and excitotoxicity, primarily leading to cell death.

JNK phosphorylates numerous important substrates, including transcription factors AP-1, c-Jun, and Fos, leading to activation of nuclear response [3–5]. Furthermore, JNK regulates intracellular signalling pathways phosphorylating many cytosolic and cell-membrane targets. Many data pointed out that the JNK activation in the brain is higher than in any other mammalian tissue [6–8], suggesting that kinase might play an important role in the central nervous system (CNS), regulating both physiological and pathological brain processes.

JNK activation, in fact, induces neuronal death in a wide range of pathological conditions, such as NMDA-induced excitotoxicity, ischemic stroke [9–11], and traumatic brain injury [12]; JNK activation has been also associated to many neurodegenerative disorders like Alzheimer [13–18], Huntington's [19], and Parkinson's disease [20, 21], but also psychiatric disorders and intellectual disabilities involving synaptic structure anomalies [22]. On the other hand, JNK plays also an important role in regulating physiological processes

such as neuronal precursors migration, axonal sprouting and polarization, dendritic branches elongation, dendritic spines stabilization, and therefore neuronal plasticity and regeneration in the adult brain.

All these evidences point out that JNK pathway in the CNS is particularly intricate, since it is not only subjected to different types of stimulations, which modulate JNK response, but it also plays different roles depending on its localization, having JNK pool distinct functions in the various cellular districts. Since neurons are exquisitely polarized cells, it is plausible that JNK activation may have different roles and functions in diverse subcellular localizations, from nuclear to cytoplasmic compartments as well as axons, dendrites, and dendritic spines. This effect is not only due to compartmentalization, but also due to the huge variety of JNK's targets. For instance c-Jun, the JNK elective target, is localized in the nucleus and regulates gene expression, while microtubule associated proteins (MAPs) are in the cytoplasm [23–26] and control axon elongation and dendrite branching [22]. More recently, JNK has been also identified at synaptic sites [27–29]; however its role in this district as well as its activation level is not completely clear yet.

Different works suggested that JNK is preferentially located in postsynaptic regions, where it interacts with key synaptic scaffold proteins (PSD-95 and PSD-93) as well as with GluR2 AMPAR subunit (GluR2L) [30]. We demonstrated that JNK has an important role in regulating glutamate receptors expression at postsynaptic density level after Aβ-induced toxicity in hippocampal neurons [17, 31]. Furthermore, many lines of evidence demonstrated the importance of NMDA receptors (NMDARs) as JNK activators in the postsynaptic compartment [9, 32], suggesting that JNK might have a crucial role in synaptic plasticity as well as in excitotoxicity. NMDA-induced excitotoxicity in fact, mediated mainly by postsynaptic NMDARs, powerfully activates JNK, and JNK specific inhibition prevents NMDAR-mediated neuronal death in cortical neurons [9, 10, 25] as well as in organotypic hippocampal slices culture [9]. However, less is known about the biological role of presynaptic NMDARs (pre-NMDARs) and their downstream pathways. The pre-NMDARs have been extensively characterized in the last years as new key-players during development and memory consolidation phases. They are mainly involved in synaptic strength modulation, through mechanisms ranging from spontaneous neurotransmitter release facilitation to LTP/LTD [33–39]. The pre-NMDARs are abundantly expressed in the developing nervous system [40–42] although their expression decreases in the adult, persisting only in most plastic brain areas (such as neocortex, cerebellum, and hippocampus) [43]. Importantly, their aberrant activation has been also associated to pathological events [44, 45]. Although JNKs presence in postsynaptic compartment has been characterized, there is lack of data for their presence and physiological role at presynaptic sites. Here we investigate JNK localization and role in presynaptic compartment. JNK identification at the presynaptic site and its activation after NMDARs stimulation may be important not only to clarify this issue, but also for the identification of new targets contributing to synaptic plasticity. Moreover, JNK downstream pathway definition could represent a new therapeutic tool to prevent synaptic dysfunction in neurodegenerative diseases.

2. Materials and Methods

2.1. Purified Synaptosomes Extraction. Purified synaptosomes from mice cortex were extracted as in Dunkley [38]. Briefly, tissues were gently homogenated with a glass-Teflon grinder in ST buffer (sucrose 320 mM, Tris 10 mM; pH 7.4) supplemented with protease and phosphatase inhibitors. Homogenates were centrifuged (1.000g 5 min 4°C) and supernatants were collected and centrifuged again (13.000 rpm, 20 min, 4°C). Pellet was resuspended in ST buffer and stratified on a 20–10–6–2% Percoll (GE Healthcare) gradient in ST buffer, prepared in ultracentrifuge tubes. Samples were then ultracentrifuged, for 8 min 33500g 4°C and interface between 10% and 20% layers, containing purified synaptosomes, was aspired. Purified synaptosomes were then precipitated 20 min 10.000g 4°C in 6 : 1 vol/vol Hepes Buffer (NaCl 140 mM, KCl 3 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.2 mM, NaH_2PO_4 1 mM, $NaHCO_3$ 3.5 mM, glucose 10 mM, Hepes 5 mM; pH 7.4, Sigma). Pellet was lysed or resuspended in the appropriate buffer.

2.2. Crude Synaptosomes Extraction. Crude synaptosomes were used for calcium currents evaluation. Brains were homogenated with a glass-Teflon grinder in SHE buffer (Hepes 4 mM pH 7.3, sucrose 320 mM, EGTA 1 mM, Sigma) with protease inhibitors and phosphatase inhibitors. After a first centrifuge (10.000g 10 min 4°C), supernatant was collected and again centrifuged (12.500g, 20 min 4°C). Pellet was resuspended in SHE buffer and subjected to a third centrifuge (12.500g, 20 min, 4°C) in order to obtain crude synaptosomal fraction pellet, which was resuspended in KRH buffer (NaCl 125 mM, KCl 5 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, Hepes NaOH pH 7.4 25 mM, glucose 6 mM, $CaCl_2$ 2 mM, Sigma) and kept at 37°C 5% CO_2 for 45 min, for functional reactivation. Crude reactivated synaptosomes were then resuspended at a final concentration of 1 mg–500 μg/ml in KRH. After resuspension, synaptosomes were kept at 37°C 5% CO_2 for 25 min for an habituation step.

2.3. Calcium Currents Evaluation. Calcium currents were measured on crude synaptosomes. Briefly, after reactivation step, synaptosomes were pelleted as described before, resuspended in 300 μl 1 : 1 mixture using KRH and calcium sensitive cell-permeating fluorophore Fluo-4 (Fluo-4 Direct™ Calcium Assay, Life Technologies). Samples were placed in a whole black 96-well (Corning Costar® 96-Well Black Polystyrene Plate) for 25 min 37°C 5% CO_2 to allow Fluo-4 to permeate cell membranes. After habituation fluorescence was measured with a Tecan F500 spectrophotometer for 180 cycles (corresponding to approximately 7 minutes) at 37°C. NMDA (100 μM, with glycine coagonist 1 μM final concentration) or KCl (50 mM final concentration) were injected 100 μl/sec at cycle 20 (45 sec). Fluorescence was recorded by a top mode reading optimizing Z-position.

2.4. Synaptosomes Stimulation. 25 minutes before stimulation D-JNKI1 peptide (Xigen) is added to the medium at a final concentration of 2 μM. Synaptosomes were treated with NMDA 100 μM (Abcam) with glycine coagonist 1 μM (Sigma) or KCl 50 mM (Sigma) for 2′, 5′, 7′, or 10′ at 37°C 5% CO_2. For NMDA stimulation $MgSO_4$ was omitted from the buffer. At the end of the stimulation synaptosomes were resuspended in lysis buffer. For western blotting analysis Bonny lysis buffer was used [25] while for SNARE complex evaluation SNARE buffer was used [37].

2.5. Immunoprecipitation. Immunoprecipitation was conducted on purified cortical synaptosomes as previously described [39]. Immunoprecipitation antibodies, all used at 1 : 100, were anti-JNK (#06-748, Upstate), anti-Syntaxin-1 (#A1238, Assay Biotech), anti-Syntaxin-2 (#110 022, Synaptic Systems), and anti-Snap25 (#331, Chemicon). After precipitation process 0.5–1 μg proteins were subjected to SDS-Page.

2.6. SDS-PAGE. 20 μg of brain homogenates, TIF (Triton-Insoluble Fraction), extracted as in [40] crude/purified synaptosomal proteins, was separated by SDS (Bio-Rad) polyacrylamide gel electrophoresis (ProtoGel, National Diagnostics). For SNARE complex detection, samples (referred to as unboiled) were kept at RT for 7 min before loading. Antibodies used were anti-p-JNK and-JNK (#9251 and #9252 both Cell Signalling, 1 : 1000), anti-Syntaxin-1 (#78539, Abcam), anti-Syntaxin-2 (#S0664, Sigma, 1 : 5000), Snap25 (Stressgen, 1 : 5000), anti-Syntaxin-1 (Abcam, 1 : 5000), anti-Vamp (#104 211, Synaptic Systems), anti-Synaptophysin (#S5768, Sigma), anti-PSD95 (#10011435, Cayman Chemicals, 1 : 1000), and anti-Actin (1501, Millipore, 1 : 5000). Blots were developed using ECL chemiluminescence system (ECL Western Blotting Substrate, Promega) and quantified by densitometry using ImageJ software.

2.7. Immunofluorescence. After extraction, purified synaptosomes were processed as in Sokolow [41]. Primary antibodies used were anti-Synaptophysin 1 : 500 (#S5768, Sigma) and anti-p-JNK 1 : 100 (#6254, Santa Cruz) while fluoresceinated secondary antibodies were Alexa Fluor 488 anti-Mouse and Alexa Fluor 594 anti-Rabbit antibodies. Images were acquired with Super-Resolution microscope (N-SIM, Nikon-Structured Illumination Microscopy). Protein colocalization was analysed using Pearson's colocalization coefficient with Imaris software.

2.8. Acute Slice Preparation. Experiments were performed on horizontal slices prepared from the somatosensory cortex of CD-1 mice of either sex, 2-3-month-old. Each animal was anaesthetized with isoflurane, USP (Abbott Laboratories, Illinois, USA), and decapitated. The whole brain was removed and rapidly immersed in ice-cold extracellular saline solution containing (mM): Tris-Hcl 72, Tris-Base 18, NaH_2PO_4 1.2, $NaHCO_3$ 30, KCl 2.5, Glucose 25, HEPES 20, $MgSO_4$ 10, Na-PIR 3, ascorbic acid 5, $CaCl_2$ 0.5, sucrose 20, pH 7.4. Horizontal somatosensory slices (200 μm thickness) were obtained using a vibratome (Vibroslice 752, Campden Instruments, Loughborough, UK). The slices were placed in a solution containing (mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 NaHCO, 20 glucose, and the pH was maintained at 7.4 for 25/30 min a 32°C and then maintained at room temperature.

2.9. Electrophysiology. After a recovery period of at least 1 h, an individual slice was transferred to a recording chamber and continuously perfused with extracellular solution at room temperature (22/25°C). Whole-cell voltage-clamp recordings were made from cortical neurons using an EPC-8 patch-clamp amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany). Pipettes of borosilicate glass, with a tip resistance between 2.0 and 3 MΩ, were used for patch-clamp recordings [42]. The intracellular solution had the following composition (mM): K-gluconate 117, KCL 13, $MgCl_2{\cdot}6H_20$ 2, Hepes 10, $CaCl_2$ 1, EGTA 11, Na_2ATP 2, Na_3GTP 0.5, pH 7.3. Voltage-clamp recordings were accepted only if the series resistance was less than 10 MΩ. Data were filtered at 3 kHz and digitized at 10 kHz using the filter and analog/digital converter of the amplifier. Digitized data were stored on computer disk using the Pulse software (HEKA Elektronik).

2.10. Statistical Analysis. At least three independent replicates have been performed for each experiment. Statistical analysis was done using Graph Pad Prism 6 program. Data were analysed using Student's t-test and one- or two-way ANOVA, followed by Dunnet/Tukey's post hoc test. All data were shown as mean ± SEM with statistical significance given at $p < 0.05$.

2.11. Computational Analysis. The pairwise alignment between Syntaxin-1 (Uniprot ID Q16623) and Syntaxin-2 (Uniprot ID P32856) was performed using Needle software available in the European Molecular Biology Open Software Suit (EMBOSS) (PMID: 10827456).

The structure of Syntaxin-1 for the docking simulations with JNK1 was extracted from the Syntaxin-1-Munc18A complex (10.1038/emboj.2008.37). The structure of JNK1 complexed with a small peptide belonging to JIP scaffold protein [43] was selected for the docking simulations. The structure of Snap25 was extracted from the 20S supercomplex [44] while the same JNK1 structure selected for the docking with Syntaxin-1 was also used. The docking simulation was performed using HADDOCK 2.2 [45] program that is one of the most suitable software to correctly predict the protein-protein complexes. The images have been obtained using the software UCSF Chimera v.1.10.1.

3. Results

3.1. JNK Is Located in the Presynaptic Compartment. To investigate JNK localization in presynaptic compartment, we used N-SIM Structured Super Resolution Microscopy on isolated cortical synaptosomes (Figure 1(a)). Synaptosomes were double stained for Synaptophysin, a constitutive and specific presynaptic protein (labeled in red) and for the JNK active form p-JNK (labeled in green, Figure 1(a)). As shown

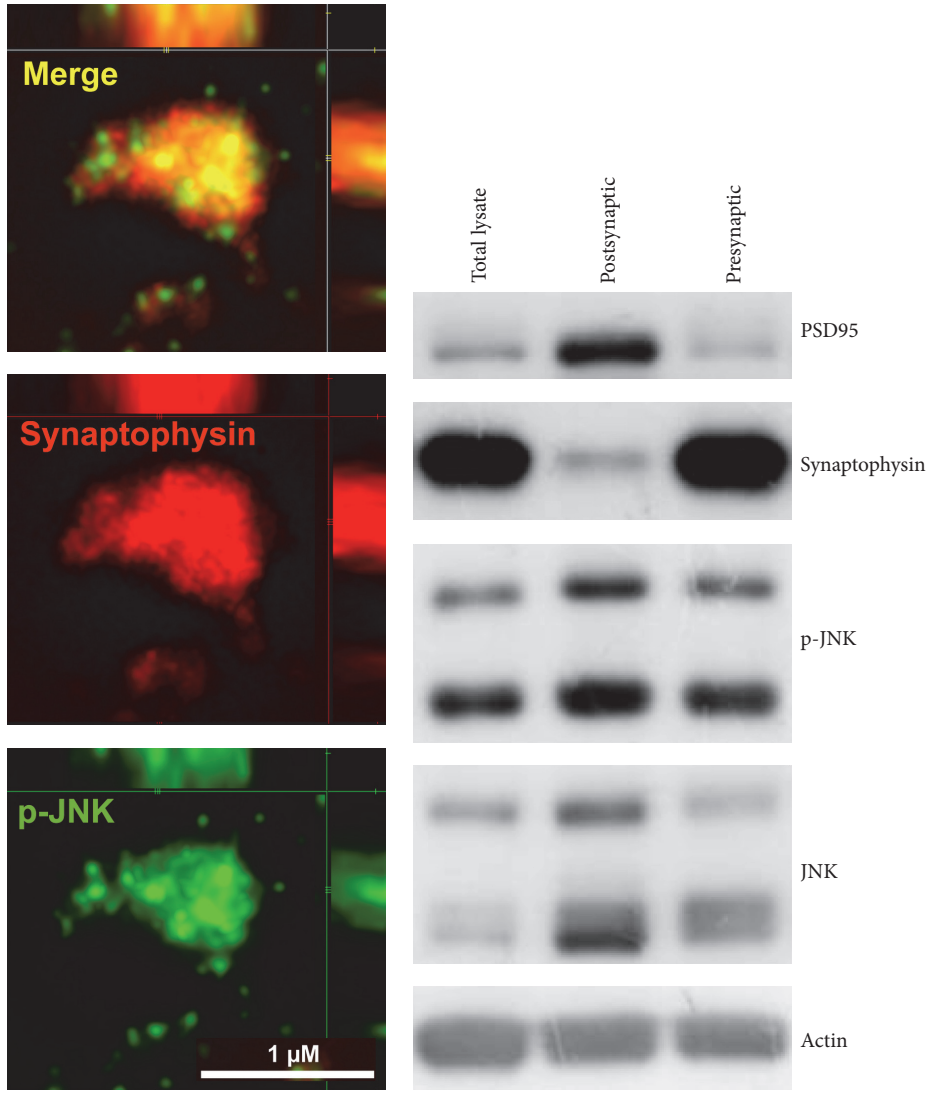

Figure 1: JNK is localized in the presynaptic compartment. (a) Immunofluorescence on mouse cortical purified synaptosomes stained for p-JNK (green) and for Syntaxin-2 (red) colocalization is visualized as yellow signal in Merge image, with Pearson's coefficient 0.54 ± 0.08. Images acquired with super resolution microscopy; scale bar 1 μm. (b) Western blotting analysis of JNK and p-JNK active form presence in 20 μg of total cortical lysates, postsynaptic density region (represented by PSD95 postsynaptic specific protein on TIF fraction) and presynaptic compartment (represented by Synaptophysin presynaptic specific protein on purified synaptosomes). JNK is localized both in postsynaptic and in presynaptic region and its phosphorylation state is comparable. Actin was used as loading control.

in Figure 1(a) (Merge panel), the JNK active form p-JNK was located in presynaptic compartment and colocalized with Synaptophysin. This evidence is also confirmed by Pearson's coefficient value, corresponding to 0.54 ± 0.08.

JNK presynaptic localization was further investigated comparing the presynaptic to the postsynaptic fraction and to total brain homogenate. JNK and activated form p-JNK were evaluated by western blotting on total cortical homogenate, cortical synaptosomal lysate (presynaptic compartment), and TIF fractionation (postsynaptic compartment; Figure 1(b)). The P-JNK activated form, as well as the JNK total form, was detectable in the presynaptic compartment (Figure 1(b)) confirming the Super Resolution Microscopy data (Figure 1(a)). In the postsynaptic TIF fractionation, identified by PSD95 scaffold protein, JNK was present as expected. The purity of the synaptosomes was confirmed by the low presence of PSD-95 in the presynaptic fraction, which as described by Evans [46] is accountable to the nonfunctional PSD structure bound to active zone. Furthermore TIF fraction purity was proved by the low presence of Synaptophysin in postsynaptic fractions.

3.2. JNK Is Rapidly Activated after Pre-NMDARs Stimulation Both in Young and in Adult Mice. To investigate pre-NMDAR-mediated JNK activation, we evaluated p-JNK/JNK ratio by western blotting on cortical synaptosomes purified

from young (p14) and adult mice after $2'$, $5'$, and $10'$ minutes of treatment with NMDA (100 μM) and glycine (1 μM).

In young mice synaptosomes (Figure 2(a)), JNK was significantly activated after 2 minutes of NMDA stimulation (75% activation increase, $^{*}p < 0.05$) and persisted after 5 minutes (70% activation increase, $^{*}p < 0.05$; Figure 2(a)). No significant alteration in the levels of presynaptic protein was detected by western blotting (Figures 2(b)–2(e)), suggesting that, at the considered time-points ($2'$, $5'$, and $10'$ minutes), JNK activation did not determine the activation of protein degradation processes.

As previously described, pre-NMDARs expression decreases during neurodevelopment [43]: to verify if pre-NMDAR-mediated JNK activation was conserved in adult mice, synaptosomes were extracted from aged mice. To test if residual pre-NMDARs expression was enough to trigger depolarization, calcium currents were measured on synaptosomes by pre-ncubation with Fluo4 cell-permeant calcium sensitive dye. Synaptosomes were stimulated with NMDA (100 μM) and glycine (1 μM) or KCl 50 mM as positive control (Figure 2(f)). Recording of calcium influx was performed with a spectrophotometer for 7 minutes, corresponding to 180 fluorescence-reading cycles, with stimulus injection at 45 sec (cycle 20, Figure 2(f) black arrow). This time-point has been chosen as a compromise to avoid aberrant fluorescence measurement due to homeostasis loss caused by lack of CO_2 and humidity control in the spectrophotometer chamber. As expected KCl injection led to a rapid and significant increase of intrasynaptosomal calcium levels lasting over time. Similarly, NMDA administration led to a comparable significant increase in fluorescence if compared to controls injected with vehicle, starting 45 sec (20 cycles) after injection and remaining stable over time. Unexpectedly we observed a drop in fluorescence level in vehicle injected synaptosomes (CTR); however, in line with previous data, NMDA+ Gly stimulation led to rapid JNK activation at 2 min (50% increase $^{***}p < 0.001$), which increases at 5 min (60% increase, $^{***}p < 0.001$) and persisted at 10 min (50% increase, $^{**}p < 0.01$; Figure 2(g)). These data suggested that pre-NMDAR-JNK axis was conserved during adult life. In fact, pre-NMDARs retained the ability to trigger excitatory response. The stimulation with NMDA (100 μM) and glycine (1 μM) induced JNK activation in both young (p14) and adult synaptosomes, and the consequent p-JNK/JNK ratio was comparable. As for young synaptosomes, also in this case, no significant protein degradation events were observed: no significant change of presynaptic proteins levels (Syntaxin-1 and Syntaxin-2, Snap25, and Vamp) at the considered time-points ($2'$ $5'$ and $10'$) was found (Figures 2(h)–2(k)).

3.3. Investigation of JNK Possible Targets. To get more insight into JNK modulation of vesicle release, JNK binding domain (JBD) was searched among presynaptic machinery proteins amino acidic sequences. In recent years, the extensive characterization of the JBDs in several JNK substrates [5] allowed identification of a canonical amino acid pattern for the JNK-substrate interaction. This canonical pattern is composed of two positively charged and two hydrophobic residues separated by two spacer regions with different length: [KR]-$X_{(0,2)}$-[KR]-$X_{(0,4)}$-[LI]-X- [LI] [47]. Among the critical steps involved in neurotransmitter release we decided to consider vesicle docking and priming steps, since previously reported data showed that JNK specific inhibition is not associated to any modification in calcium influx after NMDAR stimulation [25]. Thus it is unlikely that JNK could act on modulating this process in the presynaptic compartment. As reported in Figure 3(a), there are two possible JBDs in Syntaxin-1 and three in Syntaxin-2, respectively. Two out of three possible JBDs are conserved between Syntaxin-1 and Syntaxin-2 (Figure 3(a)). In particular, the JBD among residues 148–155 is fully conserved between the two isoforms (Figure 3(a)) making this region a very good candidate to be involved in the interaction with JNK. Analyzing also the sequence of Snap25, a unique putative JBD has been found (Figure 3(b)). The presence of putative JBDs pattern in three presynaptic machinery proteins is a strong indication of possible physical interaction of those proteins with JNK.

3.4. JNK Interacts with SNARE Proteins. The presence of JBDs sequences in three members (Syntaxin-1, Syntaxin-2, and Snap25) of the SNARE protein family allowed us to predict the possible three-dimensional (3D) organization of the JNK-SNARE protein complexes using computational approach, the protein-protein docking (Figures 3(c)-3(d)). The JBD sequence, fully conserved in both isoforms of Syntaxin (residues 148–155), has been mapped on the structure of Syntaxin-1 [48], which we have chosen as a representative structure also for Syntaxin-2, because they share a sequence identity of 64.4% and sequence similarity of 80% (Figure 3(c)). The unique JBD sequence located between residues 30 and 35 in Snap25 was also mapped on the 3D structure (Figure 3(d)).

The JBD present on the SNARE proteins and the region of JNK1 contacting the JIP peptide was used to drive the docking simulation performed with HADDOCK 2.2 software [49]. The best Syntaxin-1-JNK and Snap25-JNK complexes were reported in Figures 3(c) and 3(d) with residues belonging to the JBDs underlined. Both complexes have a favourable HADDOCK score that is a sum of different energy contribution terms (i.e., electrostatic energy, Van der Waals energy, desolvation energy, and restraints violation energies). In detail, the Syntaxin-1-JNK best complex reports an HADDOCK score of -24.9 ± 6.2 while the Snap25-JNK1 reports an HADDOCK score of -115.3 ± 0.8, underlining that the second complex formation could be more favoured. In both complexes the JBDs are involved in a dense network of hydrophobic and electrostatic interaction with JNK that are crucial for the stability of the complexes.

To prove these interactions, SNARE proteins were evaluated with western blotting, after immunoprecipitation of Syntaxin-1, Syntaxin-2, and Snap25 on purified cortical synaptosomal lysates. JNK was found in the coprecipitate; in particular the JNK protein band was more detectable with Syntaxin-2 and Syntaxin-1 and was less intensely visible with Snap25 (Figures 4(a), 4(b), and 4(c)). To confirm the interaction of JNK with Syntaxin-1, Syntaxin-2, and Snap25, the reverse immunoprecipitation on purified cortical

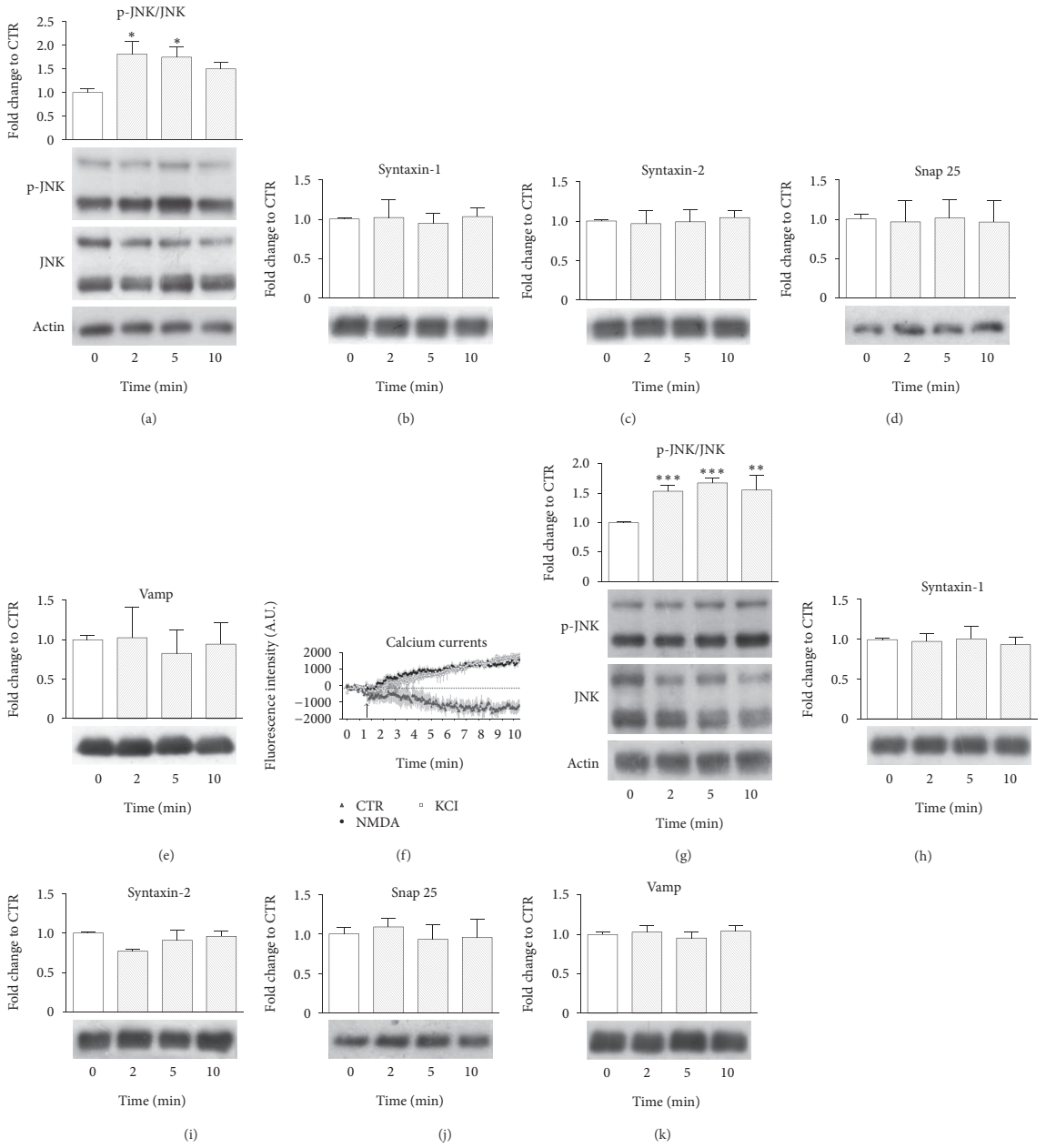

Figure 2: JNK is activated after pre-NMDARs stimulation in young mice and adult. (a–e) Western blotting and relative quantification of JNK activation (a), measured as p-JNK/JNK ratio, on p14 mice crude synaptosomes stimulated with NMDA 100 μM and glycine 1 μM for 2, 5, and 10 min. JNK activation significantly increases 2 min after treatment ($^{*}p < 0.05$) and persists at 5 min ($^{*}p < 0.05$). Western blotting and relative quantification of presynaptic proteins Syntaxin-1 (b), Syntaxin-2 (c), Snap25 (d), and Vamp (e) extracted from p14 mice crude synaptosomes stimulated with NMDA 100 μM and glycine 1 μM for 2, 5, and 10 min. Protein levels remain unchanged after treatment at the considered time-points. Actin was used as loading control (20 μg proteins loaded; $N = 5$). One-way ANOVA, *Dunnet's* post-hoc test. Data are showed as mean ± S.E.M. (f) Calcium currents, measured with calcium sensible fluorophore Fluo4, on crude synaptosomes treated with NMDA 100 μM and glycine 1 μM or with KCl 50 mM. Fluorescence was recorded with a spectrophotometric approach for 7 min, with NMDA + Gly or KCl injection at 45 sec. After both injections there was a significant increase in calcium levels starting from 45 sec after stimulation (each experimental group $N = 5$). One-way ANOVA, *Dunnet's* post hoc test. Data are shown as mean ± S.E.M. (g–k) Western blotting and relative quantification of JNK activation (g), measured as p-JNK/JNK ratio on adult mice crude synaptosomes stimulated with NMDA 100 μM and glycine 1 μM for 2, 5, and 10 min. JNK activation increases 2 min after treatment ($^{***}p < 0.001$) and persists at 5 min ($^{***}p < 0.01$) and at 10 min ($^{**}p < 0.01$). Western blotting and relative quantification of presynaptic proteins Syntaxin-1 (h), Syntaxin-2 (i), Snap25 (j), and Vamp (k) extracted from adult mice crude synaptosomes stimulated with NMDA 100 μM and glycine 1 μM for 2, 5, and 10 min. Protein levels remained unchanged after treatment for all three time-points. Actin was used as loading control (20 μg proteins loaded; $N = 4$). One-way ANOVA, *Dunnet's* post hoc test. Data are shown as mean ± S.E.M.

JNK binding domain (JBD) pattern

[K/R]-X(0, 2)-[K/R]-X(0, 4)-[L/I]-X-[L/I]

```
Syntaxin-1   1 MKDRTQELRTAKDSDDDDDVAVTVDRDRFMDEFFEQVEEIRGFIDKIAEN  50
Syntaxin-2   1 MRDRLPDL-TACRKNDDGDTVVVVEKDHFMDDFFHQVEEIRNSIDKITQY  49

Syntaxin-1  51 VEEVKRKHSAILASPNPDEKTKEELEELMSDIKKTANKVRSKLKSIEQSI 100
Syntaxin-2  50 VEEVKKNHSIILSAPNPEGKIKEELEDLNKEIKKTANKIRAKLKAIEQSF  99
                    JBD
Syntaxin-1 101 EQEEGLNRSSADLRIRKTQHSTLSRKFVEVMSEYNATQSDYRERCKGRIQ 150
Syntaxin-2 100 DQDESGNRTSVDLRIRRTQHSVLSRKFVEAMAEYNEAQTLFRERSKGRIQ 149
               JBD
Syntaxin-1 151 RQLEITGRTTTSEELEDMLESGNPAIFASGIIMDSSISKQALSEIETRHS 200
Syntaxin-2 150 RQLEITGRTTTDDELEEMLESGKPSIFTSDIISDSQITRQALNEIESRHK 199

Syntaxin-1 201 EIIKLENSIRELHDMFMDMAMLVESQGEMIDRIEYNVEHAVDYVERAVSD 250
Syntaxin-2 200 DIMKLETSIRELHEMFMDMAMFVETQGEMINNIERNVMNATDYVEHAKEE 249
                        JBD
Syntaxin-1 251 TKKAVKYQSKARRKKIMIIICCVILGIVIASTVGGIFA- 288
Syntaxin-2 250 TKKAIKYQSKARRKKWIIIAVSVVLVAIIALIIGLSVGK 287
```

(a)

