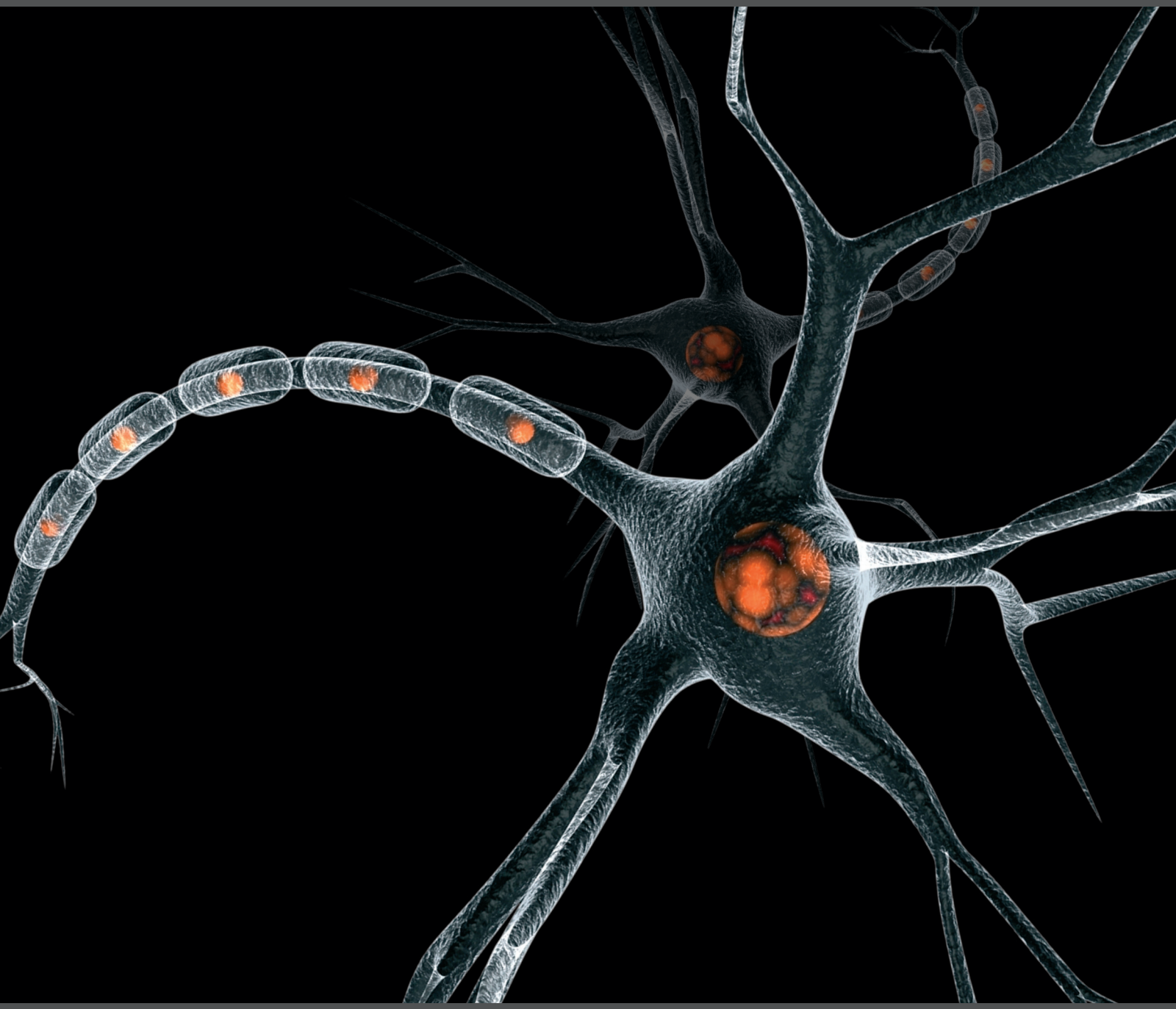
Neural Plasticity

Glia and the Synapse: Plasticity and Disease

Guest Editors: Irina Nikonenko, Emma Victoria Jones, Hubert Fiumelli, and Yann Bernardinelli



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Editorial Board

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Editorial
Glia and the Synapse: Plasticity and Disease

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Glial cells play multiple, diverse roles in the central nervous system (CNS), ranging from the basal support of neuronal function to close partnership with the synapse. Growing experimental evidence shows the importance of glia for proper brain functioning and their involvement in injury and disease. Astrocytes are the most intriguing cells among the glial family. It is well known that they provide energetic substrates to neurons, take up neurotransmitters, and maintain ion homeostasis. Recent research has revealed that they can also release gliotransmitters and signaling molecules as well as maintain and regulate the extracellular matrix. Structurally, astrocytic fine processes enwrap synaptic contacts and exhibit remarkable plasticity resulting from crosstalk between these compartments. These features endow astrocytes with the ability to sense neuronal activity and integrate and modulate synaptic transmission, revealing them as a crucial element in mechanisms of synaptic transmission and plasticity.

Indeed, astrocyte-synapse interactions are complex and dynamic and are required for normal synaptic physiology and plasticity, as well as for the development and refinement of the neuronal circuits. Although much progress has recently been made in our understanding of the cellular and molecular mechanisms that underlie neuronal-glial interactions, ongoing research is adding new information and new questions regarding the role of glial cells in CNS development, function, and disease.

In this special issue we collected research and review articles that focus on glia-synapse interactions with a particular focus on astrocytes. An important role played by these cells in regulating long-term potentiation (LTP) and memory mechanisms in hippocampus is reviewed by Y. Ota et al. In the review "*The role of astrocytes in the regulation of synaptic plasticity and memory formation*," the authors presented a summary of receptors and signaling molecules implicated in LTP. In addition, they propose an integrative model describing how astrocytes may modulate LTP at the postsynaptic site. Supported by a growing number of studies, their model confirms the involvement of the glutamatergic, cholinergic, and purinergic pathways in the neuron-astrocyte interactions taking place during synaptic plasticity. Moreover, this cellular interplay implicates also ephrin signaling and cytokines. Finally, Y. Ota and coauthors discuss the central role played by astrocytic calcium and associated gliotransmitters in hippocampal-dependent memory.

Although the primary function of astrocytes is to take up glutamate to prevent excitotoxicity, astrocytes are also able to release this neurotransmitter. This is generally well accepted even if the mechanisms of release remain uncertain. In this special issue, a novel mechanism of glutamate release from astrocytes was studied by C. Cali et al. in the manuscript "*G-protein coupled receptor-evoked glutamate exocytosis from astrocytes: role of prostaglandins*." They show the role of the proinflammatory mediator prostaglandin E_2 (PGE_2) in glutamate exocytosis from astrocytes in the intact brain. Inhibition of cyclooxygenase pathway caused a significant reduction in the total number of fusion events of VGLUT1-positive glutamate containing vesicles in astrocytes induced by activation

of purinergic and glutamatergic receptors. Prostaglandin-mediated signaling is implicated in the later, slower phase of glutamate release and requires autocrine/paracrine action of PGE_2, suggesting a physiological role for this mediator in intercellular communication, in addition to its known role in inflammatory reactions in the brain.

Gliotransmitters glutamate and D-serine have been shown to modulate NMDA receptors (NMDAR) at extrasynaptic sites, revealing neuronal NMDAR as active components of glia to neuron communication. In the review "*GluN3A: an NMDA receptor subunit with exquisite properties and functions*," L. A. Kehoe et al. discuss recent data on the GluN3A subunit which provides "nonconventional" properties to NMDA receptors. Expression of this subunit in early development helps to shape neuronal networks, but it may also be implicated in different neuropathologies. Interestingly, the presence of GluN3 subunit on perisynaptic astrocytic processes suggests its possible involvement in neuron-glia interactions, although more research is required to elucidate this question.

Another mechanism used by astrocytes to regulate intercellular interactions in the CNS is through secretion of matricellular proteins. These proteins are nonstructural molecules that regulate the extracellular matrix and cell-cell interactions. In the paper "*Astrocyte-secreted matricellular proteins in CNS remodelling during development and disease*," E. V. Jones and D. S. Bouvier review the roles of matricellular proteins secreted from developing and reactive astrocytes in CNS development, injury, and disease and discuss their potential as therapeutic targets.

In the paper "*Astrocyte-synapse structural plasticity*," Y. Bernardinelli et al. review the data on plasticity of perisynaptic astrocytic processes (PAPs). The authors discuss electron and optical microscopy data showing the distribution of fine astrocytic processes around synapses and overview growing evidence on PAP structural plasticity. Although the exact mechanisms and roles of this type of astrocytic plasticity are still not clear, recent data has revealed a requirement for neuronal activity and suggests that PAPs may be implicated in the structural support and plasticity of a synapse, control of neurotransmission and intersynaptic crosstalk, energy supply, maintenance of extracellular homeostasis, and integration of synaptic signals.

The study of glial structural plasticity (such as PAPs) requires precise quantification of fine processes and their motility. This challenging task is addressed in the paper "*Improved method for the quantification of motility in glia and other morphologically complex cells*" by M. Sild et al. who propose and describe in detail a new approach to calculate a motility index for cells with complex, dynamic morphologies.

In this special issue, only some aspects out of a broad range of topics on synapse-glia interactions are highlighted and discussed. Despite great progress made recently in our understanding of glia and their role in the CNS, there is still a long road ahead. We hope that the data presented in this issue will help the future research in this quickly growing and important field.

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Emma Victoria Jones
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Review Article

Astrocyte-Secreted Matricellular Proteins in CNS Remodelling during Development and Disease

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Matricellular proteins are secreted, nonstructural proteins that regulate the extracellular matrix (ECM) and interactions between cells through modulation of growth factor signaling, cell adhesion, migration, and proliferation. Despite being well described in the context of nonneuronal tissues, recent studies have revealed that these molecules may also play instrumental roles in central nervous system (CNS) development and diseases. In this minireview, we discuss the matricellular protein families SPARC (secreted protein acidic and rich in cysteine), Hevin/SC1 (SPARC-like 1), TN-C (Tenascin C), TSP (Thrombospondin), and CCN (CYR61/CTGF/NOV), which are secreted by astrocytes during development. These proteins exhibit a reduced expression in adult CNS but are upregulated in reactive astrocytes following injury or disease, where they are well placed to modulate the repair processes such as tissue remodeling, axon regeneration, glial scar formation, angiogenesis, and rewiring of neural circuitry. Conversely, their reexpression in reactive astrocytes may also lead to detrimental effects and promote the progression of neurodegenerative diseases.

1. Introduction

Astrocytes secrete numerous factors and active molecules [1–4], which modulate synapse development, neuronal activity, and plasticity during development and in the mature brain [5–10]. In particular, immature astrocytes produce and secrete many types of proteins that allow them to remodel the extracellular matrix (ECM) surrounding neurons and synapses [11]. In addition, astrocytes have the ability to react to any kind of insult or change in their environment, physical, viral, or chronic disease, and work side by side with microglia, the immune cells of the brain, in order to contain and to repair the brain from injuries [12, 13]. Astrocyte reactivity is associated with striking changes in gene expression and morphology [14, 15]. It has been demonstrated that reactive astrocytes revert to a partially immature molecular profile [16], which allow them to reexpress a variety of factors/proteins required for tissue remodelling around injury sites [13]. Some of these proteins have direct roles on modulation of ECM and cell to cell interactions. One mechanism used by reactive astrocytes to reshape their microenvironment is through the secretion of matricellular proteins.

Matricellular proteins are a family of structurally unrelated proteins that are secreted into the extracellular space. They act as nonstructural regulators of the ECM and cell-matrix interactions through modulation of growth factor signaling, cytokines, hormones, and proteases [17]. In general, matricellular proteins are highly expressed during development and present at a lower level during adulthood. However, their expression is upregulated following injury or disease. Interestingly, mice with homozygous-null mutations for this family of molecules do not usually exhibit gross abnormalities and generally have subtle phenotypes until challenged [18]. In contrast with other extracellular matrix proteins, matricellular proteins have been shown to have deadhesive activity, which likely contributes to their role in tissue remodeling following injury or in disease states. The matricellular protein family comprises many members to date (recently reviewed in [19]). Although widely studied in nonneuronal tissues, their roles in the CNS are

not well understood. This minireview will focus on the expression and function of matricellular proteins secreted from astrocytes of the CNS, both in development and in the context of glial reactivity and disease, namely, SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin/BM-40), Hevin (also known as SPARC-like 1 or SC1), Tenascin C (TN-C), Thrombospondins (TSP), and the CCN (CYR61/CTGF/NOV) family.

2. SPARC and Hevin/SC1

SPARC/osteonectin/BM-40 (now referred to as SPARC) and SC1/Hevin, first discovered two decades ago, are part of a larger family of SPARC family proteins, which share a common E-F hand calcium binding (EC) domain [20–23].

Of the SPARC family proteins, Hevin has the greatest homology to SPARC and both proteins have three main domains; an N-terminal acidic calcium-binding domain, a central cysteine-rich follistatin domain, and the C-terminal EC domain [24] which confers collagen-binding [25–27]. SPARC has been shown to modulate several growth factor signaling cascades (i.e., VEGF (vascular endothelial growth factor), PDGF (platelet-derived growth factor), FGF2 (fibroblast growth factor-2), and TGFβ (transforming growth factor beta) and can regulate integrin-mediated adhesion (reviewed in [28]). Mice containing targeted deletions of either SPARC or Hevin are viable and fertile and generally exhibit relatively mild phenotypes until revealed by injury or challenge [20, 29, 30]. However, recent studies of SPARC and Hevin knock-out animals have revealed important roles for these molecules in CNS development [9, 31–33].

During embryonic development, SPARC and Hevin have somewhat overlapping expression profiles and are found in radial glia and the developing vasculature (recently described in detail in [34, 35]). Indeed, Hevin was shown to be important for appropriate termination of radial glia-guided neuronal migration and proper lamination of the cerebral cortex [32].

During the first few weeks of postnatal development, SPARC and Hevin are highly expressed in astrocytes [9, 31], with Hevin also being present in neurons [36]. Secretion of SPARC and Hevin from astrocytes has been shown to play an important role during synapse development (recently reviewed in [5, 11]). Kucukdereli and colleagues [31] demonstrated that astrocyte-secreted Hevin promotes the formation of excitatory synapses in the superior colliculus. They proposed that SPARC may compete with Hevin to regulate synapse development, since SPARC was found to antagonize the synaptogenic effects of Hevin on cultured RGC neurons. In contrast, morphological synapse development in hippocampal neurons cultured with SPARC null astrocytes versus wild type astrocytes was similar [9]. However, synapses in SPARC null cultures were found to have a greater number of AMPA receptors and an increase in synaptic strength, which was rescued through application of recombinant SPARC. Regulation of AMPA receptors by SPARC required β3-integrin signaling [9]. A recent study has shown that these synaptic changes can lead to alterations in hippocampal-related behaviours in the adult. Campolongo and colleagues demonstrated that SPARC knock-out mice exhibited an increase in anxiety and antidepressant-like behaviours [29].

Taken together, these studies suggest that SPARC may play an important role in excitatory synapse development by preventing premature maturation of synapses to facilitate proper formation of neural circuitry. How Hevin and SPARC might interact to exert their effects on synapses is not yet understood.

2.1. SPARC and Hevin Are Upregulated in Reactive Astrogliosis. In the mature CNS, the expression of SPARC and Hevin is reduced, and, in the case of SPARC, its expression is confined to radial-like glia cells such as Bergmann glia and Muller glia, resting microglia at low levels in astrocytes [35, 37]. In contrast, Hevin expression in the adult brain is restricted to mature astrocytes and neurons and is not present in microglia [34, 38]. However, upon injury or disease, the expression of both proteins is upregulated in reactive astrocytes [39, 40]. Hevin is found in the cell bodies of reactive, dividing astrocytes and in hypertrophied processes surrounding neurons and blood vessels following status epilepticus in rat [41]. Hevin is also highly expressed in reactive glia following lesion injury [42, 43] and ischemic stroke [44], where Hevin expression was shown to be upregulated in astrocytes surrounding the lesion at 1 day after stroke and was sustained for up to a week. Similarly, SPARC has been shown to be upregulated in reactive astrocytes following injury [39] and stroke [45]. Interestingly, SPARC upregulation occurred in a slow sustained manner in both of these studies, suggesting that SPARC produced from reactive astrocytes may play a role in later, rather than immediate, processes of recovery and repair.

In the human brain, SPARC is expressed in reactive human astrocytes proximal to brain tumours [46] and was shown to be increased in cases of human epilepsy [47]. SPARC is expressed in several types of brain tumours, and its expression in astrocytomas and gliomas is generally associated with increased invasion, angiogenesis, and a negative prognosis [48, 49]. The role of Hevin in cancer is likely complex: its expression has been shown to be correlated with invasive gliomas [50] yet it is highly downregulated in other malignancies and has been described as a tumour suppressor gene [24, 51].

2.2. Cleavage of SPARC and Hevin and the Generation of Functional Protein Fragments. The role of Hevin and SPARC in cancer, brain injury, and disease is likely dependent on the tissue type and other molecules in the ECM environment, such as the presence of proteases. Both SPARC and Hevin have been shown to be cleaved by members of the matrix metalloproteinases (MMP) family to release functional protein fragments. Hevin can be cleaved by MMP-3 and ADAMTS4 (a disintegrin and metalloproteinase with Thrombospondin motifs) to release a shorter, C-terminal fragment that is highly homologous to SPARC, known as "SPARC-like fragment" (SLF) [38, 52]. Interestingly, the SLF was shown to oppose the synaptogenic action of full length Hevin [31, 52] and was found associated with SPARC in neovasculature of

gliomas [52]. Furthermore, it is possible that SPARC may play a role in the proteolysis of Hevin, since SPARC has been shown to induce the expression of MMPs [53, 54]. SPARC is regulated by MMP proteolysis and is cleaved by MMP-3 *in vitro* to produce protein fragments that have differential effects on cell proliferation and migration [55]. Peptides encompassing different domains of SPARC (such as would be released by MMP cleavage) have been shown to have potent effects. For example, "peptide 2.3," which contains part of the follistatin-like domain of SPARC, regulates SPARC-β-integrin interactions in nonneuronal cells [56], can stimulate angiogenesis activity [57], and rescues β-integrin and AMPA receptor overaccumulation at synapses in SPARC null hippocampal cultures [9].

MMPs and their inhibitors, TIMPs (tissue inhibitors of metalloproteinases), are upregulated in reactive astrocytes in a range of disease contexts such as following ischemic stroke or in Alzheimer's disease (AD), where they are thought to have an important role (reviewed in [58, 59]). In ischemic stroke or injury, MMP expression has been shown to have a detrimental role in the initial stages [60], but it is protective in the later recovery phases where it is important for promoting angiogenesis of new blood vessels and restoration of the blood brain barrier [61]. The role of MMPs in angiogenesis is thought to involve VEGF signaling [58], which has also been shown to be regulated by SPARC (reviewed in [28]). Given that MMP can regulate SPARC and vice versa, it would be interesting to determine whether MMP and SPARC could coordinate regulation of tissue remodeling and angiogenesis following injury to the CNS.

Currently, the role of SPARC and Hevin in the CNS has been mostly studied in the context of development. One interesting question is whether these proteins will have similar functions in the adult CNS following injury or disease. For example, are SPARC and Hevin involved in *de novo* synapse formation and circuit rewiring following CNS injury? Does their function require proteolysis? Answers to these questions will help provide insight into the functions of SPARC and Hevin produced from reactive astrocytes and their role in modulating the ECM environment.

3. Tenascin C

Tenascin C (or TN-C with C for cytotactin) is a secreted extracellular matrix glycoprotein that is part of a family of three homologs along with Tenascin R and Tenascin X. TN-C was discovered in the early 1980s and has carried numerous names like myotendinous antigen, glioma mesenchymal extracellular matrix, hexabrachion, tenascin, Jl-200/220, and cytotactin [62]. TN-C is composed of several distinct domains containing epidermal growth factor-like repeats, fibronectin type III repeats, and a segment of great homology with the α and β chains of fibrinogen [62, 63]. It is transiently expressed by neural and nonneural cells during development and plays a role in the ECM remodelling during tissue repair. During brain development, the expression of TN-C by radial glia and subpopulations of astrocytes has been shown to affect multiple processes such as cell migration and proliferation, axonal guidance, and synaptic plasticity.

3.1. Astrocyte-Secreted TN-C Is a Key Molecule in the Establishment of Neuronal Circuitry. TN-C was first described as a protein produced and secreted *in vitro* by astrocytes in culture [64, 65]. It was demonstrated that its expression was limited to specific astrocyte cell lines or subpopulations during CNS development [66, 67]. Paradoxically, TN-C was considered both as a repulsive substrate for neuronal and astrocytic growth [68, 69] and as a permissive one, by providing axonal guidance cues [66, 67, 70, 71]. Depending on the context, such as the expression of other ECM molecules, TN-C can have the opposite differential effects on neuronal growth. TN-C is also known to influence astrocyte proliferation and induces process elongation through an autocrine/paracrine mechanism [63]. In addition, TN-C has been shown to act through β1 and α9 integrin-dependent cell adhesion [69, 72] and to interact physically with the neuronal GPI—membrane-anchored adhesion glycoprotein F3/contactin of the Ig superfamily [71].

TN-C is expressed *in vivo* in various brain regions and exhibits a precise temporal and spatial distribution. In the somatosensory cortex of rodents, TN-C was detected by immunohistochemistry in populations of astrocytes delineating the boundaries of whisker barrel fields in the early postnatal development (P1–P7) [73]. By P9, TN-C expression in barrels has almost disappeared. TN-C was also reported to be transiently expressed by cortical radial glia cells [74] and by astrocytes of the optic nerves [75]. In the cerebellar cortex, TN-C was found to be highly expressed in the first four postnatal weeks before being downregulated [75]. Using *in situ* hybridization and electron microscopy, the authors showed that TN-C was mainly produced and secreted by astrocytes and epithelial cells and was only detected at the cellular surface of neurons. However, in the developing hippocampus, TN-C not only is expressed by radial glia cells and immature astrocytes but also by a subset of neurons of the stratum oriens [76]. In the spinal cord, TN-C is synthesized by a subset of gliogenic precursors in the late phase of embryogenesis and has been shown to influence proliferation and migration of subpopulation of astrocytes [77].

Surprisingly, TN-C null mice do not show a major phenotype or alteration in the gross histoarchitecture of the CNS but do display subtle morphological alterations in some subtypes of neurons [78, 79]. In addition, TN-C null mice exhibited an enhanced proliferation but a delayed migration of immature astrocytes toward the ventral spinal cord white matter [77]. Furthermore, it has been shown that the number of cells is changed in the cerebral cortex of TN-C null mice when compared to the WT animals, with an abnormally high neuronal density and increased astrogliosis but low density of parvalbumin-positive interneurons and reduced ratio of oligodendrocytes [79]. Evers and colleagues observed plasticity impairments in the hippocampus of one-month-old TN-C null mice. Indeed, intertheta burst stimulation-(TBS-) induced LTP (long term potentiation) were shown to be significantly reduced and LTD (long term depression) abolished at Schaffer collateral—CA1 synapses whereas LTP in the dentate gyrus or CA3 were described as normal [78]. The authors demonstrated a causal effect between the deficiency in TN-C and an impairment of the L-type $Ca2^{+}$

channel-dependent forms of synaptic plasticity in the CA1 that could be mediated by interactions with integrins or proteoglycans. In addition, TN-C −/− mice trained in the step-down avoidance test also showed clear deficits in contextual memory [80]. Interestingly, different domains of TN-C have distinct functional properties. Indeed, the intrahippocampal injections in wild type animals of the recombinant fragment of TN-C containing the fibronectin type III repeats 6–8, but not 3–5, blocked memory formation and suggests that TN-C can directly modulate synaptic plasticity [80]. Therefore, the reexpression of TN-C by reactive astrocytes during injuries or brain diseases could impact the processes of cell proliferation, migration, and synaptic plasticity.

3.2. TN-C in Astrogliosis and Brain Diseases. Tenascin C is poorly expressed in adult brain. However, it has been observed to be upregulated at injury sites in correlation with astrocyte reactivity and glial scar formation. Indeed, stab wound in cerebellar and cerebral cortices structures have been shown, by *in situ* hybridization and immunohistochemistry, to enhance the production of TN-C in a discrete population of GFAP-(glial fibrillary acidic protein) positive astrocytes proximal to the injury site [81]. TN-C is also intensely expressed by astrocytes in culture in scratch wound assays [82]. Interestingly, TN-C could be involved in the maintenance of astrogliosis surrounding site of severe injuries. Indeed, stab wound assays in cerebral cortex revealed that GFAP expression, which is usually correlated with severity of reactivity, was significantly weaker in TN-C null versus wild type mice, one week after the stab [83]. However, in the same study, IgG leakage persisted much longer in TN-C-deficient mice and RNA levels of proinflammatory cytokines TNFα (tumour necrosis factor alpha), IL-6 (Interleukin-6), and IL-1β levels were higher [83]. Thus, the production of TN-C may influence blood-brain barrier (BBB) repair and the regulation of inflammatory cytokine levels either directly or indirectly through modulation of BBB integrity. In addition, TN-C production in astrocytes can be modulated by inflammatory cytokines and growth factors. Indeed, astrocytes increased their production of TN-C when cultured in presence of activated macrophage- and microglial-conditioned media [84]. Smith and colleagues have also demonstrated that the synergic action of both TGF-β1 and basic fibroblast growth factor (bFGF), both factors known to be upregulated in injury, stimulates the expression of TN-C [84].

Interestingly, TN-C upregulation has been described in numerous neurodegenerative diseases. TN-C expression is induced in the hippocampi of both epileptic rat brains [85, 86] and human patients with temporal lobe epilepsy (TLE) [87]. In brains of TLE patients, the regions exhibiting a diffuse and elevated expression of TN-C were also characterized by an extended area of reactive gliosis and synaptic reorganization. In addition, TN-C was recently reported as a plasma biomarker for neurodegenerative diseases, as levels were found to be significantly increased in blood of Alzheimer's disease (AD), mild cognitive impairments patients [88, 89], and in the amniotic fluid of Down syndrome-affected pregnancies [90]. In addition, TN-C level was shown to be upregulated in the AD-like mouse model CRND8 when compared to the wild type littermates [91]. Furthermore, TN-C deficiency in transgenic CRND8 mice provoked a reduction of β and γ secretase activity, Aβ oligomerization, plaque load, and synaptic impairments. In addition, loss of TN-C in CRND8 Tg mice enhanced production of anti-inflammatory cytokines and reduced proinflammatory cytokines [91]. This contrasts with the effect of TN-C deficiency on inflammation levels in the context of physical injury [83]. Therefore, TN-C may act as a promoter or inhibitor of the inflammation depending on the type of insult. In support of this, TN-C has also been demonstrated to induce production of proinflammatory cytokines via activation of Toll-like receptor 4 (TLR4) in nonneuronal tissue [92]. Interestingly, TLR4 is known to be expressed at the surface of astrocytes [93, 94] and microglia [95] and is directly implicated in the induction of inflammation in various neurodegenerative diseases [95–97].

In conclusion, the upregulation of TN-C by reactive astrocytes in response to an insult to the brain is context specific and may be a key factor in multiple processes such as the maintenance of astrocyte reactivity, BBB repair, and the potentiation of inflammatory processes. It could also directly affect neuronal plasticity and lead to memory impairments.

4. Thrombospondins

There are five known thrombospondins (TSP, also known as THBS) in vertebrates, which can be classified into two groups: the trimeric TSP1 and -2 and the pentameric TSP3, -4, and 5. All of the TSPs have epidermal growth factor-(EGF-) like repeats followed by calcium-binding type 3 repeats, and all share a highly conserved C-terminal region [98]. Thrombospondins are secreted molecules and interact with structural components of the ECM as well as proteases, cytokines, and growth factors to modulate cell signaling, adhesion, migration, and other cellular processes [99]. TSPs have many known binding partners, including integrins, neuroligins, Reelin receptors ApoER and VLDLR (very low density lipoprotein receptor), the calcium channel subunit $\alpha 2\delta$-1, and several growth factor ligands and their receptors (summarized in [100, 101]).

The different TSPs are expressed throughout the organism at various stages of mammalian development [98]. In the CNS, TSPs 1 and 2 are expressed in cultured and developing astrocytes, with expression peaking during the first postnatal week in mice [102, 103]. In addition, high levels of TSP1 can be found in cultured human astrocytes [104, 105]. Astrocyte-secreted TSP1/2 has been shown to be important for the formation of excitatory synapses *in vitro* and *in vivo* ([102, 106], recently reviewed extensively in [11, 101]). Briefly, TSP treatment of cultured neurons led to an increase in the number of synapses [102] and rate of synaptogenesis [107]. Synapses induced by TSPs are morphologically normal but postsynaptically silent, indicating that TSP is important for the initiation of synapse formation, but other secreted factors are required for completion of synapse maturation [5, 9], reviewed in [108].

4.1. Expression of TSPs in the Mature CNS and Following Injury. In mature astrocytes, the expression of TSP1/2 is decreased, although a low level is maintained throughout the brain and is particularly concentrated in areas of neurogenesis in the adult (i.e., subventricular zone (SVZ) and subgranular zone (SGZ)) [102, 109–111]. Indeed, loss of TSP1 was associated with a decrease in the number and distribution of SVZ neural precursors entering the olfactory bulb from the rostral migratory stream [112]. In contrast to TSP1/2, expression of TSP4 is low during development but present in adulthood, where it is expressed in spinal cord astrocytes [113] and mature forebrain astrocytes [114] and was found localized to synapses at neuromuscular junctions in the peripheral nervous system [115].

Similar to the other astrocytic matricellular proteins, TSPs are upregulated in reactive astrocytes following ischemic [116, 117] and mechanical [118] injury. It is likely that several signaling pathways can stimulate TSP production in reactive astrocytes. For example, in an *in vivo* trauma model, TSP1 expression was induced by extracellular ATP (Adenosine triphosphate) via P2Y purinergic receptors and ERK (extracellular signal-regulated kinase)/p38 MAPK (mitogen-activated protein kinase) signaling [119]. Additionally, TSP1 expression was activated by collagen-stimulated integrin signaling in reactive astrocytes following brain injury [120].

Following stroke, TSP1 and -2 display a differential temporal expression pattern; TSP1 was shown to be upregulated in the penumbra (the region surrounding the lesion core) within 3 days, whereas changes in TSP2 expression were delayed and levels were only increased at one week after stroke [116, 117]. Interestingly, the peak in expression of TSP1 and TSP2 was inversely correlated with angiogenic activity in the penumbra, consistent with a role for TSP1/2 in regulation of angiogenesis [121]. Conversely, Liauw and colleagues [116] reported that loss of TSP1/2 did not affect blood vessel density after stroke. Furthermore, they suggested that TSPs play a beneficial role in synaptic recovery, since TSP1/2 null mice exhibited a reduction in synapse number and axonal sprouting (and consequent motor function) when compared to wild type mice. This is particularly interesting considering that several studies have shown that TSPs can promote neurite outgrowth of cultured neurons *in vitro* [122–125] and is expressed around regenerating axons following injury *in vivo* [126, 127]. In addition, TSPs have been shown to induce dendritic spine formation [105] and are important for synaptogenesis ([102, 107] discussed in text above). In support of this, TSP expression is downregulated in diseases characterized by synaptic loss or abnormal synapse formation, such as in AD [128] and Down syndrome [105]. Taken together, upregulation of TSP1/2 expression in reactive astrocytes may contribute to tissue recovery following injury by promoting *de novo* synapse formation and rewiring of neural circuitry.

One key question in the field is where reactive astrocytes come from? Do they develop from local mature astrocytes or do they originate from neurogenic niches and migrate to the site of injury? It is possible that both of these processes occur depending on injury context [129]. In addition, it is thought that the molecular profile of reactive astrocytes overlaps with that of immature astrocytes or radial glial cells [16]. Recently, recruitment of newborn astrocytes and neurons from the SVZ was shown to play an important role following ischemic injury and stroke [130, 131]. Importantly, TSP4 was shown to be upregulated in SVZ-derived astrocytes surrounding lesions induced by photothrombotic injury and was required for injury-induced astrogenesis of neural stem cells, which was mediated through Notch signaling. Loss of TSP4 led to an increase in hemorrhaging and changes in glial scar formation [131]. In addition, expression of TSP4 was increased following peripheral nerve injury in spinal cord astrocytes, which morphologically resembled immature astrocytes [113]. Further study of TSPs may assist in the understanding of astrocyte heterogeneity and the process of astrogliosis.

5. CCN Family

The matricellular protein family CCN, which stands for CYR61/CTGF/NOV, are a family of six homolog members containing CYR61/CCN1 (cysteine-rich 61), CTGF/CCN2 (connective tissue growth factor), NOV/CCN3 (nephroblastoma overexpressed), and WISP-1/CCN4, WISP-2/CCN5, and WISP-3/CCN6 (Wnt-inducible-secreted proteins). All CCN proteins have a modular structure, which consists of four conserved cysteine rich-domains, with sequence homology to the insulin-like growth factor-binding proteins (IGFBP), the von Willebrand factor C (VWC) domain, Thrombospondin type 1 repeat (TSR), and a C-terminal domain with a cysteine-knot motif. CCN proteins can bind to integrin receptors and coreceptors such as heparan sulfate proteoglycans (HSPGs), low-density lipoprotein receptors-related proteins (LPRs), and TRKA [132]. They have been reported to participate in multiple functions in non-CNS organs, such as in migration, proliferation, and apoptosis (reviewed in [132]). They are upregulated in chronic inflammatory diseases, where they are suggested to be important players in the modulation of inflammatory cytokines and chemokines production (reviewed in [133]). Despite established roles in nonneuronal tissues, the expression and function of CCN family proteins in the CNS remain poorly understood.

5.1. CCN2/CTGF, Astrocytes, and Brain Insults. CCN2/CTGF is currently the only CCN family member in which expression has been demonstrated *in vivo* in CNS astrocytes. CCN2 expression has been detected not only in astrocyte somas and processes but also in a subpopulation of cortical neurons in adult rat brain, in tanycytes, and in the grey matter of the spinal cord [134]. CCN2 has been reported to be highly expressed in human astrocyte cell cultures [135] but was predominantly detected in neurons in healthy human brain and partially in subtypes of glial cells such as the glia limitans [136, 137].

Furthermore, CCN2 upregulation has been widely observed in reactive gliosis adjacent to the site of mechanical

injuries caused by stab wound in rodents and also in the brain tissue of stroke patients [137] and following traumatic brain injury (TBI) [136]. Interestingly, in neurodegenerative diseases, CCN2 elevation has been detected both in neurons and reactive astrocytes. This dual expression was reported after excitotoxic damages provoked by kainic lesions in rat hippocampi [138] and in CNS tissue of multiple sclerosis (MS) [139], amyotrophic lateral sclerosis (ALS), [140] and AD [141] patients. In brains of AD patients, this increase was particularly significant around the amyloid plaques and neurofibrillary tangles [141]. CCN2 increase has also been highly correlated with glioblastoma (reviewed in [142]). Interestingly, CCN proteins are also proposed as potent modulators of cytokines and chemokines in different organs [133]. In primary cultured rat astrocytes, CCN2 mRNA levels were reported to be differentially modulated by distinct cytokines, showing an upregulation following treatment with TGFβ and downregulation by TNFα [133]. Since both TGFβ and TNFα are known to dramatically modify the glial and neuronal environment in lesioned tissue and neurodegenerative diseases [143–147], it is interesting to speculate that CCN2 production and release by reactive astrocytes may participate in the cascade of inflammatory events occurring in injuries and neurodegenerative processes. Finally, CCN2 has been demonstrated to bind to TrkA (neurotrophic tyrosine kinase receptor type 1) and p75NTR (p75 neurotrophin receptor), receptors which transduce neurotrophin signals [148]. How CCN2 elevation might influence neurotrophin signalling in brain diseases remains to be evaluated.

6. Perspectives: Astrocyte-Derived Matricellular Proteins as Tools to Reshape the ECM and Cell-Cell Interactions Following Injury and Disease

Reactive astrocytes are found in nearly all situations following central nervous system injury and disease. Astrocyte reactivity is contextual [13] and leads to numerous morphological changes, such as an increase in the number and volume of processes [149], extension and elongation [150], and polarisation of processes around sites of injury [151]. This process requires dynamic modifications in cell-cell and cell-ECM interactions and involves the recruitment of other cell types, including immune cells [13]. Furthermore, the microenvironment surrounding the insult may modulate reactive astrogliosis and formation of the glial scar [152]. In this review, we have discussed several nonstructurally related families of matricellular proteins which are upregulated in reactive astrocytes. A common feature among these proteins is that they exhibit high levels of expression during development which is reduced to a lower level in the adult. However, their expression is reactivated following injury or disease. This pattern of expression is consistent with the fact that reactive astrocytes upregulate expression of proteins (GFAP, Vimentin, BLBP (brain lipid-binding protein), and Nestin) that are also present at high levels in radial glia or immature astrocytes [153]. Despite this, recent studies have shown that reactive astrocytes exhibit significant heterogeneity and the type of astrocyte reactivity occurring is dependent on the type and extent of CNS injury and disease [154–156]. For example, there is an increase in proliferating reactive astrocytes following lesions such as stroke but not in diseases such as Alzheimer's disease [129, 157]. Matricellular proteins have been described as dynamic regulators of the ECM and cell-cell interactions and are well placed to regulate the process of astrocyte reactivity and tissue remodelling occurring in the CNS following injury and in neurodegenerative disease (see Figure 1).

Ultimately, reactive astrogliosis can progress to form a glial scar, which has been shown to have both positive and negative effects on tissue recovery. The glial scar, which contains proliferating reactive astrocytes, forms a dense physical barrier around an injury. This protects the healthy brain from damaged tissue and inflammation and has been shown to play a role in repair of the BBB [14]. It is conceivable that matricellular proteins play an important role in modulating the glial scar, perhaps through regulation of glial cell proliferation and the surrounding vasculature. For example, SPARC is antiproliferator and can regulate microglia proliferation *in vitro* and *in vivo* following photothrombotic stroke [21, 45]. Conversely, TN-C was found to induce astrocyte proliferation *in vitro* [63]. In addition, SPARC, CCN2, and TSP1/2 have been implicated in angiogenesis and leukocyte infiltration in nonneuronal tissues [101, 158, 159] and therefore may serve to repair the BBB and restrain an immune response [160–162].

It has been well documented that the glial scar can inhibit axon regeneration; reactive "scar" astrocytes produce proteoglycans and other ECM molecules to inhibit axonal outgrowth, which ultimately limits functional recovery [163]. Through modulation of structural ECM components and adhesion and growth factor signaling (e.g., integrin signaling), astrocyte-secreted matricellular proteins may control axonal growth. In support of this, TN-C has been shown to both promote and inhibit axonal outgrowth and TSPs are known to positively affect growth [164]. All four matricellular protein families modulate cell adhesion *in vitro*, where they have been reported to restructure actin-containing focal adhesions and stress fibers [165]. This may translate into an *in vivo* role of "opening up" the ECM to create a permissive microenvironment for facilitating cell migration, outgrowth, angiogenesis, synaptic remodelling, and morphological plasticity. At the same time, upregulation of matricellular proteins may have negative consequences for tissue recovery and progression of disease. For example, matricellular proteins could promote an inflammatory response [19] and chronic inflammation is thought to be detrimental in neurodegenerative diseases such as Alzheimer's disease [166–168].

Huge challenges are awaited in order to understand the secretion of matricellular proteins from reactive astrocytes and functions in the CNS. Given their potential to modulate the microenvironment surrounding regions of brain injury and disease, astrocyte-secreted matricellular

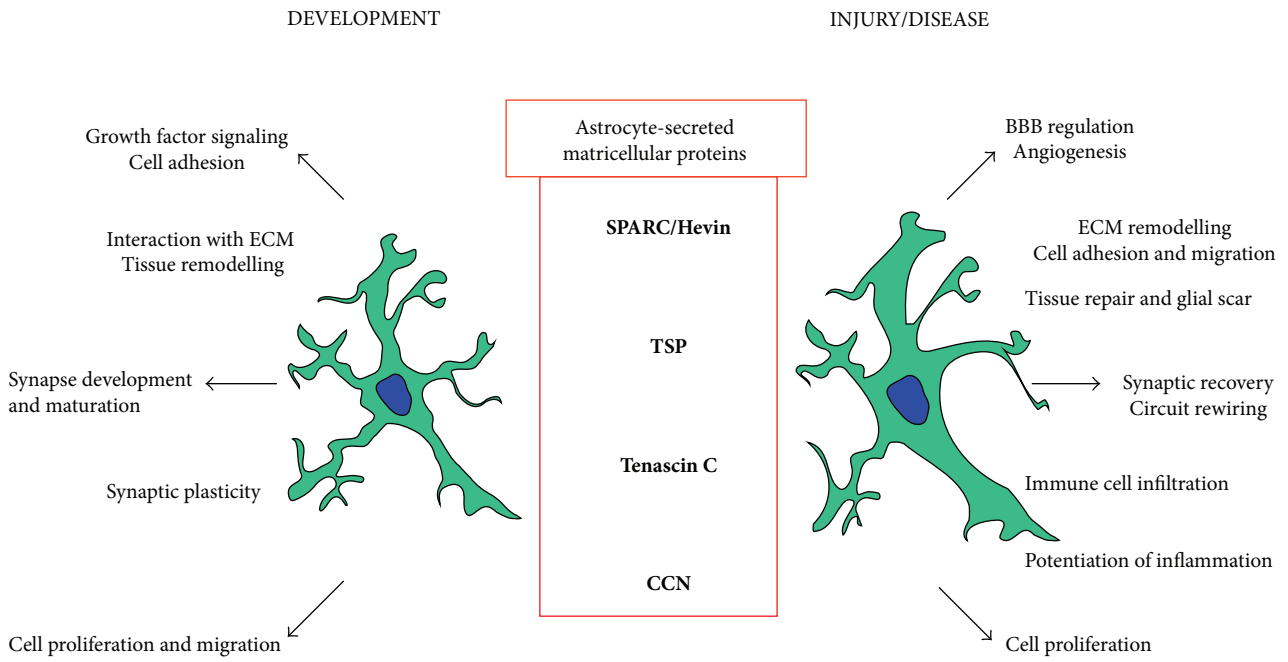

Figure 1: *Astrocyte-secreted matricellular proteins: Developmental tools for reactivity purposes.* Astrocyte-secreted matricellular proteins are highly expressed during the development of the CNS where they have multiple complex roles (left panel). In general, the expression of these molecules in the adult nervous system is reduced, but they are reexpressed at a high level in reactive astrocytes following injury or in disease states. Here, we summarise the possible roles of matricellular proteins secreted from reactive astrocytes (right panel).

proteins may represent important therapeutic targets for CNS repair.

Conflict of Interests

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

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References

[1] P. Bezzi, V. Gundersen, J. L. Galbete et al., "Astrocytes contain a vesicular compartment that is competent for regulated exocytosis of glutamate," *Nature Neuroscience*, vol. 7, no. 6, pp. 613–620, 2004.

[2] N. B. Hamilton and D. Attwell, "Do astrocytes really exocytose neurotransmitters?" *Nature Reviews Neuroscience*, vol. 11, no. 4, pp. 227–238, 2010.

[3] M. Martineau, T. Shi, J. Puyal et al., "Storage and uptake of D-serine into astrocytic synaptic-like vesicles specify gliotransmission," *Journal of Neuroscience*, vol. 33, no. 8, pp. 3413–3423, 2013.

[4] V. Schubert, D. Bouvier, and A. Volterra, "SNARE protein expression in synaptic terminals and astrocytes in the adult hippocampus: a comparative analysis," *Glia*, vol. 59, no. 10, pp. 1472–1488, 2011.

[5] N. J. Allen, M. L. Bennett, L. C. Foo et al., "Astrocyte glypicans 4 and 6 promote formation of excitatory synapses via GluA1 AMPA receptors," *Nature*, vol. 486, no. 7403, pp. 410–414, 2012.

[6] M. A. di Castro, J. Chuquet, N. Liaudet et al., "Local Ca2+ detection and modulation of synaptic release by astrocytes," *Nature Neuroscience*, vol. 14, no. 10, pp. 1276–1284, 2011.

[7] M. Haber and K. K. Murai, "Reshaping neuron-glial communication at hippocampal synapses," *Neuron Glia Biology*, vol. 2, no. 1, pp. 59–66, 2006.

[8] C. Henneberger, T. Papouin, S. H. R. Oliet, and D. A. Rusakov, "Long-term potentiation depends on release of d-serine from astrocytes," *Nature*, vol. 463, no. 7278, pp. 232–236, 2010.

[9] E. V. Jones, Y. Bernardinelli, Y. C. Tse, S. Chierzi, T. P. Wong, and K. K. Murai, "Astrocytes control glutamate receptor levels at developing synapses through SPARC-β-integrin interactions," *Journal of Neuroscience*, vol. 31, no. 11, pp. 4154–4165, 2011.

[10] A. Panatier, J. Vallée, M. Haber, K. K. Murai, J. C. Lacaille, and R. Robitaille, "Astrocytes are endogenous regulators of basal transmission at central synapses," *Cell*, vol. 146, no. 5, pp. 785–798, 2011.

[11] L. E. Clarke and B. A. Barres, "Emerging roles of astrocytes in neural circuit development," *Nature Reviews Neuroscience*, vol. 14, no. 5, pp. 311–321, 2013.

[12] C. Farina, F. Aloisi, and E. Meinl, "Astrocytes are active players in cerebral innate immunity," *Trends in Immunology*, vol. 28, no. 3, pp. 138–145, 2007.

[13] M. V. Sofroniew and H. V. Vinters, "Astrocytes: biology and pathology," *Acta Neuropathologica*, vol. 119, no. 1, pp. 7–35, 2010.

[14] M. V. Sofroniew, "Molecular dissection of reactive astrogliosis and glial scar formation," *Trends in Neurosciences*, vol. 32, no. 12, pp. 638–647, 2009.

[15] M. V. Sofroniew, "Reactive astrocytes in neural repair and protection," *Neuroscientist*, vol. 11, no. 5, pp. 400–407, 2005.

[16] J. L. Ridet, S. K. Malhotra, A. Privat, and F. H. Gage, "Reactive astrocytes: cellular and molecular cues to biological function," *Trends in Neurosciences*, vol. 20, no. 12, pp. 570–577, 1997.

[17] P. Bornstein, "Thrombospondins as matricellular modulators of cell function," *Journal of Clinical Investigation*, vol. 107, no. 8, pp. 929–934, 2001.

[18] P. Bornstein and E. H. Sage, "Matricellular proteins: extracellular modulators of cell function," *Current Opinion in Cell Biology*, vol. 14, no. 5, pp. 608–616, 2002.

[19] N. G. Frangogiannis, "Matricellular proteins in cardiac adaptation and disease," *Physiological Reviews*, vol. 92, no. 2, pp. 635–688, 2012.

[20] A. D. Bradshaw, "Diverse biological functions of the SPARC family of proteins," *International Journal of Biochemistry and Cell Biology*, vol. 44, no. 3, pp. 480–488, 2012.