JNK binding domain (JBD) parttern

[K/R]-X(0, 2)-[K/R]-X(0, 4)-[L/I]-X-[L/I]

```
                                    JBD
Snap25       1 MAEDADMRNELEEMQRRADQLADESLESTRRMLQLVEESKDAGIRTLVML  50
Snap25      51 DEQGEQLERIEEGMDQINKDMKEAEKNLTDLGKFCGLCVCPCNKLKSSDA 100
Snap25     101 YKKAWGNNQDGVVASQPARVVDEREQMAISGGFIRRVTNDARENEMDENL 150
Snap25     151 EQVSGIIGNLRHMALDMGNEIDTQNRQIDRIMEKADSNKTRIDEANQRAT 200
Snap25     201 KMLGSG 206
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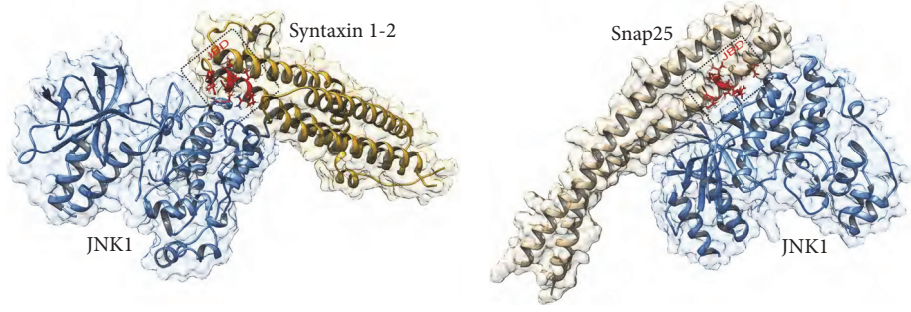
(b)



(c) (d)

Figure 3: JBD is crucial for SNARE interaction with JNK. (a) Sequence alignment between Syntaxin-1 and Syntaxin-2. The JBDs are underlined in red. (b) Sequence of Snap25 with the JBD underlined in red. On top of panels (a) and (b) the canonical JBD pattern is also reported. (c) Best JNK-Syntaxin complex resulting from the docking predictions. JNK is shown in light blue and Syntaxin in gold. The lateral chains of the residues belonging to the JBD are shown in red. (d) Best complex of the JNK-Snap25 docking results. Also in this case JNK is depicted in light blue while Snap25 is shown in light brown. The lateral chains of the residues of the JBD in Snap25 are reported in red.

synaptosomal lysates was performed. According to data previously found mostly Syntaxin-2 and Syntaxin-1 were traceable in JNK's coprecipitate (Figure 4(d)). However, a precise quantification is not possible due to the different efficiency of the antibodies used.

3.5. SNARE Proteins Interact Both with JNK2 and with JNK3. To investigate if a specific JNK isoform was involved in the interaction with SNARE protein complex, single JNK isoform precipitation was performed on cortical synaptosomal lysates. After precipitation of JNK3 [7, 50], an isoform known to be more sensitive to NMDA stimulation, and of JNK2, an isoform known to be important for neural plasticity, SNARE protein expression was evaluated by western blotting in the coprecipitate. Presence of both Syntaxin-2 and Snap25 was traceable in JNK2 coprecipitate (Figure 5, left panel); similarly

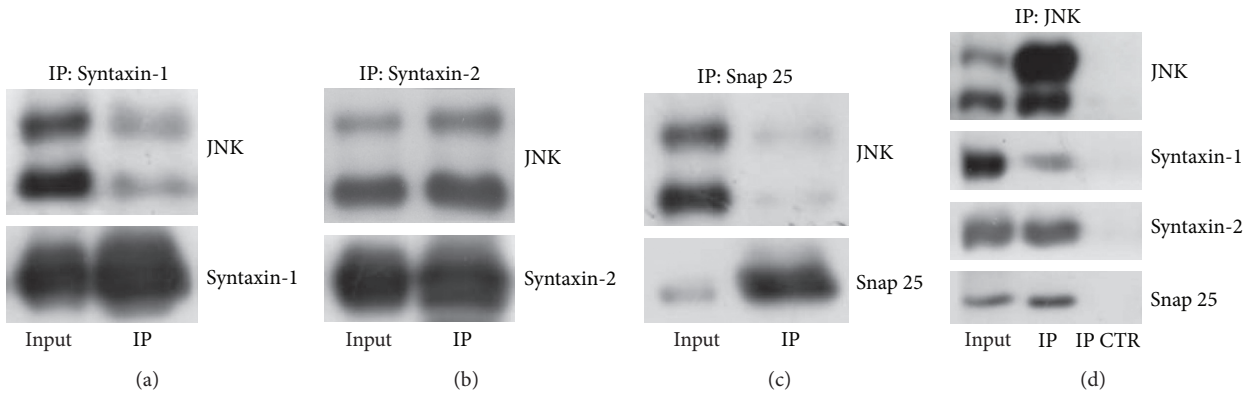


Figure 4: JNK preferentially interacts with Syntaxin-1, Syntaxin-2, and Snap25. (a–c) Western blotting after immunoprecipitation of Syntaxin-1 (a), Syntaxin-2 (b), and Snap25 (c) on purified synaptosomes lysates extracted from adult mice cortex. JNK is detectable in Syntaxin-1 and Syntaxin-2 precipitate, while immunoreactive signal is weak in Snap25 precipitate. (d) Both Syntaxin-1 and Syntaxin-2, as well as Snap25, were detectable in JNK precipitate. 500 μg of total lysates has been subjected to immunoprecipitation, while 20 μg was loaded as control. $N = 4$.

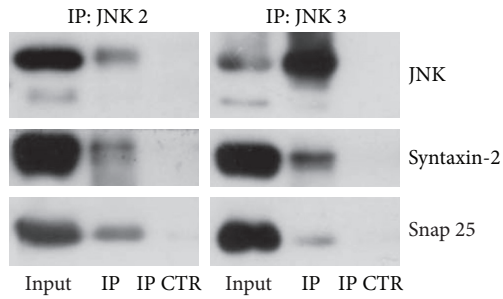


Figure 5: Both JNK2 and JNK3 interact with Syntaxin-2 and Snap25. Western blotting after immunoprecipitation of JNK2 (left) and JNK3 (right) on purified synaptosomes lysates extracted from adult mice cortex. Syntaxin-2 and Snap25 were detectable both in JNK2 and in JNK3 precipitate. 500 μg of total lysates has been subjected to immunoprecipitation, while 20 μg was loaded as control (each experimental group $N = 3$).

both SNAREs were present in JNK3 coprecipitate (Figure 5, right panel). These results indicated that both JNK isoforms, JNK3 and JNK2, interact with Syntaxin and Snap25, without having a preferential interaction with a specific isoform.

3.6. JNK Inhibition Reduces the Frequency but Not the Amplitude of mEPSCs in Cortical Slices. To determine the site of synaptic modifications induced by JNK, the miniature excitatory postsynaptic currents (mEPSCs) were examined in the presence of JNK inhibitor, D-JNKI1. Miniature synaptic events are due to the spontaneous release of glutamate vesicles, which in turn activates postsynaptic glutamate receptors. According to the quantal hypothesis, changes in the amplitudes of mEPSCs reflect a change in postsynaptic responsiveness, whereas presynaptic mechanisms result in no change in the amplitude of the mEPSCs but may cause a change in the frequency of mEPSCs recorded in the postsynaptic cell [51, 52]. The mEPSCs were, therefore, recorded in cortical slices, pretreated for 1 hr with D-JNKI1 (2 μM), in the presence of TTX (1 μM) to prevent action-potential firing, and bicuculline (20 μM) to block GABAergic currents. As shown in Figure 6(a) (sample traces of mEPSCs) and in Figures 6(b)-6(c) (quantification of mEPSCs frequency and amplitude), D-JNKI1 induced a significant decrease in the frequency of mEPSCs if compared to control conditions ($^{*}p < 0.05$, $N = 8$) but had no statistically significant effect on mEPSCs amplitude. These results support the hypothesis that JNK can act presynaptically to regulate transmitter release.

3.7. JNK Inhibition Reduces SNARE Complex Formation after NMDA Stimulation. Having demonstrated JNK interaction with Syntaxin-1/Syntaxin-2 as well as with Snap25 and that the specific inhibition of JNK, with D-JNKI1, reduced spontaneous release in an in vitro slice preparation. We here studied the effect of D-JNKI1 on JNK-SNARE complex formation in control condition and after NMDA stimulation. The SNARE complexes (Syntaxin + Snap25 + Vamp) are detergent resistant structures and can be visualized by western blotting on unboiled samples [53]. Anti-Syntaxin-1 antibody was used to visualize SNARE complex at approximately 75–100 KDa and used for densitometric quantification. Higher MW signals, ascribable to more structured complexes, enriched with other release-machinery factors, were detected too. The synaptosomes were treated with D-JNKI1 (2 μM) for 25 minutes. Inhibition of JNK did not affect SNARE complex assembly in control condition. When synaptosomes were stimulated by NMDA (100 μM NMDA plus 1 μM glycine),

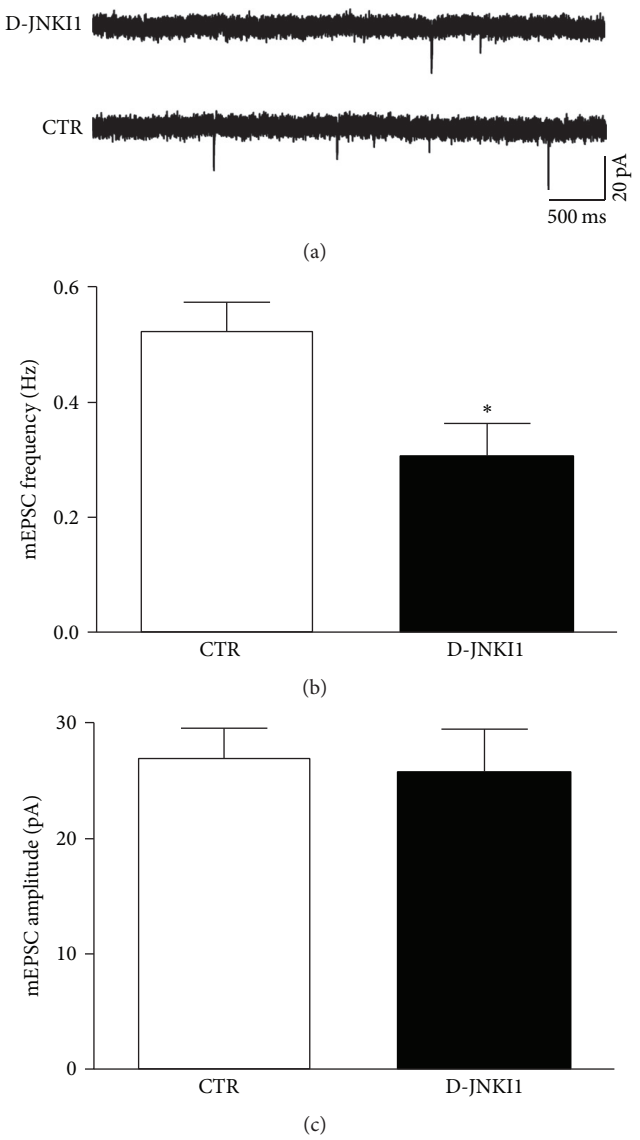


Figure 6: D-JNKI1 treatment reduced the frequency of mEPSCs in mice cortical slices. (a) Representative traces of mEPSCs recorded from cortical slices under control condition or after incubation with D-JNKI1n for 1 hr. (b-c) Bar graph (mean ± SEM) showing the mEPSC frequency (b) and amplitude (c) obtained in the first 5 min of recordings from control ($N = 8$) and D-JNKI1-treated ($N = 8$) cortical neurons. CTR versus D-JNKI1 treatment: $^{*}p < 0.05$ Student's t-test, n =8. Data are shown as mean +/− SEM.

there was a significant 50% increase of complex formation ($^{*}p < 0.05$) after 7 minutes of treatment, due to the increased vesicle release triggered by the pre-NMDARs activation (Figure 7(a)). When synaptosomes were firstly pretreated with D-JNKI1 (2 μM) for 25 minutes and then stimulated with NMDA, SNARE complex assembly was significantly prevented ($^{\#}p < 0.05$) and complex levels were restored to nonstimulated control levels (see Figure 7(a)). These results showed that JNK inhibition prevented SNARE proteins complexation, according to electrophysiology data. Finally, the levels of SNARE complex proteins were evaluated in boiled samples for all the conditions, control synaptosomes with and without D-JNKI1 as well as synaptosomes stimulated by NMDA with and without D-JNKI1. No change in protein levels was observed under these conditions (Figures 7(b)–7(d)). However we were not able to detect any reduction of noncomplexed Syntaxin-1 in unboiled NMDA treated samples, even though a significant reduction could have been expected due to Syntaxin-1 assembly in SNARE complex. This might probably be due to the huge enrichment of Syntaxin-1 in synaptosomal fraction, whose alterations in the protein levels cannot be conclusively evaluated by western blotting.

4. Discussion

JNK kinase plays an important role in regulating several functions in the central nervous system and in synaptic plasticity processes [54, 55]. In particular, a specific JNK pool in the postsynaptic site is able to modulate synaptic strength regulating both AMPA and NMDA glutamate receptors localization in the postsynaptic density region (PSD). This feature is partially due to JNK interaction with PSD scaffold protein PSD95 [56], but also by its direct interaction with NR2A/B [57, 58]. Importantly, in pathological conditions, such as Alzheimer's disease (AD) synaptopathy, JNK induces a massive removal of glutamate receptors (AMPA and NMDA) from PSD region, leading to LTP impairment, LTD increase [18, 31], and dendritic spines loss [17, 31]. JNK's role in the regulation of glutamatergic synapse dysfunction and dysmorphogenesis is intriguing and not completely established. The JNK postsynaptic function is well recognized. In fact, JNK is rapidly activated after glutamate receptors stimulation [59], and in addition plays a key role in NMDA-induced neuronal-death (excitotoxicity; see [10, 60, 61]). Despite the great amount of data supporting JNK function after NMDA activation, nothing is known by now about JNK involvement in presynaptic NMDARs (pre-NMDARs) downstream pathway. The pre-NMDARs are present at the presynaptic site in the more plastic brain regions in adult, such as cortex and hippocampus [62], where they control neurotransmitter release [63, 64]. The activation of JNK signalling after NMDA stimulation at the presynaptic site is not proven yet, and only few studies have proposed a presynaptic role for JNK [65]. Many findings in literature show that synaptosomes preparation is a valid tool to value presynaptic compartment and related receptors, as previously described. By studying pre-NMDARs stimulation in synaptosomes, we showed for the first time JNK activation in the presynaptic site. Thanks to both microscopic and biochemical approaches, in fact, we detected JNK and its activated form, p-JNK, in cortical presynaptic terminals. Notably its levels in presynaptic site seem comparable to those of postsynaptic site, previously characterized ([17], see Figures 1 and 2). We proved and examined JNK activation after pre-NMDA receptors stimulation directly at pre-synaptic site (synaptosomes) in both young and adult cortex preparations. Interestingly, JNK activation rates were comparable for young and adult preparations, indicating that pre-NMDARs stimulation induced JNK activation in the presynaptic site at both ages. In addition, this process

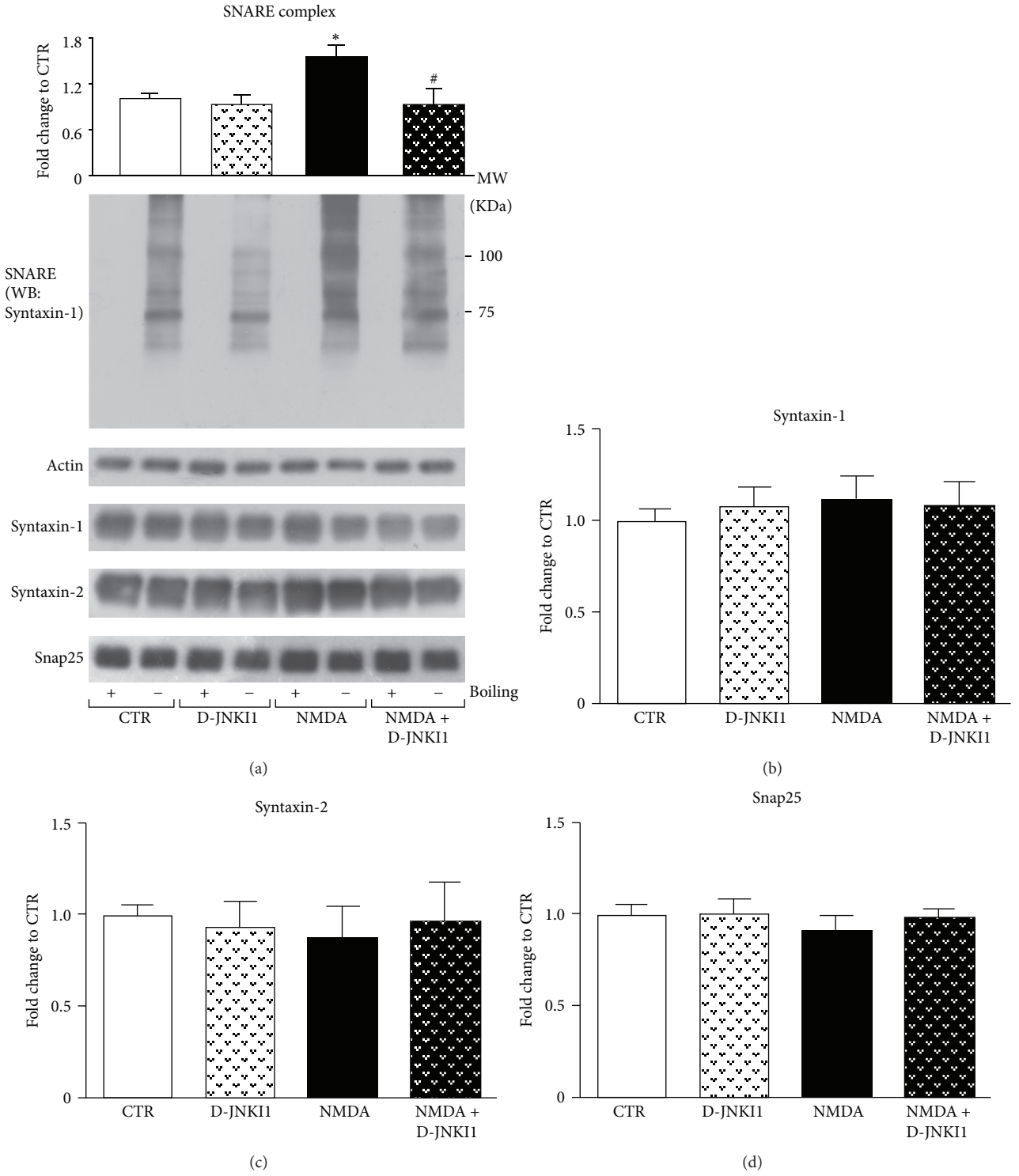


Figure 7: JNK inhibition reduces SNARE complex formation. (a) Western blotting and relative quantification of SNARE complex levels (anti-Syntaxin-1 immunoreactive bands from approximately 75 to 100 KDa) in 20 μg of crude synaptosomes stimulated with NMDA 100 μm and glycine 1 μm for 7 min in presence or absence of D-JNKI1 (2 μm), added to synaptosomes 25 min before stimulation. After NMDA/Gly exposure there was a significant increase in SNARE complexes formation ($^{*}p < 0.05$), which is totally prevented by D-JNKI1 preadministration ($^{\#}p < 0.05$). SNARE complexes are normalized on actin, $N = 6$. Data are shown as mean ± SEM, Two-way ANOVA, *Tukey's* post hoc test. (b–d) Western blotting and relative quantification of Syntaxin-1, Syntaxin-2, and Snap25 in 20 μg of crude synaptosomes stimulated with NMDA 100 μm and glycine 1 μm for 7 min in presence or absence of D-JNKI1 (2 μm), added to synaptosomes 25 min before stimulation. There was no significant variation of protein levels after NMDA/Gly exposure as well as after D-JNKI1 administration alone or in combination with NMDA/Gly. Proteins are normalized on actin, $N = 6$. Two-way ANOVA, *Tukey's* post hoc test. Data are shown as mean ± SEM.

is conserved after neurodevelopment although pre-NMDARs expression is widely decreased and persisted only in cortex and hippocampus in adult [62]. No alteration of presynaptic proteins levels was observed in synaptosomes, suggesting that JNK is not involved in protein degradation, as what occurs in the postsynaptic compartment [17, 18, 30].

Literature evidences suggest that presynaptic kinases regulate neurotransmitter release, mainly affecting three steps: stored vesicle mobilization [66], axonal ending depolarization due to calcium influx [67], and presynaptic vesicles fusion [68]. In this context the role of JNK in regulating presynaptic tasks is poorly understood: there are only few data reporting vesicle mobilization as an important step in spontaneous release [69], while JNK inhibition had already been proven not to change calcium intracellular levels [25] suggesting that it is unlikely that the kinase could modulate calcium channels or NMDA receptors permeability. Thus thanks to our modelling design finding, we examined whether JNK acts as a physiological effector of SNARE complex formation regulating vesicle release. The biochemical data confirmed the prediction of the computational analysis, suggesting the interaction of JNK with Syntaxin-1, Syntaxin-2, and Snap25 as well as with the SNARE complex formation. We have demonstrated that Syntaxin-1 and Syntaxin-2 share around 64% of sequence identity and there is only one JBD motif fully conserved in both isoforms, while we found only one complete JBD motif into the Snap25 sequence. Once we got these data from the sequence alignment, we were able to map the JBD on the Syntaxin-1/Syntaxin-2 and Snap25 three-dimensional (3D) structures in order to perform the docking simulation. The results of the docking simulations can show the possible conformations of the Syntaxin-1/Syntaxin-2-JNK and Snap25-JNK complexes. However, the electrophysiological and biochemical experiments proved the rationale of the docking hypothesis.

To get more insight into JNK presynaptic function, we examined the effects of the specific JNK inhibitor D-JNKI1 on mEPSCs recorded from cortical slices. By preventing JNK action, the frequency, but not the amplitude, of mEPSCs was strongly reduced (50%) thus confirming a modulation of presynaptic release probability by JNK. These results combined with the biochemical findings suggest that the interaction of JNK with Syntaxin-1 and Syntaxin-2 as well as Snap25 at the presynaptic level could represent an important mechanism for regulating vesicle release.

Whether the JNK binding to Snap25 is direct or mediated by Syntaxin is difficult to establish since there are many interactions among the T-SNARE proteins that form the complex, including Syntaxin and Snap25 which, in resting conditions, are partially assembled in a primed structure [70–72]. For this reason, we evaluated NMDA-mediated SNARE complex assembly in presence of specific D-JNKI1 inhibitor. As expected, JNK inhibition reduces SNAREs complexation, indicating JNK as a modulator of vesicle docking-priming, via Syntaxin/Snap25 direct interactions and probably phosphorylation. Such effect may result in a fine regulation of neurotransmitter release by JNK signalling pathway.

We then tried to clarify which JNK isoforms are more prone to interact with Syntaxin-1/ Syntaxin-2 and Snap25. The brain specific JNK3 isoform, majorly related to NMDARs activation [7, 50], is the most intriguing form, but unfortunately the antibodies are not able to discriminate well among JNK isoforms. Immunoprecipitation assay suggests a functional overlapping between the two isoforms, JNK2 and JNK3. Further studies are needed to clarify this issue. These findings all together unravel a new and important role of JNK in the presynaptic site.

5. Conclusions

The JNK stress-signalling pathway is indicated as a key player in developmental and neurodegenerative pathologies. Its implication in excitotoxicity and synaptic dysfunction/dysmorphogenesis makes its importance clear. JNK represents a therapeutic target against neurodegenerative processes, but much information is missing to manipulate its action in order to reduce unwanted adverse effects. In this work we demonstrated for the first time that JNK leads to neurotransmitter release facilitation, due to a direct interaction with SNARE proteins. Thus an important new role of JNK in controlling synaptic plasticity emerges from this study, adding another part of the story to better clarify JNK function in synapse modulation.

Competing Interests

There is no actual or potential conflict of interests.

Authors' Contributions

Silvia Biggi and Lucia Buccarello made equal contribution to this paper.

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Review Article

Emerging Synaptic Molecules as Candidates in the Etiology of Neurological Disorders

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Synapses are complex structures that allow communication between neurons in the central nervous system. Studies conducted in vertebrate and invertebrate models have contributed to the knowledge of the function of synaptic proteins. The functional synapse requires numerous protein complexes with specialized functions that are regulated in space and time to allow synaptic plasticity. However, their interplay during neuronal development, learning, and memory is poorly understood. Accumulating evidence links synapse proteins to neurodevelopmental, neuropsychiatric, and neurodegenerative diseases. In this review, we describe the way in which several proteins that participate in cell adhesion, scaffolding, exocytosis, and neurotransmitter reception from presynaptic and postsynaptic compartments, mainly from excitatory synapses, have been associated with several synaptopathies, and we relate their functions to the disease phenotype.

1. Introduction

Communication among neurons in the central nervous system (CNS) is mediated by specialized contacts named synapses that are formed by presynaptic and postsynaptic compartments. The presynapse contains the active zone (AZ), a region that concentrates proteins involved in the recruitment and fusion of synaptic vesicles (SVs), which release neurotransmitter into the synaptic cleft [1, 2] (Figure 1). The postsynaptic side contains the postsynaptic density (PSD) containing the receptors and the signaling machinery that respond to the presynaptically released neurotransmitter, propagating neuronal communication through an action potential [3] (Figure 1). Synapses form during CNS development in a space and time-dependent manner, and these structures are very dynamic in the adult, exhibiting plasticity in response to prevailing physiological requirements.

In the previous three decades, the molecular composition and the organization of the pre- and postsynaptic compartments have been greatly elucidated by a combination of biochemistry, proteomic, genetic, superresolution microscopy, and 3D electron microscopy techniques [4, 5]. Furthermore, interactors with most of the synaptic proteins have been identified, allowing the construction of an intricate protein network. Despite the latter, to translate this protein network into synapse function and efficacy is a complex task because some protein-protein interactions are more stable while others are temporal in response to plasticity events [6–8]. Furthermore, some proteins have diverse isoforms with a spatial-temporal expression pattern that sometimes partially overlaps. The abnormal expression of a synaptic protein and/or mutations and consequent perturbations in synapse physiology might produce aberrant neuronal circuits, synaptic dysfunction, and finally the development of a neurological disease [9–11].

Human genetic studies and animal models of neurological diseases have led to an emerging concept in neurobiology; the term is "synaptopathy," which refers to brain

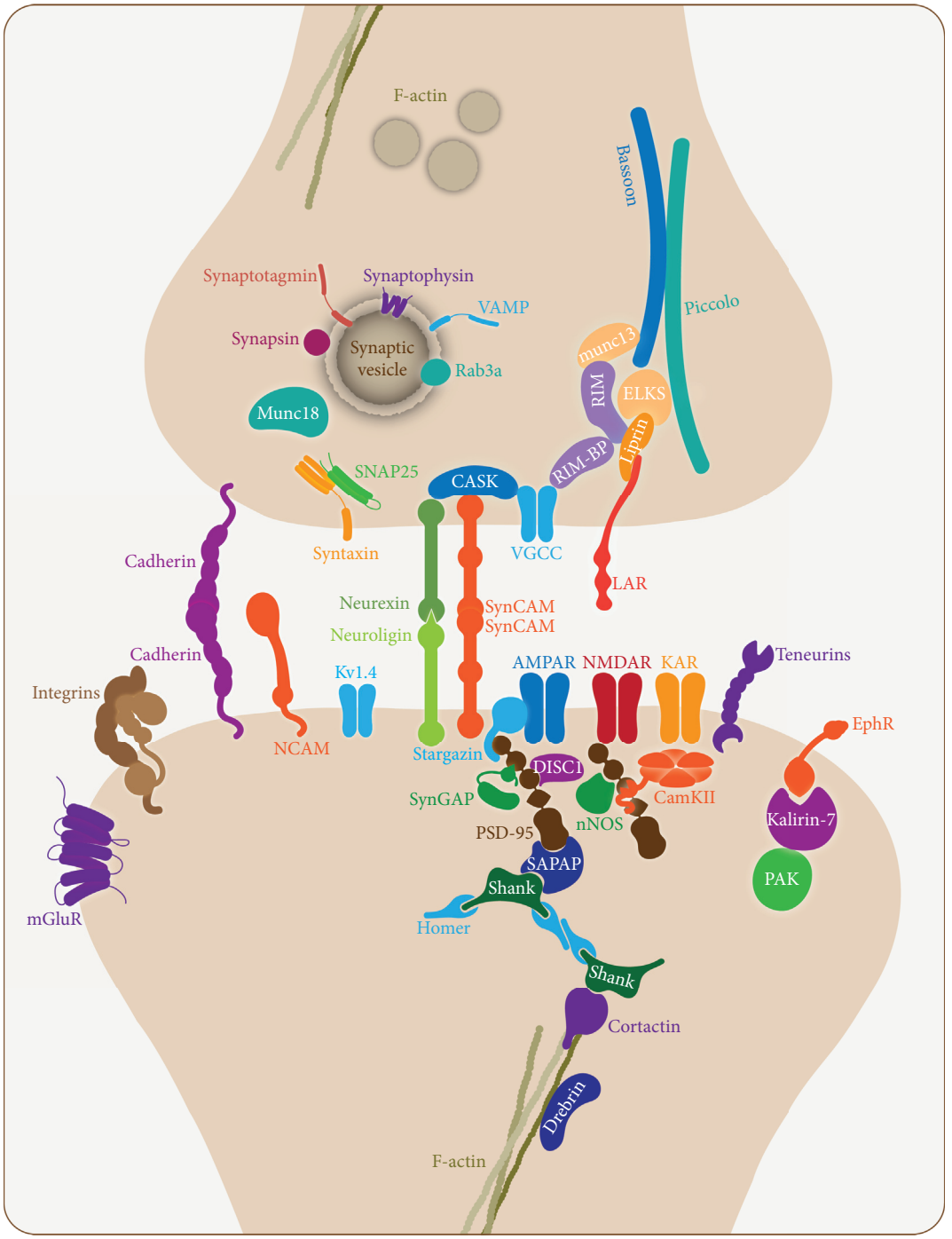

Figure 1: Molecular composition of a central chemical synapse. The image shows a typical excitatory synapse in the CNS. Pre- and postsynaptic proteins are organized in macromolecular functional complexes playing different roles in scaffolding, exocytosis, endocytosis, and signaling in their respective compartments. In addition, the most relevant adhesion molecules are represented.

disorders that have arisen from synaptic dysfunction, including neurodevelopmental (autism spectrum disorders (ASD), intellectual disability (ID), Fragile X syndrome (FXS), Down Syndrome, attention deficit hyperactivity disorder (ADHD), and epilepsy) and neuropsychiatric disorders (bipolar disorder (BPD), schizophrenia (SCZ), and major depressive disorder (MDD)) and neurodegenerative diseases (Alzheimer's disease (AD), Huntington's Disease (HD), and Parkinson's Disease) (Figure 2).

Among the neurodevelopmental disorders, ASD and FXS are synaptopathy-related diseases that are mostly determined by genetic factors. On the one hand, ASD is heritable in 80% of cases, and impaired individuals manifest a variety of intellectual deficiencies from social communication deficits

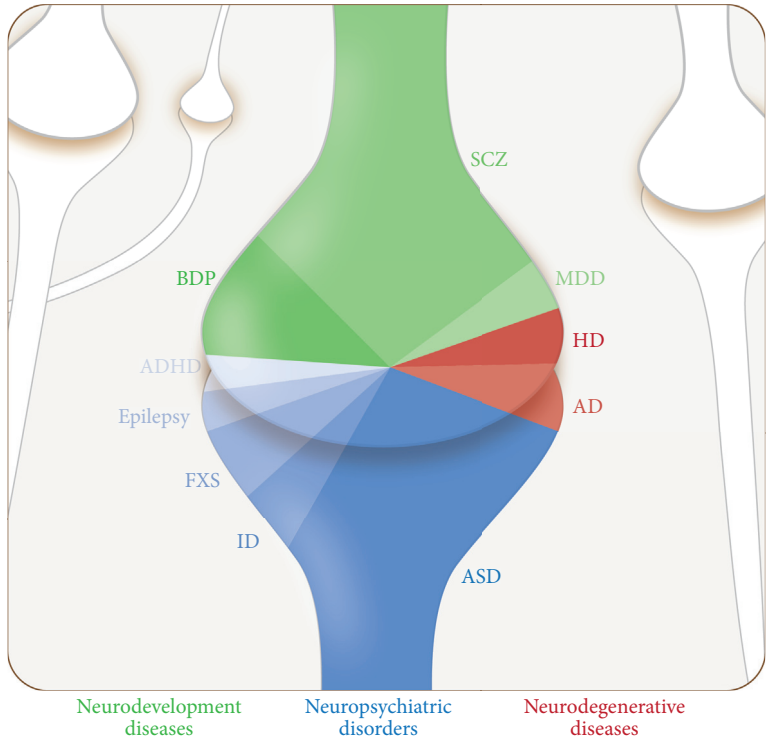

Figure 2: Schematic representation of neurological disorders associated with synaptic protein dysfunction. The image summarizes the neurological diseases described in this review represented by color code: neurodevelopmental (green spectrum), neuropsychiatric (blue spectrum), and neurodegenerative (red spectrum). The number of synaptic proteins involved in each category is proportionally illustrated. AD, Alzheimer's disease; ADHD, attention deficit hyperactivity disorder; ASD, autism spectrum disorder; BPD, bipolar spectrum disorder; FXS, Fragile X syndrome; HD, Huntington's Disease; ID, intellectual disability; MDD, major depressive disorder; SCZ, schizophrenia.

to repetitive and abnormal behaviors [12]. On the other hand, patients with FXS, which is the most common form of inherited mental retardation caused by transcriptional silencing of the fragile X mental retardation protein (FMRP), display ASD-associated symptoms such as ID, altered social interactions, and delayed speech [13]. Regarding neuropsychiatric disorders, SCZ and BPD are strongly linked to genetic and environmental factors. SCZ patients develop abnormal social behavior together with false beliefs, anxiety disorders, and confused thinking, symptoms that are pathophysiologically triggered by synaptic dysfunction resulting from a reduction in the dendritic spine density [14]. Patients affected by BPD manifest periods of depression or elevated mood associated with psychotic attacks that are often related to an elevated risk of self-harm or suicide [15]. In reference to neurodegenerative diseases, the pathology of AD is characterized by the accumulation of senile plaques in the brain, which result in the abnormal amyloid-β (Aβ) peptide processing of the amyloid precursor protein (APP), neurofibrillary tangles as a consequence of tau hyperphosphorylation, synaptic disruption, and selective neuronal loss in brain areas associated with memory and cognition. Hence, AD is considered the most prevalent neurodegenerative disease in the elderly population and the most common form of dementia [16]. HD is also a progressive neurodegenerative disorder with symptoms that include cognitive disturbances, mood disorders, and motor abnormalities caused by a mutation in the huntingtin (Htt) protein [17]. Therefore, it is evident that a unique impairment in a single component of this convoluted system, that is, the synapse, can compromise proper synapse function and result in synaptopathy. Understanding the molecular mechanisms leading to synaptic dysfunction will contribute to the development of suitable synapse-targeted therapies for neurodevelopmental, neuropsychiatric, and neurodegenerative disorders.

Here, we describe pre- and postsynaptic proteins that are involved in the pathology of neurological disease originating at chemical synapses in the CNS and are known to support synaptic function via different mechanisms, including adhesion, scaffolding, SV cycling, and signaling.

2. Presynaptic Proteins

Presynaptic sites are characterized using electron microscopy by an electrodense material that represents the AZ where specific proteins aggregate to regulate the cycle of SVs. The AZ translates an action potential into a chemical signal that induces the release of neurotransmitters into the synaptic cleft. Synaptic vesicles undergo cycles of exocytosis and endocytosis regulated by AZ proteins. AZ proteins participate in the active modulation of exocytosis according to the circuit requirements. In fact, once synapses are established, AZ undergoes molecular remodeling during their lifespan to support the requirements of synaptic activity and plasticity.

Therefore, AZ proteins have to interact coordinately to accomplish normal and dynamic synaptic functions. Altogether, AZ proteins, the cytoskeleton, and adhesion and signaling molecules maintain the integrity of the presynapse.

A group of proteins that are directly involved in the exocytosis of SVs is the SNARE (SNAP Soluble NSF Attachment Protein REceptor) complex formed by synaptobrevin, syntaxin, and synaptosomal-associated protein 25 (SNAP25), which mediates SV fusion with the AZ plasma membrane [18]. Syntaxin and SNAP25 are plasma membranes proteins and synaptobrevin is a SV protein. Other SV proteins, synaptotagmin, synaptophysin, and synapsin, participate in different steps of exocytosis. A second group of AZ proteins that form the cytomatrix at the active zone (CAZ) has been highly evolutionarily conserved: Rab3 interacting molecules (RIM Munc13, ELKS, RIM-binding protein (RIM-BP), and liprin-α) suggesting a primordial role at presynapses [2]. In fact, this group of proteins functions in SV priming, docking, calcium channel localization and clustering, and scaffolding. Other CAZ proteins are Piccolo and Bassoon, two large, highly homologous vertebrate AZ proteins with roles in scaffolding and synaptic integrity [19]. In addition, Bassoon plays a role in SV endocytosis at CNS synapses [20] and in calcium channel clustering at ribbon synapses in the retina and cochlea [21]. Interestingly, Piccolo participates in the dynamic assembly of F-actin to regulate the migration of SVs to the AZ [22]. Presynapses also contain adhesion molecules, which, in addition to mediating cell-cell contacts, also deliver intracellular signaling through trans-synaptic communication. Here, we review some presynaptic proteins that have been associated with synaptopathies in genome-wide association (GWA) studies and family linkage studies (Figure 3(a) and Table 1).

2.1. Synaptic Vesicle Proteins

2.1.1. Synapsin. Synapsins are phosphoproteins that are associated with the membrane of SVs and play a role in tethering SVs to the cytoskeleton away from the AZ. The phosphorylation of synapsin during an action potential induces the release of SVs from the reserve pool, allowing their movement toward the presynaptic AZ to release neurotransmitter. Therefore, synapsin will regulate the number of vesicles accessible for exocytosis. In vertebrates, three synapsin genes have been described (*SynI*/*SynII*/*SynIII*) [69] that are alternatively spliced to render the 2–6 protein isoforms [70, 71]. Synapsins have been implicated in several psychiatric disorders, such as BPD and SCZ [23], and specific mutations and polymorphisms in *Syn* genes cause familial epilepsy [24, 31]. Accordingly, a causal role has been attributed to *SynI* and *SynII* in the pathogenesis of ASD and epilepsy [24, 25]. Like nonsense mutations, Q555X in the *SynI* gene was found in a family presenting both ASD and epilepsy [25]. The expression levels of synapsin also seem to correlate with psychiatry disorders because a decrease in synapsin-2a and synapsin-3a has been observed in the hippocampal tissue of patients with SCZ and BPD [23, 26] and decreased protein levels of synapsin-2a were observed in the olfactory bulbs from people with SCZ [27]. In another study, a decrease in *SynII* gene expression in postmortem brain tissue of BPD patients might be explained by the presence of hypomethylated I CpG islands found in this gene [32]. The findings in humans are, in part, reproducible in animal models because a *Syn II* knock-out (KO) animal model results in a schizophrenic-like phenotype [28–30]. The third member of the family, synapsin 3, has also been implicated in SCZ because a decrease in its expression was observed in the prefrontal cortex of individuals with SCZ [33].

2.1.2. Synaptophysin. Synaptophysin is a SV glycoprotein and the most widely used synaptic marker. Interestingly, KO mice for synaptophysin are normal, but electrophysiological experiments indicate that this protein is necessary for efficient endocytosis of SV in hippocampal neurons [72]. Synaptopathies involving synaptophysin are less evident because studies investigating these diseases are discrepant. In a recent study of the CA1 region of the hippocampus derived from a postmortem individual with SCZ, synaptophysin levels were decreased together with PSD95 and Homer [34]. These molecular defects at synapses in the CA1 region of the hippocampus might explain, in part, the cognitive defects in SCZ. Synaptophysin also might participate in the pathology of BPD. Scarr et al. studied the expression of several proteins involved in SV exocytosis in Brodmann area 9 of the brain cortex of a subject with BPD [35], an area of the brain with lower levels of energy consumption in bipolar patients [73]. In that study, increases in SNAP25 and synaptophysin were observed, suggesting a role for these proteins in this disease [35].

2.2. Cytomatrix of Active Zone Proteins

2.2.1. RIMs. RIMs were first identified as Rab3-interacting molecules [74], among which there are four isoforms (RIM1-4) encoded by different genes in vertebrates. RIM proteins are represented by two long isoforms, RIM1α and RIM2α, which contain a zinc-finger domain, a PDZ, and two C-terminal C2A and C2B domains [75]. Short isoforms for RIM include RIM2γ, RIM3γ, and RIM4γ formed by the C2B domain. RIM1α participates in SV docking and priming and the recruitment of voltage-dependent Ca^{2+} channels (VDCCs) into the AZ of presynapses. RIM1α also plays a role in presynaptic long-term potentiation (LTP) in the hippocampus and cerebellum [76]. The RIM short isoform, RIM3, is postulated to have a role in the regulation of neurotransmitter release by modulating presynaptic Ca^{2+} influx [77]. Deletion of the *Rims1* and *Rims2* genes, which produce the five isoforms, RIM1α, RIM1β, RIM2α, RIM2β, and RIM2γ, in mice severely impairs the Ca^{2+} responsiveness of neurotransmitter release via a mechanism that affects Ca^{2+} channel tethering to the AZ [78]. A single gene deletion produced a mild effect, suggesting a redundant and compensatory role for these two genes [78].

Although an association with human SCZ has not been attributed to RIM1α, KO mice for this protein exhibit a phenotype similar to human SCZ [36]. Interestingly, a genetic microdeletion and a genome-wide expression profiling study revealed an association of the RIM3 isoform with ASD in children with this disease [38, 39]. Increased expression levels

Table 1: Presynaptic proteins involved in different synaptopathies and their role in physiological synaptic function.

	Function	Neurological disease	References
Synaptic vesicles proteins			
Synapsin 1	Mobilization, release, and tethering of SV to the cytoskeleton away from the AZ	BPD	[23]
		Epilepsy	[24, 25]
		ASD	[25]
Synapsin 2	SVs mobilization and regulation of the number and density of the reserve pool	SCZ	[23, 26–30]
		Epilepsy	[31]
		BPD	[23, 32]
Synapsin 3	Synaptogenesis and modulation of neurotransmitter release	SCZ	[23, 33]
		BPD	[23]
Synaptophysin	Control of SVs endocytosis	SCZ	[34]
		BPD	[35]
Cytomatrix of active zone proteins			
RIMs	Docking, SV fusion, and neurotransmitter release Synaptic plasticity	SCZ	[36, 37]
		ASD	[38, 39]
Piccolo	AZ scaffolding protein	SCZ	[37]
		MDD	[40–42]
		BPD	[43]
SNARE proteins			
SNAP25	Mediation of vesicle docking and fusion	BPD	[35, 44, 45]
		SCZ	[44, 46, 47]
		ADHD	[48–50]
Adhesion molecules			
SynCAM1	Synapse formation, synaptic plasticity, and axonal pathfinding	ASD	[51–54]
Cadherin	Selection of neuronal target, synapse formation, and plasticity	SCZ	[55, 56]
		BPD	[55]
		ASD	[55, 57–60]
		ADHD	[61]
NRXN1	Formation and maturation of the synapse	ASD	[62–65]
		SCZ	[66–68]

The table summarizes the physiological synaptic function of presynaptic proteins whose alterations result in synaptopathies related to neurodevelopmental, neuropsychiatric, and neurodegenerative diseases. ADHD, attention deficit hyperactivity disorder; ASD, autism spectrum disorder; AZ, active zone; BPD, bipolar disorder; MDD, major depressive disorder; NRXN, neurexin; RIM, Rab3a interacting molecule; SCZ, schizophrenia; SynCAMs, Synaptic adhesion molecules; SV, synaptic vesicle.

of RIM2 and RIM3 were observed in the amygdala in SCZ [37]. Recently, a role in axonal and dendritic arborization was assigned to RIM3 and RIM4 [79]; thus, the participation of these proteins in psychiatry disorders is not rare because autism and SCZ are diseases associated with aberrant dendritic growth and alterations of dendritic spine numbers [80].

2.2.2. Piccolo. Piccolo is a multidomain CAZ protein and the largest protein in the presynapse. Piccolo is a nontransmembrane protein that is transported during development into newly forming synapses in a dense core vesicle of Golgi origin [81, 82]. Piccolo interacts with several actin binding proteins, including Abp1, GIT-1, and PRA1 [19], and it is thought that through these interactions Piccolo modulates F-actin dynamics at presynapses [83]. In the AZ, Piccolo forms a macromolecular complex with other AZ proteins, including Bassoon, ELKS, RIM, and Munc13 [19]. Recent studies have shown that Piccolo and Bassoon regulate the stability of AZ proteins at presynapses [84], and hence a defect in these proteins might compromise the structure and function of synapses in synaptopathies.

In population studies, Piccolo has demonstrated an association with some psychiatric disorders. Weidenhofer et al. reported an increase in the gene expression of *PCLO*, *Rims2*, and *Rims3* in the amygdala in SCZ [37], although some variability in Piccolo expression was observed, suggesting that this protein might not be affected in some cases of schizophrenia. A GWA study conducted in the Netherlands [40] and corroborated by others [41, 42] found an association of the *PCLO* gene with MDD due to a single nucleotide

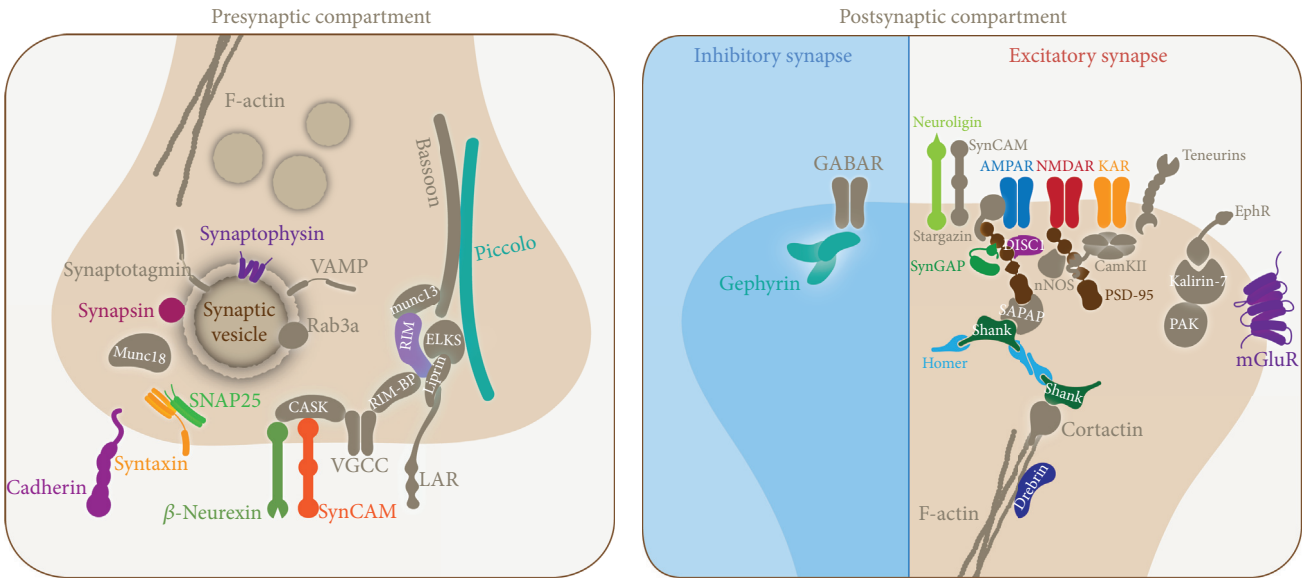

FIGURE 3: Schematic representation of synaptic proteins associated with synaptopathies. (a) Presynaptic and (b) postsynaptic proteins involved in human synaptopathies described in this review are color highlighted. Mutations in a gene or gene combination for a synaptic protein may lead to neurodevelopment, neuropsychiatric, and neurodegenerative diseases.

polymorphism (SNP), SNP rs2522833, in which a serine is replaced with alanine in the C2A domain, a region known to bind phosphatidylinositol or synaptotagmin-1. Following this finding, Furukawa-Hibi et al. [85] produced a transgenic mouse overexpressing the Piccolo C2A domain. Interestingly, the animals presented depression-like behavior, supporting the hypothesis that disruption of the interactions of the C2A domain for Piccolo with other presynaptic proteins can cause depressive behavior. The participation of Piccolo in mood disorders is also supported by another study examining the genetic variation of individuals with BPD, in which two SNPs were found in an intron of the *PCLO* gene accompanied by increased expression of the protein [43]. Therefore, mutated or unbalanced expression of Piccolo might trigger psychiatry disorders.

2.3. SNARE Proteins

2.3.1. SNAP25. SNAP25 is a t-SNARE protein and a key component of the SNARE protein complex, the machinery involved in the fusion of SVs. SNAP25 assembles with syntaxin and synaptobrevin to mediate vesicle docking and Ca^{2+}-triggered fusion.

SNAP25 has been involved in several human neuropsychiatric disorders. Abnormal levels of the protein have been found in the postmortem brain of bipolar patients [35, 44], and a SNAP25 variant was found in the prefrontal cortex of patients with early-onset BPD [45]. In a linkage study, two polymorphisms located in the 3′-untranslated region of the human *SNAP* gene were associated with ADHD [48]. Additional evidence for a role of SNAP25 in ADHD was provided by a recent meta-analysis [49]. In SCZ, the structure of the hippocampus is abnormal, and a decrease in the amount of SNAP25 has been observed in the hippocampus and areas of the cortex of patients with this disease [44, 46, 47]. The same has been observed in the hippocampus of animal models of ADHD [50]. It has been postulated that abnormal levels of SNAP25 expression affect neurotransmitter release mechanisms and short-term plasticity, which are characteristics of SCZ and ADHD.

The role of SNAP25 in neuropsychiatry disorders might not only be explained by its novel function in exocytosis because recent articles have assigned postsynaptic functions to this SNARE protein. Accordingly, SNPA25 was assigned a role in NMDAR and kainate-type receptor trafficking [86, 87]; however, no studies have demonstrated its presence at dendritic spines. The findings of Tomasoni et al. support a role for SNAP25 in postsynaptic function by demonstrating structural modifications at the PSD and immature dendritic spines upon SNAP-25 diminution [88]. Interestingly, as previously mentioned, SCZ is associated with defects in spine morphology and dynamics, and some SNAP25 mutants are related to this disease.

2.4. Adhesion Molecules

2.4.1. SynCAM1. Synaptic adhesion molecules (SynCAMs) are molecules that actively participate in synapse formation and plasticity [89, 90] and axonal pathfinding [51]. They are a subgroup of the immunoglobulin superfamily of cell adhesion molecules that are localized both pre- and postsynaptically and mediate homophilic cell-cell interactions in a Ca^{2+}-independent manner. Two missense mutations in the *SynCAM1* gene were found in DNA samples of individuals with ASD [52]. These two mutations were found in a domain necessary for trans-active interactions. In addition,

the mutant proteins were more susceptible to protease cleavage and presented abnormal intracellular trafficking [52]. The role of SynCAM1 in this pathology is strengthened by investigations of SynCAM1 KO mice showing impairment in social behavior similar to individuals with ASD [53, 54]. The role of SynCAM in axon guidance during development and its contribution to neural circuit formation might explain its participation in the pathology of ASD [51].

2.4.2. Cadherin. Cadherin is a Ca^{2+}-dependent homophilic cell adhesion molecule with a role in neuronal target selection, synapse formation, and plasticity in the vertebrate CNS. It comprises a superfamily of approximately 100 members expressed in brain. In the CNS, cadherin expression follows a spatiotemporal pattern, suggesting an important role in specific circuit development [91]. Classic cadherins are found in pre- and postsynaptic compartments, at both nascent and mature synapses [92, 93]. The structures of classic cadherins include 5 extracellular EC repeats and an intracellular binding domain, and they are encoded by 20 genes. At presynapses, the intracellular domain of classic cadherin interacts with β-catenin, which serves as a linker to F-actin [94].

Several neuropsychiatric diseases have been associated with cadherins, such as SCZ, ASD, BPD, and alcoholism [55]. Here, we provide a few examples. Genetic microdeletion of cadherin-8 and duplication of cadherin-13 were found in individuals with ASD and learning disability [57, 58]. A recent study aimed at identifying common genetic risk factors for ASD studied 780 families with children with ASD. They found six single SNPs in the cadherin-9 and cadherin-10 genes, suggesting an association of the adhesion molecules with autism [59]. In addition, the deletion of cadherin-12 and cadherin-18 genes was correlated with SCZ [56]. These observations of defects in cadherin genes in patients with autism have also been observed in population studies in which the presence of these SNPs is correlated with individuals with problems in oral communication but not spatial memory [60, 61]. These findings suggest that cadherins may be involved in the regulation of specific circuits related to verbal working memory.

2.4.3. Neurexins. NRXNs are presynaptic adhesion molecules that interact transsynaptically with postsynaptic neuroligins (NLs), an interaction that is known to be important for synaptogenesis and synapse maintenance [95]. NRXNs are encoded by three genes (*NRXN1-3*), each of which has two promoters that generate long (α) and short (β) protein isoforms with identical intracellular but different extracellular domains. The distribution of NRXNs in the CNS has been studied by in situ hybridization. The data suggest their differential expression in the embryonic nervous system but partial overlap in the mature central nervous system [96]. However, protein expression experiments that also consider splice variants are needed to decipher the precise distribution of these proteins [97, 98].

Studies conducted *in vitro* have shown that both NRXNs and NLs can induce postsynaptic or presynaptic specialization clustering, respectively [99, 100]; however, these findings have not been completely observed in loss of function animal models. Consequently, more work is necessary to understand the synaptogenic role of NRXN-NL. NRXNs interact through their intracellular domain with CASK and Mints, two proteins that are known to interact with the β-subunit of N-type Ca^{2+} channels and with P/Q-type Ca^{2+} channels in the case of Mints [101]. This interaction might link NRXNs to the SV release machinery [102].

The first report linking NRXN to a neurodevelopmental disorder was performed in a boy with ASD in whom the promoter and exons 1–5 were deleted from the *NRXN1* gene encoding NRXN-1α [103]. Other investigators have observed similar deletions in the NRXN 1α gene in ASD [62–65]. All these deletions were heterozygous for NRXN-1α. Deletions in *NRXN1* have also been identified in SCZ patients and involve the promoter and exon 1 of NRXN 1-α [66–68]. No postmortem studies have been performed in ASD and schizophrenic individuals carrying a deletion in the *NRXN-1*α gene, and thus these findings cannot be correlated to the expression levels of the protein. A homozygous KO mouse has been developed for the *NRXN1* gene [104]. Although this animal model does not represent heterozygous ASD and schizophrenic human cases, these animals showed mild behavioral deficits similar to those observed in ASD and SCZ individuals [105]. Furthermore, the *NRXN-1*α KO mice showed impairments in social interaction and communication analogous to those observed in ASD [104, 106]. Taken together, we can conclude that NRXN plays a role at the synapse and that further knowledge of the functional interactome of NRXN might improve our understanding of its specific functions and roles in synaptopathies.

3. Postsynaptic Proteins

The PSD is a dynamic lattice-like array composed of interacting proteins lining the postsynaptic membrane that organize and stabilize synaptic receptors, ion channels, structural proteins, and signaling molecules required for normal synaptic transmission and synaptic function [107, 108]. However, its composition and morphology are dynamically changing as a function of neuronal activity, and thus the PSD plays a fundamental role in regulating the strength and plasticity of excitatory synaptic neurotransmission [109, 110]. Therefore, maintenance of the architecture and composition of the PSD is considerably important for proper synaptic connections that allow the preservation of cognition, memory, and functional circuitry. In most neurological diseases, one or more of these processes are disrupted and impaired. Consequently, an understanding of the PDS molecular network and signaling pathways underlying normal synapse function is crucial to comprehend the pathological mechanisms responsible for different synaptopathies. Here, we describe some postsynaptic proteins that are involved in synaptopathies (Figure 3(b) and Table 2).

3.1. Adhesion Molecules

3.1.1. Neuroligins. NLs are postsynaptic cell adhesion proteins that participate in associations with presynaptic NRXNs in

Table 2: Postsynaptic proteins involved in different synaptopathies and their role in physiological synaptic function.

Protein	Function	Neurological disease	References
Adhesion molecules			
NL1	Memory formation and maturation of excitatory synapses	ASD	[111, 112]
		AD	[113]
		FXS	[114]
NL2	Formation and remodeling of inhibitory synapses	SCZ	[115]
		ASD	[116]
NL3	Formation and remodeling of excitatory and inhibitory synapses	ASD	[105, 117–121]
NL4	Formation and remodeling of excitatory and inhibitory synapses	ASD	[117–119, 122–126]
Glutamate receptors			
NMDARs	Regulation of synaptic plasticity and memory formation	ASD	[127–129]
		SCZ	[127, 130, 131]
		AD	[132–136]
		HD	[137, 138]
KARs	Maturation of neural circuits during development	ASD	[139–141]
		SCZ	[142]
		BPD	[142, 143]
AMPARs	Mediators of excitatory transmission and synaptic plasticity	ASD	[144]
		SCZ	[145–147]
		BPD	[148]
		MDD	[149]
		FXS	[150, 151]
		HD	[152]
mGluRs	Regulation of neuronal excitability, learning, and memory	ASD	[153–156]
		ID	[156]
		FXS	[157–159]
Scaffolding proteins			
PSD-95	Stabilization of the synapse, and regulation of synaptic strength, transmission, and plasticity	AD	[160–162]
		ASD	[163, 164]
		SCZ	[164, 165]
		HD	[166–168]
		FXS	[169–173]
Shank1	Regulation of the structural and functional organization of the dendritic spines	ASD	[174–176]
		SCZ	[177, 178]
Shank2	Synaptogenesis; regulation of the molecular structure and modulation of interacting proteins in the PSD	ASD	[179–182]
		ID	[183, 184]
		SCZ	[185]
Shank3	Synapse formation, dendritic spine maturation, and synaptic plasticity	ASD	[186–193]
		PMS	[194–196]
		SCZ	[197]
Homer	Organization, stabilization and function of the PSD, and contribution in dendritic spine morphogenesis	SCZ	[198–202]

Table 2: Continued.

Protein	Function	Neurological disease	References
SynGAP	Involvement in the cognitive development and synaptic transmission and function	SCZ	[203]
		ASD	[204, 205]
		ID	[206]
Gephyrin	Clustering and localization of glycine and GABA receptors at inhibitory synapses	ASD	[207]
		SCZ	
		Epilepsy	[208]
Other postsynaptic-associated proteins			
DISC1	Regulation of synaptic plasticity	SCZ	[209–212]
		Depression	[209, 213]
		BPD	[210]
		ASD	[214]
		AD	[215]

The table summarizes the physiological synaptic function of postsynaptic proteins whose alterations result in synaptopathies related to neurodevelopmental, neuropsychiatric, and neurodegenerative diseases. AD, Alzheimer's disease; AMPARs, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BPD, bipolar spectrum disorder; ASD, autism spectrum disorder; DISC1, disrupted in schizophrenia 1; FXS, Fragile X syndrome; HD, Huntington's Disease; ID, intellectual disability; KARs, kainate receptors; MDD, major depressive disorder; mGluRs, metabotropic glutamate receptors; NLs, neuroligins; NMDARs, *N*-methyl-D-aspartate; PMS, Phelan-McDermid syndrome; PSD-95, postsynaptic density-95; SCZ, schizophrenia.

synaptogenesis through the recruitment to synaptic sites of receptors, channels, and signaling molecules. NLs constitute a multigene family of brain-specific membrane proteins composed of different isoforms in humans, including NL1, NL2, NL3, NL4, and NL4Y (occasionally referred to as NL5) [98, 216]. Although it has been suggested that NLs may develop similar functions related to mediating recognition processes between neurons, sequence comparisons have shown that NL1, NL3, and NL4 are more similar to one another than to NL2 [12]. Several studies have reported that NL1 is particularly localized to excitatory synapses and interacts with postsynaptic density protein-95 (PSD-95), which is highly enriched at the PSD [217]. NL3 and NL4 are similarly expressed at excitatory synapses [95], but they have also been found in inhibitory ones: NL3 at γ-aminobutyric acid-(GABA-) ergic [218] and NL4 at glycinergic synapses [219]. In contrast, NL2 is located exclusively at inhibitory synapses and clusters with $GABA_A$ and glycine receptors [220, 221]. Although NL2 is present specifically at inhibitory synapses, NL2 has revealed excitatory properties in a spinal nerve ligation model of neuropathic pain through a functional shift from inhibition to excitation [222].

NLs are well-accepted molecules that participate in the pathogenic mechanism of diverse neurological diseases and exert a strong genetic influence on developmental disorders. It has been reported that an aberrant form of NL at the postsynaptic membrane, an anomalous association with NRXN, or both anomalies trigger an abnormal excitatory and inhibitory balance and the underlying development of cognitive disorders.

A proper NL1 level, especially in the hippocampus, is crucial for memory formation. Moreover, impairment of the NL1 level might induce the development of autism-related symptoms [111, 112] and also participate in the cognitive disability observed in AD [223]. Particularly during the early phases of AD, it has been reported that Aβ oligomers preferentially bind to postsynaptic regions where they might interact with *N*-methyl-D-aspartate (NMDA) receptors (NMDARs) and NL1 [224, 225]. Moreover, NL1 has been reported to act as a nucleating factor in the stabilization of Aβ accumulation *in vitro* by inducing the formation of Aβ oligomers [226]. These data suggest that NL1 can promote the targeting of Aβ oligomers to the postsynaptic sites of excitatory synapses and thereby promotes synaptic toxicity in AD. A mutation in the *NL1* gene was found in AD patients, generating a premature stop codon in the extracellular domain of *NL1* (p.Thr271fs) that blocks the function of NL1 and thus its ability to form glutamatergic synapses [113]. Interestingly, in a neuroinflammation rodent model induced by hippocampal injections of $A\beta_{1-40}$, there was a decrease in NL1 expression with subsequent impairment of synaptic function and memory [227, 228]. All of these studies indicate that altered NL1 function could underlie the molecular mechanisms associated with the memory loss and the cognitive impairment observed in AD patients.

In addition to its involvement in AD pathology, NL1 is known to participate in molecular mechanisms related to other neurological diseases [229]. The role of NL1 in Fragile X syndrome (FXS), the most common form of inherited mental retardation, is supported by a study conducted in a mouse model of the disease, in which the overexpression of NL1 improved social behavior without any observed effect on learning and memory [114].

Several studies have linked NL2 with symptoms related to neurological diseases, such as anxiety and SCZ, or alterations in normal behavior. In a genetic study of 584 SCZ patients, several mutations were found in the *NL2* gene, two of which were related to abnormal GABAergic synapse formation, suggesting a role in the onset of SCZ [115]. Studies in *NL2*-deficient mice have reported that *NL2* deletion disrupts

the inhibitory synapse function in hippocampal sections without affecting their numbers and also triggers a pronounced anxiety phenotype in those mice [230]. In addition, *NL2* deletion also affects inhibitory synapses in projection neurons of the basal amygdala, leading to their excessive activation under anxiogenic conditions [231]. Overexpression of *NL2* caused different social and emotional behaviors in rats such as reduced aggression [232], whereas transgenic mice exhibited a stereotypical jumping behavior, anxiety, and impaired social interactions, probably as a consequence of a significant increase in the density of inhibitory synapses and the subsequent morphological change in the excitatory synapse [233]. To study the role of NL2 in the pathogenesis of ASD, Wöhr et al. investigated the presence of several behavioral phenotypes observed in ASD patients in *NL2* null and heterozygote mice [116]. These mice presented some of the behavioral characteristics of ASD patients, suggesting that NL2 plays a partial role in the etiology of the disease.

For the *NL3* and *NL4* genes, point mutations, truncations, and sequence deletions in their coding regions are associated with both ASD and mental retardation [117–119]. In some patients with ASD, the Arg^{451} residue is substituted by Cys^{451} (R451C) in NL3. Interestingly, NL3 (R451C) mutant mice exhibit a deficit in social behaviors and learning abilities as a consequence of inhibited synaptic transmission in addition to a significant increase in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor- (AMPAR-) mediated excitatory synaptic transmission, enhanced NMDAR-NR2B regulation, and increased LTP. Moreover, this mutation alters dendritic branching with consequent alterations of the structure of the synapse [120]. Regarding the effect of NL3 (R451C) in GABAergic postnatal signaling in the hippocampus, it has been reported that this knock-in mutation produces enhanced GABAergic but not glutamatergic transmission, suggesting that NL3 regulates the excitatory/inhibitory balance during the development of neuronal circuits [121]. Conversely, another study showed that the NL3 (R704C) mutation triggered impaired synapse function, specifically inducing a decrease in AMPAR-mediated synaptic transmission in hippocampal slices without changing NMDA or GABAR-mediated synaptic transmission [105], thus suggesting that NL3 plays a fundamental role in synaptic transmission in excitatory synapses.

Several deletions of X-chromosomal DNA in the *NL4* locus have been found in a wide spectrum of neuropsychiatric conditions and in autistic and nonautistic mentally retarded patients [122, 123]. Moreover, *in vitro* studies have also revealed that *NL4X* deletion results in neurodevelopmental defects during the formation of neurons and their connections as well as in decreased gene expression of NL1 and NL3 [124]. The amino acid R87 is conserved in all NL isoforms, but a single amino acid substitution in NL4 (R87W) that affects the activity of NL4 in synapse formation and abolishes the functional effect of NL4 on synapse strength has been discovered in ASD patients [125]. In addition, a single mutation has been identified in NL4X (R704C) that consists of a single amino acid substitution in a conserved arginine residue. As previously mentioned, this mutation has been studied in a homologous recombination mouse NL3 model, in which the analogous arginine is conserved [105]. Furthermore, the R704C mutation, which is very close to the T707 residue that is phosphorylated in humans by protein kinase C (PKC) [126], inhibits the phosphorylation of T707 through an unknown mechanism. Additionally, the phosphomimetic mutation at T707 has demonstrated enhanced synaptogenesis, possibly via an unknown mechanism that includes glutamatergic receptors or presynaptic terminal recruitment [216].

3.2. Glutamate Receptors. Glutamate is considered the major excitatory neurotransmitter in the human brain, and the pathophysiology of several mental disorders is known to depend on glutamatergic system activity. Glutamate receptors comprise the ionotropic (iGluRs) and metabotropic glutamate receptors (mGluRs). iGluRs include NMDA, AMPA, and kainate receptors based on structural, pharmacological, and physiological properties.

3.2.1. N-Methyl-D-aspartate Receptors, NMDARs. NMDARs are formed by three subunits called GluN1-3 and different splice variants [234]. NMDARs consist of tetrameric structures with a large number of receptor subtypes that determine their pharmacological and functional properties. These receptors are crucial for neuronal communication and are recognized to have a key role in neural plasticity.

Several lines of evidence indicate that NMDARs are involved in different ASDs. De novo mutations in the GluN2B (*GRIN2B*) and GluN2A (*GRIN2A*) genes have been identified in different cases of ASD and SCZ, respectively, as well as truncation mutations in *GRIN1*, *GRIN2B,* and *GRIN2A* in ASD and SCZ patients [127]. Animal models have allowed the development of suitable ASD-related phenotypes, such as the parvalbumin-selective *NR1* KO, which results in reduced sociability and impaired ultrasonic vocalizations [128]. Moreover, the use of NMDARs antagonists, such as ketamine [235] or D-cycloserine [236], has contributed to testing therapeutic drugs in patients or animal models of ASD [129]. Typical behavioral manifestations and cognitive impairment of SCZ have been associated with dysfunctional NMDAR trafficking and regulation [130], which is indeed regulated by different genes. For example, it has been reported that the stimulation of neuregulin-1, a growth factor associated with SZC in humans [237], triggers a rapid internalization of NMDARs, suppressing their activation in the postmortem prefrontal cortex of SCZ patients [131].

In AD patients, the glutamatergic system, especially NMDAR-mediated transmission, appears to be strongly affected because NMDARs are activated by the accumulation of Aβ oligomers during the initial phases of the disease [132, 133]. In particular, it has been reported that Aβ oligomeric species activate the GluN2B subunit of NMDARs, which in turn produces an increase in intracellular Ca^{2+} levels and subsequent excitotoxicity [134]. Conversely, it has been reported that oligomeric Aβ causes a selective loss of synaptic GluN2B responses together with a subunit composition from GluN2B to GluN2A [135], potentially as an attempt to reduce Aβ-induced injury because GluN2A subunits are implicated in protective signaling pathways [238]. Several NMDAR

antagonists, such as 1-benzyl-1,2,3,4-tetrahydro-β-carboline [239] or MK-801 [240], have been generated and used as potential therapeutic drugs to prevent synaptic dysfunction in AD models. However, it is noteworthy that, considering the involvement of NMDARs in synaptic function, complete inhibition of their activity triggers important secondary effects such as severe memory impairment. Interestingly, memantine, a low-affinity NMDAR antagonist that is also employed for the treatment of dementia and depression, does not accumulate in the channel, allowing normal synaptic transmission [241]. However, it is well-accepted that the hyperphosphorylated tau protein contributes to the AD-associated neurodegeneration and is also required for the Aβ-mediated neurotoxicity [242]. Some studies utilizing transgenic mice have confirmed that both Aβ and tau are involved in the neuropathology of AD. For example, Aβ has been shown to function via NR2A to trigger dendritic spine loss, whereas tau acts through the NR2B subunit to promote neurodegeneration [136].

Similarly, several HD transgenic mouse models have indicated that NMDARs, as well as the GluN2B subunit, are involved in the pathology of HD. The motor learning deficits manifested by YAC128 mice expressing the mutated Htt (mHtt) were attenuated by chronic extrasynaptic NMDAR blockade with memantine [137, 138]. In addition, the mechanism of action of an antihistamine compound proposed for the treatment of different neurological diseases including HD, Dimebon, has been reported to occur through the inhibition of NMDAR activity [243].

3.2.2. Kainate Receptors, KARs. Kainate receptors (KARs), which are highly expressed in the cortex and hippocampus, are targeted to synapses, where they play specific roles in the maturation of neural circuits during development [244]. KARs are tetrameric receptors that form homomeric or heteromeric receptors via the combination of five subunits: GluR5 (GRIK1), GluR6 (GRIK2), GluR7 (GRIK3), KA1 (GRIK4), and KA2 (GRIK5).

Some abnormalities in genes encoding the glutamate receptor subunits of the kainate type, such as *GRIK2* and *GRIK4*, have been reported to be involved in BPD, SCZ, ASD, and mental retardation diseases [139, 142, 143]. In particular, chromosome 6q21 has been identified as an important region for autism, and a SNP was found in the glutamate receptor 6 (*GluR6* or *GRIK2*) gene related to ASD [140]. Additionally, overexpression of *GRIK4*, a gene encoding KA1, induced an altered synaptic transmission in mice that also manifested ASD-associated symptoms such as enhanced anxiety, depressive states, and impaired social interactions [141]. Despite this association of KARs with the pathology of neurological diseases, additional studies are necessary to fully understand their roles in synapse function.

3.2.3. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptors, AMPARs. AMPARs are glutamate receptors that mediate fast synaptic transmission in the CNS. They are formed by the heterotetrameric combination of four subunits: GluR1-4, which determine the functional properties of AMPARs.

A deletion mutation in the *AMPA 2* gene encoding the glutamate receptor GluR2 subunit has been found in patients with ASD [144]. Furthermore, AMPARs have been associated with SCZ through dysbindin, a widely suspected susceptibility protein in SCZ. Accordingly, enhanced AMPAR-mediated transmission has been observed in cultured hippocampal neurons from dysbindin-deficient mice [145]. However, several unsuccessful genetic studies in humans have been developed to associate different SNPs in the AMPAR subunits, GluR1, GluR2, and GluR4 (encoded by the *GRIA1*, *GRIA2,* and *GRIA4* genes, resp.), with SCZ [146], BPD [148], and major depressive disorder [149]. Similarly, although altered trafficking of AMPAR has been associated with the pathology of SCZ, no changes were observed in the expression of the AMPAR subunits GluR1-4 in the endoplasmic reticulum of SCZ patients [245].

However, a role for AMPAR in the pathology of SCZ cannot be discarded because olanzapine, an atypical antipsychotic drug, has a therapeutic effect on memory dysfunction and cognitive impairment manifested in SCZ patients through the modulation of synaptic plasticity caused by the upregulation of GluR1 Ser845 phosphorylation [147]. In addition, 7,8-dihydroxyflavone, a tropomyosin receptor kinase B (TrkB) agonist, is considered a potential pharmacotherapeutic strategy for FXS. Briefly, FXS is a common inherited cause of mental retardation, human cognitive dysfunction, and autism resulting from the transcriptional silencing of the FMR1 gene that encodes FMRP. The 7,8-dihydroxyflavone induces an increase in GluR1 subunit expression that results in improved spatial and fear memory and a decrease in morphological abnormalities in the spines of *Fmr1* KO mice [150]. Furthermore, another link between AMPARs and FXS has been reported, in which FXS-associated synaptic proteins also regulate the AMPAR subunit GluR1, supporting an important role in neuronal development and maturation [151].

Therefore, several lines of evidence indicate that the proper function of AMPARs, the major mediators of excitatory transmission in the CNS, is highly important for synaptic plasticity and cognitive functions, as evidenced by the association with several and different neurological disorders including ASDs, SCZ, AD, FXS, and HD [152].

3.2.4. Metabotropic Receptors, mGluRs. mGluRs are involved in the regulation of neuronal excitability, learning, and memory and are classified in three groups as follows: mGluR1 and mGluR5 belong to the group 1 family; mGluR2, mGluR3, and mGluR4 form the group 2 family; and mGluR6, mGluR7, and mGluR8 are included in the group 3 family. These receptors are found synaptically and extrasynaptically. Group I members, which comprise mainly postsynaptic receptors that activate neuronal depolarization and excitability, are coupled to Gq/G11 and activated phospholipase Cb to generate inositol 1,4,5-triphosphate (IP3) and diacylglycerol with the consequent mobilization of calcium and activation of protein kinase C (PKC). In contrast, groups 2 and 3 members are mostly presynaptic receptors that are localized in positions where they inhibit synaptic vesicle release through Gi/o proteins.

Some evidence indicates that mGluRs are involved in both nonsyndromic [153] and syndromic cases of autism [154]. In a valproate-induced rat model of autism, a significant reduction of mGluR2/3 protein and mRNA levels has been observed [155]. *N*-acetylcysteine, a drug that stimulates the uptake of cysteine in exchange for glutamate, which is transported to the extracellular milieu through the antiporter Xc-, reverted the social interaction and anxiety behaviors of autistic rats as a result of presynaptic mGluR2/3 [155]. In addition, mGluRs are also associated with FXS. The reduced mGluR5 expression has been observed to impede the development of abnormalities in *Fmr1* KO mice [157]. Furthermore, a recent study confirmed that mGluR5 dysfunction is associated with neurological disorders such as obsessive-compulsive disorder and autism [156]. Conversely, enhanced mGluR5 function is associated with FXS pathology. In *Fmr1* KO mice, mGluR5 was shown to have a weaker association with its scaffolding protein Homer, which targets mGlu5 to synapses, and thus disrupted mGluR5-Homer scaffolds triggered dysfunctional mGluR5 and FXS phenotypes [158]. A recent study reported that, in transgenic mice expressing mutant mGluR5 unable to bind Homer, the most representative biochemical, neurophysiological, and behavioral alterations of the illness were observed in FXS mice [159].

3.3. Scaffolding Proteins

3.3.1. PSD-95. The postsynaptic density protein 95 (PSD-95; also known as DLG4 and SAP90) is the most abundant protein in excitatory chemical synapses and the main scaffolding protein at the PSD. In fact, PSD-95 contributes to synaptic stabilization, strength, and transmission, and its proper regulation is known to be essential for accurate synaptic development and plasticity. PSD-95 belongs to the membrane-associated guanylate kinase (MAGUK) protein family. All of these proteins possess three independent PDZ (PSD-95, Dlg1, and zonula occludens-1 proteins (zo-1)) domains through which they interact with glutamate receptors, cell adhesion molecules, and cytoskeletal elements. The PDZ domain of PSD-95 binds to different postsynaptic proteins; for example, PDZ2 interacts with NR2 and NR1 of NMDARs [246] and stargazin interacts with PDZ1 and PDZ2 of PSD-95 to regulate AMPAR synaptic numbers [247]. Other proteins, such as Ras GTPase-activating protein (SynGAP), appear to interact with the three PDZ domains of PSD-95 [248]. In addition to making PSD-95 a target of multiple synaptic interactions, PDZ domains confer to this protein a high susceptibility to modification by posttranslational modifications that affect its postsynaptic localization within the PSD in dendritic spines. However, alterations of some of the signaling pathways that control these modifications may contribute to the development of neurological diseases such as AD, SCZ, HD, and FXS [249].

Synaptic alterations in AD are often correlated with cognitive changes. Regarding the association between the alterations in PSD-95 and AD, it is known that during brain aging, Aβ oligomers may bind directly to NMDARs, which in turn interact with PSD-95 [224, 225]. Moreover, an altered distribution pattern of NMDARs and PSD-95 has been observed in human AD postmortem brains [160] and a direct relationship between Aβ oligomers and PSD-95. In fact, it has been reported that Aβ oligomers colocalize with PSD-95 at excitatory synapses in AD brain tissues as well as in cultured rat hippocampal neurons exposed to Aβ oligomers [250, 251]. A deleterious effect of Aβ oligomers is known to increase with time. The latter could be explained by the time-dependent decrease in the levels of synaptic PSD-95 in excitatory synapses as the pathology advances in murine models of AD, suggesting that this PSD-95 reduction is a sign of the postsynaptic degeneration underlying long-term functional deficits [161, 162]. The specific mechanism by which PSD-95 expression is reduced in patients with AD may result from the ubiquitin-proteasomal degradation of PSD-95. It has been reported that in cells transfected with a PSD-95 mutant lacking the PEST sequence, which is essential for its ubiquitination, Aβ treatment fails to decrease the expression of PSD-95 [252]. Therefore, the regulation of PSD-95 would be a crucial step in the pathological progression mediated by Aβ oligomers. Accordingly, it has been reported that Wnt-5a, a synaptogenic ligand, decreases the synaptic disruption induced by Aβ oligomers, revealing its neuroprotective role by blocking the reduction of synaptic PSD-95 in hippocampal neurons exposed to Aβ oligomers [253]. Therefore, in AD neuropathology, the ability of PSD-95 to interact with other synaptic elements is impaired with the consequent disruption of the organization and stabilization of the PSD, resulting in a loss of NMDARs and SynGAP [254]. Therefore, molecules involved in different signaling pathways that regulate PSD-95 may have therapeutic potential for decreasing Aβ-induced synaptic loss and cognitive impairment in AD.

The role of PSD-95 in the etiology of ASD is less clear because no rare genetic mutations in PSD-95 have been associated with ASDs to date; however, *PSD-95* deletion mice exhibit behavioral and molecular abnormalities that are related to ASD symptoms, such as increased repetitive behaviors, altered social behaviors, impaired motor coordination, and anxiety [163].

Reduction of the hippocampal size is one of the characteristics of SCZ patients, and it has been reported that the CA1 region of the hippocampus plays an important role in the pathophysiology of SCZ. In postmortem brains of SCZ patients, the expression of PSD-95 is reduced together with its known interacting proteins Homer1 and mGluR1 [34]. Therefore, the molecular abnormalities in PSD-95 and its molecular interactome may contribute to the cognitive dysfunction displayed in SCZ patients [34, 164, 165]. Similarly, altered levels of PSD-95 have been observed in mouse models of HD [166, 167]. In these patients, the Htt is structurally altered and associated with a loss of function. Using a knock-in mouse model of HD, characteristic motor and cognitive deficits of the illness were observed in mutant mice, as well as altered levels of PSD-95 and other proteins associated with synaptic function [168]. All of these studies indicate that mHtt contributes negatively to synaptic plasticity and may be one of the mechanisms underlying the cognitive deficits in HD.

It is known that PSD-95 possesses a binding site for FMRP, the FXS-related protein, and that its translation depends on the absence of this protein in FXS patients [169];

moreover, FMRP is also required for the stability of PSD-95 [255, 256] and glutamate receptor mRNA in the PSD [257]. Furthermore, FMRP is essential for correct excitatory synapse elimination through the proteasomal degradation of PSD-95, and thus the defective degradation of PSD-95 might explain the excessive number of dendritic spines observed in patients with FXS [170]. Several studies conducted in FXS mouse models have reproduced the phenotype observed in FXS patients, including deficits in cognitive flexibility, attention, and inhibitory control. In addition, a strong relationship has been observed between the decreased levels of proteins involved in synaptic function, such as PSD-95, and cognitive impairment [171–173]. A recent study has reported that FMRP colocalizes with PSD-95 [258]. Therefore, PSD-95 is clearly associated with the pathobiology of FXS.

In summary, any alterations of the synaptic levels of PSD-95, a key molecule in PSD organization and function, may affect interactions with its partners and contribute to the development of several CNS diseases.

3.3.2. Shank Family Proteins. The members of the Shank/ProSAP family, Shank1, Shank2, and Shank3, are multidomain scaffold proteins located at the PSD of glutamatergic synapses that interact with a large variety of membrane and cytoplasmic proteins. Shank proteins are expressed in areas of the brain that are essential for cognition and learning and trigger a crucial role in spine formation and maturation [259]. Shanks have differential patterns of expression in the CNS: Shank2 is the first isoform expressed in the brain, followed by Shank3 and then Shank1 [260]. Shank proteins interact with a large number of postsynaptic proteins to regulate PSD function, including ionotropic-glutamate receptors, PSD-95, Homer, and components of the actin cytoskeleton [261]. Furthermore, the trans-synaptic functions of Shanks are believed to be modulated by their interaction with the NL-NRXN complex [262].

Human genetic studies have strongly linked Shank genes, including *Shank1*, *Shank2*, and *Shank3*, with Phelan-McDermid syndrome (PMS), ASD, AD, and SCZ associated with ID [250, 260, 263]. A rare inherited deletion encompassing the *Shank1* gene has been associated with autism in males, whereas only anxiety and shyness behaviors were observed in females, suggesting that *Shank1* deletion could have sex-dependent effects [174]. Several genetic mouse models have been used to study *Shank1* mutations in the pathology of autism. *Shank1*-mutant mice displayed functional alterations in synaptic transmission as a consequence of a modified protein composition and morphology of the PSD and a reduced size of dendritic spines [175]. These observations resulted in an altered cognitive and communicative function [176]. Mutant mice exhibited an enhanced anxiety behavior, a decreased long-term memory, and a deficit in motor coordination. Surprisingly, although a decrease in the long-term retention of information was observed, *Shank1*-mutant mice were able to improve their spatial learning [175, 176, 264]. Furthermore, a promoter variant in the *Shank1* gene has been related to the symptoms of patients with SCZ [177] as well as a de novo Shank1 mutation [178].

Several human genetic studies have indicated that Shank2 is involved in ASD and ID [179, 180, 183], and two simultaneous studies produced a *Shank2* null mouse model to understand the molecular mechanisms underlying this pathology [181, 182]. *Shank2* null mice presented fewer dendritic spines and reduced basal synaptic transmission [181]. The behavior of the animals was similar to ASD, with abnormalities in vocal and social behavior [181, 182]. Furthermore, Shank null mice showed reduced NMDAR functions, and the rescue of this mutation resulted in significantly improved animal social behavior [182]. The latter finding suggests that NMDAR function could be a target for patients with ASD. However, care must be taken with this approach because different levels of glutamate receptor expression are observed between *Shank2*(−/−) and *Shank3*$\alpha\beta$(−/−) mutants [181], suggesting that any treatment should consider the synaptopathic phenotype.

Shank2 rare variants have also been identified in SCZ patients [185], and one of these variants has been related to ID [184]. Therefore, several lines of evidence indicate that Shank2 protein is a potential therapeutic target for autism and SCZ synaptopathologies.

Loss of one copy of the *Shank3* gene (haploinsufficiency) is considered the most prevalent monogenic cause of ASD [186]. Moreover, ASD is characterized by de novo mutations and deletions in *Shank3*, while truncating mutations are typically observed in ID patients [187]. In addition to its involvement in ASD, SCZ, and ID, Shank3 is the main protein responsible for the neuropsychiatric symptoms that occur in PMS patients [194], which manifest common characteristics of ID to varying degrees, delayed or absent speech, ASD-related symptoms, motor delays, and epilepsy. However, the clinical features of PMS are highly variable depending on the corresponding mutation. Translocations [195], de novo or truncating mutations [196], are *Shank3* mutations associated with PMS. The *Shank3* mutations, R1117X and R536W, have been observed in patients with SCZ associated with ID [197]. To understand the function of Shank3, mutant mouse models were generated; two different mutant mice carrying a deletion of exons 4–9 (JAX 017890 [188] and JAX 017442 [189]) showed repetitive behavior and impaired motor performance and cognitive dysfunction. Other animal models of Shank3 mutations include a deletion of exons 4–7, *Shank3 (4–7)*, and a deletion of exons 13–16, *Shank3 (13–16),* in which the mutant mice manifest anxiety and altered social and repetitive behaviors [190]. Deletion of exon 21 in Shank3 (21) [191] produced some ASD-related behaviors in mice together with impaired learning and memory, and a mouse model with an exon 9 deletion, *Shank3 (9)*, manifested mildly impaired spatial memory [192].

Shank3 is a well-known PSD protein in excitatory synapses and plays an important role in synaptic plasticity and functional coupling between presynaptic neurotransmitter release and a precise and rapid postsynaptic response. However, less is known about how defects in Shank3 could participate in the pathology of diseases such as ASD and SCZ. A recent study showed that Shank3 activation and localization in rat hippocampal neuron dendritic spines is regulated by zinc [193]. Interestingly a *Shank3* mutation

(*Shank3* (R87C)) found in some ASD patients retains its zinc sensitivity but does not regulate the reliability of presynaptic neurotransmitter release. The latter does not preclude the possibility that other *Shank3* mutants or the other zinc-sensitive isoforms of *Shank2* interact abnormally with zinc, a metal that participates in synaptic plasticity and exhibits homeostatic dysregulation in ASD and other neurological disorders [265, 266].

3.3.3. Homer. Homer is a family of scaffolding proteins formed by three members with a conserved aminoterminal enabled/vasodilator-stimulated phosphoproteins homolog 1 (EVH1) domain that binds to proline-rich sequences of mGluR [267, 268], inositol 1,4,5-triphosphate receptor (IP_3R) [269], ryanodine receptors [270], transient receptor potential canonical-1 (TRPC1) ion channels [271], and Shank [272], functioning as adaptor proteins for several postsynaptic proteins [273]. The *Homer 1* gene undergoes alternative splicing to produce two isoforms, a short cytosolic version called Homer 1a, the expression of which increases after neuronal activation, mainly in frontal-subcortical neuronal circuits that are associated with neuropsychiatric disorders [274]. The long versions, Homer 1b/c, which are constitutively expressed and localized at the PSD, can form dimers through their carboxy-terminal domain [273]. These dimers bind to Shank and the metabotropic glutamate receptors mGluR1 and mGluR5, as well as indirectly to NMDAR and AMPAR through Shank. Therefore, Homer 1b/c form large protein complexes at the PSD, allowing the interaction of PSD proteins and signaling pathways. The short isoform, Homer 1a, acts as a dominant-negative of the long Homer isoforms, regulating the interactions of Homer with PSD proteins and suggesting that Homer 1 is a key organizer of the PSD, regulating the function of postsynaptic receptors and synaptic spine morphogenesis [259]. A stress condition that induces an increase in the expression of the Homer short isoform in rat limbo-corticostriatal structures [275] and hippocampus [276] suggests that this isoform regulates the consolidation of memories of stressful situations. Accordingly, *Homer 1* KO mice exhibit an exacerbated behavioral response to stressors because, in the absence of Homer 1, there is no buffering response to anxiety [198]. The latter could explain the mechanism by which the absence of Homer 1 triggers depression in humans. *Homer 1* KO mice also have behavioral and neurochemical abnormalities with a SCZ-like phenotype. Rescue experiments in those animals with Homer 1a and Homer 1c suggest different roles for this proteins in behavioral responses after stress [198]. In humans, linkage studies have identified susceptibility to SCZ in chromosomal loci containing the Homer 1 gene [199–201]. Moreover, a role for Homer 1 in SCZ in humans was validated in a recent study in which an association was found between two Homer 1 polymorphisms and the Positive and Negative Syndrome Scale (PANSS), a medical scale used for measuring the symptom severity of patients with SCZ [202]. Taken together, defects in Homer proteins will produce alterations in the architecture and function of the PSD, ultimately resulting in a neurological disease.

3.3.4. SynGAP1. SynGAP1, which is a postsynaptic component of the PSD, plays an important and essential role in the development of cognition and proper synaptic function. It has been reported that SynGAP interacts with PSD-95 [248] and is phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (CamKII), which in turn is activated by increased Ca^{2+} levels induced by NMDAR activation [277]. This phenomenon suggests that SynGAP develops an essential role in the NMDAR-dependent activation of Ras signaling pathways and in synaptic plasticity.

Mice with a heterozygous null mutation of *SynGAP* manifest impaired learning and memory associated with decreased synaptic transmission and NMDAR-mediated synaptic currents [278]. Moreover, SynGAP function has been studied in a mouse model of SCZ. As explained above, NMDARs are involved in the pathophysiology of SCZ, and it has been reported that reduced expression of SynGAP also results in abnormal behaviors, as demonstrated by abnormal functions of NMDARs such as a persistent hyperactivity and social and working memory loss, among others [203]. In addition to interacting with the aforementioned proteins and receptors, SynGAP is associated with other postsynaptic proteins such as PSD-95, the synapse-associated protein-102 (SAP-102), and PSD-93, NRXNs, and NLs, proteins that have been related to ASD [204]. The excitatory and inhibitory synaptic balance is altered in patients with ASD [279], and it has also been reported that SynGAP may regulate this synaptic balance in cortical neurons [205]. Furthermore, a recent study established a relationship between SynGAP and ID. Patients with a de novo mutation, haploinsufficiency for *SynGAP1*, were found to manifest epilepsy, hypotonia, constipation and other ID-associated symptoms [206].

3.3.5. Gephyrin. Although in this review we preferentially considered the most important pre- and postsynaptic proteins involved in synaptopathies located at the AZ and PSD, respectively, of excitatory synapses, gephyrin (gphn) is also included because it is a key scaffolding protein at the postsynaptic membrane that plays an essential role through its interactions with NL2, a previously described protein, and collybistin in the clustering and localization of glycine and the α and β subunits of $GABA_A$ receptors at inhibitory synapses [220]. It has been reported that *gphn* KO mice, however, display correct glutamate receptor localization; they manifest a loss of postsynaptic $GABA_A$ and glycine receptor clustering [280].

Interestingly, gphn is well-known to be involved in several neurological disorders including ASD, SCZ, and epilepsy because it is functionally linked to various synaptic proteins that represent a genetic risk for the development of neurological diseases such as NLs, NRXNs, and collybistin. In particular, exonic gene microdeletions in *gphn*, including hemizygous microdeletions, de novo, and paternally inherited deletions have been found in different ASD families. They displayed ASD-related symptoms such as motor and social impairment, repetitive and impulsive behaviors, anxiety, and obsessive-compulsive disorders [207]. A recent study has reported a novel exonic *gphn* microdeletion in patients with

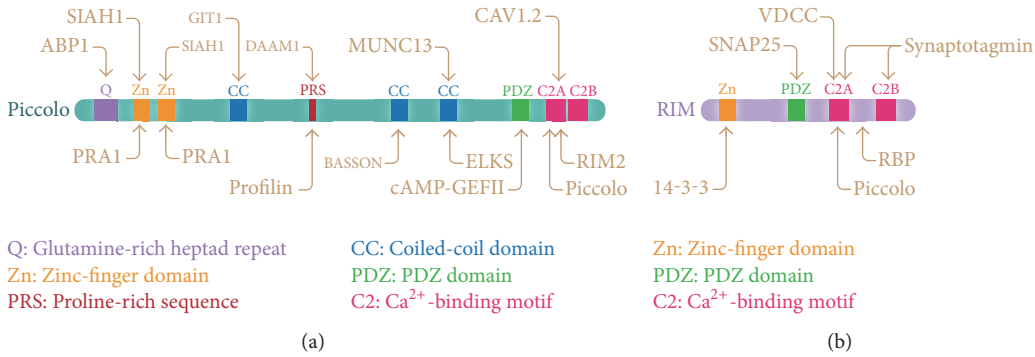

Figure 4: Domain structure of two active zone proteins associated with synaptopathies. The diagrams show the multimodular organization of (a) Piccolo and (b) RIM, and their interaction with other proteins. Arrows indicate binding reactions. Domains are shown in colored boxes and designations are indicated by standard abbreviations.

idiopathic generalized epilepsy, confirming that genetic alterations constitute a risk factor for neuropsychiatric disorders through the impairment of GABAergic inhibitory synaptic transmission [208].

3.4. Other Postsynaptic-Associated Proteins

3.4.1. Disrupted in Schizophrenia 1, DISC1. DISC1, a protein encoded by the *DISC* gene, has been found in asymmetric synapses, principally on the postsynaptic side [281]; in fact, a bioinformatics analysis of DISC1 interactions suggested that DISC1 is an essential component of the PSD and a key player in the regulation of synaptic plasticity [282]. The DISC1 gen locus has been considered a risk factor because the (1;11)(q42;q14.3) translocation was observed in different members of a Scottish family that manifested clinical phenotypes associated with BPD, SCZ, and depression [209, 210]. Moreover, a DISC1 intragenic microsatellite has been associated with autism, whereas a SNP of DISC1 has been related to Asperger syndrome in Finnish families [214].

Different mouse models have reported that DISC1 is involved in synapse function and related to neurological diseases such as depression [213]. It is known that C57BL/6J mice carry an exonic deletion in *Disc1*, which produces a truncated DISC1 protein that mimics the putative phenotypic effects of the disease-associated chromosomal translocation, resulting in memory impairment and fewer synaptic spines [211]. In contrast, the downregulation of DISC1 by shRNA in adult C57BL/6 mice elicited an accelerated dendritic development and synapse formation in both GABAergic and glutamatergic synapses of newborn neurons [283]. As previously described, SCZ is a neurological disorder characterized by disrupted synaptic connectivity. Likewise, prolonged knockdown of DISC1 has been shown to induce synaptic deterioration, and inhibition of the signaling pathways triggered by DISC1 improves the behavioral deficit manifested in the DISC1 knockdown mouse model [284].

In addition, it is noteworthy that, surprisingly, an increased amount of insoluble DISC1 oligomer aggregates was detected in the postmortem brain of SCZ patients, demonstrating a common link with other neurological disorders characterized by protein aggregation such as AD and HD [212]. A novel study has reported significantly enhanced levels of APP fragments, as well as decreased levels of $A\beta_{42}$ and $A\beta_{40}$ as a consequence of DISC1 knockdown, suggesting that DISC1 participates in the proteolytic processing of APP and thus establishing a relationship with the pathology of AD [215].

4. Piccolo and RIM, an Example of Multidomain Presynaptic Proteins with Diverse Functions

A highlight characteristic of several AZ proteins, such as Piccolo and RIM, is their multidomain structure. Piccolo and RIM form homo and hetero-oligomers at the CAZ and exert their synaptic functions through molecular interactions with different binding targets. The functions and interactions of Piccolo include (a) actin cytoskeleton dynamic (profilin, Daam1, Abp1, and GIT1), (b) exocytosis (cAMP-GEFII), (c) endocytosis (PRA1 and GIT1), (d) protein turnover (Siah1), (e) membrane trafficking (Epc2), (f) calcium signaling (L-type Ca^{2+} channel), (g) scaffolding (Bassoon, RIM, Munc13, and ELKS), and (h) SV priming (RIM, Munc13) (Figure 4(a)). RIM has prominent roles in (a) SV docking and priming (SNAP25, synaptotagmin), (b) scaffolding (Piccolo, ELKS, 14-3-3), and (c) calcium channel signaling (RBP, N- and P/Q-type Ca^{2+}-channels) (Figure 4(b)).

Therefore, mutations on Piccolo and/or RIM genes rendering a mutated protein or altered levels of protein expression could produce functional imbalances at the synapse due to abnormal interactions with their respective partners. Consequently, neuronal circuits might be impaired responding suboptimally to environmental requirements affecting synaptic plasticity, which is known to be altered in many neurological diseases.

5. Concluding Remarks

Genetic studies of human synaptopathies together with animal models have revealed that in most cases a disease cannot

be explained by the gene mutation of a single synaptic protein, and, similarly, abnormal individual expression of different synaptic proteins can trigger the same or a similar disease phenotype. Accordingly, GWA studies that have identified specific genes associated with synaptopathies sometimes do not replicate all of the symptomatology of the disease in animal models, suggesting the participation of other genes. This phenomenon is not unexpected because synaptic proteins are coupled to a highly dynamic interactome that regulates basal and plastic synapse functions. Hence, additional GWA studies are necessary to identify most of the defective gene variants and the brain region harboring the molecular alteration in a specific synaptopathy. Such findings in humans may be used to create suitable animal models that closely mimic the human defect, allowing detailed studies of the physiological alterations. Therefore, this will allow the consideration of specific pharmacological therapies for the underlying synaptopathic genotype and phenotype.

Abbreviations

Aβ: Amyloid-β
Abp1: Actin binding protein
AD: Alzheimer's disease
ADHD: Attention deficit hyperactivity disorder
AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP: Amyloid precursor protein
ASD: Autism spectrum disorders
AZ: Active zone
BPD: Bipolar disorder
CAZ: Cytomatrix at the active zone
CNS: Central nervous system
EVH1: Enabled/vasodilator-stimulated phosphoproteins homolog 1
F-actin: Filamentous actin
FMRP: Fragile X retardation protein
FXS: Fragile X syndrome
GABA: Gamma aminobutyric acid
gephyrin: gphn
GWA: Genome-wide association
HD: Huntington's Disease
Htt: Huntingtin
ID: Intellectual disability
iGluRs: Ionotropic-glutamate receptors
KARs: Kainate receptors
KO: Knock-out
LTP: Long-term potentiation
MDD: Major depressive disorder
NLs: Neuroligins
mGluRs: Metabotropic glutamate receptors
NMDA: *N*-Methyl-D-aspartate
NRXN: Neurexin
PANSS: Positive and negative syndrome scale
PMS: Phelan-McDermid syndrome
PKC: Protein kinase C
PRA1: Prenylated rab3A acceptor
PSD: Postsynaptic density
RIM: Rab3 interacting molecules
RIM-BP: RIM-binding protein
SCZ: Schizophrenia
shRNAi: Short hairpin RNA interference
SNAP25: Synaptosomal-associated protein 25
SNARE: SNAP Soluble NSF Attachment Protein Receptor
SNP: Single nucleotide polymorphism
SynCAMs: Synaptic adhesion molecules
SV: Synaptic vesicles
TrkB: Tropomyosin receptor kinase B
TRPC1: Transient receptor potential canonical-1
VDCCs: Voltage-dependent Ca^{2+} channels.

Competing Interests

All authors declare no conflict of interests.

Authors' Contributions

Viviana I. Torres and Daniela Vallejo contributed equally to this work.

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Review Article

Could Perinatal Asphyxia Induce a Synaptopathy? New Highlights from an Experimental Model

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Birth asphyxia also termed perinatal asphyxia is an obstetric complication that strongly affects brain structure and function. Central nervous system is highly susceptible to oxidative damage caused by perinatal asphyxia while activation and maturity of the proper pathways are relevant to avoiding abnormal neural development. Perinatal asphyxia is associated with high morbimortality in term and preterm neonates. Although several studies have demonstrated a variety of biochemical and molecular pathways involved in perinatal asphyxia physiopathology, little is known about the synaptic alterations induced by perinatal asphyxia. Nearly 25% of the newborns who survive perinatal asphyxia develop neurological disorders such as cerebral palsy and certain neurodevelopmental and learning disabilities where synaptic connectivity disturbances may be involved. Accordingly, here we review and discuss the association of possible synaptic dysfunction with perinatal asphyxia on the basis of updated evidence from an experimental model.

1. Introduction

The fascinating architectural organization of the brain is characterized by its exquisite anatomical and functional details and hierarchical network levels [1]. Synapses are highly specialized structures that allow electrochemical signals to convey tidily packaged information through the nervous system. Consequently, information from both the external and internal environment can be detected and processed and behaviour adjusted including body functions, memories, and emotions [2]. Thus, flawless synapses wiring and connectivity patterns must be preserved in order to keep messages communication unaltered and subside healthy neurophysiology. Actually, minimal disturbances of function are eventually expressed as brain disorders. By now, increasing evidence demonstrates the relevance of synapse dysfunction as a major determinant of many neurological diseases. Following this concept hereof, synaptopathies are by now treated as brain diseases themselves characterized by shared prevailing etiopathology [2]. Unfortunately, nervous cells do not divide neither are they replaced after they die off or get damaged except for a few cases. By the same token, from a prognostic viewpoint, synaptopathies are likely irreversible [1].

Synaptic networks enable organizing packaged and properly arranged information into, within, and out from the brain. Temporal spatial appearance, maturation, and specialization of synaptic units have played a key role in not only biological evolution across species, but the possibility of high cognitive and refined locomotor processes in vertebrates as well [3]. On the one hand, modulation of synapse activity

constitutes a major strategy to maintain brain homeostasis. On the other hand, slight though persistent disturbances in synapse physiology are likely to underlie major defects which are eventually expressed as brain disorders. The increasingly used term "synaptopathy" refers to brain disorders underlying synaptic dysfunction. The term dates back to a review by the Brundin Laboratory discussing Huntington's disease as a result of synaptic failure [4]. In this review authors suggested that distorted synaptic communication could bring on the early symptoms of Huntington's disease and trigger neuronal death in later stages of the illness [4]. In its broadest sense the term synaptopathy refers to any perturbation in which aberrant mechanisms correlate with synaptic dysfunction regardless of its pathophysiological origin. This may result in a baffled use of the term "synaptopathy" masking whether synaptic dysfunction is the cause or consequence of a particular pathophysiological sequence of events.

Synaptic dysfunction has been consistently documented as a leading determinant in several neurodegenerative diseases and neurodevelopmental disorders (NDDs) (e.g., autism spectrum disorders, Down's syndrome, startle disease, and epilepsy) [5]. The time interval around birth is usually considered the elapsed time between 5 months before and 1 month after delivery or perinatal period in man; it is critical and presents with extreme sensitivity to disturbing factors. Accordingly, the emergence of risk factors at this time may affect the normal synaptic network structure and function which may be symptomatically expressed later in life provided epigenetic environment is presented [6]. Perinatal asphyxia (PA) is an obstetric complication derived from impaired gas exchange [7] resulting in progressive hypercapnia and hypoxemia if unattended. Perinatal asphyxia inevitably leads to abnormal brain development and neurological morbidity [6–12]. Certainly, robust literature has been produced showing evidence of the etiopathogenic role of PA in epilepsy [13], cerebral palsy [14–17], mental retardation [18], attention deficit disorder [19, 20], and schizophrenia [21, 22].

Here we present a critical overview of synapse dysfunction associated with PA illustrated with key findings obtained using an experimental model. We discuss possible causes for synapse failure as putative common denominators. Comprehension of the molecular underpinnings leading to synaptic dysfunction will aid in the development of tailored synapse-targeted therapies for neurological disorders.

2. Experimental Model of Perinatal Asphyxia: Early Damage in CNS

In 1991 a global model of PA was developed at the Karolinska Institutet, Sweden. It represented a novel method to produce general asphyxia using rats which was not available by then. It consisted in performing a delayed caesarean section on pregnant rats resembling a delivery labour causing asphyctic lesions in newborns. Asphyxia was induced by immersing fetus-containing uterus horns in a water bath at 37 degrees C for 14-15 min, 15-16 min, and 16-17 min [30]. Ever since, several laboratories have used this established model of PA that induces modifications in the central nervous system (CNS) which are characterized by cortical, hippocampal, and striatal loss of neurons along with behavioural deterioration as well [30–32]. When the water bath lasts about 19 min, mortality rate increases (±40%) (Figure 1) [30, 31].

As Bjelke et al. suggested, this model is highly reproducible and easily performed. It resembles normal labour minimizing the influence of surgery, anesthesia, or drugs [30]. The main advantages of this model can be summarized as follows: (a) asphyxia takes place at the time of the delivery much the same as when umbilical cord circulation is disrupted; (b) the procedure results in systemic acidosis, hypercapnia, and hypoxia as observed in global asphyxia; (c) it is not invasive avoiding the confounding interferences of surgical procedures; (d) it induces global asphyxia which affects the whole brain (cerebral hemispheres and deep structures) making the model suitable for behavioural studies [26, 33, 34].

The immature brain is highly liable to threatening environments. Since PA is induced in newborn rats its overflowing plasticity can be certainly overwhelmed [35]. During neuronal development thin dendritic ectoplasmic pseudopods or filopodia are programmed to shield actually stable dendritic spines [36] and are determinant in the establishment of neural circuits in time [37]. Not long ago, the overexpression of M6a was reported not only to induce both neurites formation and increase in filopodium/spine density in primary cultures of rat hippocampus [38] but also to be involved in synaptogenesis [39]. Another report showed that activation by the phosphoinositide 3-kinase (a serine/threonine kinase) leading to glycogen synthase kinase 3 activation through the PI3K/Akt/GSK3 pathway was required in these processes [40]. Regulatory alterations were found in hippocampal dendritic spines cytoskeleton as prevailing features associated with memory disorders [41] whereby there is fair agreement that the hippocampus might be involved in the pathophysiology of NDDs as proposed by different studies [42, 43]. Abnormal neural network formation may be the consequence of atypical neurogenesis [44]. Many studies have focused on the analysis and discussion of cell survival and death mechanisms [45]. Nevertheless, our knowledge on the precise events triggered by PA that lead to NDDs in due time is insufficient.

Neuronal plasticity is derived from the intrinsic shapeable quality of brain tissue and it comprises neurogenesis and programmed cell death along with activity-dependent synaptic plasticity. The very well-known long-term potentiation (LTP) and long-term depression (LTD) phenomena convey the plastic response to repetitive synaptic stimulation and are associated with architectural adjustment in dendritic spines and hence in neuronal circuits [46]. Overplasticity in the developing brain could lead to disability disarranging otherwise properly organized connections and rendering maladaptive neuronal circuits [47]. Perinatal asphyxia during labour and/or delivery is doomed to cause long-term disability and embodies a worrying complication in neonatal and paediatric care [48]. In view of the not so low prevalence of this complication, several studies have reported sound information on the subject using the aforementioned experimental

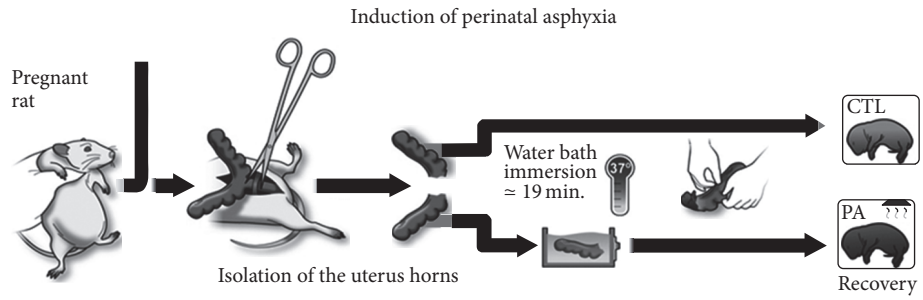

Figure 1: Illustration showing the main steps involved in the induction of PA. Adapted from [23].

model, which reproduces the pathophysiological processes of PA at the time of delivery [49].

3. Effects of Experimental Perinatal Asphyxia on Synaptic Organization

First studies using the above-mentioned model reported time-dependent loss of pyramidal cells in CA1 and CA3 brain regions. This finding evidenced that PA could affect hippocampal microarchitecture foundations and induce subsequent memory deficits. The same model of PA also led to imbalance in the dopaminergic system involved in motor behaviour and in other neurotransmission circuits as well. The results reported by Bjelke et al. in 1991 might provide an acceptable experimental approach to induce minimal brain disorder, namely, neurodevelopmental disabilities like hyperkinesia and attention deficit as usually found in children following perinatal asphyxia [30].

Several laboratories have used the Swedish experimental model. Researchers from the Netherlands, Canada, and Germany found increased density of cortical and striatal presynaptic buttons in twenty-two-month-old rats exposed to PA. Van de Berg et al. proposed that such area-specific modifications were intended to counterbalance PA-induced neuronal loss. Quite the contrary, higher presynaptic density of buttons was related to aggravation of cognitive impairment according to age [32]. The postsynaptic locus received attention too. Argentine researchers found that asphyctic rats developed an increase in striatal synapses disarrangement and thickening in postsynaptic densities (PSDs). Perinatal asphyxia also compromised hippocampal PSDs according to severity and duration of the hypoxic insult. Lowering the temperature of the experimental setting was reported to effectively prevent synaptic changes [50]. This group had previously observed that PA induced cytoarchitectural changes in the corpus striatum and that detrimental changes could be prevented by hypothermia as well [51].

In our laboratory we found long-term misfolding and aggregation of proteins in striatal PSDs of 6-month-old rats which were subjected to severe PA. Based on our findings that PSDs were highly modified and ubiquitinated, we suggested that these changes might constitute an aberrant morphological mechanism underlying synaptic dysfunction in response to PA [24]. Following our later observations, early misfolding and aggregation of striatal synaptic proteins might actually represent the triggering point of long-term neurodegenerative events. One month after induction of PA not only did we find an increment in PSDs but also it was also concomitant with high ubiquitination levels. The magnitude of the increase in PSDs was dependent on the duration and severity of the hypoxic insult [25]. In this way we were able to confirm that protein ubiquitination could serve as a useful marker for the degree of alteration of PSDs and neuronal damage as found in experimental PA [24, 25].