[21] R. A. Brekken and E. H. Sage, "SPARC, a matricellular protein: at the crossroads of cell-matrix communication," *Matrix Biology*, vol. 19, no. 8, pp. 816–827, 2001.

[22] I. G. Johnston, T. Paladino, J. W. Gurd, and I. R. Brown, "Molecular cloning of SC1: a putative brain extracellular matrix glycoprotein showing partial similarity to osteonectin/BM40/SPARC," *Neuron*, vol. 4, no. 1, pp. 165–176, 1990.

[23] I. J. Mason, A. Taylor, J. G. Williams, H. Sage, and B. L. Hogan, "Evidence from molecular cloning that SPARC, a major product of mouse embryo parietal endoderm, is related to an endothelial cell 'culture shock' glycoprotein of Mr 43,000," *The EMBO Journal*, vol. 5, no. 7, pp. 1465–1472, 1986.

[24] M. M. Sullivan and E. H. Sage, "Hevin/SC1, a matricellular glycoprotein and potential tumor-suppressor of the SPARC/BM-40/Osteonectin family," *International Journal of Biochemistry and Cell Biology*, vol. 36, no. 6, pp. 991–996, 2004.

[25] H. O. Hambrock, B. Kaufmann, S. Müller et al., "Structural characterization of TSC-36/Flik: analysis of two charge isoforms," *Journal of Biological Chemistry*, vol. 279, no. 12, pp. 11727–11735, 2004.

[26] P. Maurer, C. Hohenadl, E. Hohenester, W. Gohring, R. Timpl, and J. Engel, "The C-terminal portion of BM-40 (SPARC/osteonectin) is an autonomously folding and crystallisable domain that binds calcium and collagen IV," *Journal of Molecular Biology*, vol. 253, no. 2, pp. 347–357, 1995.

[27] T. Sasaki, N. Miosge, and R. Timpl, "Immunochemical and tissue analysis of protease generated neoepitopes of BM-40 (osteonectin, SPARC) which are correlated to a higher affinity binding to collagens," *Matrix Biology*, vol. 18, no. 5, pp. 499–508, 1999.

[28] L. B. Rivera, A. D. Bradshaw, and R. A. Brekken, "The regulatory function of SPARC in vascular biology," *Cellular and Molecular Life Sciences*, vol. 68, no. 19, pp. 3165–3173, 2011.

[29] M. Campolongo, L. Benedetti, O. L. Podhajcer, F. Pitossi, and A. M. Depino, "Hippocampal SPARC regulates depression-related behavior," *Genes, Brain and Behavior*, 2012.

[30] P. E. Framson and E. H. Sage, "SPARC and tumor growth: where the seed meets the soil?" *Journal of Cellular Biochemistry*, vol. 92, no. 4, pp. 679–690, 2004.

[31] H. Kucukdereli, N. J. Allen, A. T. Lee et al., "Control of excitatory CNS synaptogenesis by astrocyte-secreted proteins hevin and SPARC," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 32, pp. E440–E449, 2011.

[32] V. Gongidi, C. Ring, M. Moody et al., "SPARC-like 1 regulates the terminal phase of radial glia-guided migration in the cerebral cortex," *Neuron*, vol. 41, no. 1, pp. 57–69, 2004.

[33] C. Eroglu, "The role of astrocyte-secreted matricellular proteins in central nervous system development and function," *Journal of Cell Communication and Signaling*, vol. 3, no. 3-4, pp. 167–176, 2009.

[34] S. Lloyd-Burton and A. J. Roskams, "SPARC-like 1 (SC1) is a diversely expressed and developmentally regulated matricellular protein that does not compensate for the absence of SPARC in the CNS," *Journal of Comparative Neurology*, vol. 520, no. 12, pp. 2575–2590, 2012.

[35] A. J. Vincent, P. W. Lau, and A. J. Roskams, "SPARC is expressed by macroglia and microglia in the developing and mature nervous system," *Developmental Dynamics*, vol. 237, no. 5, pp. 1449–1462, 2008.

[36] S. Lively and I. R. Brown, "Localization of the extracellular matrix protein SC1 coincides with synaptogenesis during rat postnatal development," *Neurochemical Research*, vol. 33, no. 9, pp. 1692–1700, 2008.

[37] D. B. Mendis, L. Malaval, and I. R. Brown, "SPARC, an extracellular matrix glycoprotein containing the follistatin module, is expressed by astrocytes in synaptic enriched regions of the adult brain," *Brain Research*, vol. 676, no. 1, pp. 69–79, 1995.

[38] M. S. Weaver, G. Workman, M. Cardo-Vila, W. Arap, R. Pasqualini, and E. H. Sage, "Processing of the matricellular protein hevin in mouse brain is dependent on ADAMTS4," *Journal of Biological Chemistry*, vol. 285, no. 8, pp. 5868–5877, 2010.

[39] X. Liu, G. Ying, W. Wang et al., "Entorhinal deafferentation induces upregulation of SPARC in the mouse hippocampus," *Molecular Brain Research*, vol. 141, no. 1, pp. 58–65, 2005.

[40] P. J. McKinnon and R. F. Margolskee, "SC1: a marker for astrocytes in the adult rodent brain is upregulated during reactive astrocytosis," *Brain Research*, vol. 709, no. 1, pp. 27–36, 1996.

[41] S. Lively and I. R. Brown, "Analysis of the extracellular matrix protein SC1 during reactive gliosis in the rat lithium-pilocarpine seizure model," *Brain Research*, vol. 1163, no. 1, pp. 1–9, 2007.

[42] D. B. Mendis, G. O. Ivy, and I. R. Brown, "Induction of SC1 mRNA encoding a brain extracellular matrix glycoprotein related to SPARC following lesioning of the adult rat forebrain," *Neurochemical Research*, vol. 25, no. 12, pp. 1637–1644, 2000.

[43] D. B. Mendis, G. O. Ivy, and I. R. Brown, "SC1, a brain extracellular matrix glycoprotein related to SPARC and follistatin, is expressed by rat cerebellar astrocytes following injury and during development," *Brain Research*, vol. 730, no. 1-2, pp. 95–106, 1996.

[44] S. Lively and L. C. Schlichter, "SC1/hevin identifies early white matter injury after ischemia and intracerebral hemorrhage in young and aged rats," *Journal of Neuropathology & Experimental Neurology*, vol. 71, no. 6, pp. 480–493, 2012.

[45] S. M. Lloyd-Burton, E. M. York, M. A. Anwar, A. J. Vincent, and A. J. Roskams, "SPARC regulates microgliosis and functional recovery following cortical ischemia," *Journal of Neuroscience*, vol. 33, no. 10, pp. 4468–4481, 2013.

[46] H. Huang, S. Colella, M. Kurrer, Y. Yonekawa, P. Kleihues, and H. Ohgaki, "Gene expression profiling of low-grade diffuse astrocytomas by cDNA arrays," *Cancer Research*, vol. 60, no. 24, pp. 6868–6874, 2000.

[47] F. Özbas-Gerçeker, S. Redeker, K. Boer et al., "Serial analysis of gene expression in the hippocampus of patients with mesial temporal lobe epilepsy," *Neuroscience*, vol. 138, no. 2, pp. 457–474, 2006.

[48] S. A. Rempel, W. A. Golembieski, S. Ge et al., "SPARC: a signal of astrocytic neoplastic transformation and reactive response in human primary and xenograft gliomas," *Journal of Neuropathology and Experimental Neurology*, vol. 57, no. 12, pp. 1112–1121, 1998.

[49] J. N. Rich, C. Hans, B. Jones et al., "Gene expression profiling and genetic markers in glioblastoma survival," *Cancer Research*, vol. 65, no. 10, pp. 4051–4058, 2005.

[50] A. Turtoi, D. Musmeci, A. G. Naccarato et al., "Sparc-like protein 1 is a new marker of human glioma progression," *Journal of Proteome Research*, vol. 11, no. 10, pp. 5011–5021, 2012.

[51] I. Esposito, H. Kayed, S. Keleg et al., "Tumor-suppressor function of SPARC-like protein 1/hevin in pancreatic cancer," *Neoplasia*, vol. 9, no. 1, pp. 8–17, 2007.

[52] M. Weaver, G. Workman, C. R. Schultz, N. Lemke, S. A. Rempel, and E. H. Sage, "Proteolysis of the matricellular protein hevin by matrix metalloproteinase-3 produces a SPARC-like fragment (SLF) associated with neovasculature in a murine glioma model," *Journal of Cellular Biochemistry*, vol. 112, no. 11, pp. 3093–3102, 2011.

[53] J. N. Rich, Q. Shi, M. Hjelmeland et al., "Bone-related genes expressed in advanced malignancies induce invasion and metastasis in a genetically defined human cancer model," *Journal of Biological Chemistry*, vol. 278, no. 18, pp. 15951–15957, 2003.

[54] P. M. Tremble, T. F. Lane, E. H. Sage, and Z. Werb, "SPARC, a secreted protein associated with morphogenesis and tissue remodeling, induces expression of metalloproteinases in fibroblasts through a novel extracellular matrix-dependent pathway," *Journal of Cell Biology*, vol. 121, no. 6, pp. 1433–1444, 1993.

[55] E. H. Sage, M. Reed, S. E. Funk et al., "Cleavage of the matricellular protein SPARC by matrix metalloproteinase 3 produces polypeptides that influence angiogenesis," *Journal of Biological Chemistry*, vol. 278, no. 39, pp. 37849–37857, 2003.

[56] M. S. Weaver, G. Workman, and E. H. Sage, "The copper binding domain of SPARC mediates cell survival in vitro via interaction with integrin β1 and activation of integrin-linked kinase," *Journal of Biological Chemistry*, vol. 283, no. 33, pp. 22826–22837, 2008.

[57] T. F. Lane, M. L. Iruela-Arispe, R. S. Johnson, and E. H. Sage, "SPARC is a source of copper-binding peptides that stimulate angiogenesis," *Journal of Cell Biology*, vol. 125, no. 4, pp. 929–943, 1994.

[58] L. A. Cunningham, M. Wetzel, and G. A. Rosenberg, "Multiple roles for MMPs and TIMPs in cerebral ischemia," *Glia*, vol. 50, no. 4, pp. 329–339, 2005.

[59] J. S. Miners, S. Baig, J. Palmer, L. E. Palmer, P. G. Kehoe, and S. Love, "Abeta-degrading enzymes in Alzheimer's disease," *Brain Pathology*, vol. 18, no. 2, pp. 240–252, 2008.

[60] E. Dejonckheere, R. E. Vandenbroucke, and C. Libert, "Matrix metalloproteinases as drug targets in ischemia/reperfusion injury," *Drug Discovery Today*, vol. 16, no. 17-18, pp. 762–778, 2011.

[61] B. Q. Zhao, S. Wang, H. Y. Kim et al., "Role of matrix metalloproteinases in delayed cortical responses after stroke," *Nature Medicine*, vol. 12, no. 4, pp. 441–445, 2006.

[62] H. P. Erickson, "Tenascin-C, tenascin-R and tenascin-X: a family of talented proteins in search of functions," *Current Opinion in Cell Biology*, vol. 5, no. 5, pp. 869–876, 1993.

[63] T. Nishio, S. Kawaguchi, T. Iseda, T. Kawasaki, and T. Hase, "Secretion of tenascin-C by cultured astrocytes: regulation of cell proliferation and process elongation," *Brain Research*, vol. 990, no. 1-2, pp. 129–140, 2003.

[64] G. Keilhauer, A. Faissner, and M. Schachner, "Differential inhibition of neuron-neurone, neurone-astrocyte and astrocyte-astrocyte adhesion by L1, L2, and N-CAM antibodies," *Nature*, vol. 316, no. 6030, pp. 728–730, 1985.

[65] J. Kruse, G. Keilhauer, and A. Faissner, "The J1 glycoprotein—a novel nervous system cell adhesion molecule of the L2/HNK-1 family," *Nature*, vol. 316, no. 6024, pp. 146–148, 1985.

[66] S. Meiners, E. M. Powell, and H. M. Geller, "A distinct subset of tenascin/CS-6-PG-rich astrocytes restricts neuronal growth in vitro," *Journal of Neuroscience*, vol. 15, no. 12, pp. 8096–8108, 1995.

[67] E. M. Powell and H. M. Geller, "Dissection of astrocyte-mediated cues in neuronal guidance and process extension," *Glia*, vol. 26, no. 1, pp. 73–83, 1999.

[68] A. Faissner and J. Kruse, "J1/tenascin is a repulsive substrate for central nervous system neurons," *Neuron*, vol. 5, no. 5, pp. 627–637, 1990.

[69] M. R. Andrews, S. Czvitkovich, E. Dassie et al., "α9 integrin promotes neurite outgrowth on tenascin-C and enhances sensory axon regeneration," *Journal of Neuroscience*, vol. 29, no. 17, pp. 5546–5557, 2009.

[70] S. Meiners, E. M. Powell, and H. M. Geller, "Neurite outgrowth promotion by the alternatively spliced region of tenascin-C is influenced by cell-type specific binding," *Matrix Biology*, vol. 18, no. 1, pp. 75–87, 1999.

[71] F. Rigato, J. Garwood, V. Calco, N. Heck, C. Faivre-Sarrailh, and A. Faissner, "Tenascin-C promotes neurite outgrowth of embryonic hippocampal neurons through the alternatively spliced fibronectin type III BD domains via activation of the cell adhesion molecule F3/contactin," *Journal of Neuroscience*, vol. 22, no. 15, pp. 6596–6609, 2002.

[72] R. Probstmeier and P. Pesheva, "Tenascin-C inhibits β1 integrin-dependent cell adhesion and neurite outgrowth on fibronectin by a disialoganglioside-mediated signaling mechanism," *Glycobiology*, vol. 9, no. 2, pp. 101–114, 1999.

[73] D. A. Steindler, N. G. F. Cooper, A. Faissner, and M. Schachner, "Boundaries defined by adhesion molecules during development of the cerebral cortex: the J1/tenascin glycoprotein in the mouse somatosensory cortical barrel field," *Developmental Biology*, vol. 131, no. 1, pp. 243–260, 1989.

[74] M. Götz, A. Stoykova, and P. Gruss, "Pax6 controls radial glia differentiation in the cerebral cortex," *Neuron*, vol. 21, no. 5, pp. 1031–1044, 1998.

[75] S. Bartsch, U. Bartsch, U. Dorries et al., "Expression of tenascin in the developing and adult cerebellar cortex," *Journal of Neuroscience*, vol. 12, no. 3, pp. 736–749, 1992.

[76] L. Ferhat, N. Chevassus Au Louis, I. Jorquera et al., "Transient increase of tenascin-C in immature hippocampus: astroglial and neuronal expression," *Journal of Neurocytology*, vol. 25, no. 1, pp. 53–66, 1996.

[77] M. Karus, B. Denecke, C. Ffrench-Constant, S. Wiese, and A. Faissner, "The extracellular matrix molecule tenascin C modulates expression levels and territories of key patterning genes during spinal cord astrocyte specification," *Development*, vol. 138, no. 24, pp. 5321–5331, 2011.

[78] M. R. Evers, B. Salmen, O. Bukalo et al., "Impairment of L-type Ca2+ channel-dependent forms of hippocampal synaptic plasticity in mice deficient in the extracellular matrix glycoprotein

tenascin-C," *Journal of Neuroscience*, vol. 22, no. 16, pp. 7177–7194, 2002.

[79] A. Irintchev, A. Rollenhagen, E. Troncoso, J. Z. Kiss, and M. Schachner, "Structural and functional aberrations in the cerebral cortex of tenascin-C deficient mice," *Cerebral Cortex*, vol. 15, no. 7, pp. 950–962, 2005.

[80] T. Strekalova, M. Sun, M. Sibbe et al., "Fibronectin domains of extracellular matrix molecule tenascin-C modulate hippocampal learning and synaptic plasticity," *Molecular and Cellular Neuroscience*, vol. 21, no. 1, pp. 173–187, 2002.

[81] E. D. Laywell, U. Dorries, U. Bartsch, A. Faissner, M. Schachner, and D. A. Steindler, "Enhanced expression of the developmentally regulated extracellular matrix molecule tenascin following adult brain injury," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 7, pp. 2634–2638, 1992.

[82] T. Nishio, S. Kawaguchi, M. Yamamoto, T. Iseda, T. Kawasaki, and T. Hase, "Tenascin-C regulates proliferation and migration of cultured astrocytes in a scratch wound assay," *Neuroscience*, vol. 132, no. 1, pp. 87–102, 2005.

[83] H. Ikeshima-Kataoka, J. S. Shen, Y. Eto, S. Saito, and S. Yuasa, "Alteration of inflammatory cytokine production in the injured central nervous system of tenascin-deficient mice," *In Vivo*, vol. 22, no. 4, pp. 409–413, 2008.

[84] G. M. Smith and J. H. Hale, "Macrophage/microglia regulation of astrocytic tenascin: synergistic action of transforming growth factor-β and basic fibroblast growth factor," *Journal of Neuroscience*, vol. 17, no. 24, pp. 9624–9633, 1997.

[85] J. Niquet, I. Jorquera, A. Faissner, Y. Ben-Ari, and A. Represa, "Gliosis and axonal sprouting in the hippocampus of epileptic rats are associated with an increase of tenascin-C immunoreactivity," *Journal of Neurocytology*, vol. 24, no. 8, pp. 611–624, 1995.

[86] A. J. Becker, J. Chen, A. Zien et al., "Correlated stage- and subfield-associated hippocampal gene expression patterns in experimental and human temporal lobe epilepsy," *European Journal of Neuroscience*, vol. 18, no. 10, pp. 2792–2802, 2003.

[87] B. Scheffler, A. Faissner, H. Beck et al., "Hippocampal loss of tenascin boundaries in Ammon's horn sclerosis," *Glia*, vol. 19, no. 1, pp. 35–46, 1997.

[88] H. D. Soares, W. Z. Potter, E. Pickering et al., "Plasma biomarkers associated with the apolipoprotein E genotype and Alzheimer disease," *Archives of Neurology*, vol. 69, no. 10, pp. 1310–1317, 2012.

[89] J. R. Hall, L. A. Johnson, R. C. Barber, H. T. Vo, A. S. Winter, and O. 'Bryant SE, "Biomarkers of basic activities of daily living in Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 31, no. 2, pp. 429–437, 2012.

[90] C. K. J. Cho, C. R. Smith, and E. P. Diamandis, "Amniotic fluid proteome analysis from down syndrome pregnancies for biomarker discovery," *Journal of Proteome Research*, vol. 9, no. 7, pp. 3574–3582, 2010.

[91] K. Xie, Y. Liu, W. Hao et al., "Tenascin-C deficiency ameliorates Alzheimer's disease-related pathology in mice," *Neurobiology of Aging*, vol. 34, no. 10, pp. 2389–2398, 2013.

[92] K. Midwood, S. Sacre, A. M. Piccinini et al., "Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease," *Nature Medicine*, vol. 15, no. 7, pp. 774–780, 2009.

[93] R. Gorina, M. Font-Nieves, L. Márquez-Kisinousky, T. Santalucia, and A. M. Planas, "Astrocyte TLR4 activation induces a proinflammatory environment through the interplay between MyD88-dependent NFκB signaling, MAPK, and Jak1/Stat1 pathways," *Glia*, vol. 59, no. 2, pp. 242–255, 2011.

[94] T. H. Holm, D. Draeby, and T. Owens, "Microglia are required for astroglial toll-like receptor 4 response and for optimal TLR2 and TLR3 response," *Glia*, vol. 60, no. 4, pp. 630–638, 2012.

[95] S. Lehnardt, L. Massillon, P. Follett et al., "Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 14, pp. 8514–8519, 2003.

[96] J. J. Jin, H. D. Kim, J. A. Maxwell, L. Li, and K. I. Fukuchi, "Toll-like receptor 4-dependent upregulation of cytokines in a transgenic mouse model of Alzheimer's disease," *Journal of Neuroinflammation*, vol. 5, article 23, 2008.

[97] N. Stefanova, L. Fellner, M. Reindl, E. Masliah, W. Poewe, and G. K. Wenning, "Toll-like receptor 4 promotes α-synuclein clearance and survival of nigral dopaminergic neurons," *American Journal of Pathology*, vol. 179, no. 2, pp. 954–963, 2011.

[98] J. C. Adams and J. Lawler, "The thrombospondins," *Cold Spring Harbor Perspectives in Biology*, vol. 3, no. 10, Article ID a009712, 2011.

[99] J. C. Adams, "Functions of the conserved thrombospondin carboxy-terminal cassette in cell-extracellular matrix interactions and signaling," *International Journal of Biochemistry and Cell Biology*, vol. 36, no. 6, pp. 1102–1114, 2004.

[100] J. E. Murphy-Ullrich and R. V. Iozzo, "Thrombospondins in physiology and disease: new tricks for old dogs," *Matrix Biology*, vol. 31, no. 3, pp. 152–154, 2012.

[101] W. C. Risher and C. Eroglu, "Thrombospondins as key regulators of synaptogenesis in the central nervous system," *Matrix Biology*, vol. 31, no. 3, pp. 170–177, 2012.

[102] K. S. Christopherson, E. M. Ullian, C. C. A. Stokes et al., "Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis," *Cell*, vol. 120, no. 3, pp. 421–433, 2005.

[103] S. Scott-Drew and C. ffrench-Constant, "Expression and function of thrombospondin-1 in myelinating glial cells of the central nervous system," *Journal of Neuroscience Research*, vol. 50, no. 2, pp. 202–214, 1997.

[104] A. S. Asch, L. L. K. Leung, J. Shapiro, and R. L. Nachman, "Human brain glial cells synthesize thrombospondin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 83, no. 9, pp. 2904–2908, 1986.

[105] O. Garcia, M. Torres, P. Helguera, P. Coskun, and J. Busciglio, "A role for thrombospondin-1 deficits in astrocyte-mediated spine and synaptic pathology in down's syndrome," *PLoS ONE*, vol. 5, no. 12, Article ID e14200, 2010.

[106] Ç. Eroglu, N. J. Allen, M. W. Susman et al., "Gabapentin receptor $\alpha 2\delta$-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis," *Cell*, vol. 139, no. 2, pp. 380–392, 2009.

[107] J. Xu, N. Xiao, and J. Xia, "Thrombospondin 1 accelerates synaptogenesis in hippocampal neurons through neuroligin 1," *Nature Neuroscience*, vol. 13, no. 1, pp. 22–24, 2010.

[108] N. J. Allen, "Role of glia in developmental synapse formation," *Current Opinion in Neurobiology*, vol. 23, no. 6, pp. 1027–1033, 2013.

[109] J. R. Hoffman, V. M. Dixit, and K. S. O'Shea, "Expression of thrombospondin in the adult nervous system," *Journal of Comparative Neurology*, vol. 340, no. 1, pp. 126–139, 1994.

[110] M. L. Iruela-Arispe, D. J. Liska, E. H. Sage, and P. Bornstein, "Differential expression of thrombospondin 1, 2, and 3 during murine development," *Developmental Dynamics*, vol. 197, no. 1, pp. 40–56, 1993.

[111] Z. Lu and J. Kipnis, "Thrombospondin 1—a key astrocyte-derived neurogenic factor," *FASEB Journal*, vol. 24, no. 6, pp. 1925–1934, 2010.

[112] S. M. Blake, V. Strasser, N. Andrade et al., "Thrombospondin-1 binds to ApoER2 and VLDL receptor and functions in postnatal neuronal migration," *EMBO Journal*, vol. 27, no. 22, pp. 3069–3080, 2008.

[113] D. S. Kim, K. W. Li, A. Boroujerdi et al., "Thrombospondin-4 contributes to spinal sensitization and neuropathic pain states," *Journal of Neuroscience*, vol. 32, no. 26, pp. 8977–8987, 2012.

[114] J. D. Cahoy, B. Emery, A. Kaushal et al., "A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function," *Journal of Neuroscience*, vol. 28, no. 1, pp. 264–278, 2008.

[115] S. Arber and P. Caroni, "Thrombospondin-4, an extracellular matrix protein expressed in the developing and adult nervous system promotes neurite outgrowth," *Journal of Cell Biology*, vol. 131, no. 4, pp. 1083–1094, 1995.

[116] J. Liauw, S. Hoang, M. Choi et al., "Thrombospondins 1 and 2 are necessary for synaptic plasticity and functional recovery after stroke," *Journal of Cerebral Blood Flow and Metabolism*, vol. 28, no. 10, pp. 1722–1732, 2008.

[117] T. N. Lin, G. M. Kim, J. J. Chen, W. M. Cheung, Y. Y. He, and C. Y. Hsu, "Differential regulation of thrombospondin-1 and thrombospondin-2 after focal cerebral ischemia/reperfusion," *Stroke*, vol. 34, no. 1, pp. 177–186, 2003.

[118] M. D. Tran and J. T. Neary, "Purinergic signaling induces thrombospondin-1 expression in astrocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 24, pp. 9321–9326, 2006.

[119] M. D. Tran, O. Furones-Alonso, J. Sanchez-Molano, and H. M. Bramlett, "Trauma-induced expression of astrocytic thrombospondin-1 is regulated by P2 receptors coupled to protein kinase cascades," *Neuroreport*, vol. 23, no. 12, pp. 721–726, 2012.

[120] T. Yonezawa, S. Hattori, J. Inagaki et al., "Type IV collagen induces expression of thrombospondin-1 that is mediated by integrin $\alpha 1\beta 1$ in astrocytes," *Glia*, vol. 58, no. 7, pp. 755–767, 2010.

[121] P. R. Lawler and J. Lawler, "Molecular basis for the regulation of angiogenesis by thrombospondin-1 and -2," *Cold Spring Harbor Perspectives in Medicine*, vol. 2, no. 5, Article ID a006627, 2012.

[122] K. M. Neugebauer, C. J. Emmett, K. A. Venstrom, and L. F. Reichardt, "Vitronectin and thrombospondin promote retinal neurite outgrowth: developmental regulation and role of integrins," *Neuron*, vol. 6, no. 3, pp. 345–358, 1991.

[123] M. F. DeFreitas, C. K. Yoshida, W. A. Frazier, D. L. Mendrick, R. M. Kypta, and L. F. Reichardt, "Identification of integrin $\alpha 3\beta 1$ as a neuronal thrombospondin receptor mediating neurite outgrowth," *Neuron*, vol. 15, no. 2, pp. 333–343, 1995.

[124] K. S. O'Shea, L. H. J. Liu, and V. M. Dixit, "Thrombospondin and a 140 kd fragment promote adhesion and neurite outgrowth from embryonic central and peripheral neurons and from PC12 cells," *Neuron*, vol. 7, no. 2, pp. 231–237, 1991.

[125] D. J. Osterhout, W. A. Frazier, and D. Higgins, "Thrombospondin promotes process outgrowth in neurons from the peripheral and central nervous systems," *Developmental Biology*, vol. 150, no. 2, pp. 256–265, 1992.

[126] J. C. Moller, M. A. Klein, S. Haas, L. L. Jones, G. W. Kreutzberg, and G. Raivich, "Regulation of thrombospondin in the regenerating mouse facial motor nucleus," *Glia*, vol. 17, no. 2, pp. 121–132, 1996.

[127] J. R. Hoffman and K. S. O'Shea, "Thrombospondin expression in nerve regeneration II. Comparison of optic nerve crush in the mouse and goldfish," *Brain Research Bulletin*, vol. 48, no. 4, pp. 421–427, 1999.

[128] L. Buee, P. R. Hof, D. D. Roberts, A. Delacourte, J. H. Morrison, and H. M. Fillit, "Immunohistochemical identification of thrombospondin in normal human brain and in Alzheimer's disease," *American Journal of Pathology*, vol. 141, no. 4, pp. 783–788, 1992.

[129] S. Sirko, G. Behrendt, P. A. Johansson et al., "Reactive glia in the injured brain acquire stem cell properties in response to sonic hedgehog," *Cell Stem Cell*, vol. 12, no. 4, pp. 426–439, 2013.

[130] P. Thored, A. Arvidsson, E. Cacci et al., "Persistent production of neurons from adult brain stem cells during recovery after stroke," *Stem Cells*, vol. 24, no. 3, pp. 739–747, 2006.

[131] E. J. Benner, D. Luciano, R. Jo et al., "Protective astrogenesis from the SVZ niche after injury is controlled by Notch modulator Thbs4," *Nature*, vol. 497, no. 7449, pp. 369–373, 2013.

[132] J. I. Jun and L. F. Lau, "Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets," *Nature Reviews Drug Discovery*, vol. 10, no. 12, pp. 945–963, 2011.

[133] L. Kular, J. Pakradouni, P. Kitabgi, M. Laurent, and C. Martinerie, "The CCN family: a new class of inflammation modulators?" *Biochimie*, vol. 93, no. 3, pp. 377–388, 2011.

[134] Y. Kondo, T. Nakanishi, M. Takigawa, and N. Ogawa, "Immunohistochemical localization of connective tissue growth factor in the rat central nervous system," *Brain Research*, vol. 834, no. 1-2, pp. 146–151, 1999.

[135] S. Meeuwsen, C. Persoon-Deen, M. Bsibsi, R. Ravid, and J. M. Van Noort, "Cytokine, chemokine and growth factor gene profiling of cultured human astrocytes after exposure to proinflammatory stimuli," *Glia*, vol. 43, no. 3, pp. 243–253, 2003.

[136] J. M. Schwab, R. Beschorner, T. D. Nguyen, R. Meyermann, and H. J. Schluesener, "Differential cellular accumulation of connective tissue growth factor defines a subset of reactive astrocytes, invading fibroblasts, and endothelial cells following central nervous system injury in rats and humans," *Journal of Neurotrauma*, vol. 18, no. 4, pp. 377–388, 2001.

[137] J. M. Schwab, E. Postler, T. D. Nguyen, M. Mittelbronn, R. Meyermann, and H. J. Schluesener, "Connective tissue growth factor is expressed by a subset of reactive astrocytes in human cerebral infarction," *Neuropathology and Applied Neurobiology*, vol. 26, no. 5, pp. 434–440, 2000.

[138] M. Hertel, Y. Tretter, C. Alzheimer, and S. Werner, "Connective tissue growth factor: a novel player in tissue reorganization after brain injury?" *European Journal of Neuroscience*, vol. 12, no. 1, pp. 376–380, 2000.

[139] J. E. Holley, D. Gveric, J. Newcombe, M. L. Cuzner, and N. J. Gutowski, "Astrocyte characterization in the multiple sclerosis glial scar," *Neuropathology and Applied Neurobiology*, vol. 29, no. 5, pp. 434–444, 2003.

[140] W. G. M. Spliet, E. Aronica, M. Ramkema, J. Aten, and D. Troost, "Increased expression of connective tissue growth factor in amyotrophic lateral sclerosis human spinal cord," *Acta Neuropathologica*, vol. 106, no. 5, pp. 449–457, 2003.

[141] U. Ueberham, E. Ueberham, H. Gruschka, and T. Arendt, "Connective tissue growth factor in Alzheimer's disease," *Neuroscience*, vol. 116, no. 1, pp. 1–6, 2003.

[142] J. J. Halliday and E. C. Holland, "Connective tissue growth factor and the parallels between brain injury and brain tumors," *Journal of the National Cancer Institute*, vol. 103, no. 15, pp. 1141–1143, 2011.

[143] A. Buss, K. Pech, B. A. Kakulas et al., "TGF-β1 and TGF-β2 expression after traumatic human spinal cord injury," *Spinal Cord*, vol. 46, no. 5, pp. 364–371, 2008.

[144] D. Lindholm, E. Castren, R. Kiefer, F. Zafra, and H. Thoenen, "Transforming growth factor-β1 in the rat brain: increase after injury and inhibition of astrocyte proliferation," *Journal of Cell Biology*, vol. 117, no. 2, pp. 395–400, 1992.

[145] A. Logan, M. Berry, A. M. Gonzalez, S. A. Frautschy, M. B. Sporn, and A. Baird, "Effects of transforming growth factor β1 on scar production in the injured central nervous system of the rat," *European Journal of Neuroscience*, vol. 6, no. 3, pp. 355–363, 1994.

[146] S. L. Montgomery and W. J. Bowers, "Tumor necrosis factor-alpha and the roles it plays in homeostatic and degenerative processes within the central nervous system," *Journal of Neuroimmune Pharmacology*, vol. 7, no. 1, pp. 42–59, 2012.

[147] M. Santello and A. Volterra, "TNFalpha in synaptic function: switching gears," *Trends in Neurosciences*, vol. 35, no. 10, pp. 638–647, 2012.

[148] N. A. Wahab, B. S. Weston, and R. M. Mason, "Connective tissue growth factor CCN2 interacts with and activates the tyrosine kinase receptor TrkA," *Journal of the American Society of Nephrology*, vol. 16, no. 2, pp. 340–351, 2005.

[149] U. Wilhelmsson, E. A. Bushong, D. L. Price et al., "Redefining the concept of reactive astrocytes as cells that remain within their unique domains upon reaction to injury," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 46, pp. 17513–17518, 2006.

[150] I. B. Wanner, M. A. Anderson, B. Song et al., "Glial scar borders are formed by newly proliferated, elongated astrocytes that interact to corral inflammatory and fibrotic cells via STAT3-dependent mechanisms after spinal cord injury," *Journal of Neuroscience*, vol. 33, no. 31, pp. 12870–12886, 2013.

[151] S. Bardehle, M. Krüger, F. Buggenthin et al., "Live imaging of astrocyte responses to acute injury reveals selective juxtavascular proliferation," *Nature Neuroscience*, vol. 16, no. 5, pp. 580–586, 2013.

[152] S. Wiese, M. Karus, and A. Faissner, "Astrocytes as a source for extracellular matrix molecules and cytokines," *Frontiers in Pharmacology*, vol. 3, article 120, 2012.

[153] S. Robel, B. Berninger, and M. Götz, "The stem cell potential of glia: lessons from reactive gliosis," *Nature Reviews Neuroscience*, vol. 12, no. 2, pp. 88–104, 2011.

[154] L. S. Chaboub and B. Deneen, "Developmental origins of astrocyte heterogeneity: the final frontier of CNS development," *Developmental Neuroscience*, vol. 34, no. 5, pp. 379–388, 2012.

[155] V. Rusnakova, P. Honsa, D. Dzamba, A. Ståhlberg, M. Kubista, and M. Anderova, "Heterogeneity of astrocytes: from development to injury—single cell gene expression," *PLoS ONE*, vol. 8, no. 8, Article ID e69734, 2013.

[156] V. García-Marín, P. García-López, and M. Freire, "Cajal's contributions to glia research," *Trends in Neurosciences*, vol. 30, no. 9, pp. 479–487, 2007.

[157] W. Kamphuis, M. Orre, L. Kooijman, M. Dahmen, and E. M. Hol, "Differential cell proliferation in the cortex of the APPswePS1dE9 Alzheimer's disease mouse model," *Glia*, vol. 60, no. 4, pp. 615–629, 2012.

[158] R. C. Savani, Z. Zhou, E. Arguiri et al., "Bleomycin-induced pulmonary injury in mice deficient in SPARC," *American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 279, no. 4, pp. L743–L750, 2000.

[159] S. Kubota and M. Takigawa, "CCN family proteins and angiogenesis: from embryo to adulthood," *Angiogenesis*, vol. 10, no. 1, pp. 1–11, 2007.

[160] T. G. Bush, N. Puvanachandra, C. H. Horner et al., "Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice," *Neuron*, vol. 23, no. 2, pp. 297–308, 1999.

[161] J. E. Herrmann, T. Imura, B. Song et al., "STAT3 is a critical regulator of astrogliosis and scar formation after spinal cord injury," *Journal of Neuroscience*, vol. 28, no. 28, pp. 7231–7243, 2008.

[162] R. R. Voskuhl, R. S. Peterson, B. Song et al., "Reactive astrocytes form scar-like perivascular barriers to leukocytes during adaptive immune inflammation of the CNS," *Journal of Neuroscience*, vol. 29, no. 37, pp. 11511–11522, 2009.

[163] J. Silver and J. H. Miller, "Regeneration beyond the glial scar," *Nature Reviews Neuroscience*, vol. 5, no. 2, pp. 146–156, 2004.

[164] C. S. Barros, S. J. Franco, and U. Müller, "Extracellular matrix: functions in the nervous system," *Cold Spring Harbor Perspectives in Biology*, vol. 3, no. 1, Article ID a005108, 2011.

[165] J. E. Murphy-Ullrich, "The de-adhesive activity of matricellular proteins: is intermediate cell adhesion an adaptive state?" *Journal of Clinical Investigation*, vol. 107, no. 7, pp. 785–790, 2001.

[166] F. Zipp and O. Aktas, "The brain as a target of inflammation: common pathways link inflammatory and neurodegenerative diseases," *Trends in Neurosciences*, vol. 29, no. 9, pp. 518–527, 2006.

[167] T. Wyss-Coray and L. Mucke, "Inflammation in neurodegenerative disease—a double-edged sword," *Neuron*, vol. 35, no. 3, pp. 419–432, 2002.

[168] T. Wyss-Coray and J. Rogers, "Inflammation in Alzheimer disease-a brief review of the basic science and clinical literature," *Cold Spring Harbor Perspectives in Medicine*, vol. 2, no. 1, Article ID 006346, 2012.

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Research Article

G-Protein Coupled Receptor-Evoked Glutamate Exocytosis from Astrocytes: Role of Prostaglandins

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Astrocytes are highly secretory cells, participating in rapid brain communication by releasing glutamate. Recent evidences have suggested that this process is largely mediated by Ca^{2+}-dependent regulated exocytosis of VGLUT-positive vesicles. Here by taking advantage of VGLUT1-pHluorin and TIRF illumination, we characterized mechanisms of glutamate exocytosis evoked by endogenous transmitters (glutamate and ATP), which are known to stimulate Ca^{2+} elevations in astrocytes. At first we characterized the VGLUT1-pHluorin expressing vesicles and found that VGLUT1-positive vesicles were a specific population of small synaptic-like microvesicles containing glutamate but which do not express VGLUT2. Endogenous mediators evoked a burst of exocytosis through activation of G-protein coupled receptors. Subsequent glutamate exocytosis was reduced by about 80% upon pharmacological blockade of the prostaglandin-forming enzyme, cyclooxygenase. On the other hand, receptor stimulation was accompanied by extracellular release of prostaglandin E_2 (PGE_2). Interestingly, administration of exogenous PGE_2 produced *per se* rapid, store-dependent burst exocytosis of glutamatergic vesicles in astrocytes. Finally, when PGE_2-neutralizing antibody was added to cell medium, transmitter-evoked exocytosis was again significantly reduced (by about 50%). Overall these data indicate that cyclooxygenase products are responsible for a major component of glutamate exocytosis in astrocytes and that large part of such component is sustained by autocrine/paracrine action of PGE_2.

1. Introduction

The morphology and the location of astrocytes place them in a unique position to be able to listen and respond to neuronal activity [1–5]. Astrocytes express a wide variety of functional neurotransmitter receptors essential for sensing neuronal activity [6]. Many of these receptors are G-protein-coupled receptors (GPCRs) that, upon activation, stimulate phospholipase C and form inositol (1,4,5)-triphosphate (IP3) which increases the intracellular calcium (Ca^{2+}) concentration through the release of Ca^{2+} from intracellular stores [6]. The intracellular cascade resulting in Ca^{2+} rise in astrocytes is the main mechanism these cells use to transduce synaptic activity. It is well established that the GPCR- mediated Ca^{2+} variations in astrocytes can trigger release of chemical substances [7, 8] such as excitatory amino acids (D-serine, glutamate) [2, 9, 10], ATP, and related nucleotides and nucleosides [11–13] or proinflammatory mediators such as eicosanoids (prostaglandins or PG) [2, 14] and tumor necrosis factor alpha (TNFα; [5, 15–17]). Interestingly, prostaglandin E_2 (PGE_2) and TNFα have been described to play an important role in the modulation of the regulated secretion of glutamate [5, 15–17]. PGE_2 and TNFα at pathological concentrations appear to exert a potent control on Ca^{2+}-dependent glutamate release from astrocytes [15, 18] and therefore could directly influence glial cells potentially resulting in complex changes in the brain network. Thus, when a local inflammatory reaction is triggered in the brain, the increased levels of such proinflammatory mediators can deeply alter the properties of glial network and thus of neuronal network [7]. However, PGE_2 and TNFα are also present in the normal brain, albeit at much lower levels than during inflammatory reactions. Constitutive levels of TNFα, in particular, have been implicated in control of the stability of neuronal networks in response to prolonged changes in activity via the phenomenon of synaptic scaling [19, 20] and play a role in controlling the strength of

excitatory synaptic transmission by promoting the insertion of AMPA receptors at the surface [21, 22]. The involvement of TNFα in regulating glutamate release from astrocytes during physiological conditions has been found in TNFα- and TNF receptor 1 knockout mice, pointing to a permissive role for the cytokine in the exocytosis of glutamate from astrocytes [17]. Recently, it has been discovered the way how TNFα modulates glutamate release from astrocytes and how this impinges on the astrocytic modulation of synaptic activity [5]. Much less information is available about the mechanism by which PGs can control glutamate release in response to activation of GPCRs [2, 15]. Here by taking advantage of a construct containing the vesicular glutamate transporter 1 and a pH-sensitive fluorescent marker of fusion (VGLUT1-pHluorin) and of total internal reflection fluorescence (TIRF) microscopy, we investigated the role of PGs in the glutamate exocytosis processes in astrocytes. We initially characterized secretory organelles expressing VGLUT1-pHluorin in astrocytes and found that the VGLUT1-pHluorin-expressing vesicles contain glutamate and belong to the family of small synaptic-like microvesicles (SLMVs) and not of other larger secretory organelles (such as dense core granules or lysosomes). Then we found that the exocytosis of such glutamatergic SLMVs, elicited by two endogenous mediators, as diverse as glutamate and ATP, are strongly depressed by pharmacological inhibition of cyclooxygenase (COX). We also provide evidence that PGE_2 exerts most of its activity in amplifying exocytosis of glutamate after it is released in the extracellular medium. We conclude that activation of COX pathway should be regarded as a crucial step in the modulation of the GPCR mediated glutamate exocytosis from astrocytes.

2. Material and Methods

2.1. Pharmacological Agents, Constructs, and Transfection. All agents (acetylsalicylic acid, indomethacin, prostaglandin E2, adenosine 5′ triphosphate disodium salt (ATP), (+)-α-Methyl-4-carboxyphenylglycine (MCPG), adenosine-3′-phospho-5′-phosphosulfate (A3P5PS), 2-methylthioadenosine 5′ diphosphate trisodium salt (2MeSADP), and (S)-3,5-dihydroxyphenylglycine hydrate (DHPG)) were from Sigma (St. Louis, USA), unless otherwise indicated. 2-Methyl-6-(phenylethynyl)-pyridine (MPEP) and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium salt (PPADS) are from Tocris Cookson (Bristol, UK). Anti-PGE_2 antibody ($AbPGE_2$) was from Cayman Chemical (Liestal, Switzerland); cyclopiazonic acid (CPA, CalBiochem, USA); Alexa 568-conjugated transferrin (Life Technologies, USA); plasmid containing the VGLUT1-pHluorin constructs was prepared as previously described [24]. The plasmid (0.5 μg for single transfection experiments) was transfected into primary rat cortical astrocytes cultures with FuGene6 (3 μL, Roche Diagnostics, Switzerland).

2.2. Astrocyte Cultures for Imaging Experiments. Astrocyte cultures containing >99% GFAP-positive cells (≤8% of which were positive for the neural precursor marker LeX) were obtained from newborn rats. They were prepared as described [9], plated (2.5×10^4 cells) on glass coverslip, and transfected 6–8-days later with VGLUT1-pHluorin. From 2 to 5 days after transfection, and coverslips were mounted in the open laminar flow perfusion incubator at 37°C (Harvard Apparatus, USA) on the stage of a Zeiss Axiovert 200 fluorescence inverted microscope modified for TIRF experiments (Visitron System, Germany). The experimental chamber (250 μL volume) was perfused at a rate of 1–1.5 mL/min. The stimulus (DHPG, ATP, tACPD/AMPA, PGE_2, 2MeSADP) was applied rapidly (2 sec) via a software-controlled microperfusion fast-step device (100 μL/min, Warner Instruments Corp., USA). Cells were perfused at 37°C in a HEPES-KRH buffer containing (in mM) NaCl 120, KCl 3.1, $MgCl_2$ 2, $CaCl_2$ 1.8, NaH_2PO_4 1.25, HEPES-Na 25 (buffered to pH 7.4), and glucose 4. In experiments with CPA, PPADS, A3P5PS, MCPG, MPEP, INDO, ASA, and $AbPGE_2$ the drugs were diluted in HEPES-KRH and incubated for 15 min before the application of the stimulus.

2.3. Optical Imaging. TIRF illumination (TIRFi) was used for our experiments. The expanded beam of a 488/568 nm argon/krypton multiline laser (20 milliwatts, Laserphysics, Germany) passed through an AOTF laser wavelength selector (VisiTech International, UK) synchronized with a SNAP-HQ CCD camera (Roper Scientific, Germany) under Metafluor software (Universal Imaging, USA) control and was introduced to the coverslip from the high numerical aperture objective lens (Zeiss α-plan FLUAR 100X). Light entered the coverslip and underwent total internal reflection at the glass-cell interface. In our experimental conditions, penetration depth of TIRFi was calculated to be about 90 nm [17, 25]. In single-wavelength TIRFi experiments (488 nm) the laser beam was filtered via the Zeiss filter set 10 and images were acquired at 20–40 Hz (Zeiss, Switzerland). In dual-wavelength TIRF illumination (488/568 nm), laser beams were combined by a dichroic mirror from the Zeiss filter 24 at 20–40 Hz. The pixel size was 126 nm (at binning 2).

2.4. Image Analysis. Video images, digitized with MetaFluor, were analyzed with MetaMorph software (Universal Imaging, USA). The fusion events of VGLUT-pHluorin positive vesicles were manually selected and counted in areas of 6000 pixels on cell surface as already reported [9, 26, 27]. A fluorescent spot was counted as "fusion event" when the pHluorin fluorescence signal of a single SLMV increased over basal by ≥4-fold.

2.5. Immunocytochemistry. Astrocytes were plated on glass coverslips coated with 2 mg/mL poly-L-lysine and 33 mg/mL laminin and cultured for 2 days. The cells were rinsed with phosphate-buffered saline (PBS) and fixed in ice-cold methanol for 15 min. After two washes in ice-cold PBS, the coverslips were incubated for 10 min with PBS containing 0.5% saponin (PBS-S) and rinsed three times for 5 min with PBS. They were then incubated for 30 min in PBS-S containing 1% bovine serum albumin (BSA) and for 1 hour at room temperature in the presence of the primary

antibodies diluted in PBS-S plus 1% BSA. The cells were rinsed with PBS, incubated 1 hour with the secondary antibody, and mounted for confocal microscopy (Leica SP5 AOBS Confocal Microscope). Primary antibodies were rabbit GFP (1 : 500, Chemicon), mouse VGLUT1 (1 : 500, Chemicon), VGLUT2 (1 : 2000, gift Robert Edwards, USA), VAMP3, (1 : 1000, Synaptic System), glutamate (1 : 3000, gift Vidar Gundersen, Oslo), VAMP2 (1 : 1000, Synaptic System), phogrin (1: 500, gift Romano Regazzi, Lausanne), LAMP1 (1 : 100, Calbiochem), EAA1 (1 : 100, BD Transduction Lab), and Tf receptor (1 : 100, Invitrogen). Secondary antibodies were Cy3 or FITC-conjugated (1 : 200, Molecular Probes).