Early synaptic alterations could be associated with striatal cytoskeletal changes induced by PA. Accumulation of F-actin was observed in dendritic spines of 1-month-old asphyctic rats. It was correlated with an increment in β-actin in PSDs and the number of mushroom-shaped spines and with a reduction in MAP-2 immunohistochemical labelling and the number of neurons. Therefore F-actin accumulation might represent a key cellular mechanism underlying neuronal death [26]. These striatal cytoskeletal changes were replicated 2 months after PA and could be blocked by hypothermia, which has proved to be an important therapeutic clinical tool for the outcome of PA [27]. Moreover actin modifications were observed in hippocampal cytoskeleton 4 months after induction of PA together with an increment in PSDs and the extent of ubiquitination. This finding suggested that cytoskeletal actin might play a role in PSDs alterations and ubiquitination. In addition, the number of hippocampal dendritic spines which were positive for F-actin stain decreased 4 months after PA [28] and increased 1 month after PA along with enhanced filopodium formation and synaptogenesis [29]. These findings put forward the possibility that such early attempt to rescue neural tissue via synaptogenesis and F-actin augmentation might not be actually effective. Certainly if this is so, it might lead to the late readaptive reduction in F-actin levels. These early new synapses might not be functional. Thus overplasticity might affect the proper establishment of neural circuits causing cognitive deficits as observed in behavioural tests [29]. Figure 2 shows some of these data as observed in hippocampus. Table 1 summarizes our results in this subject.

An Austrian group was also interested in protein derangements during postnatal development following PA and attempted to identify differentially expressed proteins that might represent potential biomarkers and pharmacological targets [52]. They decided to study hippocampal proteins following their own report of postnatal changes during brain

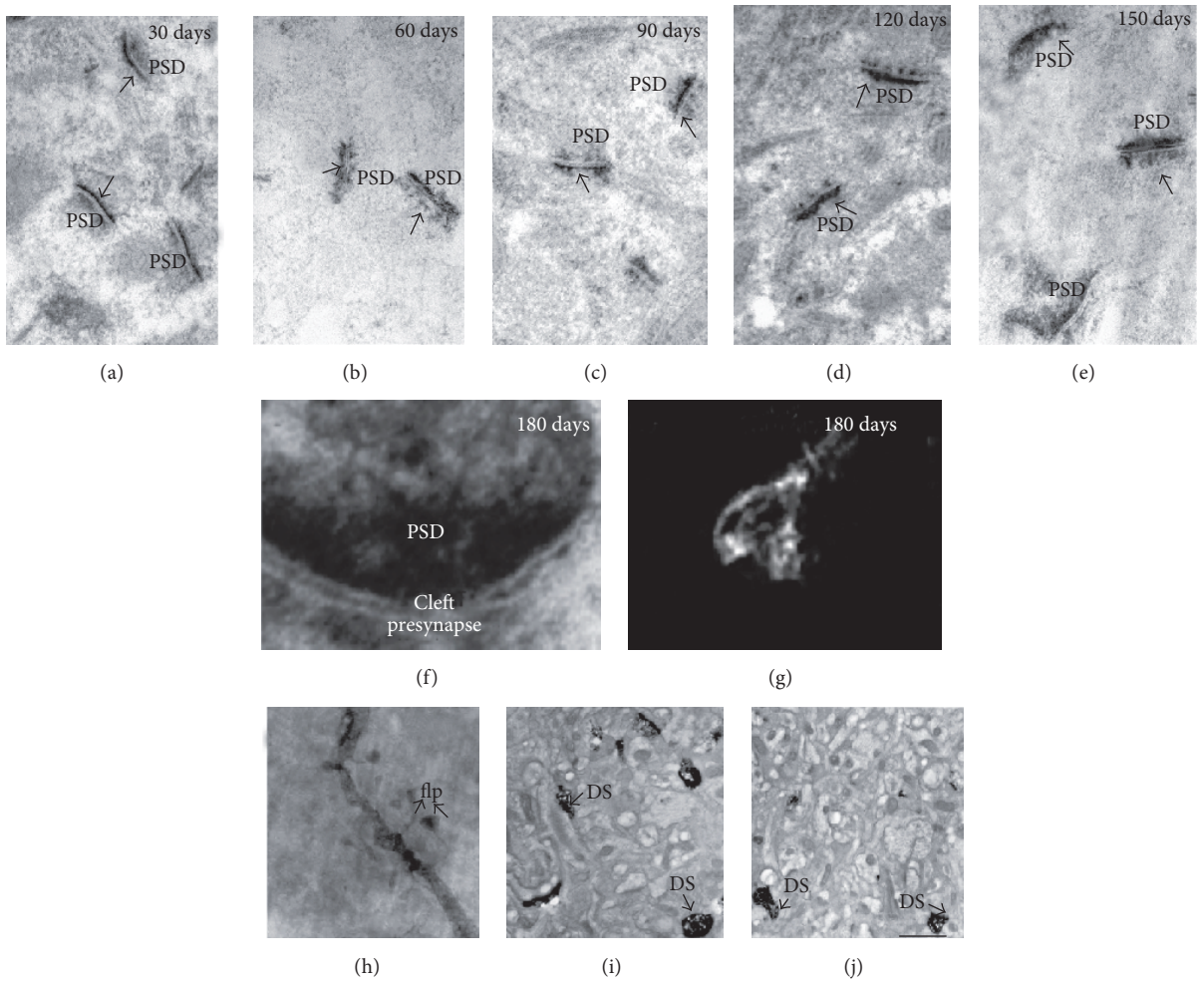

Figure 2: Microphotographs of synaptic terminals of the hippocampus CA1 area from animals subjected to PA. (a–e) Hippocampal synapses stained with E-PTA showing increased thickness in PSDs. (f) More consistent changes were observed in PSDs after 180 days of PA. Three-dimensional reconstruction of the asphyctic postsynaptic domain (g) showed clear signs of degeneration. (h) Dendritic shaft injected with Golgi silver staining showed filopodium after 30 days of PA (arrows). (i-j) DS stained with phalloidin eosin and photoconverted. A higher number of DS are observed at 30 days compared with 180 days after PA. PSDs = postsynaptic densities; E-PTA = ethanolic phosphotungstic acid; DS = dendritic spines. Scale bar 0.5 μm.

development [53]. Hippocampal protein levels were determined by a gel-based proteomic method. Results revealed an impairment of brain protein machinery as a consequence of PA. Several protein levels were altered at specific time points after the insult. Interestingly synapsin IIb levels were increased. Weitzdörfer et al. interpreted this finding as a result of both altered neurotransmitter release and compensatory synaptogenesis following PA [52]. The Swedish experimental model was selected for this study for its suitability to evaluate morphology, metabolism, neurochemistry, and long-term effects of PA [54].

A Chilean group referred to their pragmatic approach when choosing the Swedish experimental model because it induced asphyxia at the time of delivery and mimicked relevant features of the process [55]. In fact, PA is frequently associated with problematic or long-lasting delivery and the aforementioned experimental model reproduces a delayed caesarean section [30]. Furthermore this model also allows performing in vitro studies as an approach to assess particular issues elicited by the insult, such as the interference with normal neuronal network development [56].

The Chilean laboratory investigated hippocampal plasticity after PA by measuring postnatal apoptosis and neurogenesis. They suggested that PA induces short- and long-term plastic changes which are regionally specific perhaps providing a framework for functional recovery of the hippocampus. These plastic changes include delayed cell death and neurogenesis involving mitogenic proteins and also pro- and anti-apoptotic proteins [57]. Recently, they investigated cell proliferation and neurogenesis as potential compensatory mechanisms for delayed cell death associated with PA using hippocampus and subventricular zone (SVZ) organotypic cultures in vitro. According to their results neurogenesis appears to be mediated by dopamine receptors and PA could hinder

Table 1: Summary of PA-induced changes from our laboratory.

Reference	Time after PA	Brain area	Main findings	Concluding remarks
Capani et al. 2009 [24]	6 months	Striatum	Thickening in PSDs and high ubiquitination levels related to injury duration and severity. Hypothermia prevented changes.	Long-term protein misfolding/aggregation in PSDs may drive synaptic dysfunction/neuronal damage.
Grimaldi et al. 2012 [25]	1 month	Striatum	Thickening in PSDs and high ubiquitination levels related to injury duration and severity.	Early misfolding/aggregation of synaptic proteins could induce long-term changes and neurodegeneration.
Saraceno et al. 2012 [26]	1 month	Striatum	Accumulation of cytoskeletal F-actin in dendritic spines. Increased number of mushroom-shaped spines. Reduced number of neurons.	Early synaptic alteration/neuronal damage might be linked to cytoskeletal F-actin accumulation.
Muñiz et al. 2014 [27]	2 months	Striatum	Increased number of mushroom-shaped F-actin dendritic spines. Hypothermia prevented changes.	Sustained synaptic and cytoskeletal changes were found.
Saraceno et al. 2012 [28]	4 months	Hippocampus	Thickening in PSDs and high ubiquitination levels. Reduced number of F-actin stained spines.	Long-term actin cytoskeleton might play a role in PA-induced PSD alterations.
Saraceno et al. 2016 [29]	1 month	Hippocampus	Thickening in PSDs and increased number of F-actin stained spines. Enhanced filopodium formation and synaptogenesis. Habituation memory changes.	Likely dysfunctional synapses might result in late readaptive decrease in F-actin levels. Overplasticity might affect the adequate establishment of neural circuits.

PSDs: postsynaptic densities. See text for more details.

this mechanism in certain areas. They hypothesized that PA might cause delayed cell death by disrupting postnatal plasticity rather than triggering a neurotoxic cascade. In other words PA might modulate postnatal neurogenesis. Therefore dopamine agonists might have neuroprotective potential via facilitation of postnatal neurogenesis and restoration of damaged circuitries [58]. Certainly, in vitro studies revealed a selective decrease in the number, neurite length, and branching of dopamine neurons as a consequence of PA [59]. Likewise a reduction in neurite length and branching was observed in the hippocampus 1 month after PA along with reduced expression of pre- and postsynaptic markers (resp., synaptophysin and postsynaptic density protein 95, PSD95) [60]. Following this evidence the Chilean group has apparently confirmed and extended Bjelke et al.'s original findings about PA-induced alteration of dopamine circuitries and hippocampal architecture. In fact, the leader of the Chilean group Herrera-Marschwitz had previously worked with Bjelke and Andersson at the Karolinska Institutet, where they published several papers about the effects of PA on rat brain and the dopamine system in particular.

Researchers from the Netherlands were also interested in the effect of PA on postnatal plasticity during the first weeks of life. They used the Swedish experimental model in order to study the ontogeny of neurotrophic factors involved in the regulation of developmental plastic changes [61]. They had previously reported a delayed increase in cellular proliferation in the hippocampus 5 days after PA, likely related to an increase in neurotrophic factors caused by the injury [62]. In contrast to that observed in the adult injured brain PA induced opposing changes in nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) content in a spatiotemporal-dependent fashion. On these bases, further studies should attempt to elucidate the mechanism of action of neurotrophic factors in the developing brain after PA [61].

Most studies using the Swedish experimental model of PA have focused on grey matter pathology. However as myelin interferes with nerve conduction velocity and may affect

synaptic transmission, studies on the effects of PA on myelination are relevant. The aforementioned Austrian researchers reported long-term myelination deficits in hippocampus and cerebellum of rats subjected to PA. These deficits were regularly accompanied by neuronal loss which was measured by a decrease in neurofilament immunoreactivity. This finding suggests that grey matter damage is linked to myelination deficits which should also be considered when studying mechanisms involved in the pathogenesis of NDDs following PA [63].

4. Conclusion

Our aim was to shed some light on the synaptic dysfunction linking PA with NDDs. Perinatal asphyxia occurs when the brain is still immature and the insult may affect initial plasticity required for the establishment of circuitries and synapses. The consumption of extra energy for the reestablishment of homeostasis might compete with the demands required for circuitries and synapsis consolidation [55]. Several studies using the Swedish experimental model have reported distinct alterations induced by PA such as thickening of both pre- [32] and postsynaptic densities [24–29, 50]; protein misfolding, aggregation, and ubiquitination [24–29]; interruption of postnatal neurogenesis [58]; reduction in neurite length and branching [59, 60]; myelination deficits [63]; and modifications in the levels of synapsin [52, 60] and neurotrophic factors [61]. Some researchers have suggested the existence of plastic changes in an attempt to counterbalance neuronal loss [29, 32, 52, 57]. However, compensatory mechanisms, namely, overplasticity, might not always be functional and may result in aggravation of cognitive impairment [32]. The inadequacy of neural circuits is irreparably accompanied by behavioural deficits [29].

Further studies should be designed in order to dissect molecular changes involved in synaptic alterations induced by PA. The Swedish experimental model of PA appears to be a useful tool of clinical relevance [26, 30, 33, 34, 54–56]. Also electrophysiological evidence is needed so as to clarify the relevance of new dysfunctional synapses and long-term neurodegeneration leading to synaptopathy following PA.

Hopefully this information will help to design new therapeutic tools, certainly a challenge for medical research in this field.

Competing Interests

The authors declare that they have no conflict of interests.

Authors' Contributions

Herrera and Otero-Losada share authorship and contributed equally to this work.

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Review Article

Dynamic Control of Synaptic Adhesion and Organizing Molecules in Synaptic Plasticity

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Synapses play a critical role in establishing and maintaining neural circuits, permitting targeted information transfer throughout the brain. A large portfolio of synaptic adhesion/organizing molecules (SAMs) exists in the mammalian brain involved in synapse development and maintenance. SAMs bind protein partners, forming *trans*-complexes spanning the synaptic cleft or *cis*-complexes attached to the same synaptic membrane. SAMs play key roles in cell adhesion and in organizing protein interaction networks; they can also provide mechanisms of recognition, generate scaffolds onto which partners can dock, and likely take part in signaling processes as well. SAMs are regulated through a portfolio of different mechanisms that affect their protein levels, precise localization, stability, and the availability of their partners at synapses. Interaction of SAMs with their partners can further be strengthened or weakened through alternative splicing, competing protein partners, ectodomain shedding, or astrocytically secreted factors. Given that numerous SAMs appear altered by synaptic activity, in vivo, these molecules may be used to dynamically scale up or scale down synaptic communication. Many SAMs, including neurexins, neuroligins, cadherins, and contactins, are now implicated in neuropsychiatric and neurodevelopmental diseases, such as autism spectrum disorder, schizophrenia, and bipolar disorder and studying their molecular mechanisms holds promise for developing novel therapeutics.

1. Synapses

It is estimated that there are more than one hundred billion neurons in the human brain, connected to one another by hundreds of trillions of contact points called synapses. These synaptic connections wire select neurons into functional neuronal circuits, enabling the brain to process and transfer information. Each synapse consists of a patch of "*presynaptic membrane*" from one neuron (typically an axon terminus) adhered to a patch of "*postsynaptic membrane*" from a second connecting neuron (typically a dendrite), and the space between them called the "*synaptic cleft.*" At chemical synapses, which comprise the vast majority of synapses in the brain, action potentials (i.e., electrical activity) from the presynaptic neuron trigger the release of neurotransmitters into the synaptic cleft, provoking molecular and cellular responses in the postsynaptic neuron in a process referred to as "*synaptic activity.*" The presynaptic side of the synapse hosts the molecular machinery needed to release and recycle synaptic vesicles containing these neurotransmitters. The postsynaptic side of the synapse responds to the released vesicle contents via receptors and channels and triggers downstream cellular responses. It is still not well understood how neurons wire together into specific circuits and, in particular, how correct synaptic connections are established and maintained over time. Remarkably, synaptic connections are dynamic and can change. In response to synaptic activity, they undergo structural and functional alterations as part of a process called "*synaptic plasticity.*" Synaptic plasticity can involve changes to the molecular components present at a synapse, the location of these components at a synapse, the efficiency with which a synapse can communicate, and even whether a synapse is maintained or completely disappears; for excellent recent reviews see [1–3]. Mechanisms of synaptic plasticity are widely believed to be involved in long term memory [1–3]. Alterations at synapses have commonly been monitored through two important phenomena: *long term potentiation* (LTP) and *long term depression* (LTD), processes

that cause an increase or decrease in synaptic strength, respectively (as gauged by the electrical output produced by the postsynaptic neuron in response to synaptic stimulation). LTP and LTD are also thought to be involved in memory and learning. Synapses, thus, through their role in mediating connections between neurons and their ability to change through mechanisms of synaptic plasticity play an essential role in proper brain function.

2. Protein Networks at Synapses and Their Relation to Disease

Synapses contain a staggering number of proteins. Extensive proteomics studies and review of the literature estimate that there are ~1,900 to more than ~2,700 proteins localized at synapses [4–6]. The synaptic proteins identified include ones involved in exocytosis and recycling of synaptic vesicles, receptors for different neurotransmitters, ion channels, extracellular matrix proteins, cell adhesion molecules, cytoskeletal proteins, scaffolding proteins, membrane transporters, GTPases, phosphatases, and molecules involved in protein degradation. However, where the exact boundary of a synapse lies is vague (i.e., where it starts and stops), so scientists have typically relied on the ability of a protein to be co-isolated with synaptosomal membrane fractions and/or microscopy to designate a synaptic identity. Also it is not known which of these many different proteins are found at a particular synapse or how their distribution and expression level varies over the different synapse types.

More than a decade ago, it was suggested that defects at synapses would underlie many neurodevelopmental and neuropsychiatric diseases [7]. Hundreds of genes are now implicated in diseases like schizophrenia, autism spectrum disorder, and other behavioral and cognitive disorders, and many of them indeed encode synaptic proteins ([8–11]; https://sfari.org/resources/sfari-gene). For this reason, the term "synaptopathies" is increasingly used to refer to neurodevelopmental, neurodegenerative, and neuropsychiatric disorders that involve the disruption of synaptic proteins [12, 13]. Given the myriad of proteins found at synapses, synapses can best be viewed as large protein interaction networks that are plastic and change in response to synaptic activity; in addition, disruption of these synaptic networks contributes to the pathology of many neurological disorders.

3. Synaptic Adhesion Molecules in the Synaptic Cleft

One large class of proteins found at synapses contains the so-called *synaptic adhesion molecules* (SAMs). Some SAMs are also called "*synaptic organizers*" because they coordinate protein interaction networks. SAMs are tethered to the presynaptic or postsynaptic membrane by a transmembrane segment or GPI anchor and extend their extracellular domains into the synaptic cleft. Their ectodomains range in size from relatively small (just a single domain) to very large (10 or more domains). Examples of prominent SAM families with putative adhesive or organizing functions are shown in Figure 1. Some families of SAMs are composed of members sharing very similar domain compositions (e.g., cadherins with five EC domains), while other families contain members that have very divergent extracellular regions (e.g., the immunoglobulin super family, IGSFs). Strikingly, many of the well-known SAM families use a limited number of modules to compose their extracellular domain, alternating, for example, immunoglobulin, fibronectin type 3, cadherin, laminin G, and leucine rich repeat domains in different ways (Figure 1).

How many SAMs are found in the human brain? Large databases cataloguing synaptic proteins such as SynaptomeDB [6] and SynProt [5] provide a tremendous starting point to derive an estimate. However, these databases are still incomplete. For instance, validated SAMs like calsyntenins and sidekicks (Figure 1) are absent in SynaptomeDB and SynProt, likely because they have evaded detection in the mass spectrometry-based proteomic studies around which these databases were heavily constructed [14, 15]. On the other hand, a multitude of potential SAMs have been identified by proteomic and genomic studies, for which precise function and synaptic localization have yet to be validated. In addition, many large families containing validated SAMs also contain many members that are as yet uncharacterized with respect to their adhesive and organizing roles and synaptic localization, so it is not known if these also function as SAMs. Therefore, though likely in the hundreds or even thousands (if considering splice variants), the number of *bona fide* SAMs in the human brain is not accurately known.

What are the functions of SAMs? Traditionally, SAMs have been evaluated according to their adhesive function, assessed typically via their ability to aggregate cells in cell-based assays, copurify with synaptosomal membrane fractions, and localize to synapses in electron microscopy images. However, in recent years, a dramatically expanded and more nuanced view has emerged for the function of these molecules. It has become clear that SAMs can support a bewilderingly large number of different kinds of protein interactions at synapses. Through their extracellular domains, SAMs can bind protein partners in the synaptic cleft. They can form strict homophilic interactions with identical molecules, semihomophilic interactions with related family members, and/or heterophilic interactions with members of other adhesion molecule families. Via their cytoplasmic tails, SAMs can bind partners intracellularly, integrating into the presynaptic and/or postsynaptic machinery. When SAMs bind partners tethered to the opposing membrane, a "*trans*-complex" is formed that generates a macromolecular bridge spanning the synaptic cleft. At the same time, the components recruited to their cytoplasmic tails from the presynaptic and postsynaptic machineries align. These *trans*-complexes support the adhesive function observed for SAMs in cell-based assays. However, SAMs are increasingly being recognized for essential roles beyond simply adhering the presynaptic and postsynaptic membranes together. They can play a role in neuron-neuron recognition and generate scaffolds onto which additional proteins can bind, and some SAMs likely signal to the presynaptic and/or postsynaptic membranes (Figure 2). For example, some SAMs can bind partners tethered to the same membrane in a side-by-side fashion forming

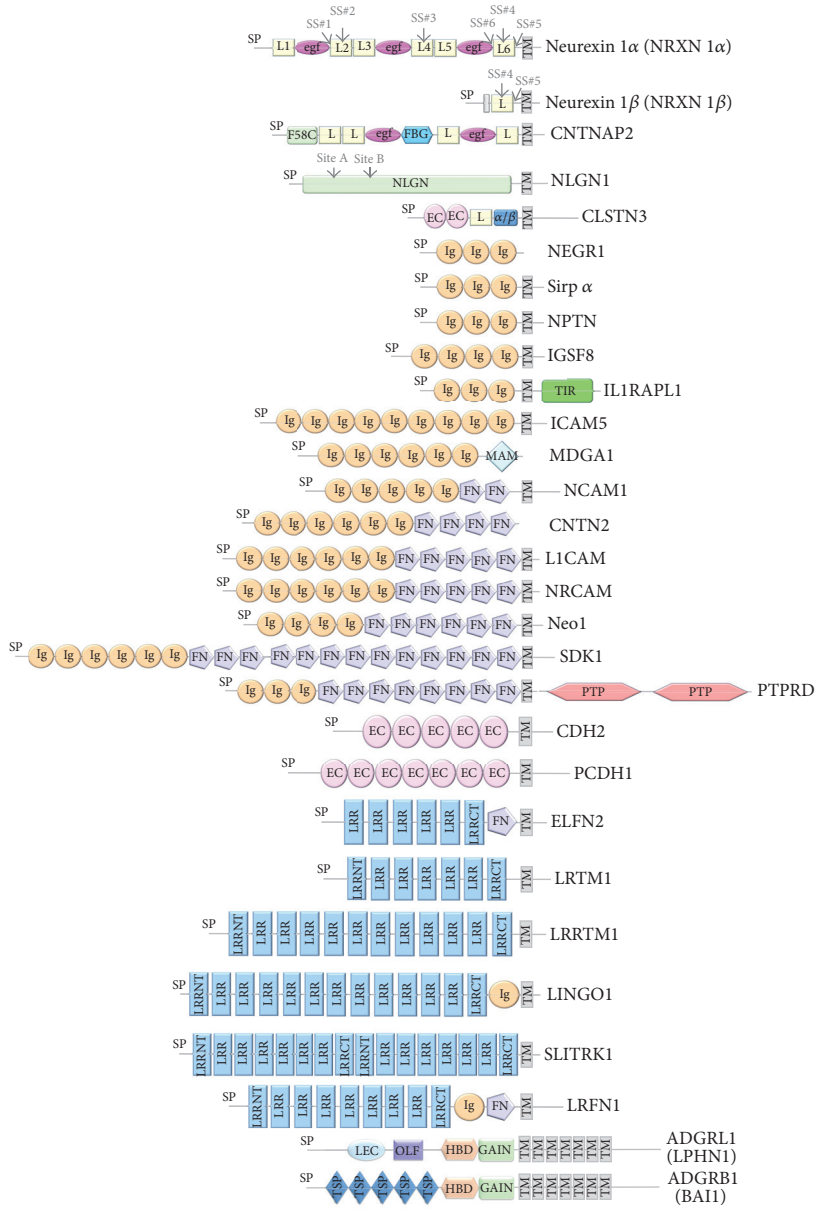

Figure 1: SAMs. Prominent families of SAMs with putative synaptic localization and function are shown. Prototypes used to depict the domain organization are indicated. From top to bottom the following is listed: neurexin 1α and neurexin 1β (NRXN1α and NRXN1β; neurexins); CNTNAP2 (contactin associated protein-like); NLGN1 (neuroligins); CLSTN3 (calsyntenins); NEGR1 (Iglons which include NEGR1, NTM, LSAMP, and OPCML); Sirp α (signal regulatory proteins); NPTN (neuroplastin); IGSF8 (immunoglobulin superfamily); IL1RAPL1 (interleukin 1 receptor accessory protein-like); ICAM5 (intercellular adhesion molecules); MDGA1 (MAM domain containing glycosylphosphatidylinositol anchor); NCAM1 (neural cell adhesion molecules); CNTN2 (contactins); L1CAM (L1 cell adhesion molecules); NRCAM (neuronal cell adhesion molecules); Neo1 (neogenin); SDK1 (sidekick cell adhesion molecules); PTPRD (protein tyrosine phosphatase receptor types D, F, and S); CDH2 (cadherins); PCDH1 (protocadherins); ELFN2 (extracellular leucine rich repeat and fibronectin type III domain containing); LRTM1 (leucine rich repeats and transmembrane domains); LRRTM1 (leucine rich repeat transmembrane neuronal); LINGO1 (leucine rich repeat and Ig domain containing); SLITRK1 (SLIT and NTRK-like family member); LRFN1 (leucine rich repeat and fibronectin type III domain containing); ADGRL1 (adhesion G protein-coupled receptor type L; previously known as latrophilins); ADGRB1 (adhesion G protein-coupled receptor type B, previously known as brain-specific angiogenesis inhibitor). Several large polymorphic families including the ephrin receptors, integrins, and plexins are not shown. The domain abbreviations used in the text are for laminin G or laminin G/neurexin/sex hormone binding globulin or LNS domains (L); epidermal growth factor repeat (EGF); coagulation factor 5/8 type C (F58C); fibrinogen-like (FBG); extracellular cadherin (EC); alpha/beta (α/β); immunoglobulin (Ig); Toll/Il-1 receptor homology (TIR); meprin, A-5 protein, receptor protein tyrosine phosphatase mu (MAM); fibronectin type 3 (FN), protein tyrosine phosphatase (PTP); leucine rich repeat (LRR), N-terminal leucine rich repeat (LRRNT); C-terminal leucine rich repeat (LRRCT); galactose binding lectin domain (LEC); olfactomedin-like domain (OLF); hormone binding domain (HBD); GPCR-autoproteolysis inducing (GAIN); thrombospondin (TSP). Other abbreviations are signal peptide (SP) and transmembrane segment (TM). Alternative splice insert sites are indicted for the SAMs NRXN1α, NRXN1β, and NLGN1, as they are referred to in the text.

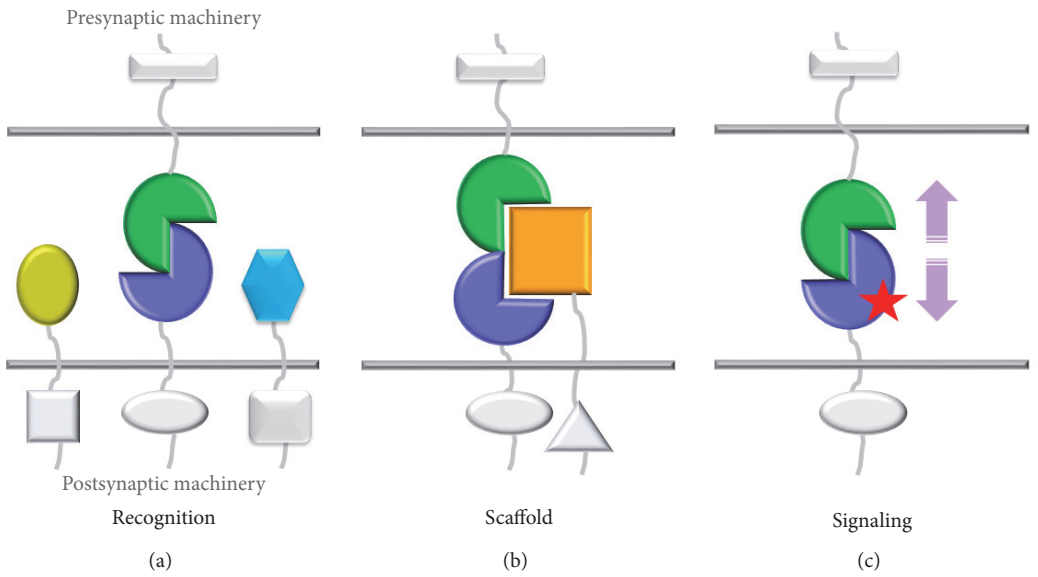

Figure 2: SAM function and mechanisms. SAMs can recruit and organize protein interaction networks in the synaptic cleft by (a) generating mechanisms to recognize specific SAM partners, but not others by binding through direct interactions; (b) binding other SAMs to generate a scaffold onto which a third protein can dock and this mechanism also supports the binding of SAMs through indirect interactions; (c) binding a partner and inducing a signaling event, for example, through (allo)steric mechanisms.

a "*cis*-complex." Such *cis*-protein assemblies are often regulatory in nature, altering, binding, or forming an essential precomplex onto which a third partner can dock to yield the final *trans*-synaptic bridge, for example, MDGA1 binding to neuroligin 2 (NLGN2) [16, 17], the CNTNAP2-CNTN2 tripartite system [18], and SALM4 binding to SALM3 which inhibits *trans*-synaptic SALM3-LAR adhesion [19]. SAMs can also interact with nonadhesion molecules like receptors, channels, and secreted factors in the synaptic cleft working to recruit and organize synaptic protein networks (or "*synaptic interactomes*"). These different modes of interaction enable SAMs to play important roles in synapse specification, not only organizing the protein interaction networks at a synapse, but also specifying functional properties, for example, by altering presynaptic release probabilities, and/or neurotransmitter receptor and channel properties; for an excellent review see [20].

A vast body of experimental work has demonstrated that SAMs play a key role in promoting the formation, development, maturation, stabilization, and eventual elimination of synapses. Through their roles in forming and maintaining synaptic connections, SAMs therefore intimately impact the wiring of neurons into circuits. The monumental task to identify, characterize, and validate each of the many SAMs and their family members has become increasingly pressing with the discovery that so many of these molecules are implicated in neurodevelopment and neuropsychiatric diseases, for example, the neurexins, neuroligins, LRRTMs, CNTNAPs, contactins, cadherins, and protocadherins (Figure 1; for recent reviews see [20–26]). So while initially recognized purely for their ability to adhere cells together, the functions of SAMs are now recognized to be much more broad, nuanced, and subject to complex regulation.

4. Plastic Interactions within the Synaptic Cleft

SAMs are strategically positioned to contribute to synaptic plasticity, given that they can alter synapse structure and function through their ability to sculpt and regulate synaptic protein interaction networks. Below we highlight several important mechanisms that have come to light that regulate SAMs, their diversity, and their functions in a synaptic activity-dependent way. We further present supporting examples to illustrate the general themes (Figure 3).

4.1. Alteration of SAM Protein Levels in the Synaptic Cleft. It has long been held that synaptic protein abundance is implicated in synaptic plasticity. In particular, altering the abundance of a specific SAM at a synapse could fundamentally impact the development, maintenance, and ultimate elimination of that synapse. A number of studies have used quantitative proteomics of synaptosomal fractions to correlate synaptic protein abundance (including those of SAMs) to events implicated in synaptic plasticity, for example, the long term synaptic adaptions that accompany the administration of drugs of abuse. Repeated morphine administration robustly downregulated CNTN1, L1CAM, neurocan, and OPCML in striatal presynaptic fractions [27], while in a second study neurexin, NCAM, and NTM protein levels decreased more than 40% in rat forebrain synaptosomal fractions, though in this case OPCML protein levels were unaltered [28]. Importantly, these studies showed that the abundance of synaptic proteins was altered in a highly selectively way. Of 175 proteins that could be identified proteomically, only 30 were robustly and consistently altered by morphine treatment

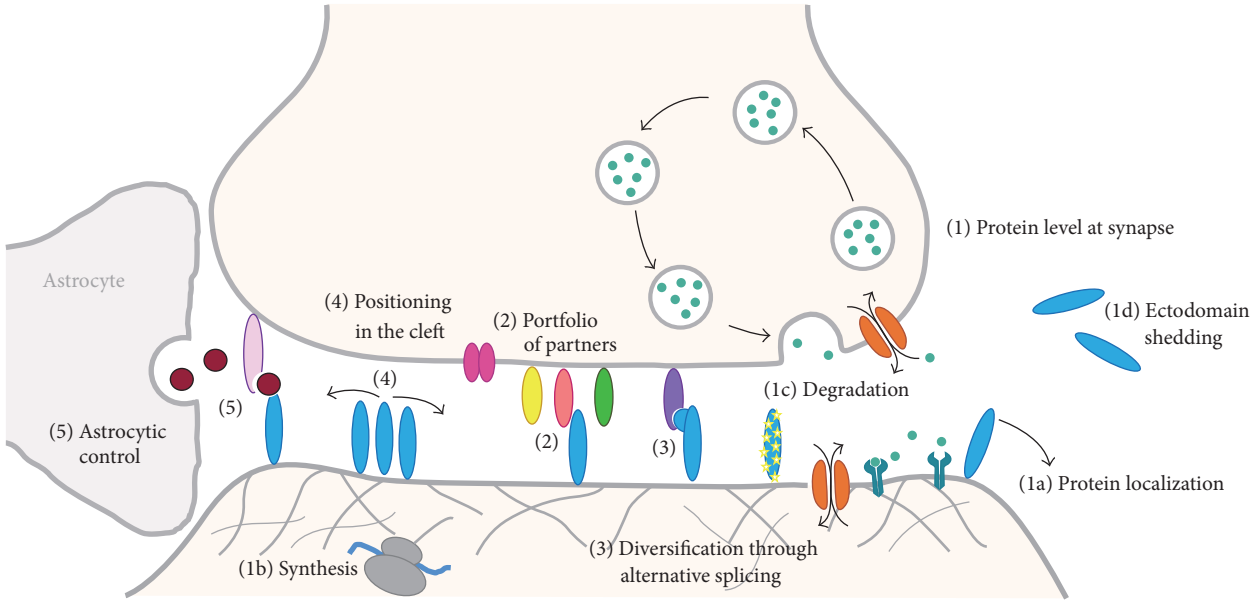

Figure 3: SAMs can contribute to synaptic plasticity. SAM function can be regulated by synaptic activity through different processes. Protein levels can change (1) as a result of altered localization targeting a protein to or away from the synaptic membrane surface (1a), protein synthesis (1b), protein degradation (1c), and ectodomain shedding (1d). The availability of members within a broad portfolio of potential partners can be altered (2). SAMs can be diversified through alternative splicing (3). SAMs can be repositioned in the synaptic cleft (4). Protein interactions supported by SAMs can be modulated by astrocytic factors (5). Details are as discussed in the text.

(i.e., 17%), indicating that the SAMs that were altered represented highly significant changes [27]. In other studies, experience dependent plasticity induced in animals by trimming their whiskers to cause sensory deprivation resulted in ~20% to ~30% lower levels for the SAMs Pcdh1, ICAM5, Plexin-A1, and Lphn 3 in juvenile mice (a period where synaptogenesis peaks) [29]. Also in this latter study, the protein abundance was only very selectively altered; only a small number of proteins were affected which included specific SAMs, while 95% of the 7000 tentative synaptic proteins examined showed no significant changes [29]. The above proteomic studies signify that the protein abundance of SAMs can change in response to events triggering synaptic plasticity. However, several caveats exist. These proteomic approaches offer only a global view of protein abundance, profiling changes in protein levels averaged over a large, heterogeneous population of synapses pooled together from many different kinds of neurons and supporting glial cells. In addition, only those proteins that are technically accessible were monitored, that is, only those proteins which were extracted in sufficiently abundant quantities to enable their detection and analysis by mass spectrometric methods [30].

How do protein levels for a specific SAM change in response to synaptic activity at a specific, single synapse or just a small subset of select synapses? Several processes have been identified that modulate SAM protein levels at the level of a single synapse, altering synapse morphology and stabilizing (or destabilizing) synaptic strength on a very local scale in response to synaptic activity (see (1a)–(1d) in Figure 3).

(i) SAMs can accumulate or be depleted from membrane surfaces in the synaptic cleft as a result of altered stability, for example, due to loss of stabilizing partners, recruitment, trafficking, internalization, and/or phosphorylation of cytoplasmic tails. For instance, levels of neurexin 1β at the synaptic membrane rise in response to neural activity, apparently due to an increase in stability (or suppressed dynamics) at the synaptic terminal [31]. NLGN1 and NLGN3 have increased surface membrane levels upon chemically induced LTP and decreased levels after LTD as a result of being dynamically exchanged at the postsynaptic membrane through active cytoskeleton transport [32]. In addition, surface expression of NLGN1 is also increased through CAMKII phosphorylation of its cytoplasmic tail in response to synaptic activity [33]. Other SAMs such as OPCML, CNTN1, and cadherins also display decreasing or increasing protein levels in the synaptic cleft in response to synaptic activity as a result of internalization into the cell or mobilization to the synaptic membrane surface [34–37].

(ii) Protein levels can rise in the synaptic cleft as a result of activity-induced expression via local protein synthesis at the synapse (recently reviewed in [38]). For example, expression of LRRTM1 and LRRTM2 (synaptic organizers that induce presynaptic differentiation) increases as a function of synaptic activity because influx of Ca2+ into the postsynaptic neuron following NMDA-receptor activation induces nuclear Ca2+-dependent transcription [39]. α-Dystroglycan expression is also upregulated by prolonged increased neuronal activity at inhibitory synapses in the CNS elevating its protein levels [40]. In addition, local

translation of DSCAM in dendrites has been shown to be rapidly induced by synaptic activity [41].

(iii) SAM levels can also decrease at synapses as a result of degradation, thereby regulating synapse development and survival. Evidence is building that highly targeted protein degradation takes place at synapses locally and that it can be regulated by synaptic activity (for recent review see [38]). Intriguingly, elegant studies have revealed that the C. elegans SAM, SYG-1, can locally inhibit an E3 ubiquitin ligase complex that tags proteins for degradation, protecting adjacent synapses from elimination [42].

(iv) One particular form of proteolysis, ectodomain shedding, is now widely documented to regulate SAM protein levels in the synaptic cleft. During shedding, the extracellular domain of a SAM is proteolytically released from its transmembrane segment or its GPI anchor that tethers it to the synaptic membrane. Liberating the SAM ectodomain permits the protein interactions and extracellular matrix to be remodeled within the synaptic cleft. Ectodomain shedding is involved in structural as well as functional synaptic plasticity and impacts key processes like LTP and LTD (for recent reviews see [43, 44]). Exactly where the released ectodomains end up is unclear. Do they remain in the synaptic cleft, binding and blocking their normal protein partners from forming trans-synaptic interactions? Or are the shed ectodomains lost from the synaptic cleft, diffusing outwards to affect other neighboring synapses? Alternatively, are they perhaps simply degraded locally?

Both presynaptic as well as postsynaptic SAMs have been demonstrated to undergo ectodomain shedding in vitro and in vivo. Activity-dependent proteolytic release has been shown for many well-known SAMs, including neuroligins, neurexins, calsyntenins, SIPRα, ICAMs, LARs, Slitrks, and nectins, and their release is executed by various proteases including matrix metalloproteases, ADAM proteases, and alpha/gamma-secretases [14, 43, 45–53]. The downstream consequence of ectodomain shedding varies. In the case of the postsynaptic adhesion molecule NLGN1, shedding destabilizes the presynaptic partner neurexin 1β at synapses and decreases the presynaptic release probability of synaptic vesicles, thereby depressing synaptic transmission [48, 50]. Ectodomain release of NLGN1 has relevance for disease, because it is promoted by epileptic seizures [50]. Release of the Sirp α ectodomain has a completely different consequence, because it promotes synapse maturation [51]. Likewise, ectodomain release of CLSTN1, which is found on the postsynaptic membrane of inhibitory and excitatory synapses, permits the transmembrane stub and Ca2+-binding cytoplasmic domain to be internalized and accumulate in the spine apparatus where it is thought to carry out a role in postsynaptic Ca2+- signaling [54].

Taken together, multiple processes exist that regulate protein levels of specific SAMs in the synaptic cleft of single synapses in response to synaptic activity.

4.2. Availability of a Broad Portfolio of Different SAMs Containing Variable, Synergistic, and Competing Partners. It is estimated that there are more than 470 putative cell adhesion molecules in humans [55], although how many of these are expressed in the brain and are synaptic is not known. Nevertheless, a broad portfolio of SAMs has been validated to date and it provides a powerful mechanism to generate a myriad of different possible interactions, some of which can be affected by synaptic activity, thereby contributing to mechanisms of synaptic plasticity (see (2) in Figure 3). Diversity is achieved in several ways. Most SAMs are modular in nature and use a combinatorial approach to build up their extracellular region by alternating different structural modules, for example, Ig domains, FN3 domains, and cadherin EC domains (Figure 1). In addition, most SAM families contain several members that, while sharing a conserved domain structure, vary in amino acid sequence. In some families, individual members are diversified even further through alternative splicing of their mRNA, inserting, deleting, or exchanging anywhere from one to more than a hundred amino acids in the encoded protein. For instance, more than a thousand splice variants have been demonstrated for neurexins (discussed below).

The portfolio of SAMs can be expanded even further on a functional level in two key ways. First, SAMs can assume evolving functions over time, carrying out one function during the early stages of brain development, while connectivities are being formed, and then switching to another function in the mature adult brain. For example, early during synapse development, neuroligins and LRRTMs appear to compensate for one another; however once synapses have formed, neuroligins and LRRTMs affect excitatory synaptic transmission differently [56]. Likewise, during early development cadherins are important for synapse adhesion, stabilization, and synaptogenesis in young neurons; however once mature synapses have formed, they no longer are needed to keep neuronal and synaptic structures in place but appear to play a role in signaling, structural plasticity, and cognitive function [34]. Second, certain SAMs appear to work together synergistically, generating new functions that do not extend to the individual members alone. Case in point, different combinations of protocadherin family members form dimeric *cis*-complexes that oligomerize into larger tetrameric *trans*-complexes; the functional roles of these different species are still being worked out [57, 58]. Members of different families can also interact with each other in a mix-and-match approach. For example, cadherins bind each other to form *trans*-complexes spanning the synaptic cleft, but they also can bind protocadherins side-by-side forming *cis*-complexes [34].

Given such a broad portfolio of SAMs, how different are the proteins functionally or are many of them redundant? The extent to which different SAMs carry out substantially different functions or are redundant is controversial. Some SAMs clearly have discrete and different biological functions. For example, NLGN1 can induce synapse formation in young

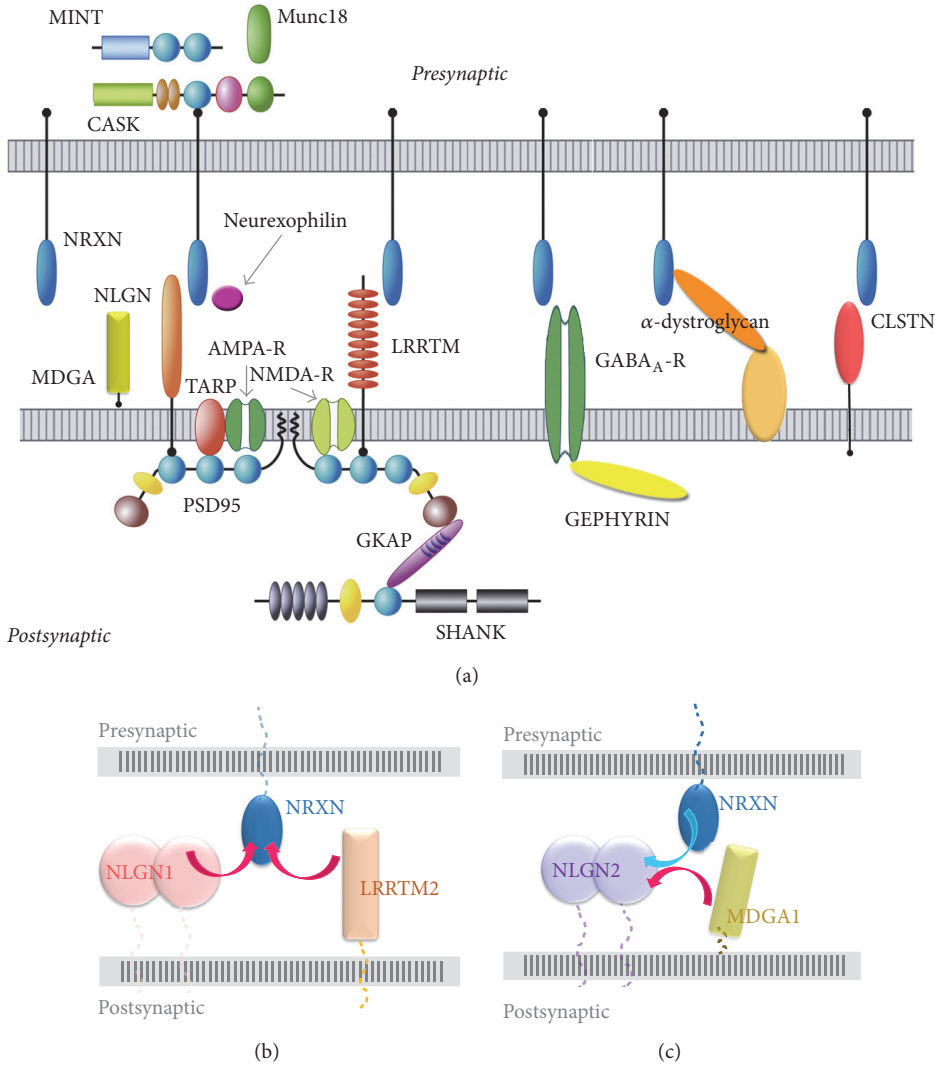

FIGURE 4: Synaptic protein interaction network coordinated by neurexins. (a) Neurexins (blue ovals) bind many protein partners tethered to the postsynaptic membrane including neuroligins, LRRTMs, α-dystroglycan, calsyntenins (CLSTN), and the GABA$_A$-receptor, as well as partners that are secreted such as neurexophilins. (b) NLGN1 and LRRTM2 can both bind neurexins at an overlapping binding site generating two competing *trans*-interactions. (c) Neurexins and MDGA1 can both bind NLGN2 at an overlapping binding site generating competing *cis*- and *trans*-interactions.

primary hippocampal cultures, but SynCAM1 cannot [59]. Members of the same SAM family can also have dramatically different roles; NLGN2 is found exclusively at inhibitory synapses, while NLGN1 is found predominantly at excitatory synapses [23]. Likewise, Slitrk1 and Slitrk3 promote excitatory versus inhibitory synapse formation, respectively [60, 61] However, equally so, SAMs can also demonstrate functionally redundant actions. For instance, LRRTM1, LRRTM2, NLGN1, and NLGN3, proteins that increase synapse numbers in vitro, appear functionally redundant because only knockdown of all four proteins together decreases the number of formed synapses significantly [62]. Thus, though many SAMs exist, their exact functional roles and the extent to which these are unique or overlap needs to be further investigated, both alone and in the broader context of the synaptic cleft.

The power of a broad portfolio of SAMs binding each other and sculpting interactomes within the synaptic cleft is beautifully illustrated by the complex interaction network that has been revealed centered on neurexins. Presynaptically tethered neurexins reach across the synaptic cleft to bind postsynaptic ligands such as the neuroligins, LRRTMs, and α-dystroglycan, forming *trans*-synaptic bridges (Figure 4(a)). Neurexins also recruit calsyntenins, though whether this interaction is direct or indirect is debated [14, 63, 64]. At excitatory synapses, neurexins extend across the synaptic cleft to bind LRRTMs or NLGN1 promoting excitatory synapse development [23, 65–67] (Figure 4(b)). Because these postsynaptic partners utilize the same or an overlapping binding surface on neurexin, LRRTM2 and NLGN1, for example, compete with each other for neurexin binding, though the

functional consequences are not clear [56, 65, 66]. In contrast, at inhibitory synapses, neurexins form a *trans*-synaptic interaction with NLGN2 promoting inhibitory synapse development [23]. However, competing with this interaction, the postsynaptic adhesion molecule MDGA1 binds NLGN2 tightly side-by-side forming a *cis*-complex on the dendritic surface that prevents the neurexin:NLGN2 *trans*-synaptic bridge, thereby decreasing inhibitory synapse development [16, 17] (Figure 4(c)). Also at inhibitory synapses, neurexin 1α binds to α-dystroglycan or neurexophilin 1 (NXPH1) in a mutually exclusive manner. One consequence of α-dystroglycan engaging the neurexin 1α L2 domain is that it prevents binding of neuroligins to the distant neurexin 1α L6 domain suggesting that an (allo)steric mechanism regulates these protein partner interactions [68], (refer back to Figure 1). Therefore, the neurexin-centered interactome provides examples of how SAMs can compete with each other for binding partners *in cis* or *in trans* and also be subject to (allo)steric mechanisms that regulate protein partner interactions.

It is possible that different SAMs interact with each other in a series of sequential and concerted steps to develop and regulate synapses; see also recent review by [69]. Revealing such a playbook of interactions will be no easy task because it is complex to accurately assess SAM function. Protein interactions that occur in vitro in a controlled experimental setting may not occur in vivo in the synaptic cleft or only under select circumstances. Likewise, SAM functions may exist in vivo that are not easily measurable in vitro. By way of illustration, neurexins are synaptogenic in vitro in coculture assays suggesting they are essential to form synapses, yet, in vivo, triple knockout of all three alpha- or beta-neurexins does not prevent synapse formation [70, 71]. Likewise, CNTNAP2, considered a *bona fide* SAM, does not appear important for synapse formation in and of itself, rather it prevents the elimination of new synapses in some way based on live imaging studies through cranial windows in mice [72]. Taken together, the broad portfolio of SAMs present in mammalian brain appears critical to generate diverse, adaptable protein interaction networks that mediate the different stages of a synapse, starting from its initial formation to its ultimate elimination, and to permit activity-dependent regulation once it has formed.

4.3. Diversification of SAMs through Alternative Splicing. One important mechanism to generate diversity of SAMs in the nervous system that deserves special attention is the process of alternative splicing, which has been shown to be regulated by synaptic activity in some cases (see (3) in Figure 3). Alternative splicing provides a very efficient and genetically "cost-effective" mechanism to generate a large panel of proteins that share a common scaffold but each differ from one another to some extent. Alternative splicing of mRNA transcripts result in insertions, deletions, and substitutions of amino acids in the encoded protein and can involve single residues, small inserts, or even complete domains. Several well-known SAM families undergo alternative splicing of their mRNAs generating a portfolio of protein molecules with altered properties and function.

Neurexins form one of the best studied families of SAMs diversified through alternative splicing. Neurexins are encoded by three genes (1, 2, and 3) that each produce a short beta form and a long alpha form, by virtue of two different promoters [23]; see also Figure 1. Single molecule mRNA sequencing of tens of thousands of neurexin mRNAs has demonstrated that there are at least ~1,400 variants by one report and more than 2,000 variants by another in the adult mouse brain [73, 74], though the transcripts are not all equally abundant [74]. Alternative splice inserts can be incorporated at six places in the extracellular region of neurexin 1α (SS#1 through SS#6), adding polypeptide inserts of up to 30 amino acids at five of these insertion sites; see Figure 1 and [23, 74]. Incorporation of splice inserts has functional consequences because several inserts have been shown to regulate the interaction of neurexins with different postsynaptic partners. For example, incorporation of SS#2 in the L2 domain of neurexin 1α decreases its binding to α-dystroglycan, while SS#4 regulates the affinity of neurexins to postsynaptic partners such as neuroligins, LRRTMs, α-dystroglycan, cerebellin precursor protein, and latrophilin/ADGRL (recently reviewed by [23, 68, 75]). Proteomic quantitation has confirmed that distinct neurexin splice variants bind different amounts of protein partners, corroborating a mechanism whereby alternative splicing regulates the binding affinity of neurexins for different ligands in vivo [76]. From a biochemical and protein structural perspective, SS#2 and SS#4 change the affinity of Ca^{2+}-binding sites central to protein interaction sites on the L2 and L6 domains, while SS#4 also induces structural plasticity because it can adopt multiple conformations [77–79]. From a functional perspective, mice engineered to constitutively include SS#4 in neurexin 3α show a decrease in synaptic strength and impaired LTP in vivo because postsynaptic AMPA-receptor levels are decreased at the synapse (as a result of increased AMPA-receptor endocytosis), although the underlying mechanism is not clear [80]. For most neurexin splice inserts, however, their effects on protein structure and function are not well delineated. Likewise, the function of rare neurexin splice variants, in which multiple domains are deleted, is also not known, nor if these yield functional proteins in the first place [74].

The very large portfolio of neurexin alternative splice forms is strategically positioned to play an important role in synaptic plasticity. In the mammalian brain, specific neurexin splice forms demonstrate cell type specific distributions and brain region specific expression both at the mRNA as well as the protein levels [73, 76, 81]. Importantly, incorporation of certain splice inserts is neuronal activity dependent, and an altered splicing profile can be reversed [82–84]. For example, analysis of mRNAs in single medium spiny neuron cells (MSNs) demonstrated that neurexin 1α and neurexin 1β are prevalent in D_1R-MSNs, but much less so in D_2R-MSNs, and mostly contain the SS#4 insert [81]. However, exposure to repeated cocaine administration, a circumstance triggering synaptic plasticity, reduces neurexin 1 mRNA levels in D_2R-MSNs even further and alters the profile of splice forms [81]. Therefore, alternative splicing of neurexins generates diversity of protein structure and function, and it can be regulated by events linked to synaptic plasticity.

Other SAM families are regulated by alternative splicing in their extracellular domain as well, altering the affinity with which they bind protein partners in the synaptic cleft. These include the neuroligins where splice inserts regulate interactions with neurexins (refer back to Figure 1, [85–88]); PTPδ and PTPσ where splicing regulates binding to Slitrks, interleukin-1 receptor accessory protein (IL1RAP), and SALM3 [89–94]; and the family of adhesion GPCRs where alternative splicing alters the domain composition of the extracellular region and consequently the profile of interacting protein partners [95].

4.4. Altered Location of SAMs within the Synaptic Cleft. The advent of powerful high resolution microscopy techniques has revealed that SAMs can be redistributed within the synaptic cleft in response to synaptic activity (see (4) in Figure 3). Recent studies show that the synaptic cleft is made up of structurally distinct subcompartments and SAMs can segregate to different regions of the cleft. Upon synaptic activity, however, certain molecules can move within or to the periphery of the synaptic cleft. The impact of these redistributions on synaptic function, however, is not clear. For instance, SynCAM1 and EphB2 receptor tyrosine kinase (EphB2) are two postsynaptic SAMs with different roles. SynCAM1 induces synapse formation and subsequently also maintains excitatory synapses, while EphB2 promotes excitatory synaptogenesis in the rapid early phase of synaptogenesis before neurons mature. By tracking SynCAM1 and EphB2 in the synaptic cleft at excitatory synapses, Perez de Arce and coworkers demonstrated that SynCAM1 is located around the cleft's edge while EphB2 is embedded deeper within the central PSD region [96]. Strikingly, upon application of an LTD paradigm, SynCAM1 underwent redistribution on the surface of the synaptic membrane forming puncta of increasing size, an intriguing finding given that SynCAM1 regulates LTD in vivo and suggesting this redistribution has functional significance [96]. Another SAM, N-cadherin, forms *trans*-synaptic bridges with N-cadherin molecules tethered to the opposing synaptic membrane. N-cadherin plays an important role presynaptically by regulating synaptic vesicle recruitment and recycling, and postsynaptically in spine remodeling and trafficking of AMPA-Rs, which is important for hippocampal LTP [97]. Superresolution microscopy has shown that N-cadherin localizes predominantly as puncta at the periphery of synapses and to a much lesser extent along the synaptic cleft in unstimulated cultured hippocampal neurons [97]. However, upon synaptic stimulation followed by a rest period, N-cadherin distributes broadly throughout the synaptic cleft [97]. Thus an increasing body of work shows that SAMs can be redistributed as a result of synaptic activity, likely altering protein interactomes in the synaptic cleft. How different SAMs are redistributed and the impact of such redistribution on synaptic function remain to be further elucidated.