2.6. Monitoring of Extracellular PGE_2 Formation. Extracellular PGE_2 was measured using a sensitive EIA kit (Prostaglandin E2 EIA kit-Monoclonal, Cayman Chemical Company, Ann Arbor). Cultured astrocytes plated on Petri dishes were washed twice with a KRH buffer containing (in mM) NaCl 120, KCl 3.1, $MgCl_2$ 2, $CaCl_2$ 1.8, NaH_2PO_4 1.25, and HEPES-Na 25 (buffered to pH 7.4). Subsequently they were stimulated (3 min) with agents dissolved in the same buffer also containing an antiPGE_2 antibody (AbPGE_2, Cayman Chemical) at concentration buffering >1000 pg/mL PGE_2. At the end of stimulations, the extracellular medium was rapidly collected, lyophilized and kept at −80°C until performing the EIA assay according to instructions.

2.7. Statistical Analysis. The experiments were analyzed using the SAS statistical package (SAS Inc., Cary, NC, USA). Statistical differences were tested by t-test and P values of 0.01^{**} or 0.05^{*}.

3. Results

Glutamatergic vesicles in astrocytes have been highlighted by transfecting cultured cells with the fluorescent construct VGLUT1-pHluorin, consisting of vesicular glutamate transporter 1 (VGLUT1) fused to a pH sensitive GFP mutant (pHluorin; [28]). Overexpression of VGLUT1-pHluorin in primary cortical astrocytes produced a punctate pattern of fluorescence (Figure 1). Astrocytes, similar to specialized secretory cells, contain three types of secretory organelles, the glutamate containing synaptic-like microvesicles (SLMVs) [9, 29, 30], the peptide containing large dense-core granules (LDCGs; [31, 32]), and the lysosomes [11–13]. These secretory organelles can be distinguished by immunocytochemistry and confocal analysis in primary cultured cells by using antibodies directed against endogenous markers [33]. In order to characterize which population of secretory organelles expressed VGLUT1-pHluorin, we performed a series of immunolabeling and confocal analysis. The VGLUT1-expressing vesicles were well colocalized with anti-VGLUT1 antibody (92 ± 3.5% for $n = 7$ cells, Figure 1(a)) but not with anti-VGLUT2 antibody (5 ± 2.7% for $n = 5$ cells, Figure 1(b)), indicating that VGLUT1-pHluorin is expressed on a particular set of intracellular glutamatergic vesicles. The VGLUT1-expressing vesicles showed a large co-localization with markers of SLMVs [9] such as VAMP3 (or cellubrevin, 94±5.5% for $n = 5$ cells, Figure 1(c)) and glutamate (92 ± 6.2% for $n = 5$ cells, Figure 1(d)). Interestingly, the VGLUT1-associated vesicles showed only a small co-localization with endogenous VAMP2 (17 ± 2% for $n = 5$ cells, Figure 1(e)) and with markers of other secretory organelles such as LDCGs (phogrin, 2.3 ± 1.7% for $n = 5$ cells, Figure 1(f)) or lysosomes, (LAMP1, 3 ± 1.2% for $n = 5$ cells, Figure 1(g)). We also checked the co-localization of VGLUT1-positive vesicles with other lysosomal markers, including early endosomes with EAA1 (13 ± 7.3% for $n = 5$ cells, Figure 1(h)) [34] and the recycling endosomes with transferrin receptor (18 ± 7% for $n = 5$ cells, Figure 1(i), [35]). Early endosomes and recycling endosomes represent two distinct populations of endosomes that significantly colocalized with VGLUT1-pHluorin (co-localization about 20%). In order to clarify whether the organelles double positive for VGLUT1-pHluorin and the marker of early endosomes (EAA1) represent a population different from SLMVs, we estimated the average fluorescence profiles (radial sweep; [23]) of fluorescent vesicles from the double immunofluorescent labeling experiments shown in Figure 1(h). For analysis, we compared the half maximum values (FWHM) of the curve obtained from isolated green fluorescent dots representing VGLUT1-pHluorin which colocalize with EAA1 with the corresponding value of radial sweep curves of fluorescent beads of different diameters (40 nm and 200 nm). We found that the VGLUT1-pHluorin dots colocalized with EAA1 had a FWHM value similar to that of 200 nm beads (490 ± 5 nm, $n = 20$; 506 ± 6 nm, $n = 20$, resp., Figures 2(a) and 2(b)). Conversely, the green fluorescent dots of VGLUT1-pHluorin that do not colocalize with EAA1 had FWHM comparable to that of fluorescent beads of 40 nm of diameter (349 ± 7 nm and 361 ± 6 nm, resp., $n = 20$ for each, Figures 2(c) and 2(d)). Thus, the organelles double positive for pHluorin and EAA1 were clearly a different population of organelles from those expressing only VGLUT1-pHluorin and most probably represent part of VGLUT1-pHluorin-positive organelles undergoing endosomal/lysosomal recycling pathway. In a parallel set of experiments, in order to rigorously determine whether population of VGLUT1-pHluorin positive vesicles also positive for marker of recycling endosomes was able to undergo regulated exocytosis, we monitored the exocytosis processes evoked by an agonist of group I metabotropic glutamate receptors (mGluR), dihydroxyphenylglycine (DHPG) [9, 29]. Primary astrocytes transfected with VGLUT1-pHluorin and preincubated with a specific marker of recycling endosomes (Alexa 568-conjugated transferrin, [35]) have been challenged with DHPG (100 μM) for 2 seconds (s). The VGLUT1-pHluorin- and the Alexa568-positive vesicles have been followed in real time with the dual wavelength TIRF experiments (488 nm and 568 nm laser TIRF). Two seconds of DHPG application evoked a burst of exocytosis of VGLUT1-pHluorin vesicles as previously reported (Figure 2(e); [9]) and only 16% of fusion events of VGLUT1-pHluorin/Alexa-568 double positive vesicles (Figure 2(f)). Overall, these data showed that VGLUT1-pHluorin can be used as a surrogate marker for glutamatergic SLMVs in astrocytes.

To analyze the role of prostaglandins (PGs) in the DHPG-evoked exocytosis of glutamatergic vesicles in astrocytes,

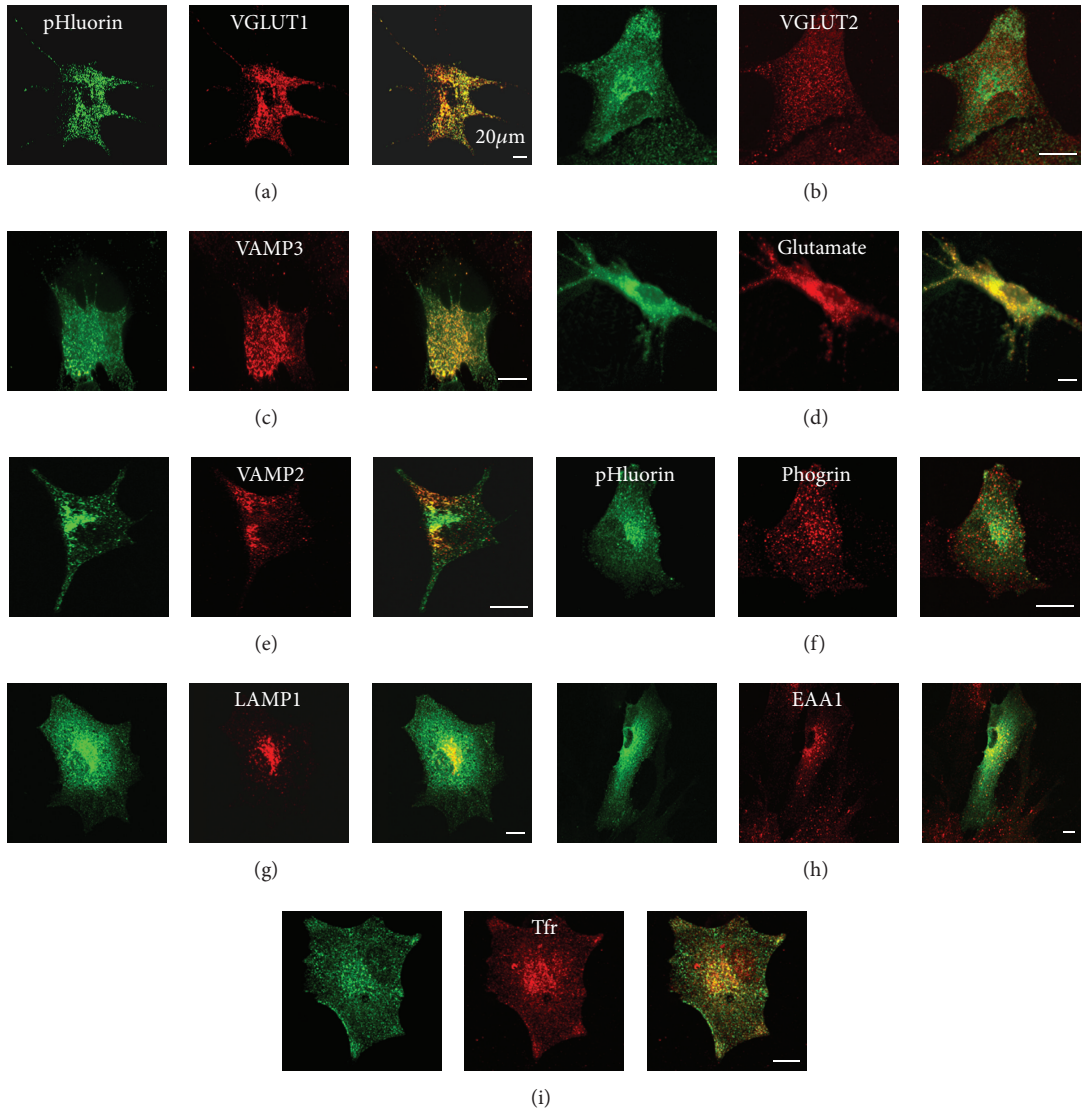

Figure 1: VGLUT1-pHluorin is mainly expressed on a specific population of glutamatergic synaptic like microvesicles. In the figure the left panels (in green) show astrocytes transfected with VGLUT1-pHluorin construct revealed by rabbit antibody against GFP. The middle panels (in red) show the markers of the intracellular secretory organelles, revealed by mouse antibodies against specific markers of ((a)–(e)) synaptic like microvesicles ((a) VGLUT1, (b) VGLUT2, (c) VAMP3, (d) glutamate, (e) VAMP2), of (f) dense core granules (phogrin), of (g) late endosomes, multivesicular bodies and lysosomes (LAMP1), of (h) early endosomes (EAA1), and of (i) recycling endosomes (transferrin receptor, Tfr). The right panels show the merged images. Bars: 20 μm.

we started by monitoring single exocytic events of VGLUT1-pHluorin-associated vesicles (Figures 3(a) and 3(b); [9]), evoked by two distinct protocols of receptor stimulation. When either purinergic receptor or glutamatergic receptor agonists were locally administrated (ATP, 100 μM, or coapplication of t-ACPD and AMPA each at 50 μM), rapid burst of exocytosis was elicited (Figures 3(c) and 3(e)), suggesting that the two stimulation protocols shared similar excitation-secretion coupling mechanism. The nature of the receptors activated by the three protocols of stimulation was then investigated by pharmacological agents with known selectivity. The response to ATP was abolished by pretreatment with PPADS (−86%, 100 μM), an agonist of most P2 purinergic receptors, as well as with A3P5PS (−83%, 100 μM), a selective $P2Y_1$ antagonist (Figure 3(d); [36]). Consistent with these results, 2MeSADP, a $P2Y_1$ agonist, potently stimulated glutamate exocytosis (data not shown), (see Supplementary Figure 1; in Supplementary Material available online at http://dx.doi.org/10.1155/2014/254574 [5, 17]), suggesting that $P2Y_1$ receptors are the predominant mediators of the glutamate exocytosis action of ATP. The presence of $P2Y_1$ receptors in astrocytes and their role in Ca^{2+} signaling pathway leading to modulation of synaptic activity have recently been reported [5, 37]. As for glutamate, it has been established that the potent glutamate releasing effect of t-ACPD+AMPA in astrocytes mainly depends on the

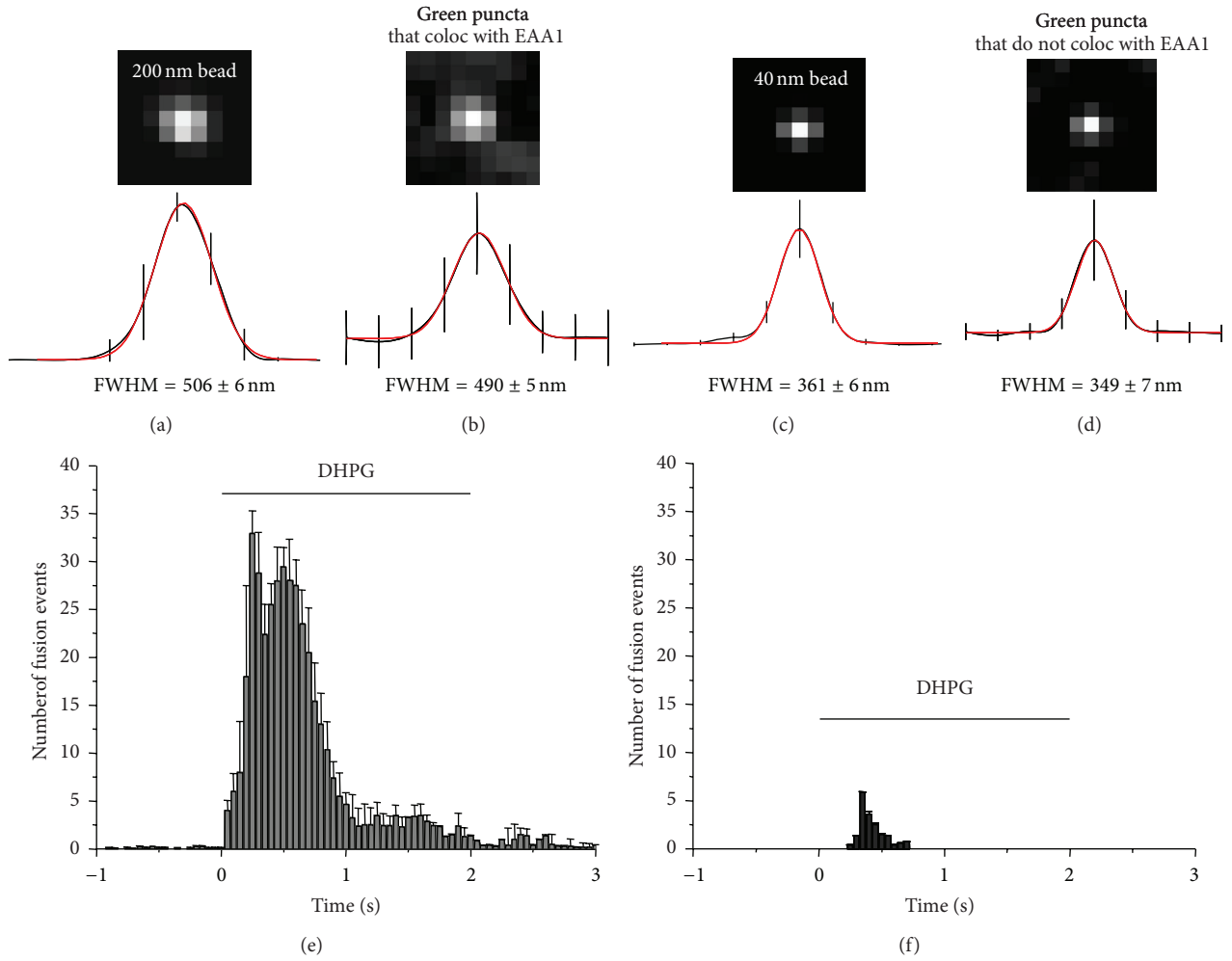

Figure 2: Analysis of VGLUT1-pHluorin vesicles that colocalize with markers of early or recycling endosomes. (a)–(d) Estimation of the size of vesicles expressing VGLUT1-pHluorin. Analysis of individual vesicle was performed in confocal images of VGLUT1-pHluorin-expressing astrocytes by plotting fluorescence intensity of pHluorin spots against distance from the centre of the spot (black curve ± SD). Such an analysis provided an estimation of the average fluorescence profile otherwise called "radial sweep" [23]. The fluorescence intensity values obtained from the radial sweep were well fitted by a one-dimensional Gaussian function (red curve). Such a curve represents the average radial sweep value obtained from 20 vesicles. Note that the half maximum value of pHluorin-expressing vesicle positive for EAA1 ((b), marker of early endosomes, 490 ± 5 nm) is similar to that of 200 nm fluorescent beads ((a), 506 ± 6 nm) and the half maximum value of pHluorin-expressing vesicle that do not express EAA1 ((d), 349 ± 7 nm) is similar to that of 40 nm fluorescent beads ((c), 361 ± 6 nm). (e), (f) Temporal distribution of VGLUT1-pHluorin and Alexa-Tf 568 fusion events evoked by DHPG application. (e) Each individual histogram represents the number (mean ± SD) of fusion events detected from VGLUT1-pHluorin vesicles in a 50 ms-long frame (n = 5 cells). (f) Fusion events (mean ± SD) detected from VGLUT1-pHluorin and Alexa-Tf568 double positive vesicles in the same cells as in (e). Each histogram represents the number of fusion events detected in a 50 ms-long frame (n = 5 cells).

simultaneous activation of group I mGluRs and ionotropic receptors of the AMPA-preferring subtype [2]. Here, we confirm that the response to t-ACPD+AMPA (each at 50 μM) is reduced to less than 40% by administration of MCPG (−78%, 500 μM), a nonselective mGluR antagonist, and by MPEP (−82%, 200 nM), a specific antagonist selective for mGluR5 (Figure 3(f)). On the whole, pharmacological profile identified mGluR5 as the metabotropic receptor subtype that is implicated in the glutamate release response to glutamatergic stimulation. Consistent with these results, DHPG, an agonist of the group I of mGluR, evoked glutamate exocytosis in astrocytes [9, 29]. The two receptors here identified as mediators of the exocytosis of glutamatergic vesicles in astrocytes belong to the G protein-coupled family, which are known to be expressed in astrocytes and to release Ca^{2+} from internal stores via IP3 pathway [38].

Glutamatergic stimulation of astrocytes is known to promote rapid, phospholipase A2-dependent activation of the arachidonic acid cascade [2, 39]. Pharmacological inhibition of the different eicosanoid-forming pathways indicates that cyclooxygenase (COX) but not other arachidonate metabolic enzymes is involved in the mechanism leading to glutamate release [2, 15, 17]. COX is an enzyme that is responsible for the formation of prostanoids [40]. The three main groups

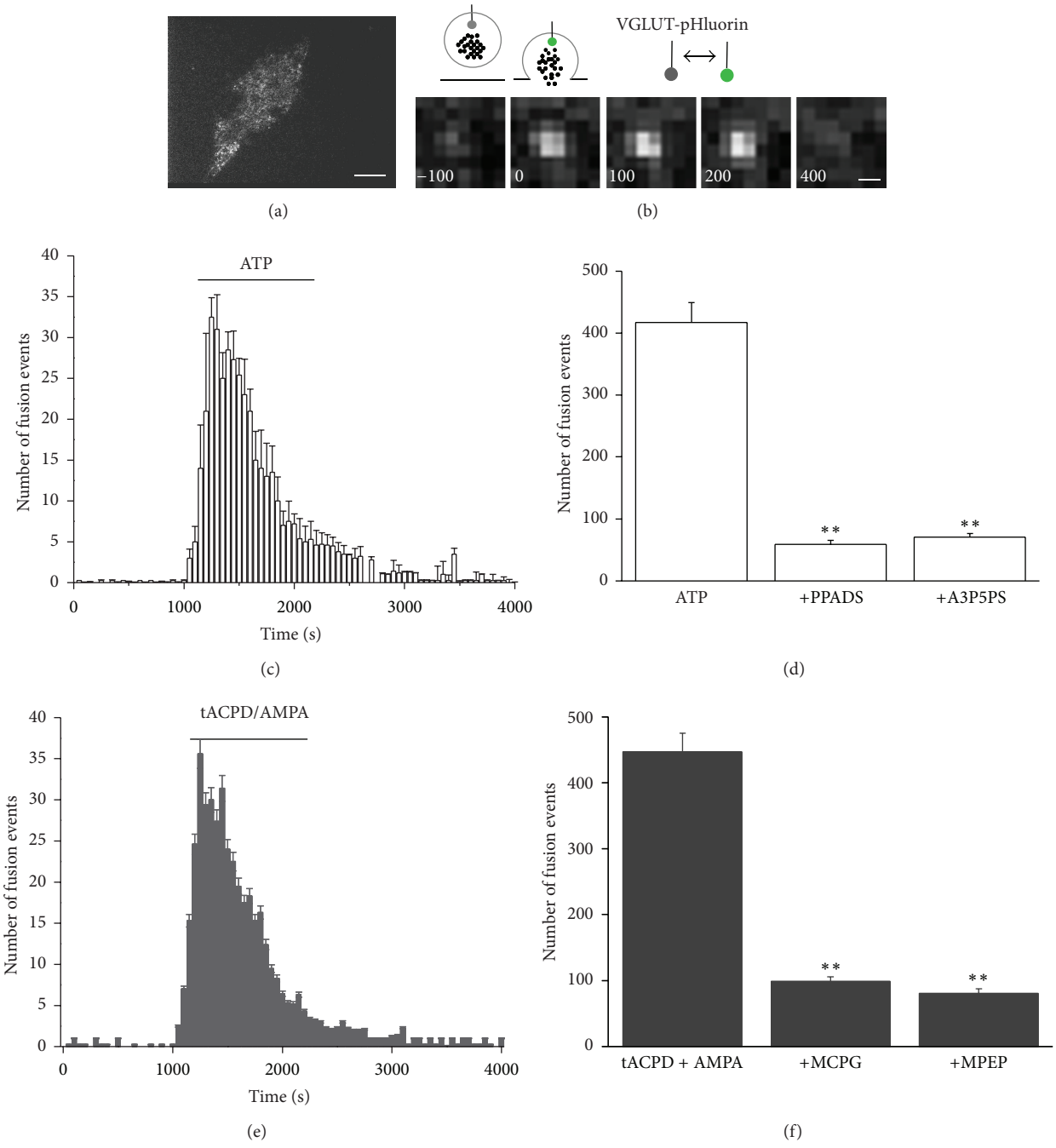

Figure 3: Pharmacological characterization of the receptor subtypes mediating exocytosis of VGLUT1-pHluorin positive vesicles in response to ATP and glutamate agonists. (a) TIRF image showing an astrocyte transfected with VGLUT1-pHluorin. Bar 20 nm. (b) Stereotyped sequence of pHluorin destaining reveals exocytosis of a VGLUT1-pHluorin positive vesicle. The sequential gray scale micrographs represent the fate of pHluorin before (−100 ms) and during (100, 200, 400 ms) the fusion event. Bars: 380 nm. The scheme shows the behaviour of pHluorin before and after fusion event. Note that the color code for the pHluorin fluorescence signal is gray when the signal is off and green when it is on. (c), (d) $P2Y_1$ receptors mediate the ATP-evoked exocytosis. (c) Temporal distribution of fusion events evoked by ATP (100 μM). (d) Histograms represent the total number of fusion events evoked by ATP (417.14 ± 32.4) that is strongly inhibited in the presence of the P2 purine antagonists PPADS (100 μM, 58.6 ± 7) as well as of the $P2Y_1$-selective compound, A3P5PS (100 μM, 70.2 ± 5.8). Data are ± SEM of 4 cells. (e), (f) mGluR5 mediates the response to t-ACPD, in the presence of AMPA. (e) Temporal distribution of fusion events evoked by 50 μM t-ACPD+50 μM AMPA. (f) Histograms represent the total number of fusion events evoked by t-ACPD+AMPA (447.1 ± 28.7) that is strongly inhibited in the presence of the mGluR antagonists, including the subtype-nonselective MCPG (500 μM, 98.3 ± 7.4) and the mGluR5-selective MPEP (200 nM, 80.1 ± 7). Data are ± SEM of 4 cells. Statistical significance of inhibition with receptor antagonists was calculated using t-test ($^{**}P < 0.01$).

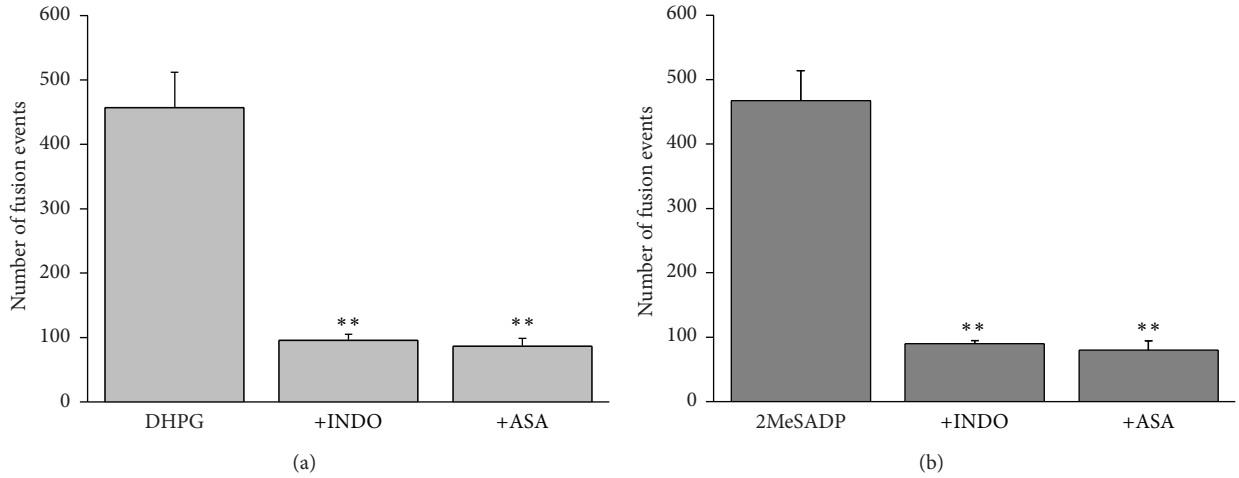

FIGURE 4: COX blockers strongly inhibit the exocytosis of glutamate evoked by activation of group I mGluR and of purinergic $P2Y_1$ receptor. (a), (b) Quantitative histograms represent the total number of fusion events evoked by either DHPG (100 μM, 456.7 ± 54.8) or 2MeSADP (20 μM, 467.6 ± 46.8) in the presence of COX blockers, INDO (1 μM; 94.9 ± 9.6, 88.8 ± 5.4, resp.) or ASA (10 μM, 86.4 ± 11.7, 79.5 ± 14.4, resp.). Data are ± SEM of 4 cells. Statistical significance of inhibition with receptor antagonists was calculated using t-test ($^{**}P < 0.01$).

of prostanoids (prostaglandins, prostacyclins, and thromboxanes) are each involved in the inflammatory response but prostaglandins (PGs) can control release of glutamate from astrocytes. We studied the involvement of PGs in the DHPG-evoked exocytosis of glutamatergic SLMVs by preincubating cells with two COX inhibitors, indomethacin (INDO, 1 μM) and aspirin (ASA, 10 μM). We found that the two anti-inflammatory drugs decreased by about 80% the releasing effect of DHPG and of 2MeSADP (Figures 4(a) and 4(b)). This evidence is consistent with a scenario in which the excitation-secretion pathway leading to glutamate exocytosis from astrocytes is controlled, at some level, by the COX pathway.

In light of the above results, we wanted to examine the mechanism by which COX inhibitors decreased glutamate exocytosis in astrocytes. It is well known that a COX product, prostaglandin E_2 (PGE_2), is formed and released in extracellular space after activation of mGluRs [2] and that it causes by itself intracellular Ca^{2+} elevations leading to glutamate release in astrocytes [2, 14]. Interestingly, we also found that the time course analysis of PGE_2 accumulation in response to activation of glutamatergic or purinergic receptors was almost maximal within the first 3 seconds of stimulation (Figure 5(a); [2, 17]). Therefore, the kinetics of the PGE_2 release is fully compatible with a potential role of this COX metabolite in rapid cell signaling. We wanted to investigate the effect of PGE_2 on the intracellular pathways leading to exocytic burst of glutamatergic SLMVs. We found that administration of PGE_2 (50 μM) to astrocytes caused a rapid burst of exocytosis that did not depend on Ca^{2+} influx from outside of the cells but on Ca^{2+} release from internal store (Figure 5(b)). In fact incubation with cyclopiazonic acid (1 μM), which causes depletion of Ca^{2+} from internal stores by blocking SERCA, abolished the response to PGE_2 (Figure 5(b) inset). Since PGE_2 is released upon receptor activation and promotes *per se* Ca^{2+} elevations and glutamate release, we specifically investigated whether extracellular PGE_2 contributes to the physiological response of astrocytes to glutamate exocytosis. We blocked extracellular PGE_2 with a specific antibody ($AbPGE_2$). Figure 5(d) shows that when $AbPGE_2$ was present in the bath, a condition in which PGE_2 is rapidly and efficiently sequestered ($AbPGE_2$ buffering capacity is >1000 pg/mL PGE_2), DHPG- evoked exocytosis of glutamatergic SLMVs was significantly reduced (−43 ± 12%, $n = 6$ cells). Similar results were obtained for 2MeSADP (Supplementary Figure 1(b); −51 ± 14%, $n = 4$ cells, resp.). Interestingly, the action of $AbPGE_2$ was specific and could not be reproduced by the boiled protein (not shown). Therefore, the whole body of evidence suggests that extracellular PGE_2 accounts for a significant component of mGluR5- and $P2Y_1$-dependent exocytosis of glutamatergic SLMVs in astrocytes.

4. Discussion

Astrocytes play an important role in the integration of rapid chemical signaling in the brain [41]. They function as signal integrators, since they generate outputs with variable timing in response to particular signals received from surrounding neuronal cells to communicate with the same neurons and/or with other cellular components of the brain circuits. A crucial element that facilitates the integrating functions of astrocytes is the regulated exocytosis of chemical substances [9, 29, 42–44]. By this process, astrocytes exert modulatory influences on neighboring cells and are thought to participate in the control of synaptic circuits and cerebral blood flow [45, 46]. Exocytosis is an evolutionary trait of eukaryotic cells that leads in a given secretory cell to a release of chemical content by a fast mechanism into the extracellular space and thus to communication with neighboring cells. In neurons, exocytosis represents one of the fastest biological events known. Similar to neurons or neurosecretory cells, astrocytes express at least three different secretory organelles:

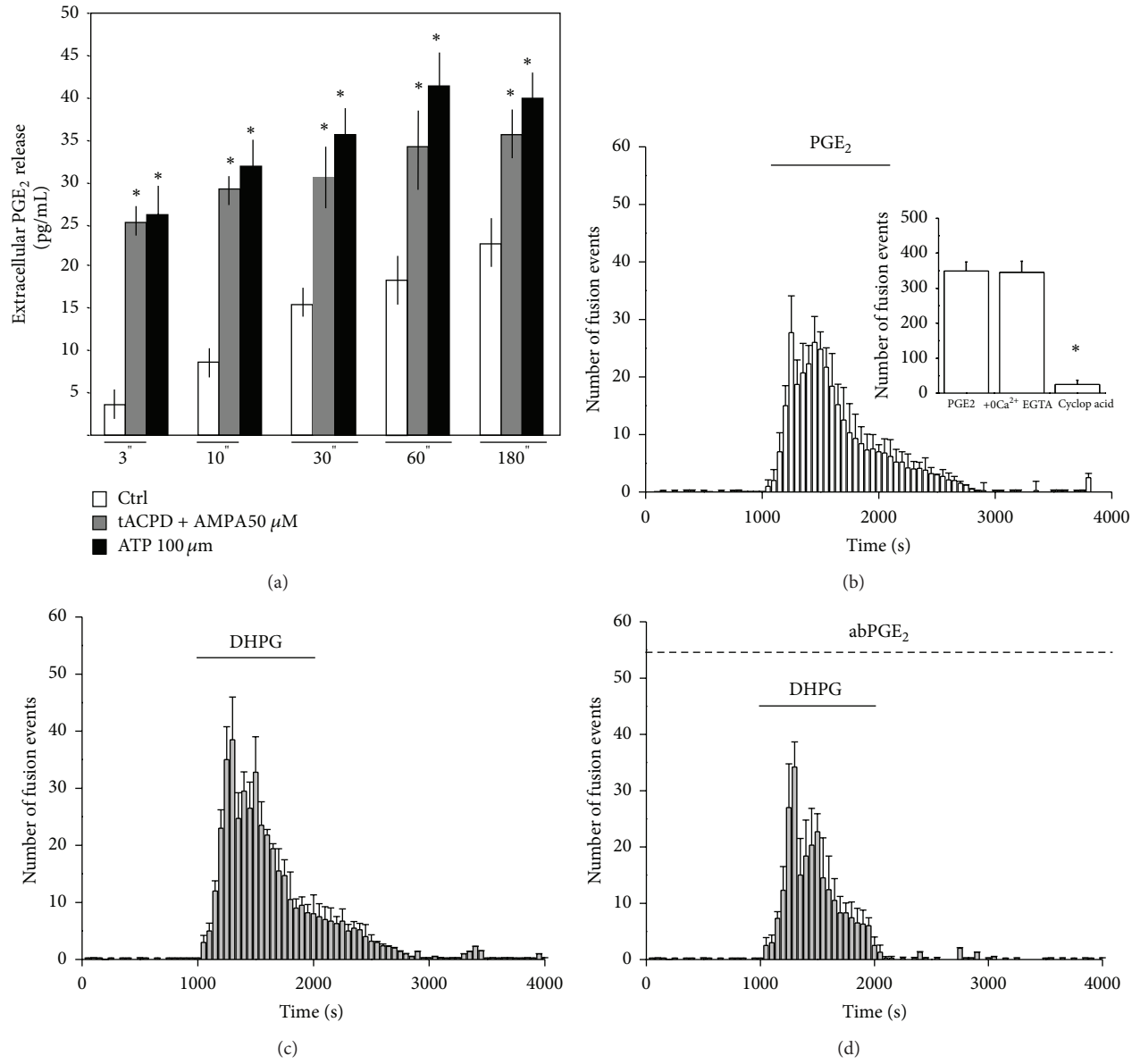

Figure 5: Extracellular PGE_2: accumulation in response to various stimuli and effects on exocytosis of glutamatergic vesicles. (a). Extracellular accumulation of PGE_2 (expressed as pg/mL) in response to 3 min stimulation with either t-ACPD+ AMPA (each at 50 μM) or ATP (100 μM). Each point represents the average ± SEM of two experiments in triplicate with each stimulus. (b) Temporal distribution of fusion events evoked by PGE_2 (50 μM). Inset histograms represent the total number of fusion events evoked by PGE_2 (349 ± 26) in the presence of 0 mM Ca^{2+} and 5 mM of EGTA (345 ± 32) or cyclopiazonic acid (CPA, 10 μM, 25 ± 12). (c) Temporal distribution of fusion events evoked by DHPG (100 μM). (d) Inhibitory effect of $AbPGE_2$ (buffering capacity >1000 pg/mL PGE_2) on exocytosis of glutamatergic vesicles evoked by DHPG (100 μM). Histograms represent temporal distribution of fusion events evoked by DHPG in the presence of $AbPGE_2$. Statistical significance was calculated using t-test ($^*P < 0.05$).

the small synaptic like microvesicles (SLMVs) [29, 30, 47], the large dense core granules (LDCGs) [31, 32, 48] which store and release distinct cargo, and lysosomes [49, 50]. In neurons and specialized secretory cells, these organelles have specialized physiological functions, are typically found in different regions of the cell and are regulated by different intracellular signaling pathways of calcium. Interestingly, in our immunolabeling and confocal analysis performed in cultured astrocytes we found that small VAMP3-, VGLUT1-, and glutamate-positive SLMVs, large phogrin-positive DCGs, and lysosomes positive for markers of multivesicular bodies and late endosomes or early and recycling endosomes exist in the same astrocytes. Our results confirmed that the task of identifying a specific population of secretory organelles in astrocytes is very difficult. Moreover, studying properties of exocytosis constitutes a challenge because the cell biological basis of this process is very incompletely defined in these cells. For example, researchers have only recently started to

understand that astrocytic exocytosis relies on multiple populations of secretory vesicles, which calls for the definition of adequate criteria to recognize the distinct populations and study them in isolation. In fact, most of the early studies of astrocyte exocytosis could not distinguish such heterogeneity as they used generic exocytosis reporters (such as synthetic fluorescent dyes). Thus, these studies did not allow an accurate description of the dynamics and properties of astrocytic secretion because they mixed contributions by more than one exocytic organelle population. As a result, the types of vesicles used in Ca^{2+}-regulated exocytosis in astrocytes are under debate. A good experimental strategy involves trying to specifically live-stain a selected population (e.g., by transfecting the cells with a population-specific fluorescent reporter). This approach must, however, be validated by subsequent co-localization analysis with markers of the intracellular organelles, which will provide additional useful information on the nature of the stained organelles. By combining TIRF experiments and transfection of a fluorescent tool used to visualize exo/endocytosis processes in living neurons (VGLUT1-pHluorin) [28], we visualized VGLUT1-positive SLMVs. We therefore characterized vesicles expressing VGLUT1 and found that they have diameter similar to that of fluorescent beads of 40 nm, contain glutamate, and express at least one of the VAMP proteins necessary for regulated exocytosis (VAMP3).

Once confirmed that VGLUT1-pHluorin was a correct tool for studying glutamatergic SLMVs in astrocytes, we then investigated the activity of the two endogenous mediators that participate in brain intercellular chemical communication: glutamate and ATP. Glutamate and ATP are well-recognized brain signaling agents that are released in response to neuronal or glial cell stimulation and that mediate intercellular signaling [7, 51]. Independently of their origin, both glutamate and ATP were previously shown to activate astrocytes causing intracellular Ca^{2+} increase followed by glutamate release [2, 52, 53]. The Ca^{2+}-dependent exocytosis of glutamate in astrocytes is controlled by multiples mediators, all acting via receptors that belong to the GPCR super family stimulating IP3 productions ($P2Y_1$ for ATP, mGluR5 for glutamate). Stimulation of GPCR also promoted stimulation of the COX pathway of the arachidonic acid metabolism with the ensuing production and release of PGs being critical for the full efficiency of the excitation-secretion coupling in astrocytes. We in fact found that COX inhibition caused a dramatic reduction in the total number of fusion events of glutamatergic SLMVs caused by activation of both purinergic and glutamatergic receptors. By analyzing the temporal distribution of the exocytic events, we found an intriguing aspect of the mode of action of PGs. Inhibition of the PGs by either COX inhibitors or a neutralizing antibody specific for PGE_2 produced a significant modification of the temporal characteristics of the exocytic burst. In particular, while the fast initial component (0–400 ms) was most often left intact, the second component of the burst (500 ms–1.6 s) was often suppressed or significantly slowed down, suggesting that PG-mediated signaling is heavily implicated in this later, slower phase of the release. Interestingly, the rapid phase of the exocytic burst was sustained almost exclusively by "resident" vesicles, whereas the slow phase was mainly sustained by "newcomer" vesicles [9]. This duality is reminiscent of observations previously done in neurons where only readily releasable synaptic vesicles are rapidly recycled and reused [54]. As we do not know whether the "newcomer" and the "resident" vesicles represent distinct population of SLMVs, it is possible that the "newcomers" may indeed represent the same population of "resident" vesicles that undergoes a second round of exocytosis upon rapid recycling. In this context PGE_2 seemed to amplify the second round of exocytosis. Since it is known that PGE_2 is immediately released from astrocytes upon stimulation [2, 17] and that exogenous PGE_2 induced rapid, Ca^{2+}-dependent burst of glutamatergic SLMVs, it follows that a component of the burst of exocytosis in response to glutamate or ATP requires an autocrine/paracrine action of PGE_2. The comparison effects of the COX blockers (about −80%) and $AbPGE_2$ (about −45%) also indicate that extracellular PGE_2 cannot be solely responsible for all the observed COX-dependent events. At present we do not have a specific explanation for these differences. It is possible that other PGs could be released together with PGE_2. For instance, it has been shown that intracellular Ca^{2+} elevations in astrocytes are known to stimulate the production of a full range of COX derivates [55] and some of them have been shown to cause Ca^{2+} rises and glutamate release from astrocytes [2, 56]. The present data confirm and extend our previous finding that PGE_2 participates to glutamate-dependent cell-cell communication [2], suggesting a physiological function for this mediator in the intact brain. The autocrine/paracrine action of PGE_2 may thus participate in the spatial control of astrocytic signal propagation. Therefore, it is possible that alterations in PGs synthesis during brain inflammations and other pathological states have a profound impact on the chemical communication in the brain. PGE_2 stimulation, for instance, acts downstream of CXCL12, as well as TNFα pathways. Although both these pathways have been suggested to be involved in the physiological glutamate-mediated brain signalling [5, 26], it is likely that PGE_2 synthesis will be largely amplified during inflammatory conditions, following the fate of its upstream inflammatory messengers [18]. As the whole pathway increases glutamate release from astroglial cells, much higher levels of PGE_2 might represent the ultimate messenger leading to pathological increase of extracellular glutamate levels.

Conflict of Interests

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

Authors' Contribution

Corrado Cali and Jan Lopatar contributed equally to this work.

Acknowledgments

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References

[1] L. Pasti, A. Volterra, T. Pozzan, and G. Carmignoto, "Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes *in situ*," *The Journal of Neuroscience*, vol. 17, no. 20, pp. 7817–7830, 1997.

[2] P. Bezzi, G. Carmignoto, L. Pasti et al., "Prostaglandins stimulate calcium-dependent glutamate release in astrocytes," *Nature*, vol. 391, no. 6664, pp. 281–285, 1998.

[3] A. Araque, E. D. Martín, G. Perea, J. I. Arellano, and W. Buño, "Synaptically released acetylcholine evokes Ca^{2+} elevations in astrocytes in hippocampal slices," *The Journal of Neuroscience*, vol. 22, no. 7, pp. 2443–2450, 2002.

[4] G. Perea and A. Araque, "Properties of synaptically evoked astrocyte calcium signal reveal synaptic information processing by astrocytes," *The Journal of Neuroscience*, vol. 25, no. 9, pp. 2192–2203, 2005.

[5] M. Santello, P. Bezzi, and A. Volterra, "TNFα controls glutamatergic gliotransmission in the hippocampal dentate gyrus," *Neuron*, vol. 69, no. 5, pp. 988–1001, 2011.

[6] A. Verkhratsky, R. K. Orkand, and H. Kettenmann, "Glial calcium: homeostasis and signaling function," *Physiological Reviews*, vol. 78, no. 1, pp. 99–141, 1998.

[7] P. Bezzi and A. Volterra, "A neuron-glia signalling network in the active brain," *Current Opinion in Neurobiology*, vol. 11, no. 3, pp. 387–394, 2001.

[8] A. Volterra and J. Meldolesi, "Astrocytes, from brain glue to communication elements: the revolution continues," *Nature Reviews Neuroscience*, vol. 6, no. 8, pp. 626–640, 2005.

[9] J. Marchaland, C. Calì, S. M. Voglmaier et al., "Fast subplasma membrane Ca^{2+} transients control exo-endocytosis of synaptic-like microvesicles in astrocytes," *The Journal of Neuroscience*, vol. 28, no. 37, pp. 9122–9132, 2008.

[10] M. T. Martineau Shi, J. Puyal, A. M. Knolhoff et al., "Storage and uptake of D-serine into astrocytic synaptic-like vesicles specify gliotransmission," *The Journal of Neuroscience*, vol. 33, no. 8, article U3605, pp. 3413–3423, 2013.

[11] T. Liu, L. Sun, Y. F. Xiong et al., "Calcium triggers exocytosis from two types of organelles in a single astrocyte," *The Journal of Neuroscience*, vol. 31, no. 29, pp. 10593–10601, 2011.

[12] C. Verderio and M. Matteoli, "ATP in neuron-glia bidirectional signalling," *Brain Research Reviews*, vol. 66, no. 1-2, pp. 106–114, 2011.

[13] M. Oya, T. Kitaguchi, Y. Yanagihara et al., "Vesicular nucleotide transporter is involved in ATP storage of secretory lysosomes in astrocytes," *Biochemical and Biophysical Research Communications*, vol. 438, no. 1, pp. 145–151, 2013.

[14] Sanzgiri, R. P. A. Araque, and P. G. Haydon, "Prostaglandin E_2 stimulates glutamate receptor-dependent astrocyte neuromodulation in cultured hippocampal cells," *Journal of Neurobiology*, vol. 41, no. 2, pp. 221–229, 1999.

[15] P. Bezzi, M. Domercq, L. Brambilla et al., "CXCR4-activated astrocyte glutamate release via TNFa: amplification by microglia triggers neurotoxicity," *Nature Neuroscience*, vol. 4, no. 7, pp. 702–710, 2001.

[16] D. Rossi, L. Brambilla, C. F. Valori et al., "Defective tumor necrosis factor-α-dependent control of astrocyte glutamate release in a transgenic mouse model of Alzheimer disease," *The Journal of Biological Chemistry*, vol. 280, no. 51, pp. 42088–42096, 2005.

[17] M. Domercq, L. Brambilla, E. Pilati, J. Marchaland, A. Volterra, and P. Bezzi, "P2Y1 receptor-evoked glutamate exocytosis from astrocytes—control by tumor necrosis factor-α and prostaglandins," *The Journal of Biological Chemistry*, vol. 281, no. 41, pp. 30684–30696, 2006.

[18] D. Rossi, F. Martorana, and L. Brambilla, "Implications of gliotransmission for the pharmacotherapy of CNS disorders," *CNS Drugs*, vol. 25, no. 8, pp. 641–658, 2011.

[19] D. Stellwagen and R. C. Malenka, "Synaptic scaling mediated by glial TNF-α," *Nature*, vol. 440, no. 7087, pp. 1054–1059, 2006.

[20] G. G. Turrigiano, "The self-tuning neuron: synaptic scaling of excitatory synapses," *Cell*, vol. 135, no. 3, pp. 422–435, 2008.

[21] E. C. Beattie, D. Stellwagen, W. Morishita et al., "Control of synaptic strength by glial TNFα," *Science*, vol. 295, no. 5563, pp. 2282–2285, 2002.

[22] D. Stellwagen, E. C. Beattie, J. Y. Seo, and R. C. Malenka, "Differential regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor-α," *The Journal of Neuroscience*, vol. 25, no. 12, pp. 3219–3228, 2005.

[23] D. Zenisek, J. A. Steyer, M. E. Feldman, and W. Almers, "A membrane marker leaves synaptic vesicles in milliseconds after exocytosis in retinal bipolar cells," *Neuron*, vol. 35, no. 6, pp. 1085–1097, 2002.

[24] S. M. Voglmaier, K. Kam, H. Yang et al., "Distinct endocytic pathways control the rate and extent of synaptic vesicle protein recycling," *Neuron*, vol. 51, no. 1, pp. 71–84, 2006.

[25] S. Chevlet, P. Bezzi, R. Ivarsson et al., "Tomosyn-1 is involved in a post-docking event required for pancreatic β-cell exocytosis," *Journal of Cell Science*, vol. 119, no. 14, pp. 2912–2920, 2006.

[26] C. Calì, J. Marchaland, R. Regazzi, and P. Bezzi, "SDF 1-alpha (CXCL12) triggers glutamate exocytosis from astrocytes on a millisecond time scale: imaging analysis at the single-vesicle level with TIRF microscopy," *Journal of Neuroimmunology*, vol. 198, no. 1-2, pp. 82–91, 2008.

[27] A. Bergeron, P. Bezzi, and R. Regazzi, "Analysis of synaptic-like microvesicle exocytosis of beta-cells using a life imaging technique," *PloS ONE*. In press.

[28] S. M. Voglmaier and R. H. Edwards, "Do different endocytic pathways make different synaptic vesicles?" *Current Opinion in Neurobiology*, vol. 17, no. 3, pp. 374–380, 2007.

[29] P. Bezzi, V. Gundersen, J. L. Galbete et al., "Astrocytes contain a vesicular compartment that is competent for regulated exocytosis of glutamate," *Nature Neuroscience*, vol. 7, no. 6, pp. 613–620, 2004.

[30] L. H. Bergersen and V. Gundersen, "Morphological evidence for vesicular glutamate release from astrocytes," *Neuroscience*, vol. 158, no. 1, pp. 260–265, 2009.

[31] S. Coco, F. Calegari, E. Pravettoni et al., "Storage and release of ATP from astrocytes in culture," *The Journal of Biological Chemistry*, vol. 278, no. 2, pp. 1354–1362, 2003.

[32] I. Prada, J. Marchaland, P. Podini et al., "REST/NRSF governs the expression of dense-core vesicle gliosecretion in astrocytes," *The Journal of Cell Biology*, vol. 193, no. 3, pp. 537–549, 2011.

[33] P. Bezzi and A. Volterra, "Monitoring exocytosis in astrocytes with total internal reflection fluorescence microscopy," in *Optical Imaging Neurons: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2011.

[34] B. Borgonovo, E. Cocucci, G. Racchetti, P. Podini, A. Bachi, and J. Meldolesi, "Regulated exocytosis: a novel, widely expressed system," *Nature Cell Biology*, vol. 4, no. 12, pp. 955–962, 2002.

[35] M. Park, J. M. Salgado, L. Ostroff et al., "Plasticity-induced growth of dendritic spines by exocytic trafficking from recycling endosomes," *Neuron*, vol. 52, no. 5, pp. 817–830, 2006.

[36] V. Ralevic and G. Burnstock, "Receptors for purines and pyrimidines," *Pharmacological Reviews*, vol. 50, no. 3, pp. 413–492, 1998.

[37] S. R. Fam, C. J. Gallagher, and M. W. Salter, "$P2Y_1$ purinoceptor-mediated Ca^{2+} signaling and Ca^{2+} wave propagation in dorsal spinal cord astrocytes," *The Journal of Neuroscience*, vol. 20, no. 8, pp. 2800–2808, 2000.

[38] A. Verkhratsky, "Calcium signalling between neurones and glia," *European Journal of Neuroscience*, vol. 10, pp. 322–322, 1998.

[39] N. Stella, M. Tencé, J. Glowinski, and J. Prémont, "Glutamate-evoked release of arachidonic acid from mouse brain astrocytes," *The Journal of Neuroscience*, vol. 14, no. 2, pp. 568–575, 1994.

[40] W. L. Smith and L. J. Marnett, "Prostaglandin endoperoxide synthase: structure and catalysis," *Biochimica et Biophysica Acta*, vol. 1083, no. 1, pp. 1–17, 1991.

[41] V. Parpura, B. J. Baker, M. Jeras, and R. Zorec, "Regulated exocytosis in astrocytic signal integration," *Neurochemistry International*, vol. 57, no. 4, pp. 451–459, 2010.

[42] Q. Zhang, M. Fukuda, E. Van Bockstaele, O. Pascual, and P. G. Haydon, "Synaptotagmin IV regulates glial glutamate release," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 25, pp. 9441–9446, 2004.

[43] D. Crippa, U. Schenk, M. Francolini et al., "Synaptobrevin2-expressing vesicles in rat astrocytes: insights into molecular characterization, dynamics and exocytosis," *The Journal of Physiology*, vol. 570, no. 3, pp. 567–582, 2006.

[44] T. Pangršič, M. Potokar, M. Stenovec et al., "Exocytotic release of ATP from cultured astrocytes," *The Journal of Biological Chemistry*, vol. 282, no. 39, pp. 28749–28758, 2007.

[45] C. M. Anderson and M. Nedergaard, "Astrocyte-mediated control of cerebral microcirculation," *Trends in Neurosciences*, vol. 26, no. 7, pp. 340–344, 2003.

[46] P. G. Haydon and G. Carmignoto, "Astrocyte control of synaptic transmission and neurovascular coupling," *Physiological Reviews*, vol. 86, no. 3, pp. 1009–1031, 2006.

[47] P. Jourdain, L. H. Bergersen, K. Bhaukaurally et al., "Glutamate exocytosis from astrocytes controls synaptic strength," *Nature Neuroscience*, vol. 10, no. 3, pp. 331–339, 2007.

[48] P. Ramamoorthy and M. D. Whim, "Trafficking and fusion of neuropeptide Y-containing dense-core granules in astrocytes," *The Journal of Neuroscience*, vol. 28, no. 51, pp. 13815–13827, 2008.

[49] J. K. Jaiswal, M. Fix, T. Takano, M. Nedergaard, and S. M. Simon, "Resolving vesicle fusion from lysis to monitor calcium-triggered lysosomal exocytosis in astrocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 35, pp. 14151–14156, 2007.

[50] Z. J. Zhang, G. Chen, W. Zhou et al., "Regulated ATP release from astrocytes through lysosome exocytosis," *Nature Cell Biology*, vol. 9, no. 8, article U115, pp. 945–953, 2007.

[51] R. D. Fields and B. Stevens, "ATP: an extracellular signaling molecule between neurons and glia," *Trends in Neurosciences*, vol. 23, no. 12, pp. 625–633, 2000.

[52] M. L. Cotrina, J. H.-C. Lin, A. Alves-Rodrigues et al., "Connexins regulate calcium signaling by controlling ATP release," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 26, pp. 15735–15740, 1998.

[53] J. Xu, H. Peng, N. Kang et al., "Glutamate-induced exocytosis of glutamate from astrocytes," *The Journal of Biological Chemistry*, vol. 282, no. 33, pp. 24185–24197, 2007.

[54] N. C. Harata, A. M. Aravanis, and R. W. Tsien, "Kiss-and-run and full-collapse fusion as modes of exo-endocytosis in neurosecretion," *Journal of Neurochemistry*, vol. 97, no. 6, pp. 1546–1570, 2006.

[55] K. Oomagari, B. Buisson, A. Dumuis, J. Bockaert, and J.-P. Pin, "Effect of glutamate and ionomycin on the release of arachidonic acid, prostaglandins and HETEs from cultured neurons and astrocytes," *European Journal of Neuroscience*, vol. 3, no. 10, pp. 928–939, 1991.

[56] J. Kitanaka, K. Takuma, K. Kondo, and A. Baba, "Prostanoid receptor-mediated calcium signaling in cultured rat astrocytes," *Japanese Journal of Pharmacology*, vol. 71, no. 1, pp. 85–87, 1996.

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Review Article

Astrocyte-Synapse Structural Plasticity

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The function and efficacy of synaptic transmission are determined not only by the composition and activity of pre- and postsynaptic components but also by the environment in which a synapse is embedded. Glial cells constitute an important part of this environment and participate in several aspects of synaptic functions. Among the glial cell family, the roles played by astrocytes at the synaptic level are particularly important, ranging from the trophic support to the fine-tuning of transmission. Astrocytic structures are frequently observed in close association with glutamatergic synapses, providing a morphological entity for bidirectional interactions with synapses. Experimental evidence indicates that astrocytes sense neuronal activity by elevating their intracellular calcium in response to neurotransmitters and may communicate with neurons. The precise role of astrocytes in regulating synaptic properties, function, and plasticity remains however a subject of intense debate and many aspects of their interactions with neurons remain to be investigated. A particularly intriguing aspect is their ability to rapidly restructure their processes and modify their coverage of the synaptic elements. The present review summarizes some of these findings with a particular focus on the mechanisms driving this form of structural plasticity and its possible impact on synaptic structure and function.

1. Introduction

Since the earliest studies on glial cells in the 19th century, Ramón y Cajal, Camillo Golgi, and their contemporary colleagues have described astrocytes as very particular cells in intimate contact with neurons and capillaries. Based on these observations, they made different hypotheses on their physiological function, ranging from passive space filling in the neuropil to active energy supply for neurons [1]. Almost 150 years later, the neurophysiological role of astrocytes is still a subject of intense debate, although increasing data suggest that they are active players in mechanisms of synaptic transmission and plasticity [2]. Numerous data demonstrate that thin astrocytic processes infiltrate brain tissue [3]. The most commonly used name for these thin processes is "peripheral astrocytic processes," as it is often difficult to distinguish, with light microscopy, their exact position with regard to different neuropil elements. However, in this review we will mostly focus on the data, obtained with various techniques, concerning fine astrocytic processes that are in close association with synaptic contacts, and thus the term "perisynaptic astrocytic process" (PAP [4]) will be applied throughout the review.

Close structural relationship between synaptic structures and PAPs makes astrocytes an important partner of neurons in the organization and functioning of synaptic connections. Astrocytes take up glutamate from the synaptic cleft [5], control the amount of glutamate spillover that activates extrasynaptic receptors or enables intersynaptic crosstalk [6], control the ion and water homeostasis through selective transmembrane movements of inorganic and organic molecules and the equilibration of osmotic gradients [7], and provide energy substrates to neurons in the form of lactate [8]. In addition, recent studies have revealed astrocytic processes as structural entities able to modulate neuronal function at the synaptic level through the calcium-dependent release of gliotransmitters [9, 10]. Indeed, astrocytes are able to sense neuronal activity by elevating their intracellular calcium concentration through Gq-coupled protein mechanisms [11]. Even if this calcium excitability phenomenon is well accepted, its exact role is still controversial [12]. Finally, PAPs have been described by several studies as plastic structures able to change their morphology within minutes, thus modifying their coverage of pre- and postsynaptic elements [13]. We review here these observations and discuss the physiological

role that structural changes in PAPs could have on both synaptic and astrocytic function.

2. Astrocytic Processes Embrace Synapses

A main structural characteristic of astrocytes is the star-shaped arborization of their stem processes. However, at the ultrastructural level, protoplasmic astrocytes have much more complex shapes consisting of thousands of very fine elaborated protrusions that make them look more like sponges than stars [14–16]. Detailed morphological descriptions and quantitative analyses provided by electron microscopy (EM), especially those based on three-dimensional (3D) reconstructions from serial ultrathin sections, have allowed the identification of astroglia and their interactions with neurons and synapses. Big stem processes of astrocytes containing typical bundles of intermediate filaments (8-9 nm in diameter) that have glial fibrillary acidic protein (GFAP) as their main constituent represent around 15% of the total volume of an astrocyte [17]. These processes ramify progressively to finally generate a dense matrix of thin elaborate terminal processes, which infiltrate brain tissue and closely associate with neuropil elements and particularly with synapses. These fine astrocytic processes account for 70–80% of the astrocytic plasma membrane [18] and often surround spine synapses, sometimes completely encapsulating them (Figure 1). Astrocytic processes are found in close proximity of synapses at all synapses investigated so far with EM in different brain regions. These small astrocytic processes are devoid of GFAP filaments, have a cytoplasm that is light in appearance, are highly irregular in outline, forming flat or lamellar sheets or conforming to the shapes of surrounding neuropil elements (Figure 1), and have large surface/volume ratios, as shown with 3D EM reconstructions [19, 20] (reviewed in [13]). In these very fine processes, mitochondria, microtubules, and endoplasmic reticulum are usually absent, but ribosomes and glycogen granules are common [21]. They also contain actin filaments [22], together with actin-binding proteins [4, 23, 24]. The contacts between astrocytic processes and dendritic spines can be quite tight, sometimes with puncta adhaerens between the nonsynaptic surface of dendritic spines and astrocytic processes [25].

PAPs are found in all brain regions, but the proportion of synapses having them and the level of synaptic coverage vary significantly. For example, PAPs embrace most of the synapses in cerebellum but only 29–56% of the synapses in the neocortex, according to different EM estimations [18, 26]. In layer IV of adult mouse somatosensory cortex, 3D EM demonstrated high heterogeneity of synaptic coverage, with around 10% of spine synapses having no contact with PAPs, while the rest of the spines have varying portion of their surface enwrapped, with a majority of them (around 68%) surrounded by astrocytic process at the axon-spine interface (ASI; Figure 1). In CA1 stratum radiatum of adult rat hippocampus, PAPs are also distributed nonuniformly. They are present near approximately 62% of synapses, with a preference towards large synapses, especially those with perforated (complex) PSDs that are considered as morphological correlates of strengthened synapses (up to 90%; [27]). Some CA1

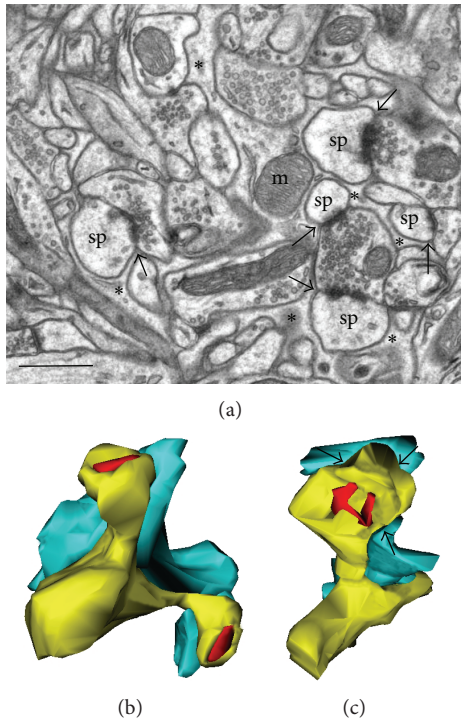

Figure 1: Electron microscopy images and 3D reconstructions illustrating glial coverage of excitatory spine synapses in neocortex. (a) EM micrograph of the neuropil in the adult mouse cortex with dendritic spine synapses (sp) surrounded by astrocytic processes marked with asterisks. Note mitochondria (m) in the branching point of astrocytic processes and perisynaptic astrocytic processes (PAPs) contacting axon-spine interface (ASI, arrows). (b) 3D reconstruction of the PAP near spine synapses with simple PSDs in adult rat hippocampus. Dendritic segment with two spines is in yellow, PSDs are in red, and PAP is in blue. (c) The same but for the big spine with complex PSD. Arrows point at ASI covered by the PAP. Note that in both reconstructions a large fraction of ASI is devoid of PAPs (original data). Scale bar: 1 μm.

synapses are completely devoid of PAPs, while others could be covered only around the dendritic spine (7%), or around the presynaptic bouton (8%), or, most often, around the ASI. In CA1 area of rat organotypic hippocampal slice cultures, the majority (85%) of spine synapses are also contacted by glial processes that do not cover synaptic surface completely. 97% of big spine synapses with a complex PSD are partially enveloped by glia but only 78% of simple synapses [28]. In rat hippocampus, less than half (43%) of the synaptic perimeter is surrounded by PAPs, and this parameter is highly variable from synapse to synapse ([29]; see Figures 1(b) and 1(c)). In general, 99.5% of CA1 synapses have some portions of ASI free from glial processes, and only a fraction of the ASI perimeter (on average, 38%) is enwrapped by astroglia. In contrast, in CA3 hippocampal region, large mossy fiber synapses are surrounded by astrocytic processes that physically isolate these synaptic complexes from the neighboring synapses, although PAPs never reach individual active zones [30].

In cerebellum, a mossy fiber (MF) excitatory terminal has hundreds of release sites that establish synaptic contacts

with different granule cell dendrites positioned close together forming a multisynaptic arrangement or glomerulus. Only a small fraction (around 15%) of these excitatory terminals is contacted by glial processes, as shown by 3D EM [31]. In contrast, excitatory inputs to Purkinje cell (PC) spines are much better isolated by the Bergman glia (BG) processes [32]. Synapses formed by climbing fibers (CF) have the highest level of astrocytic coverage, with an average degree of enwrapping around 87%. In more than half of these synapses, at least 90% of their surface is covered by PAPs. Parallel fiber (PF) synapses have a bit less of perisynaptic glial coverage (around 65% of the synapse perimeter [32]). Serial 3D EM of labeled lateral BG processes that represent up to 90% of the BG total membrane surface area demonstrated their very complex and irregular morphology, with high surface to volume ratio [19]. Another peculiar morphological organization revealed by serial 3D EM of labeled lateral BG processes is the so-called BG microdomain, a repetitive unit on the stem process consisting of a thin stalk and a complex cabbage-like head packed with thin leaflets that wrap around individual synapses or groups of synapses and constitutes an autonomous functional unit.

In the Nucleus Tractus Solitarii (NTS), a sensory nucleus in the caudal medulla that receives primary afferent visceroceptive inputs, but also afferent fibers from various brain regions, volume fraction of astrocytic processes and the density of astrocyte membranes are rather high, equal to 15% and 2.8 $\mu m^2/\mu m^3$, respectively. This nucleus contains simple one-to-one, mostly axodendritic, synapses but is also enriched in divergent (one axonal terminal innervating several postsynaptic dendrites or spines) and convergent (several axon terminals making clustered synaptic contacts on the same dendritic segment) multisynaptic arrangements or glomeruli. Like in other brain regions, astrocytic coverage of synapses is generally not complete and highly variable, ranging from no coverage to almost complete enwrapping, and constitutes, on average, 47% of synapse perimeter. At simple glutamatergic synapses, astrocytic processes cover 58% of their perimeter, while, in multisynaptic arrangements, glial coverage is restricted to the nonshared, outer part of synaptic perimeters and amounts around 50% of this outer part [33]. For more examples of PAP distribution and synaptic ensheathment see also [3, 13].

Although electron microscopy provides the most precise estimations of PAP morphology, distribution, localization, and degree of synaptic coverage, this technique is limited to static observations as it requires fixation and embedding of tissue samples. Current light microscopy approaches use PAP labeling with different fluorescent probes, static or time-lapse observations with confocal microscopy followed by off-line image treatment (e.g., deconvolution helping to overcome the limits of confocal resolution, often lower than the size of the smallest PAPs), and 3D/4D reconstructions [34, 35]. These data will be discussed in the following sections. Development of superresolution light microscopy imaging techniques [36] will further improve the precision of PAP observations.

3. PAP Distribution Is Related to the Patterns of Neuronal Connectivity in Different Brain Regions

The distribution of astrocytic processes shows high variability between different brain regions and even subregions. One of the first indications was provided by a light microscopy study that assessed the orientation of hippocampal astrocytic processes [37]. They found that during development, hippocampal astrocytes become polarized. Their orientation in CA1 is almost perpendicular to the stratum pyramidale, while in stratum lacunosum-moleculare they transiently orient parallel to the fissure. This particular orientation pattern seems to correlate with neuronal projections in the hippocampus [37].

Confocal and electron microscopy studies in hippocampus [17, 38] and neocortex [15] have shown that astrocytes cover their own exclusive spatial domains where they interact with neuronal elements, with only limited overlap in narrow peripheral zones. This typical domain organization helps the demarcation of functional compartments and adequate neurovascular regulation. However, in some cortical regions this organization is compromised. In posterior piriform cortex (PPC) of adult rats, surface-associated astrocytes (SAA) are directly opposed to the cortical surface, send large-caliber processes into layer I, and give rise to an extensive network of superficial processes that form a continuous sheet at the surface of PPC and lack the domain organization typical of neocortical astrocytes [39]. In PPC, connectivity and patterns of activation by individual odorants are widely distributed, overlap extensively, and are modulated by context and behavior, and thus SAA also form extensively overlapping processes [39].

In hippocampus, 99.5% of CA1 synapses have some portions of ASI free from glial processes, thus favoring glutamate spillover and synaptic crosstalk. Interestingly, the fraction of synaptic perimeter covered with PAPs is inversely proportional to the synapse size, so that large synapses, often with complex PSDs, have more of ASI open and are more susceptible to the transmitter spillover ([27, 29]; see Figures 1(b) and 1(c)). Importantly, only 33% of the neighboring synapse pairs in the neuropil have an astrocytic process intruding along the shortest path between them, thus further favoring spillover and crosstalk [29]. In CA1 synapses, a significant asymmetry characterizes the distribution of perisynaptic glia with PAPs being threefold more present on the postsynaptic side than on the presynaptic side [40]. 3D modeling using these EM data predicted that extrasynaptic actions of glutamate near these synapses would favor presynaptic feedback and preserve specificity transfer of information to the postsynaptic site.

In cerebellum, the morphology of mossy fiber divergent glomeruli, mostly without glial fingers between the granule cell dendrites contacting an MF excitatory terminal, favors glutamate spillover. This organization enhances the efficacy of fast synaptic transmission and contributes to the time course of MF synaptic currents and to the desensitization of postsynaptic AMPA receptors (AMPAR) during short-term depression [31]. Bergman glia constitutes a large volume fraction in cerebellum (33%), more than three times that of

hippocampus and cortex [29, 40, 41]. Unlike neocortex and hippocampus, functional microdomains from different BG cells intermingle within a given space of the neuropil [19, 42]. Intermingling of microdomains from different BG cells covering PF and CF synapses may provide an independent microenvironment designed to compartmentalize calcium signals, contribute to independent activation of individual dendritic spines or ensembles of spines on PC, and ensure selectivity in synapse modulation and efficient clearance through glutamate transporters expressed in the fine BG processes [19, 43].

In the NTS synapses, incomplete glial ensheathment of all synaptic types would provide enough routes for glutamate spillover, although the crosstalk between distant synapses is probably reduced, due to the low synaptic density and high volume fraction of astrocytic processes. At the same time, individual synapses in multisynaptic glomeruli in NTS can strongly influence each other thanks to the direct apposition of neighboring synaptic elements mostly devoid of intervening glial processes that generally cover nonshared, outer part of synaptic perimeters. Consequently, these glomeruli may be viewed as individual computing units providing processing of visceral information already at this level, especially given that sensory afferent terminals in the NTS are frequently involved in divergent arrangements. On the other hand, convergent multisynaptic arrangements may also add to information processing by occlusion phenomenon between sensory inputs converging onto the same NTS neuron [33].

These examples demonstrate that the astrocytic spatial organization varies in different brain regions and may be related to the corresponding patterns of neuronal connectivity helping to establish specific functional and structural architectures. This suggests that astrocytes show sensitivity to neuronal activity and could adapt their morphology to the activity in their environment.

4. PAPs Exhibit Significant Structural Plasticity

The heterogeneity of PAPs around different synapses described above suggests the ability of astrocytes to control the level of synaptic coverage and, consequently, implies that PAPs might be as plastic as their neuronal counterparts.

Indeed, the first examples of PAP structural plasticity were described a long time ago, in EM studies of oxytocin (OXT) secreting magnocellular neurons in supraoptic (SON) and paraventricular (PVN) nuclei of hypothalamus [44]. Under basal conditions, somata and dendrites of magnocellular secretory neurons are mostly separated by neuropil elements and especially by fine processes of both stellate and radial-like astrocytes [45–47]. Under conditions of stimulation, including parturition, lactation, osmotic stimulation, and stress, astrocytes respond by rapid, within few hours, and reversible structural remodeling leading to a reduced coverage [46, 48]. Even more striking rhythmic ultrastructural rearrangements take place in suprachiasmatic nucleus (SCN) of the rat hypothalamus where glial and neuronal structural plasticity follows 24 hours light/dark (L/D) cycles [49]. These observations revealed an important potential for structural plasticity in astrocytes.

A technique of choice to observe real time movements of astrocytes is time-lapse light microscopy. In cultured hippocampal or cortical astrocytes, highly motile astrocytic filopodia-like processes were detected, moving or growing in the time course of minutes or even seconds [4, 24, 50]. Whether these filopodia-like structures are comparable to *in situ* PAPs is uncertain, although they contain the actin binding protein ezrin that is specifically localized to PAPs [4]. Thus, the mechanisms driving filopodia movements in cultured astrocytes could be common with those of PAPs *in vivo*. Using a transgenic mice line expressing green fluorescent protein (GFP) under a GFAP promoter, Hirrlinger et al. were able to image astrocytic processes adjacent to synapses in acute brain slices and demonstrate that these processes were motile [51]. However, because of the use of a nonmembrane targeted GFP that limited the possibility to detect very fine astrocytic processes in this and other studies [52, 53], it was difficult to identify whether these processes were indeed PAPs. *In situ* studies using membrane targeted fluorescent protein transfected biolistically in organotypic cultures allowed to visualize very fine astrocytic structures in a time-lapse manner and confirmed that those structures were dynamic [14]. Unfortunately, in this study astrocytic membranes were not coimaged with synaptic elements, again limiting a conclusion as to whether these processes were indeed PAPs. Haber and coworkers were the first to demonstrate convincing movements of PAPs *in situ* by performing dual labeling of dendritic spines and astrocytic processes through viral gene delivery of membrane targeted fluorescent proteins [34]. They nicely showed that PAPs are plastic structures that can engage and disengage from a dendritic spine in hippocampal slices. PAP motility have been confirmed by other researchers in hippocampus ([35]; see also Figure 2) as well as in BG contacting PC in acute slices of cerebellum [54, 55].

Time-lapse confocal studies have definitively demonstrated that PAPs are highly dynamic as they can modify their structure in a time course of minutes. PAP movements were observed in primary and organotypic cultures as well as in the acute slice model. Whether PAP motility occurs only during development and synaptogenesis or is persistent in adulthood and how this remodeling occurs *in vivo* remain, however, to be determined.

5. Driving Force and Mechanisms of PAP Structural Plasticity

Already early EM observations of astrocytic structural plasticity in rat hypothalamus suggested a dependence on environmental cues and stimulation, such as parturition, lactation, osmotic stimulation, and stress [44–48]. An EM study in the visual cortex of rats reared in a complex environment revealed a specific and significant increase in the ensheathment of synapses by astrocytic processes that accompanied structural and functional synaptic changes [56]. Chronic whisker stimulation in adult mice induced PAP structural plasticity in the corresponding barrel of the somatosensory

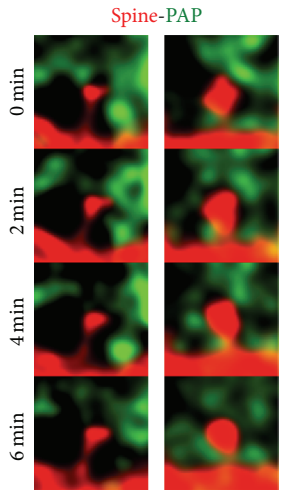

Figure 2: Perisynaptic astrocytic processes (PAPs) are motile. Z-stack projection of confocal images showing hippocampal CA1 dendritic spines (mcherryf, red) and PAPs (eGFPf, green) recorded in the stratum radiatum of organotypic hippocampal slice cultures. Neurons and astrocytes were virally infected with membrane targeted fluorescent proteins by injection. The labeled structures were monitored every 2 min to reveal PAP movements around spines. Square size: $3 \times 3\,\mu$m (original data).

cortex. Serial EM analyses have revealed that stimulation leads to a significant increase of the perimeter of excitatory synapse covered by PAPs, particularly at the axon-spine interface [41]. In dentate gyrus of hippocampus, the spatial relationship between astrocytic processes and synapses was analyzed at different time-points after induction of long-term potentiation (LTP) in adult rats *in vivo* [57]. This study reported a significant increase in the number and surface density of astrocytic processes 8 h after induction of LTP. At this time-point, synaptic complexes became increasingly more enveloped by PAPs. In organotypic hippocampal slice cultures, the morphological remodeling of CA1 excitatory spine synapses underlying induction of synaptic potentiation by theta burst stimulation of Schaffer collaterals was accompanied by an increase in the glial coverage of both pre- and postsynaptic elements, particularly of the large spine synapses with complex PSDs. Importantly, the increase in coverage was NMDA receptor-dependent [28]. In the same hippocampal pathway, a growth of astrocytic processes related to kindling and ischemic preconditioning has also been reported [58]. Additionally, EM studies in both hippocampus and cortex revealed that PAPs are preferentially present near synapses with big and often complex-shaped PSDs that are considered as morphological correlates of strengthened synapses [27–29]. These data, together with an observation that the proportion of cortical astrocyte coverage is correlated with the size of the PSD [41] also strongly suggest that PAP structural organization can be regulated by synaptic activity. The EM data available so far show that structural and functional pre- and postsynaptic changes due to experience-related paradigms and LTP (read [59]) are accompanied by structural changes in adjacent PAPs.

PAP movements observed with time-lapse confocal microscopy in hippocampal slices appeared to be coordinated with the extent of spine movements suggesting that the two phenomena are linked [34]. Because spine dynamics is dependent upon neuronal activity, the same assumption could then be made for PAPs. Interestingly, by assessing PAP movements around spines using an index of motility, Haber and coworkers revealed a high heterogeneity of this parameter [34], reminiscent of the synaptic coverage heterogeneity described above. This could mean that synaptic coverage is intimately linked to PAP motility. The fast motility of PAPs could then represent the movements accomplished by PAPs to increase or decrease synaptic coverage.

Increase of neuronal activity in hippocampal slices by application of the GABAR inhibitor bicuculline failed to elevate PAP motility [34], and PAP movements still occurred in the presence of TTX [35]. Nevertheless, glutamate application induced PAP remodeling, and inhibition of glutamate transporter under TTX prevented coordinated PAP-spine movements, suggesting, as shown in the cultures, that glutamate could be involved in PAP structural plasticity *in situ* [35].

Other indications also suggest an activity-dependence of PAP structural plasticity. A well-established feature of astrocytes is their ability to respond with an intracellular calcium elevation ($Ca^{2+}{}_i$) to neurotransmitters such as glutamate [50], GABA [60], ATP [61], endocanabinoids [62], and others. This increase can be evoked by neuronal activity both *in situ* [63] and *in vivo* [64, 65]. Glutamate stimulates filopodia outgrowth on cultured astrocytes and these effects are mimicked by both kainate and quisqualate agonists [50], as well as mGluR agonist [4]. mGluR antagonists abolish this structural remodeling, while an NMDAR agonist [50] does not influence it. Astrocytes were shown to express Ca^{2+}-permeable AMPAR [66, 67] and mGluR [68], the latter specifically in PAPs [4]. This suggests the involvement of non-NMDA ionotropic glutamate receptors (possibly AMPAR) and mGluR in the mechanisms driving astrocytic filopodia outgrowth. Consistent with this, BG retracted from PC spines when Ca^{2+}-impermeant AMPAR were overexpressed in BG [54]. However, it should be mentioned that the subunit composition of AMPAR is regulated developmentally, with Ca^{2+}-permeable AMPAR subunits (GluA2) decreasing during development [69]. Moreover, the question about the glial cell type that expresses AMPAR is still open, as NG2 cells and astrocytes share common GFAP marker [70]. mGluR 3 and 5 subtypes are localized in both filopodia from cultured astrocytes and in PAPs of hippocampal astrocytes *in situ* [4].

Although astrocytes are lacking voltage-gated channels, are not electrically excitable, and cannot generate action potentials, they can sense neuronal activity through calcium excitability, particularly thanks to the close apposition of PAPs to synapses [9, 10]. The most widely accepted mechanism for $Ca^{2+}{}_i$ signaling is the Gq protein coupled receptor-induced calcium release from intracellular stores, specifically from the endoplasmic reticulum [71]. Interestingly, calcium could be central to the mechanisms that control actin movements in astrocytes in the vicinity of the synapse [24, 72]. Calcium uncaging experiments in astrocytic cultures confirmed

that Ca^{2+} elevation triggers filopodia outgrowth [24]. In acute slices from transgenic mice that have an attenuated IP_3 pathway, kinetics of Ca^{2+} signals in hippocampal astrocytic processes following mGluR agonist application was reduced. EM analysis of the hippocampus from these mice revealed reduced PAP coverage of synapses as well as an elevated proportion of uncovered synapses [72]. This suggests that IP_3-mediated Ca^{2+} signals may be part of the mechanism driving morphological changes in PAPs.

Actin-based movements and remodeling are sensitive to Ca^{2+} changes which are affecting a plethora of actin binding proteins. Fine astrocytic processes contain mostly actin cytoskeleton, and the actin-binding protein alpha-actinin aggregates at the tip of growing filopodia in cultured astrocytes [22]. Inhibitors of actin polymerization block PAP motility [34]. Filopodia growth requires the redistribution of cytoskeletal proteins but no *de novo* synthesis or degradation of respective proteins [22], suggesting that astrocytes are capable of rapid movements in response to their environment. In both cultures [22] and slices [52, 53], astrocytic processes can adapt their morphology through a mechanism involving the rac-1 member of the ras superfamily of GTPases. Small GTPases are also involved in the motility of Bergman glia in the cerebellum [55]. Interestingly, a dominant negative form of the actin binding protein profilin-1 abolished Ca^{2+}-dependent filopodia outgrowth and movement [24]. Immunocytochemistry in rat brain sections demonstrated that the actin-binding protein ezrin and the mGluRs 3 and 5 are compartmentalized to PAPs but not to the main processes containing GFAP [4]. The experiments using ezrin siRNA or dominant-negative ezrin in primary astrocytes indicated that filopodia formation and motility require ezrin [4].

Astrocytic processes are subject to swelling (see review [13]). Even if swelling is often associated with pathological conditions, subtle volumetric changes can occur under physiological conditions, typically during osmotic pressure changes following synaptic transmission [73]. Thus, the swelling- and actin-based mechanisms for PAP structural plasticity could occur simultaneously [13].

Recent data indicate also the existence of extracellular mechanisms that could influence synapse-related PAP plasticity. Several astrocyte-neuron adhesion molecules were described, although there is still a lack of information about the presence of these molecules at the PAP-synapse interface. Among astrocyte-neuron adhesion molecules that could be regulated by activity and potentially localized at synapses the best candidate is the EphA4 receptor tyrosine kinase that is enriched in dendritic spines of hippocampal pyramidal neurons [74] (reviewed in [75]). The EphA4 ligand, ephrin-A3, appears to be localized on PAP membranes [76]. Binding of glial ephrin-A3 to neuronal EphA4 maintains normal spine morphology [76] while preventing the interaction results in unstable spines with disrupted shapes [53]. Interestingly, exogenous and endogenous ephrin-A induce astrocytic processes outgrowth and extension through their binding to astrocytic EphA ligands [52]. Concomitantly, calcium signals in astrocytes are perturbed by EphA, suggesting the implication of intracellular calcium in the mechanism regulating EphA4/ephrin-A3 adhesion and the subsequent regulation of individual dendritic spines.

Neuroligins are adhesion molecules known to be associated with synapses [77]. The expression of neuroligins 1 and 2 in the CNS is restricted to excitatory and inhibitory synapses, respectively. Neuroligin 3 is expressed by neurons but also by glial cells, in particular in the ensheathing glia in olfactory bulb as well as in retinal astrocytes [78]. Mutations of the genes coding for neuroligins and neurexins are associated with autism [79], a brain disorder characterized by anomalies of dendritic spine morphology. It was shown recently that astrocytes in autistic patients also exhibit significantly altered morphology, particularly of their processes. In addition, astrocytes of the neuroligin knockdown mice showed similar alterations [80]. This is suggesting implication of neuroligins in the maintenance of synaptic structures, including the astrocytic component. Very little is known about intracellular interaction partners of neuroligin 3. However, at glutamatergic synapses, neuroligin 1 binds to postsynaptic scaffolding protein PSD-95 that links to GKAP which in turn binds Shank, and this complex may further recruit other postsynaptic proteins to the excitatory synaptic junctions (read [81] for a review on neuroligins). Typically, mGluR receptors as well as ionotropic glutamate receptors are associated with Shank at postsynaptic sites, thereby providing a functional link between synaptic activity, intracellular calcium, and neuroligins (read [82] for a review on Shank).

Another potential candidate is the neuron-glia cell adhesion molecule (Ng-CAM). Ng-CAM has been implicated in binding between neurons and between neurons and glia [83]. SynCAM1, an adhesion molecule involved in synaptic differentiation and organization, is also expressed in astroglial cells where it mediates astrocyte-to-astrocyte and glial-neuronal adhesive communication [84]. NCAM is probably the most extensively studied cell adhesion molecule and is thought to intervene in most cell interactions via modulation of cell adhesivity and intracellular signaling. It is especially conspicuous in hypothalamo-neurohypophysial astrocytes (reviewed in [85]).

N-cadherin adhesion molecule is implicated in the establishment and stability of neuronal connections through activity-dependent mechanisms [86, 87]. The most important mechanism is the modification in extracellular calcium concentration, which affects the structure of the ectodomain and cadherin-mediated cell adhesion. Also, the cytoplasmic domain binds to various cytosolic and membrane proteins, including heterotrimeric G proteins. Moreover, the C-terminal domain mainly binds to catenin and anchors the protein to the actin cytoskeleton [88]. Interestingly, a member of the cadherin family, protocadherin-gammaC5 that is implicated in activity-regulated synaptic stability, is expressed in both astrocytes and neurons. In the brain, significant numbers of Pcdh-gammaC5 clusters are located at contact points between neuronal synaptic components and PAPs, suggesting that Pcdh-gammaC5 is involved in neuron-astrocyte interactions at the synaptic level [89].

Together, these data suggest that perisynaptic astrocytic processes possess the machinery to both sense neuronal

activity and remodel their actin filaments in an activity-dependent manner. These mechanisms are regulated by intracellular calcium, through the IP3 pathway, and could directly contribute to activity-dependent PAP structural plasticity. The data on adhesion molecules expressed in PAPs suggest their participation in remodeling, repositioning, and stabilization of perisynaptic astrocytic arrangements, although further research is needed to elucidate their exact roles.

6. What Are the Consequences for a Synapse?

Astrocytic ensheathment of the synapses may constitute a physical barrier for transmitter spillover from the synaptic cleft leading to extra- and heterosynaptic signaling. The consequences of the heterogeneity of the synaptic coverage by PAPs observed so far in different brain regions have been discussed in several studies. This heterogeneity suggests that glutamate or other neurotransmitters escape nonuniformly from synapses in a given brain area and that at certain synapses and/or under certain conditions synaptic crosstalk will be favored through a better spillover [40]. Structural plasticity of PAPs might significantly contribute to these mechanisms. A good example is provided by the rapid and reversible structural remodeling of astrocytes in hypothalamus under conditions of stimulation, leading to a reduced coverage of the somata and dendrites of magnocellular secretory neurons [46, 48]. These anatomical modifications result in astrocytic-dependent synaptic metaplasticity [90]. For example, in the SON of lactating rats astrocytic coverage undergoes extensive reduction that leads to an increase in directly juxtaposed surfaces of OXT magnocellular neurons [91, 92] and the change of both tortuosity and volume fraction of ECS [93]. Consequently, the number of synaptic contacts is increased and diffusion of neuroactive substances is facilitated. At the same time, glutamate clearance is delayed resulting in an increased negative feedback of glutamate on its own release and facilitated heterosynaptic depression of GABA release through glutamate spillover [93, 94]. Also, astrocytes in SON provide D-serine, an endogenous ligand for NMDARs, so that astrocytic withdrawal in lactating animals leads to a decrease in synaptic responses mediated by NMDARs and in availability of these receptors for activation, thus influencing the direction and magnitude of long-term synaptic plasticity [95]. Similar structural modifications of neuronal-glial interactions under conditions of enhanced neurosecretion occur in parallel in the neurohypophysis, the major projection site of magnocellular neurons, leading to facilitation of hormone release to blood circulation [46].

In the SCN of the hypothalamus, EM analysis of glial and axonal coverage of the somata and dendrites of the neurons expressing vasoactive intestinal peptide (VIP) or arginine vasopressin (AVP), the two main sources of SCN efferents, revealed significant variations during the L/D cycle [96]. The glial coverage of VIP dendrites increases at night, in concomitance with a decrease in the coverage of the somata and dendrites of these neurons by axon terminals. Conversely, glial coverage of the AVP dendrites drops during nighttime with no change in axonal coverage but with a striking increase in the somatal and dendritic appositions thus helping nocturnal facilitation of the intercellular synchronization involving these neurons [49].

Synapse modeling studies focused on the glial perisynaptic environment suggested profound effects of glial coverage on activation profiles of perisynaptic receptors. Rusakov has estimated that if one-half of the synaptic perimeter is covered with PAPs, it leads to almost a twofold increase of the glutamate concentration inside the glial sheath and two- to fourfold decrease outside. If around 95% of the synapse is covered, the concentration difference between inner and outer sides can reach 10- to 100-fold. Synaptic coverage may have stronger effects on synaptic transmission and Ca^{2+} depletion in the cleft at smaller synapses, especially with slower kinetics of perisynaptic ion transients [97]. Obviously, modifications of synaptic coverage due to PAP structural plasticity may have significant impact on spillover effects. Quantitative topological analysis of the extracellular space (ECS) based on 3D EM reconstructions of the CA1 neuropil of the adult rat allowed to decompose ECS into sheets and tunnels that are built by the surface patches of the axonal/dendritic/glial membranes [98]. These sheets and tunnels are distributed nonuniformly, with axons surrounded by more tunnel-like ESC, astrocytic processes accompanied by more sheet-like volumes, while around dendritic spines, the ECS is more tunnel-like than elsewhere. Diffusion simulations indicated that release of bioactive molecules to ECS would produce higher concentration peaks and maximum rate of concentration change, as seen by cell surface receptors, in the narrower sheet regions than in the larger tunnel spaces. These spatial irregularities would imply that ECS sheets may be specialized into enhancing signaling through concentration changes, while volume transmission of large molecules would be ensured through tunnels. Given that glia swelling under different physiological and pathological conditions may modulate extracellular volume fraction and that astrocytic processes are preferentially accompanied by ECS sheets, astroglia are well positioned to regulate volume communication in neuropil and provide a selective and specialized control of perisynaptic ECS volume around a synapse [98].

As shown by light and EM immunochemistry, perisynaptic astrocytic processes (PAPs) surrounding excitatory spine synapses express a plethora of different proteins, among which glutamine synthetase [99], astrocytic glutamate transporters [100], and aquaporins that may regulate "adaptive" swelling of PAPs [101], as well as actin associated molecules [4, 23], metabotropic glutamate receptors [4], and cell adhesion molecules [46]. Thus, by enwrapping a synapse, astroglia not only constitute a physical barrier for transmitter diffusion and help to retain a high level of neurotransmitter around a synapse but also, through the expression of specific proteins in perisynaptic processes, can participate in both sensing and responding to synaptic activity, actively take up glutamate from the synaptic cleft [5], control the amount of glutamate spillover that activates extrasynaptic receptors or enable inter-synaptic crosstalk [6], buffer potassium following neuronal depolarization [102], and provide energy substrates to neurons [103]. Remarkably, astrocytes are also able to release so-called gliotransmitters in a calcium-dependent manner,

among which are D-serine [104], glutamate [105], and ATP [106]. There is now extensive evidence that astrocytes can modulate synaptic transmission and plasticity through gliotransmission [62, 95, 107–115]. However, the exact mechanisms taking place during calcium-dependent neuron-glia interactions are still a matter of debate as studies using different approaches to selectively increase astrocytic $Ca^{2+}{}_i$ yielded conflicting results [12, 116, 117]. Gliotransmission is not the only consequence of calcium events in astrocytes. Calcium can regulate the expression level of glutamate transporters on astrocytes [118]. Intracellular calcium is also known to take part in the mechanism of neurometabolic coupling with neurons by either modulating the energetic response [119] or participating to the propagation of metabolic signals across the astrocytic network [120]. As discussed above, calcium signaling, often related to synaptic activity, could be central to the mechanisms that control PAP remodeling in the vicinity of the synapse [24, 72], which could in turn optimize the positioning of glial glutamate transporters to provide more efficient clearance of glutamate [5], modify the positioning of the release sites of gliotransmitters close to extrasynaptic neuronal receptors [2] or release of energetic substrates such as lactate in front of neuronal monocarboxylate transporters [121], and regulate ionic content of the extrasynaptic space [122].

Another aspect of PAP structural plasticity is its possible impact on the synaptic structure [40]. In concert with this concept, it was shown that in hippocampal slices the probability of a spine to disappear is higher when PAPs are not present around it [53]. Inhibition of astrocytic processes motility through interference with Rac1 results in an increase in dendritic filopodia [53], the protrusions known to be immature and nonpersistent [123]. Interfering with EphA4 or ephrin-A3 induces growth of astrocytic processes in cell culture [52]. The same treatments in slice cultures perturb the contact-dependent ephrin/Eph signaling by astrocytes, shorten the lifetime of dendritic protrusions contacted by astrocytic processes, and decrease the probability for the newly formed spines to be stabilized [53]. Consistent with this, BG coverage of PC spines seems to be required for synapse formation and regulation [54, 55]. Some hippocampal dendritic spines are able to extend spine head protrusions to neighboring functional presynaptic boutons [124]. Interestingly, the volume overlap between spines and PAPs decreased during the formation of spine head protrusion, suggesting an inverse correlation between spine coverage by PAPs and the creation of new synaptic connections [35]. These data point at the role of PAPs in regulation of new spine stability.

7. Discussion

Astroglia show intricate morphological interactions with neurons. Astrocytes are well positioned to influence synaptic transmission as they send fine processes that ensheath a synapse, forming a tripartite complex with pre- and postsynaptic neuronal components. About 60% of neuronal synaptic structures are partially enwrapped by PAPs, and the level of synaptic coverage increases with neuronal activity, LTP, and during experience-related paradigms. Moreover, PAPs can display rapid remodeling by extending and retracting from dendritic spines in a time scale of minutes. This rapid astrocytic motility appears to be influenced by neuronal activity and is coordinated with morphological changes in spines. In hippocampus, cortex, and cerebellum, glial processes exhibit two distinct structural plasticity phenomena: a highly dynamic movement referred to as motility and changes in their coverage of a synapse. Whether these two forms of plasticity are linked is still unclear, as well as the precise impact of such structural plasticity on synaptic function, structure, and plasticity.