4.5. Astrocytic Control of SAMs. A fascinating development has been the demonstration that astrocytes (a type of glial cell found interspersed between neurons which can ensheath synapses) secrete factors that modulate the action of SAMs (see (5) in Figure 3). During the development of the nervous system, astrocytes regulate synapse formation and remodeling, impacting synapse number through their ability to promote the formation and elimination of synapses [98]. A single mouse astrocyte can ensheath more than 100,000 synapses [99]. In the mature brain, astrocytes also can modulate synaptic plasticity [98]. Immature astrocytes secrete thrombospondin 1 and thrombospondin 2 (TSP-1 and TSP-2), large, trimeric extracellular matrix proteins that promote the formation of silent synapses in vitro and in vivo (i.e., synapses that are presynaptically active, but postsynaptically silent because they lack functional AMPA-Rs) [100]. TSP1 can bind postsynaptic neuroligins, increasing the speed of excitatory synapse formation at early stages in cultured rat hippocampal neurons, although not the final density of the synapses formed in mature neurons [101]. Hevin, another protein secreted by astrocytes, can modify the interaction between two SAMs in the synaptic cleft by working as an adaptor protein [102]. Hevin binds directly to neurexin 1α and NLGN1(+B), a pair of SAMs that normally do not interact, and engages them in a *trans*-synaptic bridge promoting excitatory synapse formation [102]. It is thought that the nine-amino-acid splice insert at site B in NLGN1(+B) sterically blocks the interaction between NLGN1 and the sixth LNS domain of neurexin 1α (L6) (refer back to Figure 1) thereby forming a key component of the "neurexin-neuroligin splice code," reviewed in [23]. The bridging of neurexin 1α and NLGN1(+B) by hevin, overriding the splice code, was shown to be critical to form thalamocortical connections in the developing visual cortex in vivo [102]. Therefore, astrocytes by secreting proteins that interact with *bona fide* SAMs can modify their interactions and regulate protein interactomes in the synaptic cleft.

4.6. Novel Mechanisms to Regulate SAMs. It is likely that additional novel mechanisms exist that regulate SAMs, impacting their function in synaptic activity-dependent ways. One tantalizing mechanism is that SAMs undergo protein structural changes in response to synaptic activity. Perhaps mechanisms will be validated confirming that SAMs can sense synaptic activity in the synaptic cleft and adjust their protein interactions in response via (allo)steric mechanisms. Certainly, incorporation of an alternative splice insert in a SAM in response to synaptic activity (as discussed above) would be one way to induce a protein conformational change. Such a splice insert driven conformational change would have the potential to alter protein interactions within the synaptic cleft. The splice inserts SS#1 and SS#6 in neurexin 1α are of interest in this respect because they integrate into molecular hinges within the neurexin ectodomain and are poised to alter the conformation of domains with respect to one another. However, it is not known yet if these splice inserts are subject to activity-dependent incorporation [74, 103]. A novel protein conformation or interaction site in a SAM might also be induced upon binding of a protein partner and controlled though synaptic activity-induced expression of that partner (refer back to Figures 2(b) and 2(c)). Neuronal activity-induced expression of α-dystroglycan [40], which binds the L2 domain of neurexin 1α and appears to sterically block the interaction of neurexin 1α with neuroligins via the L6

domain, is a prime example [68] (refer back to Figure 1). Synaptic stimulation also appears to induce homodimerization of N-cadherin, an event altering the overall protein architecture [104]. Lastly, the protein conformation of a SAM containing Ca^{2+}-binding sites might also be altered by changes in Ca^{2+} levels in the synaptic cleft as a result of synaptic activity, affecting its interactions with protein partners. Experimental evidence is accumulating that Ca^{2+} levels decrease in the synapse cleft in response to (prolonged) synaptic activity, a result of Ca^{2+} flooding into the presynaptic terminal during synaptic vesicle release and/or into the postsynaptic terminal upon NMDA-receptor activation [105, 106]. It has been suggested that the extracellular Ca^{2+}-level in the synaptic cleft is ~1 mM and can drop significantly, maybe as much as 30–60% as presynaptic and postsynaptic channels open [107]. Studies on *trans*-complexes of cadherins have shown that their interactions depend in part on extracellular Ca^{2+} levels and are rapidly decreased when extracellular Ca^{2+} is depleted [108]. Thus, additional and novel mechanisms to regulate SAMs in response to synaptic activity may be validated in the near future.

5. SAMs Are Implicated in Neuropsychiatric and Neurodevelopmental Diseases

Many SAMs, including neurexins, neuroligins, LRRTMs, and other leucine rich repeat containing proteins, contactins, CNTNAPs, and cadherins are now implicated in neuropsychiatric and neurodevelopmental diseases, such as autism spectrum disorder, schizophrenia, bipolar disorder, epilepsy, and mental retardation [8, 20, 22, 24, 26, 109]. Initially, it was speculated that these molecules played crucial roles in the formation of synapses, and their lesion would lead to large scale disruption of synapse formation. Nevertheless, it was puzzling why deficits in such molecules, if indeed so essential for synapse formation, were selectively linked to cognitive and behavioral disorders, leaving other brain functions such as the coordination of movement or the processing of auditory and visual information apparently undisturbed. It is now recognized that there is a very large portfolio of SAMs in the mammalian brain, and there is not one single SAM, which when deleted, is sufficient to prevent synapse formation on a large scale given their partially redundant and overlapping functions. Furthermore, we now realize that the function of SAMs is much more complex and nuanced than purely the adhesion of presynaptic and postsynaptic membranes, as discussed in this review. It is also clear that various SAMs have discrete localization to very select groups of synaptic contacts, imparting their functional role in a synapse selective way. Recent work is focusing on unravelling the exact contribution of different SAM family members at specific synaptic contacts in order to understand how they mediate select neural circuits; see, for example, [110]. In addition, increasing attention is being paid to SAMs that selectively localize to excitatory or inhibitory synapses, respectively. An imbalance in excitatory (E) versus inhibitory (I) synaptic transmission has been speculated to play a role in the pathogenesis of neuropsychiatric disease, though whether this is a root cause or a result of other molecular processes that have been disrupted is not clear; for a recent review see [111]. Importantly, altering the level of select SAMs in animal models alters excitatory and/or inhibitory transmission (affecting the E/I balance) and leads in parallel to cognitive and social deficits, recently reviewed in [20, 112, 113]. In summary, it is still largely unknown how exactly the different SAMs contribute to the molecular mechanisms that underlie the pathogenesis of neuropsychiatric and neurodevelopmental diseases. Certainly, determining for each implicated SAM (1) at which synapses the SAM is present, (2) what its role is in developing, as well as mature brain, and (3) how the SAM is dynamically regulated will provide vital information to assess the role of that particular SAM in cognitive and behavioral disorders.

6. Conclusion

SAMs play a key role in establishing and maintaining synapses; they are involved in synapse formation, development, maturation, and elimination. Through their roles at synapses, SAMs are in position to impact the flow of information throughout the brain and beyond. Exciting work is being done to investigate the extent to which SAMs respond to synaptic activity modifying their protein interactions and function. Because SAMs are implicated in neuropsychiatric and neurodevelopmental disorders, studying their precise molecular mechanisms and interaction modes with their partners holds promise that this information can eventually be leveraged to design completely novel therapeutic strategies that regulate aberrant synaptic communication.

Competing Interests

The author declares that they have no competing interests.

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Research Article

Pilocarpine-Induced Status Epilepticus Is Associated with Changes in the Actin-Modulating Protein Synaptopodin and Alterations in Long-Term Potentiation in the Mouse Hippocampus

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Epilepsy is a complex neurological disorder which can severely affect neuronal function. Some patients may experience status epilepticus, a life-threatening state of ongoing seizure activity associated with postictal cognitive dysfunction. However, the molecular mechanisms by which status epilepticus influences brain function beyond seizure activity remain not well understood. Here, we addressed the question of whether pilocarpine-induced status epilepticus affects synaptopodin (SP), an actin-binding protein, which regulates the ability of neurons to express synaptic plasticity. This makes SP an interesting marker for epilepsy-associated alterations in synaptic function. Indeed, single dose intraperitoneal pilocarpine injection (250 mg/kg) in three-month-old male C57BL/6J mice leads to a rapid reduction in hippocampal SP-cluster sizes and numbers (in CA1 stratum radiatum of the dorsal hippocampus; 90 min after injection). In line with this observation (and previous work using SP-deficient mice), a defect in the ability to induce long-term potentiation (LTP) of Schaffer collateral-CA1 synapses is observed. Based on these findings we propose that status epilepticus could exert its aftereffects on cognition at least in part by perturbing SP-dependent mechanisms of synaptic plasticity.

1. Introduction

Epilepsy subsumes a group of serious disorders of the central nervous system characterized by a predisposition to recurrent unprovoked seizures, that is, abnormal excessive synchronous neural activity [1]. Several forms of epilepsy are currently distinguished, with temporal lobe epilepsy being among the most resistant ones to pharmacological treatment [2, 3]. If not treated (or not responding to medication), some patients with epilepsy will experience an episode of prolonged seizure activity, status epilepticus (SE), which is a life-threatening condition [4, 5]. While SE represents an exacerbation or even initial manifestation of epileptic disorders, it can also result from other brain pathologies, such as traumatic brain injury or stroke [6, 7]. Although aggressive treatment may halt SE, surviving patients experience severe postictal cognitive dysfunctions, such as memory deficits and spatial/temporal disorientation [2, 8–11].

Animal models of SE have been employed to demonstrate that ongoing epileptic activity in the brain can affect both excitatory and inhibitory synapses and thus neuronal plasticity [12–15]. However, the molecular mechanisms and cellular targets through which SE affects synaptic plasticity remain not well understood. Here, we employed the well-established model of pilocarpine-induced status epilepticus, which can be linked to alterations in memory performance [11], to study the effects of SE on synaptopodin (SP [16]) and synaptic plasticity.

SP is an actin-modulating protein expressed in cortical principal neurons [16]. It is a marker and essential component for the spine apparatus organelle and has been firmly linked to the ability of neurons to express synaptic plasticity (e.g., [17–19]). Hence, in an attempt to establish SP as a marker for epilepsy-associated alterations in synaptic plasticity, we tested whether SE-induced alterations in synaptic plasticity (e.g., [20]) are accompanied by *in vivo* changes of SP.

2. Materials and Methods

2.1. Animals and Seizure Staging. Animal handling was approved by the Institutional Animal Care and Use Committee at the Chaim Sheba Medical Center, which adheres to the national law and NIH rules. Briefly, SE was induced in 3-month-old male C57BL/6J mice by a single intraperitoneal (ip) injection of 250 mg/kg pilocarpine hydrochloride. In order to avoid side effects induced by peripheral cholinergic activation, mice were treated with atropine sulphate monohydrate (1 mg/kg, ip) 30 minutes before pilocarpine injection, while diazepam (3 mg/kg; ip) was used to halt convulsions prior to experimental assessment (90 min after pilocarpine injection; Figure 1(a)). After pilocarpine injection, behavioral seizure activity was documented every 10 minutes by an investigator blind to experimental conditions using the modified Racine's stages (c.f. [20]; 0 = no seizures, 1 = freezing, 2 = single twitches, 3 = orofacial seizures, 4 = clonic seizures, 5 = tonic seizures, and 6 = death). Control groups received atropine, diazepam, and vehicle-only (instead of pilocarpine). Animals were subjected to further experimental assessment 3–5 min after diazepam injection.

2.2. Immunostaining and Microscopy. Deeply anaesthetized mice were transcardially perfused with 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS, 0.1 M, pH 7.4) for 20 min. Brains were removed and postfixed in the same fixative for at least 24 h. Serial coronal sections (50 μm) were cut with a vibratome (VT 1000S, Leica, Bensheim, Germany) and stained with an antibody against SP as previously described [19, 21]. Briefly, sections containing the dorsal hippocampus were incubated for 1 h with 10% (v/v) normal goat serum (NGS) in 0.5% (v/v) Triton X-100 containing PBS to reduce unspecific staining and subsequently incubated for 48 h at 4°C in rabbit anti-SP antibody (1 : 1000 in 10% (v/v) NGS, 0.1% (v/v) Triton X-100; SE-19, Sigma Aldrich). Sections were washed and incubated for 3 h with Alexa 488-labeled donkey anti-rabbit antibody (1 : 1000, 10% (v/v) NGS, 0.1% (v/v) Triton X-100; Invitrogen). TO-PRO® (Invitrogen) nuclear stain was used to visualize cytoarchitecture (1 : 5000 in PBS; 10 min). Sections were washed again, transferred onto glass microscope slides, and mounted with antifading mounting medium (DAKO Fluoromount). Confocal images were acquired using a Nikon Eclipse C1si laser-scanning microscope equipped with a 60x oil-immersion objective lens (NA 1.4; Nikon). All high resolution images (60x objective lens; 7x scan zoom) were acquired at tissue levels ~5 μm below the surface using the exact same settings at the microscope (detector gain and amplifier were initially set to obtain pixel densities within a linear range).

2.3. RNA Extraction and Quantitative PCR (qPCR). Mice were deeply anaesthetized using ketamine/xylazine (100 mg/kg and 10 mg/kg, resp.) and rapidly decapitated. Whole hippocampi were isolated and immediately frozen in liquid nitrogen. Tissue was transferred into ice-cold TRIzol® and homogenized. RNA was extracted via phenol-chloroform phase separation and eluted with Bio-Rad Aurum 732-6820 kit (Bio-Rad, CA, USA). 1 μg of total RNA was used for reverse transcription using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Rhenium, Israel). qPCR was performed using the StepOnePlus™ Real-Time PCR system (Applied Biosystems). Targets were amplified using SYBR Green with Hypoxanthine Guanine Phosphoribosyltransferase as reference gene (HPRT: forward primer: 5′-TGAAAGACTTGCTCGAGATGTCA-3′; reverse primer: 5′-CACACAGAGGGCCACAATGT-3′; SP: forward primer: 5′-GTCTCCTCGAGCCAAGCA-3′; reverse primer: 5′-CACACCTGGGCCTCGAT-3′). A standard qPCR protocol was used: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The average CT values (mean ± standard deviation) of synaptopodin gene expression in control and SE were 21.65 ± 0.35 and 21.76 ± 0.15, respectively.

2.4. Electrophysiology. Mice were deeply anaesthetized with ketamine/xylazine (100 mg/kg and 10 mg/kg, resp.) before rapid decapitation. After removing the brain, 400 μm coronal slices containing the dorsal hippocampus, which expresses robust long-term potentiation under physiological conditions (c.f. [22, 23]), were prepared using a vibratome (World Precision Instruments). Slices were consecutively incubated for 1.5 h in a humidified, carbogenated (5% CO_2 and 95% O_2) gas atmosphere at 33 ± 1°C and were superfused with artificial CSF (containing (in mM) 124 NaCl, 2 KCl, 26 $NaHCO_3$, 1.24 KH_2PO_4, 2.5 $CaCl_2$, 2 $MgSO_4$, and 10 glucose, pH 7.4) in a standard interface chamber. Recordings were made with a glass pipette containing 0.75 M NaCl (4 MΩ) placed in the stratum radiatum CA1. Stimulation was evoked using a Master 8 pulse stimulator (A.M.P.I., Jerusalem, Israel) and was delivered through a set of bipolar nichrome electrodes placed on a side of the recording electrode. LTP was induced by high-frequency stimulation consisting of 100 pulses at twice the test intensity, delivered at a frequency of 100 Hz (HFS; 100 Hz, 1 s; [24]). Before applying the tetanic stimulation, baseline values were recorded at a frequency of 0.033 Hz. Responses were digitized at 5 kHz and stored on a computer.

2.5. Quantification and Statistics. qPCR data were analyzed using HPRT as reference gene. Analysis was performed according to Pfaffl [25]. The qPCR assay efficiency was calculated with the StepOnePlus software (Applied Biosystems, USA) based on dilution series of five samples for each assay. Values were normalized to vehicle-treated controls. Immunolabeled SP-clusters were analyzed in stratum radiatum of area CA1 using the Image J software package (available from http://rsb.info.nih.gov/ij) [26, 27]. SP-clusters were assessed in single plane confocal images by setting the same threshold value and minimal pixel size for all images using the "analyze particles" function of ImageJ software. Three visual fields in CA1 stratum radiatum were analyzed in each slice and SP-cluster sizes and numbers were averaged for each hippocampus. Values were normalized to the results obtained in age- and time-matched vehicle-treated control animals.

Electrophysiological data were analyzed offline using Spike 2 software. Field excitatory postsynaptic potential fEPSP slope changes after tetanic stimulation were calculated with respect to baseline. Statistical comparisons between the two groups were performed using a t-test (unpaired, two-tailed). Immunostainings and qPCR analysis were statistically compared using the nonparametric Mann-Whitney test, since normal distribution of these data cannot be assured. P values smaller 0.05 were considered a significant difference. In the text and figure values represent mean ± standard error of the mean (SEM), unless indicated otherwise. Values from seizure staging represent median ± interquartile range. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; nonsignificant differences are indicated by "NS."

3. Results

Adult 3-month-old C57BL/6J animals were injected with atropine (1 mg/kg; i.p.) 30 min before a single dose of pilocarpine (250 mg/kg; ip) to induce stable SE. Behavioral seizure staging was documented using the modified Racine's stages (Figure 1). Intraperitoneal injection of 250 mg/kg pilocarpine induced rapid-onset and stable behavioral seizure activity (Figure 1(a)). After 90 minutes, 42.3% (11 animals out of 26) of all animals were in stages 1–3 and 38.5% (10 animals out of 26) were in stage 4 or 5 of the modified Racine's stages. 5 out of 26 animals died during experimental procedure (Figure 1(b); these animals were not included in further analysis). After 90 minutes, diazepam was used (3 mg/kg; ip) to halt convulsions prior to experimental assessment. We did not observe any apparent correlation between seizure stage and both effects on LTP-alterations and changes in synaptopodin cluster properties. Age- and time-matched control animals were treated in the same way except for pilocarpine injection, which was replaced by injecting the exact same volume of vehicle-only. Changes in SP were assessed in CA1 stratum radiatum of the dorsal hippocampus and LTP was probed at Schaffer collateral-CA1 synapses to correlate SE-induced changes in SP with alterations in associative synaptic plasticity.

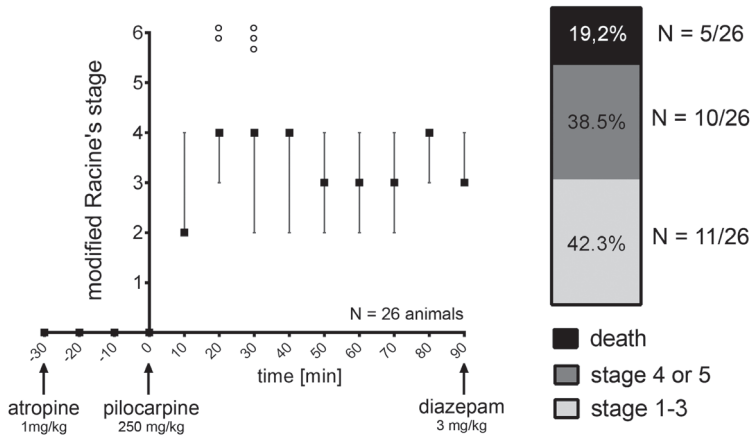
3.1. Pilocarpine-Induced Status Epilepticus Decreases Synaptopodin Cluster Sizes and Numbers. Anatomically matched coronal brain slices containing the dorsal hippocampus were immunostained for SP (Figure 2(a)) to assess SE-induced changes in SP-cluster properties. Sizes and numbers of SP-clusters were determined in confocal images of CA1 stratum radiatum in pilocarpine-treated animals and controls (Figure 2(b); cf., LTP of Schaffer collateral-CA1 synapses, Figure 3). Indeed, a significant reduction in both cluster sizes and numbers following pilocarpine-induced status epilepticus was observed in these experiments (Figure 2(c)). Particularly, the decrease in SP-cluster numbers was prominent (−80%). These results disclose that SP-clusters are severely affected after SE-induction.

3.2. Synaptopodin mRNA Levels Are Not Changed after Pilocarpine-Induced SE. We next speculated that SE-induced alterations in SP-cluster properties could be accompanied by changes in SP-mRNA levels. Hence, we repeated SE-experiments (and vehicle-injections) in a different set of mice and isolated the hippocampi to assess differences in mRNA levels using qPCR (Figure 2(d)). HPRT served as a reference gene in these experiments. Although a trend toward reduced SP-mRNA levels was observed, the change did not reach the level of significance (Figure 2(d); Mann-Whitney test; $P = 0.63$). We conclude that the SE-induced changes in SP-cluster sizes and numbers are not the result of a strong reduction in SP-mRNA levels.

3.3. SE-Induced Changes in Synaptopodin Clusters Reflect SE-Induced Alterations in LTP. Based on our previous work, which disclosed an interdependency between SP and the ability of neurons to express synaptic plasticity [17–19, 21], we hypothesized that SE-induced changes in SP-cluster sizes/numbers will be associated with alterations in the expression of synaptic plasticity. Hence, LTP at Schaffer collateral-CA1 synapses was probed in anatomically matched dorsal hippocampal slices of pilocarpine- and vehicle-injected animals (Figure 3(a)). Notably, baseline synaptic transmission was not affected after SE-induction: both fiber volleys and input/output properties were not significantly different between the two groups (Figure 3(b)). After obtaining stable baseline recordings, tetanic stimulation (1 s; 100 Hz) was used to induce LTP. Indeed, we observed an LTP-defect in SE-treated animals as predicted by the reduction in SP-clusters (Figure 3(c)) and our previous investigations [17, 18, 21]. We conclude that SE-induced changes in SP are accompanied by a deficit in the ability of neurons to express associative synaptic plasticity.

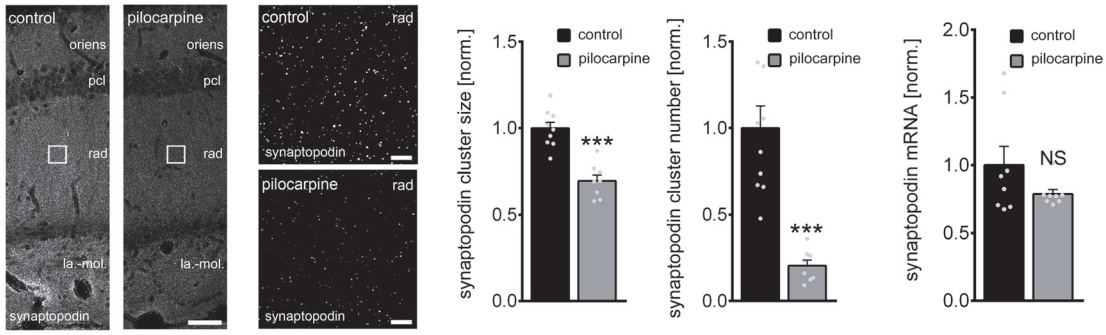
4. Discussion

SP has been firmly linked to the ability of neurons to express synaptic plasticity [28–31]: SP-deficient mice exhibit a deficit in the ability to induce LTP [17] as well as homeostatic synaptic plasticity [19]. Moreover, evidence has been provided that SP affects dendritic spine plasticity [18, 30]. Despite these intriguing phenotypes, SP-deficiency seems not to affect spine numbers and baseline synaptic transmission, since both



(a) (b)

Figure 1: Intraperitoneal pilocarpine injection leads to stable behavioral seizure activity. (a) After a single dose of intraperitoneal pilocarpine injection (250 mg/kg), behavioral seizure activity was documented every 10 minutes using the modified Racine's stages. Stage 6 animals (indicated by black circles) were not included in the analysis.(stage 0 = no seizures, 1 = freezing, 2 = single twitches, 3 = orofacial seizures, 4 = clonic seizures, 5 = tonic seizures, and 6 = death). (b) 90 minutes after pilocarpine injection 42.3% of the animals were in stages 1–3 and 38.5% were in stage 4 or 5 of the modified Racine's stages. 5 out of 26 animals died during experimental procedure and were not included in further analysis (N = 26 animals; stages 1–3: 11/26 animals, stage 4 or 5: 10/26 animals, death: 5/26 animals).



(a) (b) (c) (d)

Figure 2: Pilocarpine-induced status epilepticus (SE) affects synaptopodin clusters in CA1 stratum radiatum. (a–c) Synaptopodin cluster sizes and numbers were assessed in CA1 stratum radiatum of anatomically matched coronal sections containing the dorsal hippocampus from pilocarpine- or vehicle-only-treated animals. $N_{control}$ = 10 hippocampal slices of 5 animals, $N_{pilocarpine}$ = 8 hippocampal slices of 4 animals; both hippocampi in each animal analyzed; averaged values from 3 images obtained from each slice (stratum oriens, oriens; stratum pyramidale, pcl; stratum radiatum, rad; stratum lacunosum-moleculare, la.-mol.). Values normalized to control. Gray dots indicate individual data points (one data point outside the axis limits for control synaptopodin cluster number). Mann-Whitney test; $^{***}P < 0.001$. Scale bars, in (a) 50 μm, in (b) 4 μm. (d) Synaptopodin mRNA levels are not significantly changed after single episode of SE. The results are normalized to HPRT gene expression within the same cDNA sample and are represented as the relative levels of the mean ± SEM versus control. N = 8 hippocampi from 8 animals of each group. Mann-Whitney test; P = 0.63, NS, not significant difference.

input/output properties of field potential recordings and single cell excitatory synaptic current recordings are not altered in SP-deficient preparations [17, 19, 32]. Hence, SP reflects the ability of neurons to express synaptic plasticity and not basic synaptic properties. This suggestion is supported by the results of the present study on unaltered baseline synaptic transmission after SE-induction (Figure 3(b)), despite severe changes in SP-cluster sizes/numbers in response to a single episode of SE (Figure 2(c)).

The signals which regulate SP-expression and SP-clustering under physiological and pathological conditions remain not well understood. While evidence has been provided that the induction of LTP leads to an NMDA-R dependent increase in SP-mRNA and SP-protein levels [28, 29], an inverse interrelation between synaptic activity and SP has also been demonstrated [19]. In this earlier study prolonged inhibition of NMDA-Rs (or voltage sensitive Ca^{2+}-channels) caused an increase in SP-cluster sizes without affecting baseline synaptic transmission [19]. Hence, SP appears to be regulated by Ca^{2+}-dependent positive- and negative-feedback mechanisms. Although it remains to be shown how distinct temporal (and spatial) Ca^{2+}-signals

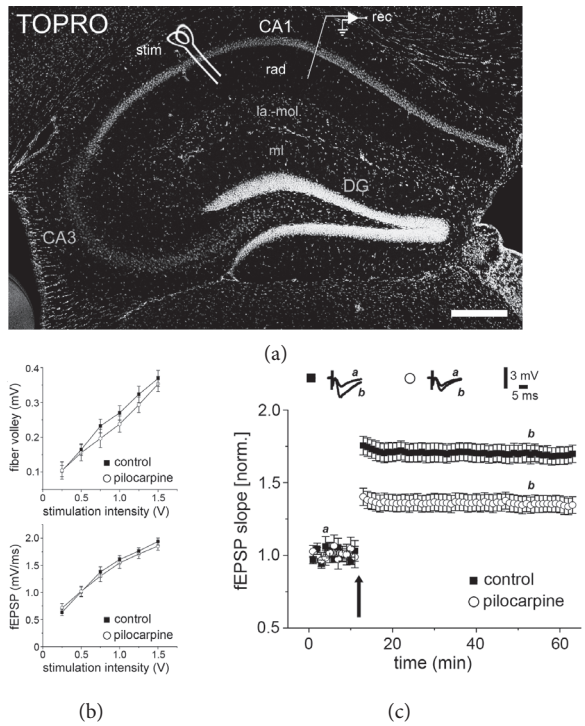


Figure 3: Pilocarpine-induced status epilepticus affects long-term potentiation (LTP) of Schaffer collateral-CA1 synapses. (a–c) LTP of Schaffer collateral-CA1 synapses was probed in acute dorsal hippocampal slices. A single 100 Hz tetanus (1 s) is applied (stim), to induce LTP (recordings of field excitatory postsynaptic potentials; fEPSP) in slices prepared from either pilocarpine- or vehicle-injected animals (stratum radiatum, rad; stratum lacunosum-moleculare, la.-mol.; stratum moleculare, mL; dentate gyrus, DG; nuclear stain, TO-PRO; scale bar: 200 μm). While no significant difference in fiber volleys and input/output properties is detected (b), a deficit in the ability to induce LTP at Schaffer collateral-CA1 synapses is observed in the pilocarpine group (c). Values normalized to baseline fEPSP slope. $\text{control}_{50\,\text{min}} = 1.69 \pm 0.06$, $\text{pilocarpine}_{50\,\text{min}} = 1.35 \pm 0.05$; $P = 0.001$; $N = 9$ slices (1 slice per animal) of each group. t-test (unpaired, two-tailed).

affect SP, the results of the present study are consistent with a homeostatic, that is, negative-feedback mechanism: prolonged seizure activity leads to a rapid reduction in SP-cluster sizes/numbers. Notably, previous work has demonstrated increased NMDA-R currents following pilocarpine-induced SE, which may support our hypothesis on Ca^{2+}-dependent negative-feedback mechanisms [33–35]. In addition, we have recently reported a similar effect on SP-clusters in a model of systemic inflammation using ip injection of lipopolysaccharides (LPS) [21]. Considering that SE-induction has been linked to neuroinflammation [36], it will be important to compare SE- and LPS-induced changes in SP and to determine the role of cytokines in SP-cluster regulation.

The consequences of SE-induced alterations in SP-cluster properties and LTP-induction need to be addressed in future studies. On one hand, alterations in LTP may underlie cognitive dysfunction seen in the context of SE [11, 12]. On the other hand, it is important to also consider that a reduction in the ability of neurons to express LTP may protect the network from maladaptive changes and circuit reverberation. Hence, it will be helpful to better understand whether restoring the ability of neurons to express LTP is detrimental or beneficial for the course of the disease. Thus, it is interesting to speculate that SE-induced alterations in LTP may protect neuronal networks from the development of epilepsy at the expense of cognitive function. However, at this point we have to concede that we do not know if SE-induced alterations in SP-expression are observed also in other brain regions, for example, in the ventral hippocampus, and whether changes in SP strictly reflect only alterations in associative synaptic plasticity [19, 27].

Regardless of these considerations the results of the present study are in line with an earlier report on the effects of kainic acid-induced SE on SP [37]. In this previous study, a semiquantitative assessment of postictal SP-changes in CA1 disclosed no changes in SP-mRNA levels but reduced SP-immunoreactivity upon ip kainic acid-injection. Apparently, SE-induced changes in SP are not only seen in the pilocarpine-induced model of temporal lobe epilepsy.

5. Conclusion

The results of the present study demonstrate that SE induces the remodeling of SP-clusters and leads to alterations in associative synaptic plasticity. Based on these results and previous work on the role of SP in synaptic plasticity and behavioral learning [17, 38], we propose that SP could be one of the neuronal targets through which prolonged seizures affect the ability of neurons to express synaptic plasticity. Although more work is required to unravel the precise mechanisms which regulate SP under SE conditions, and to better understand the role of SP (and synaptic plasticity) in epilepsy, we are confident to propose that SP may serve as a marker molecule for seizure-induced alterations in the ability of neurons to express synaptic plasticity, that is, SE-related synaptopathies.

Disclosure

Andreas Vlachos and Nicola Maggio are joint senior authors.

Competing Interests

The authors declare that there are no competing interests.

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Review Article

The Contribution of α-Synuclein Spreading to Parkinson's Disease Synaptopathy

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Synaptopathies are diseases with synapse defects as shared pathogenic features, encompassing neurodegenerative disorders such as Parkinson's disease (PD). In sporadic PD, the most common age-related neurodegenerative movement disorder, nigrostriatal dopaminergic deficits are responsible for the onset of motor symptoms that have been related to α-synuclein deposition at synaptic sites. Indeed, α-synuclein accumulation can impair synaptic dopamine release and induces the death of nigrostriatal neurons. While in physiological conditions the protein can interact with and modulate synaptic vesicle proteins and membranes, numerous experimental evidences have confirmed that its pathological aggregation can compromise correct neuronal functioning. In addition, recent findings indicate that α-synuclein pathology spreads into the brain and can affect the peripheral autonomic and somatic nervous system. Indeed, monomeric, oligomeric, and fibrillary α-synuclein can move from cell to cell and can trigger the aggregation of the endogenous protein in recipient neurons. This novel "prion-like" behavior could further contribute to synaptic failure in PD and other synucleinopathies. This review describes the major findings supporting the occurrence of α-synuclein pathology propagation in PD and discusses how this phenomenon could induce or contribute to synaptic injury and degeneration.

1. Introduction

Pathological accumulation of α-synuclein in the brain is a typical neuropathological hallmark of Parkinson's disease (PD), a complex neurodegenerative disorder characterized by motor disability that derives from the neurodegeneration of nigrostriatal dopaminergic neurons. The presence of Lewy bodies (LB) and Lewy neurites (LN), proteinaceous inclusions whose main constituent is fibrillary-aggregated α-synuclein, is a defining neuropathological alteration observed in the brain of affected patients. In the last few years, it has become evident that PD may be considered as a synaptopathy [1, 2]. Indeed, striatal dopaminergic terminal loss appears to precede neurodegeneration in the substantia nigra [3, 4] and the deposition of α-synuclein, which is considered as a causative factor for the onset of the disorder, mainly affects synaptic terminals in its early stages [5, 6]. Nonetheless, the exact molecular mechanisms that determine the selective vulnerability of nigrostriatal synapses to α-synuclein deposition are still enigmatic. One of the reasons for the reduced resilience of dopaminergic terminals to α-synuclein accumulation may be the fact that dopamine neurons of the substantia nigra show relatively large axonal lengths with 10–100 times the number of synapses of neighboring neuronal cells [7]. This implies that these cells require an elevated energy demand to efficiently sustain the trafficking of organelles and vesicles to allow proper synapse functioning [8]. Hence, they might result particularly susceptible even to subtle homeostatic changes at synaptic sites, which constitute their major source of energy consumption.

Compelling evidence indicates that α-synuclein can spread from diseased to healthy cells, thus contributing to disease worsening [9]. Indeed, graft-derived dopamine neurons can develop LB pathology several years after transplantation [10–12], an event that can be responsible for the loss of beneficial effects of cell therapy along with time. In addition,

both neurons and astrocytes have been found to internalize disease-associated α-synuclein in the postmortem brain of subjects with Lewy pathology, suggesting prion-like spread of α-synuclein by uptake from surrounding structures [13].

It is worth noting that the progression of PD symptoms seems to correlate with the topographical brain propagation of α-synuclein pathology between anatomically interconnected brain areas [14, 15], suggesting the occurrence of trans-synaptic spreading of pathology. This is not surprising when considering that α-synuclein is a synaptic-vesicle associated protein [16]. Studies on experimental models of PD have confirmed the occurrence of trans-synaptic transmission of pathological α-synuclein *in vivo* [17–20]. In light of evidences supporting host-to-graft α-synuclein passage, as well as the ability of the protein to propagate protein misfolding in recipient cells, a "prion-like" hypothesis of PD has been postulated [21–29]. Indeed, prions are transmissible misfolded conformers of the prion protein, PrP, which seed further generation of infectious proteins [30]. However, the mechanisms mediating α-synuclein release, uptake, and behavior in recipient cells deserve further investigation to definitely assert that α-synuclein behaves like a prion. For instance, not all the postmortem analyses on PD cases seem to confirm the caudo/rostral spread of α-synuclein pathology throughout the brain described by Braak [14, 15]. Moreover, the ability to seed aggregation in recipient cells has been found to be dependent on high concentration of aggregates in the face of the need of low amount of aggregates to induce cytotoxicity [31]. These evidences pose into question both the trans-synaptic spreading hypothesis and the prion-like behavior of α-synuclein. Nonetheless, cell-to-cell transmission of α-synuclein can occur with Ca^{2+}-dependent exosome-mediated release [32] or nonclassical exocytosis [33] as the most plausible candidate mechanisms for α-synuclein ejection. Finally, what could be the transmissible form of the protein that can mediate toxicity and/or function as seed for "misfolding" propagation in recipient cells between monomeric, oligomeric, and fibrillary α-synuclein still needs to be clarified. In this review, we describe the features of α-synuclein pathology spreading in PD and discuss how this could contribute to synaptic damage.

2. Alpha-Synuclein Function at the Dopamine Synapse

Alpha-synuclein was first identified in the synaptic vesicles and nuclei of the electric organ of *Torpedo Californica* [34]. The protein is highly expressed in presynaptic terminals of the brain and peripheral nervous system, where it associates with the synaptic vesicle apparatus [35–37]. The expression of α-synuclein is elevated within the synapses of nigral dopamine neurons [38]. There, the protein can modulate synaptic dopamine release by directly modulating the dopamine transporter (DAT), synapsin III, the small GTP-binding protein Rab3A, and the soluble N-ethylmaleimide sensitive fusion attachment protein receptor (SNARE) protein member vesicle associated membrane protein-2 (VAMP-2) [6]. Indeed, it catalyzes the entry of VAMP-2 into the SNARE complex [39] and enhances DAT localization at the plasma membrane [40], which consistently is impaired by α-synuclein aggregation [41, 42]. The toxicity exerted by α-synuclein deposition in dopaminergic neurons is rescued by Rab3A expression, suggesting that this protein is relevant for its normal function [43]. Conversely, α-synuclein membrane association is regulated by the Rab3A recycling machinery and presynaptic activity [44]. Finally, synapsin III distribution and expression in dopaminergic neurons is regulated by α-synuclein, while synapsin III gene silencing inhibits α-synuclein aggregation [4].

In the synapse, likewise in the other neuronal compartments, α-synuclein exists in a dynamic equilibrium between a soluble state and a membrane-bound state, with its affinity for synaptic vesicles being higher than that for cell membranes [45–47] (Figure 1(a)). However, when interacting with cell membranes, the protein presents higher affinity for lipid rafts [48]. The interaction between α-synuclein and lipid membranes is also relevant for the protein to exert its functions [49]. This, notwithstanding, α-synuclein presents elevated structural plasticity and its effective conformation in soluble and membrane-bound state is matter of debate. While some evidences support the existence of a stable cytosolic tetrameric form of the protein, other studies have shown that it can be found as a disordered monomer in the central nervous system (CNS) and other mammalian cells or that these forms both coexist in a dynamic equilibrium [50, 51]. More recently, the existence of a stable dimer has been suggested by computational evidences [52]. As for the aminoacidic sites involved in the interaction with membranes, numerous studies have reported a key role for the ones located at the N-terminal portion of α-synuclein [53]. Jiang and coauthors [54], by coupling neutron reflectometry (NR) and fluorescence spectroscopy analysis, have found that the N- and C-terminal regions near positions 4 and 94 are anchored to the membrane, while the putative linker spanning residue 39 samples multiple conformations, which are sensitive to the chemical nature of the membrane surface. The mechanism of α-synuclein binding to lipid membranes has been found to be primarily dependent on the surface charge density of the lipid bilayer and the phase state of the lipids, with α-synuclein possessing lipid ordering effect and thermally stabilizing vesicles [55]. These findings suggest that this process might be tunable by environmental changes.

Collectively, these evidences support that subtle changes in α-synuclein structural folding, likewise the formation of oligomers or insoluble aggregates, can severely compromise the activity of dopaminergic neurons, with the adjunct of cell-to-cell transmission likely worsening and perpetrating the related injury.

3. Central and Peripheral Localization of Neuronal α-Synuclein Pathology in PD: Trans-Synaptic or Systemic Spreading?

The presence of different forms of α-synuclein in cerebrospinal fluid (CSF), blood plasma [56–58], and saliva [59] coupled to the discovery of α-synuclein pathology in

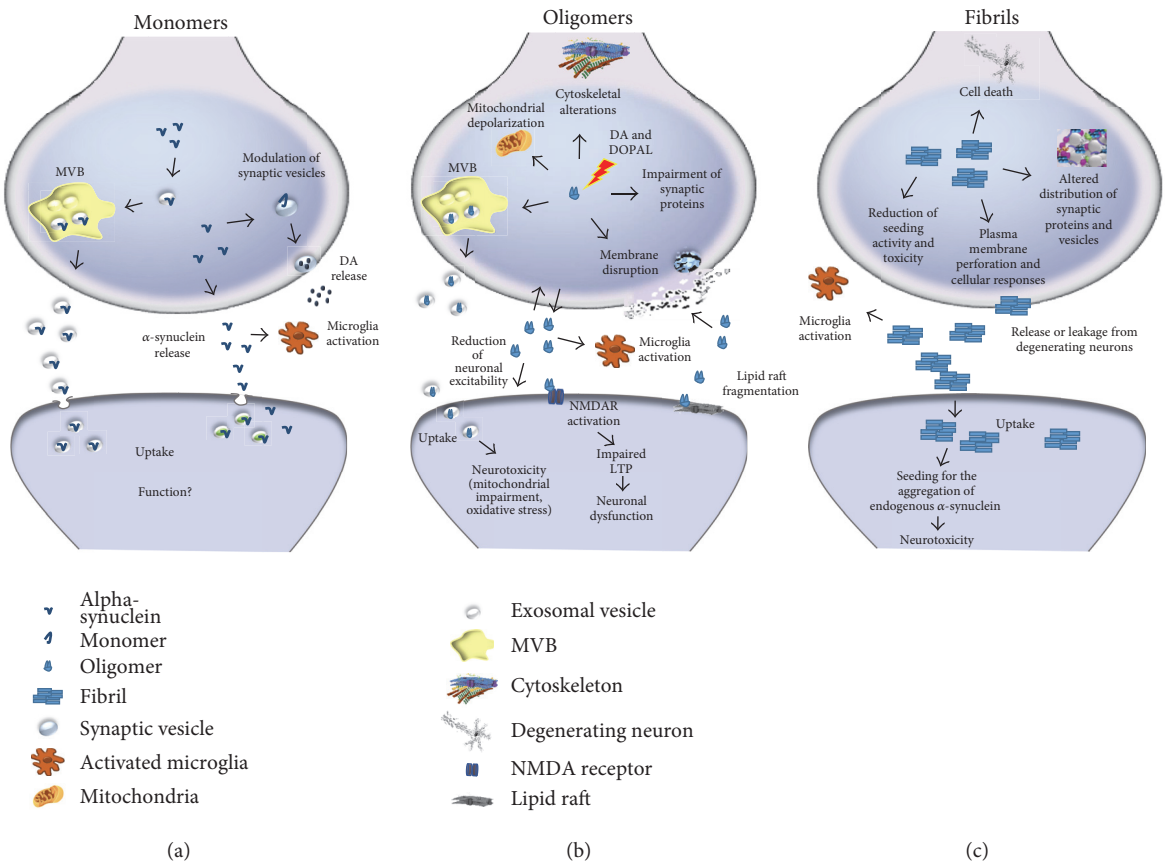

Figure 1: Monomeric, oligomeric, and fibrillary α-synuclein at the synaptic terminal. (a) Monomeric α-synuclein modulates synaptic function by controlling synaptic vesicle release. This form of the protein can be released in association with exosomes, activates microglial cells, and can be internalized at postsynaptic sites. (b) Oligomeric α-synuclein formation is enhanced by interaction of monomeric protein with DA. Alpha-synuclein oligomers can form a stable adduct with the toxic dopamine metabolite DOPAL. Oligomers can be released in association with extracellular vesicles and then activate microglia. Alpha-synuclein oligomers can disrupt synaptic vesicles membranes as well as presynaptic and postsynaptic membranes. Exogenous α-synuclein oligomers can damage lipid rafts and affect LTP by activating NMDA receptors. Intracellular α-synuclein oligomers with endogenous or exogenous origin impair mitochondrial functions and cytoskeletal architecture. (c) Fibrillary-aggregated α-synuclein alters synaptic vesicle release by clustering synaptic vesicles and by perforating plasma membrane. Extracellular fibrils deriving from degenerating neurons in the PD brain can activate microglial cells and actively contribute to alpha-synuclein pathology spreading. The formation of endogenous α-synuclein fibrils can reduce seeding activity and toxicity although exogenous α-synuclein fibrils function as a seed for the aggregation of endogenous α-synuclein in recipient cells.

embryonic nigral transplants of PD patients [10, 11, 60] suggested that α-synuclein can move from cell to cell and can initiate pathology in recipient neurons. It is well established that α-synuclein can be secreted [61, 62], a process that is thought to be mainly mediated by exosomal vesicles [63, 64].

Compelling evidence indicates that in PD α-synuclein pathology is not confined within the brain. LB-like aggregates of the protein have been found in the dorsal root ganglia, as well as in several tracts of the peripheral nervous system such as gastrointestinal innervation, motor nerves innervating the pharyngeal muscles, cranial and spinal nerves, skin nerves, and olfactory epithelium [8, 65–70]. The presence of these peripheral aggregates has been proposed to associate with typical PD premotor symptoms [71] and could contribute to disease onset in the brain, as supported by evidences indicating that vagotomy diminishes the risk to develop the disorder [20]. As hypothesized by Braak and colleagues [14], caudo-rostral spreading of α-synuclein has been found to occur through vagal connections in experimental models of PD [17]. Resection of the vagal autonomic projection impedes the peripheral-to-CNS diffusion of pathological α-synuclein and the onset of PD-like motor phenotype in the chronic intragastric rotenone model of PD [19], supporting a causative role for protein spreading in the onset of the disorder. Vagotomy eliminates most, but not all, α-synuclein-positive neurites in the plexus, thus providing a candidate pathway for the retrograde transport of putative PD pathogens or toxins from the enteric nervous system to the central nervous system [67]. However, even vagal nerve impairment per se has been found to induce dopamine functional damage, therefore suggesting that the vagal degeneration occurring in the PD brain [72, 73] might be pivotally involved in PD pathogenesis independently from α-synuclein pathological spreading. Studies in rodents have shown that

vagal afferent endings in the myenteric plexus and the gastrointestinal smooth muscle do not express α-synuclein, whereas virtually all vagal preganglionic projections to the gut show α-synuclein immunopositivity both in axons and in terminal varicosities in apposition with myenteric neurons.

However, some other studies raise some concerns about its validity as α-synuclein accumulation within the peripheral nervous system can occur also in neurologically intact subjects with aging [74]. Not by chance, PD and aging have been proposed to be a unique entity and the disease has been postulated to manifest in all subjects whether they could live long enough [75]. In addition, typical manifestations of aging such as frailty that interestingly has been found to associate with brain neuropathological accumulation of LB and nigral neuronal loss [76, 77] also characterize PD [78]. Other studies, failing to detect either the typical pattern of topographical distribution of α-synuclein pathology in the postmortem brain of affected patients [79, 80] or the development of pathology spreading following preformed fibril-inoculation *in vivo*, cast further confusion over the prion-like hypothesis.

Recent studies indicate that α-synuclein oligomers are increased in red blood cells and CSF of PD subjects and could serve as biomarkers of disease [81, 82]. The levels of α-synuclein are also increased in peripheral lymphocytes [83] as well as in plasma and CNS-derived exosomes of affected individuals [84]. The systemic spreading of the protein could thus also involve exosomal-mediated release and biological fluids. Indeed exosomes are small membrane vesicles which result from exocytosis of multivesicular bodies. They function as mediators of intercellular communication, as they transfer specific proteins, lipids, microRNAs, and DNA between cells. Because of their small size, exosomes can move from the site of discharge by diffusion and reach several biological fluids, such as blood, CSF, urine, and synovial fluid [85]. Consistently, plasma exosomal α-synuclein is likely CNS-derived and increased in PD [84] and CSF exosomes have been found to induce α-synuclein aggregate formation in recipient healthy cells [86]. This suggests that the circulatory system could also mediate α-synuclein systemic spreading, with the choroid plexus being likely involved in the passage of alpha-synuclein from the blood to the brain and vice versa. Notably, the presence of increased α-synuclein immunoreactivity at this site has been described in PD [13]. However, it still remains to be determined whether a link effectively exists between peripheral and brain α-synuclein deposition. In the case that they might constitute a unique phenomenological entity, defining what might come first between central and peripheral deposition could be crucial for determining the causes of PD.

4. Toxicity of α-Synuclein Oligomers and Fibrils: A Still Unresolved Dichotomy

Aggregation of α-synuclein is a critical step in the etiology of PD, with prefibrillar oligomers of the protein that might constitute the direct precursors of fibrils being involved in neurodegenerative process [87, 88]. Even if the injection of fibrils into the rat brain is found to be more toxic than that of oligomers and ribbons, as it induces neurodegeneration and motor impairment, all these species can self-amplify *in vivo* and lead to PD/multiple system atrophy-like alterations in the injected animals [89]. This suggests that strain-specific prion-like infectivity and symptomatology characterize different α-synuclein conformers, whose biochemical nature is still unknown. Therefore, asserting which, between fibrils or oligomers, are the most toxic species is still difficult at present. Nonetheless, the analysis of both oligomers' and fibrils' structure could help to elucidate this conundrum. In addition, data supporting that monomeric, oligomeric, and fibrillary α-synuclein can activate microglial cells [90–93] suggest that all these forms of the protein could affect neuronal homeostasis by modulating microglia function that could be either protective or detrimental in PD [94].

As for oligomers, those formed by a peptide derived from residues 36–55 of α-synuclein were recently characterized by X-ray crystallography [95]. The authors showed that this specific peptide is able to adopt a β-hairpin structure, which assembles in a hierarchical fashion. Three β-hairpins then assemble to form a triangular trimer and three copies of the triangular trimer then assemble to form a basket-shaped nonamer that couple to form an octadecamer. Following molecular modeling analysis, these authors also proposed that full-length α-synuclein might be able to assemble in this fashion. Circular dichroism spectroscopy demonstrated that the peptide 36–55 interacts with anionic lipid bilayer membranes, like oligomers of full-length α-synuclein, and is found to be toxic in neuronal cell models. Other cryoelectron microscopy studies have shown that oligomers isolated during fibril formation possess a hollow cylindrical architecture with similarities to certain types of amyloid fibril [96].

The formation of α-synuclein dimers has been described to initiate aggregation and neurotoxicity [97]. Computer modeling and cell-based studies have revealed that upon interaction with plasma membranes α-synuclein rapidly penetrates them, changing its conformation from α-helical toward a coiled structure. This penetration facilitates the incorporation of additional α-synuclein monomers to the complex and subsequent displacement of phospholipids and formation of oligomers in the membrane [98]. Consistently, α-synuclein oligomers neurotoxicity *in vivo* has been found to be mediated by membrane disruption [99].

Chen and coauthors described that stable toxic α-synuclein oligomeric species with a hollow cylindrical architecture with similarities to certain types of amyloid fibril can be trapped during fibril formation [96]. Their study showed that the β-sheet geometry acquired in the early stages of the self-assembly process plays a key role in dictating the kinetic stability and the pathological nature of individual oligomeric species. Spectroscopy studies have also shown that whereas fibrils adopt a parallel arrangement oligomers adopt an antiparallel β-sheet structure [100], thus suggesting that differences in the toxicity of these species might rely on their diverse conformations. The neurotoxicity of oligomers has been demonstrated in different experimental conditions. Alpha-synuclein overexpression in neuroblastoma cells causes the formation of α-synuclein oligomeric

species, whose presence is associated with mitochondrial fragmentation and autophagic-lysosomal pathway activation in live cells [101]. The accumulation of oligomeric and fibrillar forms of α-synuclein has a negative impact on mitochondria function by inhibiting mitochondria complexes IV and V in the striatum [102], reducing mitochondrial Ca^{2+} release and NADH oxidation [103]. Permeabilization of mitochondria membranes can also be induced by α-synuclein oligomers [104–107]. The accumulation of toxic α-synuclein oligomers in the endoplasmic reticulum (ER) has been described as a feature of α-synucleinopathies [108, 109]. Recently, α-synuclein oligomers have been found to interact with metal ions to induce oxidative stress and neuronal death in PD [110]. Interestingly, the oligomer-induced reactive oxygen species (ROS) production was independent of several known cellular enzymatic sources and relied solely on the presence of free metal ions.