One hypothesis that we would like to propose is that the activity-dependent structural plasticity of PAPs may allow astrocytes to control both the structural and functional properties of the synapse. Astrocytic contacts around synapses may provide structural support to a synapse while controlling the diffusion of molecules within the ECS and participating in extracellular homeostasis. Astrocytic processes in the vicinity of the synapse may control the amount of glutamate spillover that activates extrasynaptic receptors, enabling intersynaptic crosstalk and allowing for modified or synchronized neurotransmission. Similarly, it could help to locally provide energy substrates to neurons during energy demanding processes such as plasticity.

There are however many questions that still remain concerning the mechanisms of PAP structural plasticity, about how it is governed and controlled. Recent data provide indications that it could be controlled by neuronal activity, probably through glial metabotropic receptors and intracellular calcium. As Ca^{2+} signaling in astrocytic processes is synaptically evoked and can occur locally in the perisynaptic astrocytic environment without propagating to the cell body or other processes, PAP motility might be required to control the established "tripartite" connections in place and to coordinate their plastic adaptations [10]. Moreover, synaptic calcium signals in astrocytes associated with PAP structural plasticity could indicate that a given astrocyte can differentiate between the around 100 000 synapses it is enwrapping [17]. Thus, PAPs could be seen as structural as well as functional [125] entities able to integrate synaptic signals.

In the CNS, excitatory synapses exhibit multiple forms of plasticity that play a central role in information processing by neural networks. They involve changes in synaptic strength but also structural reorganization of synaptic connections that can occur during the whole lifetime [126]. These mechanisms of synaptic structural plasticity are now believed to be crucial for memory processes in the CNS. Consistent with this, abnormal synaptic morphologies are linked to various developmental psychiatric diseases including mental retardation, schizophrenia, and autism [127]. An important question therefore is to better understand the mechanisms regulating these structural aspects of plasticity. As PAP remodelling seems to be important for synapse function, formation, and stability, astrocytes might play an important role in these processes. Further studies, however, are needed to evaluate the functional and structural consequences of PAP structural plasticity in CNS function and disease.

Abbreviations

3D: Three dimensional
ASI: Axon-spine interface
AVP: Arginine vasopressin
AMPA: 2-Amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid
AMPAR: AMPA receptor
BG: Bergman glia
$Ca^{2+}{}_i$: Intracellular calcium concentration
CF: Climbing fibers
ECS: Extracellular space
EM: Electron microscopy
EphA: Ephrin-A receptor
EphB: Ephrin-B receptor
GABA: Gamma-aminobutyric acid
GABAR: GABA receptor
GFAP: Glial fibrillary acidic protein
GFP: Green fluorescent protein
GKAP: Guanylate kinase-associated protein
L/D cycle: Light/dark cycle
LTP: Long-term potentiation
MF: Mossy fibers
mGluR: Metabotropic glutamate receptor
NMDA: n-Methyl-D-aspartic acid
NMDAR: NMDA receptor
NTS: Nucleus Tractus Solitarii
OXT: Oxytocin
PAP: Perisynaptic astrocytic process
PC: Purkinje cell
PF: Parallel fibers
PPC: Posterior piriform cortex
PVN: Paraventricular nucleus of hypothalamus
SAA: Surface-associated astrocytes
SCN: Suprachiasmatic nucleus of the hypothalamus
SON: Supraoptic nucleus of hypothalamus
VIP: Vasoactive intestinal peptide.

Conflict of Interests

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

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References

[1] H. Kettenmann and B. R. Ransom, *Neuroglia*, Oxford University Press, Oxford, UK, 2005.

[2] A. Volterra and J. Meldolesi, "Astrocytes, from brain glue to communication elements: the revolution continues," *Nature Reviews Neuroscience*, vol. 6, no. 8, pp. 626–640, 2005.

[3] T. I. Chao, M. Rickman, and J. R. Wolff, "The synapse-astrocyte boundary: an anatomical basis for an integrative role of glia in synaptic transmission," in *Tripartite Synapse: Glia in Synaptic Transmission*, A. Volterra, P. J. Magistretti, and P. G. Haydon, Eds., vol. 1, pp. 3–23, Oxford University Press, Oxford, UK.

[4] M. Lavialle, G. Aumann, E. Anlauf, F. Pröls, M. Arpin, and A. Derouiche, "Structural plasticity of perisynaptic astrocyte processes involves ezrin and metabotropic glutamate receptors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 31, pp. 12915–12919, 2011.

[5] N. C. Danbolt, "Glutamate uptake," *Progress in Neurobiology*, vol. 65, no. 1, pp. 1–105, 2001.

[6] D. M. Kullmann and F. Asztely, "Extrasynaptic glutamate spillover in the hippocampus: evidence and implications," *Trends in Neurosciences*, vol. 21, no. 1, pp. 8–14, 1998.

[7] V. Benfenati and S. Ferroni, "Water transport between CNS compartments: functional and molecular interactions between aquaporins and ion channels," *Neuroscience*, vol. 168, no. 4, pp. 926–940, 2010.

[8] I. Allaman, M. Bélanger, and P. J. Magistretti, "Astrocyte-neuron metabolic relationships: for better and for worse," *Trends in Neurosciences*, vol. 34, no. 2, pp. 76–87, 2011.

[9] A. Panatier, J. Vallée, M. Haber, K. K. Murai, J. C. Lacaille, and R. Robitaille, "Astrocytes are endogenous regulators of basal transmission at central synapses," *Cell*, vol. 146, no. 5, pp. 785–798, 2011.

[10] M. A. Di Castro, J. Chuquet, N. Liaudet et al., "Local Ca^{2+} detection and modulation of synaptic release by astrocytes," *Nature Neuroscience*, vol. 14, no. 10, pp. 1276–1284, 2011.

[11] G. Perea and A. Araque, "Glial calcium signaling and neuron-glia communication," *Cell Calcium*, vol. 38, no. 3-4, pp. 375–382, 2005.

[12] C. Agulhon, T. A. Fiacco, and K. D. McCarthy, "Hippocampal short- and long-term plasticity are not modulated by astrocyte Ca^{2+} signaling," *Science*, vol. 327, no. 5970, pp. 1250–1254, 2010.

[13] A. Reichenbach, A. Derouiche, and F. Kirchhoff, "Morphology and dynamics of perisynaptic glia," *Brain Research Reviews*, vol. 63, no. 1-2, pp. 11–25, 2010.

[14] A. M. Benediktsson, S. J. Schachtele, S. H. Green, and M. E. Dailey, "Ballistic labeling and dynamic imaging of astrocytes in organotypic hippocampal slice cultures," *Journal of Neuroscience Methods*, vol. 141, no. 1, pp. 41–53, 2005.

[15] M. M. Halassa, T. Fellin, H. Takano, J. H. Dong, and P. G. Haydon, "Synaptic islands defined by the territory of a single astrocyte," *Journal of Neuroscience*, vol. 27, no. 24, pp. 6473–6477, 2007.

[16] E. Shigetomi, E. A. Bushong, M. D. Haustein et al., "Imaging calcium microdomains within entire astrocyte territories and endfeet with GCaMPs expressed using adeno-associated viruses," *Journal of General Physiology*, vol. 141, pp. 633–647, 2013.

[17] E. A. Bushong, M. E. Martone, Y. Z. Jones, and M. H. Ellisman, "Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains," *Journal of Neuroscience*, vol. 22, no. 1, pp. 183–192, 2002.

[18] J. R. Wolff, "Quantitative aspects of astroglia," in *Proceedings of the 6th international Congress of Neuropathology*, pp. 327–352, 1970.

[19] J. Grosche, V. Matyash, T. Möller, A. Verkhratsky, A. Reichenbach, and H. Kettenmann, "Microdomains for neuron-glia interaction: parallel fiber signaling to Bergmann glial cells," *Nature Neuroscience*, vol. 2, no. 2, pp. 139–143, 1999.

[20] K. Hama, T. Arii, E. Katayama, M. Marton, and M. H. Ellisman, "Tri-dimensional morphometric analysis of astrocytic processes with high voltage electron microscopy of thick Golgi preparations," *Journal of Neurocytology*, vol. 33, no. 3, pp. 277–285, 2004.

[21] A. Peters, S. L. Palay, and H. D. Webster, *The Fine Structure of the Nervous System: Neurons and Their Supporting Cells*, Oxford University Press, New York, NY, USA, 1991.

[22] M. Gorath, T. Stahnke, T. Mronga, O. Goldbaum, and C. Richter-Landsberg, "Rapid morphological changes in astrocytes are accompanied by redistribution but not by quantitative changes of cytoskeletal proteins," *Glia*, vol. 36, no. 1, pp. 102–115, 2001.

[23] A. Derouiche and M. Frotscher, "Peripheral astrocyte processes: monitoring by selective immunostaining for the actin-binding ERM proteins," *Glia*, vol. 36, no. 3, pp. 330–341, 2001.

[24] D. Molotkov, S. Zobova, J. M. Arcas, and L. Khiroug, "Calcium-induced outgrowth of astrocytic peripheral processes requires actin binding by Profilin-1," *Cell Calcium*, vol. 53, no. 5-6, pp. 338–348, 2013.

[25] J. Spacek and K. M. Harris, "Three-dimensional organization of cell adhesion junctions at synapses and dendritic spines in area CA1 of the rat hippocampus," *Journal of Comparative Neurology*, vol. 393, pp. 58–68, 1998.

[26] J. Spacek, "Relationships between synaptic junctions, puncta adhaerentia and the spine apparatus at neocortical axo-spinous synapses. A serial section study," *Anatomy and Embryology*, vol. 173, no. 1, pp. 129–135, 1985.

[27] M. R. Witcher, S. A. Kirov, and K. M. Harris, "Plasticity of perisynaptic astroglia during synaptogenesis in the mature rat hippocampus," *Glia*, vol. 55, no. 1, pp. 13–23, 2007.

[28] I. Lushnikova, G. Skibo, D. Muller, and I. Nikonenko, "Synaptic potentiation induces increased glial coverage of excitatory synapses in CA1 hippocampus," *Hippocampus*, vol. 19, no. 8, pp. 753–762, 2009.

[29] R. Ventura and K. M. Harris, "Three-dimensional relationships between hippocampal synapses and astrocytes," *Journal of Neuroscience*, vol. 19, no. 16, pp. 6897–6906, 1999.

[30] A. Rollenhagen, K. Sätzler, E. P. Rodríguez, P. Jonas, M. Frotscher, and J. H. R. Lübke, "Structural determinants of transmission at large hippocampal mossy fiber synapses," *Journal of Neuroscience*, vol. 27, no. 39, pp. 10434–10444, 2007.

[31] M. A. Xu-Friedman and W. G. Regehr, "Ultrastructural contributions to desensitization at cerebellar mossy fiber to granule cell synapses," *Journal of Neuroscience*, vol. 23, no. 6, pp. 2182–2192, 2003.

[32] M. A. Xu-Friedman, K. M. Harris, and W. G. Regehr, "Three-dimensional comparison of ultrastructural characteristics at depressing and facilitating synapses onto cerebellar Purkinje cells," *Journal of Neuroscience*, vol. 21, no. 17, pp. 6666–6672, 2001.

[33] K. Chounlamountry and J. P. Kessler, "The ultrastructure of perisynaptic glia in the nucleus tractus solitarii of the adult rat: comparison between single synapses and multisynaptic arrangements," *Glia*, vol. 59, no. 4, pp. 655–663, 2011.

[34] M. Haber, L. Zhou, and K. K. Murai, "Cooperative astrocyte and dendritic spine dynamics at hippocampal excitatory synapses," *Journal of Neuroscience*, vol. 26, no. 35, pp. 8881–8891, 2006.

[35] D. Verbich, G. A. Prenosil, P. K. Y. Chang, K. K. Murai, and R. A. Mckinney, "Glial glutamate transport modulates dendritic spine head protrusions in the hippocampus," *Glia*, vol. 60, no. 7, pp. 1067–1077, 2012.

[36] N. Ji, H. Shroff, H. Zhong, and E. Betzig, "Advances in the speed and resolution of light microscopy," *Current Opinion in Neurobiology*, vol. 18, no. 6, pp. 605–616, 2008.

[37] B. E. Nixdorf-Bergweiler, D. Albrecht, and U. Heinemann, "Developmental changes in the number, size, and orientation of GFAP-positive cells in the CA1 region of rat hippocampus," *Glia*, vol. 12, no. 3, pp. 180–195, 1994.

[38] K. Ogata and T. Kosaka, "Structural and quantitative analysis of astrocytes in the mouse hippocampus," *Neuroscience*, vol. 113, no. 1, pp. 221–233, 2002.

[39] S. L. Feig and L. B. Haberly, "Surface-associated astrocytes, not endfeet, form the glia limitans in posterior piriform cortex and have a spatially distributed, not a domain, organization," *Journal of Comparative Neurology*, vol. 519, no. 10, pp. 1952–1969, 2011.

[40] K. R. Lehre and D. A. Rusakov, "Asymmetry of glia near central synapses favors presynaptically directed glutamate escape," *Biophysical Journal*, vol. 83, no. 1, pp. 125–134, 2002.

[41] C. Genoud, C. Quairiaux, P. Steiner, H. Hirling, E. Welker, and G. W. Knott, "Plasticity of astrocytic coverage and glutamate transporter expression in adult mouse cortex," *PLoS Biology*, vol. 4, no. 11, article e343, 2006.

[42] J. Grosche, H. Kettenmann, and A. Reichenbach, "Bergmann glial cells form distinct morphological structures to interact with cerebellar neurons," *Journal of Neuroscience Research*, vol. 68, no. 2, pp. 138–149, 2002.

[43] K. Yamada, M. Fukaya, T. Shibata et al., "Dynamic transformation of Bergmann glial fibers proceeds in correlation with dendritic outgrowth and synapse formation of cerebellar Purkinje cells," *Journal of Comparative Neurology*, vol. 418, pp. 106–120, 2000.

[44] D. T. Theodosis, D. A. Poulain, and J. D. Vincent, "Possible morphological bases for synchronisation of neuronal firing in the rat supraoptic nucleus during lactation," *Neuroscience*, vol. 6, no. 5, pp. 919–929, 1981.

[45] C. Montagnese, D. A. Poulain, J. D. Vincent, and D. T. Theodosis, "Synaptic and neuronal-glial plasticity in the adult oxytocinergic system in response to physiological stimuli," *Brain Research Bulletin*, vol. 20, no. 6, pp. 681–692, 1988.

[46] D. T. Theodosis, "Oxytocin-secreting neurons: a physiological model of morphological neuronal and glial plasticity in the adult hypothalamus," *Frontiers in Neuroendocrinology*, vol. 23, no. 1, pp. 101–135, 2002.

[47] D. T. Theodosis, A. Trailin, and D. A. Poulain, "Remodeling of astrocytes, a prerequisite for synapse turnover in the adult brain? Insights from the oxytocin system of the hypothalamus," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 290, no. 5, pp. R1175–R1182, 2006.

[48] A. K. Salm, "Mechanisms of glial retraction in the hypothalamo-neurohypophysial system of the rat," *Experimental Physiology*, vol. 85, 2000.

[49] D. Becquet, C. Girardet, F. Guillaumond, A. M. François-Bellan, and O. Bosler, "Ultrastructural plasticity in the rat suprachiasmatic nucleus. Possible involvement in clock entrainment," *Glia*, vol. 56, no. 3, pp. 294–305, 2008.

[50] A. H. Cornell-Bell, P. G. Thomas, and S. J. Smith, "The excitatory neurotransmitter glutamate causes filopodia formation in cultured hippocampal astrocytes," *Glia*, vol. 3, no. 5, pp. 322–334, 1990.

[51] J. Hirrlinger, S. Hülsmann, and F. Kirchhoff, "Astroglial processes show spontaneous motility at active synaptic terminals in situ," *European Journal of Neuroscience*, vol. 20, no. 8, pp. 2235–2239, 2004.

[52] M. W. Nestor, L. P. Mok, M. E. Tulapurkar, and S. M. Thompson, "Plasticity of neuron-glial interactions mediated by astrocytic EphARs," *Journal of Neuroscience*, vol. 27, no. 47, pp. 12817–12828, 2007.

[53] H. Nishida and S. Okabe, "Direct astrocytic contacts regulate local maturation of dendritic spines," *Journal of Neuroscience*, vol. 27, no. 2, pp. 331–340, 2007.

[54] J. J. Lippman Bell, T. Lordkipanidze, N. Cobb, and A. Dunaevsky, "Bergmann glial ensheathment of dendritic spines regulates synapse number without affecting spine motility," *Neuron Glia Biology*, vol. 6, no. 3, pp. 193–200, 2010.

[55] J. J. Lippman, T. Lordkipanidze, M. E. Buell, S. O. Yoon, and A. Dunaevsky, "Morphogenesis and regulation of Bergmann glial processes during Purkinje cell dendritic spine ensheathment and synaptogenesis," *Glia*, vol. 56, no. 13, pp. 1463–1477, 2008.

[56] T. A. Jones and W. T. Greenough, "Ultrastructural evidence for increased contact between astrocytes and synapses in rats reared in a complex environment," *Neurobiology of Learning and Memory*, vol. 65, no. 1, pp. 48–56, 1996.

[57] J. Wenzel, G. Lammert, U. Meyer, and M. Krug, "The influence of long-term potentiation on the spatial relationship between astrocyte processes and potentiated synapses in the dentate gyrus neuropil of rat brain," *Brain Research*, vol. 560, no. 1-2, pp. 122–131, 1991.

[58] N. Hawrylak, F. L. F. Chang, and W. T. Greenough, "Astrocytic and synaptic response to kindling in hippocampal subfield CA1. II: synaptogenesis and astrocytic process increases to in vivo kindling," *Brain Research*, vol. 603, no. 2, pp. 309–316, 1993.

[59] P. Caroni, F. Donato, and D. Muller, "Structural plasticity upon learning: regulation and functions," *Nature Reviews Neuroscience*, vol. 13, pp. 478–490, 2012.

[60] J. Kang, L. Jiang, S. A. Goldman, and M. Nedergaard, "Astrocyte-mediated potentiation of inhibitory synaptic transmission," *Nature Neuroscience*, vol. 1, no. 8, pp. 683–692, 1998.

[61] J. T. Neary, M. McCarthy, Y. Kang, and S. Zuniga, "Mitogenic signaling from P1 and P2 purinergic receptors to mitogen-activated protein kinase in human fetal astrocyte cultures," *Neuroscience Letters*, vol. 242, no. 3, pp. 159–162, 1998.

[62] M. Navarrete and A. Araque, "Endocannabinoids potentiate synaptic transmission through stimulation of astrocytes," *Neuron*, vol. 68, no. 1, pp. 113–126, 2010.

[63] J. T. Porter and K. D. McCarthy, "Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals," *Journal of Neuroscience*, vol. 16, no. 16, pp. 5073–5081, 1996.

[64] J. Schummers, H. Yu, and M. Sur, "Tuned responses of astrocytes and their influence on hemodynamic signals in the visual cortex," *Science*, vol. 320, no. 5883, pp. 1638–1643, 2008.

[65] X. Wang, N. Lou, Q. Xu et al., "Astrocytic Ca^{2+} signaling evoked by sensory stimulation in vivo," *Nature Neuroscience*, vol. 9, no. 6, pp. 816–823, 2006.

[66] M. Iino, K. Goto, W. Kakegawa et al., "Glia-synapse interaction through Ca^{2+}-permeable AMPA receptors in Bergmann glia," *Science*, vol. 292, no. 5518, pp. 926–929, 2001.

[67] G. Seifert, M. Weber, J. Schramm, and C. Steinhäuser, "Changes in splice variant expression and subunit assembly of AMPA receptors during maturation of hippocampal astrocytes," *Molecular and Cellular Neuroscience*, vol. 22, no. 2, pp. 248–258, 2003.

[68] W. Sun, E. McConnell, J. F. Pare et al., "Glutamate-dependent neuroglial calcium signaling differs between young and adult brain," *Science*, vol. 339, pp. 197–200, 2013.

[69] S. J. Liu and I. Savtchouk, "Ca^{2+} permeable AMPA receptors switch allegiances: mechanisms and consequences," *Journal of Physiology*, vol. 590, no. 1, pp. 13–20, 2012.

[70] D. E. Bergles, R. Jabs, and C. Steinhäuser, "Neuron-glia synapses in the brain," *Brain Research Reviews*, vol. 63, no. 1-2, pp. 130–137, 2010.

[71] M. L. Cotrina and M. Nedergaard, "Intacellular calcium control mechanism in glia," in *Neuroglia*, H. Kettenmann and B. R. Ransom, Eds., vol. 2, pp. 229–239, Oxford University Press, Oxford, UK, 1st edition.

[72] M. Tanaka, P. Y. Shih, H. Gomi et al., "Astrocytic Ca^{2+} signals are required for the functional integrity of tripartite synapses," *Molecular Brain*, vol. 6, p. article 6, 2013.

[73] A. A. Mongin and H. K. Kimelberg, "ATP regulates anion channel-mediated organic osmolyte release from cultured rat astrocytes via multiple Ca^{2+}-sensitive mechanisms," *American Journal of Physiology—Cell Physiology*, vol. 288, no. 1, pp. C204–C213, 2005.

[74] M. È. Tremblay, M. Riad, S. Chierzi, K. K. Murai, E. B. Pasquale, and G. Doucet, "Developmental course of EphA4 cellular and subcellular localization in the postnatal rat hippocampus," *Journal of Comparative Neurology*, vol. 512, no. 6, pp. 798–813, 2009.

[75] K. K. Murai and E. B. Pasquale, "Eph receptors, ephrins, and synaptic function," *Neuroscientist*, vol. 10, no. 4, pp. 304–314, 2004.

[76] K. K. Murai, L. N. Nguyen, F. Irie, Y. Yu, and E. B. Pasquale, "Control of hippocampal dendritic spine morphology through ephrin-A3/EphA4 signaling," *Nature Neuroscience*, vol. 6, no. 2, pp. 153–160, 2003.

[77] S. Y. Jung, J. Kim, O. B. Kwon et al., "Input-specific synaptic plasticity in the amygdala is regulated by neuroligin-1 via postsynaptic NMDA receptors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 10, pp. 4710–4715, 2010.

[78] M. Gilbert, J. Smith, A. J. Roskams, and V. J. Auld, "Neuroligin 3 is a vertebrate gliotactin expressed in the olfactory ensheathing glia, a growth-promoting class of macroglia," *Glia*, vol. 34, no. 3, pp. 151–164, 2001.

[79] B. Chih, H. Engelman, and P. Scheiffele, "Control of excitatory and inhibitory synapse formation by neuroligins," *Science*, vol. 307, no. 5713, pp. 1324–1328, 2005.

[80] F. Cao, A. Yin, G. Wen et al., "Alteration of astrocytes and Wnt/beta-catenin signaling in the frontal cortex of autistic subjects," *Journal of Neuroinflammation*, vol. 9, no. 1, article 223, 2012.

[81] M. L. Bang and S. Owczarek, "A matter of balance: role of neurexin and neuroligin at the synapse," *Neurochemical Research*, vol. 38, no. 6, pp. 1174–1189, 2013.

[82] Y. H. Jiang and M. D. Ehlers, "Modeling autism by SHANK gene mutations in mice," *Neuron*, vol. 78, pp. 8–27, 2013.

[83] M. Grumet, S. Hoffman, C. M. Chuong, and G. M. Edelman, "Polypeptide components are binding functions of neuron-glia cell adhesion molecules," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 24, pp. 7989–7993, 1984.

[84] U. S. Sandau, Z. Alderman, G. Corfas, S. R. Ojeda, and J. Raber, "Astrocyte-specific disruption of SynCAM1 signaling results in ADHD-like behavioral manifestations," *PLoS ONE*, vol. 7, no. 4, Article ID e36424, 2012.

[85] D. T. Theodosis, R. Piet, D. A. Poulain, and S. H. R. Oliet, "Neuronal, glial and synaptic remodeling in the adult hypothalamus:

functional consequences and role of cell surface and extracellular matrix adhesion molecules," *Neurochemistry International*, vol. 45, no. 4, pp. 491–501, 2004.

[86] G. W. Huntley, A. M. Elste, S. B. Patil, O. Bozdagi, D. L. Benson, and O. Steward, "Synaptic loss and retention of different classic cadherins with LTP-associated synaptic structural remodeling in vivo," *Hippocampus*, vol. 22, no. 1, pp. 17–28, 2012.

[87] P. Mendez, M. de Roo, L. Poglia, P. Klauser, and D. Muller, "N-cadherin mediates plasticity-induced long-term spine stabilization," *Journal of Cell Biology*, vol. 189, no. 3, pp. 589–600, 2010.

[88] S. Hirano and M. Takeichi, "Cadherins in brain morphogenesis and wiring," *Physiological Reviews*, vol. 92, no. 2, pp. 597–634, 2012.

[89] Y. Li, D. R. Serwanski, C. P. Miralles et al., "Synaptic and nonsynaptic localization of protocadherin-γc5 in the rat brain," *Journal of Comparative Neurology*, vol. 518, no. 17, pp. 3439–3463, 2010.

[90] S. H. R. Oliet, A. Panatier, R. Piet, J. P. Mothet, D. A. Poulain, and D. T. Theodosis, "Neuron-glia interactions in the rat supraoptic nucleus," *Progress in Brain Research*, vol. 170, pp. 109–117, 2008.

[91] G. I. Hatton, "Function-related plasticity in hypothalamus," *Annual Review of Neuroscience*, vol. 20, pp. 375–397, 1997.

[92] D. T. Theodosis and D. A. Poulain, "Activity-dependent neuronal-glial and synaptic plasticity in the adult mammalian hypothalamus," *Neuroscience*, vol. 57, no. 3, pp. 501–535, 1993.

[93] R. Piet, D. A. Poulain, and S. H. R. Oliet, "Contribution of astrocytes to synaptic transmission in the rat supraoptic nucleus," *Neurochemistry International*, vol. 45, no. 2-3, pp. 251–257, 2004.

[94] S. H. R. Oliet, R. Piet, and D. A. Poulain, "Control of glutamate clearance and synaptic efficacy by glial coverage of neurons," *Science*, vol. 292, no. 5518, pp. 923–926, 2001.

[95] A. Panatier, D. T. Theodosis, J. P. Mothet et al., "Glia-Derived d-Serine Controls NMDA Receptor Activity and Synaptic Memory," *Cell*, vol. 125, no. 4, pp. 775–784, 2006.

[96] L. M. Prolo, J. S. Takahashi, and E. D. Herzog, "Circadian rhythm generation and entrainment in astrocytes," *Journal of Neuroscience*, vol. 25, no. 2, pp. 404–408, 2005.

[97] D. A. Rusakov, "The role of perisynaptic glial sheaths in glutamate spillover and extracellular Ca^{2+} depletion," *Biophysical Journal*, vol. 81, no. 4, pp. 1947–1959, 2001.

[98] J. P. Kinney, J. Spacek, T. M. Bartol, C. L. Bajaj, K. M. Harris, and T. J. Sejnowski, "Extracellular sheets and tunnels modulate glutamate diffusion in hippocampal neuropil," *Journal of Comparative Neurology*, vol. 521, pp. 448–464, 2013.

[99] A. Derouiche and M. Frotscher, "Astroglial processes around identified glutamatergic synapses contain glutamine synthetase: evidence for transmitter degradation," *Brain Research*, vol. 552, no. 2, pp. 346–350, 1991.

[100] A. Derouiche and T. Rauen, "Coincidence of L-glutamate/L-aspartate transporter (GLAST) and glutamine synthetase (GS) immunoreactions in retinal glia: evidence for coupling of GLAST and GS in transmitter clearance," *Journal of Neuroscience Research*, vol. 42, no. 1, pp. 131–143, 1995.

[101] E. A. Nagelhus, T. M. Mathiisen, and O. P. Ottersen, "Aquaporin-4 in the central nervous system: cellular and subcellular distribution and coexpression with KIR4.1," *Neuroscience*, vol. 129, no. 4, pp. 905–913, 2004.

[102] E. A. Newman, "Inward-rectifying potassium channels in retinal glial (Muller) cells," *Journal of Neuroscience*, vol. 13, no. 8, pp. 3333–3345, 1993.

[103] P. J. Magistretti, L. Pellerin, D. L. Rothman, and R. G. Shulman, "Energy on demand," *Science*, vol. 283, no. 5401, pp. 496–497, 1999.

[104] M. Martineau, T. Shi, J. Puyal et al., "Storage and uptake of D-serine into astrocytic synaptic-like vesicles specify gliotransmission," *Journal of Neuroscience*, vol. 33, pp. 3413–3423, 2013.

[105] P. Bezzi, V. Gundersen, J. L. Galbete et al., "Astrocytes contain a vesicular compartment that is competent for regulated exocytosis of glutamate," *Nature Neuroscience*, vol. 7, no. 6, pp. 613–620, 2004.

[106] D. N. Bowser and B. S. Khakh, "Vesicular ATP is the predominant cause of intercellular calcium waves in astrocytes," *Journal of General Physiology*, vol. 129, no. 6, pp. 485–491, 2007.

[107] H. R. Parri, T. M. Gould, and V. Crunelli, "Spontaneous astrocytic Ca^{2+} oscillations in situ drive NMDAR-mediated neuronal excitation," *Nature Neuroscience*, vol. 4, no. 8, pp. 803–812, 2001.

[108] T. A. Fiacco and K. D. McCarthy, "Intracellular astrocyte calcium waves in situ increase the frequency of spontaneous AMPA receptor currents in CA1 pyramidal neurons," *Journal of Neuroscience*, vol. 24, no. 3, pp. 722–732, 2004.

[109] A. Serrano, N. Haddjeri, J. C. Lacaille, and R. Robitaille, "GABAergic network activation of glial cells underlies hippocampal heterosynaptic depression," *Journal of Neuroscience*, vol. 26, no. 20, pp. 5370–5382, 2006.

[110] P. Jourdain, L. H. Bergersen, K. Bhaukaurally et al., "Glutamate exocytosis from astrocytes controls synaptic strength," *Nature Neuroscience*, vol. 10, no. 3, pp. 331–339, 2007.

[111] M. Navarrete, G. Perea, D. F. de Sevilla et al., "Astrocytes mediate in vivo cholinergic-induced synaptic plasticity," *PLoS Biology*, vol. 10, no. 2, Article ID e1001259, 2012.

[112] C. Henneberger, T. Papouin, S. H. R. Oliet, and D. A. Rusakov, "Long-term potentiation depends on release of d-serine from astrocytes," *Nature*, vol. 463, no. 7278, pp. 232–236, 2010.

[113] R. Min and T. Nevian, "Astrocyte signaling controls spike timing-dependent depression at neocortical synapses," *Nature Neuroscience*, vol. 15, no. 5, pp. 746–753, 2012.

[114] G. R. J. Gordon, K. J. Iremonger, S. Kantevari, G. C. R. Ellis-Davies, B. A. MacVicar, and J. S. Bains, "Astrocyte-mediated distributed plasticity at hypothalamic glutamate synapses," *Neuron*, vol. 64, no. 3, pp. 391–403, 2009.

[115] A. V. Gourine, V. Kasymov, N. Marina et al., "Astrocytes control breathing through pH-dependent release of ATP," *Science*, vol. 329, no. 5991, pp. 571–575, 2010.

[116] T. A. Fiacco, C. Agulhon, S. R. Taves et al., "Selective stimulation of astrocyte calcium in situ does not affect neuronal excitatory synaptic activity," *Neuron*, vol. 54, no. 4, pp. 611–626, 2007.

[117] J. Petravicz, T. A. Fiacco, and K. D. McCarthy, "Loss of IP3 receptor-dependent Ca^{2+} increases in hippocampal astrocytes does not affect baseline CA1 pyramidal neuron synaptic activity," *Journal of Neuroscience*, vol. 28, no. 19, pp. 4967–4973, 2008.

[118] P. Devaraju, M. Y. Sun, T. L. Myers, K. Lauderdale, and T. A. Fiacco, "Astrocytic group I mGluR dependent potentiation of astrocytic glutamate and potassium uptake," *Journal of Neurophysiology*, vol. 109, no. 9, pp. 2404–2414, 2013.

[119] O. H. Porras, I. Ruminot, A. Loaiza, and L. F. Barros, "Na^+-Ca^{2+} cosignaling in the stimulation of the glucose transporter GLUT1 in cultured astrocytes," *Glia*, vol. 56, no. 1, pp. 59–68, 2008.

[120] Y. Bernardinelli, P. J. Magistretti, and J. Y. Chatton, "Astrocytes generate Na^+-mediated metabolic waves," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 41, pp. 14937–14942, 2004.

[121] A. Suzuki, S. A. Stern, O. Bozdagi et al., "Astrocyte-neuron lactate transport is required for long-term memory formation," *Cell*, vol. 144, no. 5, pp. 810–823, 2011.

[122] M. Simard and M. Nedergaard, "The neurobiology of glia in the context of water and ion homeostasis," *Neuroscience*, vol. 129, no. 4, pp. 877–896, 2004.

[123] M. de Roo, P. Klauser, and D. Muller, "LTP promotes a selective long-term stabilization and clustering of dendritic spines," *PLoS Biology*, vol. 6, no. 9, article e219, 2008.

[124] D. A. Richards, J. M. Mateos, S. Hugel et al., "Glutamate induces the rapid formation of spine head protrusions in hippocampal slice cultures," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 17, pp. 6166–6171, 2005.

[125] M. Navarrete and A. Araque, "Basal synaptic transmission: astrocytes rule!," *Cell*, vol. 146, no. 5, pp. 675–677, 2011.

[126] Y. Zuo, A. Lin, P. Chang, and W. B. Gan, "Development of long-term dendritic spine stability in diverse regions of cerebral cortex," *Neuron*, vol. 46, no. 2, pp. 181–189, 2005.

[127] P. J. Harrison, "The hippocampus in schizophrenia: a review of the neuropathological evidence and its pathophysiological implications," *Psychopharmacology*, vol. 174, no. 1, pp. 151–162, 2004.

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Review Article

GluN3A: An NMDA Receptor Subunit with Exquisite Properties and Functions

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N-methyl-D-aspartate receptors (NMDAR) are pivotal for synaptic plasticity and memory formation. Conventional NMDAR consist of heterotetrameric structures composed of GluN1 and GluN2 subunits. A third subunit, GluN3, can also assemble with NMDAR subunits giving a remarkable modification of their heteromeric structure, forming a "nonconventional" NMDAR. As a consequence, the stoichiometry and kinetic properties of the receptors are dramatically changed. Among the GluN3 family, the GluN3A subunit has been the focus of a large amount of studies during recent years. These studies reveal that GluN3A is transiently expressed during development and could play a role in the fine tuning of neuronal networks as well as associated diseases. Moreover, GluN3A distribution outside the postsynaptic densities, including perisynaptic astrocytes, places it at a strategic position to play an important role in the interactions between neurons and glial cells. This review highlights GluN3A properties and addresses its role in neurophysiology and associated pathologies.

1. Introduction

The glutamatergic network during postnatal development is under a tight regulation controlled by activity. This activity is mediated by postsynaptic ionotropic glutamate receptors (iGluR), NMDAR, and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors (AMPAR) as well as metabotropic glutamate receptors (mGluR) [1]. Indeed, activation of NMDAR promotes the insertion of AMPAR to the synapse, inducing long-term potentiation (LTP) [2]. In contrast, a reduction in NMDAR activation promotes the removal of AMPAR, provoking long-term depression (LTD) [3]. These functional synaptic plasticity properties are tightly linked with structural modifications such as enlargements and reductions in dendritic spine size or even formation and elimination of synapses [4–6]. These mechanisms are directly influenced by postsynaptic calcium (Ca^{2+}) [7], and Ca^{2+} influx is strongly controlled by NMDAR subunit composition [8, 9]. While GluN1 and GluN2 are the main subunits forming functional NMDAR [10–12], a third member of the family, GluN3, provides entirely new properties to NMDAR kinetics, especially with regard to Ca^{2+} permeability [13, 14]. When coassembled with GluN1 and GluN2 subunits, GluN3A exerts a dominant-negative effect on NMDAR properties [13, 15, 16]. Its presence dominates the properties of NMDAR resulting in a negative action on NMDAR, that is, insensitivity to magnesium (Mg^{2+}) and reduced Ca^{2+} influx. Predominately expressed during post-natal development, GluN3A has a strong impact on dendritic spine densities [17, 18] and is consequently a key player in controlling glutamatergic synaptic development and plasticity [19].

Recent advances in understanding glutamatergic synapse function and structure have also revealed astrocytes to be an active component of the synapse [20, 21]. Astrocytes participate in the modulation of synaptic transmission as well as in LTP through the release of glutamate and D-Serine in a Ca^{2+}-dependent manner [22, 23]. These gliotransmitters can act on neuronal GluN3-containing NMDAR located at extrasynaptic sites [22, 24]. Moreover, astrocytes also express GluN3-containing NMDAR [25] that could influence their intracellular Ca^{2+} homeostasis. Thus GluN3 could be crucial

for the bidirectional communication between astrocytes and neurons.

This review provides an up-to-date overview of recent findings on GluN3A. Moreover, particular focus will be made on its role in synapse dynamics, disease, and interactions between neurons and astrocytes.

2. Conventional NMDAR Channel Properties

NMDAR are comprised of seven subunits divided into three main subfamilies: the obligatory GluN1 subunit, four GluN2 subunits (A-D), and two GluN3 subunits (A-B). GluN1 and GluN2-containing receptors are the most abundant NMDAR complexes throughout the central nervous system (CNS). The GluN2 subunits determine channel properties and the subcellular localization of the receptor. NMDAR have characteristic properties unlike that of AMPAR and kainate receptor family, exhibiting a strong voltage-dependent block by Mg^{2+}, high permeability to Ca^{2+}, and slow gating kinetics. However, these properties can vary dramatically depending on the expression and the composition of the subunits within the receptor.

The activation of NMDAR requires the coordinated binding of glycine and glutamate to the ligand binding domain (LBS) site on GluN1 and GluN2 subunits, respectively. This triggers the conformation change of the receptor, allowing the flow of ions and depolarization of the postsynaptic site [26]. GluN2A-containing receptors display faster kinetics, a higher open probability, and faster deactivation kinetics compared with GluN2B-containing receptors [26, 27]. In addition, GluN2C-containing NMDAR exhibit unique properties of low conductance, low open probability, and low sensitivity to Mg^{2+} [28–30]. Furthermore, the presence of GluN2D-containing NMDAR leads to extremely slow decay times [26, 31]. In contrast, GluN3A-containing NMDAR bring about distinct nonconventional properties to the NMDAR complex.

3. GluN3A "Nonconventional" Properties

Since its discovery in 1995 by two independent groups [15, 16], GluN3A (then termed Chi-1 and NMDAR-L) has sparked great interest due to its particular properties within the NMDAR family. Although it shares a low sequence homology with GluN1 and GluN2 (~27%), it holds specific characteristics of the NMDAR family [15, 16]. Such features consist of (i) a large N-terminal extracellular domain with multiple glycosylation sites, (ii) three transmembrane domains (M1, M3 and M4), with M2 being a reentrant loop, (iii) a hydrophobic sequence just upstream of M1 within the N-terminal domain (NTD) known as the S1 region giving rise to the LBD, (iv) a large extracellular domain between M3 and M4 housing the S2 region, and (v) a unique C-terminal domain (CTD) of GluN3A that holds specific glycosylation and phosphorylation sites, differing from GluN1 and GluN2 subunits [15, 16]. More specifically, the M2 region has been described as controlling the ion selectivity of the glutamate-gated channels [32] due to the presence of a QNR site (glutamine(Q)/asparagine(N)/arginine(R)). In the case of GluN3A the presence of an arginine is adjacent to this site [15, 16]. Site directed mutagenesis indicates that the QNR site influences the flow of divalent ions, specifically controlling the permeability of Ca^{2+}, and influencing the Mg^{2+} block of recombinant NMDAR.

As with all other subunits of NMDAR, GluN3A also exhibits a bilobed extracellular domain, formed by the S1 region of the NTD and the S2 segment of the extracellular loop between M3 and M4 domains. This S1S2 segment forms the LBD for all subunits [33]. Both GluN1 and GluN3 subunits bind the coagonist glycine for NMDAR activation. GluN3A binds glycine at a much higher affinity than GluN1, almost 650 times better than GluN1 [33], which gives a unique profile that is selectively different from GluN1. In addition to glycine, D-serine also acts as an agonist at GluN3A subunits, again with a higher affinity than GluN1.

When GluN1 and GluN2 subunits coassemble, they form a core which regulates the channels permeation to ions [34–37]. When GluN3A is coexpressed with GluN1, this channel permeation pathway is also formed. The alignment of a ring of polar threonine residues in both GluN1/3A assemblies forms a constriction in the outer vestibule of the channel. This disturbs the chain of Ca^{2+} binding sites that usually facilitate the Ca^{2+} flux, thus reducing Ca^{2+} permeability [37], a characteristic of GluN3A-containing NMDAR.

3.1. Triheteromeric NMDAR Containing GluN3A. The distinct properties of NMDAR comprised of GluN1, GluN2, and GluN3A subunits have been consistently reported in various recombinant and transgenic systems [13, 38]. GluN3A forms stable biochemical complexes with the other NMDAR subunits [38]. NMDAR comprised of GluN1/2A or 2B in both low and physiological extracellular Ca^{2+} conditions induce just one large conductance state. The presence of GluN3A results in two distinct independent conductance states: the typical large conductance state, similar to what is found in conventional receptors, and the second significantly smaller, which exhibits a slight increase in mean opening time [14, 17, 38]. Ca^{2+} permeability in GluN3A-containing NMDAR is significantly reduced [13, 14, 38] due to the constriction of the outer vestibule [37]. Another prominent feature of GluN3A-containing receptors is the insensitivity to Mg^{2+} block at hyperpolarized potential: even with varying concentrations of Mg^{2+}, GluN3A prevents a Mg^{2+} block [13, 38] (Figure 1); these properties are further characterized in other reviews on GluN3; see [39–41]. Overall, these properties promote a reduction in NMDA-induced currents. In contrast, the amplitudes of NMDA-induced currents in GluN3A KO neurons are larger in cerebral cortical neurons compared to WT neurons [17]. However, this is only detected during development as in adulthood the levels reach normal values.

Functional NMDAR require the combination of one or two GluN1 subunits with either one or two of the GluN2 subunits; and/or a combination of GluN3 subunits [42, 43]. GluN1 is the obligatory subunit which is always present in functional NMDAR complexes [12, 29]. The tetrameric structure of NMDAR was suggested to be arranged in a couple of dimers in a 1-1-2-2 orientation (i.e., GluN1-GluN1

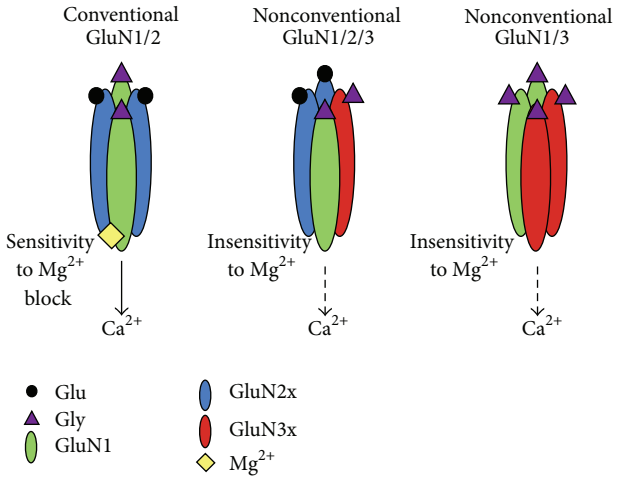

Figure 1: Schematic illustrating the conventional NMDAR containing GluN1 and GluN2 subunits (left), a nonconventional heterotrimeric NMDAR containing all three subunits (middle), and a glycine diheteromeric NMDAR containing GluN1 and GluN3 subunits (right). The main properties that are changed in these nonconventional NMDAR are the agonist binding, the insensitivity to Mg^{2+}, and the low permeability to Ca^{2+}. Thus GluN3-containing NMDAR exert a dominant-negative effect on NMDAR properties. Glu: glutamate, Gly: Glycine, Mg^{2+}: magnesium, and Ca^{2+}: calcium.

and GluN2-GluN2) heavily dependent on the final transmembrane domain and the CTD [44]. However, evidence now suggests that the GluN1 subunits are arranged in dimers and the GluN2 subunits are simply added to form the tetramer [45]. It still remains unclear how the addition of the GluN3 subunit fits into this stoichiometric formation. Although GluN3A can form functional NMDAR complexes with GluN1 and GluN2 subunits [46], it has been proposed that neither GluN2 or GluN3A subunits can form homo-oligomers, unlike the GluN1 subunit [47]. Evidence proposes a two step assembly process for NMDAR containing GluN3A; first a GluN1 subunit associates with either a GluN2 or GluN3 subunit into heterodimers. Secondly, these two heterodimers form the final tetrameric subunit arrangement [47]. The precise stoichiometry remains largely unknown, and it could differ in brain region, cell type and during development. In addition to the heterotrimeric glutamate receptors, evidence suggests a possible excitatory glycine receptor in the form of a diheteromeric GluN1/3 receptor.

3.2. Diheteromeric NMDAR Containing GluN3A. Interestingly, GluN1 can coassemble with either GluN3A and/or GluN3B and form functional excitatory glycine receptors [46, 48]. These NMDAR are not activated by glutamate. However, activation of these receptors by glycine leads to extremely low permeability to Ca^{2+}, low sensitivity to Mg^{2+}, and triggers bursts of firing [49, 50]. As both the GluN1 and GluN3 subunits have a glycine binding site, it would be expected that binding of glycine to both subunits is sufficient for receptor activation. However, coapplication of Zinc (Zn^{2+}), which usually acts on GluN2 subunits [51], may also act as both a potent positive modulator and an agonist at these GluN1/3 NMDAR [52]. The full extent of expression of these GluN1/3A glycine excitatory receptors *in vivo* remains largely unknown and very little literature has focused on the impact of the GluN3B subunit on NMDAR function. Two possibilities proposed in a review by Pachernegg et al., [40] suggested that glycine could be saturated at these receptors and provoke a more depolarized state, implicating action potentials and synaptic transmission. Furthermore, their effects presynaptically could reduce firing frequency and postsynaptically induce an increase in evoked potentials [40]. Further studies are needed to elucidate a role of these GluN1/3 receptors *in vivo*.

3.3. Membrane Targeting of GluN3A. Correct assembly and trafficking of the NMDAR complex are critical for functional surface expression. As with GluN2 subunits, GluN3A subunits are dependent on the coassembly with GluN1 subunits to be expressed at the surface membrane [14, 53]. Furthermore, GluN2 subunits contain a PDZ domain in their CTD that interacts with PSD95/SAP102 scaffolding proteins [26] influencing NMDAR anchoring and stabilization within the postsynaptic density. To date it appears that like GluN1 [54], GluN3A does not contain a PDZ domain and therefore would require the coassembly with GluN2 subunits to be targeted to the postsynaptic density (PSD).

GluN3A and GluN1-1a subunits are assembled at early stages in the biosynthetic pathway at the level of the endoplasmic reticulum (ER). Only when GluN3A is coassembled with GluN1-1a can it then exit the ER and be trafficked to the surface. In the absence of GluN1-1a, GluN2 and GluN3 are retained in the ER, strongly suggesting that GluN1-1a is necessary for receptor trafficking and efficient membrane insertion [14].