Studies on human α-synuclein transgenic mice have shown that the accumulation of oligomers mainly occurs at synaptic sites and is crucial for the induction of synaptic and neuronal degeneration [111]. Consistently, the formation of α-synuclein soluble oligomers can reduce neuronal excitability of neocortical pyramidal cells, suggesting that it could impact on network activity [112]. Oligomers have been found to affect synaptic function by changing lipid raft composition and increasing N-methyl-D-aspartate (NMDA) receptor activation at postsynaptic membranes [113, 114].

Alpha-synuclein oligomers could thus easily impinge on synaptic resilience by disrupting membranes, inducing mitochondrial depolarization, altering cytoskeleton dynamics, impairing protein clearance pathways, and enhancing oxidative stress [115] (Figure 1(b)). In addition, the preferential degeneration of dopaminergic neurons in PD [116, 117] led to the hypothesis that dopamine may play an important role in α-synuclein oligomerization [118]. The oxide forms of dopamine, generated by oxidative stress, accelerate formation of α-synuclein oligomers as an endogenous protofibril stabilizer, demonstrating the connection between dopamine and α-synuclein oligomer formation [119, 120]. The toxic dopamine metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL) can compromise the membrane-binding affinity of α-synuclein to synaptic vesicles, as well as its fibrillation, by forming an adduct with the protein, thus reducing its functional ability to modulate synaptic vesicles [121].

Fibrils exert their toxic actions by activating other detrimental processes in neuronal cells. This may be related to the different conformation of oligomers and fibrils. Consistently, Curtain and coauthors [122], by using small angle X-ray scattering and ensemble optimization modeling studies, were able to demonstrate that α-synuclein oligomers and fibrils originate in two distinct conformer pools, with E53T and E45K mutations enhancing the tendency to form fibrils, while the A30P conferring propensity toward oligomer assembly. This is in line with previous data reporting that A30P mutant α-synuclein forms different fibril structures [123] and that mutations in the KTKE(Q)GV imperfect amino acid repeats in the N-terminal part of the protein can also affect its tendency toward fibril formation [124]. Mutant α-synuclein preferentially shifts from monomer to fibrils, thus suggesting that the formation of these species plays a crucial role in the pathophysiology of early-onset PD [125, 126]. Whether the lipid bound α-helical form or the unfolded state initiates protein aggregation remains to be determined.

X-ray and electron diffraction of the α-synuclein aggregates showed that the fibrils consist of a β-sheet structure in which the β-strands run perpendicularly to the long fiber axis [127]. A flexible break close to residues 52–55 has been found to be relevant for fibril formation [128], while the negative charges and aromatic residues at the C-terminal region play a modulatory role on fibrillation [129, 130]. In addition, molecular dynamics simulations allowed observing that residues 36–55 of the nonamyloid component (NAC) domain, in the central region of the protein, are important for the formation of β-hairpin and that the point mutations stabilize this β-hairpin that represented the first step of α-synuclein aggregation [95].

Alpha-synuclein fibrils can exert toxicity by disrupting the normal distribution of membrane proteins and synaptic vesicles [6], perforating plasma membranes [131, 132], triggering cellular responses [133], and causing death in cell systems [134] (Figure 1(c)). However, the stabilization of fibril clusters can prevent fragmentation and reduces seeding activity and toxicity [135]. In addition, α-synuclein structure is strictly dependent on membrane interactions that can both accelerate [136, 137] and inhibit [136, 138, 139] fibril formation.

5. Mechanisms of α-Synuclein Release

Since 2003, Braak and colleagues had speculated that, in idiopathic PD, LB pathology could spread from the enteric nervous system or the olfactory bulb to precise brain regions during the progression of the disease [14], thus suggesting the occurrence of cell-to-cell α-synuclein transmission. The existence of this phenomenon was confirmed thanks to post-mortem studies carried out on the brain of PD patients who had received transplants of mesencephalic neurons 14 years prior to the demise. Indeed, the analysis of autopsy samples showed that grafted neurons had developed LB pathology [10, 60]. Host-to-graft transmission was then corroborated in different experimental models *in vivo* [140–142].

The first evidence in favor of α-synuclein release was provided by Dixon and colleagues that demonstrated that, in yeasts, the protein can be delivered to the extracellular space through the classical ER-to-Golgi secretory pathway [143]. However, these findings were not confirmed in SH-SY5Y cells, where α-synuclein has been observed to be mediated by ER/Golgi-independent unconventional exocytosis pathway [33, 62] and in a Ca^{2+}-dependent manner by exosomes [63], respectively. Protein overexpression, misfolding, and posttranslational modifications can promote α-synuclein accumulation and release, caused by impaired intracellular degradation. Several cellular mechanisms have been found to mediate α-synuclein degradation, including the ubiquitin-proteasome system (UPS) and lysosome-mediated digestion [144]. In PD, dysfunction of the UPS

causes the accumulation of misfolded α-synuclein and is thought to be highly implicated in the pathogenesis of PD [145–149]. However, ubiquitination of α-synuclein in LB has been found to constitute a pathological event not associated with impairment of proteasomal function [150]. Alpha-synuclein can also be cleared by the autophagy-lysosome pathway (ALP). Indeed, Cuervo [151] showed that wild type α-synuclein is translocated into lysosome for degradation. Conversely, aggregated α-synuclein can cause a malfunction in lysosomal degradation pathway [152]. Remarkably, also aging may represent another important factor related to the dysfunction of protein control systems [145, 151, 153–155] causing a vicious circle that exacerbate the accumulation of toxic forms of α-synuclein.

The oxidized forms of α-synuclein are preferentially secreted [62] and studies in induced pluripotent stem cell-derived neurons harboring α-synuclein gene locus triplications showed that increased intracellular levels of the protein foster its release [156]. However, the amount of released α-synuclein deriving from injured neurons is likely very low as neuronal damage and neurodegeneration do not exacerbate its propagation [18].

At the subcellular level, it has been observed that, in neurons, α-synuclein can be found in the three endosomal compartments and translocate across them (early, late, and recycling endosomes) [32]. This pathway could be involved in the process of protein release [32]. Moreover, treatments that could disturb the homeostasis of the endosomal system alter α-synuclein spreading [63, 157]. From the early endosomes, a portion of the protein can be translocated to the recycling endosomal compartment, which can fuse with the plasma membrane to allow the release of soluble forms of α-synuclein. This process seems to be mediated by the interaction between α-synuclein and Rab GTPases [158]. The portion of protein that remains in the endosomal compartment is targeted to multivesicular bodies (MVBs), which usually fuse with lysosomes for protein degradation. In diseased neurons, where the accumulation of toxic α-synuclein aggregates could perturb the activity of protein clearance mechanisms, MBVs can release the intraluminal vesicles in the extracellular space as exosomes [159]. Remarkably, numerous findings [63, 157, 160, 161] support that α-synuclein can be released in association with exosomes, small vesicles (40–100 nm in diameter) originating from the endosomal compartment. Exosomes can be secreted and interact with cell membranes in a cell type-dependent manner and recipient cells can internalize them through different endocytic mechanisms [162]. In several neurodegenerative diseases, exosomes seem to play the role of "garbage" carrier, acting as an alternative pathway of protein elimination when intracellular mechanisms are engulfed [163, 164]. The process through which α-synuclein is targeted to the endosomal compartment is still matter of study, with a recent work suggesting posttranslational modifications of the protein being involved. In particular, the conjugation of Small Ubiquitin like Modifier (SUMO) also defined as SUMOylation of α-synuclein triggers its internalization in exosomes [160]. To date, SUMOylation serves to regulate the solubility of aggregation-prone proteins [165, 166], as well as a ubiquitin-independent endosomal sorting complex required for transport (ESCRT) sorting signal, regulating the extracellular vesicle release of α-synuclein [160]. Interestingly, ubiquitinated forms of α-synuclein have been found to be present in LB [167, 168] and the ubiquitination of the protein has been found to increase its aggregation propensity and neurotoxicity *in vitro* [169, 170]. Taken together, these findings suggest that α-synuclein ubiquitination and SUMOylation may be coexisting phenomena whose equilibrium regulates the folding state and the localization of the protein. In particular, the fact that SUMOylation facilitates the exosomal-mediated release of α-synuclein [160] supports the hypothesis that exosomes might behave as possible "way out" to eliminate exceeding amount of the protein that cannot be degraded by conventional mechanisms such as proteasome or lysosome digestion. Further studies are needed to corroborate the role of exosomes in toxic α-synuclein spreading and in the progression of PD. Nonetheless, exosomes and their cell-to-cell transmission mechanisms could represent novel intriguing therapeutic targets to lessen or block the evolution of α-synuclein pathology.

6. Uptake of Aberrant α-Synuclein from Recipient Cells: Consequences on Synaptic Functions

In the brain, neurons can show differential vulnerability to α-synuclein accumulation. Indeed, even though it has been reported that in PD subjects LB pathology first affects the vagal nuclei, locus coeruleus, and olfactory bulbs, the motor symptoms appear when α-synuclein deposition reaches nigrostriatal dopamine neurons. In particular, since the striatal synaptic accumulation of the protein is much higher than its deposition in the cell bodies of the substantia nigra [171], it may be hypothesized that mechanisms promoting its synaptic translocation could be enhanced in the early phases of disease. When taking into account the trans-synaptic spreading hypothesis by Braak, it is feasible that nigrostriatal dopamine neurons might interpret the uptake of α-synuclein pathological seeds as a signal that prompts them to further promote α-synuclein trans-synaptic transfer as a response. In line with this idea, the injection of synthetic fibrils and LB extracts from PD subjects in the mouse brain has been found to induce a rapid and progressive synucleinopathy between anatomically interconnected brain regions [9, 164, 172–174]. Nonetheless, other studies have reported considerable difficulties in inducing a widespread induction of α-synuclein pathology following intracerebral administration of amyloidogenic forms of the protein in mice [175]. Furthermore, several mechanisms, such as neuroinflammation, have been found to act synergistically or independently to promote the spread of pathology following fibrillary amyloidogenic and nonamyloidogenic α-synuclein [175]. This evidence calls into question that extracellular α-synuclein can catalyze aggregation and spread of intracellular protein only through a nucleation dependent conformational templating mechanism. Worthy of note, besides the fact that fragmented amyloid-like aggregates of short α-synuclein fibrils can function as seeds that trigger prion-like conversion

[176], the transmission of mature fibrils between cells might be difficult in light of their dimensions and stability. It is easier to speculate that they might derive from degenerating neurons in the parenchyma. On this line, brain propagation of α-synuclein has been found to involve nonfibrillar protein species and to be enhanced in α-synuclein null mice [177]. In addition, PD-causing α-synuclein missense mutations shift native tetramers to monomers as a mechanism for disease initiation [51]. Extracellular oligomeric species that are also transmissible through exosomal vesicles [178] are highly abundant in the PD brain [115] and can promote α-synuclein aggregation in recipient cells [179]. Transmitted electron microscopy studies in the postmortem human brain of subjects affected by synucleinopathies, reporting the presence of oligomeric α-synuclein within the early-endosomal compartment of neuronal cells, are also in line with the idea that oligomers might be the transmissible species [13]. However, what could be the cause of their increased accumulation and how they trigger aggregation of endogenous α-synuclein in recipient cells still needs elucidation. The release of oligomers might serve to eliminate exceeding levels of the protein, or it could depend on the activation of plastic structural adaptive mechanisms in neurons as the protein is involved in synaptic plasticity [180, 181]. Otherwise, protein oligomers could behave as a transmissible neuronal messenger between neighboring neurons. Remarkably, the higher stability of α-synuclein oligomers and fibrils renders these species more suitable to be secreted in the extracellular space when compared to monomeric protein. Indeed, the conformation of this latter could be modulated even by subtle homeostatic changes in the microenvironment.

Alpha-synuclein uptake could very well contribute to synaptic damage. Indeed, the protein could accumulate at synapses by altering the function of endogenous proteins and engulfing the retrograde transport from the terminals that receive α-synuclein to the cell bodies. Alternatively, recipient cells might collapse as they fail to degrade internalized α-synuclein efficiently. It is feasible that the accumulation of extracellular α-synuclein in dopaminergic synaptic terminals can easily initiate synaptic failure given the relevance of the protein in the regulation of dopamine release [182]. Folding and misfolding of endogenous α-synuclein can be modulated by exogenous pathological α-synuclein forms and then affect, or be affected by, their interaction with lipid membranes [183]. Alpha-synuclein binding to lipid membranes can be either detrimental or protective to neuronal cells [184]. For instance, A30P, E46K, and A53T disease variants of α-synuclein show increased lipid binding affinity [185] although they have distinct membrane permeabilization properties [186] and can thus differentially affect membrane structure [187]. In line with this idea, exogenous α-synuclein has been found to induce lipid raft fragmentation thus leading to both pre- and postsynaptic alterations [114] and α-synuclein oligomers can impair long term potentiation (LTP) and impair synaptic transmission [113].

Finally, a recent study showed that different α-synuclein conformers can cross the blood brain barrier and distribute to the CNS after intravenous injection [89]. This novel evidence suggests that the diffusion of α-synuclein pathology might also be mediated by mechanisms other than the simple trans-synaptic spreading of the protein among interconnected brain regions.

Looking at the mechanisms of α-synuclein uptake, while the soluble monomeric protein can cross the plasma membrane or can be captured by Rab5a-dependent [188] and dynamin-dependent endocytosis [140], high order assembly can enter into recipient cells by using different endocytic pathways [169]. Among the possible mechanisms of uptake, macropinocytosis has also been explored [189]. Other authors reported that extracellular α-synuclein uptake by microglial cells is mediated by the GM1 ganglioside as well as by hitherto-unknown protein receptors in clathrin-, caveolae-, and dynamin-independent, but lipid raft-dependent processes [90]. However, the caveolae-specific protein caveolin-1 has been found to interact with and to mediate α-synuclein toxicity in neuroblastoma cells [190] thus suggesting that the possibility that the uptake of the protein might be mediated by caveolae-mediated endocytosis at least in neuronal cells deserves further investigation. More recently, mesenchymal stem cells were identified as blockers of the clathrin-mediated endocytosis of extracellular α-synuclein, a process that is controlled by the interaction with NMDA receptor [191]. In addition, the interaction between preformed α-synuclein fibrils and immune receptor lymphocyte activation gene 3 (LAG3) has been found to be essential for initiating the transneuronal propagation of α-synuclein [192]. This study clearly opens new avenues for PD therapy as LAG3 antibodies are already being tested as cancer treatments [193] and suggested that LAG3 might mediate both immune system activation and systemic spreading of pathological fibrillary α-synuclein species.

The fact that α-synuclein can be released in association with exosomes and extracellular vesicles (EV) strongly suggests that these might constitute the primary vehicles of cell-to-cell transmission of the protein, preserving it from degradation by extracellular enzymes and facilitating its correct targeting toward recipient cells. Indeed, given the high structural instability of the protein and its small size, it is quite unlikely that α-synuclein could easily survive in the brain parenchyma environment in a free and soluble form.

Collectively, these evidences strongly support that α-synuclein spreading could very well contribute to synaptic impairment in PD although the biological factors determining the selective vulnerability of nigrostriatal neurons, even on top of the "prion-like" hypothesis, are not yet clear.

7. Concluding Remarks

At present, the spreading hypothesis of α-synuclein pathology is still matter of debate. Indeed, although a plethora of research studies seem to confirm that α-synuclein can diffuse throughout the nervous system, is transmitted from cell to cell, and could induce toxicity and function as a seed for the aggregation of endogenous protein, other evidences seem to confute these findings (Table 1). This, notwithstanding, data on the transmissibility of the protein as well as on the misfolding-inducing ability of α-synuclein

Table 1

First author	Title	Year	Journal
	Evidences supporting α-synuclein spreading		
Braak	"Staging of Brain Pathology Related to Sporadic Parkinson's Disease"	2003	Neurobiol. Aging
Del Tredici	"Sporadic Parkinson's Disease: Development and Distribution of Alpha-Synuclein Pathology"	2016	Neuropathol. Appl. Neurobiol.
Iljina	"Kinetic Model of the Aggregation of Alpha-Synuclein Provides Insights into Prion-Like Spreading"	2016	Proc. Natl. Acad. Sci. USA
Oh	"Mesenchymal Stem Cells Inhibit Transmission of α-Synuclein by Modulating Clathrin-Mediated Endocytosis in a Parkinsonian Model"	2016	Cell Rep.
Helwig	"Brain Propagation of Transduced Alpha-Synuclein Involves Non-Fibrillar Protein Species and Is Enhanced in Alpha-Synuclein Null Mice"	2016	Brain
Bernis	"Prion-Like Propagation of Human Brain-Derived Alpha-Synuclein in Transgenic Mice Expressing Human Wild-Type Alpha-Synuclein"	2015	Acta Neuropathol. Commun.
Illes-Toth	"Distinct Higher-Order Alpha-Synuclein Oligomers Induce Intracellular Aggregation"	2015	Biochem. J.
Ulusoy	"Neuron-to-Neuron α-Synuclein Propagation *In Vivo* Is Independent of Neuronal Injury"	2015	Acta Neuropathol. Commun.
Stuendl	"Induction of α-Synuclein Aggregate Formation by CSF Exosomes from Patients with Parkinson's Disease and Dementia with Lewy Bodies"	2016	Brain
Prusiner	"Evidence for α-Synuclein Prions Causing Multiple System Atrophy in Humans with Parkinsonism"	2015	Proc. Natl. Acad. Sci. USA
Masuda-Suzukake	"Pathological Alpha-Synuclein Propagates through Neural Networks"	2014	Acta Neuropathol. Commun.
Sacino	"Brain Injection of Alpha-Synuclein Induces Multiple Proteinopathies, Gliosis, and a Neuronal Injury Marker"	2014	J. Neurosci.
Kovacs	"Intracellular Processing of Disease-Associated α-Synuclein in the Human Brain Suggests Prion-Like Cell-to-Cell Spread"	2014	Neurobiol. Dis.
Recasens	"Lewy Body Extracts from Parkinson Disease Brains Trigger α-Synuclein Pathology and Neurodegeneration in Mice and Monkeys"	2014	Ann. Neurol.
Ulusoy	"Caudo-Rostral Brain Spreading of α-Synuclein through Vagal Connections"	2013	EMBO Mol. Med.
Masuda-Suzukake	"Prion-Like Spreading of Pathological α-Synuclein in Brain"	2013	Brain
Angot	"Alpha-Synuclein Cell-to-Cell Transfer and Seeding in Grafted Dopaminergic Neurons *In Vivo*"	2012	PLoS One
Luk	"Intracerebral Inoculation of Pathological Alpha-Synuclein Initiates a Rapidly Progressive Neurodegenerative Alpha-Synucleinopathy in Mice"	2012	J. Exp. Med.
Luk	"Pathological Alpha-Synuclein Transmission Initiates Parkinson-Like Neurodegeneration in Nontransgenic Mice"	2012	Science
Kordower	"Transfer of Host-Derived Alpha Synuclein to Grafted Dopaminergic Neurons in Rat"	2011	Neurobiol. Dis.
Hansen	"Alpha-Synuclein Propagates from Mouse Brain to Grafted Dopaminergic Neurons and Seeds Aggregation in Cultured Human Cells"	2011	J. Clin. Invest.
Danzer	"Seeding Induced by Alpha-Synuclein Oligomers Provides Evidence for Spreading of Alpha-Synuclein Pathology"	2009	J. Neurochem.
Kordower	"Transplanted Dopaminergic Neurons Develop PD Pathologic Changes: A Second Case Report"	2008	Mov. Disord.
Kordower	"Lewy Body-Like Pathology in Long-Term Embryonic Nigral Transplants in Parkinson's Disease"	2008	Nat. Med.
Li	"Lewy Bodies in Grafted Neurons in Subjects with Parkinson's Disease Suggest Host-to-Graft Disease Propagation"	2008	Nat. Med.
	Evidences confuting α-synuclein spreading		
Sumikura	"Distribution of Alpha-Synuclein in the Spinal Cord and Dorsal Root Ganglia in an Autopsy Cohort of Elderly Persons"	2015	Acta Neuropathol. Commun.
Sacino	"Amyloidogenic α-Synuclein Seeds Do Not Invariably Induce Rapid, Widespread Pathology in Mice"	2014	Acta Neuropathol.
Halliday	"The Progression of Pathology in Parkinson's Disease"	2010	Ann. N. Y. Acad. Sci.
Jang	"Non-Classical Exocytosis of Alpha-Synuclein Is Sensitive to Folding States and Promoted under Stress Conditions"	2010	J. Neurochem.
Hawkes	"Parkinson's Disease and Aging: Same or Different Process?"	2008	Mov. Disord.
Kalaitzakis	"Controversies over the Staging of Alpha-Synuclein Pathology in Parkinson's Disease"	2008	Acta Neuropathol.

oligomers and fibrils suggested that these features might easily perturb synaptic homeostasis, especially in dopamine neurons. It could be feasible that α-synuclein release, coupled to fibrillary insoluble inclusion formation, could deprive dopamine synaptic terminals from the modulatory action of the protein. In parallel, the exosome-mediated exchange of α-synuclein oligomers between neighboring terminals could overwhelm intracellular trafficking by encumbering on the endosomal system. Much work still needs to be done to define the contribution of α-synuclein spreading to PD synaptopathy. However, the determination of what can be considered as a transmissible pathological form of α-synuclein as well as the mechanisms through which this entity can be transmitted from a diseased presynaptic terminal to a healthy postsynaptic ending can help us to understand much more on PD neurobiology and to identify novel effective therapeutic strategies to cure this disorder. Indeed, whether PD is primarily a disorder of the synapse, novel effective therapeutic approaches should both heal diseased synapses and block the cell-to-cell transmission of toxic α-synuclein species.

Disclosure

PierFranco Spano is Professor Emeritus of Pharmacology, University of Brescia.

Competing Interests

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

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Research Article

Early Transcriptional Changes Induced by Wnt/β-Catenin Signaling in Hippocampal Neurons

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Wnt/β-catenin signaling modulates brain development and function and its deregulation underlies pathological changes occurring in neurodegenerative and neurodevelopmental disorders. Since one of the main effects of Wnt/β-catenin signaling is the modulation of target genes, in the present work we examined global transcriptional changes induced by short-term Wnt3a treatment (4 h) in primary cultures of rat hippocampal neurons. RNAseq experiments allowed the identification of 170 differentially expressed genes, including known Wnt/β-catenin target genes such as Notum, Axin2, and Lef1, as well as novel potential candidates Fam84a, Stk32a, and Itga9. Main biological processes enriched with differentially expressed genes included neural precursor (GO:0061364, p-adjusted = 2.5×10^{-7}), forebrain development (GO:0030900, p-adjusted = 7.3×10^{-7}), and stem cell differentiation (GO:0048863 p-adjusted = 7.3×10^{-7}). Likewise, following activation of the signaling cascade, the expression of a significant number of genes with transcription factor activity (GO:0043565, p-adjusted = 4.1×10^{-6}) was induced. We also studied molecular networks enriched upon Wnt3a activation and detected three highly significant expression modules involved in glycerolipid metabolic process (GO:0046486, p-adjusted = 4.5×10^{-19}), learning or memory (GO:0007611, p-adjusted = 4.0×10^{-5}), and neurotransmitter secretion (GO:0007269, p-adjusted = 5.3×10^{-12}). Our results indicate that Wnt/β-catenin mediated transcription controls multiple biological processes related to neuronal structure and activity that are affected in synaptic dysfunction disorders.

1. Introduction

The Wnt signaling cascade plays an essential role during embryogenesis and adult tissue homeostasis. Wnts are lipid modified secreted glycoproteins that signal through three major cellular pathways: the Planar Cell Polarity, the Wnt/Ca^{2+}, and the Wnt/β-catenin signaling pathway, also referred to as the canonical Wnt signaling pathway [1–3]. The canonical cascade initiates with the binding of a Wnt ligand to Frizzled (FZD) receptors and LRP5/6 coreceptors located at the cellular membrane [4]. Wnt binding leads to the inhibition of the β-catenin destruction complex consisting of Axin, adenomatous polyposis coli (APC) [5], casein kinase 1 (CK1), and glycogen synthase kinase 3β (GSK3β) [6], which ultimately results in the stabilization of β-catenin protein in the cytosol and its subsequent nuclear translocation where it interacts with members of the T-cell factor/lymphoid enhancing factor (TCF/LEF) family of transcription factors to enhance transcription of Wnt/β-catenin target genes [3]. Conversely, in the absence of Wnt ligand activation, Axin and APC facilitate the sequential phosphorylation of β-catenin by CK1 and GSK3β [6] tagging this protein for ubiquitination and subsequent proteasome mediated degradation [7].

Throughout mammalian brain development the activity of the Wnt cascade is spatially confined to specialized regions such as the olfactory bulb, frontal cortex, hippocampal formation, and the cerebellum [8–11]. In these brain domains Wnt/β-catenin signaling participates in diverse biological processes including neurogenesis [12], axonal remodeling and patterning [13, 14], and development and maturation of

functional synapses within the CNS [15–20]. Indeed, Wnt1, Wnt3a, Wnt7a, and Wnt8 are ligands known to activate Wnt/β-catenin signaling and are involved in brain development and synaptogenesis [21, 22]. Wnt3a is essential in early development of hippocampal structures and participates in the establishment of long term potentiation events [23, 24]. Wnt7a and Wnt8a have also been shown to regulate excitatory synaptic formation [17, 25]. Furthermore a recent study suggests that LRP6, Wnt/β-catenin signaling coreceptor, is critical for the development of functional synapses in vivo [25]. Therefore, given its multiple roles in synaptic function and brain homeostasis, Wnt/β-catenin signaling is a functional and positional candidate to understand complex prevalent neurological conditions in the human population.

At the presynaptic region, canonical Wnt ligands such as Wnt7a and Wnt3a enhance the clustering and recycling of synaptic vesicles (SVs) in primary cultures of rat hippocampal neurons [26]. Consistently, loss of Wnt7a function inhibits SVs clustering, an effect that is mimicked by loss-of-function of Dishevelled 1 (DVL1) signaling downstream of Wnt ligands [19]. Interestingly, the Wnt7a/Dvl1 double mutant shows defects in spine morphogenesis and excitatory synaptic neurotransmission [17], which parallels behavioral abnormalities with a disrupted presynaptic assembly and excitatory/inhibitory balance. Wnt/β-catenin signaling also seems to trigger neurotransmitter release and SV trafficking by modulating SV-associated phosphoproteins. While Wnt7a and Wnt3a enhance the clustering [27] and phosphorylation [28] of synapsin 1 at the synaptic button prior to neurotransmitter release, Dvl1 is involved in neurotransmitter release through direct binding to synaptotagmin I in differentiated neurons [29].

Experience driven plasticity is highly dependent on proper synaptic transmission and is mainly modulated by Ca^{2+} related pathways. In this regard, Wnt noncanonical and canonical pathways have been extensively related to Ca^{2+} homeostasis and signaling [19, 28, 30, 31]. For instance, ligands such as Wnt3a [28], Wnt5a [30], and Wnt7a [19] have all been shown to increase Ca^{2+} influxes to stimulate excitatory synaptic strength in hippocampal neurons or in peripheral nerves to alter pain sensitivity [32]. Other mechanisms modulating the activity of the synaptic terminal involve the function of cell adhesion proteins, most notably transsynaptic cadherin-β-catenin interactions that have an essential function during the recruitment and clustering of SVs to synapses [33–37]. Importantly, the effect on synaptic function, plus the biological control of neuronal polarity and axon outgrowth/navigation to their final synaptic targets, is mainly accomplished through rapid posttranslational changes affecting the cytoskeletal machinery [38, 39].

While Wnt/β-catenin ligands have a profound effect on the modulation of synaptic terminals; however the transcriptional program elicited by the signaling cascade in neurons has received little attention. Therefore, given that the integration of differentially expressed genes into biological networks provides a wider vision for the transcriptional landscape of a particular cell-type and considering that others and we have previously observed that Wnt/β-catenin target gene expression is rapidly induced (2–6 h) upon activation of the signaling cascade [40–44], here we use genome-wide transcriptional data to identify target genes and main biological processes directed by transient Wnt3a exposure (4 h) in primary cultures of rat hippocampal neurons. We expect that understanding of the genetic program maintained by β-catenin/TCF-LEF complexes in hippocampal cells will provide molecular mechanisms to couple activity, structure, and function of synaptic networks and circuits in this important brain region, which is severely affected in neurodegenerative and neurodevelopmental disorders.

2. Materials and Methods

2.1. Primary Cultures of Rat Hippocampal Neurons. Hippocampal neurons were dissociated and maintained as described before [28]. Briefly, neurons were taken from 18-day pregnant Sprague-Dawley rats and maintained for 14 days in vitro (DIV) on 12-well culture plates (500,000 cells/well) coated with poly-L-lysine (Sigma) and supplemented with neurobasal/B27 media (Gibco). The culture was placed on a shelf in a 37°C humidified CO_2 incubator, and the medium was changed every 2 days.

2.2. Wnt3a Purification. Wnt3a is a specific Wnt/β-catenin signaling agonist that can be efficiently recovered from conditioned medium from Wnt3a-secreting L-cells (ATCC, Rockville, MD; see also the Wnt homepage at http://web.stanford.edu/group/nusselab/cgi-bin/wnt/). Wnt3a purification was carried out as previously described [28, 57, 58]. The presence of the Wnt3a protein was detected with an anti-Wnt3a antibody (R & D Systems, Minneapolis, MN). Purity was analyzed by SDS-PAGE (8%), stained with Coomassie Blue G250, and analyzed through densitometry by using software ImageJ [59].

2.3. RNAseq Experiments. Rat hippocampal neurons were transiently exposed with Wnt3a for 4 h and then total RNA was extracted (3 control and 3 Wnt3a samples matched by experiment). RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA integrity numbers (RIN) obtained were above 7.5 and suitable for sequencing. RNA was processed using the Illumina TruSeq Stranded mRNA kit to generate 280 bp size paired-end libraries. Libraries were sequenced using the Illumina HiSeq 2000 benchtop sequencer. Raw reads (average depth of 100.62 M 2 × 80) were aligned to the Rnor6 build for rat genome reference, using Bioconductor R Package (v3.1; [60]). Differential expression was statistically analyzed with DESeq v1.20 following author recommendations [61]. Heterogeneity across the samples was examined visualizing reads distribution plots before and after DESeq normalization. Pearson's correlation analysis showed $r2$ values above 0.93 between all samples, within the standards (i.e., 0.92 and 0.98) suggested by ENCODE [62]. The complete pipeline of our study is presented in Supplementary Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/4672841. Summary statistics for the sequencing process and alignment are provided in

Supplementary Tables 1 and 2, respectively. Likewise, read normalization results are presented in Supplementary Figure 2.

2.4. Gene Ontology (GO) Enrichment Analysis. We tested the gene ontology (GO; http://geneontology.org/) [63] structure and annotations using the package Ontologizer [64], considering categories with less than 500 members to avoid associations to major categories that are less informative (i.e., signaling) and excluding the ones "Inferred by Electronic Annotation" (IEA), from "Reviewed Computational Analysis" (RCA) and with "No biological Data available" (ND), which are characterized by a high rate of false positives [65, 66]. We used the parent-child-union algorithm to call for overrepresentation adjusting the p values with Benjamini-Hochberg multiple testing correction, to avoid false positives [67]. We considered GO terms significantly overrepresented with an adjusted p value below 1×10^{-6} for further analyses.

2.5. Functional Protein Association Network (FPAN). To assay for known interactions between the differentially expressed genes we retrieved high confidence functional interactions from the STRING 10.0 database (search tool for the retrieval of interacting genes/proteins; http://string-db.org/) [68] which contains curated interactions from different evidence sources (i.e., genomic context, coexpression, and curated literature). We kept only "highest confidence" interactions (i.e., the interactions with a combined score >0.9 provided by STRING). The final FPAN was composed of 9,443 nodes (rat genes) and 309,728 nonredundant edges (interactions). All network and subnetworks were visualized with Cytoscape software [69].

2.6. Module Search. All gene-wise p values obtained with DESeq were introduced in to the FPAN as a floating-point attribute. A fold change greater than 1.25 (25%) was used as a threshold for inclusion (n = 2,892 genes). Module search was carried out with the Cytoscape JActiveModules plugin [70] with a gene overlap threshold of 20% following the method reported previously [66, 71]. Briefly, the program searches for significant highly connected subnetworks or modules enriched with expression information (p value). Starting from one random node (Monte Carlo procedure) modules grow in comparison with an expected background distribution created by the software from 10,000 internal randomizations, obtaining a specific standard deviation score (S score). Modules with $S > 3$ (3 standard deviation above the mean of randomized scores) and with a gene number between 10 and 50 were considered significant [72]. To acquire a mean S score and standard deviation (SD) for each resulting module the search was performed 10 times. Finally, the same procedure was conducted with permuted p values over the entire genes present in the FPAN (permuted analysis). Statistical differences between permuted and real (Wnt3a) analyses were assessed through one-sided Student's t-test.

Table 1: Top 20 nominal significant Wnt3a-upregulated genes in hippocampal neurons.

Symbol ID	FC	p value	Wnt pathway	References
Fam84a	2.4	2.2×10^{-6}	—	—
Notum	14.1	1.5×10^{-5}	Wnt target gene	[45]
Lef1	2.1	1.7×10^{-5}	Wnt target gene	[46]
Axin2	3.5	1.2×10^{-4}	Wnt target gene	[47]
Prkg2	2.7	1.4×10^{-4}	Functional	[48]
Cxcl3	4.6	5.2×10^{-4}	Wnt target gene	[49]
Ahr	2.1	1.0×10^{-3}	Wnt target gene	[50]
Stk32a	1.8	1.3×10^{-3}	—	—
Itga9	2.0	1.7×10^{-3}	—	—
Tnfresf19	2.4	1.8×10^{-3}	Wnt target gene	[51]
Tmem72	1.7	2.0×10^{-3}	—	—
Fras1	1.9	2.2×10^{-3}	—	—
Fam167a	1.8	2.2×10^{-3}	—	—
Gata2	2.8	2.3×10^{-3}	Wnt target gene	[52]
Id2	1.3	3.0×10^{-3}	Wnt target gene	[53]
Msx2	2.1	3.9×10^{-3}	Wnt target gene	[54]
Sp5	10.3	4.2×10^{-3}	Wnt target gene	[55]
Cd83	2.1	4.4×10^{-3}	—	—
Cgnl1	1.5	4.9×10^{-3}	—	—
Hunk	1.5	5.3×10^{-3}	Wnt target gene	[56]

Functional: known to modulate Wnt pathway transcriptional activity. FC: fold change.

3. Results

3.1. Differentially Expressed Wnt/β-Catenin Target Genes and Ontological Categories. To observe a direct transcriptional effect of Wnt/β-catenin signaling in primary cultures of hippocampal neurons and thus avoid noise due to activation of secondary unrelated pathways [40–44], we compared the whole transcriptome of cells transiently incubated with purified Wnt3a (4 h, $n = 3$) with cells from the same batch under control conditions (Wnt3a vehicle; $n = 3$; see Material and Methods). We identified 170 differentially expressed genes with a nominal p value of <0.05 (Figure 1). Raw read counts per sample, in ascending order according to their respective fold change (FC), are shown in Figures 1(a) and 1(b). Smear plots between treatment and control conditions revealed that differentially expressed genes (red dots) displayed an inverse relationship between the observed FC and the mean of normalized counts (Figure 1(c)). Overall, we found that 1% of the total rat genes included in our analyses (170 out of 16,252; Figure 1(d)) showed a differential response upon Wnt3a stimulation and that 88% of these genes were upregulated (149 genes) (Supplementary Table 3). Functional characterization of differentially expressed genes revealed that transcription factors (18.1%), proteins with nucleic acid binding activity (14.8%), and signaling related molecules (8.7%) represent main protein categories activated upon Wnt3a stimulation (Figure 1(e)).

We observed that the strongest upregulated gene in Wnt3a treated neurons was Fam84a (Gene ID: 313969; nominal p value = 2.2×10^{-6}; FC = 2.4; Table 1). Likewise,

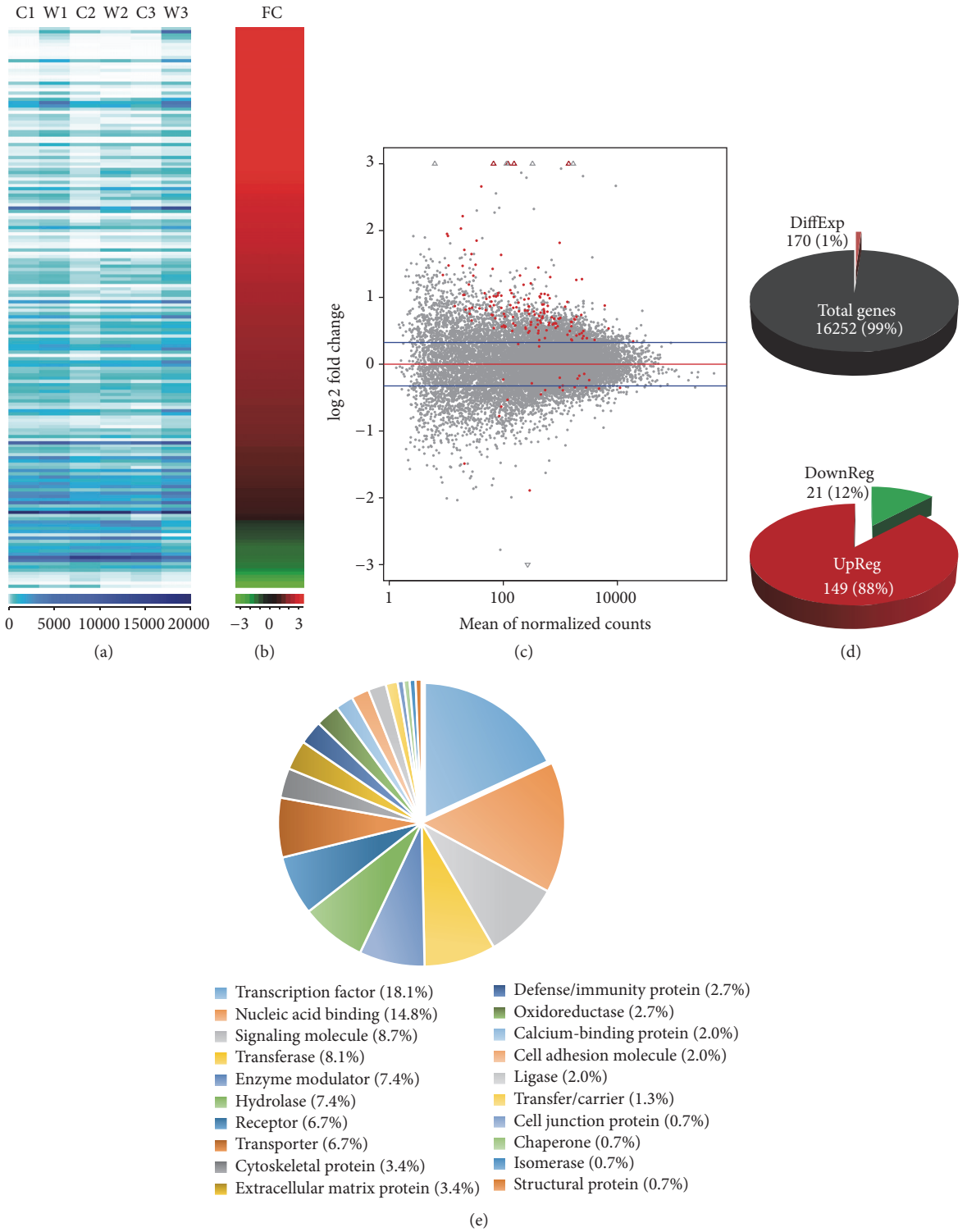

Figure 1: Transcriptome analysis, differentially expressed genes, and their functional classification. (a) Rat genes total amount of raw reads is displayed for each of the samples sequenced (C1: Control 1, W1: Wnt1, C2: Control 2, W2: Wnt2, C3: Control 3, and W3: Wnt3). Dark to light blue color scale denotes a higher to lower number of counts per gene, respectively. (b) Fold change observed for the cluster analysis of the 170 differentially expressed rat genes in red to green scale for up- to downregulation, respectively. (c) Smear plot. log 2 fold change (y axis) values versus normalized read counts per gene (x axis) between treatment and control conditions. A total of 170 confident transcript calls over the threshold of 0.05 p value (red dots) are shown. (d) Comparison of the 170 genes found differentially expressed and the total genes analyzed. (e) For all genes over threshold, we determined their protein class with the Panther software (v11.0).

Table 2: GO categories overrepresented in the 170 differentially expressed genes.

Category	GO ID	GO Name	GIP	GIS	p-adjusted (BH)
BP	GO:0061351	Neural precursor cell proliferation	122	14	1.6×10^{-10}
	GO:0030900	Forebrain development	266	18	2.2×10^{-09}
	GO:0048863	Stem cell differentiation	267	18	2.3×10^{-09}
	GO:0007420	Brain development	418	22	1.0×10^{-06}
	GO:0048732	Gland development	289	18	1.6×10^{-06}
	GO:0048864	Stem cell development	225	16	1.6×10^{-06}
	GO:0021872	Forebrain generation of neurons	58	9	3.3×10^{-06}
	GO:0021953	Central nervous system neuron differentiation	138	12	7.0×10^{-06}
	GO:0045165	Cell fate commitment	173	13	9.6×10^{-06}
MF	GO:0043565	Sequence-specific DNA binding	452	22	4.1×10^{-06}

BP: biological processes; CC: cellular components; MF: molecular function; GO ID: gene ontology term ID; GIP: genes in population; GIS: genes in study; BH: Benjamini-Hochberg correction multiple testing correction.

among the top 20 upregulated genes we found 11 Wnt/β-catenin target genes (Table 1 and Supplementary Table 3), including the palmitoleoyl-protein carboxylesterase Notum (ID: 303743; nominal p value = 1.5×10^{-5}; FC = 14.1), Lef1 (ID: 161452; nominal p value = 1.7×10^{-5}; FC = 2.1), Axin2 (ID: 29134; nominal p value = 1.2×10^{-4}; FC = 3.5), and the chemokine (C-X-C motif) ligand 3 Cxcl3 (ID: 171551; nominal p value = 5.2×10^{-4}; FC = 4.6). Interestingly, novel Wnt/β-catenin targets genes ranked between known targets and included the serine/threonine kinase 32A Stk32a (ID: 364858; nominal p value = 1.3×10^{-3}; FC = 1.8), the integrin subunit alpha 9 Itga9 (ID: 586004; nominal p value = 1.7×10^{-3}; FC = 2.0), and the transmembrane protein 72 TMEM72 (ID: 362424; nominal p value = 2.0×10^{-3}; FC = 1.7), among others (Table 1). These results indicate that after transient treatment with Wnt3a we can readily identify nascent RNA messages derived from activating the transcriptional program controlled by Wnt/β-catenin signaling in these hippocampal neurons. As to noncanonical Wnt signaling components being expressed in primary neurons as a consequence of Wnt3a treatment, we intersected the 170 nominally differentially expressed genes with the reactome pathway "beta catenin independent Wnt signaling" including 143 genes belonging to PCP and/or Ca^{2+} noncanonical pathways (DOI: 10.3180/REACT_172694.1). Only Lef1, Tcf7, and Prkg2 were found in both gene sets (Supplementary Figure 3a). While Lef1 and Tcf7 are widely acknowledged as major canonical components, Prkg2 is recognized only as a functional modulator of the canonical pathway (Table 2) through the inhibition of GSK3β activity [48]. Thus we conclude that Wnt3a did not enhance the expression of noncanonical components.

At a global level, we found that differentially expressed genes after Wnt3a treatment define gene ontology (GO) categories that have essential roles during brain development and homeostasis (Table 2). We observed that major biological process (BP) categories enriched with differentially expressed genes included neural precursor cell proliferation (GO:0061351; adjusted p value = 1.6×10^{-10}), forebrain development (GO:0030900; adjusted p value = 2.2×10^{-9}), and stem cell differentiation (GO:0048863; adjusted p value = 2.3×10^{-9}). Similarly, sequence-specific DNA binding activity (GO:0043565; adjusted p value = 4.1×10^{-6}) was the main molecular function (MF) category overrepresented in our analysis. All 170 differential expressed genes and their functional relationships are shown in Figure 2. A high degree of overlap was observed between previous GO terms with 37 genes (7 of which were among top 20 upregulated; Figure 2, hexagonal nodes) responsible for the enrichment given by 89 ontological associations (Figure 2, color edges). Additionally, we detected 11 high confidence interactions connecting 21 out of the 170 genes using the entire rat functional protein association network (FPAN) extracted from STRING database (see Materials and Methods).

3.2. Molecular Interaction Networks Activated by Wnt/β-Catenin Signaling. While differential gene expression analysis allows for the identification of disease altered genes in specific tissues by analyzing one gene at the time, this approach does not take into account the functional relationships between genes [73]. Therefore, since the convergence or aggregation of additional expression signals in a set of genes can become significant in a network context, we searched for highly connected subnetworks or modules displaying significant changes in expression upon Wnt signaling activation. We performed a module search using 2,892 upregulated genes (DESeq FC > 1.25) on the entire rat FPAN composed of a total of 9,443 genes with at least 1 high confidence interaction defined by STRING database (edge score above 900, see Materials and Methods) [68]. We observed a significantly higher number of modules (p value = 5.00×10^{-4}) using actual Wnt3a transcriptomic data (average = 17.00; SD = 0.982) in comparison with modules arising from chance using permuted data (average = 11.00, SD = 0.987) (Figure 3(a)). While the average score of the first fourth modules derived from Wnt3a data was significantly higher compared to their permuted counterpart (M1–M4; Figure 3(b)), the average score for M4 was below the highest random score and therefore M4 was not considered for further analysis. Altogether, these results show that the number and structure of modules remained consistent across iterations and that they could not be reached by chance,

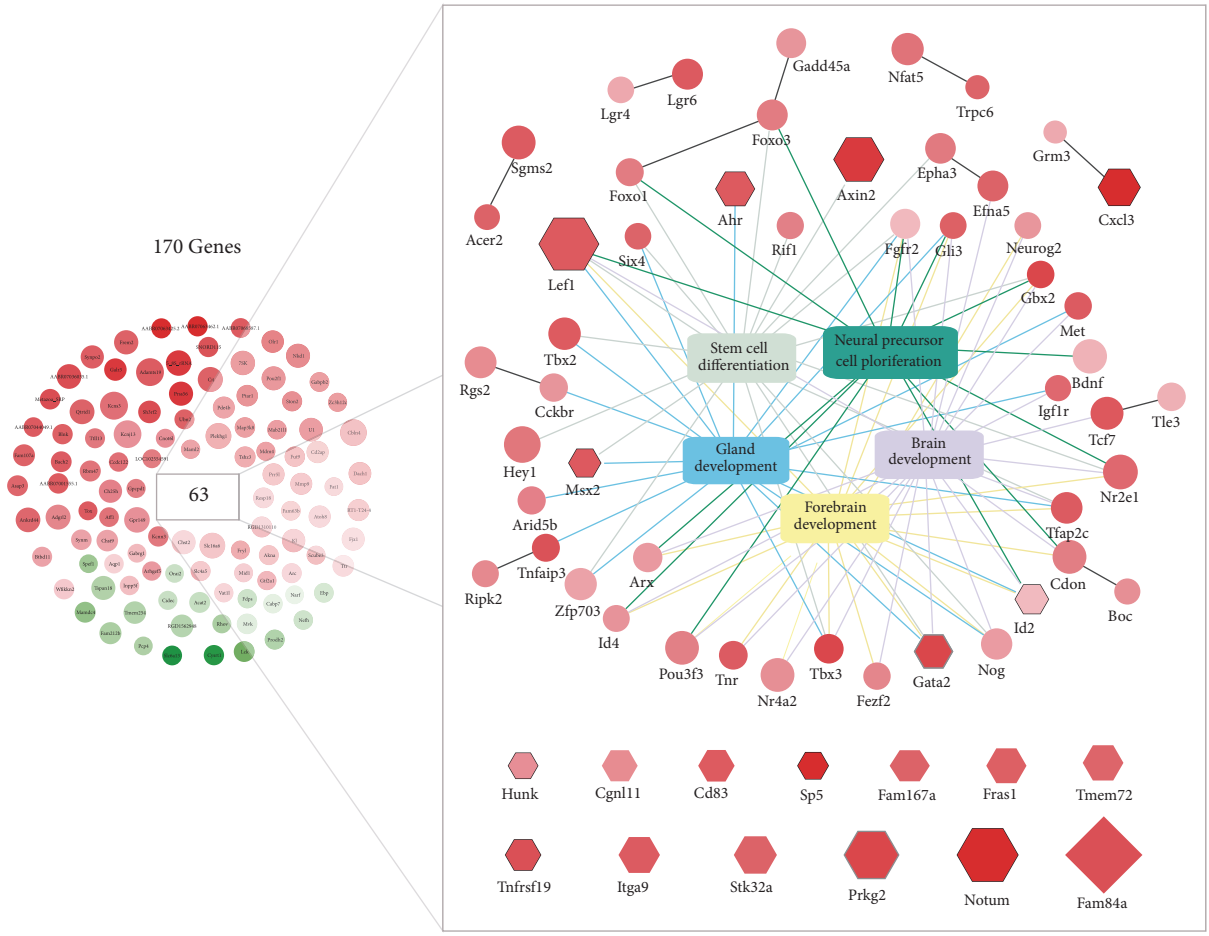

FIGURE 2: Functional relationships of Wnt differentially expressed genes. Nominal Wnt-induced differentially expressed genes are presented (n = 170) alongside their mayor functional interactions. Node color represents the observed fold change (FC) upon Wnt3a treatment in a gradient from green (downregulation) to red (upregulation) values (i.e., green: FC < 1; red FC > 1; white: FC = 1). Similarly, node size is proportional to the $-\log 10$ p value assigned by DESeq to the gene. Central boxes denote the top 5 overrepresented GO categories and their respective gene associations (color edges) which are presented for neural precursor (GO:0061364), forebrain development (GO:0030900), stem cell differentiation (GO:0048863), brain development (GO:0007420), and gland development (GO:0048732) categories. Direct functional interactions extracted from STRING are shown with black edges. Hexagon-shaped nodes show top 25 genes differentially expressed. Diamond-shaped node highlight top 1 differentially expressed gene (Fam68a). Black and grey node borders denote top 20 Wnt target or functional evidence, respectively.

supporting the idea that Wnt3a-derived subnetworks M1, M2, and M3 are indeed biologically meaningful. Components and interactions of M1, M2, and M3 subnetworks are provided in Figure 4. GO analysis further revealed that M1 was highly enriched with genes belonging to the glycerolipid metabolic process category (Table 3; GO:0046486; adjusted p value = 4.5×10^{-19}) and with lipase activity (GO:0016298; adjusted p value = 5.2×10^{-9}); M2 was mainly involved in learning or memory processes (Table 3; GO:0007611; adjusted p value = 4.0×10^{-5}) with genes related to cell adhesion molecule binding activity (GO:0050839; adjusted p value = 1.1×10^{-6}); and M3 was overrepresented with genes belonging to neurotransmitter secretion process (Table 3; GO:0007269; adjusted p value = 5.3×10^{-12}) and with syntaxin-1 binding activity (GO:0017075; adjusted p value = 1.2×10^{-12}). The complete list of the 93 genes belonging to M1, M2, and M3 subnetworks is presented in Supplementary Table 4 with their observed FC and p value.

4. Discussion

The transcriptional program directed by Wnt/β-catenin signaling has been examined mainly in cancer models and has allowed for the identification of novel target genes and a better understanding of the oncogenic properties of this pathway [74–76]. Conversely, much remains to be elucidated about the transcriptional program of the cascade in the central nervous system. In this context, initial attempts to discover novel Wnt/β-catenin target genes functioning in the brain relied on in silico analyses of proximal gene promoters enriched with TCF/LEF binding sites [77, 78]. More recently, bioinformatics and transcriptomic approaches have been

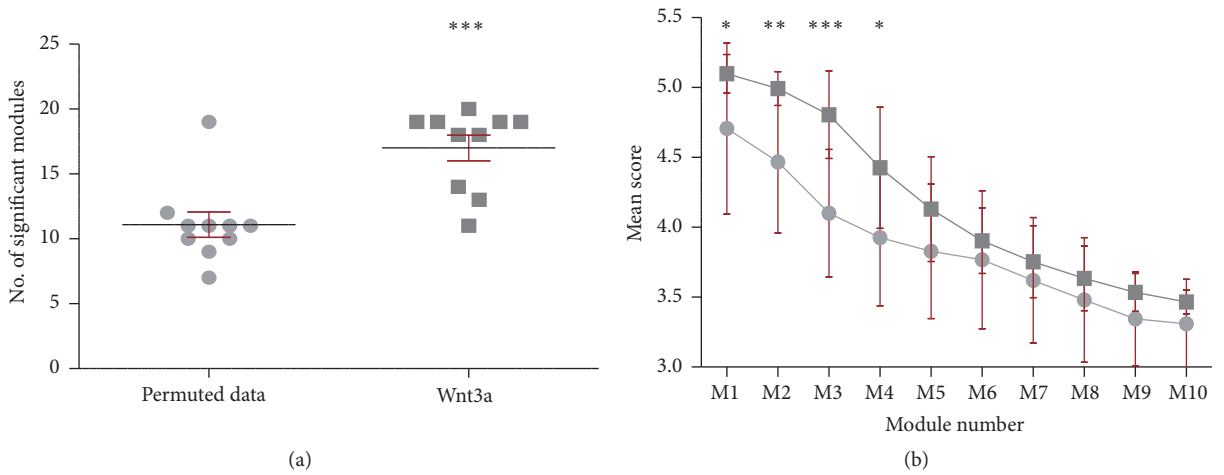

Figure 3: Module search results over the rat FPAN. (a) The number of significant modules (size between 10 and 50 genes with a SD score >3) obtained with nominal data (Wnt3a, dark grey) and with the same values permuted across the FPAN (permuted data, light grey). (b) Individual SD score comparison of the top 10 modules obtained in the corresponding module searches presented in (a). Asterisk denotes the Student t-test p value significance ($^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$).

Table 3: Top 3 GO categories overrepresented in Wnt3a-induced M1, M2, and M3 subnetworks.

Module	Category	GO ID	GO Name	GIP	GIS	p-adjusted (BH)
M1	BP	GO:0046486	Glycerolipid metabolic process	193	18	4.5×10^{-19}
		GO:0006644	Phospholipid metabolic process	166	17	1.1×10^{-18}
		GO:0006650	Glycerophospholipid metabolic process	134	16	1.2×10^{-18}
	MF	GO:0016298	Lipase activity	59	8	5.2×10^{-09}
		GO:0016788	Hydrolase activity, acting on ester bonds	430	14	1.2×10^{-08}
		GO:0004620	Phospholipase activity	47	7	1.7×10^{-08}
M2	BP	GO:0007611	Learning or memory	181	7	4.0×10^{-05}
		GO:0050890	Cognition	200	7	4.4×10^{-05}
	CC	GO:0043256	Laminin complex	8	4	2.0×10^{-07}
		GO:0005610	Laminin-5 complex	4	3	2.9×10^{-06}
		GO:0005605	Basal lamina	18	4	2.9×10^{-06}
	MF	GO:0050839	Cell adhesion molecule binding	131	7	1.1×10^{-06}
		GO:0001948	Glycoprotein binding	74	5	1.7×10^{-05}
		GO:0005178	Integrin binding	76	5	1.7×10^{-05}
M3	BP	GO:0007269	Neurotransmitter secretion	82	10	5.3×10^{-12}
		GO:0006887	Exocytosis	176	12	5.3×10^{-12}
		GO:0016079	Synaptic vesicle exocytosis	37	8	1.2×10^{-11}
	CC	GO:0044456	Synapse part	373	14	2.6×10^{-11}
		GO:0043679	Axon terminus	131	10	7.7×10^{-11}
		GO:0044306	Neuron projection terminus	139	10	9.4×10^{-11}
	MF	GO:0017075	Syntaxin-1 binding	16	7	1.2×10^{-12}
		GO:0000149	SNARE binding	69	8	8.0×10^{-10}
		GO:0019905	Syntaxin binding	46	7	1.7×10^{-09}

BP: biological processes; CC: cellular components; MF: molecular function; GO ID: gene ontology term ID; GIP: genes in population; GIS: genes in study; BH: Benjamini-Hochberg correction multiple testing correction.

used in thalamic neurons [79], or human neural progenitor cells [41], to identify Wnt/β-catenin target genes involved in neuronal excitation or neurodegenerative diseases, including Alzheimer's disease. In this regard, multiple lines of evidence support a functional role for Wnt/β-catenin signaling in prevalent neurological disorders related to synaptic dysfunction including autism, Alzheimer's disease, or epilepsy [80–83]. Nevertheless, although these studies have resulted in hundreds of potential novel candidates, the expression of those target genes shows minimal overlap (less than 2%) in

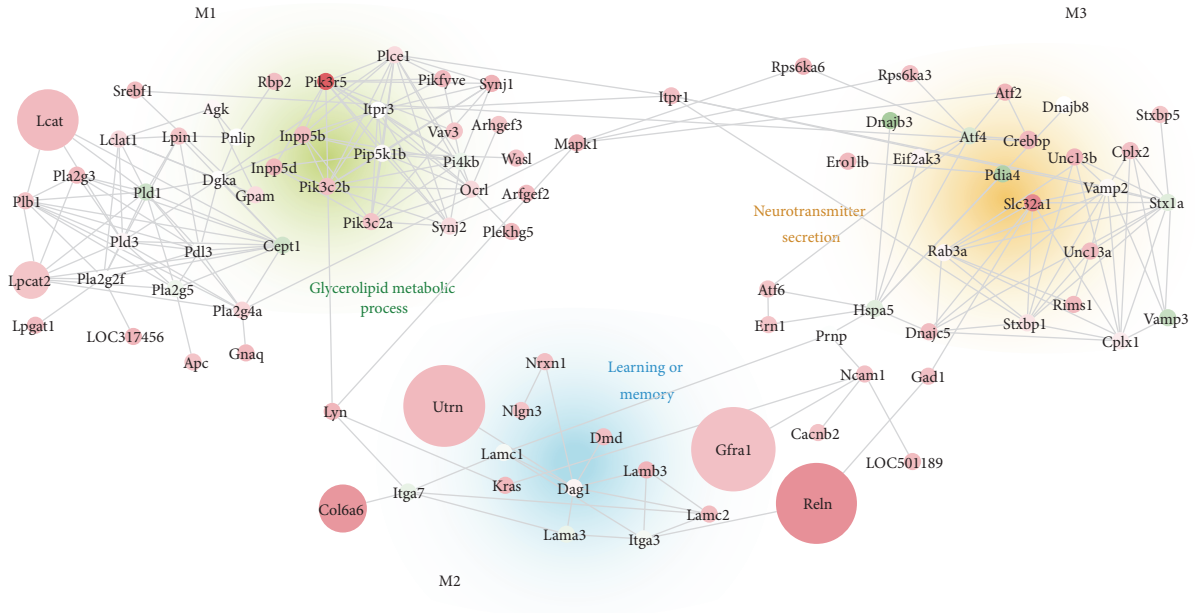

FIGURE 4: Molecular networks induced by Wnt3a treatment. Gene composition (nodes) and interactions (edges) are shown for modules M1 (upper right, green background), M2 (middle bottom, light blue background), and M3 (upper right, orange background). Node color represents the observed fold change (FC) upon Wnt3a treatment in a gradient from green (downregulation) to red (upregulation) values (i.e., green, FC < 1; red, FC > 1; white, FC = 1). Similarly, node size is proportional to the $-\log 10$ p value assigned to the gene behavior. For each module the main GO category associated is shown: M1 = glycerolipid metabolism process (GO:0046486), M2 = learning and memory (GO:0007611), and M3 = neurotransmitter secretion (GO:0007269). Module structure: M1 is composed of 44 genes with 132 interactions; M1 is composed of 25 genes with 32 interactions; and M1 is composed of 30 genes with 62 interactions (see also Supplementary Table 4).

different cell backgrounds (Supplementary Figure 3b), likely due to cell-type specific transcriptional and/or translational mechanisms.

Ultimately the goal of Wnt/β-catenin signaling in neuronal cells is to modulate changes in gene expression that are manifested in diverse cellular processes, including neurogenesis, axonal pathfinding, dendritic development, synaptic formation, and plasticity [39, 84]. These changes are likely accomplished by transcription factors that either interact or are activated downstream of β-catenin transcriptional complexes. Interestingly, we found that a large proportion (18.1%, $n = 28$) of Wnt/β-catenin targets in hippocampal neurons are genes coding for transcription factors (Figure 1). Indeed, within the top 20 Wnt3a upregulated genes we observed 6 transcription factors: Lef1, Ahr, Gata2, Id2, Msx2, and Sp5, that are target genes or functional partners with components of the signaling cascade and that play a role in synaptic development. For instance TCF/LEF transcription factors are instrumental in Wnt/β-catenin target gene expression [85], which include target genes participating in synaptic development and function [79]. Similarly, Ahr participates in the expression and membrane presentation of NMDA receptors [86]. Gata2 overexpression in hippocampal neurons leads to a decrease in synaptic spines density and depressive behavior in rats [87]. Id family genes including Id2 are upregulated in Rett syndrome [88], a neurodevelopmental disorder which features altered synaptic plasticity [89]; Id2 is also asymmetrically expressed in human embryonic brain hemispheres suggesting a role for this transcription factor in cortical specialization [90]. Additionally, several of these proteins also modulate Wnt pathway activation in other tissues [91–94]. While further experimental validation including proteomics and TCF/LEF transcription factor occupancy at a genome-wide level (ChIP-seq) is needed to confirm these potential candidates as Wnt/β-catenin targets in neuronal and nonneuronal cells, we note that none of the early Wnt3a-upregulated genes seems to be the result of downstream transcription factors or secondary transcriptional waves (Supplementary Figure 3c).

GO analysis results from differential expression data revealed that Wnt/β-catenin signaling enhances transcription of genes involved in brain development, particularly forebrain development, stem cell differentiation, and neural precursor cell proliferation (Figure 2). In this regard, a role for Wnt/β-catenin components in forebrain development has been clearly established [95] and recently this issue has received considerable attention mainly associated with the onset of autism [96]. Similarly, Wnt signaling is well known for enhancing cell renewal of neural stem cells and is a key modulator of neurogenesis [97]. Notably, Axin2, a classic Wnt target gene [47] and β-catenin destruction complex scaffold protein [98], has been recently shown to control the switch of intermediate progenitors from proliferative to differentiate status in the developing cerebral cortex [99]. Indeed, enhancing Axin expression in neuronal progenitors leads to an enlarged neocortex, an excess of excitatory synapses and autistic like behaviors [100].

Network based approaches have been widely used in the study of neurological diseases where usually the number of genetic markers that exceeds the significance threshold is very small; thus most of the identified markers are neglected [73]. The integration of these below-threshold markers into biologically significant networks has successfully identified novel modules of interaction in multiple sclerosis, Alzheimer's disease, autism, and other disorders [66, 71, 96, 101]. Our network analysis of Wnt3a differentially expressed pathways extends the notion that Wnt/β-catenin signaling has an essential function in neurotransmitter secretion, learning, and memory (Figure 4, modules M2 and M3). We also found a highly significant module comprising components of glycerolipid metabolism that is differentially expressed in neurons exposed to Wnt3a (module M1). M1 is enriched with several lipid-modifying enzymes families, such as PI(3)Ks, PLAs, PLCs, and PLDs whose products are readily found in synaptic membranes enhancing vesicle docking and release [102]. For instance, PLAs produce arachidonic acid, a precursor which will be further processed by COX2, a known Wnt/β-catenin target gene [103], during the generation of several eicosanoids which mediate the inflammatory response [104]. Arachidonic acid released from the postsynaptic terminal is known to potentiate synaptic transmission by inhibiting presynaptic potassium channels [105]. Mutations in PLAs have been described in both Alzheimer's disease and autism [104, 106]. Likewise, the production of phosphatidic acid by PLDs modulates several aspects of Wnt/β-catenin signaling in cancer and is recognized as a critical regulator of cell proliferation and tumorigenesis [107].

The M2 subnetwork, involved in learning or memory, is enriched in extracellular matrix components such as integrins and laminins. First, Itga9 (integrin subunit alpha 9) is among the 10 top upregulated genes reported in this study. Integrins are differentially expressed in specific regions in the adult brain [108] and they interact with Reln (Reelin) to activate cortical lamination [109]. Interestingly, the Reelin pathway, which crosstalks with Wnt/β-catenin signaling throughout brain development [110], has been associated with Alzheimer's disease [111] and autism [112]. Second, the enrichment of laminin subunits is particularly interesting since laminins are necessary for synaptic ultrastructure [113] and have been proven to prevent beta amyloid aggregation [114]. Third, neuronal cell adhesion molecules Neuroligin (Nlgn) and Neurexin (Nrxn) are essential for synaptic structure and function in the synaptic cleft and Nlgn3 and Nrxn1 have been associated with autism [115]. Finally, Wnt3a-derived data also allowed the identification of M3 subnetwork related to neurotransmitter secretion enriched with transcription factors belonging to the ATF/cAMP response element binding proteins (CREB) family of proteins, whose function is involved in synaptic plasticity and memory [116, 117]. Similarly, M3 includes several genes whose products are involved in membrane fusion, synaptic exocytosis, and presynaptic dynamics [118], including SNARES (Vamp2, Vamp3, and Stx1A), STX binding proteins (Stxbp1 and Stxbp5), and Rab signaling molecules (Rab3a and Rims1), which may account for synaptic effects induced by the crosstalk between canonical Wnt pathway and calcium signaling components and that may be important in synaptic dysfunction disorders.

5. Conclusion

Transcriptomics and network analyses are highly useful for identifying novel targets that can be used to better understand cellular changes during the onset or progression of neurodegenerative and neurodevelopmental disorders. Our results provide novel insights into the early transcriptional program and molecular networking directed by Wnt/β-catenin signaling in hippocampal neurons and warrant further investigation.

Ethical Approval

All procedures involving experimentation on animals were approved by the Bioethical Committee of Universidad Andres Bello and were conducted in accordance with the guidelines of the National Fund for Scientific and Technological Research (FONDECYT; Chile).

Disclosure

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Competing Interests

The authors declare no conflict of interests.

Authors' Contributions

Eduardo Pérez-Palma, Víctor Andrade, Giorgia D. Ugarte, and Giancarlo V. De Ferrari conceived and designed the experiments. Eduardo Pérez-Palma, Víctor Andrade, Bernabé I. Bustos, Camilo Villaman, Matías A. Medina, Miguel E. Ávila, and Giorgia D. Ugarte performed experiments or analyzed the data. Eduardo Pérez-Palma, Víctor Andrade, Mario O. Caracci, and Giancarlo V. De Ferrari wrote the paper. All authors read and approved the final manuscript. Eduardo Pérez-Palma and Víctor Andrade contributed equally to this work.

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Research Article

Neuregulin-1 Regulates Cortical Inhibitory Neuron Dendrite and Synapse Growth through DISC1

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Cortical inhibitory neurons play crucial roles in regulating excitatory synaptic networks and cognitive function and aberrant development of these cells have been linked to neurodevelopmental disorders. The secreted neurotrophic factor Neuregulin-1 (NRG1) and its receptor ErbB4 are established regulators of inhibitory neuron connectivity, but the developmental signalling mechanisms regulating this process remain poorly understood. Here, we provide evidence that NRG1-ErbB4 signalling functions through the multifunctional scaffold protein, Disrupted in Schizophrenia 1 (DISC1), to regulate the development of cortical inhibitory interneuron dendrite and synaptic growth. We found that NRG1 increases inhibitory neuron dendrite complexity and glutamatergic synapse formation onto inhibitory neurons and that this effect is blocked by expression of a dominant negative DISC1 mutant, or DISC1 knockdown. We also discovered that NRG1 treatment increases DISC1 expression and its localization to glutamatergic synapses being made onto cortical inhibitory neurons. Mechanistically, we determined that DISC1 binds ErbB4 within cortical inhibitory neurons. Collectively, these data suggest that a NRG1-ErbB4-DISC1 signalling pathway regulates the development of cortical inhibitory neuron dendrite and synaptic growth. Given that NRG1, ErbB4, and DISC1 are schizophrenia-linked genes, these findings shed light on how independent risk factors may signal in a common developmental pathway that contributes to neural connectivity defects and disease pathogenesis.

1. Introduction

Proper functioning of the central nervous system requires a fine balance between excitatory and inhibitory neurotransmission [1]. Cortical inhibitory neurons, classified by their expression of the inhibitory neurotransmitter gamma aminobutyric acid (GABA), comprise 10–25% of neurons in the cortex and are the primary source of inhibition [2]. Cortical inhibitory neurons play major roles in neural development and are important for processes such as fine-tuning of glutamatergic synapse formation and function and defining the timing of critical periods of experience-dependent neural plasticity in the developing brain [3, 4]. Cortical inhibitory neurons are also regulators of high frequency gamma oscillations, which are thought to underlie cognitive processes such as working memory and attention [5–7]. There is also abundant evidence that deficits in the development and function of cortical inhibitory neurons are involved in neurodevelopmental disorders such as epilepsy, schizophrenia, and autism spectrum disorders (ASDs) [1, 8–13]. Therefore, understanding the molecular pathways that regulate inhibitory neuron development may shed light on how their function is disrupted in these disorders. In this regard, the morphological development of cortical inhibitory neurons is governed by both extracellular (e.g., neuronal activity [14] and NRG1 [15–17]) and intracellular signalling molecules (e.g., the distal-less homeobox (Dlx) family of transcription factors [18]), which regulate the branching of dendrites and formation of synapses. However, the underlying signalling pathways governing inhibitory neuron development and, consequently, how these processes may be affected in neurodevelopmental disorders are still poorly understood.

Multiple studies have implicated a crucial role for the Neuregulin-1- (NRG1-) ErBb4 signalling pathway in the

development of cortical inhibitory neurons. Furthermore, several linkage and genetic association studies have identified the genes encoding both of these proteins as risk factors for schizophrenia [19–23]. NRG1 is a neurotrophic factor that binds to and activates the ErbB family of receptor tyrosine kinases on target neurons [24, 25]. In the mouse cortex, ErbB4 is predominantly expressed in GABA-ergic inhibitory neurons, with lower expression levels in excitatory neurons [17, 26–28]. Biological functions of the NRG1-ErbB4 signalling pathway in inhibitory neuron development include processes such as neuronal migration, dendrite growth, synapse formation, and neurotransmitter receptor expression [15, 16, 29–31]. For example, application of NRG1 to cortical neuronal cultures results in increased dendrite growth and excitatory synaptogenesis onto inhibitory neurons [15, 16], and inhibitory neuron-specific ErbB4 knockout mice display decreased excitatory synaptogenesis onto cortical inhibitory neurons [30]. One of the mechanisms by which NRG1-ErbB4 signalling regulates these processes is through activation of Kalirin-7, a gene previously implicated in schizophrenia [15, 32, 33]. However, there is little known about other signalling molecules downstream of NRG1-ErbB4 in this context. Disrupted in Schizophrenia 1 (DISC1) is another putative schizophrenia risk gene [34–41], and many lines of evidence suggest that it may functionally and/or physically interact with the NRG1-ErbB4 signalling pathway [15, 17, 30, 40–47]. DISC1 was first identified as a balanced translocation between chromosomes 1 and 11 (1q42.1; 11q14.8) in a Scottish pedigree with a high prevalence of schizophrenia and other psychiatric disorders [32, 33]. The functional consequence of this translocation is unknown. Previous studies suggest that it may work as a dominant negative protein [48–50], while another study suggests the disease mechanism may be haploinsufficiency [51] with the possibility of novel transcripts being generated due to the translocation [52]. The DISC1 gene encodes a scaffold protein that is expressed in the developing and adult brain and shares many roles in neurodevelopment with the NRG1-ErbB4 pathway [15, 46, 48, 50, 53, 54]. Conditional inhibitory neuron-specific ErbB4 knockout mice and DISC1 genetic mouse models display similar morphological deficits in brain development as well as behavioural phenotypes such as abnormal sensorimotor gating, working memory, and sociability [17, 30, 40–43, 50]. Additionally, ErbB4 and DISC1 share common binding partners at the postsynaptic density of excitatory synapses (e.g., postsynaptic density-95 (PSD95) and Kalirin-7) suggesting that they may physically or functionally interact [15, 44, 45]. A study by Seshadri and colleagues demonstrated that treatment of primary mouse cortical neurons with NRG1 increased DISC1 expression in the neurites of cortical neurons [49]. However, this effect was primarily mediated by ErbB2/3, suggesting that a novel NRG1-ErbB2/3 pathway regulates DISC1 expression in cortical excitatory neurons [49]. More recently, a study by the Sawa laboratory demonstrated that, in the mature mouse cortex, there is a functional relationship between NRG1, ErbB4, and DISC1 in the regulation of synaptic plasticity in inhibitory neurons [55]. However, whether this relationship is established during inhibitory neuron development and how the Scottish DISC1 mutation impacts this process have not been experimentally interrogated.

Here, we show that NRG1 functions through DISC1 to regulate the development of dendrite growth and excitatory synapse formation onto inhibitory neurons using inhibitory neuron-specific expression of a dominant negative DISC1 mutant that models the Scottish mutation. Furthermore, we provide evidence that treatment of primary mouse cortical cultures with NRG1 increases DISC1 levels and localization to glutamatergic synapses in the primary dendrites of inhibitory neurons. Finally, we provide evidence that ErbB4 binds to DISC1, suggesting that, in developing inhibitory neurons, NRG1-ErbB4 signals through DISC1. Together these results show that two candidate schizophrenia risk pathways functionally interact to regulate the development of cortical inhibitory neuron morphology.

2. Materials and Methods

2.1. Antibodies and Constructs. The following primary antibodies were used in this study: goat anti-DISC1 N-terminus (N-16) (Santa Cruz Biotechnology; IF/PLA 1 : 100, WB 1 : 500), rabbit anti-ErbB4 C-terminus (C-18) (Santa Cruz Biotechnology; PLA 1 : 100, WB 1 : 100), guinea pig anti-VGLUT1 (EMD Millipore; IF 1 : 1000, WB 1 : 3000), anti-GAD65&67 (Millipore; 1 : 1000), mouse anti-GFP (Santa Cruz Biotechnology; IP 1 : 1000), rabbit anti-β-actin (Cell Signaling Technologies; IB 1 : 1000), anti-mouse IgG (IP 1 : 1000), and chicken anti-GFP (Aves Labs Inc.; IF 1 : 1000). All secondary antibodies (anti-goat cy5, anti-guinea pig cy3, and anti-chicken 488; Jackson ImmunoResearch; IF 1 : 500, anti-rabbit-HRP, anti-mouse-HRP; GE Life Sciences; IB 1 : 5000) were raised in donkey.

The DLX5/6-GFP construct was a gift from De Marco García et al. [14]. The control shRNA, DISC1 shRNA, and DISC1-GFP constructs were created as described previously [56]. The P_{G67}-GFP construct was a gift from Di Cristo [57, 58]. The P_{G67}-DISC1FL and P_{G67}-DISC1DN constructs were generated by GeneArt (Life Technologies). The full-length mouse DISC1 gene (DISC1FL) (RefSeq NC_000074.6) and a C-terminal truncated mutant in which the C-terminal 257 amino acids are deleted (DISC1DN) [50] were assembled from synthetic oligonucleotides and/or PCR products. Each fragment was cloned separately into the P_{G67}-GFP vector (kanR) using PacI and PmeI cloning sites, resulting in constructs containing the promoter of GAD67 upstream of the DISC1FL or DISC1DN coding sequence. The plasmid DNA was then purified from transformed bacteria. The ErBb4 plasmid and ErbB4 KD plasmid were gifts from Yardena Samuels (pcDNA3.1-ErbB4: Addgene plasmid #29527, pcDNA3.1-ErbB4 kinase dead: Addgene plasmid #29533).

2.2. Cell Culture, Transfection, and Treatment. Primary cortical neurons were cultured as follows. Cortices were dissected out of CD1 mouse (Charles River) embryonic brains at E16. Dissociation was aided by incubation in 0.3 mg/mL Papain (Worthington Biochemical)/400 U/mL DNase I (Invitrogen) in 1x Hanks Buffered Saline Solution (HBSS) for 20 minutes at 37°C, followed by light trituration. Cells were seeded onto

0.1 mg/mL Poly-D-Lysine (BD Sciences)/3.3 μg/mL Laminin (Sigma)-coated cover slips (Matsunami) in 12-well plates at a density of ~0.8–1 × 10^6 cells/well in plating media containing Neurobasal medium, 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin, and 2 mM L-Glutamine (Invitrogen). After 1.5 hours, media was changed to serum-free feeding media containing Neurobasal medium, 2% B27 supplement, 1% Penicillin/Streptomycin, and 2 mM L-Glutamine. At DIV2–4, cultures were treated with 1 μM Cytosine β-D-arabinofuranoside hydrochloride (Ara-C) (Sigma) to inhibit glial cell proliferation. Cultures were maintained at 37°C, 5% CO_2. All media components were from Gibco unless otherwise specified. Transfections were performed at DIV7 using Lipofectamine LTX and Plus reagents (Invitrogen) according to the manufacturer's instructions.

HEK 293 FT cells were cultured in Dulbecco's Modified Eagle Medium (Fisher Scientific) supplemented with 10% FBS and 1% Glutamax (Fisher Scientific) and were passaged every 2–4 days. HEK 293 FT cell transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Primary neurons were treated with 5 nM Recombinant Human NRG1β1/HRG1β1 EGF Domain (R&D Systems) dissolved in phosphate-buffered saline (PBS) on DIV19 and 20. An equal volume of PBS was used as a vehicle control. For western blotting, primary cortical cultures were treated with 5 nM NRG1 on DIV3 and 4, and scraped into lysis buffer on DIV5. For Duolink Proximity Ligation Assays, cells were treated with NRG1β1 or PBS for 5 minutes prior to fixation. HEK 293 FT cells were treated with 10 nM Human NRG1β1/HRG1β1 EGF Domain for 5 minutes at 37°C. Following treatment, cells were placed on ice, washed with ice-cold PBS, and scraped into lysis buffer.

2.3. Coimmunoprecipitation and Western Blotting. Protein lysates were prepared by cell scraping in lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris-Cl, and cOmplete mini protease inhibitor cocktail (Roche). 25 μL protein G dynabeads (Fisher Scientific) were incubated with 5 μg primary antibody or IgG control antibody for 1 h at 4°C. Lysates were then incubated with the bead-Ab conjugate for 1 h at 4°C. The beads were then washed three times with lysis buffer and then boiled in sample buffer for 5 minutes. For western blotting, 20 μL of sample was loaded in a 8% Tris-Glycine gel and run at room temperature, followed by transfer to a PVDF membrane (Thermo Scientific). Membranes were blocked for 1 h in 3% milk in 1x TBST and incubated with primary antibody overnight and then with secondary antibody (donkey anti-mouse or anti-rabbit HRP, GE Healthcare) for 1 h at room temperature before exposure using a ChemiDoc MP system (BioRad).

2.4. Immunocytochemistry and Quantification. On DIV21, cells on glass cover slips were fixed in 4% formaldehyde in PBS for 20 minutes at room temperature. Cells were washed in PBS, followed by blocking in Blocking/Permeabilization solution consisting of 10% Donkey Serum (Cedarlane) and 0.3% Triton X-100 (Fisher Scientific) in PBS for 1 hour at room temperature. Incubation in primary antibodies was performed at 4°C overnight with gentle agitation. Cells were then washed in PBS, followed by incubation with secondary antibodies in 50% Blocking/Permeabilization solution at room temperature with gentle agitation for 1.5 hours. Cells were then washed in PBS and were mounted on VistaVision glass microscope slides (VWR) using Prolong Gold antifade reagent (Life Technologies). Cover slips were allowed to dry overnight before being imaged on a Zeiss LSM700 confocal microscope. For puncta analyses, images were manually thresholded using ImageJ such that each image within an experiment was thresholded to the same value. The "Particle Analysis" tool in ImageJ was used to count the number of individual puncta from the cell body and two to three dendritic sections per cell (10–40 μm^2) of primary dendrites adjacent to the cell body. Sholl analysis was performed in ImageJ. The Straight Line tool was used to draw a line 200 μm in length starting from the centre of the soma. The Sholl analysis plugin (http://labs.biology.ucsd.edu/ghosh/software/ShollAnalysis.pdf) was used to make concentric circles increasing at a constant radius of 10 μm and to count the number of intersections.

2.5. Duolink Proximity Ligation Assay (PLA). The PLA was performed using Duolink *In Situ* Red reagents (Sigma). Cortical neurons were seeded onto poly-D-Lysine/Laminin-coated cover slips in 24-well plates (~3.5 × 10^5 cells/well) or 12-well plates (~1 × 10^6 cells/well). After treatment on DIV21, the cortical neurons were fixed with 4% formaldehyde in PBS at room temperature for 20 minutes. Cells were washed in 1x PBS 3 times, 8 minutes each, followed by blocking in Blocking/Permeabilization solution consisting of 10% Donkey Serum (Cedarlane) and 0.3% Triton X-100 (Fisher Scientific) in PBS for 1 hour at room temperature. Incubation in primary antibodies was performed at 4°C overnight with gentle agitation. Primary antibodies were omitted in the control PLA condition. Samples were washed in 1x Wash Buffer A (supplied with the kit) at room temperature 2 times, 5 minutes each, followed by incubation with a mixture containing the two PLA probes diluted in 50% Blocking/Permeabilization Solution in a humidified chamber at 37°C for 1 hour. The cells were again washed in 1x Wash Buffer A at room temperature 2 times, for 5 minutes. The ligation reaction was performed in a humidified chamber at 37°C for 30 minutes, followed by washing in 1x Wash Buffer A 2 times, 5 minutes each. The cells were then incubated with the amplification-polymerase solution for 100 minutes at 37°C in a darkened humidified chamber. The cells were then washed with 1x Buffer B (supplied with the kit) 2 times, 10 minutes each, followed by a 1 minute wash with 0.01x buffer B at room temperature. Cover slips were then mounted onto VistaVision glass microscope slides (VWR) using mounting media with DAPI (supplied with the kit). Images were acquired on a Zeiss LSM700 confocal microscope using a 63x objective. The PLA signal density (identified as red dots) was quantified in the cell body and 3 primary dendrites per cell from manually thresholded maximum intensity projections of three to seven Z-stacks (1 μm step size) per image using ImageJ.

2.6. Statistical Analysis. Quantified data are presented as mean ± SEM and analyzed using GraphPad PRISM 6.

Statistical comparisons between two groups were made using unpaired student's *t*-tests. Comparisons between multiple groups were made using one-way analysis of variance (ANOVA), with Tukey's *post hoc* tests to identify significant differences between groups. Probability (p) values of less than 5% were considered significant.

3. Results

3.1. shRNA Knockdown of DISC1 Inhibits NRG1-Mediated Dendrite and Excitatory Synapse Growth of Cortical Inhibitory Neurons. We used mixed primary cortical neuron cultures derived from E16/17 mouse embryos as our model system, which contains both excitatory and inhibitory neurons. To label and identify cortical inhibitory neuron, we transfected cultured neurons with a plasmid that expresses green fluorescent protein (GFP) under an enhancer element of the distal-less homeobox (DLX) 5 gene, which is expressed in the majority of forebrain inhibitory neurons [14]. We then cotransfected previously validated control shRNA or DISC1 shRNA plasmids together with DLX5/6-GFP into day *in vitro* (DIV) 7 neurons [56]. We treated cultures with NRG1 (or PBS control) for two days beginning at DIV19 and analyzed cells at DIV21. We found that knocking down DISC1 expression caused no change in the puncta density of the excitatory presynaptic marker, vesicular glutamate transporter 1 (VGLUT1), in both the cell body and primary dendrites compared to control shRNA-treated neurons (Supplementary Figures 1A–C in Supplementary Material available online at http://dx.doi.org/10.1155/2016/7694385). Furthermore, we found that the control shRNA-expressing neurons treated with NRG1 showed an increase in VGLUT1 puncta density in the primary dendrites, in line with a previous report [16] (Figures 1(a) and 1(c)). To determine if DISC1 plays a role in this process, we knocked down DISC1 in neurons treated with NRG1 and discovered that the NRG1-mediated increase in VGLUT1 puncta density was completely abolished (Supplementary Figures 1A–C).

Next we determined if NRG1 regulates dendritic growth of cortical inhibitory neurons through DISC1. Using the same cultures for analysis, we imaged the complete dendritic morphology of individual GFP-labelled cortical inhibitory neurons. Using Sholl analysis, we determined that knocking down DISC1 led to a decrease in dendritic morphology in PBS-treated cells (Supplementary Figures 1D–F). Furthermore, we determined that NRG1 treatment for two days led to an increase in dendritic morphology, which was abolished when DISC1 expression was decreased using shRNA (Supplementary Figures 1D–F). Taken together, these results suggest that NRG1 regulates the dendritic and synaptic growth of cortical inhibitory neurons and requires DISC1 expression to mediates these effects.

3.2. NRG1 Regulates DISC1 Expression and Localization to Glutamatergic Synapses in Cortical Inhibitory Neurons. The results in Supplementary Figure 1 suggest that NRG1 regulates cortical inhibitory dendrite and synapse growth; however, a caveat of these experiments is that DISC1 was knocked down nonspecifically in both excitatory and inhibitory neurons since we used cultures. Therefore, in our subsequent experiments we specifically manipulated DISC1 levels in cortical inhibitory neurons with a construct that uses the glutamic acid decarboxylase (GAD67) promoter to drive separate expression of GFP and DISC1 (P_{G67}-GFP). GAD67 is expressed in all forebrain GABA-ergic neurons as it is the rate-limiting enzyme in the conversion of glutamate to GABA [59]. Furthermore, it has been reported that the majority of DLX5-expressing cortical inhibitory neurons also express GAD67 [14]. Immunostaining of cortical cultures transfected with P_{G67}-GFP confirmed that GFP-positive neurons expressed endogenous GAD67 (Figure 1(a)).

Given the potential relationship between NRG1 and DISC1 we uncovered, we wanted to determine if NRG1 treatment specifically regulates DISC1 expression in cortical inhibitory neurons. It has been previously shown that NRG1 treatment of cortical neuron cultures leads to an increase in DISC1 levels via an ErbB2/3-mediated mechanism, most likely reflecting DISC1 levels in excitatory neurons as they make up 80–90% of cortical neuron cultures [49]. Therefore, we hypothesized that NRG1 also regulates DISC1 expression levels and localization specifically within inhibitory neurons. Using quantitative immunofluorescence, we first detected that two days of NRG1 treatment (starting at DIV19) of developing cultures caused a significant increase in DISC1 levels in the primary dendrites and the cell body of DIV21 cortical inhibitory neurons compared to vehicle treatment (PBS) (Figures 1(b), 1(c), and 1(f)), suggesting that the growth effects of NRG1 on inhibitory neurons may require DISC1. Given this result, we next asked whether the NRG1-induced increase in DISC1 expression is localized to excitatory synapses on inhibitory neurons by staining for VGLUT1. The numbers of VGLUT1 or double-positive DISC1/VGLUT1 puncta on the cell body and primary dendrites of P_{G67}-GFP positive inhibitory neurons were quantified. We found that NRG1 treatment led to a significant increase in the number of VGLUT1-positive excitatory synapses on both the cell body and primary dendrites (Figures 1(b), 1(d), and 1(g)). Furthermore, we found a significant increase in double-positive DISC1/VGLUT1 puncta on the cell body and primary dendrites on cortical inhibitory neurons (Figures 1(b), 1(e), and 1(h)). These data indicate that NRG1 stimulation is sufficient to increase DISC1 levels and localize its expression to excitatory synapses formed on inhibitory neurons. Additionally, western blotting of cultured cortical neurons treated with NRG1 on DIV3 and 4 showed a slight increase in VGLUT1 and DISC1 levels compared to vehicle (PBS) treated cultures, although this was not significant (Figure 1(i)).

3.3. NRG1 Functions through DISC1 to Regulate Glutamatergic Synaptogenesis onto Cortical Inhibitory Neurons. In the mouse brain, the NRG1 receptor ErbB4 is primarily localized to GABA-ergic interneurons in the postsynaptic densities receiving glutamatergic input, where it regulates excitatory synapse formation and maturation [17]. To investigate whether DISC1 works downstream of NRG1-ErbB4 to regulate excitatory synapse formation onto cortical inhibitory neurons, we examined VGLUT1 immunofluorescence in primary cortical cultures. Cortical cultures were transfected with P_{G67}-GFP on DIV 7 and treated with NRG1 or PBS

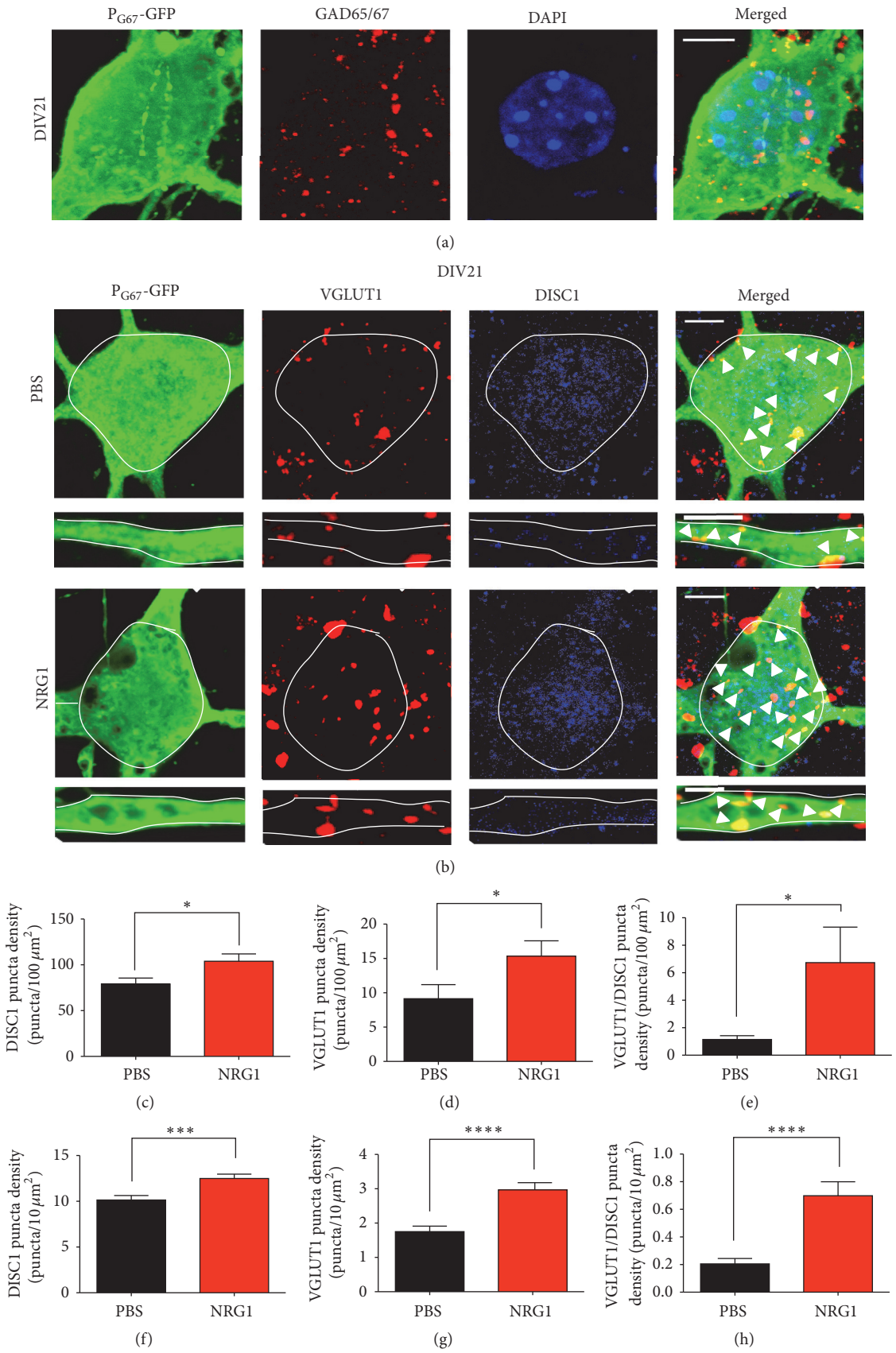


Figure 1: Continued.

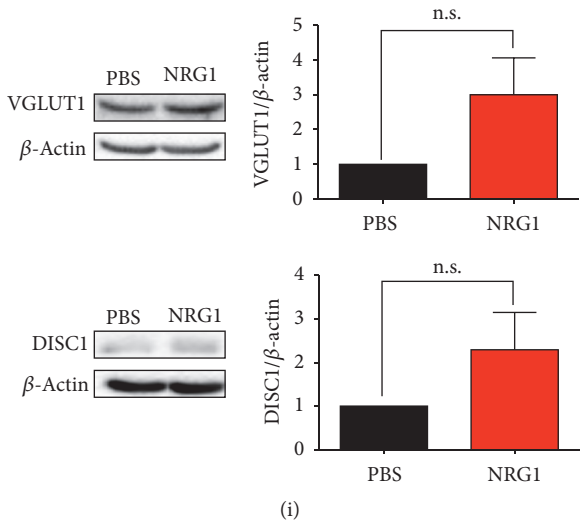
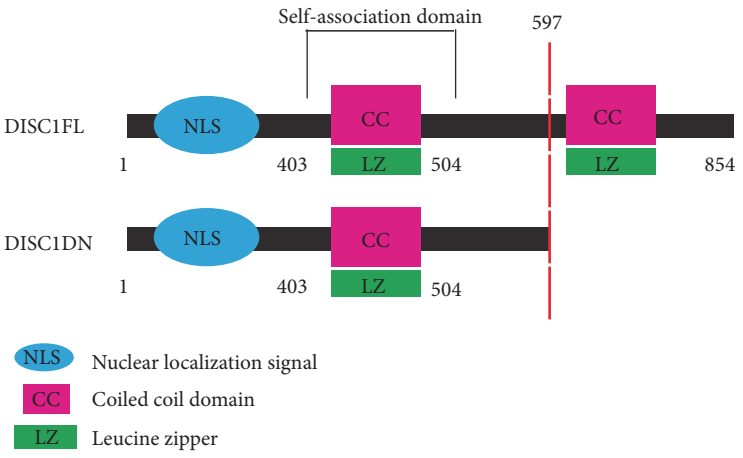


Figure 1: NRG1 regulates DISC1 expression and localization to excitatory synaptic terminals in cortical inhibitory neurons. (a) Representative image of GAD65&67 staining (red) in DIV21 cortical inhibitory neurons transfected with P_{G67}-GFP on DIV7. Images were acquired at 63x. Scale bar = 5 μm. (b) Representative images of immunofluorescent staining of DISC1 (blue) and VGLUT1 (red) in DIV21 cortical inhibitory neurons transfected with P_{G67}-GFP on DIV7 and treated with NRG1β or PBS for 2 days. Cultures were also stained for GFP to enhance the GFP signal (green). Images were acquired at 63x. Scale bars = 5 μm (cell body zoom image) and 2 μm (dendrite zoom image). Arrowheads indicate double-positive colocalized VGLUT1/DISC1 puncta. NRG1 treatment results in an increase in VGLUT1 puncta density, DISC1 puncta density, and double-positive colocalized VGLUT1/DISC1 puncta density in the cell body (c–e) and in the primary dendrites (f–h). Significance determined using an unpaired student's t-test. Error bars represent standard error of the mean, $n = 25$ cells (2-3 primary dendrites/cell) per condition from 5 experiments, $^{*}p < 0.05$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. (i) Western blot for VGLUT1 (top) and DISC1 (bottom) in cultured cortical neurons treated with NRG1 for 2 days starting at DIV3 and then lysed at DIV5. $N = 3$ separate mouse litters. Student's t-test.

for two days, starting on DIV 19. Cultures were then fixed and analyzed on DIV21. Quantification of discrete puncta of VGLUT1 immunoreactivity in DIV21 cortical inhibitory neurons expressing P_{G67}-GFP revealed that NRG1 treatment caused a significant increase in puncta density on both the cell body and primary dendrites (Figures 2(b)–2(d)). Coexpression of P_{G67}-GFP with a plasmid expressing full length mouse DISC1 under control of the GAD67 promoter (P_{G67}-DISC1FL) revealed that expression of DISC1FL in inhibitory neurons at baseline conditions (PBS) had no effect on VGLUT1 puncta density compared to P_{G67}-GFP-only controls (Figures 2(b)–2(d)). To study the Scottish DISC1 mutation, we used a C-terminal truncated mouse DISC1 mutant (DISC1DN) (Figure 2(a)). The stop codon of this mutant occurs at the orthologous region of the translocation breakpoint found in the human DISC1 Scottish pedigree [50]. When overexpressed in mice, this mutant has been shown to act in a dominant negative manner by binding to and redistributing wild-type (WT) DISC1, causing defects in neural migration, dendrite formation, and reduced cortical parvalbumin levels [50, 53, 54]. We cotransfected P_{G67}-GFP with a plasmid expressing DISC1DN under control of the GAD67 promoter (P_{G67}-DISC1DN) and compared its expression to DISC1FL in cortical inhibitory neurons and found no gross differences in expression levels (Figure 2(c)). In subsequent experiments with the DISC1FL and DISC1DN plasmids, we found that expression of DISC1DN in inhibitory neurons at baseline conditions (PBS) significantly decreased VGLUT1 puncta on the primary dendrites, but not in the cell body (Figures 2(d) and 2(e)), suggesting that the DISC1 Scottish mutation impairs excitatory synaptogenesis onto cortical inhibitory neurons at baseline conditions. We then performed the same experiment in the presence of NRG1 stimulation for 2 days (starting at DIV19). We discovered that expression of P_{G67}-DISC1DN completely blocked the NRG1-induced increase in VGLUT1 puncta density on both primary dendrite shafts and the cell body (Figures 2(d) and 2(e)). These data indicate that inhibiting DISC1 specifically in cortical inhibitory neurons blocks NRG1-induced effects on glutamatergic synaptogenesis. Taken together, these results implicate a cell-autonomous role for NRG1-DISC1 signalling in developing cortical inhibitory neurons. However, it is important to note that while the truncated DISC1 mimics the Scottish mutation discovered in patients, our overexpression paradigm does not recapitulate allele heterozygosity as patients have one intact DISC1 allele.

3.4. NRG1 Functions through DISC1 to Regulate Dendrite Growth in Cortical Inhibitory Neurons. Given our identification of a developmental relationship between NRG1 and DISC1 in excitatory synaptogenesis on inhibitory neurons, we examined whether this extends to neuronal morphology. Although both NRG1 and DISC1 have been found to independently regulate dendrite growth in cortical neurons, it is still unknown whether they regulate this process together [15, 48]. Therefore, to elucidate a functional interaction between NRG1 and DISC1 in cortical inhibitory neurons dendrite growth, we examined the effects of expression of P_{G67}-DISC1FL or P_{G67}-DISC1DN at baseline (PBS) and NRG1 treatment conditions. Cortical cultures were cotransfected



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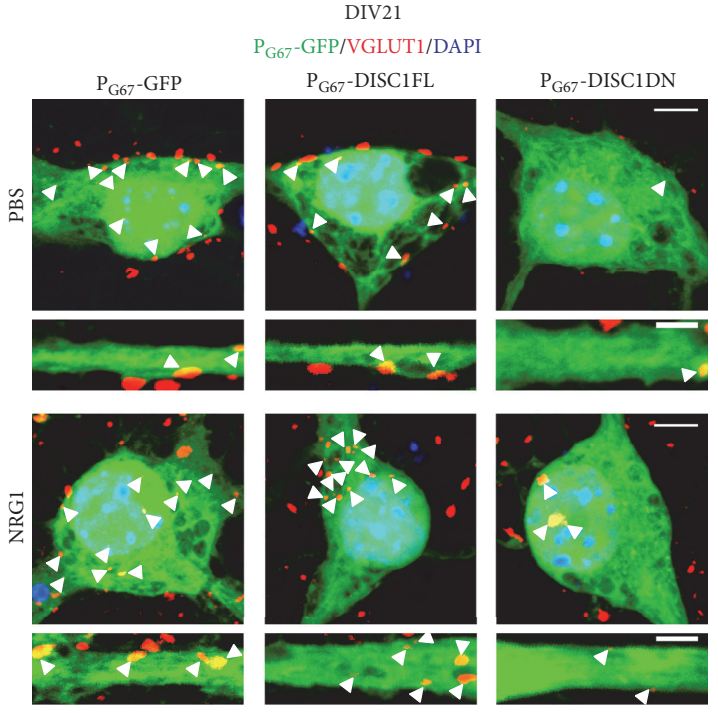

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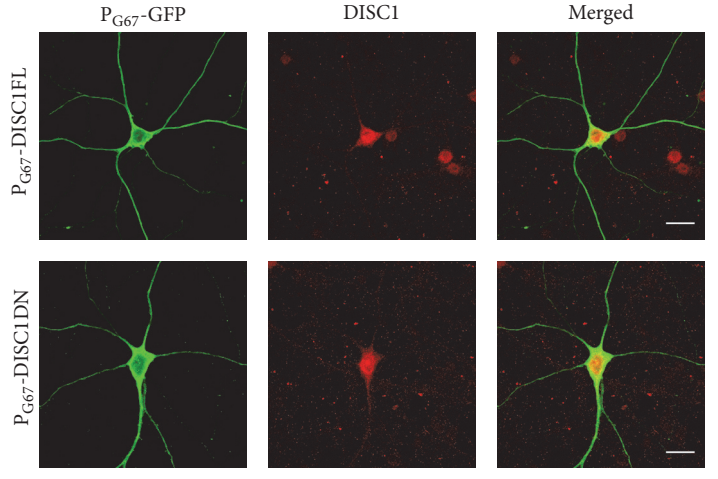

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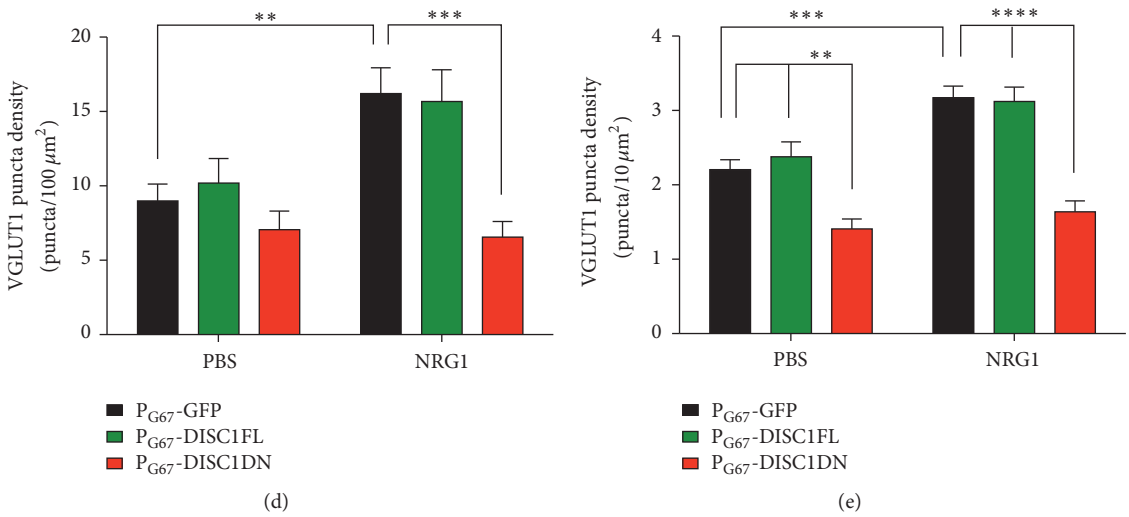


Figure 2: NRG1 functions through DISC1 to regulate glutamatergic synaptogenesis onto cortical inhibitory neurons. (a) Schematic of the mouse DISC1FL protein and DISC1DN truncated mutant protein. (b) Representative images of immunofluorescence staining of VGLUT1 in DIV21 cortical inhibitory neurons cotransfected with P_{G67}-GFP and P_{G67}-DISC1FL or P_{G67}-DISC1DN on DIV7. Cells were treated with NRG1β or PBS for 2 days. Cultures were stained for GFP to enhance the GFP signal. Images were acquired at 63x. Scale bars = 5 μm (cell body zoom image), 2 μm (dendrite zoom image). Arrowheads indicate VGLUT1 puncta colocalized with P_{G67}-GFP. (c) Representative images of immunofluorescent staining of DISC1 in DIV21 cortical inhibitory neurons transfected with P_{G67}-GFP and P_{G67}-DISC1FL or P_{G67}-DISC1DN on DIV7. NRG1β treatment caused a significant increase in VGLUT1 puncta density in the cell body (d) and primary dendrites (e) which was blocked by expression of P_{G67}-DISC1DN. Significance determined using a one-way analysis of variance (ANOVA) with Tukey's *post hoc* tests. Error bars represent standard error of the mean, $n = 27$–50 cells (2-3 primary dendrites/cell) per condition from 3 experiments, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

with P_{G67}-GFP and either P_{G67}-DISC1FL or P_{G67}-DISC1DN at DIV7. Cultures were then treated with either NRG1 or PBS on DIV19 and fixed and analyzed on DIV21. Sholl analysis revealed that, at baseline conditions, expression of P_{G67}-DISC1FL had no significant effect on dendrite growth, whereas P_{G67}-DISC1DN expression significantly decreased dendrite growth (Figures 3(a)–3(c)). Similar to previous reports, we found that stimulation of cultures with NRG1 increased inhibitory neuron dendrite growth and complexity (Figures 3(a)–3(c)). We next asked whether this NRG1-dependent effect requires DISC1 function. Sholl analysis revealed that expression of P_{G67}-DISC1FL or P_{G67}-DISC1DN blocked the NRG1-induced effects on dendrite growth, causing a significant decrease in dendrite growth compared to the P_{G67} control under NRG1 treatment conditions (Figures 3(a)–3(c)). These data suggest that the DISC1DN mutant affects dendrite growth specifically in cortical inhibitory neurons, implicating a cell-autonomous role of DISC1 in regulating dendrite growth in this cell type. In addition, the observation that overexpression of either full-length DISC1 or mutant truncated DISC1 inhibited NRG1-induced dendrite growth demonstrates the complexities of NRG1 signalling.

3.5. ErbB4 and DISC1 Interact in Cortical Inhibitory Neurons. Our data thus far suggest that NRG1 requires DISC1 for certain aspects of inhibitory neuron dendrite and glutamatergic synapse growth; however, we do not know whether DISC1 functions directly downstream of ErbB4, the receptor for NRG1. DISC1 and ErbB4 share many binding partners at the postsynaptic density [15, 44, 45]; therefore, we hypothesized that DISC1 may physically interact with ErbB4. A recent study demonstrated that DISC1 plays a role in regulating the interaction between ErbB4 and the postsynaptic protein, PSD95 particularly in the mature cortex [55]. However, whether DISC1 binds the ErbB4 receptor specifically within developing inhibitory neurons, and if NRG1 regulates this process, remains unknown. We first took a biochemical approach to test this using a heterologous cell system (HEK293 FT cells). We expressed ErbB4, kinase dead ErbB4 (ErbB4 KD), or DISC1-GFP alone or DISC1-GFP + ErbB4 or DISC1-GFP + ErbB4 KD in HEK293 cells, immunoprecipated for GFP, and used an ErbB4 antibody to determine binding to DISC1. We found that when DISC1-GFP and ErbB4 were expressed together in HEK293 cells, we detected an interaction between the two proteins, demonstrating that they can direct bind one another (Figure 4(a), asterisks). Interestingly, we found this interaction was reduced when a kinase dead version of ErbB4 was expressed, indicating DISC1 may require NRG1 activation of ErbB4 for intracellular binding (Figure 4(a)). However, the interaction between DISC1 and ErbB4 was not changed in the presence of NRG1 stimulation likely because the overexpressed ErbB4 receptor self-dimerizes, causing transactivation [60]. While these experiments indicate ErbB4 can bind DISC1, these results do not extend to inhibitory neurons. Considering that only 10–25% of cultured cortical neurons are inhibitory neurons, traditional coimmunoprecipitation experiments would not be able to detect interactions specifically within inhibitory neurons.

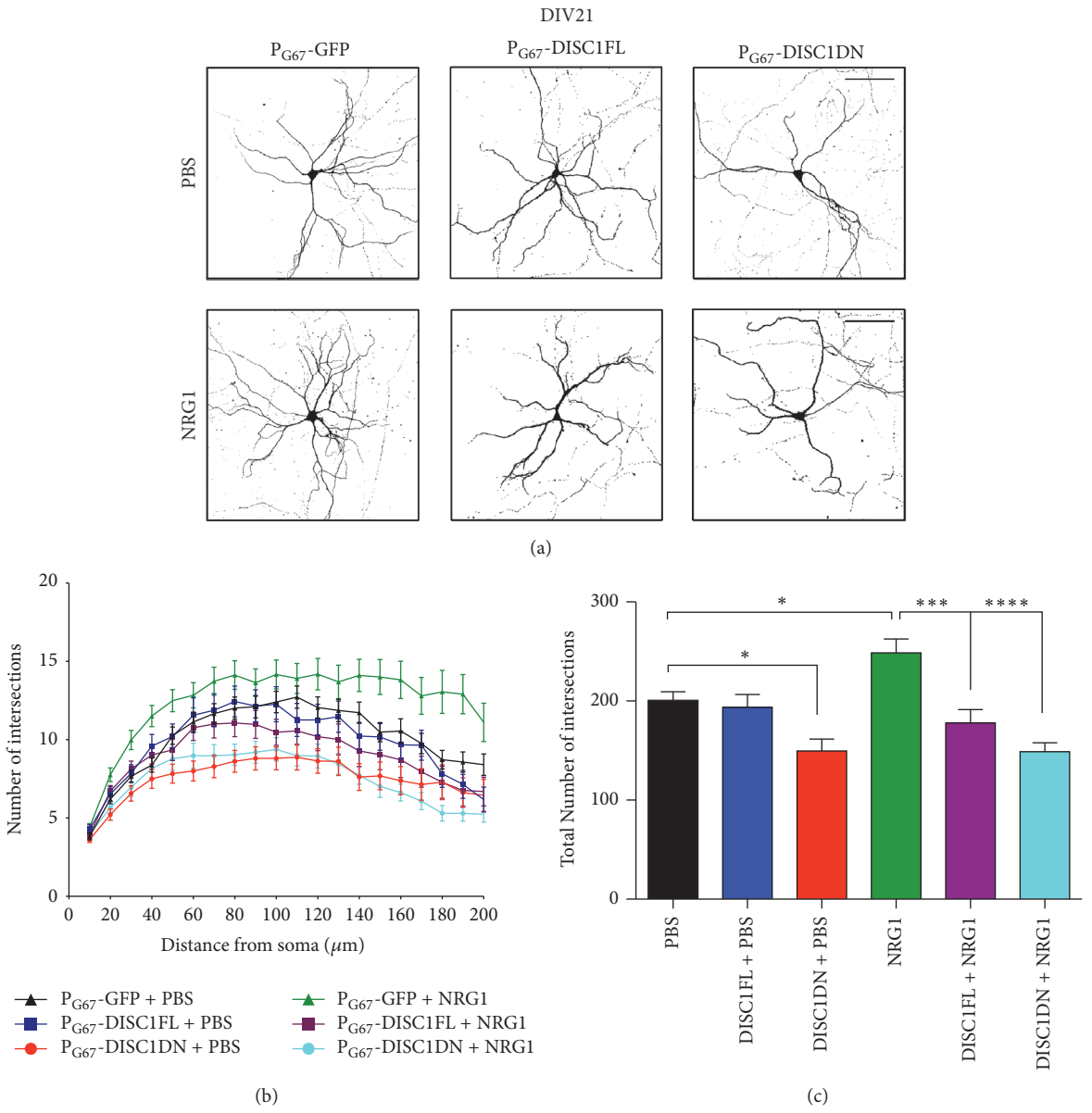
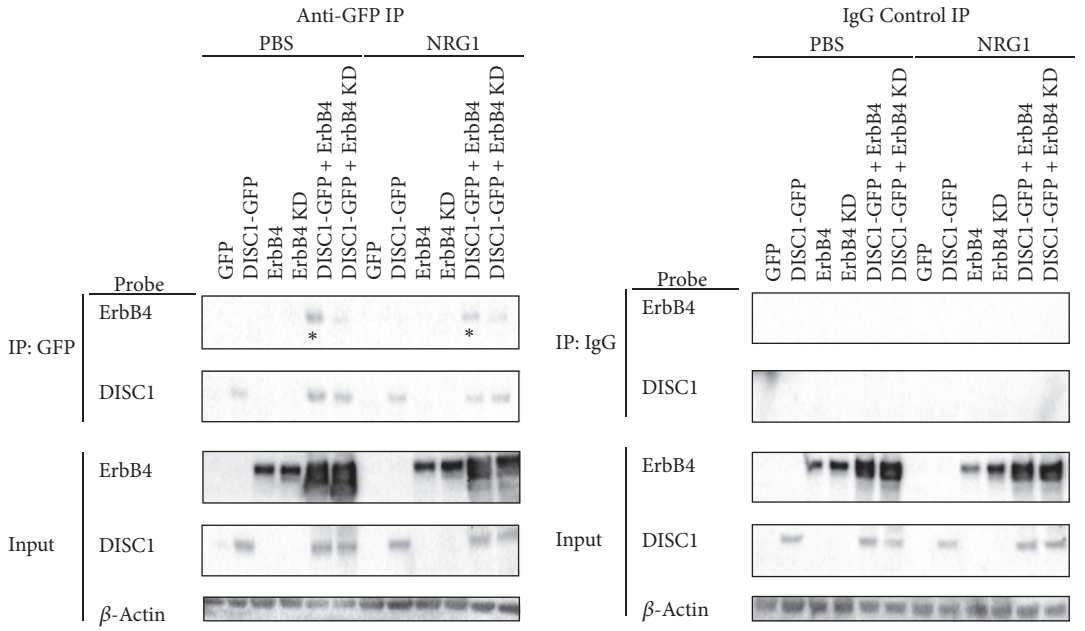


FIGURE 3: NRG1 functions through DISC1 to regulate cortical inhibitory neuron dendrite growth. (a) Representative images of DIV21 cortical inhibitory neurons cotransfected with P_{G67}-GFP and P_{G67}-DISC1FL or P_{G67}-DISC1DN on DIV7. Cells were treated with PBS (top panels) or NRG1β (bottom panels) for 2 days. Cultures were stained for GFP to enhance to GFP signal. Images were acquired at 20x. Scale bar = 100 μm. (b) Dendrite growth was analyzed by Sholl analysis using ImageJ. (c) At baseline conditions (PBS treatment), expression of P_{G67}-DISC1DN resulted in a significant decrease in total dendrite growth compared to P_{G67}-GFP controls; expression of P_{G67}-DISC1FL had no effect compared to P_{G67}-GFP controls. In cells treated with NRG1β, both P_{G67}-DISC1FL and P_{G67}-DISC1DN caused a significant decrease in total dendrite growth compared to P_{G67}-GFP controls. Significance determined using a one-way analysis of variance (ANOVA) with Tukey's *post hoc* tests. Error bars represent standard error of the mean, n = 34–54 cells per condition from 3 experiments; $^{*}p < 0.05$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

Therefore we used an alternative technique to overcome this problem and examine the interaction specifically in P_{G67}-GFP-positive cultured inhibitory neurons.

To do this, we performed a Proximity Ligation Assay (PLA) on DIV21 cortical cultures transfected with P_{G67}-GFP on DIV7 (Figures 4(b)–4(d)). PLA is a method that allows for visualization of endogenous protein-protein interactions in fixed cells and results in a punctate fluorescent signal where the proteins are within 40 nm of each other. Analysis of PLA signal density in cortical inhibitory neurons expressing P_{G67}-GFP revealed that NRG1 caused a significant increase in PLA signal density compared to PBS-treated controls in both the cell body and primary dendrites (Figures 4(b)–4(d)). Signal density in PBS treated cells was no different than that of the control PLA condition, in which primary antibodies were omitted (Figures 4(b)–4(d)). We also detected PLA signal outside of the GFP-inhibitory neuron, which we attribute to DISC1 binding to the low levels of ErbB4 in excitatory



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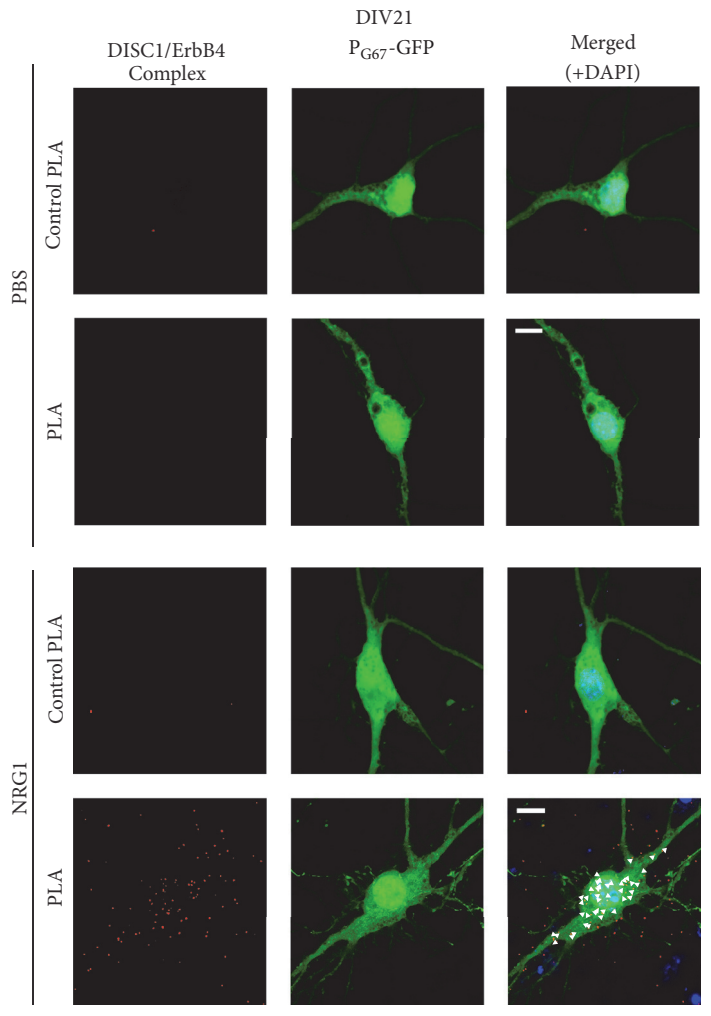

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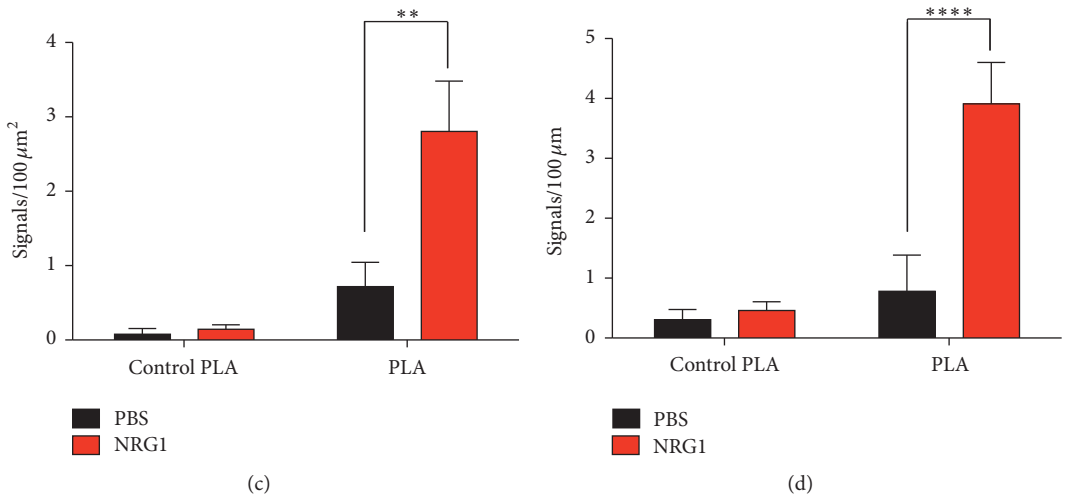

Figure 4: ErbB4 and DISC1 physically interact in cortical inhibitory neurons. (a) Co-IP of DISC1-GFP and ErbB4 in lysates from HEK293 FT cells transfected with ErbB4, ErbB4 KD, or DISC1-GFP alone or with DISC1-GFP + ErbB4 or DISC1-GFP + ErBb4 KD, with or without NRG1 treatment. Left panel: western blot for ErbB4 and DISC1-GFP in anti-GFP (DISC1) precipitates and input. Right panel: western blot for ErbB4 and DISC1-GFP in anti IgG control precipitates and input. DISC1-GFP binds ErbB4 in both PBS and NRG1 conditions (left panel, asterisks). Binding is reduced with DISC1-GFP and kinase dead ErbB4 (ErbB4 KD) (left panel). No binding was observed in the IgG control precipitates (right panel). (b) Proximity Ligation Assay (PLA) was performed in DIV21 cortical inhibitory neurons transfected with P_{G67}-GFP on DIV7 and treated with NRG1β or PBS for 5 min prior to fixation. Primary antibodies were omitted in the control PLA condition. Representative images were acquired at 63x. Scale bars = 10 μm. NRG1β treatment significantly increased the number of PLA signals in the cell body. Arrowheads indicate PLA signals colocalized with P_{G67}-GFP positive neurons (c) and the primary dendrites (d). Significance determined using a one-way analysis of variance (ANOVA) with Tukey's *post hoc* tests. Error bars represent standard error of the mean, n = 6–15 cells (2-3 primary dendrites/cell) per condition from 3 experiments; $^{**}p < 0.01$, $^{****}p < 0.0001$.

neurons (Figure 4(b), lower left panel). Taking the biochemical and PLA results together, they demonstrate that ErbB4 binds to DISC1 and that NRG1 stimulation increases this interaction in developing cortical inhibitory neurons. This suggests that DISC1 may be recruited to activated ErbB4 upon NRG1 binding to ErbB4 and is a part of the initial signalling cascade downstream of NRG1-ErbB4 during development.

4. Discussion

The development of cortical inhibitory neurons is crucial for normal cognitive processes, and disrupted development and function of these cells are strongly implicated in neurodevelopmental and psychiatric disorders. However, since their development is not well understood, it is important to gain a better understanding of the signalling mechanisms that regulate inhibitory dendrite and synapse growth. Our study reveals that NRG1-ErbB4 signalling functions through DISC1 to regulate dendrite growth and excitatory synapse formation on cortical inhibitory neurons. Specifically, we found that NRG1 stimulation increases DISC1 levels and its localization to excitatory synapses in the primary dendrites of cortical inhibitory neurons, a mechanism that may underlie the developmental effects of NRG1 on dendrite growth and excitatory synaptogenesis onto cortical inhibitory neurons. Furthermore, we show that NRG1-ErbB4 signals through DISC1 to developmentally regulate excitatory synaptogenesis onto cortical inhibitory neurons. Third, we show that NRG1-ErbB4 signals through DISC1 to regulate the development of dendrite growth in cortical inhibitory neurons. Finally, we show that NRG1 stimulation promotes binding of ErbB4 to DISC1 in cortical inhibitory neurons.

The results from this study are consistent with other *in vitro* NRG1 studies, which show that NRG1 regulates dendrite growth and excitatory synaptogenesis onto cortical inhibitory interneurons [15, 16]. *In vivo* data from two different conditional neocortical inhibitory neuron-specific ErbB4 knockout mouse models displaying decreased VGLUT1 puncta density on hippocampal interneurons further corroborates our findings [17, 30]. The data in the present study provide a potential mechanism mediating the effects of NRG1 signalling in cortical inhibitory neuron development, whereby DISC1 functions downstream of NRG1-Erbb4 signalling. A previous study by Cahill et al. in 2012 elucidated a mechanism whereby NRG1-ErbB4 signalling regulates dendrite growth in cortical inhibitory interneurons by disinhibiting the RAC1-GEF Kalirin-7 [15]. Interestingly, Kalirin-7 is also a binding partner of DISC1 at the postsynaptic density (PSD) [46], suggesting that ErbB4, DISC1, and Kalirin-7 may form a functional complex in cortical inhibitory neurons to regulate dendrite growth, and provides an avenue for further research into the downstream mechanisms of NRG1 signalling.

In this study, we examined a potential mechanism by which DISC1 mediates the effects of NRG1-ErbB4 signalling, in which NRG1-ErbB4 signalling regulates DISC1 levels in the primary dendrites of cortical inhibitory neurons. This is consistent with a study by Seshadri and colleagues in 2010 which showed that treatment of primary mouse cortical

neurons with NRG1 increased the expression of the 130 kDa isoform of DISC1 in the primary dendrites of cortical neurons [49]. However, this effect was found to be mediated by ErbB2/3 heterodimers and likely reflects the large numbers of excitatory neurons from cortical cultures (~80–90%) since they did not isolate inhibitory neurons [49]. Furthermore, because ErbB4 expression is much higher in inhibitory neurons than in excitatory neurons [17, 26–28], it is not surprising that NRG1 regulation of DISC1 levels in excitatory neurons would require ErbB2/3 and not ErbB4. We have also shown that NRG1 stimulation increases colocalization of DISC1 with VGLUT1 in cortical inhibitory neurons, suggesting that NRG1 stimulation localizes DISC1 to developing excitatory synapses contacting inhibitory neurons. Therefore, our study provides the first evidence that NRG1 regulates DISC1 expression and localization in developing cortical inhibitory neurons. However, whether this is carried out at the transcriptional, translational, or posttranslational level remains to be elucidated in future studies.