All NMDAR subunits contain some form of an ER retention signal in their CTD. It could be speculated that the correct folding and coassembly of the subunits mask the ER retention signals allowing for GluN1 to guide the trafficking of the receptors to the surface [14].

4. Unique Expression Pattern

NMDAR subunits have distinct expression patterns [26] that tightly regulate the development of synapses and drive synaptic plasticity to refine the neuronal network. GluN3A is no exception and perhaps is an influential mediator in shaping synaptic connections. It has a unique developmental expression pattern within several brain regions [55]. Initially expressed in the thalamus, entorhinal cortex, subiculum and several layers of the neocortex, its expression intensifies during the first postnatal weeks in the CA1 field of the hippocampus and in the thalamus. It has also been detected at varying degrees in the spinal cord, medulla, pons, tegmentum and hypothalamus [15, 16, 56]. Its endogenous natural expression pattern has been thoroughly described in rodent, human and macaques [15, 57–59]. Detected as early as embryonic day 15, its expression increases in the first two weeks of postnatal development and thereafter sharply declines from

P16 and remains low in adulthood [15, 16, 38, 55]. The retina, olfactory tract, amygdala, and some regions of the cortex do retain low levels of GluN3A into adulthood [58, 60], but its functions remain unknown. This is suggesting that GluN3A could control NMDAR function in a time-dependent manner during critical periods of development. In recent years, studies have confirmed GluN3A expression postsynaptic sites within the PSD but situated at the periphery, at perisynaptic, extrasynaptic, and presynaptic sites, as well as on astrocytes [14, 25, 61] (its functions on astrocytes will be discussed later in Section 8). GluN3B has different spatial and temporal expression patterns and can act independently from GluN3A [42, 49, 50, 62]. GluN3B mRNA expression levels are elevated through development and maintained into adulthood, with a distribution in the pons, midbrain, medulla and spinal cord [42]. Therefore it appears that GluN3A and GluN3B have distinctly different roles in the brain during development and adulthood.

5. Which Intracellular Proteins Interact with GluN3A?

The CTD of GluN3A is different from that of GluN1 or GluN2. Its dominant-negative properties on receptor kinetics could have downstream effects on intracellular signaling pathways, protein translation and cytoskeletal protein arrangement. One protein that directly interacts with GluN3A is Protein Phosphatase 2A (PP2A) [63, 64]. PP2A is one of the major serine-threonine phosphatases existing as a heterotrimeric enzyme complex in neurons [65]. This interaction between PP2A and GluN3A drives an increase in the activity levels of the enzyme [64]. This tight interaction is abolished upon NMDAR stimulation, causing a dissociation of PP2A from GluN3A and resulting in the dephosphorylation of Ser 897 on the GluN1 subunit [64]. As PP2A has been implicated in LTD, this interaction between PP2A and GluN3A could implicate the level of LTD by maintaining a high level of activity of PP2A. However, the effects of overexpressing GluN3A in transgenic mice showed no changes in LTD [18]. Interestingly, PP2A expression, like GluN3A, is developmentally regulated, peaking around P8 and declining from P12 to a low level in adulthood.

GluN3A also interacts with MAP1B [66] and MAP1-s [67]. MAP1 family proteins are important in the development of axons and dendrites [68]. By binding to the microtubule lattice, they could drive the trafficking of GluN3A-containing NMDAR to peri- and extrasynaptic sites. However, their binding site on the CTD of GluN3A overlaps with PP2A binding site [63], suggesting that there could be a potential competition or a reciprocal binding pattern of the two to GluN3A. MAP1 proteins could traffic GluN3A to its synaptic location and once it dissociates, PP2A can bind to exert its effects. Future experiments are needed to confirm the role of these interactions.

A third report also demonstrates the interaction of plectrin, CARP-1 (cell cycle and apoptosis regulatory protein 1, a perinuclear phosphoprotein [69]) and GPS2 (G protein suppressor 2) with GluN3A, but the exact role these three proteins exert on GluN3A function is still unknown [70]. As plectrin is a large scaffolding protein that binds to several cytoskeletal proteins, it may play a role in the distribution, localization and clustering of GluN3A to appropriate synaptic sites. Although experimental evidence is lacking, we could suggest GPS2 to be linked with NMDAR activation through the suppression of RAS/MAPK-mediated signaling. CARP-1 function in the CNS has never been confirmed outside the CNS and it has been linked to breast carcinoma cells as a protein increasing apoptosis [70]. Again, further experiments will clarify the precise role of these interactions.

Interestingly, GluN3A interacts with the small GTPase Ras homologue enriched in brain (Rheb). This member of the Ras superfamily of GTP-binding proteins stimulates the activity of the mTOR signaling complex 1 (mTORC1), resulting in protein synthesis [71]. This pathway is heavily involved in the fragile X syndrome, in which the absence of the fragile X mental retardation protein (FMRP) causes cognitive deficits in humans [72, 73]. FMRP activity is dependent on its phosphorylation, under the bidirectional control of ribosomal protein S6 kinases and PP2A, in which the activity of both proteins are modulated by the mTOR pathway [74, 75]. It appears that Rheb interacts to the same region of the CTD of GluN3A [73], as PP2A [63, 64]. It is possible that GluN3A could sequester synaptic Rheb, and therefore act as a break on mTOR activity. Consequently, overexpressing GluN3A could prevent the activation of mTOR, and in contrast silencing GluN3A would promote the activity of mTOR. This could have important regulatory consequences on spine and synapse dynamics.

5.1. Intracellular Proteins Targeting GluN3A Endocytosis. GluN3A expression sharply declines after P16 in rodents. Its endocytosis is therefore tightly regulated. A report by Pérez-Otaño et al., [24] in 2006 was the first to highlight the activity dependent interaction of PACSIN1/syndapin, an endocytotic adaptor protein, with GluN3A [24]. More recently, identification of a specific motif within the CTD of GluN3A has also been linked to the endocytotic complex [76].

PACSIN1 is a brain-derived protein involved in synaptic vesicle endocytosis. The conserved Src homology 3 (SH3) domain on PACSIN1 enables protein-protein interactions with endocytotic proteins such as N-WASP and dynamin [77, 78]. The presence of a coiled-coil alpha helical domain on PACSIN1 allows for homodimeric and homotetrameric interactions, this provides multiple SH3 domains that can bind with several endocytotic proteins simultaneously [79, 80]. Activity drives the interaction of PACSIN1 with the CTD of GluN3A, promoting its internalization [24]. Disrupting PACSIN1 expression consequently disrupts the surface expression levels of GluN3A. PACSIN1 phosphorylation has also been linked to Rac1 activation, affecting spine formation [81]. This could occur when Rheb and PP2A are dissociating from GluN3A, exposing the CTD for PACSIN1, leading to activation of the mTOR and Rac1 pathway. PACSIN1 offers a mechanistic way to disrupt GluN3A surface expression.

Another unique portion of the CTD is a conserved YWL motif. Src phosphorylates the tyrosine Y971 residue, promoting the interaction with the $\mu 2$ subunit of AP2 (activating protein 2, involved in clathrin-mediated endocytosis), recruiting the endocytotic machinery and consequently triggering the removal of GluN3A from the surface [76]. By mutating this YWL motif, endocytosis of GluN3A is dramatically prevented, enhancing the surface expression of GluN3A. In contrast, stimulation of endogenous Src via PACAP-38 (pituitary adenylate cyclase activating peptide shown to activate Src in CA1 cells [76, 82]) promotes the internalization of GluN3A and an overall decrease in surface expression [76]. This motif does not conform to previously identified tyrosine-based endocytotic motifs, usually comprising YXXØ (where X is any amino acid and Ø a bulky hydrophobic residue [76]) and dileucine motifs that bind to AP2. GluN2B contains such a motif and phosphorylation of the tyrosine residue by Fyn inhibits AP2 binding and prevents GluN2B internalization [83]. Conversely phosphorylation by Src also inhibits the endocytosis of GluN2A, thus suggesting that tyrosine phosphorylation drives the removal of GluN3A while maintaining the surface expression of GluN2 subunits. What remains unclear is that if PACSIN1 and the phosphorylation of the YWL motif act as two independent systems or if they work in conjunction with one another to assist the removal of GluN3A.

6. GluN3A Effects on Dendritic Spine Dynamics

Dendritic spines are very dynamic structures that undergo a continuous process of formation and elimination that is particularly active during development [84]. These mechanisms are also modulated by neuronal activity and notably regulated in an NMDAR-dependent manner [85–87]. An implication of GluN3A in these mechanisms was not unexpected as GluN3A expression peaks at a time when this structural plasticity is most distinguished.

Indeed, in the wake of GluN3A discovery, Das et al., 1998 [17] reported that mice lacking GluN3A showed an increase in spine density in cortical neurons at P19, with a tendency for spine heads to be enlarged and elongated [17]. This coincided with an enhancement of NMDAR responses and an absence of the smaller conductance state seen in GluN3A positive neurons. This was the first evidence to suggest that the absence of GluN3A during its endogenous expression window can affect spine dynamics.

In contrast, ten years after this initial paper Roberts et al., 2009 [18] showed in a transgenic mouse model overexpressing GluN3A (beyond its natural time window), that spine density is reduced. This reduction mostly concerns mature mushroom-shaped spines that also exhibit slightly smaller PSD length [18].

A detailed review by Henson et al. in 2010 [41] proposed two hypotheses to account for these observations: the "synaptic brake hypothesis" and the "synaptic elimination hypothesis." However, these two hypotheses are still to be explored. The synaptic brake hypothesis suggests that GluN3A-containing receptors limit synapse formation, and its dominant-negative mechanisms on current and Ca^{2+} influx prevent synapse plasticity [41]. Only their time- and activity dependent removal will then allow conventional NMDAR to drive the maturation of the synapse. This interplay between GluN3A-containing receptors and mature GluN1/GluN2-containing receptors will firstly prevent early maturation of synapses, and secondly only strengthen and stabilize the appropriate synapses, leading to a properly formed neuronal circuitry [41].

On the contrary, the synaptic elimination hypothesis proposes that GluN3A-containing receptors act as a tagging mechanism to label weak and inactive synapses, which will promote the retraction, and final elimination of the spine and synapse [41]. Those spines receiving sufficient activity will therefore drive the removal of GluN3A via its internalization mechanisms and drive the insertion of mature NMDAR.

These two hypotheses are still open and future experiments should determine (i) whether GluN3A does in fact prevent spine formation, or (ii) whether expression of GluN3A promotes spine instability and elimination, and prevents plasticity at individual spines. Additionally, the actual downstream mechanisms underlying GluN3A influence on spine formation or elimination still remain to be identified.

Understanding the influence of GluN3A on spine dynamics could provide important new insights on its implications in neurodegenerative diseases.

7. GluN3A in Disease

Abnormalities in dendritic spine density, turnover, formation and elimination have been implicated in disorders from mental retardation to Huntington's disease (HD) and Alzheimer's disease [88]. Furthermore, NMDAR hyperactivity or hypoactivity are associated with several neurological conditions such as Alzheimer's disease, Parkinson's disease, schizophrenia, depression and ischemia [26, 89–91]. Growing evidence in recent years has implicated GluN3A in various disorders of the CNS. With respect to NMDAR hypofunction and dendritic spine abnormalities, see reviews [40, 41]. In the past year, GluN3A has been directly implicated in HD and in cocaine addiction, furthermore there are additional reports confirming GluN3A neuroprotective properties in ischemia and in striatal lesions.

HD is a debilitating neurodegenerative disease in which patients carry a mutation in the Huntingtin (mHtt) protein [92, 93]. An expansion of the polyglutamine chain in mHtt forms aggregates leading to synaptic failure and neuronal death, predominately in medium spiny neurons (MSN) of the striatum [94, 95]. Htt has numerous binding partners associated with roles in transcriptional regulation, intracellular trafficking and cytoskeletal organization [96]. PACSIN1 is one such protein, which has a high affinity to interact with mHtt, the longer the polyQ chain the stronger the interaction [97]. This interaction sequesters PACSIN1 from its usual synaptic location. The redistribution of PACSIN1 promotes the reinsertion of GluN3A-containing NMDAR at the synaptic plasma membrane in HD mouse models [97].

This is consistent with analyses of postmortem tissue from human HD patients, in which there is an increase in GluN3A levels [97]. Motor and cognitive deficits as well as decreases in spine density and striatal atrophy of MSNs are rescued in HD mouse models lacking GluN3A [97]. This can provide options to target either GluN3A or PACSIN1 as a therapy in early stages of HD.

An interesting new report has also linked GluN3A with altered NMDAR transmission in cocaine-induced addiction. Cocaine exposure drives a redistribution of AMPAR and NMDAR on dopamine neurons in the ventral tegmental area [98–100]. Emphasis in recent years has focused on the AMPAR switch from Ca^{2+}-impermeable GluA2-containing receptors to Ca^{2+}-permeable GluA2-lacking receptors induced by a single injection of cocaine [101]. This also coincides with an increase in AMPAR/NMDAR ratio, caused by enlarged AMPAR-mediated EPSC amplitudes together with reduced amplitudes of NMDAR-EPSCs [100]. The reduction of NMDAR-EPSCs amplitude is the result of the reinsertion of GluN2B as well as GluN3A-containing NMDAR [102], this accounts for the reduction in outward rectification and alteration in Mg^{2+} block. Indeed, GluN3A KO mice lack this effect. In addition, DA neurons from these mice and from neurons transfected with a shRNA for GluN3A failed to exhibit cocaine-evoked plasticity of NMDA and AMPA receptors [102]. Furthermore, it was found that NMDAR transmission could be reestablished by changing the ratio of GluN1/2B/3A to GluN1/2A through activation of mGluR1 [102]. This offers prospects for targeting either GluN3A or mGluR1 receptors to restore normal synaptic transmission in drug-addictive behaviour.

As previously discussed, GluN3A could implicate some neuroprotective properties [41, 103]. Excitotoxicity that occurs in disease states is often the result of an overactivation of Ca^{2+}-permeable NMDAR. The dominant-negative effects of GluN3A on Ca^{2+} permeability could be of interest to reduce Ca^{2+} influx and consequently prevent cell death.

Indeed, in transgenic GluN3A overexpressing mice model, striatal MSN-induced death via the neurotoxin 3-nitropropionic acid (3-NP) was significantly prevented [104]. These mice also show less dystonia and an improvement in hindlimb clasping and locomotor ability. Synaptic ver sus extrasynaptic NMDAR promote different survival or cell death pathways, respectively [26]. Synaptic GluN2A-containing NMDAR predominately protect against cell death [105]. However, extrasynaptic GluN2B-containing NMDAR appear to promote cell death pathways [106]. This study shows a tendency for the formation of GluN1/2B/3A-containing receptors to be located at extrasynaptic sites, and the presence of GluN3A prevents cell death signaling. This is further characterised by a decrease in the activation of calpains, proteases that cleave fodrin, and striatal-enriched protein tyrosine phosphatases that trigger major cell death signaling pathways [104]. However, GluN3A cannot account for complete neuroprotection as only 52% of cells survived in this mouse model [104]. Ischemia and hypoxia induce an endogenous upregulation of GluN3A in rat hippocampal and prefrontal neurons [107], further supporting the hypothesis that GluN3A-containing NMDAR have neuroprotective properties. Expression of GluN3A reduces the Ca^{2+} influx as well as hydroxyl radicals and nitric oxide levels after glutamate insult.

These studies provide evidence that GluN3A can act in a neuroprotective manner [103]. This subunit that is usually expressed only during development, appears to be upregulated in response to toxic insult induced by excessive glutamate activation of NMDAR. Perhaps the therapeutic benefit of this could be to target a quick and efficient increase in GluN3A expression that can alleviate the neuronal death by preventing Ca^{2+} influx and cell death.

8. Perspectives: GluN3A and Astrocytes

In addition to neurons, GluN3A has also been reported to be expressed in astrocytes and could consequently participate in their bidirectional communication. Astrocytes are the most important glial cell type interacting with neurons, especially at glutamatergic synapses [20]. Astrocytes send fine cellular processes in the vicinity of the synapses. These so-called perisynaptic astrocytic processes express glutamate receptors and transporters important for the bidirectional communication with neurons [108]. In particular, they express mGluR [109, 110] that are mainly responsible for the astrocyte-to-neuron transmission [111]. mGluR activation following synaptic release of glutamate triggers intracellular Ca^{2+} elevation in astrocytes [111, 112] which in turn can induce the release of several transmitters from astrocytes (e.g., gliotransmitters) [20]. Among these gliotransmitters glutamate [113] and D-serine [114] have been identified and both of them can bind to neuronal iGluR including NMDAR [22, 23] giving rise to the concept of a tripartite synapse [115]. The effects of gliotransmitters probably occur in the periphery of the synapse on extrasynaptic receptors [22, 111]. This fits with the localization of GluN3A which is primarily expressed at extrasynaptic sites [24]. These extrasynaptic GluN3A-containing receptors could therefore participate in the mechanisms of gliotransmission.

In addition to mGluR, astrocytes also express iGluR. The presence of AMPA and Kainate receptors on astrocytes have already been described [116]. More recently, NMDAR have been detected on astrocytes as well [117]. However, the exact role of iGluR on astrocytes remains unclear, although we can suggest their participation in neuron-to-glia communication. Glutamate application onto astrocytes evokes three types of responses: an AMPAR, an NMDAR and a glutamate transporter response [118]. Furthermore, axonal stimulations in cortical layer IV–VI give rise to inward currents in astrocytes in layer II, demonstrating that iGluR are involved in neuronal-to-astrocyte communication [118]. All seven subunits of NMDAR were confirmed to be expressed at varying levels in both fetal and adult human astrocytes [25].

Supporting the notion that mGluR mediates a majority of glutamatergic Ca^{2+} signaling in cortical astrocytes [111, 119], activation of NMDAR can facilitate a rise in intracellular Ca^{2+} in astrocytes [25, 120]. However, Palygin et al. in 2011

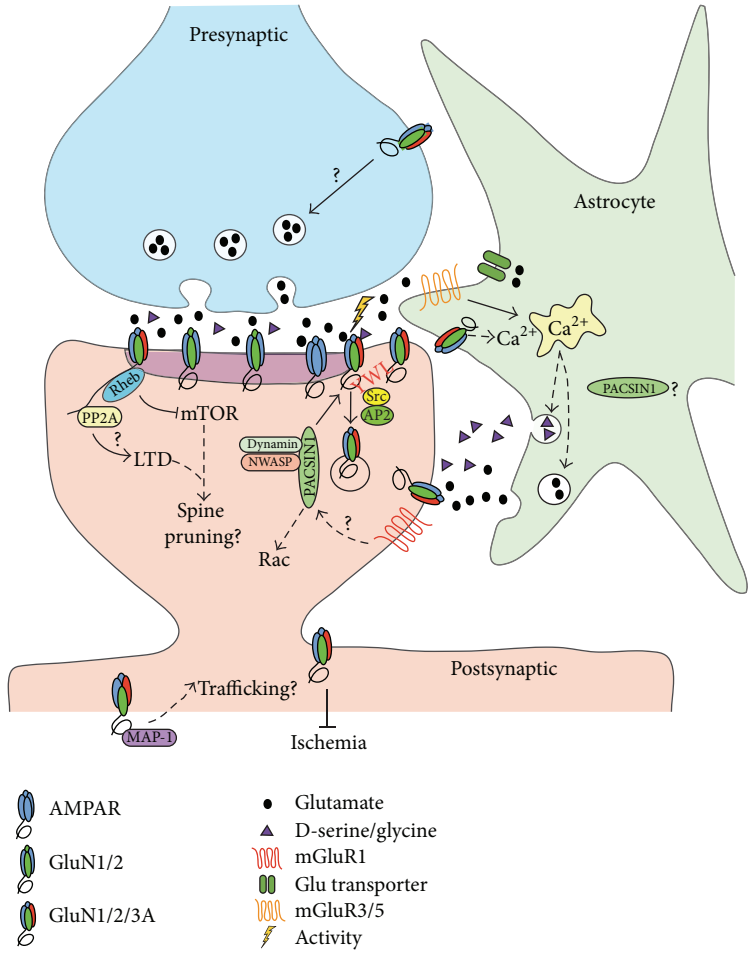

FIGURE 2: Schematic of the proposed distribution of GluN3A-containing NMDAR in the pre- and postsynaptic sites as well as on astrocytic processes. Endocytosis of GluN3A is activity dependent, driving PACSIN1 binding and Src phosphorylation of the YWL motif to selectively internalize GluN3A-containing NMDAR. Other molecular interactions with GluN3A could play roles in trafficking of the receptor subunit and dendritic spine dynamics. The roles of GluN3A in astrocytic processes still remain unclear but it could potentially exert an attenuating effect on calcium entry in a reciprocal manner to mGluR calcium entry.

[120] reported that these NMDAR on astrocytes display lower Ca^{2+} permeability and weak Mg^{2+} block, suggesting the presence of a GluN3 subunit. Thus, the stoichiometry of these NMDAR could contain GluN1/GluN2C or 2D and GluN3A subunits. The application of D-serine or glycine evoked only small responses, while the responses to NMDA and D-serine are large, this outrules the presence of excitatory glycine GluN1/3 receptors and suggests a heterotrimeric composition of astrocytic NMDAR [120].

Why would GluN3A-containing NMDAR be expressed on astrocytes? One important property of GluN3A-containing NMDAR is their lack of Mg^{2+} sensitivity. As astrocytes and astrocytic processes are hyperpolarized, it therefore might be important to express NMDAR that are not fully blocked under resting conditions. In addition, as mentioned above, Ca^{2+} excitability of astrocytes is pivotal for their interaction with neurons and NMDAR are known to potentiate Ca^{2+} responses in astrocytes [111]. It is generally accepted that astrocytic Ca^{2+} signals evoked by glutamatergic activity are mainly mediated by mGluRs. However, mGluR expression in astrocytes decreases during development [110] as does GluN3A. It might be plausible that GluN3A exert an attenuating effect on Ca^{2+} when mGluR are high and inversely modulate astrocytic Ca^{2+} signals through development.

Furthermore, perisynaptic astrocytic processes appear to be highly plastic structures, see [21, 121]. This form of structural plasticity suggests astrocytes to be active players in the mechanisms of synapse formation, stabilization [122, 123] and maturation [124]. Interestingly, NMDAR are important in this process [123], as well as in the increased synaptic coverage by perisynaptic astrocytic processes observed after LTP [125]. In fact, the Rac1 pathway in hippocampal astrocytes [122] as well as in Bergman glia (astrocytes of the cerebellum) [126] have been identified as the mechanism driving astrocytic movements [127]. Rac1 is in turn well known to regulate spine dynamics [128, 129]. As described above, Rac1 could be indirectly linked with GluN3A via PACSIN1, suggesting a possible role for astrocytic GluN3A in mediating effects

of synaptic structure, and function through the dynamics of astrocytic processes.

9. Discussion

Our understanding of the role of GluN3A in neurons, in astrocytes, and of its role in the tripartite synapse in general is slowly emerging (Figure 2).

What does the future hold for GluN3A? Pharmacological agents that specifically act on GluN3A do not yet exist. To achieve this, more research would be needed to confirm the precise stoichiometry of GluN3A-containing NMDAR in neurons and astrocytes. This is a difficult issue as the stoichiometry can be dependent on splice variants. Furthermore, GluN1 has eight functionally distinct splice variants in total, in which GluN1-1a appears to easily coassemble with GluN3A [14]. In fact, there is an additional splice variant of GluN3A, a longer version that consists of additional 60 amino acids in the CTD and is only detected at present in rodent [56]. The precise role for this variant is unknown.

Gathering further information on intracellular proteins that interact with GluN3A could also be beneficial in terms of our understanding of plasticity and dendritic spine dynamics. The most encouraging results to date are the interactions with PP2A and Rheb, both of which can have significant downstream effects, notably regarding LTD or LTP. Furthermore, while Rheb is bound to GluN3A the mTOR pathway is being suppressed, potentially affecting protein translation and preventing the maturation of the synapse. Further research is required to determine if astrocytic GluN3A drives the same interactions. PACSIN1, which has been implicated in GluN3A activity dependent removal and has a specific role in the pathology of HD, is also expressed in astrocytes [130]. Understanding its function in astrocytes could shed new light on the possible role of GluN3A in gliotransmission. Finally, elucidating the role of GluN3A in spine dynamics could reveal its role in structural and functional plasticity. Enhanced GluN3A expression promotes a decrease in spine density but the actual mechanism responsible for this spine density reduction is not known. As with HD, it appears that the reinsertion of GluN3A can promote spine density reduction even in the adult brain. It would be interesting to understand whether the reactivation or reinsertion of GluN3A into the synapse could reactivate a critical period as seen in early development. Furthermore, it would be important to know whether the expression of GluN3A is able to affect neuron-glia interactions and whether this is modulated during development as already seen with mGluR [110].

Overall, the glutamatergic tripartite synapse concept has an exciting future ahead. Piecing together the role of the ionotropic and metabotropic glutamate receptor families as well as transporters and transmitters will offer potential therapeutic interventions to target many synaptopathies.

Conflict of Interests

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

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References

[1] R. C. Malenka and R. A. Nicoll, "NMDA-receptor-dependent synaptic plasticity: multiple forms and mechanisms," *Trends in Neurosciences*, vol. 16, no. 12, pp. 521–527, 1993.

[2] R. Malinow, Z. F. Mainen, and Y. Hayashi, "LTP mechanisms: from silence to four-lane traffic," *Current Opinion in Neurobiology*, vol. 10, no. 3, pp. 352–357, 2000.

[3] R. C. Malenka and M. F. Bear, "LTP and LTD: an embarrassment of riches," *Neuron*, vol. 44, no. 1, pp. 5–21, 2004.

[4] Q. Zhou, K. J. Homma, and M.-M. Poo, "Shrinkage of dendritic spines associated with long-term depression of hippocampal synapses," *Neuron*, vol. 44, no. 5, pp. 749–757, 2004.

[5] M. Matsuzaki, N. Honkura, G. C. R. Ellis-Davies, and H. Kasai, "Structural basis of long-term potentiation in single dendritic spines," *Nature*, vol. 429, no. 6993, pp. 761–766, 2004.

[6] N. Toni, P.-A. Buchs, I. Nikonenko, C. R. Bron, and D. Muller, "LTP promotes formation of multiple spine synapses between a single axon terminal and a dendrite," *Nature*, vol. 402, no. 6760, pp. 421–425, 1999.

[7] C. J. Mcbain and M. L. Mayer, "N-methyl-D-aspartic acid receptor structure and function," *Physiological Reviews*, vol. 74, no. 3, pp. 723–760, 1994.

[8] E. M. Quinlan, D. H. Olstein, and M. F. Bear, "Bidirectional, experience-dependent regulation of N-methyl-D-aspartate receptor subunit composition in the rat visual cortex during postnatal development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 22, pp. 12876–12880, 1999.

[9] A. Barria and R. Malinow, "Subunit-specific NMDA receptor trafficking to synapses," *Neuron*, vol. 35, no. 2, pp. 345–353, 2002.

[10] M. Sheng, J. Cummings, L. A. Roldan, Y. N. Jan, and L. Y. Jan, "Changing subunit composition of heteromeric NMDA receptors during development of rat cortex," *Nature*, vol. 368, no. 6467, pp. 144–147, 1994.

[11] R. Dingledine, K. Borges, D. Bowie, and S. F. Traynelis, "The glutamate receptor ion channels," *Pharmacological Reviews*, vol. 51, no. 1, pp. 7–61, 1999.

[12] S. F. Traynelis, L. P. Wollmuth, C. J. McBain et al., "Glutamate receptor ion channels: structure, regulation, and function," *Pharmacological Reviews*, vol. 62, no. 3, pp. 405–496, 2010.

[13] G. Tong, H. Takahashi, S. Tu et al., "Modulation of NMDA receptor properties and synaptic transmission by the NR3A subunit in mouse hippocampal and cerebrocortical neurons," *Journal of Neurophysiology*, vol. 99, no. 1, pp. 122–132, 2008.

[14] I. Pérez-Otaño, C. T. Schulteis, A. Contractor et al., "Assembly with the NR1 subunit is required for surface expression of NR3A-containing NMDA receptors," *Journal of Neuroscience*, vol. 21, no. 4, pp. 1228–1237, 2001.

[15] A. M. Ciabarra, J. M. Sullivan, L. G. Gahn, G. Pecht, S. Heinemann, and K. A. Sevarino, "Cloning and characterization of χ-1: a developmentally regulated member of a novel class of the

ionotropic glutamate receptor family," *Journal of Neuroscience*, vol. 15, no. 10, pp. 6498–6508, 1995.

[16] N. J. Sucher, S. Akbarian, C. L. Chi et al., "Developmental and regional expression pattern of a novel NMDA receptor-like subunit (NMDAR-L) in the rodent brain," *Journal of Neuroscience*, vol. 15, no. 10, pp. 6509–6520, 1995.

[17] S. Das, Y. F. Sasaki, T. Rothe et al., "Increased NMDA current and spine density in mice lacking the NMDA receptor subunit NR3A," *Nature*, vol. 393, no. 6683, pp. 377–381, 1998.

[18] A. C. Roberts, J. Díez-García, R. M. Rodriguiz et al., "Downregulation of NR3A-containing NMDARs is required for synapse maturation and memory consolidation," *Neuron*, vol. 63, no. 3, pp. 342–356, 2009.

[19] T. Yuan and C. Bellone, "Glutamatergic receptors at developing synapses: the role of GluN3A-containing NMDA receptors and GluA2-lacking AMPA receptors," *European Journal of Pharmacology*, 2013.

[20] A. Volterra and J. Meldolesi, "Astrocytes, from brain glue to communication elements: the revolution continues," *Nature Reviews Neuroscience*, vol. 6, no. 8, pp. 626–640, 2005.

[21] A. Reichenbach, A. Derouiche, and F. Kirchhoff, "Morphology and dynamics of perisynaptic glia," *Brain Research Reviews*, vol. 63, no. 1-2, pp. 11–25, 2010.

[22] T. Fellin, O. Pascual, S. Gobbo, T. Pozzan, P. G. Haydon, and G. Carmignoto, "Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors," *Neuron*, vol. 43, no. 5, pp. 729–743, 2004.

[23] A. Panatier, D. T. Theodosis, J.-P. Mothet et al., "Glia-derived d-serine controls NMDA receptor activity and synaptic memory," *Cell*, vol. 125, no. 4, pp. 775–784, 2006.

[24] I. Pérez-Otaño, R. Luján, S. J. Tavalin et al., "Endocytosis and synaptic removal of NR3A-containing NMDA receptors by PACSIN1/syndapin1," *Nature Neuroscience*, vol. 9, no. 5, pp. 611–621, 2006.

[25] M.-C. Lee, K. K. Ting, S. Adams, B. J. Brew, R. Chung, and G. J. Guillemin, "Characterisation of the expression of NMDA receptors in human astrocytes," *PLoS ONE*, vol. 5, no. 11, Article ID e14123, 2010.

[26] A. Sanz-Clemente, R. A. Nicoll, and K. W. Roche, "Diversity in NMDA receptor composition: many regulators, many consequences," *Neuroscientist*, vol. 19, no. 1, pp. 62–75, 2013.

[27] J. A. Gray, Y. Shi, H. Usui, M. J. During, K. Sakimura, and R. A. Nicoll, "Distinct modes of AMPA receptor suppression at developing synapses by GluN2A and GluN2B: single-cell NMDA receptor subunit deletion in vivo," *Neuron*, vol. 71, no. 6, pp. 1085–1101, 2011.

[28] M. Farrant, D. Feldmeyer, T. Takahashi, and S. G. Cull-Candy, "NMDA-receptor channel diversity in the developing cerebellum," *Nature*, vol. 368, no. 6469, pp. 335–339, 1994.

[29] H. Monyer, R. Sprengel, R. Schoepfer et al., "Heteromeric NMDA receptors: molecular and functional distinction of subtypes," *Science*, vol. 256, no. 5060, pp. 1217–1221, 1992.

[30] T. Ishii, K. Moriyoshi, H. Sugihara et al., "Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits," *Journal of Biological Chemistry*, vol. 268, no. 4, pp. 2836–2843, 1993.

[31] S. L. C. Brothwell, J. L. Barber, D. T. Monaghan, D. E. Jane, A. J. Gibb, and S. Jones, "NR2B- and NR2D-containing synaptic NMDA receptors in developing rat substantia nigra pars compacta dopaminergic neurones," *Journal of Physiology*, vol. 586, no. 3, pp. 739–750, 2008.

[32] B. Sommer, M. Köhler, R. Sprengel, and P. H. Seeburg, "RNA editing in brain controls a determinant of ion flow in glutamate-gated channels," *Cell*, vol. 67, no. 1, pp. 11–19, 1991.

[33] Y. Yao and M. L. Mayer, "Characterization of a soluble ligand binding domain of the NMDA receptor regulatory subunit NR3A," *Journal of Neuroscience*, vol. 26, no. 17, pp. 4559–4566, 2006.

[34] T. Kuner, L. P. Wollmuth, A. Karlin, P. H. Seeburg, and B. Sakmann, "Structure of the NMDA receptor channel M2 segment inferred from the accessibility of substituted cysteines," *Neuron*, vol. 17, no. 2, pp. 343–352, 1996.

[35] A. I. Sobolevsky, L. Rooney, and L. P. Wollmuth, "Staggering of subunits in NMDAR channels," *Biophysical Journal*, vol. 83, no. 6, pp. 3304–3314, 2002.

[36] A. I. Sobolevsky, C. Beck, and L. P. Wollmuth, "Molecular rearrangements of the extracellular vestibule in NMDAR channels during gating," *Neuron*, vol. 33, no. 1, pp. 75–85, 2002.

[37] A. Wada, H. Takahashi, S. A. Lipton, and H.-S. V. Chen, "NR3A modulates the outer vestibule of the "NMDA" receptor channel," *Journal of Neuroscience*, vol. 26, no. 51, pp. 13156–13166, 2006.

[38] Y. F. Sasaki, T. Rothe, L. S. Premkumar et al., "Characterization and comparison of the NR3A subunit of the NMDA receptor in recombinant systems and primary cortical neurons," *Journal of Neurophysiology*, vol. 87, no. 4, pp. 2052–2063, 2002.

[39] C.-M. Low and K. S.-L. Wee, "New insights into the not-so-new NR3 subunits of N-methyl-D-aspartate receptor: localization, structure, and function," *Molecular Pharmacology*, vol. 78, no. 1, pp. 1–11, 2010.

[40] S. Pachernegg, N. Strutz-Seebohm, and M. Hollmann, "GluN3 subunit-containing NMDA receptors: not just one-trick ponies," *Trends in Neurosciences*, vol. 35, no. 4, pp. 240–249, 2012.

[41] M. A. Henson, A. C. Roberts, I. Pérez-Otaño, and B. D. Philpot, "Influence of the NR3A subunit on NMDA receptor functions," *Progress in Neurobiology*, vol. 91, no. 1, pp. 23–37, 2010.

[42] K. Matsuda, Y. Kamiya, S. Matsuda, and M. Yuzaki, "Cloning and characterization of a novel NMDA receptor subunit NR3B: a dominant subunit that reduces calcium permeability," *Molecular Brain Research*, vol. 100, no. 1-2, pp. 43–52, 2002.

[43] H. Furukawa, S. K. Singh, R. Mancusso, and E. Gouaux, "Subunit arrangement and function in NMDA receptors," *Nature*, vol. 438, no. 7065, pp. 185–192, 2005.

[44] S. Schorge and D. Colquhoun, "Studies of NMDA receptor function and stoichiometry with truncated and tandem subunits," *Journal of Neuroscience*, vol. 23, no. 4, pp. 1151–1158, 2003.

[45] P. T. Atlason, M. L. Garside, E. Meddows, P. Whiting, and R. A. J. McIlhinney, "N-methyl-D-aspartate (NMDA) receptor subunit NR1 forms the substrate for oligomeric assembly of the NMDA receptor," *Journal of Biological Chemistry*, vol. 282, no. 35, pp. 25299–25307, 2007.

[46] R. A. Al-Hallaq, B. R. Jarabek, Z. Fu, S. Vicini, B. B. Wolfe, and R. P. Yasuda, "Association of NR3A with the N-methyl-D-aspartate receptor NR1 and NR2 subunits," *Molecular Pharmacology*, vol. 62, no. 5, pp. 1119–1127, 2002.

[47] T. Schüler, I. Mesic, C. Madry, I. Bartholoma, and B. Laube, "Formation of NR1/NR2 and NR1/NR3 heterodimers constitutes the initial step in N-methyl-D-aspartate receptor assembly," *Journal of Biological Chemistry*, vol. 283, no. 1, pp. 37–46, 2008.

[48] M. H. Ulbrich and E. Y. Isacoff, "Rules of engagement for NMDA receptor subunits," *Proceedings of the National Academy*

of Sciences of the United States of America, vol. 105, no. 37, pp. 14163–14168, 2008.

[49] J. E. Chatterton, M. Awobuluyi, L. S. Premkumar et al., "Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits," *Nature*, vol. 415, no. 6873, pp. 793–798, 2002.

[50] N. A. Cavara, A. Orth, and M. Hollmann, "Effects of NR1 splicing on NR1/NR3B-type excitatory glycine receptors," *BMC Neuroscience*, vol. 10, article 32, 2009.

[51] P. Paoletti, A. M. Vergnano, B. Barbour, and M. Casado, "Zinc at glutamatergic synapses," *Neuroscience*, vol. 158, no. 1, pp. 126–136, 2009.

[52] C. Madry, H. Betz, J. R. P. Geiger, and B. Laube, "Supralinear potentiation of NR1/NR3A excitatory glycine receptors by Zn^{2+} and NR1 antagonist," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 34, pp. 12563–12568, 2008.

[53] R. A. J. McIlhinney, E. Molnár, J. R. Atack, and P. J. Whiting, "Cell surface expression of the human N-methyl-D-aspartate receptor subunit 1a requires the co-expression of the NR2A subunit in transfected cells," *Neuroscience*, vol. 70, no. 4, pp. 989–997, 1996.

[54] V. A. Alvarez, D. A. Ridenour, and B. L. Sabatini, "Distinct structural and ionotropic roles of NMDA receptors in controlling spine and synapse stability," *Journal of Neuroscience*, vol. 27, no. 28, pp. 7365–7376, 2007.

[55] H.-K. Wong, X.-B. Liu, M. F. Matos et al., "Temporal and regional expression of NMDA receptor subunit NR3A in the mammalian brain," *Journal of Comparative Neurology*, vol. 450, no. 4, pp. 303–317, 2002.

[56] L. Sun, F. L. Margolis, M. T. Shipley, and M. S. Lidow, "Identification of a long variant of mRNA encoding the NR3 subunit of the NMDA receptor: its regional distribution and developmental expression in the rat brain," *FEBS Letters*, vol. 441, no. 3, pp. 392–396, 1998.

[57] D. J. Goebel and M. S. Poosch, "NMDA receptor subunit gene expression in the rat brain: a quantitative analysis of endogenous mRNA levels of NR1(Com), NR2A, NR2B, NR2C, NR2D and NR3A," *Molecular Brain Research*, vol. 69, no. 2, pp. 164–170, 1999.

[58] H. T. Mueller and J. H. Meador-Woodruff, "Distribution of the NMDA receptor NR3A subunit in the adult pig-tail macaque brain," *Journal of Chemical Neuroanatomy*, vol. 29, no. 3, pp. 157–172, 2005.

[59] M. Eriksson, A. Nilsson, S. Froelich-Fabre et al., "Cloning and expression of the human N-methyl-D-aspartate receptor subunit NR3A," *Neuroscience Letters*, vol. 321, no. 3, pp. 177–181, 2002.

[60] N. J. Sucher, K. Kohler, L. Tenneti et al., "N-methyl-D-aspartate receptor subunit NR3A in the retina: developmental expression, cellular localization, and functional aspects," *Investigative Ophthalmology and Visual Science*, vol. 44, no. 10, pp. 4451–4456, 2003.

[61] R. S. Larsen, R. J. Corlew, M. A. Henson et al., "NR3A-containing NMDARs promote neurotransmitter release and spike timing-dependent plasticity," *Nature Neuroscience*, vol. 14, no. 3, pp. 338–344, 2011.

[62] D. W. McClymont, J. Harris, and I. R. Mellor, "Open-channel blockade is less effective on GluN3B than GluN3A subunit-containing NMDA receptors," *European Journal of Pharmacology*, vol. 686, no. 1–3, pp. 22–31, 2012.

[63] K. M. On and N. J. Sucher, "Molecular interaction of NMDA receptor subunit NR3A with protein phosphatase 2A," *NeuroReport*, vol. 15, no. 9, pp. 1447–1450, 2004.

[64] S. F. Chan and N. J. Sucher, "An NMDA receptor signaling complex with protein phosphatase 2A," *Journal of Neuroscience*, vol. 21, no. 20, pp. 7985–7992, 2001.

[65] G. N. Barnes, J. T. Slevin, and T. C. Vanaman, "Rat brain protein phosphatase 2A: an enzyme that may regulate autophosphorylated protein kinases," *Journal of Neurochemistry*, vol. 64, no. 1, pp. 340–353, 1995.

[66] M. Eriksson, H. Samuelsson, S. Björklund et al., "MAP1B binds to the NMDA receptor subunit NR3A and affects NR3A protein concentrations," *Neuroscience Letters*, vol. 475, no. 1, pp. 33–37, 2010.

[67] M. Eriksson, H. Samuelsson, E.-B. Samuelsson et al., "The NMDAR subunit NR3A interacts with microtubule-associated protein 1S in the brain," *Biochemical and Biophysical Research Communications*, vol. 361, no. 1, pp. 127–132, 2007.

[68] S. Halpain, A. Hipolito, and L. Saffer, "Regulation of F-actin stability in dendritic spines by glutamate receptors and calcineurin," *Journal of Neuroscience*, vol. 18, no. 23, pp. 9835–9844, 1998.

[69] Y. Jiang, V. T. Puliyappadamba, L. Zhang et al., "A novel mechanism of cell growth regulation by Cell Cycle and Apoptosis Regulatory Protein (CARP)-1," *Journal of Molecular Signaling*, vol. 5, article 7, 2010.

[70] M. Eriksson, A. Nilsson, H. Samuelsson et al., "On the role of NR3A in human NMDA receptors," *Physiology and Behavior*, vol. 92, no. 1-2, pp. 54–59, 2007.

[71] A. M. Arsham and T. P. Neufeld, "Thinking globally and acting locally with TOR," *Current Opinion in Cell Biology*, vol. 18, no. 6, pp. 589–597, 2006.

[72] M. F. Bear, G. Dölen, E. Osterweil, and N. Nagarajan, "Fragile X: translation in action," *Neuropsychopharmacology*, vol. 33, no. 1, pp. 84–87, 2008.

[73] N. J. Sucher, E. Yu, S. F. Chan et al., "Association of the small GTPase Rheb with the NMDA receptor subunit NR3A," *NeuroSignals*, vol. 18, no. 4, pp. 203–209, 2011.

[74] U. Narayanan, V. Nalavadi, M. Nakamoto et al., "FMRP phosphorylation reveals an immediate-early signaling pathway triggered by group I mGluR and mediated by PP2A," *Journal of Neuroscience*, vol. 27, no. 52, pp. 14349–14357, 2007.

[75] U. Narayanan, V. Nalavadi, M. Nakamoto et al., "S6K1 phosphorylates and regulates fragile X mental retardation protein (FMRP) with the neuronal protein synthesis-dependent mammalian target of rapamycin (mTOR) signaling cascade," *Journal of Biological Chemistry*, vol. 283, no. 27, pp. 18478–18482, 2008.

[76] D. Chowdhury, S. Marco, I. M. Brooks et al., "Tyrosine Phosphorylation Regulates the Endocytosis and Surface Expression of GluN3A-Containing NMDA Receptors," *Journal of Neuroscience*, vol. 33, no. 9, pp. 4151–4164, 2013.

[77] B. Qualmann, J. Roos, P. J. DiGregorio, and R. B. Kelly, "Syndapin I, a synaptic dynamin-binding protein that associates with the neural Wiskott-Aldrich syndrome protein," *Molecular Biology of the Cell*, vol. 10, no. 2, pp. 501–513, 1999.

[78] F. Simpson, N. K. Hussain, B. Qualmann et al., "SH3-domain-containing proteins function at distinct steps in clathrin-coated vesicle formation," *Nature Cell Biology*, vol. 1, no. 2, pp. 119–124, 1999.

[79] M. M. Kessels and B. Qualmann, "Syndapin oligomers interconnect the machineries for endocytic vesicle formation and actin

polymerization," *Journal of Biological Chemistry*, vol. 281, no. 19, pp. 13285–13299, 2006.

[80] A. Halbach, M. Mörgelin, M. Baumgarten, M. Milbrandt, M. Paulsson, and M. Plomann, "PACSIN 1 forms tetramers via its N-terminal F-BAR domain," *FEBS Journal*, vol. 274, no. 3, pp. 773–782, 2007.

[81] S. Schael, J. Nuechel, S. Mueller et al., "Casein kinase 2 phosphorylation of PACSIN 1 regulates neuronal spine formation," *Journal of Biological Chemistry*, 2013.

[82] D. S. Macdonald, M. Weerapura, M. A. Beazely et al., "Modulation of NMDA receptors by pituitary adenylate cyclase activating peptide in CA1 neurons requires Gαq, protein kinase C, and activation of Src," *Journal of Neuroscience*, vol. 25, no. 49, pp. 11374–11384, 2005.

[83] T. Nakazawa, S. Komai, T. Tezuka et al., "Characterization of Fyn-mediated tyrosine phosphorylation sites on GluRε2 (NR2B) subunit of the N-methyl-D-aspartate receptor," *Journal of Biological Chemistry*, vol. 276, no. 1, pp. 693–699, 2001.

[84] P. Caroni, F. Donato, and D. Muller, "Structural plasticity upon learning: regulation and functions," *Nature Reviews Neuroscience*, vol. 13, no. 7, pp. 478–490, 2012.

[85] M. Maletic-Savatic, R. Malinow, and K. Svoboda, "Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity," *Science*, vol. 283, no. 5409, pp. 1923–1927, 1999.

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

[87] M. de Roo, P. Klauser, and D. Muller, "LTP promotes a selective long-term stabilization and clustering of dendritic spines," *PLoS Biology*, vol. 6, no. 9, article e219, 2008.

[88] A. T. U. Schaefers and G. Teuchert-Noodt, "Developmental neuroplasticity and the origin of neurodegenerative diseases," *The World Journal of Biological Psychiatry*, 2013.

[89] H. T. Mueller and J. H. Meador-Woodruff, "NR3A NMDA receptor subunit mRNA expression in schizophrenia, depression and bipolar disorder," *Schizophrenia Research*, vol. 71, no. 2-3, pp. 361–370, 2004.

[90] M. A. Snyder and W. J. Gao, "NMDA hypofunction as a convergence point for progression and symptoms of schizophrenia," *Frontiers in Cellular Neuroscience*, vol. 7, article 31, 2013.

[91] D. C. Mathews, I. D. Henter, and C. A. Zarate Jr., "Targeting the glutamatergic system to treat major depressive disorder," *Drugs*, vol. 72, no. 10, pp. 1313–1333, 2012.

[92] R. A. C. Roos, "Huntington's disease: a clinical review," *Orphanet Journal of Rare Diseases*, vol. 5, no. 1, article 40, 2010.

[93] M. DiFiglia, M. Sena-Esteves, K. Chase et al., "Therapeutic silencing of mutant huntingtin with siRNA attenuates striatal and cortical neuropathology and behavioral deficits," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 43, pp. 17204–17209, 2007.

[94] M. DiFiglia, E. Sapp, K. O. Chase et al., "Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain," *Science*, vol. 277, no. 5334, pp. 1990–1993, 1997.

[95] G. Schaffar, P. Breuer, R. Boteva et al., "Cellular toxicity of polyglutamine expansion proteins: mechanism of transcription factor deactivation," *Molecular Cell*, vol. 15, no. 1, pp. 95–105, 2004.

[96] J.-Y. Li, M. Plomann, and P. Brundin, "Huntington's disease: a synaptopathy?" *Trends in Molecular Medicine*, vol. 9, no. 10, pp. 414–420, 2003.

[97] S. Marco, A. Giralt, M. M. Petrovic et al., "Suppressing aberrant GluN3A expression rescues synaptic and behavioral impairments in Huntington's disease models," *Nature Medicine*, vol. 19, pp. 1030–1038, 2013.

[98] M. A. Ungless, J. L. Whistler, R. C. Malenka, and A. Bonci, "Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons," *Nature*, vol. 411, no. 6837, pp. 583–587, 2001.

[99] B. Schilström, R. Yaka, E. Argilli et al., "Cocaine enhances NMDA receptor-mediated currents in ventral tegmental area cells via dopamine D5 receptor-dependent redistribution of NMDA receptors," *Journal of Neuroscience*, vol. 26, no. 33, pp. 8549–8558, 2006.

[100] M. Mameli, C. Bellone, M. T. C. Brown, and C. Lüscher, "Cocaine inverts rules for synaptic plasticity of glutamate transmission in the ventral tegmental area," *Nature Neuroscience*, vol. 14, no. 4, pp. 414–416, 2011.

[101] C. Bellone and C. Lüscher, "Cocaine triggered AMPA receptor redistribution is reversed in vivo by mGluR-dependent long-term depression," *Nature Neuroscience*, vol. 9, no. 5, pp. 636–641, 2006.

[102] T. Yuan, M. Mameli, E. C. O'Connor et al., "Expression of cocaine-evoked synaptic plasticity by GluN3A-containing NMDA receptors," *Neuron*, vol. 80, no. 4, pp. 1025–1038, 2013.

[103] N. Nakanishi, S. Tu, Y. Shin et al., "Neuroprotection by the NR3A subunit of the NMDA receptor," *Journal of Neuroscience*, vol. 29, no. 16, pp. 5260–5265, 2009.

[104] R. Martínez-Turrillas, E. Puerta, D. Chowdhury et al., "The NMDA receptor subunit GluN3A protects against 3-nitropropionic-induced striatal lesions via inhibition of calpain activation," *Neurobiology of Disease*, vol. 48, no. 3, pp. 290–298, 2012.

[105] Y. Terasaki, T. Sasaki, Y. Yagita et al., "Activation of NR2A receptors induces ischemic tolerance through CREB signaling," *Journal of Cerebral Blood Flow and Metabolism*, vol. 30, no. 8, pp. 1441–1449, 2010.

[106] Y. Liu, P. W. Tak, M. Aarts et al., "NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo," *Journal of Neuroscience*, vol. 27, no. 11, pp. 2846–2857, 2007.

[107] H. Wang, H. Yan, S. Zhang, X. Wei, J. Zheng, and J. Lee, "GluN3A subunit exerts a neuroprotective effect in brain ischemia and hypoxia process," *ASN Neuro*, vol. 5, no. 4, Article ID e00120, 2013.

[108] A. Derouiche, E. Anlauf, G. Aumann, B. Mühlstädt, and M. Lavialle, "Anatomical aspects of glia-synapse interaction: the perisynaptic glial sheath consists of a specialized astrocyte compartment," *Journal of Physiology Paris*, vol. 96, no. 3-4, pp. 177–182, 2002.

[109] M. Lavialle, G. Aumann, E. Anlauf, F. Pröls, M. Arpin, and A. Derouiche, "Structural plasticity of perisynaptic astrocyte processes involves ezrin and metabotropic glutamate receptors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 31, pp. 12915–12919, 2011.

[110] W. Sun, E. McConnell, J.-F. Pare et al., "Glutamate-dependent neuroglial calcium signaling differs between young and adult brain," *Science*, vol. 339, no. 6116, pp. 197–200, 2013.

[111] A. Panatier, J. Vallée, M. Haber, K. K. Murai, J.-C. Lacaille, and R. Robitaille, "Astrocytes are endogenous regulators of basal transmission at central synapses," *Cell*, vol. 146, no. 5, pp. 785–798, 2011.

[112] J. T. Porter and K. D. McCarthy, "Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals," *Journal of Neuroscience*, vol. 16, no. 16, pp. 5073–5081, 1996.

[113] P. Bezzi, V. Gundersen, J. L. Galbete et al., "Astrocytes contain a vesicular compartment that is competent for regulated exocytosis of glutamate," *Nature Neuroscience*, vol. 7, no. 6, pp. 613–620, 2004.

[114] J.-P. Mothet, L. Pollegioni, G. Ouanounou, M. Martineau, P. Fossier, and G. Baux, "Glutamate receptor activation triggers a calcium-dependent and SNARE protein-dependent release of the gliotransmitter D-serine," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 15, pp. 5606–5611, 2005.

[115] A. Araque, V. Parpura, R. P. Sanzgiri, and P. G. Haydon, "Tripartite synapses: glia, the unacknowledged partner," *Trends in Neurosciences*, vol. 22, no. 5, pp. 208–215, 1999.

[116] E. Brand-Schieber and P. Werner, "AMPA/kainate receptors in mouse spinal cord cell-specific display of receptor subunits by oligodendrocytes and astrocytes and at the nodes of Ranvier," *Glia*, vol. 42, no. 1, pp. 12–24, 2003.

[117] R. Káradóttir, P. Cavelier, L. H. Bergersen, and D. Attwell, "NMDA receptors are expressed in oligodendrocytes and activated in ischaemia," *Nature*, vol. 438, no. 7071, pp. 1162–1166, 2005.

[118] U. Lalo, Y. Pankratov, F. Kirchhoff, R. A. North, and A. Verkhratsky, "NMDA receptors mediate neuron-to-glia signaling in mouse cortical astrocytes," *Journal of Neuroscience*, vol. 26, no. 10, pp. 2673–2683, 2006.

[119] C. Agulhon, J. Petravicz, A. B. McMullen et al., "What is the role of astrocyte calcium in neurophysiology?" *Neuron*, vol. 59, no. 6, pp. 932–946, 2008.

[120] O. Palygin, U. Lalo, and Y. Pankratov, "Distinct pharmacological and functional properties of NMDA receptors in mouse cortical astrocytes," *British Journal of Pharmacology*, vol. 163, no. 8, pp. 1755–1766, 2011.

[121] M. Haber and K. K. Murai, "Reshaping neuron-glial communication at hippocampal synapses," *Neuron Glia Biology*, vol. 2, no. 1, pp. 59–66, 2006.

[122] H. Nishida and S. Okabe, "Direct astrocytic contacts regulate local maturation of dendritic spines," *Journal of Neuroscience*, vol. 27, no. 2, pp. 331–340, 2007.

[123] M. W. Nestor, L.-P. Mok, M. E. Tulapurkar, and S. M. Thompson, "Plasticity of neuron-glial interactions mediated by astrocytic EphARs," *Journal of Neuroscience*, vol. 27, no. 47, pp. 12817–12828, 2007.

[124] D. Verbich, G. A. Prenosil, P. K.-Y. Chang, K. K. Murai, and R. A. Mckinney, "Glial glutamate transport modulates dendritic spine head protrusions in the hippocampus," *Glia*, vol. 60, no. 7, pp. 1067–1077, 2012.

[125] I. Lushnikova, G. Skibo, D. Muller, and I. Nikonenko, "Synaptic potentiation induces increased glial coverage of excitatory synapses in CA1 hippocampus," *Hippocampus*, vol. 19, no. 8, pp. 753–762, 2009.

[126] J. J. Lippman, T. Lordkipanidze, M. E. Buell, S. O. Yoon, and A. Dunaevsky, "Morphogenesis and regulation of Bergmann glial processes during Purkinje cell dendritic spine ensheathment and synaptogenesis," *Glia*, vol. 56, no. 13, pp. 1463–1477, 2008.

[127] D. Kalman, S. N. Gomperts, S. Hardy, M. Kitamura, and J. M. Bishop, "Ras family GTPases control growth of astrocyte processes," *Molecular Biology of the Cell*, vol. 10, no. 5, pp. 1665–1683, 1999.

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

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

[130] M. Plomann, R. Lange, G. Vopper et al., "PACSIN, a brain protein that is upregulated upon differentiation into neuronal cells," *European Journal of Biochemistry*, vol. 256, no. 1, pp. 201–211, 1998.

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Review Article

The Role of Astrocytes in the Regulation of Synaptic Plasticity and Memory Formation

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Astrocytes regulate synaptic transmission and play a role in the formation of new memories, long-term potentiation (LTP), and functional synaptic plasticity. Specifically, astroglial release of glutamate, ATP, and cytokines likely alters the survivability and functioning of newly formed connections. Among these pathways, regulation of glutamate appears to be most directly related to the promotion of LTP, which is highly dependent on the synchronization of synaptic receptors through the regulation of excitatory postsynaptic potentials. Moreover, regulation of postsynaptic glutamate receptors, particularly AMPA receptors, is dependent on signaling by ATP synthesized in astrocytes. Finally, cytokine signaling is also implicated in regulating LTP, but is likely most important in plasticity following tissue damage. Despite the role of these signaling factors in regulating LTP and functional plasticity, an integrative model of these factors has not yet been elucidated. In this review, we seek to summarize the current body of evidence on astrocytic mechanisms for regulation of LTP and functional plasticity, and provide an integrative model of the processes.

1. Introduction

The long-term storage of information in the form of memory is one of the principal functions of the developed nervous system. The ability to utilize this information provides evolutionary advantages in adapting and responding to situations in a given environment. The method for the formation of memories and the process of functional specialization in the brain during development has been found to be mediated by both structural and functional plasticity, including long-term potentiation between neurons [1]. While much attention has been given to these processes on a neuronal level, less attention has been given to what role glial cells, particularly astrocytes, may have in the underlying mechanisms.

While astrocytes were formerly thought to serve mostly as housekeeping cells, they have recently gained attention as an integral part of the chemical synapse. In addition to their structural and metabolic roles, astrocytes are now thought to be heavily involved in synaptogenesis and in regulating the communication between already formed connections [2]. Several studies have demonstrated that astrocytes utilize both ionotropic and metabotropic systems in order to regulate neuron to neuron communication [3–5], and that they may have specific mechanisms for regulating the formation of memories. Here, we review recent evidence for the importance of astrocytes in both structural and functional synaptic plasticity, specifically long-term potentiation, the key chemical transmitters that are involved (Table 1), as well as the underlying mechanisms by which astrocytes may regulate these processes.

2. Glial Cells: Astrocytes

Glial cells are nonneuronal cells that are now believed to constitute 50% of the cells in the whole brain in humans and other primates [6–9], although other reports have suggested that glia may outnumber neurons 10 : 1 [10–12]. Astrocytes, as their name suggests, appear to be star-shaped when Golgi stained or immunostained for glial fibrillary acidic protein [5]. However, the morphology and physiology of astrocytes differ depending on the type [13]. Typically, astrocytes have a complex structure that is highly branched with many small protrusions that contact the synaptic cleft [14, 15]. With their unique morphology, astrocytes form the blood brain barrier, have a role in ion homeostasis, and form the tripartite

Table 1: Summary of receptors/signaling molecules and related mechanisms.

Signaling molecule	Receptor(s)	Mechanism of action in plasticity/memory formation
Acetylcholine	Muscarinic Ach-R's	Causes an increase in $[Ca^{2+}]$ activating mGluR's
Adenosine	A1 Receptors	Inhibition of cAMP dependent transcription
ATP	P2Y Receptors	Enhances concentration of AMPA receptors
Cytokines (i) TNF-α (ii) CCL2 (iii) Interleukin-1	(i) CXCR4 (ii) NMDA Receptors (iii) IL1 Receptors	(i) Glutamate release and the insertion of AMPA receptors (ii) Inhibits NMDA receptor activity (iii) Unknown
D-Serine	NMDA Receptors	Coagonist of receptors
Ephrin (i) Ephrin-A (ii) Ephrin-B	(i) EphA Receptors (ii) EphB Receptors	(i) Promotes retraction of dendritic spines (ii) Regulates D-serine release
Glutamate	AMPA receptors, NMDA receptors, mGluR's	Increased EPSP, upregulation of AMPA receptors
Lactate	MCT2	Provides additional metabolic energy for growth/plasticity
Nicotine	nAchR	Releases Ca^{2+} and promotes the release of D-serine

synapse [16]. The blood brain barrier is made up of capillary endothelial cells, vascular pericytes, and the perivascular endfeet of astrocytes. Together, they create a highly selective barrier that allows oxygen and hormones to permeate into the brain while preventing the passage of other molecules due to possible harmful effects.

Astrocytes also maintain homeostasis of various ions such as sodium, potassium, chloride, and hydrogen [17]. For instance, astrocytes play a critical role in regulating extracellular K^+ levels. When the extracellular concentration of K^+ is high, astrocytes uptake the ion using transporters or channels and transfer it to adjacent astrocytes via gap junctions by a process called spatial buffering [18–21]. Due to this process, astrocytes prevent extracellular concentrations of K^+ from reaching toxic levels.

In the tripartite conceptualization of the synapse, perisynaptic astrocytes are present along with the standard presynaptic and postsynaptic neurons [15, 22–24]. Contact made by perisynaptic astrocytes with the synaptic cleft depends on the type and location of synapses [13, 15, 25]. In the hippocampus for instance, 64% of synapses are contacted by perisynaptic astrocytes at the synaptic cleft [26]. The intricate arborization and ramifications of astrocytes allow them to tightly enwrap the synaptic terminal in order to modulate synaptic processes [14, 15, 25]. Previous studies suggest that astrocytes respond to neurotransmitter release by increasing their intracellular calcium levels and controlling neuronal excitability through the release of gliotransmitters [2]. Based on findings that explain the functioning of the tripartite synapse, more attention has been given to the potential role of how astrocytes aid memory. In areas known for synaptic plasticity, such as the hippocampus, astroglial membranes appear to surround the majority of larger axo-dendritic synapses, and around 60% of all synapses in the hippocampus [27, 28]. In astrocytes that are part of a tripartite synapse, calcium peaks, which correspond to calcium oscillations tuned to neuronal activity, cause the proximal and distal release of glutamate from the astrocyte to neighboring neurons [5].

Excess glutamate is taken up by astrocytes and further regulated through a shunting cycle by which it is broken down into glutamine, repackaged, sent to the presynaptic-neuron, and finally converted back into glutamate [16]. Astroglial glutamatergic regulation is so widespread that it is estimated that only 20% of synaptic glutamate is taken up by transporters on the postsynaptic neuron, while the other 80% is processed by transporters such as the glutamate aspartate transporter (GLAST) on the membrane of the associated astrocyte [29]. Additionally, astrocytes have the ability to swell and shrink in size through the use of aquaporin channels, and this may allow them to reduce the leakage of neurotransmitters, increasing the active concentration in the synapse, and preventing spillover in the case of damage [30, 31]. However, transmitters can also be released through these channels when exposed to a hypotonic bath solution, ischemia, or a traumatic brain injury [32–34]. Besides their role in signaling, astrocytes have also been implicated in controlling the development of the nervous system through factors such as axon guidance and synaptogenesis, as discussed below.

3. Plasticity in the Hippocampus

It is now well known that the hippocampus, located in the inferior temporal lobe, is responsible for the formation and storage of memory [35, 36]. The hippocampal structure has distinct functional areas implicated in memory formation, that is, the CA1, CA3, and the dentate gyrus. Various parts of the brain display some form of synaptic plasticity, but the hippocampus is one of the structures that has received much attention due to its overall functional importance.

Synaptic plasticity refers to experience mediated structural and functional changes to the connections between neurons that results in changes to neural circuits [37–39]. These neural circuits are often developed (synaptogenesis) and strengthened through the reinforcement of some connections and the removal of others (synaptic stripping), which can occur in response to environmental experience. During early development, plasticity can occur through large scale dendritic and axonal conformational changes, and while these processes are observed in the adult mammalian

brain, the scale on which they take place and the efficacy of regulatory processes involved are inhibited [40]. While less overall change is observed in the adult brain, early developmental plasticity in children, as well as memory formation and learning in adults, are both likely dependent on structural changes in the functioning of the synapse itself [41–43]. Recent evidence shows that even small structural changes to the dendritic spines can drastically alter the overall output/input of synaptic protein receptors, which in adults is likely more important in determining neuronal activity than dendritic spine density [41–43]. Due to a high concentration of synapses in the brain, tuning of activity could be accomplished through regulating synaptic function with relatively little conformational change, which is important in being able to learn and store large quantities of information without negatively impacting other signaling pathways.

The tuning of synaptic activity associated with functional plasticity, or changes in synaptic strength [44], has been demonstrated through the modulation of membrane receptors by enzymatic activity such as phosphorylation [39, 45–47]. However, changes to the chemical environment within the synapse are likely more influential in the associated changes to neuronal firing and receptor concentrations. The changes in dendritic spines and synaptic activity in relation to plasticity have been found to be most closely linked to synaptic glutamate receptors and changes in both internal and external calcium in neurons [48]. It is consistently demonstrated that neuron-glia interactions are essential to this type of environmental regulation, with astrocytes being paramount in regulating signaling molecules such as glutamate which are particularly important in the plasticity and learning processes [49, 50]. As astrocytes are in part often responsible for regulating synapse formation and synaptic activity, there is a strong possibility that their activity plays an integral role in plasticity and learning.

Among the various forms of functional synaptic plasticity, long-term potentiation (LTP) has received much attention in the hippocampus due to its role in memory [51]. LTP is the process of a long-lasting enhancement in synaptic strength. This was first observed in electrophysiological studies, using high-frequency stimulation (100 Hz) of neurons in the perforant pathway and recording the activity at the dentate gyrus [52]. Electrode recordings followed by tetanic stimulation exhibited a longer lasting excitatory postsynaptic potential (EPSP) of the postsynaptic neuron in the dentate gyrus. The mechanism of LTP differs depending on the location of the hippocampus. For example, N-methyl D-aspartate (NMDA) receptor dependent LTP occurs at the Schaffer collateral region while NMDA receptor independent LTP is observed at the mossy fibers of the CA3 region [53–57]. Despite various forms of LTP that occur in the hippocampus, NMDA receptor dependent LTP is heavily studied. Several studies blocking NMDA receptor activity showed impairment in different types of memory in mice, implicating NMDA receptors in memory formation [58–61]. However, these studies do not indicate that LTP causes memory, as LTP may be an underlying process that helps form memory but does not directly cause it.

For NMDA receptor dependent LTP to occur, glutamate binding to NMDA receptors and depolarization of the neuron is required. Activation of NMDA receptors allows calcium to stimulate cyclic adenosine monophosphate (cAMP) release, causing a cascade of signaling mechanisms involving protein kinase A, cAMP response element binding protein (CREB), cAMP response element (CRE), mitogen activated protein kinase, and calcium calmodulin dependent protein kinase II [62–64]. These factors lead to the upregulation of transcription. Therefore, synthesis of new proteins underlies the mechanism for long-term memory. While much attention has been given to the regulation of hippocampal neurons by these factors, there is a growing body of evidence that astrocytic support is more critical in the regulation and function of many LTP related compounds and mechanisms than previously thought.

4. LTP Associated Gliotransmitters

Astrocytes release and regulate several neuroactive molecules that can affect neuronal activity and modulate plasticity and LTP. These compounds (summarized in Table 1) include glutamate, ATP, cytokines, and several other key signaling molecules like D-serine, adenosine, and lactate [65]. Glutamate plays a key role in the regulation of synaptic activity and causes a response in astrocytes [66, 67]. Importantly, astrocytes actively sequester up to 90% of glutamate that is released into the extracellular space between neurons [68, 69]. Glutamate causes a wide range of effects in astrocytes via metabotropic glutamate receptors (mGluR), NMDA receptors, and α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors.

Although cortical astrocytes generally express functional NMDA receptors, this does not appear to be the case for hippocampal astrocytes [70–72]. Hippocampal astrocytes do not exhibit activation upon standard NMDA receptor agonists [70, 72]. Functional AMPA receptors, on the other hand, are expressed by hippocampal astrocytes [73, 74]. Additionally, hippocampal astrocytes change the properties of their AMPA receptors during postnatal development. At the beginning stages of postnatal development, low levels of AMPA receptor currents were observed and significantly increased around P12 [75]. Immature astrocytes also had a prolonged activation of the AMPA receptor, which induced an influx of Na^+ and Ca^{2+}. As astrocytes matured, glutamate responses greatly increased as well.

Astrocytes are likely able to synchronize with neuronal activity and subsequently regulate glutamate transmission between neurons [76–79]. For example, astrocytic glutamate release activates presynaptic NMDA receptors and promotes increased excitatory communication between neurons [80]. These NMDA receptors are also subjected to further regulation by endogenous concentrations of D-serine, which serves as a coagonist, specifically in the hippocampal region, suggesting its potential importance in new memory formation [81–84].

In addition to the ionotropic glutamate receptors, astrocytes also use mGluRs. In the hippocampus, mGluR1 [85, 86],

mGluR5 [87, 88], and mGluR3 [89, 90] are expressed and functionally important in astrocytes and the modulation of neuronal activity. However, astrocytes of 1-week-old mice, but not older mice, express high levels of mGluR5 [90]. Furthermore, astrocytes of adult mice did not exhibit an increase in Ca^{2+} when stimulated with an mGluR5 agonist. These results suggest developmental changes in the expression of mGluRs in astrocytes. Contradictory to the observations by [90], there has been research demonstrating mGluR5's role in Ca^{2+} elevation in adults. Further research must be done to clarify these opposing findings.

Astrocytic activation can result in the release of gliotransmitters that can affect neuronal activity. Gliotransmitter release is Ca^{2+} dependent and involves the following mechanism. Astrocytes express a neurotransmitter receptor called G-protein coupled metabotropic receptor (GPCR). Specifically, the G-protein G_q, coupled to phospholipase C (PLC), is involved in elevating intracellular Ca^{2+} levels in astrocytes [91, 92]. When G_q is stimulated, PLC is activated to break down phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) [91, 92]. By breaking down PIP_2, the endoplasmic reticulum can release stored Ca^{2+} to stimulate gliotransmitter release.

In gliotransmission, astrocytes release vesicles that are packed with gliotransmitters via the process of exocytosis. Astrocytes express proteins that are known to be involved in vesicle fusion such as soluble NSF attachment protein receptor (SNARE), synaptotagmin, complexin2, and Munc18a, which are critical for gliotransmitter release [5, 92]. For example, altering the SNARE complex resulted in a failure of glutamate release from astrocytes [93]. Glutamate release also requires vacuolar type of proton ATPase to exchange the proton gradient from the vesicular lumen with glutamate [94, 95]. The same mechanism was observed for D-serine in hippocampal astrocytes [94]. In addition, in hippocampal astrocytes, synaptic-like microvesicles (SLMV) were found with the R-type SNAP receptor (R-SNARE), which is known to govern exocytosis [5]. Together, these proteins that are expressed by hippocampal astrocytes package gliotransmitters for release.

Additionally, ATP signaling regulates Ca^{2+}-dependent glutamate release via astrocytic P2Y receptors [96]. ATP released from astrocytes also interacts directly with pre- and postsynaptic neurons, serving to regulate their own glutamatergic transmission and to also enhance the concentration of AMPA receptors, which facilitates the release of neuropeptides including oxytocin and vasopressin [65]. Additionally, some of the ATP released by astrocytes is converted directly to adenosine, which can act as both an agonist and antagonist for specific K^+ and Ca^{2+} channels [97].

Cytokines and chemokine receptors are also implicated in the regulation of Ca^{2+} stores, glutamatergic transmission, and synaptic plasticity as a whole. In astrocytes, the CXCR4-CXCL12 signaling axis has been implicated in both modulating glutamate exocytosis, and in causing the release of the cytokine TNF-α [98]. TNF-α is also linked to regulating both glutamate release and the insertion of AMPA receptors into neighboring neurons [99, 100]. Finally, cytokine signaling in astrocytes, as well as microglia, plays a role in the response to physically sensing pain and responding to damage, with chemokine (C-C motif) ligand 2 (CCL2) released from astrocytes having a strong regulatory effect on the activity of NMDA receptors [101].

Despite the evidence indicating the significance of Ca^{2+} in the release of gliotransmitters, there have been controversial findings that challenge this assertion. Some studies have observed that blocking Ca^{2+} in hippocampal astrocytes located at the CA1 region *in situ* does not change Ca^{2+} levels in neurons, change spontaneous excitatory postsynaptic current, result in astrocytic glutamate release, or NMDA receptor mediated slow inward currents in pyramidal neurons [102–104]. These findings may suggest that a mechanism not dependent on Ca^{2+} release may lead to gliotransmitter release in astrocytes.

Although the gliotransmitters discussed above are important in regulating LTP, another crucial gliotransmitter to postsynaptic neurons is lactate. Memory formation is the result of a cascade of cellular and molecular processes and thus, to ensure the proper functionality of a neuron, astrocytes provide neurons with lactate, a usable form of energy [105–107]. Through glycogenolysis, astrocytes convert stored glycogen into lactate and release it into the synapse through the MCT1 or MCT4 transporter [107]. The neuron is then able to take up lactate via an MCT2 transporter, which has been confirmed through blocking MCT2 with either 4-CIN or MCT2-oligodeoxynucleotides [106, 107]. Rats showed memory impairment in inhibitory avoidance and spatial memory tasks when glycogenolysis, MCT1, MCT4, or MCT2 were inhibited [106, 107]. Thus, it is clear that the metabolism of astrocytes is critical in hippocampal dependent memory.

5. Ephrin Signaling and Glutamate Transporters

Ephrin signaling, consisting of ephrin-As and ephrin-Bs, is known for its involvement in neural development by inhibiting axonal and dendritic growth via actin rearrangement [108–114]. The interaction between ephrin-A3 and EphA4, which are expressed by astrocytes and dendritic spines of neurons, respectively, is involved in decreasing levels of GLAST and glutamate transporter 1 (GLT-1) for proper synapsing to occur [115–118].

Astrocytes express both EphB receptors and ephrin-B ligands, ephrinB3 being the most active during LTP [119]. EphrinB3 enhances D-serine release by regulating serine racemase (SR), an enzyme responsible for the conversion of L-serine to D-serine, and an SR-interacting protein, protein kinase C (PKCα). Specifically, ephrinB3 downregulates PKCα in order to increase the interaction between SR and Protein Interacting with C-kinase (PICK1), causing D-serine release [119]. Moreover, ephrinB3 is able to bind to both EphB3 and EphA4 receptors [120]. By measuring D-serine levels in EphB3 and EphA4 knockouts in cultured astrocytes, both receptors were necessary for D-serine release [119]. Thus, while ephrin-A signaling regulates levels of GLT-1, ephrin-B

signaling regulates levels of D-serine release for activation of NMDA receptors.

6. Cholinergic Signaling

Nicotine influences memory by inducing synaptic transmission at acetylcholinergic synapses [121–123]. In Alzheimer's disease, patients treated with nicotine had improved cognitive functioning [124]. Astrocytes express nicotinic acetylcholine receptors (nAChR), implicating nicotine's role in cholinergic dependent memory. This effect on memory is dependent on glutamatergic NMDA receptors, which requires binding of D-serine released by astrocytes [81–83, 125]. As described previously, D-serine binds to NMDA receptors, allowing the influx of ions to induce LTP. Therefore, nicotine binding to the nAChR on astrocytes stimulates the release of D-serine by increasing internal calcium concentrations, allowing NMDA receptors on the postsynaptic neuron to induce LTP [123–125]. Similar to nAChR, activation of muscarinic AChR (mAChR) also increases internal calcium concentrations [126–128].

7. Other Receptors

7.1. Adenosine Receptors. There are other receptors thought to be involved in astrocyte-neuron communication. The adenosine A1 receptor is expressed on presynaptic neurons, and activation of the receptor activates the inhibitory metabotropic g-protein (G_i) pathway. Memory deficits in mice that underwent 6 hours of sleep deprivation were prevented by pharmacologically blocking the A1 receptor [129]. Furthermore, astrocytes modulate levels of adenosine during 12 hours of sleep deprivation in mice [130]. Interestingly, A1 receptor activation in astrocytes can also modulate sleep in a rodent model of inflammation [131].

7.2. Interleukin-1. The cytokine interleukin-1 (IL-1) also plays a key role in hippocampal dependent memory. Blocking activity of IL-1 receptors resulted in the poor performance of learning with the Morris water maze and fear conditioning, as well as reduced LTP [132–135]. Although IL-1 receptors can be expressed by many cells, it is prominently expressed on astrocytes [136–140]. IL-1 receptor knockout mice that did not express IL-1 receptors on astrocytes exhibit memory deficits that can be rescued with transplantation of neural precursor cells from wild-type mice that express IL-1 receptors [139]. The underlying mechanism of IL-1 has yet to be determined in the context of memory.

8. Discussion

Research on synaptic plasticity and memory has traditionally been neuron-centric, yet it is crucial to not ignore the astrocytic role in these processes since they are now known to modulate neuronal activity. Not only do astrocytes regulate the extracellular concentration of neurotransmitters, they also regulate the activity and expression of receptors on the postsynaptic neuron through gliotransmitter activity, and play a role in dampening activity and promoting the removal of nonadvantageous connections [141]. The evidence reviewed here shows that astrocytes have an ongoing role in the regulation of neuronal activity through the release of gliotransmitters and the expression of transporters/receptors on their extracellular surface. Based on these findings, we propose a mechanism of astrocyte-to-postsynaptic neuron interaction that supports the induction of LTP (see Figure 1). Here, the influx of intracellular Ca^{2+} caused by the activation of cholinergic receptors and mGluRs allows multiple gliotransmitters (e.g, glutamate, D-serine, TNF-α, and ATP) to be released. These gliotransmitters then bind to their respective receptor to regulate the influx of ions on the postsynaptic neuron, which causes a cascade of molecular mechanisms that initiate transcription. Ephrin B signaling may also contribute to gliotransmitter release by increasing intracellular Ca^{2+}. Moreover, for the cellular and molecular changes of the postsynaptic neuron, lactate must be provided by astrocytes for energy to protect neurons from cytotoxic death, and GLT-1 regulates the extracellular glutamate concentration during the late phase of LTP.

The purpose of the proposed mechanism is to represent how astrocytes may regulate the postsynaptic neuron during LTP. Behavioral studies used to determine that the role of astrocytes are known to be hippocampal dependent tasks. However, this by no means allows us to determine which part of the hippocampus the mechanism takes place in, nor the type of LTP. More importantly, there are various kinds of memory such as episodic memory, procedural memory, associative memory, and fear conditioned memory. Moreover, it is important to note that this model only examines astrocyte to postsynaptic terminal communication: it is well known that astrocytes are also able to modulate presynaptic terminal [142, 143].

Although we have explained detailed evidence of how astrocytes regulate the postsynaptic neuron, we must also consider how astrocytes affect activity of the presynaptic neuron as well. Hippocampal astrocytes are able to detect synaptic activity at distinct locations via mGluR5 and increase intracellular Ca^{2+} levels for a prolonged time span, which results in alteration of basal synaptic transmission [144, 145]. The mechanism also involves astrocytic release of purines to activate A2A receptors expressed by the presynaptic neuron. Calcium activity was also observed to not only be involved in gliotransmission, but neurotransmission as well. Synaptic transmission in neighboring synapses was reduced when blocking Ca^{2+} in astrocytes, suggesting the ability of astrocytes to modulate the activity of presynaptic neurons.

The engraftment of human astrocytes in mice enhances LTP and significantly increases the release of the cytokine TNF-α [146]. Since a xenograft of human astrocytes can functionally modulate the activity of mice neurons, it may be possible that a xenograft from another species would facilitate LTP if placed into a human patient. There is still much to research in glial neurobiology in order to fully understand the underlying mechanisms of neural networks that are involved in plasticity and memory. For instance, since astrocytes are physically connected with other astrocytes

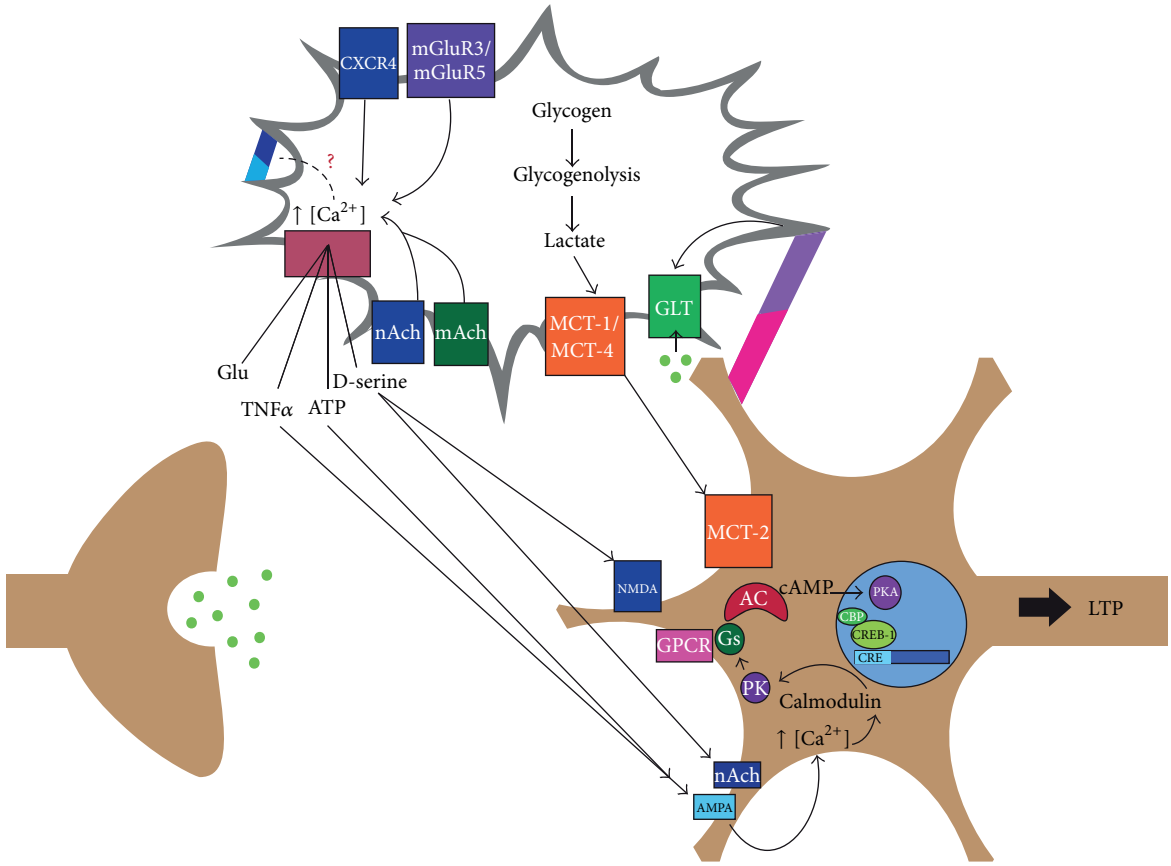

Figure 1: Integrative model of postsynaptic glia-neuron communication during memory formation.

through gap junctions to form a glial syncytium, it is crucial to further examine how astrocytic signaling may regulate neuronal activity and therefore, underlie LTP. It is now clear that astrocytes play an important part in learning and memory, and continuing to elucidate astrocytic processes that are involved in learning and memory will help advance our understanding of the dynamic role of these glial cells in modulating LTP.

Conflict of Interests

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

Authors' Contribution

All authors equally contributed to this work.

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References

[1] S. J. Martin, P. D. Grimwood, and R. G. M. Morris, "Synaptic plasticity and memory: an evaluation of the hypothesis," *Annual Review of Neuroscience*, vol. 23, no. 1, pp. 649–711, 2000.

[2] J. S. Bains and S. H. R. Oliet, "Glia: they make your memories stick!," *Trends in Neurosciences*, vol. 30, no. 8, pp. 417–424, 2007.

[3] N. B. Hamilton and D. Attwell, "Do astrocytes really exocytose neurotransmitters?" *Nature Reviews*, vol. 11, no. 4, pp. 227–238, 2010.

[4] U. Lalo, A. Verkhratsky, and Y. Pankratov, "Ionotropic ATP receptors in neuronal-glial communication," *Seminars in Cell & Developmental Biology*, vol. 22, no. 2, pp. 220–228, 2011.

[5] A. Volterra and J. Meldolesi, "Astrocytes, from brain glue to communication elements: the revolution continues," *Nature Reviews*, vol. 6, no. 8, pp. 626–640, 2005.

[6] F. A. C. Azevedo, L. R. B. Carvalho, L. T. Grinberg et al., "Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain," *Journal of Comparative Neurology*, vol. 513, no. 5, pp. 532–541, 2009.

[7] S. M. Dombrowski, C. C. Hilgetag, and H. Barbas, "Quantitative architecture distinguishes prefrontal cortical systems in the rhesus monkey," *Cerebral Cortex*, vol. 11, no. 10, pp. 975–988, 2001.

[8] S. Herculano-Houzel and R. Lent, "Isotropic fractionator: a simple, rapid method for the quantification of total cell and neuron numbers in the brain," *The Journal of Neuroscience*, vol. 25, no. 10, pp. 2518–2521, 2005.

[9] C. C. Hilgetag and H. Barbas, "Are there ten times more glia than neurons in the brain?" *Brain Structure & Function*, vol. 213, no. 4-5, pp. 365–366, 2009.

[10] E. R. Kandel, J. H. Schwartz, and T. M. Jessell, *Principles of Neural Science, Nerve Cells and Behavior*, McGraw-Hill, New York, NY, USA, 4th edition, 2000.

[11] A. Nishiyama, Z. Yang, and A. Butt, "Astrocytes and NG2-glia: what's in a name?" *Journal of Anatomy*, vol. 207, no. 6, pp. 687–693, 2005.

[12] B. Pakkenberg and H. J. G. Gundersen, "Total number of neurons and glial cells in human brain nuclei estimated by the disector and the fractionator," *Journal of Microscopy*, vol. 150, no. 1, pp. 1–20, 1988.

[13] N. A. Oberheim, S. A. Goldman, and M. Nedergaard, "Heterogeneity of astrocytic form and function," *Methods in Molecular Biology*, vol. 814, pp. 23–45, 2012.

[14] A. Derouiche, E. Anlauf, G. Aumann, B. Mühlstädt, and M. Lavialle, "Anatomical aspects of glia-synapse interaction: the perisynaptic glial sheath consists of a specialized astrocyte compartment," *Journal of Physiology*, vol. 96, no. 3-4, pp. 177–182, 2002.

[15] A. Reichenbach, A. Derouiche, and F. Kirchhoff, "Morphology and dynamics of perisynaptic glia," *Brain Research Reviews*, vol. 63, no. 1-2, pp. 11–25, 2010.

[16] A. Verkhratsky and A. Butt, *Neuronal-Glial Interactions, in Glial Neurobiology: A Textbook*, John Wiley & Sons, Chichester, UK, 2007.

[17] M. Simard and M. Nedergaard, "The neurobiology of glia in the context of water and ion homeostasis," *Neuroscience*, vol. 129, no. 4, pp. 877–896, 2004.

[18] K. Holthoff and O. W. Witte, "Directed spatial potassium redistribution in rat neocortex," *Glia*, vol. 29, no. 3, pp. 288–292, 2000.

[19] P. Kofuji and E. A. Newman, "Potassium buffering in the central nervous system," *Neuroscience*, vol. 129, no. 4, pp. 1045–1056, 2004.

[20] R. K. Orkand, J. G. Nicholls, and S. W. Kuffler, "Effect of nerve impulses on the membrane potential of glial cells in the central nervous system of amphibia," *Journal of Neurophysiology*, vol. 29, no. 4, pp. 788–806, 1966.

[21] W. Walz, "Role of astrocytes in the clearance of excess extracellular potassium," *Neurochemistry International*, vol. 36, no. 4-5, pp. 291–300, 2000.

[22] A. Araque, V. Parpura, R. P. Sanzgiri, and P. G. Haydon, "Tripartite synapses: glia, the unacknowledged partner," *Trends in Neurosciences*, vol. 22, no. 5, pp. 208–215, 1999.

[23] M. M. Halassa, T. Fellin, and P. G. Haydon, "Tripartite synapses: roles for astrocytic purines in the control of synaptic physiology and behavior," *Neuropharmacology*, vol. 57, no. 4, pp. 343–346, 2009.

[24] M. Santello, C. Calì, and P. Bezzi, "Gliotransmission and the tripartite synapse," *Advances in Experimental Medicine and Biology*, vol. 970, pp. 307–331, 2012.

[25] V. Matyash and H. Kettenmann, "Heterogeneity in astrocyte morphology and physiology," *Brain Research Reviews*, vol. 63, no. 1-2, pp. 2–10, 2010.

[26] M. R. Witcher, S. A. Kirov, and K. M. Harris, "Plasticity of perisynaptic astroglia during synaptogenesis in the mature rat hippocampus," *Glia*, vol. 55, no. 1, pp. 13–23, 2007.

[27] E. A. Bushong, M. E. Martone, Y. Z. Jones, and M. H. Ellisman, "Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains," *The Journal of Neuroscience*, vol. 22, no. 1, pp. 183–192, 2002.

[28] R. Ventura and K. M. Harris, "Three-dimensional relationships between hippocampal synapses and astrocytes," *The Journal of Neuroscience*, vol. 19, no. 16, pp. 6897–6906, 1999.

[29] R. A. Swanson, "Astrocyte neurotransmitter uptake," in *Neuroglia*, H. Kettenmann and B. R. Ransom, Eds., pp. 346–354, Oxford University Press, Oxford, UK, 2005.

[30] C. Iacovetta, E. Rudloff, and R. Kirby, "The role of aquaporin 4 in the brain," *Veterinary Clinical Pathology*, vol. 41, no. 1, pp. 32–44, 2012.

[31] M. C. Papadopoulos and A. S. Verkman, "Aquaporin water channels in the nervous system," *Nature Reviews*, vol. 14, no. 4, pp. 265–277, 2013.

[32] H. K. Kimelberg, "Astrocytic swelling in cerebral ischemia as a possible cause of injury and target for therapy," *Glia*, vol. 50, no. 4, pp. 389–397, 2005.

[33] H. K. Kimelberg, S. K. Goderie, S. Higman, S. Pang, and R. A. Waniewski, "Swelling-induced release of glutamate, aspartate, and taurine from astrocyte cultures," *The Journal of Neuroscience*, vol. 10, no. 5, pp. 1583–1591, 1990.

[34] E. M. Rutledge and H. K. Kimelberg, "Release of [^{3}H]-D-aspartate from primary astrocyte cultures in response to raised external potassium," *The Journal of Neuroscience*, vol. 16, no. 24, pp. 7803–7811, 1999.

[35] M. C. W. Kroes and G. Fernández, "Dynamic neural systems enable adaptive, flexible memories," *Neuroscience & Biobehavioral Reviews*, vol. 36, no. 7, pp. 1646–1666, 2012.

[36] W. B. Scoville and B. Milner, "Loss of recent memory after bilateral hippocampal lesions," *Journal of Neurology, Neurosurgery, and Psychiatry*, vol. 20, no. 1, pp. 11–21, 1957.

[37] L. F. Abbott and S. B. Nelson, "Synaptic plasticity: taming the beast," *Nature Neuroscience*, vol. 3, no. 11s, pp. 1178–1183, 2000.

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

[39] H.-K. Lee, "Synaptic plasticity and phosphorylation," *Pharmacology & Therapeutics*, vol. 112, no. 3, pp. 810–832, 2006.

[40] K. Lehmann, A. Steinecke, and J. Bolz, "GABA through the ages: regulation of cortical function and plasticity by inhibitory interneurons," *Neural Plasticity*, vol. 2012, Article ID 892784, 11 pages, 2012.

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

[42] K. J. Harms and A. Dunaevsky, "Dendritic spine plasticity: looking beyond development," *Brain Research*, vol. 1184, no. 1, pp. 65–71, 2007.

[43] R. Yuste and T. Bonhoeffer, "Morphological changes in dendritic spines associated with long-term synaptic plasticity," *Annual Review of Neuroscience*, vol. 24, pp. 1071–1089, 2001.

[44] K. Okamoto, M. Bosch, and Y. Hayashi, "The roles of CaMKII and F-Actin in the structural plasticity of dendritic spines: a potential molecular identity of a synaptic tag?" *Physiology*, vol. 24, no. 6, pp. 357–366, 2009.

[45] K. Deisseroth, H. Bito, and R. W. Tsien, "Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity," *Neuron*, vol. 16, no. 1, pp. 89–101, 1996.

[46] J. A. Esteban, S.-H. Shi, C. Wilson, M. Nuriya, R. L. Huganir, and R. Malinow, "PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity," *Nature Neuroscience*, vol. 6, no. 2, pp. 136–143, 2003.

[47] L. A. Raymond, W. G. Tingley, C. D. Blackstone, K. W. Roche, and R. L. Huganir, "Glutamate receptor modulation by protein phosphorylation," *Journal of Physiology*, vol. 88, no. 3, pp. 181–192, 1994.

[48] C. Lüscher, R. A. Nicoll, R. C. Malenka, and D. Muller, "Synaptic plasticity and dynamic modulation of the postsynaptic membrane," *Nature Neuroscience*, vol. 3, no. 6, pp. 545–550, 2000.

[49] N. J. Allen and B. A. Barres, "Signaling between glia and neurons: focus on synaptic plasticity," *Current Opinion in Neurobiology*, vol. 15, no. 5, pp. 542–548, 2005.

[50] R. D. Fields and B. Stevens-Graham, "Neuroscience: new insights into neuron-glia communication," *Science*, vol. 298, no. 5593, pp. 556–562, 2002.

[51] A. Citri and R. C. Malenka, "Synaptic plasticity: multiple forms, functions, and mechanisms," *Neuropsychopharmacology*, vol. 33, no. 1, pp. 18–41, 2008.

[52] T. Lømo, "The discovery of long-term potentiation," *Philosophical Transactions of the Royal Society B*, vol. 358, no. 1432, pp. 617–620, 2003.