The role of DISC1 in psychiatric disorders remains controversial; however, many biological studies have shown that DISC1 plays important roles in cortical development [44, 46, 50, 55, 56]. There have been few studies examining the function of DISC1 in cortical inhibitory neurons [61, 62]; therefore what role it plays in their development is still not well understood. Our study provides the first report of DISC1 regulating dendrite growth and glutamatergic synapse formation specifically in cortical inhibitory neurons during neurodevelopment. We show using a P_{G67}-DISC1DN construct, which expresses a dominant negative form of DISC1 in GABA-ergic neurons, that DISC1 regulates dendrite growth in a cell-autonomous fashion. Furthermore, inhibitory-specific expression of a dominant negative DISC1 mutant and inhibitory-specific overexpression of full length DISC1 were both able to abolish NRG1-induced effects on dendritic arborisation, suggesting that an optimal level of NRG1-ErbB4 signalling is necessary for proper dendrite growth. This hypothesis is supported by a study in which two mutant NRG1 mouse strains, one with elevated cysteine-rich domain- (CRD-) NRG1 levels in cortical pyramidal neurons and one with reduced CRD-NRG1 levels, were both able to disrupt excitatory-inhibitory balance of neurotransmission [63]. In contrast, expression of the dominant negative DISC1 mutant, but not full-length DISC1, was able to block NRG1-induced effects on glutamatergic synaptogenesis onto cortical inhibitory neurons in the present study. This suggests that NRG1-ErbB4-DISC1 signalling may mediate its effects on dendrite growth and excitatory synapse development via two different mechanisms in cortical inhibitory neurons. NRG1-ErbB4 signalling has been found to mediate synapse maturation and dendrite growth via two distinct mechanisms in hippocampal mouse cultures [57]. Specifically, regulation of the maturity of synapses contacting ErbB4-positive hippocampal neurons by ErbB4 was dependent on the extracellular domain and PDZ motif, whereas the tyrosine kinase domain was not required [57]. In contrast, ErbB4 regulated dendrite growth via its tyrosine kinase domain and PI3 kinase signalling [57]. DISC1 may carry out different functions downstream of NRG1-ErbB4 stimulation depending on its interaction with different ErbB4 domains (PDZ or tyrosine kinase domain) or its interaction with other ErbB4 binding proteins that preferentially bind to either the PDZ or tyrosine kinase domain.

ErbB4 and DISC1 share common binding partners at the postsynaptic density, including PSD95 and Kalirin-7 [15, 46, 47]. However, a physical interaction between ErbB4 and DISC1 has not been examined thus far in primary inhibitory neurons due to the difficulty in isolating a large number of purified cells (devoid of excitatory neurons). Here, using an alternative technique (Proximity Ligation Assay), we have shown that NRG1 stimulation promotes binding of ErbB4 to DISC1 in cortical inhibitory neurons. Further investigation is needed to determine which protein domains are important for this interaction and for NRG1-induced effects on cortical inhibitory neuron development and whether Kalirin-7 is also involved in this complex. Additionally, it will be important to understand the potential mechanisms underlying a developmental switch for DISC1 regulating ErbB4 signalling during development versus the mature cortex, as these roles may be opposite.

While our study provides a mechanism for NRG1 function during inhibitory neuron development, it highlights potential differences with NRG1 signalling in the mature cortex. Seshadri et al. recently reported that DISC1 negatively regulates ErbB4 signalling, where, upon removal of DISC1, there is increased phosphorylation of ErbB4 and binding to PSD95 [55]. These results are in contrast to the results of our study, which suggests that DISC1 positively regulates ErbB4 signalling. However this can potentially be explained by a difference in the time point examined in brain function, since we examined neurodevelopmental ages while the Seshadri et al. study examined inhibitory neuron function in the mature cortex. Furthermore, the difference in approach to disrupt DISC1 function could also explain potential differences. For example, our study used a dominant negative form of DISC1 that models the Scottish mutation, whereas the Seshadri et al. study used shRNA and a DISC1 knockout transgenic mouse model. This also highlights that the Scottish mutation may not be accurately modeled by a complete loss of DISC1 function. Future studies are necessary to tease apart the exact mechanism of NRG1-ErbB4 regulation by DISC1 across different developmental and adult time points of inhibitory neuron function.

5. Conclusion

In conclusion, this study elucidated the novel convergence of NRG1-ErbB4 signalling and DISC1 onto a common signalling pathway regulating the development of cortical inhibitory neurons. As NRG1, ErbB4, and DISC1 are all candidate schizophrenia-associated risk genes [19–21, 23, 34–41], the results of this study not only shed light on the molecular mechanisms governing the normal development of cortical inhibitory neurons but also may provide insight into the aberrant processes underlying psychiatric disorders.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Cellular Zinc Homeostasis Contributes to Neuronal Differentiation in Human Induced Pluripotent Stem Cells

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Disturbances in neuronal differentiation and function are an underlying factor of many brain disorders. Zinc homeostasis and signaling are important mediators for a normal brain development and function, given that zinc deficiency was shown to result in cognitive and emotional deficits in animal models that might be associated with neurodevelopmental disorders. One underlying mechanism of the observed detrimental effects of zinc deficiency on the brain might be impaired proliferation and differentiation of stem cells participating in neurogenesis. Thus, to examine the molecular mechanisms regulating zinc metabolism and signaling in differentiating neurons, using a protocol for motor neuron differentiation, we characterized the expression of zinc homeostasis genes during neurogenesis using human induced pluripotent stem cells (hiPSCs) and evaluated the influence of altered zinc levels on the expression of zinc homeostasis genes, cell survival, cell fate, and neuronal function. Our results show that zinc transporters are highly regulated genes during neuronal differentiation and that low zinc levels are associated with decreased cell survival, altered neuronal differentiation, and, in particular, synaptic function. We conclude that zinc deficiency in a critical time window during brain development might influence brain function by modulating neuronal differentiation.

1. Introduction

Zinc is an essential trace metal interacting with a plethora of proteins. It plays a functional role in structural, regulatory, and signaling processes and thus is essential for a healthy brain. However, abnormally high levels of zinc are cytotoxic. Therefore, zinc levels have to be highly regulated during embryogenesis and development of the central nervous system (CNS). It is thus not surprising that zinc deficiencies can contribute to the occurrence of numerous human birth defects involving CNS malformation [1, 2]. On a mechanistic point of view, zinc has many roles in the developing and adult brain [3]. For example, zinc is an essential catalytic component of many different mammalian enzymes, such as DNA and RNA polymerases and histone deacetylases [4] needed for DNA replication and cellular proliferation. Additionally, zinc-dependent enzymes such as metalloproteinases and zinc-binding proteins such as metallothioneins (MTs) have a function in metabolism and zinc signaling [5]. Furthermore, many protein-protein interactions and DNA-binding properties of receptors [6] and transcription factors known to regulate key genes involved in cellular proliferation and neurogenesis are mediated by zinc-finger motifs [7, 8]. Intriguingly, maternal zinc deficiency has been identified as a risk factor for the development of autism in the offspring [9]. Further, mice exposed to zinc deficiency during brain development display autism like behavior later in life [10, 11]. Therefore, zinc signaling might play a crucial role during brain development, in particular neurogenesis and synaptogenesis, and by that ultimately mediate correct circuit formation.

Cellular zinc homeostasis is regulated by transporters, such as DMTs (divalent metal transporters), ZnTs (zinc transporters of the SLC30A family), and ZIP (Zrt-Irt-like proteins

of the SLC39A family), and intracellular zinc-binding proteins, in particular metallothioneins (MTs). Transmembrane transporters mediate the uptake and removal of zinc and transport of zinc into and out of intracellular organelles. ZnT proteins transport zinc out of the cytosol and ZIP proteins move zinc into the cytosol. Zinc binding in the cytosol is mostly regulated by proteins of the MT family (MT-1, MT-2, and MT-3), which bind zinc transiently and are therefore able to provide zinc for signaling processes [12, 13].

It was reported that zinc may play a role in the control of both developmental and adult neurogenesis mediated by proliferating adult stem cells in the subgranular zone of the dentate gyrus [14]. However, on a cellular level, the underlying mechanisms that regulate zinc homeostasis in differentiating neurons and the influence of different zinc levels on differentiation efficacy and nerve cell function after differentiation are so far not well understood.

Here, we used human induced pluripotent stem cells (hiPSC) as model system for neuronal differentiation to determine the cellular consequences of altered zinc levels. To that end we used iPS cells from keratinocytes of two healthy controls [15]. iPS cells are somatic cells that can be reprogrammed to a pluripotent state by gene transfer [16–18]. As pluripotent stem cells, they can be differentiated into several lineages, of which we choose a neuronal fate. We differentiated iPS cells into neuronal precursor cells (NPCs) and neurons using conditions that favor the generation of motor neurons and evaluated the differential expression of zinc homeostasis genes and outcomes of altered zinc levels.

2. Methods

2.1. Materials. DMEM/F12 + GlutaMAX, DPBS without Ca^{2+}/Mg^{2+}, GlutaMAX, NEAA antibiotic-antimycotic, natural essential amino acids, knockout serum replacement, BDNF, GDNF, IGF-1, B27, FBS, and N2 were purchased from Gibco/Life Technologies. DPBS with Ca^{2+}/Mg^{2+} was obtained from PAA. mTeSR1 stem cell medium and dispase were purchased from Stemcell Technologies. Purmorphamine was obtained from Calbiochem. Insulin was obtained from SAFC. ROCK inhibitor was purchased from Ascent Scientific and β-mercaptoethanol was purchased from Millipore. HESC qualified matrigel was obtained from BD Biosciences. Retinoic acid, poly-L-ornithine, laminin, heparin sodium salt, sodium selenite, apotransferrin, putrescin, progesterone, acetylcholine, glutamate, GABA, glycine, and NMDA were purchased from Sigma-Aldrich. Cyclothiazide was obtained from Tocris. Ultra-low attachment flasks were purchased from Corning Costar and μ-dishes (35 mm, low) were purchased from Ibidi. Chelex 100 resin was purchased from Bio-Rad. Tissue-Tek® was obtained from Sakura. The Apoptosis/Necrosis/Healthy Cell Detection kit was purchased from PromoKine and ApoTox-Glo Triplex Assay was purchased from Promega. Triton X-100 was procured from Roche. RNeasy Mini and Micro kit, Quantitect primers, QuantiFast SYBR Green RT-PCR kit, RT^2 First Strand kit, and customized RT^2 profiler rotor-disks were obtained from Qiagen. Primary antibodies were purchased from Synaptic Systems (Homer1 1 : 500, Synaptophysin 1 : 500, and GRIA3 1 : 500), Abgent (CHRNA3 1 : 500), UC Davis/NIH NeuroMab Facility (GABA-A-Rα1 1 : 500), Covance (SMI-32 (NEFH) 1 : 1000), R&D Systems (active caspase-3 1 : 1000), and Sigma-Aldrich (NMDAR1 1 : 500). Alexa Fluor-conjugated secondary antibodies were purchased from Invitrogen (1 : 1000). Unless otherwise indicated, all other chemicals were obtained from Sigma-Aldrich.

2.2. Cell Culture. IPS cell lines were generated by Linta et al. as previously described [15]. iPS cells were cultured in mTeSR1 medium at 37°C, 5% O_2, and 5% CO_2. Differentiation into motor neurons was performed as previously described by Hu and Zhang [19]. For the formation of embryoid bodies (EB), iPS cells were cultured in suspension in hESC medium (DMEM/F12 + 20% knockout serum replacement + 1% NEAA + 1% β-mercaptoethanol + 1% antibiotic-antimycotic) in ultra-low attachment flasks (Corning Costar). ROCK inhibitor was added for the first 48 h. Neurodifferentiation was induced by changing the medium to (DMEM/F12 + 24 nM sodium selenite + 16 nM progesterone + 0.08 mg/mL apotransferrin + 0.02 mg/mL insulin + 7.72 μg/mL putrescin + 1% NEAA, 1% antibiotic-antimycotic + 50 mg/mL heparin + 10 μg/mL of the neurotrophic factors BDNF, GDNF, and IGF-1) at e03. At e07 EB were plated on laminin coated 12-well plates. 0.1 μM of retinoic acid (RA) was added at e09. At e14 neural rosettes (NR) were detached and cultured in suspension with addition of 1 μM purmorphamine. 2-3 neural stem cell (NSC) spheres were plated on a 35 mm μ-dish (Ibidi) coated with poly-L-ornithine (PLO) and laminin. Retinoid acid was reduced to 0.05 μM. Medium was changed 1x/week. Motor neurons were analyzed at d21 and d42 after final plating.

2.3. Establishment of Different Zinc Conditions. Zinc deficient medium was generated by depletion of all divalent cations using Chelex 100 resin (Bio-Rad) as described by the manufacturer and as previously published [20]. To that end mTeSR1, hESC medium, and basal differentiation medium were incubated with Chelex 100 beads that were subsequently removed by centrifugation, a cell strainer, and sterile filtering with 0.2 μm sterile filters. Original cation concentrations were reestablished for all divalent cations present in neurobasal medium (0.247 μM $Fe_2(NO_3)_3$, 0.81 mM $MgCl_2$, and 1.8 mM $CaCl_2$) except for $ZnSO_4 \cdot 4H_2O$. The original pH of the medium was readjusted with HCl (mTeSR1 pH 8.2; hESC basal pH 8.0; basal differentiation medium pH 7.5). Supplemental factors were added to the zinc depleted medium right before use. As a control condition, the original zinc concentration of zinc depleted medium was reestablished. Zinc supplemented medium was prepared by addition of 10 μM $ZnCl_2$ to the original medium.

2.4. Preparation of Cryosections of NSC Spheres. NSC spheres were fixed with 4% paraformaldehyde/10% saccharose and washed three times with PBS and resuspended in 100 μL PBS. Tissue-Tek in small aluminium foil containers was snap-frozen until it was viscous. NSC spheres were transferred to the viscous Tissue-Tek and snap-frozen until everything

was solid. 30 μm cryosections were made at the cryostat. Alternatively, NSC were live-stained using the Apoptosis/Necrosis/Healthy Cell Detection kit (PromoKine) according to the manufacturer's instructions for suspension cells before fixing. Staining time was enlarged to 45 min to ensure penetration of NSC spheres. Fixing and washing were performed in buffers containing 1.25 mM calcium-chloride to ensure annexin V binding to phosphatidylserine.

2.5. Immunocytochemistry. For immunofluorescence, the cultures were fixed with 4% paraformaldehyde/4% sucrose/PBS at 4°C for 20 min and processed for immunocytochemistry. After washing 3 × 5 min with 1x PBS, cells were permeabilized with 0.2% Triton X-100 for 5 min at RT. Blocking was performed with 5% FBS in 1x PBS for 1 h at RT, followed by primary antibody incubation with at 4°C overnight. After a 3 × 5 min washing-step with 1x PBS, incubation with the Alexa Fluor-coupled secondary antibody followed for 1 h. Cells were mounted with ProLong® Gold antifade reagent either with or without DAPI.

2.6. Metabolic Apoptosis Assay. Embryoid bodies (EB) grown in zinc depleted or zinc replete conditions were harvested and transferred to an opaque 96-well plate in different dilutions with zinc depleted or zinc replete DMEM/F12 + 24 nM sodium selenite + 16 nM progesterone + 0.08 mg/mL apotransferrin + 0.02 mg/mL insulin + 7.72 μg/mL putrescin + 1% NEAA, 1% antibiotic-antimycotic + 50 mg/mL heparin + 10 μg/mL of the neurotrophic factors BDNF, GDNF, and IGF-1 in a final volume of 100 μL/well. Ethanol-treated EB from the zinc repletion condition were used as a positive control, and medium without cells was used as a blank. The ApoTox-Glo Triplex Assay (Promega) was performed as described in the manufacturer's protocol. 20 μL of combined substrates cell-permeant GF-AFC and cell-impermeant bis-AAF-R110 in assay buffer was added to the cell solution and incubated for 45 min at 37°C. Fluorescence was read in a PerkinElmer 2030 Explorer at 405 nm (excitation) and 460 nm (emission) for viability and 485 nm (excitation) and 535 nm (emission) for cytotoxicity. 100 μL of Caspase-Glo® 3/7 Substrate in Caspase-Glo® 3/7 Buffer was added to the wells and incubated at 37°C for 2 h. Luminescence was read in a PerkinElmer 2030 Explorer. Viability/cytotoxicity ratios were not significantly different. Apoptotic cells were normalized to number of viable cells.

2.7. Quantitative Real-Time PCR. Isolation of total RNA from iPS cells in different stages of motor neuron differentiation was performed using the RNeasy Mini kit (Qiagen) as described by the manufacturer including all additional purification steps. For the reverse transcriptase-mediated PCR studies, first strand synthesis and real-time quantitative RT-PCR amplification were carried out in a one-step, single-tube format using the QuantiFast SYBR Green RT-PCR kit. Thermal cycling and fluorescent detection were performed using the Rotor-Gene-Q real-time PCR machine (model 2-Plex HRM) (Qiagen). The qRT-PCR was assayed in 0.1 mL strip tubes in a total volume of 20 μL reaction mixture containing 1 μL of undiluted total RNA, 2 μL of QuantiTect Primer Assay oligonucleotides, 10 μL of 2x QuantiFast SYBR Green RT-PCR Master Mix supplemented with ROX (5-carboxy-X-rhodamine) dye, and 6.8 μL of RNase-free water and 0.2 μL of QuantiFast RT Mix. Amplification conditions were as follows: 10 min at 55°C and 5 min at 95°C, followed by 40 cycles of PCR for 5 s at 95°C for denaturation and 10 s at 60°C for annealing and elongation (one-step). The SYBR Green I reporter dye signal was measured against the internal passive reference dye (ROX) to normalize non-PCR-related fluctuations. Resulting data were analyzed utilizing the hydroxymethylbilane synthase (HMBS) gene as an internal standard to normalize transcript levels through all stages of differentiation. Cycle threshold (ct) values were calculated by the Rotor-Gene Q software (version 2.0.2). All qRT-PCR reactions were run in biological triplicate for each cell line. To ascertain primer specificity a melting curve was obtained for the amplicon products to determine their melting temperatures. Real-time quantitative PCR was carried out using oligonucleotides allowing to investigate expression of NEFH as well as MT2A, MT3, MTF1, ProSAP1/Shank2, ProSAP2/Shank3, Shank1, ZnT1, ZnT2, ZnT3, ZnT4, ZnT5, ZnT6, ZIP1, and ZIP3 (validated primer pairs, Quantitect primer assay, Qiagen). The set of zinc homeostasis and other zinc-related genes included in the analyses of this study was chosen based on a literature search for reports on the expression and function of specific zinc transporters and zinc-binding genes in the brain and spinal cord [21–25].

For high-throughput screening of synaptic genes total RNA was isolated using the RNeasy Mini kit according to the manufacturer's manual. All additional purification steps were performed and RNA eluted in a total of 15 μL RNAse-free water. Genomic DNA elimination and cDNA synthesis were performed with 800 ng RNA using the RT^2 First Strand Kit (Qiagen) as described by the manufacturer. Quantitative real-time PCR screening was performed using the RT^2 SYBR Green qPCR Mastermix and customized RT^2 profiler rotor-disks with preimmobilized primers (Qiagen #CAPA9600-12:CAPH13069R) according to the manufacturer's protocol in a reaction volume of 20 μL. Ct values were calculated by the Rotor-Gene Q software as previously described and normalized to NEFH as an internal standard for mature motor neurons.

2.8. Microscopy. Fluorescence images were obtained with an upright Axioscope microscope equipped with a Zeiss CCD camera (16 bits; 1280 × 1024 ppi) using Axiovision software (Zeiss) and analyzed using Axiovision and ImageJ (v1.49o) software.

2.9. Electrophysiology. Single cell patch-clamp electrophysiology of motor neurons d42 after plating was performed using intracellular buffer (140 mM KCl, 2 mM $MgCl_2$, 4 mM EGTA, 4 mM ATP·2Na, and 10 mM HEPES; pH 7.2) and extracellular buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 6 mM glucose, and 12 mM HEPES; pH 7.4). Standard parameters like resting membrane potential, membrane resistance, serial resistance, cell size (capacitance using

the c-slow compensation method and the "sine + dc" method according to Lindau and Neher [26]), synaptic currents (voltage clamp mode), synaptic events (current clamp mode), and half-width as well as peak height after AP induction were assessed. In addition cells were stimulated either with 100 μM acetylcholine, 500 μM glutamate, 10 μM GABA, and 500 μM glutamate along with 100 μM cyclothiazide or with 50 μM NMDA along with 10 μM glycine (switch to Mg free buffer).

2.10. Statistics. Statistical analysis was performed using Microsoft Excel, GraphPad Prism 5, and SPSS with a significance level set to 0.05 ($^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$). According to the parameters tested, values were analyzed for normal distribution and Student's t-test or 1-way ANOVA with subsequent post hoc analysis (Tukey's multiple comparison test) was performed. Only in case no iPSC line specific differences were detected, values from both lines were pooled.

3. Results

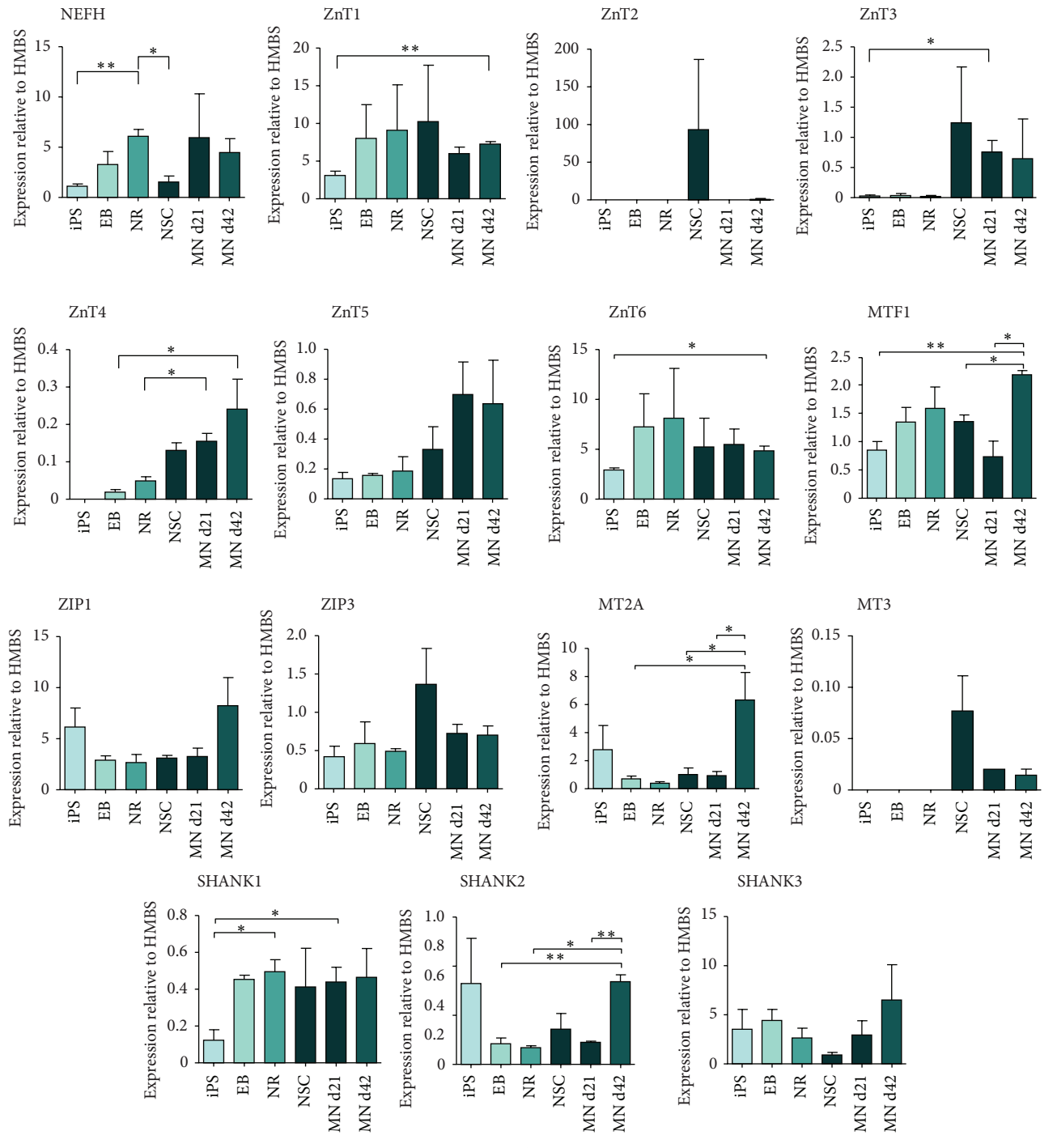
3.1. Expression of Zinc Homeostasis Genes during Stem Cell Differentiation into Motor Neurons. iPS cells from two control cell lines were differentiated to motor neurons using a well-characterized protocol [19, 27] (Figure 1(a)). Identity of motor neurons was confirmed by the motor neuronal marker NEFH. To investigate whether specific zinc homeostasis genes are developmentally regulated, we determined the mRNA expression levels of selected zinc homeostasis genes and the zinc-dependent SHANK genes that have been associated with autism spectrum disorders (ASD) [28] during neuronal differentiation in various stages (iPS cells, embryoid bodies, neural rosettes, neural stem cells, and motor neurons) (Figure 1(b)). Several zinc transporters show developmental stage-dependent expression as well as altered expression levels during differentiation. While ZIP1 and ZIP3 are expressed on similar level throughout all stages of motor neuron differentiation, the expression of transporters from the ZnT family shows more variations. In particular, an increase in ZnT4 expression from iPS cells to motor neurons can be detected. In addition, the expression of ZnT1 is significantly increased in motor neurons at d42 compared to the iPS cell stage. The expression of ZnT3 is very low in early stages but also significantly increases in immature motor neurons and remains elevated in mature motor neurons. Similarly, no expression of ZnT4 was detected in iPS cells, but mRNA levels gradually increase from EB to motor neurons, which can also be observed for ZnT5 expression. Additionally, mRNA levels of ZnT6 were found to be significantly higher in motor neurons compared to iPS cells. In contrast, ZnT2 expression was only detected in NSC (Figure 1(b)).

Zinc homeostasis is regulated not only by the influx and efflux of zinc via transporters, but also by buffering of zinc due to zinc-binding proteins. Metallothioneins (MTs) thereby play a crucial role. The expression of MTs is regulated in response to the zinc levels present inside cells. This process is mediated by the transcription factor MTF1. Interestingly, the mRNA level of MTF1 itself is significantly higher in motor neurons compared to iPS cells.

The expression of MT2A, apart from some variance in iPS cells, is low during all stages of motor neuron development compared to adult motor neurons at d42. Thus, MT2A expression very specifically significantly increases in motor neurons between d21 and d42. The expression of MT3 in turn was only detected in NSC and motor neurons but not in earlier stages, which is in line with the CNS specific expression pattern of MT3 [29]. No significant difference was detected between young and old motor neurons (Figure 1(b)). Finally, proteins of the SHANK family have been shown to bind zinc (Shank2 and Shank3) [10, 30, 31] and are key proteins of synapses in the CNS and also occur at neuromuscular junctions [32]. Interestingly, although SHANK proteins are associated with synapses that only occur in differentiated motor neurons, their expression can be already detected in iPS cells and later stages of motor neuron differentiation, hinting towards an additional role outside postsynaptic densities (PSDs) [33]. Particularly SHANK3 seems to be expressed on similar level throughout differentiation. In contrast, although also expressed in each stage, mRNA levels of SHANK1 and SHANK2 seem to increase towards later stages (Figure 1(b)).

Comparing not only the expression of a single gene between different developmental stages but also the general expression levels between genes (Figure 1(c)), expression levels of ZnT1 and ZnT6 were the highest compared to other ZnTs across all stages of motor neuron differentiation. Similarly, ZIP1 expression was comparably high throughout all phases of differentiation. Zinc supplementation (10 μM $ZnCl_2$ during all stages) does not significantly alter the expression of the aforementioned genes compared to untreated controls (see Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/3760702) with the exception of MT2A in old motor neurons.

3.2. Zinc Deficiency Affects Expression of Zinc Homeostasis Genes during Stem Cell Differentiation into Motor Neurons. Given that, under physiological conditions, the blood brain barrier (BBB) tightly regulates brain zinc levels protecting the brain from abnormally high zinc concentrations, zinc deficiency might likely occur during pregnancy [34], leading to alterations in brain development of the offspring [35, 36]. Thus, in the next set of experiments, we compared expression levels of the zinc homeostasis genes previously investigated under zinc depleted and zinc replete (control) conditions. To that end cells were exposed to medium that was previously zinc depleted using Chelex-100 beads or zinc replete medium as a control condition throughout all stages of neurodifferentiation (Figure 2). Our results show that zinc deficiency leads to gene and differentiation stage-dependent changes. Zinc deficiency leads to a decrease of ZnT1 expression in motor neurons at d42, and a significant upregulation of ZnT5 expression in motor neurons at d21 and less so at d42. The expression of ZIP1 was significantly decreased in the NSC stage under zinc deficient conditions, while the expression of MT3 significantly increased. Again expression levels of SHANK proteins were sensitive for altered zinc concentrations in the medium. The expression of SHANK1 significantly increased in motor neurons at d21



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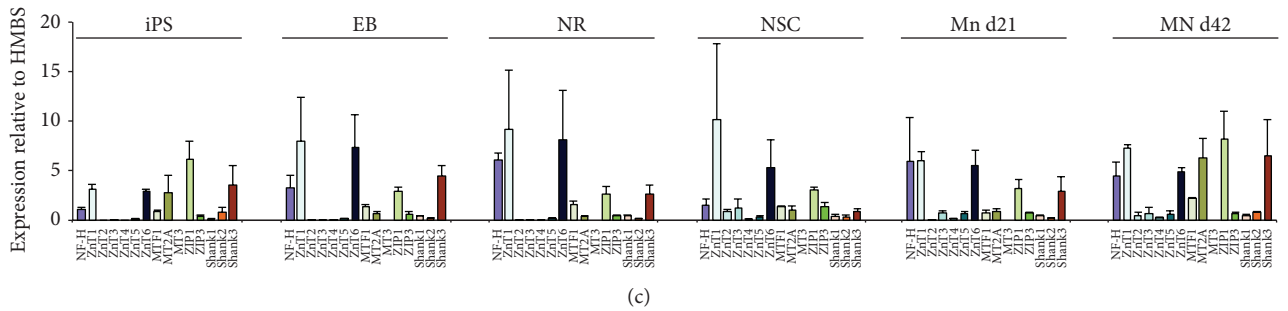


(c)

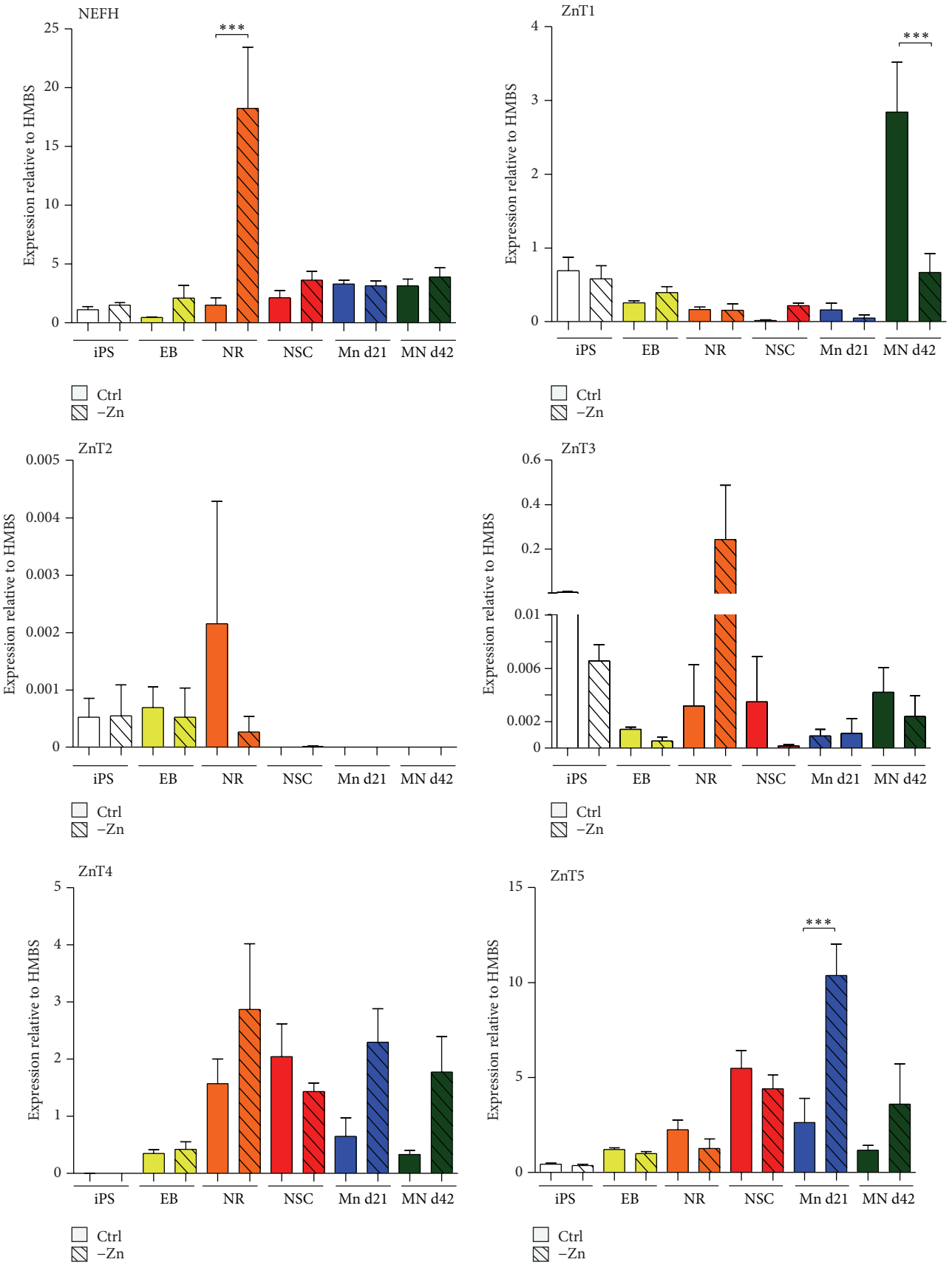
Figure 1: Expression of zinc homeostasis genes during stem cell differentiation into motor neurons. (a) Quantitative evaluation of mRNA expression levels normalized to HMBS of selected zinc homeostasis and the zinc-dependent SHANK genes. Analyses were performed in triplicate ($n = 3$) and the mean normalized expression is shown. Neurofilament H (NEFH) expression was used to control successful differentiation and increases significantly in NRs and rises again after a significant decrease in NSC in suspension (iPSC versus NR: $p = 0.0028$; NR versus NSC: $p = 0.0175$). Several zinc transporters show developmental stage-dependent expression as well as altered expression levels during differentiation. Expression of ZnT1 is significantly increased in motor neurons at d42 compared to the iPS cell stage ($p = 0.0086$). ZnT2 expression in turn was only detected above background in NSC. Expression of ZnT3 is very low in early stages (iPS, EB, and NR) but increases in NSC and becomes significant in motor neurons (iPSC versus MN d21: $p = 0.0239$) in which ZnT3 levels remain elevated. No expression of ZnT4 was detected in iPS cells, but mRNA levels gradually increase from EB to motor neurons (EB versus MN d42: $p = 0.0352$; NR versus MN d21: $p = 0.0384$). This is also observed for ZnT5 expression; however it is only seen as trend. Additionally, the expression of ZnT6 was found to be significantly higher in motor neurons compared to iPS cells (iPS versus MN d42: $p = 0.0178$). Along with the increase in expression of some zinc transporters during motor neuron differentiation, the mRNA levels of MTF1 are significantly higher in motor neurons compared to iPS cells (iPSC versus MN d42: $p = 0.0074$; NSC versus MN d42: $p = 0.0170$; MN d21 versus MN d42: $p = 0.0263$). While ZIP1 and ZIP3 are expressed on similar level through all stages of motor neuron differentiation, the expression of MT2A shows some variance in iPSC but otherwise is significantly higher in motor neurons compared to most previous stages and significantly increases in motor neurons between d21 and d42 (EB versus MN d42: $p = 0.0347$; NSC versus MN d42: $p = 0.0474$; MN d21 versus MN d42: $p = 0.0397$). The expression of MT3 in turn could only be detected in NSC and motor neurons but not in earlier stages. Although SHANK proteins are associated with synapses that only occur in motor neurons, their expression can be already detected in iPS cells and later stages of motor neuron differentiation. SHANK1 and SHANK2 expression increases towards later stages (SHANK1: iPSC versus NR: $p = 0.0225$; iPSC versus MN d21: $p = 0.0327$) (SHANK2: EB versus MN d42: $p = 0.0061$; NR versus MN d42: $p = 0.0132$; MN d21 versus MN d42: $p = 0.0017$), although the expression of SHANK2 in iPS cells shows high variability. SHANK3 is expressed on similar level throughout differentiation. (b) Representative images of hiPSC undergoing differentiation to motor neurons (MN) via the generation of embryoid bodies (EB), neural rosettes (NR), and neural stem cells (NSC). (c) Comparison of mRNA expression levels across different zinc homeostasis genes. In general, expression levels of ZnT1 and ZnT6 were the highest compared to other ZnTs across all developmental stages. Similarly, ZIP1 expression was high throughout all phases of motor neuron differentiation, while MTF1 and MT2A expression increased in old motor neurons. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$.

and of SHANK3 in neural rosettes under zinc deficient conditions.

3.3. Zinc Deficiency Significantly Impairs iPS Cell Differentiation. To understand the biological consequences of the observed alterations, we analyzed the capacity of stem cells to undergo successful neuronal differentiation under zinc deficient conditions in neuronal stem cells (NSC) and embryoid bodies (EB) (Figure 3). While the average size of NSC clusters was not significantly altered upon zinc deficiency (Figure 3(a)), the number of NSC clusters was significantly reduced in zinc deficient conditions (Figure 3(b)) indicating a reduction in cell survival. However, we could not detect a significant increase in markers for apoptosis or necrosis at the NSC stage (Figure 3(c)). Apoptotic cells were identified using annexin V labeled with FITC, necrotic cells by ethidium homodimer III, and the total number of cells was assessed using Hoechst labeling. The analysis of the amount of cleaved caspase-3 shows an increase of cleaved caspase-3 in NSC grown under zinc deficient conditions, which is seen as a trend (Figure 3(d)). Activation of caspase-3 requires cleavage of its inactive form into activated p17 and p12 fragments, activating downstream processes of apoptosis. The amount of cleaved caspase-3 thus provides a read-out for initiated apoptosis.

Since we did not detect significantly increased cell death at the NSC stage, cell survival seems impaired already at earlier stages. This was confirmed by analysis of EB (Figure 3(e)). Here, we found a significant increase in the number of cells undergoing apoptosis in cells grown under zinc deficient conditions compared to controls.

3.4. Altered Zinc Levels Affect Glutamatergic Neuronal Differentiation and Neuronal Function. Finally, to investigate whether zinc deficiency does influence not only cell survival but also the fate and developmental endpoints of cells during differentiation, we first analyzed differentiated motor neurons regarding their morphology (Figure S2) and functionality using electrophysiological read-outs (Figure 4). We stained motor neurons for the motor neuronal marker NEFH, the postsynaptic marker Homer1, and the presynaptic marker synaptophysin as well as DAPI for nuclear labeling. Morphological analysis of motor neurons derived from iPS cells grown under control and zinc supplemented conditions did



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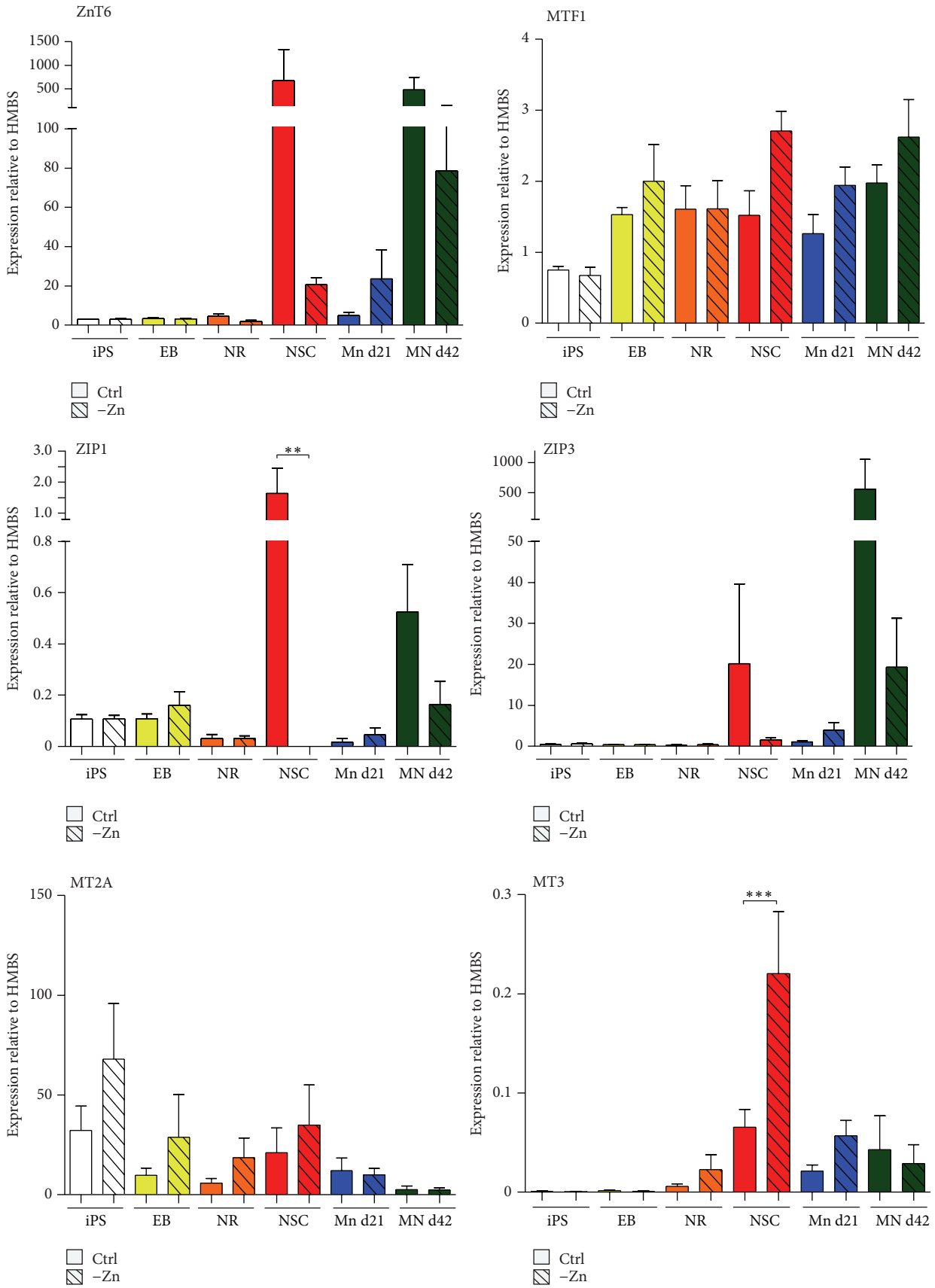

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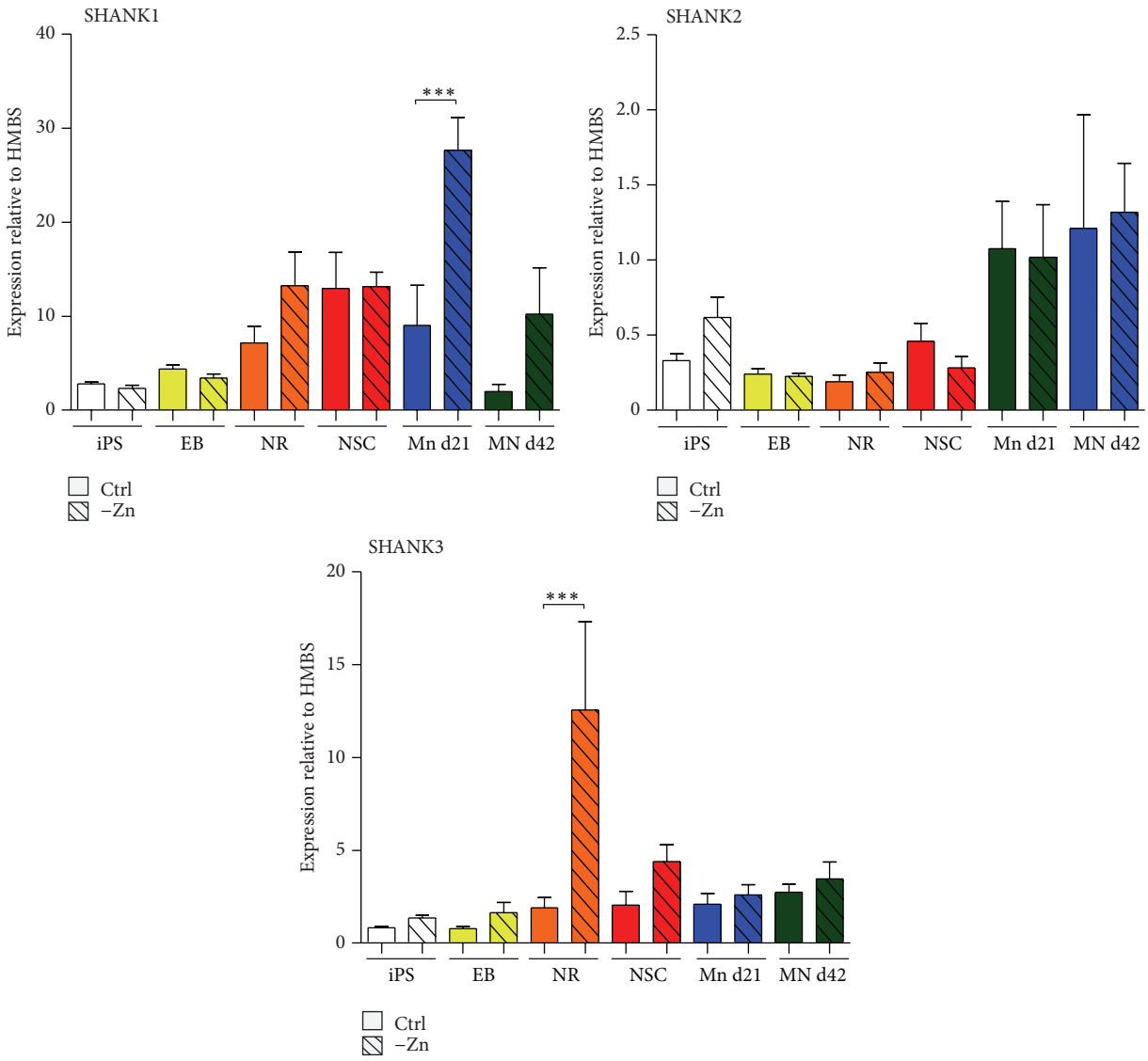

Figure 2: Zinc deficiency alters the expression of zinc homeostasis genes during stem cell differentiation into motor neurons. Quantitative evaluation of mRNA expression levels normalized to HMBS of selected zinc homeostasis and the zinc-dependent SHANK genes. Data shows the average normalized gene expression based on $n = 6$ measurements. Neurofilament H (NEFH) expression was used to control successful differentiation. For statistical analysis, a 1-way ANOVA was used followed by Tukey's multiple comparison test. Several zinc transporters show expression levels depending on the availability of zinc in the medium. Comparing cells grown in zinc depleted medium with cells grown in medium which was resupplemented with zinc in the amount used in control medium after zinc depletion, a significant difference in ZnT1 expression was found in d42 motor neurons (one-way ANOVA: $p < 0.0001$; MN d42 Ctrl versus −Zn: $p < 0.05$). No significant difference was detected regarding the expression of ZnT2, ZnT3, ZnT4, ZnT6, and MTF1 comparing controls and zinc deficient conditions. The expression of ZnT5 was significantly increased in motor neurons at d21 under zinc deficient conditions (one-way ANOVA: $p < 0.0001$; MN d21 Ctrl versus −Zn: $p < 0.05$). The expression of ZIP1 was significantly reduced in neuronal stem cells under zinc deficient conditions (one-way ANOVA: $p = 0.0012$; NSC Ctrl versus −Zn: $p < 0.05$). The expression of ZIP3 was not found to be altered, similar to the expression levels of MT2A. MT3 expression was significantly higher in neuronal stem cells under zinc deficient conditions (one-way ANOVA: $p < 0.0001$; NSC Ctrl versus −Zn: $p < 0.05$). The expression of SHANK1 was increased in motor neurons at d21 under zinc deficient conditions (one-way ANOVA: $p < 0.0001$; MN d21 Ctrl versus −Zn: $p < 0.05$) and the expression of SHANK3 in neural rosettes under zinc deficient conditions (one-way ANOVA: $p < 0.0001$; NR Ctrl versus −Zn: $p < 0.05$). No change was observed for SHANK2 expression. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

not reveal any significant differences regarding soma size (Figure S2A), the number of primary dendrites (Figure S2B), and the number of synapses (Figure S2C). Using single cell patch-clamp electrophysiology, a significant difference in resting potentials was detected between cells grown under zinc deficient and zinc supplemented conditions (Figure 4(a)) as well as a significant difference in cell size assessed by capacitance measurements using the *c*-slow compensation method and the "sine + dc" method according to Lindau and Neher [26] (Figure 4(b)) and the number of ion channels

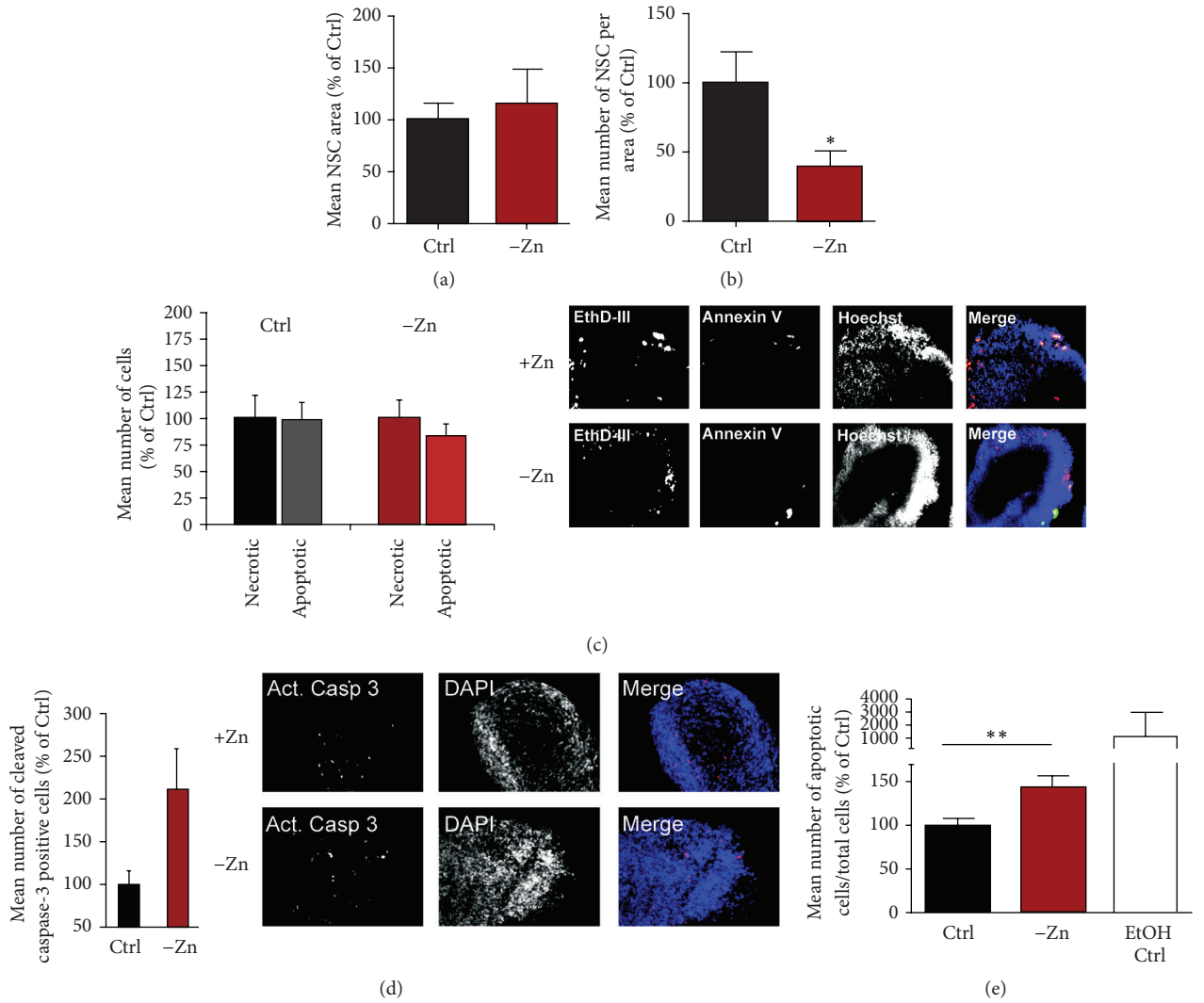

Figure 3: Zinc deficiency significantly impairs iPS cell differentiation. (a) The average size of NSC clusters is not significantly altered upon zinc deficiency. (b) The mean number of NSC clusters per area measured on a 10 cm petri dish is significantly reduced in zinc deficient conditions (t-test, $p = 0.0281$; $n = 8$). (c) Apoptosis and necrosis were evaluated for the NSC stage during differentiation. No difference in the number of cells labeled with markers for apoptosis or necrosis was found between zinc deficient and zinc sufficient media (t-test, $n = 20$). Right panel: exemplary images of NSC stained with annexin V labeled with FITC (apoptotic cells) and ethidium homodimer III (necrotic cells) and DAPI (total number of cells). (d) The mean number of cells showing signals specific for cleaved caspase-3 was increased under zinc deficient conditions seen as trend (t-test, $p = 0.09$; $n = 20$). Right panel: exemplary images of NSC stained with anti-active caspase-3 antibody and DAPI. (e) A significant increase in apoptotic cells was detected in embryoid bodies grown under zinc deficient conditions using the ApoTox-Glo™ Triplex Assay (t-test, $p = 0.0098$; $n = 12$). $^{*}p \leq 0.05$; $^{**}p \leq 0.01$.

active or existent as assessed by membrane resistance, both hinting towards a slightly more immature phenotype (Figure 4(c)). There was an increase in resting potential negativity in zinc depleted cells. The cells did not show any significant differences in the half-width of induced action potentials and sodium currents that could confirm a larger difference in maturity (Figures 4(d) and 4(e)).

During electrophysiological measurements, all cells were measured within the same buffer with physiological zinc concentration. We detected a significant increase in acetylcholine (ACh) induced currents in cells grown under zinc deficient conditions (Figure 4(f)). In contrast, a decrease in glutamate (Glut) induced currents can be seen under zinc deficient conditions (Figure 4(g)).

To further elucidate the alterations in glutamate signaling, we investigated AMPAR and NMDAR currents in the cells grown under different zinc levels. We observed a reduction in both AMPAR and NMDAR currents (Figures 4(h) and 4(i)) and also detected a reduction in GABAR currents (Figure 4(j)) after differentiation of cells in zinc depletion conditions. In line with these results, the number of cells showing ACh induced currents was higher in zinc deficient conditions (Figure 4(k)), while the number of cells showing Glut induced currents was significantly less when compared

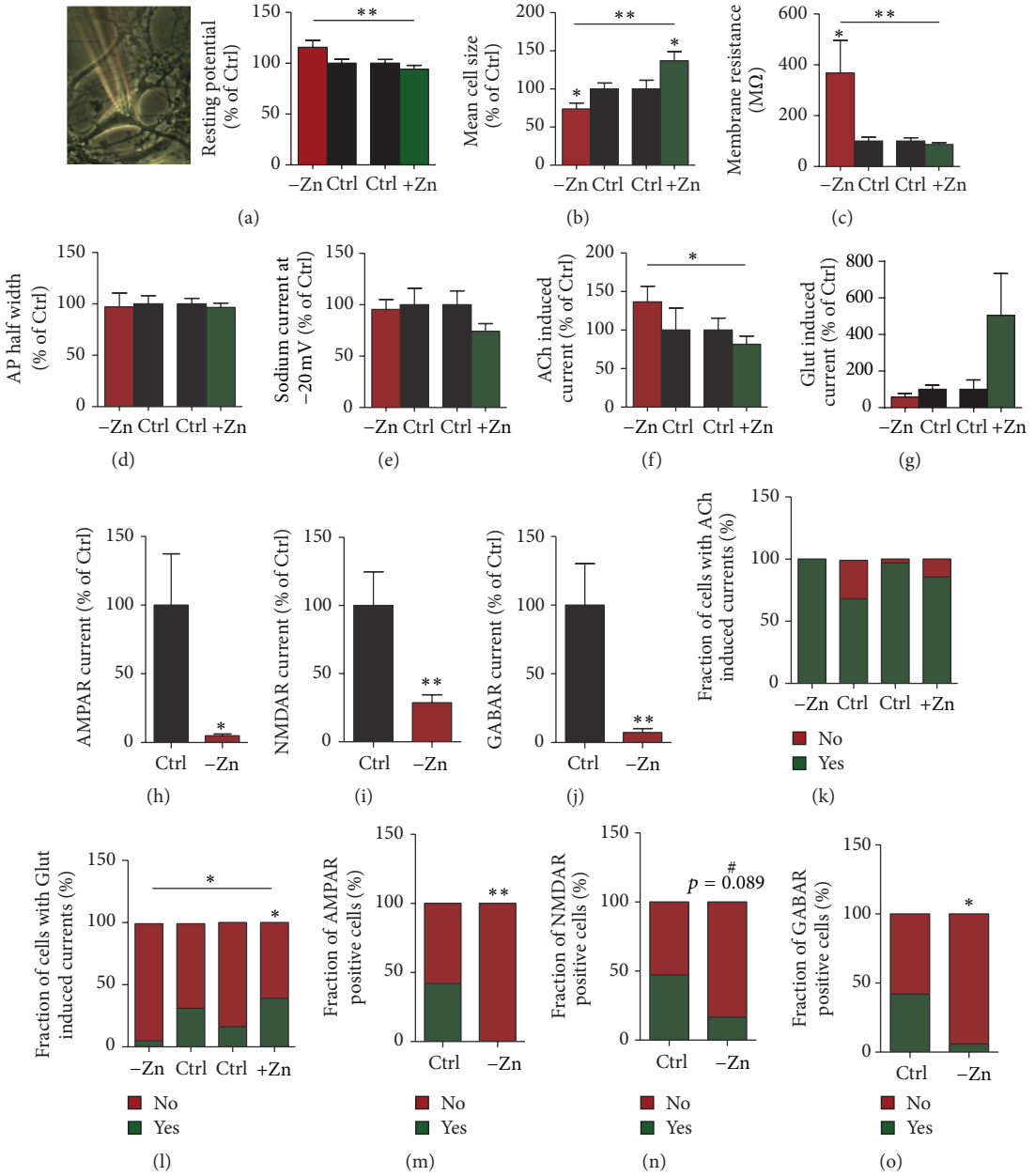

Figure 4: Altered zinc levels affect glutamatergic signaling. (a) Representative image of a neuron used for patch-clamp electrophysiological studies (left panel). Right panel: a significant difference in resting potentials was detected between cells grown under zinc deficient and zinc supplemented conditions (t-test, $p = 0.0028$; $n = 19$: $\text{Ctrl}_{-\text{Zn}}$ and −Zn, $n = 32$: $\text{Ctrl}_{+\text{Zn}}$, and $n = 42$: +Zn). (b) A significant difference in membrane capacity was detected between cells grown under zinc deficient and zinc supplemented conditions (t-test, $\text{Ctrl}_{-\text{Zn}}$ versus −Zn, $p = 0.0228$; $\text{Ctrl}_{+\text{Zn}}$ versus +Zn, $p = 0.0273$; −Zn versus +Zn, $p = 0.0016$; $n = 19$: $\text{Ctrl}_{-\text{Zn}}$ and −Zn; $n = 32$: $\text{Ctrl}_{+\text{Zn}}$, $n = 42$: +Zn). (c) A significant difference in membrane resistance was detected between cells grown under zinc deficient and zinc supplemented conditions (t-test, $\text{Ctrl}_{-\text{Zn}}$ versus −Zn, $p = 0.0445$; −Zn versus +Zn, $p = 0.0018$; $n = 19$: $\text{Ctrl}_{-\text{Zn}}$ and −Zn; $n = 32$: $\text{Ctrl}_{+\text{Zn}}$; $n = 42$: +Zn). (d) Single cell patch-clamp electrophysiology did not show any significant differences in the half-width of induced action potentials (AP) and sodium currents (e). (f) A significant increase in acetylcholine (ACh) induced currents was observed in zinc deficient cells when compared to zinc supplemented cells (t-test, −Zn versus +Zn, $p = 0.050$; $n = 19$: $\text{Ctrl}_{-\text{Zn}}$ and −Zn, $n = 32$: $\text{Ctrl}_{+\text{Zn}}$, and $n = 42$: +Zn). (g) A decrease in glutamate (Glut) induced currents can be seen under zinc deficient conditions as trend. (h) AMPAR currents and NMDAR currents (i) as well as GABAR currents (j) were significantly decreased after differentiation of cells in zinc depletion conditions (t-test, AMPAR, $p = 0.0186$; NMDAR, $p = 0.0099$; GABAR, $p = 0.0067$; $n = 19$: $\text{Ctrl}_{-\text{Zn}}$, $n = 18$: −Zn, $n = 31$: $\text{Ctrl}_{+\text{Zn}}$, and $n = 41$: +Zn). (k) The number of cells showing ACh induced currents was higher in zinc deficient conditions. (l) The number of cells showing Glut induced currents was significantly less under zinc deficient conditions when compared to zinc supplemented cells (contingency (Chi square) test, $p = 0.0113$). The fraction of cells with Glut induced currents was also significantly increased in zinc supplemented cells compared to controls (contingency (Chi square) test, $p = 0.04$) ($n = 19$: $\text{Ctrl}_{-\text{Zn}}$, $n = 18$: −Zn, $n = 31$: $\text{Ctrl}_{+\text{Zn}}$, and $n = 41$: +Zn). (m) The fraction of cells with AMPAR currents (m) and NMDAR currents (n) as well as GABAR currents (o) was decreased after differentiation of cells in zinc depletion conditions (contingency (Chi square) test, AMPAR, $p = 0.0031$; NMDAR, $p = 0.089$; GABAR, $p = 0.0198$; $n = 19$: $\text{Ctrl}_{-\text{Zn}}$; $n = 17$-18: −Zn). All currents were normalized to cell size. $^{\#}$trend (p value between 0.05 and 0.1). $^{*}p \leq 0.05$; $^{**}p \leq 0.01$.

to zinc supplemented cells (Figure 4(l)). In addition, the fraction of cells displaying AMPAR, NMDAR (as a trend), or GABAR currents was significantly decreased in cells of the zinc depletion condition (Figures 4(m)–4(o)).

Given that the observed shift hints towards a reduction in glutamatergic and GABAergic neurotransmission favoring ACh signaling, we evaluated expression of synaptic receptors. A screening for the expression of genes associated with glutamatergic and cholinergic neurotransmission as well as synapse plasticity revealed a decrease of ACh receptors, mainly CHRNA3, CHRNA5, CHRNA7, CHRNA9, and CHRNA10, while an increase in metabotropic glutamate receptor expression (GRM2, GRM3, and GRM5) was observed (Figure 5(a)). As the alterations might reflect a compensatory mechanism in response to reduced zinc levels and are not reflected by the functional read-outs, we further investigated whether the altered gene expression is translated into changes on protein levels. To that end, we performed analyses of fluorescent signal intensity and measured the amount of immunoreactive signals after immunocytochemistry (Figures 5(b) and 5(c)). Under reduced zinc conditions, we could detect a significant decrease in the number of gamma-aminobutyric acid (GABA) receptor 1 (GABRA1) signals per dendrite and a decrease in GABRA1 fluorescent signal intensity correlating with the amount of protein present. Further, we could detect a reduction in the protein concentration of ionotropic glutamate (AMPA) receptor 3 (GRIA3), which however was only found significant in one iPSC line. No alterations were detected in GRIN1 (ionotropic glutamate (NMDA) receptor 1) and CHRNA3 (nicotinic cholinergic receptor, alpha 3).

4. Discussion

The global prevalence of zinc deficiency with estimated 31% [37] may have significant effects on brain development, cognition, and neurological diseases, in particular those that might be caused by an impaired glutamatergic signaling such as autism spectrum disorders [38, 39].

Here, we found that the expression of genes mediating zinc homeostasis and signaling is endogenously regulated during motor neuronal differentiation and provide a detailed data-set for gene expression of a subset of zinc transporter genes and zinc-binding proteins during stem cell differentiation into motor neurons. These regulatory processes are likely both to occur as reaction to altered demands for zinc in specific differentiation stages and to enable zinc signaling, which, for example, is involved in processes such as apoptosis. In line with this, we could show that insufficient zinc supply has an effect on cell survival and by that reduces the number of neuronal stem cells differentiating from iPSC. It has been previously reported that zinc deficiency can affect neuronal cell precursor proliferation by the induction of apoptosis via p53-mediated processes [3, 40, 41].

Given this important role of adequate zinc supply for neurogenesis, it is not surprising that physiological responses exist to tightly regulate zinc levels during neuronal differentiation. Indeed, we observed a response in gene expression of zinc homeostasis genes under zinc deficient conditions. For example, a reduction of the zinc exporter ZnT1 as observed in mature motor neurons will lead to increased retention of zinc inside the neuron. Reduced ZnT1 levels were already observed in neonatal rats exposed to zinc deficiency [42]. Similarly, reduced expression of ZnT5 in young motor neurons may reduce zinc export into cell organelles and thereby ameliorate the decrease in cytoplasmic zinc. Such a responsiveness of ZnT5 to zinc deficiency was also shown with in THP1 cells [43]. In contrast, an increase in MT3 as observed in NSC under zinc depletion might increase the ability to retain and buffer zinc inside the cell. A moderate increase of MT3 expression in response to zinc deficiency was reported before in rats [44]. However, a great variability of these alterations during the different stages of neuronal differentiation hints towards highly dynamic compensatory processes and/or further regulatory processes on protein level.

In case of an inability to compensate low zinc levels such as in our experimental conditions, not only is the number of neuronal stem cells decreased but also differentiated motor neurons show significant differences when compared to motor neurons that differentiated under zinc adequate conditions. The differences we observed cannot be explained by alterations of NMDAR, AMPAR, GABAR, or acetylcholine esterase by the neuromodulatory function of zinc, as all measurements were performed using the same buffer that contained physiological zinc concentrations. Thus, the differences must be based on developmentally acquired variances and are not due to acute alterations in zinc levels. In particular, we observed an increase in ACh signaling under zinc deficient conditions. This increase was seen both on the level of ACh induced currents and in the total number of cells responsive to ACh stimulation. More prominent, glutamatergic signaling was impaired under zinc deficient conditions. Again both a reduction in glutamate induced currents (both AMPAR and NMDAR currents) and a reduction in the total number of cells responding to glutamate stimulation were observed under zinc deficient conditions. In addition, GABAR currents and the fraction of cells displaying GABAR currents were significantly reduced. The results can be explained by an increase in the number of cholinergic synapses per neuron and a reduction of glutamatergic and GABAergic synapses per neuron and/or a strengthening of existing synapses. Indeed, we found a decrease of GABRA1 immunoreactive signals per dendrite as well as a reduction of GRIA3 levels per synapse supporting this model. We did not observe a significant increase in CHRNA3 signals. However, the CHRNA family alone consists of 10 members encoding for different subunits and more detailed analyses are needed in future studies. For example, on mRNA level, we observed mixed effects of neuronal differentiation under zinc deficient conditions on the cholinergic system. Here, the expression of many CHRNA family members was decreased under zinc deficient conditions, while the expression of CHRNA2 and 6 and CHRNB3 was increased.

Gene transcription was also found to be increased for some members of the glutamatergic and GABAergic system, such as GABRA1 and GRIA3 under zinc deficient conditions, where we detected a decrease of synaptic signals on protein level. It might be speculated that neuronal differentiation

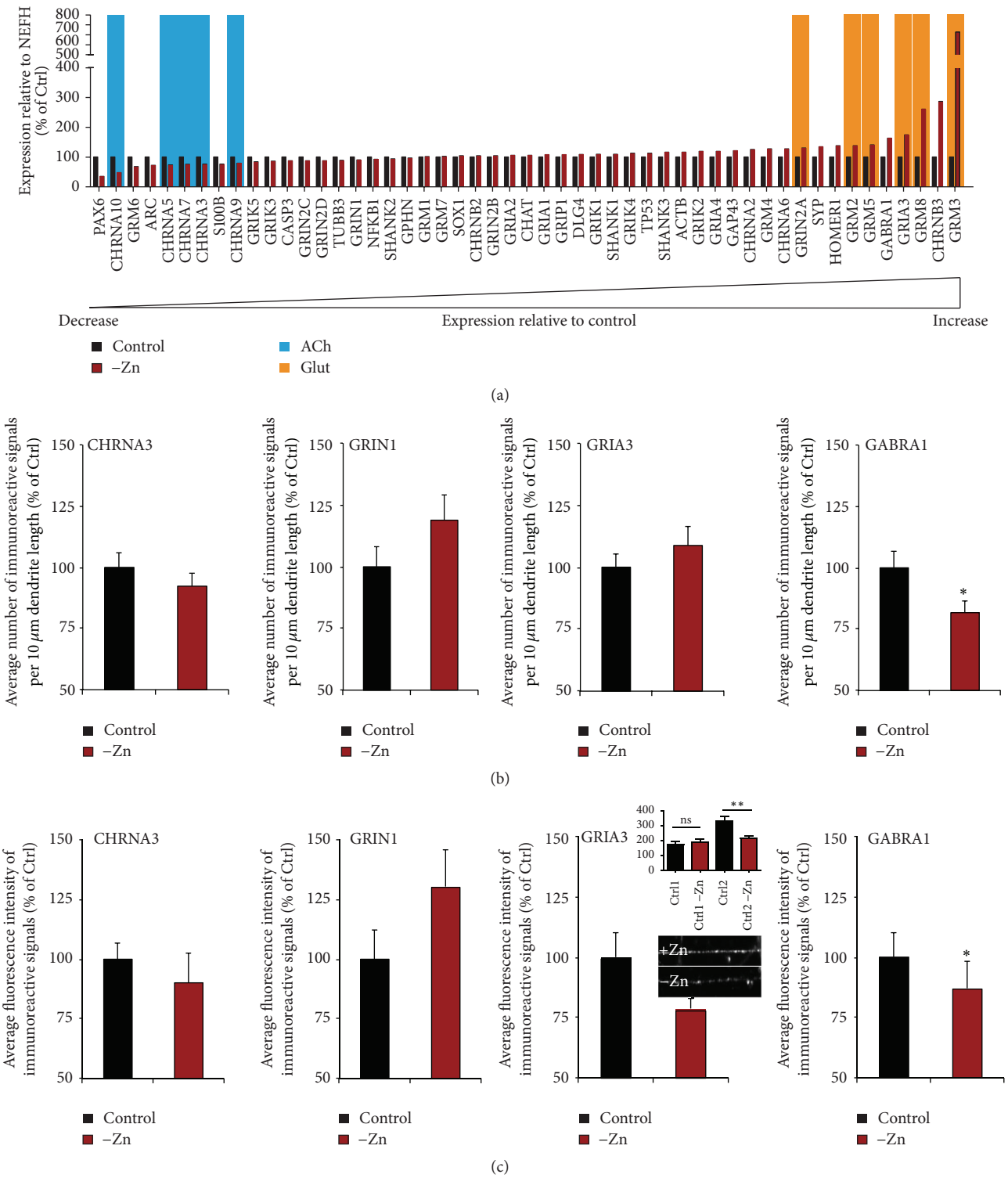

Figure 5: Cells differentiated under different zinc levels show altered gene expression and protein levels of neurotransmitter receptors. (a) A screening for the expression of genes associated with glutamatergic and cholinergic neurotransmission as well as synapse plasticity reveals a decrease of ACh receptors, mainly CHRNA3, CHRNA5, CHRNA7, CHRNA9, and CHRNA10, while an increase in glutamate receptor expression (GRM2, GRM3, and GRM5; GRIA3; GRIN2A) was observed. (b) Immunocytochemical analysis of $n = 20$ cells per condition. The mean number of signals per dendrite length was assessed. No differences were detected for CHRNA3, GRIN1, and GRIA3. A significant reduction was seen for GABRA1 signals (t-test, $p = 0.05$). (c) Immunocytochemical analysis of $n = 20$ cells per condition. The mean signal intensity per fluorescent puncta was assessed. No differences were detected for CHRNA3 and GRIN1. A significant reduction of GABRA1 signal intensity (t-test, $p = 0.022$) was observed. GRIA3 signal intensity was reduced only in one cell line (t-test, $p = 0.0045$). ns: not significant. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$.

under zinc deficient conditions may result in a mislocalization of the proteins rather than decrease of total protein that cannot be overcome even upon increased gene transcription. Here, we only assessed synaptic protein concentrations. It is known, for example, that some synaptic glutamate receptors are dependent on a zinc sensitive PSD scaffold of SHANK2 and SHANK3 proteins for synaptic localization [10, 28].

Thus, it might be possible that zinc signals play a role in the establishment of neuronal subtype identity. Intriguingly, an excess of cholinergic neurons was found in the basal forebrain of autistic children [45] and nicotinic cholinergic antagonists have been reported beneficial in autism [46]. Although there are similarities in neuronal subtypes as previously discussed, here we used motor neurons as a model-neuron. It is possible that zinc signaling is regulated and involved in the differentiation of stem cells into other neuronal subtypes in alternative fashion.

Taken together, neurogenesis is an essential first step of the development of the CNS. The influence of zinc ions on cell differentiation and neuronal function can be complex and manifold and might span the whole spectrum from cell differentiation into mature neurons, followed by synaptogenesis, to synaptic pruning. In addition, programmed cell death is an important factor contributing to CNS development. Insufficient supply with zinc thereby might influence processes such as DNA replication, transcriptional control, mRNA translation, apoptosis, and microtubule stability [3, 47, 48]. Thus, zinc signaling and zinc levels need tight control by zinc transporters and buffering proteins. We conclude that sufficient supply with the essential trace metal zinc might be especially relevant during the time window of brain development and maternal zinc status might be a critical factor to secure healthy mental functioning. However, these findings might also be relevant to adult neurogenesis, as dietary zinc deprivation, zinc chelation, and depletion of vesicular zinc in ZnT3 knockout mice all lead to a significant decrease in hippocampal progenitor cell proliferation also in adult animal models [49].

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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