[53] G. Barrionuevo, S. R. Kelso, D. Johnston, and T. H. Brown, "Conductance mechanism responsible for long-term potentiation in monosynaptic and isolated excitatory synaptic inputs to hippocampus," *Journal of Neurophysiology*, vol. 55, no. 3, pp. 540–550, 1986.

[54] G. L. Collingridge, S. J. Kehl, and H. McLennan, "Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus," *Journal of Physiology*, vol. 334, pp. 33–46, 1983.

[55] E. W. Harris and C. W. Cotman, "Long-term potentiation of guinea pig mossy fiber responses is not blocked by N-methyl D-aspartate antagonists," *Neuroscience Letters*, vol. 70, no. 1, pp. 132–137, 1986.

[56] E. W. Harris, A. H. Ganong, and C. W. Cotman, "Long-term potentiation in the hippocampus involves activation of N-methyl-D-aspartate receptors," *Brain Research*, vol. 323, no. 1, pp. 132–137, 1984.

[57] R. C. Malenka and M. F. Bear, "LTP and LTD: an embarrassment of riches," *Neuron*, vol. 44, no. 1, pp. 5–21, 2004.

[58] D. M. Bannerman, M. A. Good, S. P. Butcher, M. Ramsay, and R. G. M. Morris, "Distinct components of spatial learning revealed by prior training and NMDA receptor blockade," *Nature*, vol. 378, no. 6553, pp. 182–186, 1995.

[59] I. Lee and R. P. Kesner, "Differential contribution of NMDA receptors in hippocampal subregions to spatial working memory," *Nature Neuroscience*, vol. 5, no. 2, pp. 162–168, 2002.

[60] K. Nakazawa, M. C. Quirk, R. A. Chitwood et al., "Requirement for hippocampal CA3 NMDA receptors in associative memory recall," *Science*, vol. 297, no. 5579, pp. 211–218, 2002.

[61] J. Z. Tsien, P. T. Huerta, and S. Tonegawa, "The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory," *Cell*, vol. 87, no. 7, pp. 1327–1338, 1996.

[62] E. R. Kandel, "The molecular biology of memory storage: a dialogue between genes and synapses," *Science*, vol. 294, no. 5544, pp. 1030–1038, 2001.

[63] M. Mayford, S. A. Siegelbaum, and E. Kandel, "Synapses and memory storage," *Cold Spring Harbor Perspectives in Biology*, vol. 4, no. 6, 2012.

[64] E. Miyamoto, "Molecular mechanism of neuronal plasticity: Induction and maintenance of long-term potentiation in the hippocampus," *Journal of Pharmacological Sciences*, vol. 100, no. 5, pp. 433–442, 2006.

[65] D. T. Theodosis, D. A. Poulain, and S. H. R. Oliet, "Activity-dependent structural and functional plasticity of astrocyte-neuron interactions," *Physiological Reviews*, vol. 88, no. 3, pp. 983–1008, 2008.

[66] A. H. Cornell-Bell, P. G. Thomas, and S. J. Smith, "The excitatory neurotransmitter glutamate causes filopodia formation in cultured hippocampal astrocytes," *Glia*, vol. 3, no. 5, pp. 322–334, 1990.

[67] S. R. Glaum, J. A. Holzwarth, and R. J. Miller, "Glutamate receptors activate Ca^{2+} mobilization and Ca^{2+} influx into astrocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 9, pp. 3454–3458, 1990.

[68] D. E. Bergles and C. E. Jahr, "Synaptic activation of glutamate transporters in hippocampal astrocytes," *Neuron*, vol. 19, no. 6, pp. 1297–1308, 1997.

[69] D. A. Rusakov, K. Zheng, and C. Henneberger, "Astrocytes as regulators of synaptic function: a quest for the Ca^{2+} master Key," *Neuroscientist*, vol. 17, no. 5, pp. 513–523, 2011.

[70] F. Conti, S. DeBiasi, A. Minelli, and M. Melone, "Expression of NR1 and NR2A/B subunits of the NMDA receptor in cortical astrocytes," *Glia*, vol. 17, no. 3, pp. 254–258, 1998.

[71] U. Lalo, Y. Pankratov, F. Kirchhoff, R. A. North, and A. Verkhratsky, "NMDA receptors mediate neuron-to-glia signaling in mouse cortical astrocytes," *The Journal of Neuroscience*, vol. 26, no. 10, pp. 2673–2683, 2006.

[72] C. Steinhäuser and V. Gallo, "News on glutamate receptors in glial cells," *Trends in Neurosciences*, vol. 19, no. 8, pp. 339–345, 1996.

[73] D. Fan, S. Y. Grooms, R. C. Araneda et al., "AMPA receptor protein expression and function in astrocytes cultured from hippocampus," *Journal of Neuroscience Research*, vol. 57, pp. 557–571, 1999.

[74] M. Zhou and H. K. Kimelberg, "Freshly isolated hippocampal CA1 astrocytes comprise two populations differing in glutamate transporter and AMPA receptor expression," *The Journal of Neuroscience*, vol. 21, no. 20, pp. 7901–7908, 2001.

[75] G. Seifert, M. Zhou, and C. Steinhäuser, "Analysis of AMPA receptor properties during postnatal development of mouse hippocampal astrocytes," *Journal of Neurophysiology*, vol. 78, no. 6, pp. 2916–2923, 1997.

[76] M. Amiri, F. Bahrami, and M. Janahmadi, "Functional contributions of astrocytes in synchronization of a neuronal network model," *Journal of Theoretical Biology*, vol. 292, pp. 60–70, 2012.

[77] M. Amiri, N. Hosseinmardi, F. Bahrami, and M. Janahmadi, "Astrocyte-neuron interaction as a mechanism responsible for generation of neural synchrony: a study based on modeling and experiments," *Journal of Computational Neuroscience*, vol. 34, no. 3, pp. 489–504, 2013.

[78] M. C. Angulo, A. S. Kozlov, S. Charpak, and E. Audinat, "Glutamate released from glial cells synchronizes neuronal

activity in the hippocampus," *The Journal of Neuroscience*, vol. 24, no. 31, pp. 6920–6927, 2004.

[79] L. K. Bekar, M. E. Loewen, K. Cao et al., "Complex expression and localization of inactivating Kv channels in cultured hippocampal astrocytes," *Journal of Neurophysiology*, vol. 93, no. 3, pp. 1699–1709, 2005.

[80] P. Jourdain, L. H. Bergersen, K. Bhaukaurally et al., "Glutamate exocytosis from astrocytes controls synaptic strength," *Nature Neuroscience*, vol. 10, no. 3, pp. 331–339, 2007.

[81] C. Henneberger, T. Papouin, S. H. R. Oliet, and D. A. Rusakov, "Long-term potentiation depends on release of D-serine from astrocytes," *Nature*, vol. 463, no. 7278, pp. 232–236, 2010.

[82] J.-P. Mothet, A. T. Parent, H. Wolosker et al., "D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 9, pp. 4926–4931, 2000.

[83] M. J. Schell, M. E. Molliver, and S. H. Snyder, "D-serine, an endogenous synaptic modulator: Localization to astrocytes and glutamate-stimulated release," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 9, pp. 3948–3952, 1995.

[84] M. Shleper, E. Kartvelishvily, and H. Wolosker, "D-serine is the dominant endogenous coagonist for NMDA receptor neurotoxicity in organotypic hippocampal slices," *The Journal of Neuroscience*, vol. 25, no. 41, pp. 9413–9417, 2005.

[85] M. Bernstein, T. Behnisch, D. Balschun, K. G. Reymann, and G. Reiser, "Pharmacological characterisation of metabotropic glutamatergic and purinergic receptors linked to Ca^{2+} signalling in hippocampal astrocytes," *Neuropharmacology*, vol. 37, no. 2, pp. 169–178, 1998.

[86] M. E. Gibbs and D. N. Bowser, "Astrocytes and interneurons in memory processing in the chick hippocampus: roles for G-coupled protein receptors, GABA(B) and mGluR1," *Neurochemical Research*, vol. 34, no. 10, pp. 1712–1720, 2009.

[87] Z. Cai, G. P. Schools, and H. K. Kimelbert, "Metabotropic glutamate receptors in acutely isolated hippocampal astrocytes: developmental changes of mGluR5 mRNA and functional expression," *Glia*, vol. 29, pp. 70–80, 2000.

[88] R. J. Cormier, S. Mennerick, H. Melbostad, and C. F. Zorumski, "Basal levels of adenosine modulate mGluR5 on rat hippocampal astrocytes," *Glia*, vol. 33, no. 1, pp. 24–35, 2001.

[89] E. Aronica, J. A. Gorter, H. Ijlst-Keizers et al., "Expression and functional role of mGluR3 and mGluR5 in human astrocytes and glioma cells: Opposite regulation of glutamate transporter proteins," *European Journal of Neuroscience*, vol. 17, no. 10, pp. 2106–2118, 2003.

[90] W. Sun, E. McConnell, J. F. Pare et al., "Glutamate-dependent neuroglial calcium signaling differs between young and adult brain," *Science*, vol. 339, no. 6116, pp. 197–200, 2013.

[91] S. B. Achour, L. Pont-Lezica, C. Béchade, and O. Pascual, "Is astrocyte calcium signaling relevant for synaptic plasticity?" *Neuron Glia Biology*, vol. 6, no. 3, pp. 147–155, 2010.

[92] V. Parpura and R. Zorec, "Gliotransmission: exocytotic release from astrocytes," *Brain Research Reviews*, vol. 63, no. 1-2, pp. 83–92, 2010.

[93] Q. Zhang, T. Pangršič, M. Kreft et al., "Fusion-related release of glutamate from astrocytes," *Journal of Biological Chemistry*, vol. 279, no. 13, pp. 12724–12733, 2004.

[94] M. Martineau, T. Shi, J. Puyal et al., "Storage and uptake of D-serine into astrocytic synaptic like vesicles specify gliotranmission," *The Journal of Neuroscience*, vol. 33, no. 8, pp. 3413–3423, 2013.

[95] A. Wilhelm, W. Volknandt, D. Langer, C. Nolte, H. Kettenmann, and H. Zimmermann, "Localization of SNARE proteins and secretory organelle proteins in astrocytes in vitro and in situ," *Neuroscience Research*, vol. 48, no. 3, pp. 249–257, 2004.

[96] P. B. Guthrie, J. Knappenberger, M. Segal, M. V. L. Bennett, A. C. Charles, and S. B. Kater, "ATP released from astrocytes mediates glial calcium waves," *The Journal of Neuroscience*, vol. 8, pp. 1078–1086, 1999.

[97] E. A. Newman, "Glial cell inhibition of neurons by release of ATP," *The Journal of Neuroscience*, vol. 23, no. 5, pp. 1659–1666, 2003.

[98] C. Calì and P. Bezzi, "CXCR4-mediated glutamate exocytosis from astrocytes," *Journal of Neuroimmunology*, vol. 224, no. 1-2, pp. 13–21, 2010.

[99] E. C. Beattie, D. Stellwagen, W. Morishita et al., "Control of synaptic strength by glial TNFα," *Science*, vol. 295, no. 5563, pp. 2282–2285, 2002.

[100] M. Santello, P. Bezzi, and A. Volterra, "TNFα controls glutamatergic gliotransmission in the hippocampal dentate gyrus," *Neuron*, vol. 69, no. 5, pp. 988–1001, 2011.

[101] Y.-J. Gao and R.-R. Ji, "Chemokines, neuronal-glial interactions, and central processing of neuropathic pain," *Pharmacology & Therapeutics*, vol. 126, no. 1, pp. 56–68, 2010.

[102] C. Agulhon, T. A. Fiacco, and K. D. McCarthy, "Hippocampal short- and long-term plasticity are not modulated by astrocyte Ca^{2+} signaling," *Science*, vol. 327, no. 5970, pp. 1250–1254, 2010.

[103] T. A. Fiacco, C. Agulhon, S. R. Taves et al., "Selective stimulation of astrocyte calcium in situ does not affect neuronal excitatory synaptic activity," *Neuron*, vol. 54, no. 4, pp. 611–626, 2007.

[104] J. Petravicz, T. A. Fiacco, and K. D. McCarthy, "Loss of IP_3 receptor-dependent Ca^{2+} increases in hippocampal astrocytes does not affect baseline CA1 pyramidal neuron synaptic activity," *The Journal of Neuroscience*, vol. 28, no. 19, pp. 4967–4973, 2008.

[105] M. E. Gibbs and L. Hertz, "Inhibition of astrocytic energy metabolism by D-lactate exposure impairs memory," *Neurochemistry International*, vol. 52, no. 6, pp. 1012–1018, 2008.

[106] L. A. Newman, D. L. Korol, and P. E. Gold, "Lactate produced by glycogenolysis in astrocytes regulates memory processing," *PLoS ONE*, vol. 6, no. 12, Article ID e28427, 2011.

[107] A. Suzuki, S. A. Stern, O. Bozdagi et al., "Astrocyte-neuron lactate transport is required for long-term memory formation," *Cell*, vol. 144, no. 5, pp. 810–823, 2011.

[108] C. Bourgin, K. K. Murai, M. Richter, and E. B. Pasquale, "The EphA4 receptor regulates dendritic spine remodeling by affecting β1-integrin signaling pathways," *Journal of Cell Biology*, vol. 178, no. 7, pp. 1295–1307, 2007.

[109] M. Hruska and M. B. Dalva, "Ephrin regulation of synapse formation, function and plasticity," *Molecular and Cellular Neuroscience*, vol. 50, no. 1, pp. 35–44, 2012.

[110] R. Klein, "Eph/ephrin signaling in morphogenesis, neural development and plasticity," *Current Opinion in Cell Biology*, vol. 16, no. 5, pp. 580–589, 2004.

[111] A. Martínez and E. Soriano, "Functions of ephrin/Eph interactions in the development of the nervous system: emphasis on the hippocampal system," *Brain Research Reviews*, vol. 49, no. 2, pp. 211–226, 2005.

[112] K. K. Murai, L. N. Nguyen, F. Irie, Y. Yu, and E. B. Pasquale, "Control of hippocampal dendritic spine morphology through ephrin-A3/EphA4 signaling," *Nature Neuroscience*, vol. 6, no. 2, pp. 153–160, 2003.

[113] P. A. Yates, A. L. Roskies, T. McLaughlin, and D. D. M. O'Leary, "Topographic-specific axon branching controlled by ephrin-As is the critical event in retinotectal map development," *The Journal of Neuroscience*, vol. 21, no. 21, pp. 8548–8563, 2001.

[114] L. Zhou, S. J. Martinez, M. Haber et al., "EphA4 signaling regulates phospholipase Cγ1 activation, cofilin membrane association, and dendritic spine morphology," *The Journal of Neuroscience*, vol. 27, no. 19, pp. 5127–5138, 2007.

[115] M. A. Carmona, K. K. Murai, L. Wang, A. J. Roberts, and E. B. Pasqualea, "Glial ephrin-A3 regulates hippocampal dendritic spine morphology and glutamate transport," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 30, pp. 12524–12529, 2009.

[116] L. E. Clarke and B. A. Barres, "Emerging roles of astrocytes in neural circuit development," *Nature Reviews*, vol. 14, pp. 311–321, 2013.

[117] A. Filosa, S. Paixo, S. D. Honsek et al., "Neuron-glia communication via EphA4/ephrin-A3 modulates LTP through glial glutamate transport," *Nature Neuroscience*, vol. 12, no. 10, pp. 1285–1292, 2009.

[118] S. Paixao and R. Klein, "Neuron-astrocyte communication and synaptic plasticity," *Signaling Mechanisms*, vol. 20, no. 4, pp. 466–473, 2010.

[119] Z. Zhuang, B. Yang, M. H. Theus et al., "EphrinBs regulate D-serine synthesis and release in astrocytes," *The Journal of Neuroscience*, vol. 30, no. 47, pp. 16015–16024, 2010.

[120] C. T. Blits-Huizinga, C. M. Nelersa, A. Malhotra, and D. J. Liebl, "Ephrins and their receptors: binding versus biology," *IUBMB Life*, vol. 56, no. 5, pp. 257–265, 2004.

[121] C. M. Hernandez and A. V. Terry Jr., "Repeated nicotine exposure in rats: effects on memory function, cholinergic markers and nerve growth factor," *Neuroscience*, vol. 130, no. 4, pp. 997–1012, 2005.

[122] A. N. Placzek, T. A. Zhang, and J. A. Dani, "Nicotinic mechanisms influencing synaptic plasticity in the hippocampus," *Acta Pharmacologica Sinica*, vol. 30, no. 6, pp. 752–760, 2009.

[123] G. Sharma and S. Vijayaraghavan, "Nicotinic receptor signaling in nonexcitable cells," *Journal of Neurobiology*, vol. 53, no. 4, pp. 524–534, 2002.

[124] P. Newhouse, K. Kellar, P. Aisen et al., "Nicotine treatment of mild cognitive impairment: a 6-month double-blind pilot clinical trial," *Neurology*, vol. 78, no. 2, pp. 91–101, 2012.

[125] M. López-Hidalgo, K. Salgado-Puga, R. Alvarado-Martínez et al., "Nicotine uses neuron-glia communication to enhance hippocampal synaptic transmission and long-term memory," *PLoS ONE*, vol. 7, no. 11, 2012.

[126] M. Navarrete, G. Perea, D. F. de Sevilla et al., "Astrocytes mediate in vivo cholinergic-induced synaptic plasticity," *PLoS Biology*, vol. 10, no. 2, Article ID e1001259, 2012.

[127] M. K. Shelton and K. D. McCarthy, "Hippocampal astrocytes exhibit Ca^{2+}-elevating muscarinic cholinergic and histaminergic receptors in situ," *Journal of Neurochemistry*, vol. 74, no. 2, pp. 555–563, 2000.

[128] N. Takata, T. Mishima, C. Hisatsune et al., "Astrocyte calcium signaling transforms cholinergic modulation to cortical plasticity in vivo," *The Journal of Neuroscience*, vol. 31, no. 49, pp. 18155–18165, 2011.

[129] C. Florian, C. G. Vecsey, M. M. Halassa, P. G. Haydon, and T. Abel, "Astrocyte-derived adenosine and A1 receptor activity contribute to sleep loss-induced deficits in hippocampal synaptic plasticity and memory in mice," *The Journal of Neuroscience*, vol. 31, no. 19, pp. 6956–6962, 2011.

[130] D. J. Hines, L. I. Schmitt, R. M. Hines, S. J. Moss, and P. G. Haydon, "Antidepressant effects of sleep deprivation require astrocyte-dependent adenosine mediated signaling," *Translational Psychiatry*, vol. 3, article e212, 2013.

[131] A. Nadjar, T. Blutstein, A. Aubert, S. Laye, and P. G. Haydon, "Astrocyte-derived adenosine modulates increased sleep pressure during inflammatory response," *Glia*, vol. 61, pp. 724–731, 2013.

[132] A. Avital, I. Goshen, A. Kamsler et al., "Impaired interleukin-1 signaling is associated with deficits in hippocampal memory processes and neural plasticity," *Hippocampus*, vol. 13, no. 7, pp. 826–834, 2003.

[133] I. Goshen, T. Kreisel, H. Ounallah-Saad et al., "A dual role for interleukin-1 in hippocampal-dependent memory processes," *Psychoneuroendocrinology*, vol. 32, no. 8-10, pp. 1106–1115, 2007.

[134] O. Ben Menachem-Zidon, I. Goshen, T. Kreisel et al., "Intrahippocampal transplantation of transgenic neural precursor cells overexpressing interleukin-1 receptor antagonist blocks chronic isolation-induced impairment in memory and neurogenesis," *Neuropsychopharmacology*, vol. 33, no. 9, pp. 2251–2262, 2008.

[135] R. Yirmiya, G. Winocur, and I. Goshen, "Brain interleukin-1 is involved in spatial memory and passive avoidance conditioning," *Neurobiology of Learning and Memory*, vol. 78, no. 2, pp. 379–389, 2002.

[136] E. M. Ban, L. L. Sarlieve, and F. G. Haour, "Interleukin-1 binding sites on astrocytes," *Neuroscience*, vol. 52, no. 3, pp. 725–733, 1993.

[137] E. T. Cunningham Jr., E. Wada, D. B. Carter, D. E. Tracey, J. F. Battey, and E. B. De Souza, "In situ histochemical localization of type I interleukin-1 receptor messenger RNA in the central nervous system, pituitary, and adrenal gland of the mouse," *The Journal of Neuroscience*, vol. 12, no. 3, pp. 1101–1114, 1992.

[138] W. J. Friedman, "Cytokines regulate expression of the type 1 interleukin-1 receptor in rat hippocampal neurons and glia," *Experimental Neurology*, vol. 168, no. 1, pp. 23–31, 2001.

[139] O. Ben Menachem-Zidon, A. Avital, Y. Ben-Menahem et al., "Astrocytes support hippocampal-dependent memory and long-term potentiation via interleukin-1 signaling," *Brain, Behavior, and Immunity*, vol. 25, no. 5, pp. 1008–1016, 2011.

[140] T. Toshihiro, D. E. Tracey, W. M. Mitchell, and E. B. De Souza, "Interleukin-1 receptors in mouse brain: characterization and neuronal localization," *Endocrinology*, vol. 127, no. 6, pp. 3070–3078, 1990.

[141] O. Pascual, K. B. Casper, C. Kubera et al., "Neurobiology: astrocytic purinergic signaling coordinates synaptic networks," *Science*, vol. 310, no. 5745, pp. 113–116, 2005.

[142] J. W. Dani, A. Chernjavsky, and S. J. Smith, "Neuronal activity triggers calcium waves in hippocampal astrocyte networks," *Neuron*, vol. 8, no. 3, pp. 429–440, 1992.

[143] J. T. Porter and K. D. McCarthy, "Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals," *The Journal of Neuroscience*, vol. 16, no. 16, pp. 5073–5081, 1996.

[144] M. A. di Castro, J. Chuquet, N. Liaudet et al., "Local Ca^{2+} detection and modulation of synaptic release by astrocytes," *Nature Neuroscience*, vol. 14, no. 10, pp. 1276–1284, 2011.

[145] A. Panatier, J. Vallée, M. Haber, K. K. Murai, J.-C. Lacaille, and R. Robitaille, "Astrocytes are endogenous regulators of basal transmission at central synapses," *Cell*, vol. 146, no. 5, pp. 785–798, 2011.

[146] X. Han, M. Chen, F. Wang et al., "Forebrain engraftment by human glial progenitor cells enhances synaptic plasticity and learning in adult mice," *Cell Stem Cell*, vol. 12, no. 3, pp. 342–353, 2013.

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Research Article

Improved Method for the Quantification of Motility in Glia and Other Morphologically Complex Cells

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Cells such as astrocytes and radial glia with many densely ramified, fine processes pose particular challenges for the quantification of structural motility. Here we report the development of a method to calculate a motility index for individual cells with complex, dynamic morphologies. This motility index relies on boxcar averaging of the difference images generated by subtraction of images collected at consecutive time points. An image preprocessing step involving 2D projection, edge detection, and dilation of the raw images is first applied in order to binarize the images. The boxcar averaging of difference images diminishes the impact of artifactual pixel fluctuations while accentuating the group-wise changes in pixel values which are more likely to represent real biological movement. Importantly, this provides a value that correlates with mean process elongation and retraction rates without requiring detailed reconstructions of very complex cells. We also demonstrate that additional increases in the sensitivity of the method can be obtained by denoising images using the temporal frequency power spectra, based on the fact that rapid intensity fluctuations over time are mainly due to imaging artifact. The MATLAB programs implementing these motility analysis methods, complete with user-friendly graphical interfaces, have been made publicly available for download.

1. Introduction

The development of advanced imaging methods has revealed that living cells exhibit highly dynamic structural remodeling [1, 2]. The rate and structural details of cellular motility can change over development or in response to the environment in ways that reveal important details about cellular signaling mechanisms [3–5]. Useful commercial and public domain programs are available to identify and measure the movements of relatively large cell structures like amoeboid pseudopodia [6–8] as well as fine features like growth cone filopodia [9], astrocytic protrusions [10], and dendritic spines [11, 12]. Analysis of these kinds of sparsely distributed cellular protrusions may be amenable to the use of image skeletonization or active contour tracing to select the features of interest and to track end-tips for motility analysis. An alternative approach is to measure perimeter shape changes of an entire cell by analyzing the difference between an original image and an "eroded" image from which all fine processes have been filtered away [10]. However, if the features of interest are very dense and spindly, overlapping and oriented in many directions as in the case of astrocytes and radial glia [13, 14], quantitative measurements of motility become particularly challenging for any of the above methods.

We have previously introduced a method for quantifying motility in cells with densely packed, fine processes as part of a study analyzing dynamic remodeling of midbrain radial glia [15]. After collecting a time series of 2-photon z-stacks, maximum intensity projections (i.e., maximum intensity value at each x-y position through the z-stack is used to generate a 2D projection) are aligned to correct for drift and then images are preprocessed by binarization using Sobel edge detection, followed by pixel dilation to remove gaps generated by the edge detection procedure. The algorithm then calculates a motility index, defined as the mean number of redistributed (added or lost) pixels between processed images at sequential time points. To deal with the wide range of different cell sizes, indices are normalized for size by dividing by cell area (in pixels).

One potential limitation of this approach is that normalizing pixel counts to the 2D projection area of each cell can distort the results due to variations in cell shapes and sizes. For example, z-projections of very long, narrow cells like radial glia will give dramatically different measurements of 2D projected area depending on whether a cell is viewed parallel or perpendicular to its radial axis. Although this index is still highly sensitive for measuring relative changes in motility of individual cells imaged from the same angle over time in response to different treatment conditions, the area normalization used to calculate the motility index introduces a source of artifact that limits its usefulness for comparing multiple cells that might be imaged at a range of different orientations.

In this paper, we introduce several significant improvements to our original method that make important advances toward correcting these limitations. The new approach offers greater sensitivity for cell motility analysis while decreasing the impact of total cell area on measurements by using an algorithm based on spatial boxcar averaging of redistributed pixels. This new motility index provides a value that better correlates with the mean elongation and retraction speeds of moving filopodial processes, eliminating the need to normalize by total cell area. Boxcar averaging measures motility preferentially in the dynamic parts of the image by giving more weight to pixel redistributions that occur in close proximity to other redistributions and are thus more likely to be part of real restructuring events. In addition, for high-temporal-resolution imaging, we have added a noise filter based on the temporal frequency power spectra of the pixels, optimized to preserve biological activity while removing imaging artifacts. We present these new tools as part of an open-source program for the popular MATLAB platform with a customizable user interface that can easily be applied to measure motility of a wide variety of cell types and structures.

2. Materials and Methods

2.1. Imaging EGFP-F-Transfected Radial Glia Cells. The cells examined for this study are radial glia in the optic tectum of albino Xenopus laevis tadpoles at developmental stage 47. Tadpoles were staged according to the criteria of Nieuwkoop and Faber [16]. Radial glia have many fine filopodial processes that undergo continuous structural remodeling over the timescale of minutes (Figure 1). The optic tectum in stage 45 tadpoles was bulk electroporated [17] with a DNA plasmid encoding membrane-targeting farnesylated EGFP (EGFP-F). Tectal radial glia cells were imaged two days later using a custom-built two-photon laser-scanning microscope with a 60×1.1 NA water-immersion objective (Olympus). A MaiTai-BB femtosecond pulsed Ti : sapphire laser (Newport/Spectra Physics) was used to generate excitation light at 910 nm. In preparation for imaging, tadpoles were paralyzed by immersion in 0.2 mM pancuronium bromide (Sigma) solution in 0.1x Modified Barth's solution with HEPES (VWR). Images of individual EGFP-F-expressing cells in live albino tadpoles were collected as z-series stacks of 50–150 optical sections sized 512 pixels × 512 pixels (79.5 μm × 79.5 μm) μm at 1 μm step size. In a typical experiment, 3 images were collected at 5 min intervals as the baseline. Subsequently, MK-801 maleate (100 μM, Tocris) was injected intraventricularly into the tadpoles at the level of the optic tectum. After a 20 min incubation, another 3 images were captured at 5 min intervals to assess the effects of the treatment on motility. In another set of experiments to estimate the contribution of sample drift and noise artifact, animals were euthanized in 0.2% MS222 (Sigma) and fixed by immersion in 4% paraformaldehyde (PFA, VWR) for 2 hours prior to imaging.

2.2. Image Registration for Analysis. Two-photon z-series stacks were processed by blind deconvolution using AutoQuant software. As all images of cells were captured *in vivo*, surrounding neurons and skin cells expressing EGFP as well as autofluorescent melanophores were sometimes present in a subset of z-series images, above or below the cell of interest where they could interfere with the automatic motility assessment. These non-glia objects were removed manually using ImageJ from the z-series stacks [18]. The careful removal of extraneous objects from the stacks is critical for obtaining accurate measurements and typically takes about 30 minutes per 100 optical sections.

The inherent voxel anisotropy in 2-photon microscopy—axial resolution is always poorer than resolution within the focal plane—makes perfect 3D registration of image stacks impractical. We therefore designed our motility analysis for 2D time series and took precautions to minimize rotation during image acquisition. Tadpoles were mounted in form-fitting polydimethylsiloxane (PDMS) imaging chambers sealed with a coverslip to minimize rotation of the samples over time. Z-series stacks were converted to 2D maximum intensity projections and realigned to correct for slight lateral drift over time using the rigid body setting of the EPFL StackReg plugin for ImageJ (http://bigwww.epfl.ch/thevenaz/StackReg/) [19].

2.3. Analysis of Morphological Dynamics. All subsequent processing and analysis steps in this paper were performed using custom-made scripts that can be run under the MATLAB (MathWorks) platform. These routines have been made available under a modified version of the GNU General Public License v3 (see details in program files) as part of a set package controlled with a user-friendly graphical user interface available at http://ruthazerlab.mcgill.ca/downloads/motility_GUIv2.zip.

After optimizing the alignment of the maximum intensity projections for each time point as described above, images were subjected to preprocessing by binarization using Sobel edge detection to identify edges, followed by pixel dilation (Figure 2). Pixel dilation expands the edges of the binarized images, largely eliminating discontinuity artifacts that sometimes occur in the edge detection step due to variations in the pixel intensities at fine processes in the raw images (e.g., arrowhead in Figure 2). A dilation radius of 6 pixels was used, as this was determined empirically to remove this artifact efficiently while still maintaining adequate resolution to detect small movements of the cell surface.

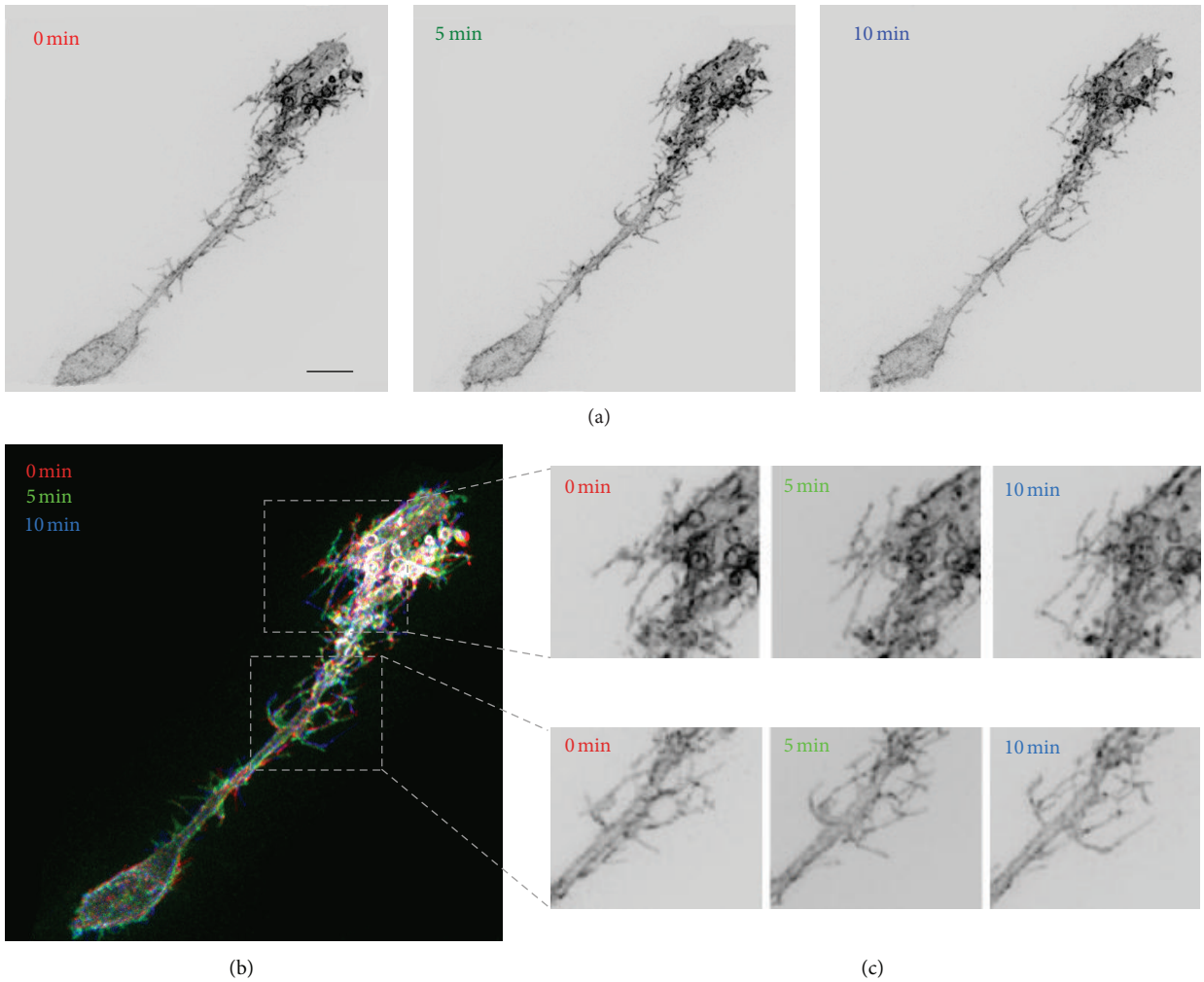

FIGURE 1: Radial glia process movement *in vivo* is apparent over a timescale of minutes. (a) Maximum intensity projections of two-photon microscope z-series images of a radial glia cell expressing farnesylated EGFP in the intact tadpole brain. (b) RGB overlay of the time-lapse images of a radial glia cell where red denotes the position of the cell at 0 minutes, green after 5 minutes, and blue after 10 minutes of imaging. (c) Magnified images of several highly dynamic sites from the radial glial cell shown in (b). Scale bar = 10 μm.

To assess the motility of the cell we subtracted the processed images of consecutive time points (Figure 3(a)) from one another. We define the binary intensity value of an image with dimensions x and y as $I_{xy}(t)$ where t is the time index of the image ranging from 1 to T in integral steps, with T being the number of time points captured. The redistribution of pixels between two time points, for each pixel defined by (x, y), is calculated as

$$\delta_{xy}(t) = I_{xy}(t+1) - I_{xy}(t). \quad (1)$$

The absolute value of this subtraction result generates an image highlighting pixels where a change has taken place from one time point to another, showing pixel redistribution (Figure 3(b)). In the original dynamics assessment approach of Tremblay et al. (2009), which we here term Method 1, the average total area of the images is taken into consideration. The area of an image with time index t is defined by

$$A(t) = \sum_{x,y} I_{xy}(t). \quad (2)$$

Sum of the redistributed pixels between time points is calculated:

$$R(t) = \sum_{xy} \delta_{xy}(t). \quad (3)$$

Motility index M_1 between two time points using Method 1 is defined as

$$M_1(t) = \frac{R(t)}{\overline{A}}, \quad (4)$$

where $\overline{A}$ is the average total area of the cell over all time points.

In the new, improved algorithm, here referred to as Method 2, the difference images calculated by formula (1) are subjected to boxcar averaging, often referred to as taking a "moving average", (Figure 3(c)) as defined by

$$\widehat{\delta}_{xy}(t) = \frac{1}{w^2} \sum_{m=x-h}^{x+h} \sum_{n=y-h}^{y+h} \delta_{mn}(t), \quad (5)$$

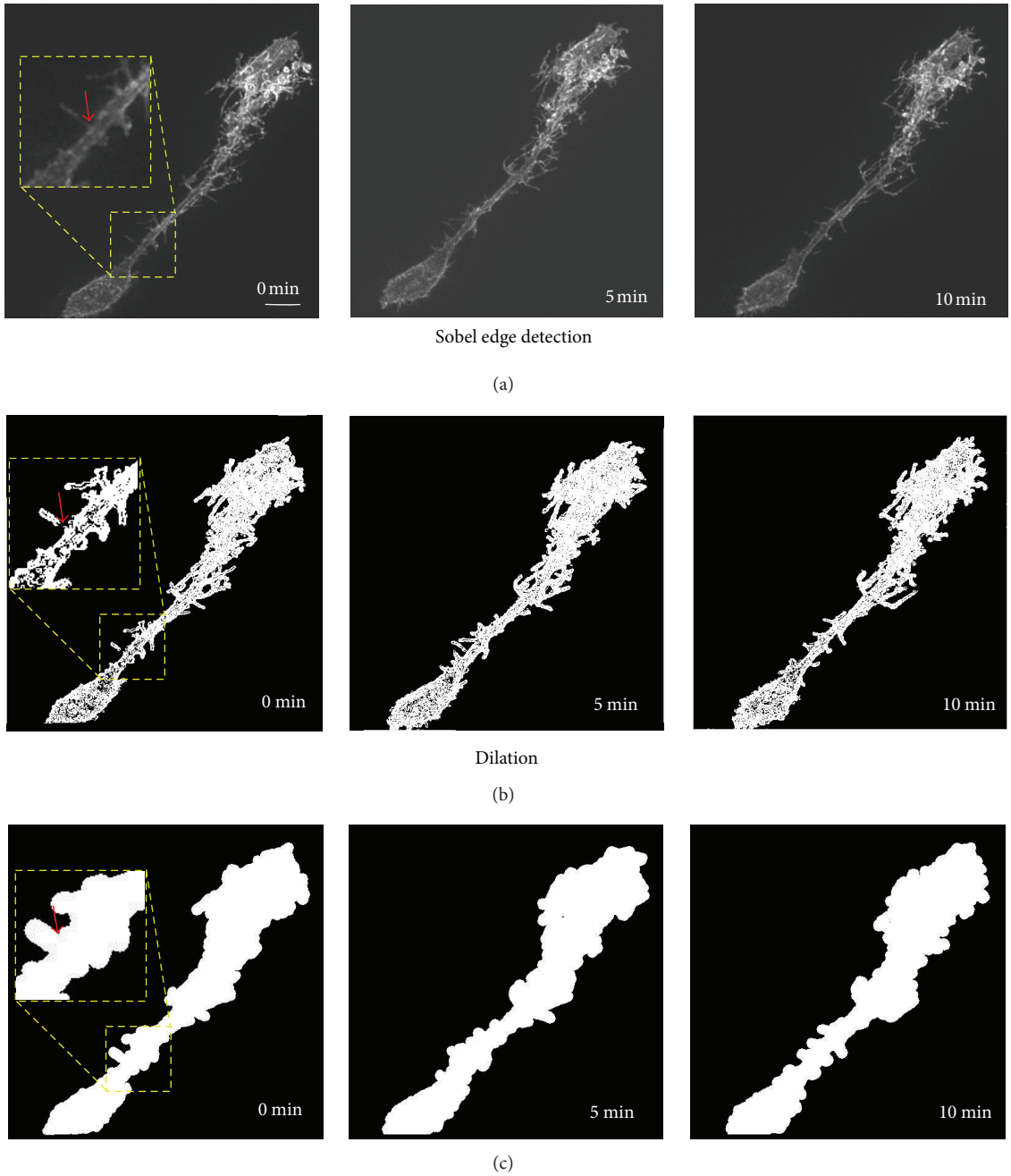

FIGURE 2: Image preprocessing for analysis. (a) Maximum intensity projections of two-photon images of a radial glia cell expressing farnesylated EGFP *in vivo* collected at 0, 5, and 10 minutes. (b) Same images after Sobel edge detection and binarization. (c) Binarized images underwent dilation with a 6-pixel radius. Pixel dilation helps eliminate artifactual discontinuities in the images (arrowhead in insets) resulting from the binarization step. Scale bar = 10 μm.

where w denotes the boxcar window width and $h = (w-1)/2$. The choice of the boxcar window size for averaging should be determined empirically depending on the dimensions of the structures imaged, taking into consideration that important details may be lost as the boxcar size is expanded. We set the boxcar size to $w = 9$ (9×9 pixel square) for our analysis. Next, the boxcar-averaged image (Figure 3(c)) is multiplied with the image showing redistribution of pixels between time points (Figure 3(b)) to obtain a final boxcar-weighted image $\theta_{xy}(t)$ (Figure 3(d)):

$$\theta_{xy}(t) = \widehat{\delta}_{xy}(t)\,\delta_{xy}(t). \tag{6}$$

This last step has the useful effect of blunting the contributions of the large number of sparse individual pixels found along the edges of the boxcar-averaged images, thus

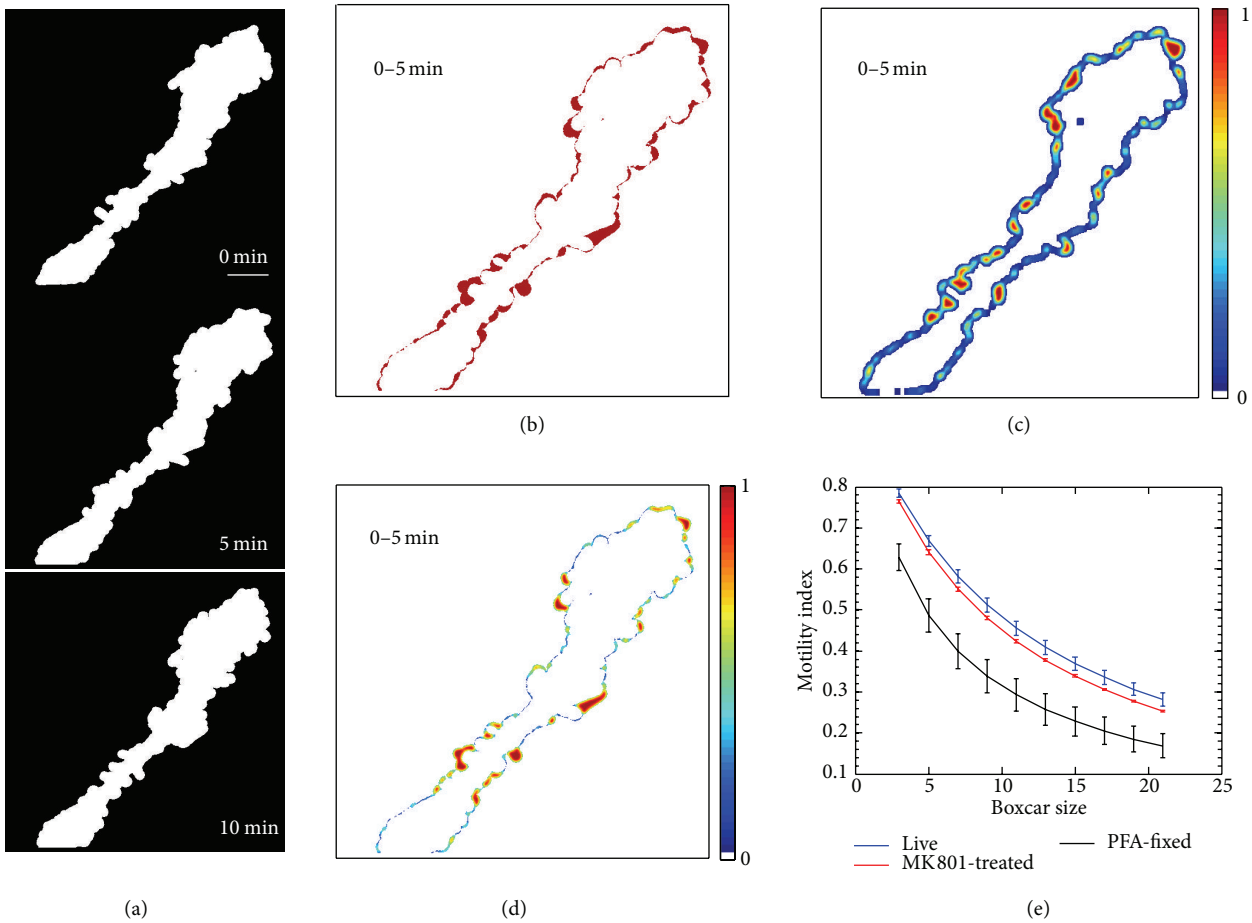

FIGURE 3: Analysis of pixel redistribution using boxcar averaging. (a) Preprocessed images from 0, 5, and 10 minutes time points. (b) Subtracting consecutive images from one another creates a map of the redistributed pixels between time points, shown in red. (c) Boxcar averaging of the pixel redistribution images from (b) using 9×9 pixel window. (d) Result of multiplication of the images in (b) and (c). The average of the nonzero pixel values in this image is used to calculate the cell motility index. (e) Plot of motility index values obtained for the datasets in Figures 5(c), 5(d), 6(c), and 6(d) using a range of different boxcar sizes. Motility values in the different conditions are most clearly separated when a 9×9 pixel boxcar size is used. Scale bar = 10 μm.

weighting the analysis in favor of dynamic "hot spots" where large groups of pixels change over time. These highly dynamic zones are expected to reflect biologically relevant pixel redistributions whereas the sparse intermittent changes along the periphery are more likely to arise from nonbiological artifact such as specimen drift, photobleaching, and detector noise.

Finally, the mean pixel value M_2 of the filtered boxcar averaged pixel redistribution image $\theta_{xy}(t)$ is calculated by

$$M_2(t) = \frac{1}{N}\sum_{x,y}\theta_{x,y}(t), \tag{7}$$

where N is the count of nonzero pixels. We thus define M_1 and M_2, respectively, as the original and improved motility indices measured between two time points. For the overall motility value of a cell under a certain condition, the mean motility index $\overline{M_1}$ or $\overline{M_2}$ over all time points was used.

The size of the boxcar window used for analysis is a customizable parameter in our software. The optimal setting depends in large part on the dimensions of the most motile processes extending out from the cell and the density with which they occur at the cell surface. We recommend using an independent dataset to empirically determine the parameters that give the greatest sensitivity for the cell type being studied. For example, in Figure 3(e), we systematically varied the boxcar window size and found that although the absolute motility index values decrease with larger boxcar sizes, the greatest difference between experimental groups is obtained with an intermediate boxcar size, in this case 9 pixels.

2.4. Temporal Frequency-Based Filtering of Spurious Motility. In another experiment, images of an active cell were taken at the higher rate of once every 20 seconds over the 15 to 18 minutes normally used for dynamics analysis (Figure 4(a)). This sampling rate (0.05 Hz) constitutes temporal oversampling for the cellular motility behavior in which we are interested. For technical reasons only a subsection of the cell was measured in order to achieve the necessary temporal resolution. Images were preprocessed as described above, resulting in dilated binary images as in Figure 2(c). Three

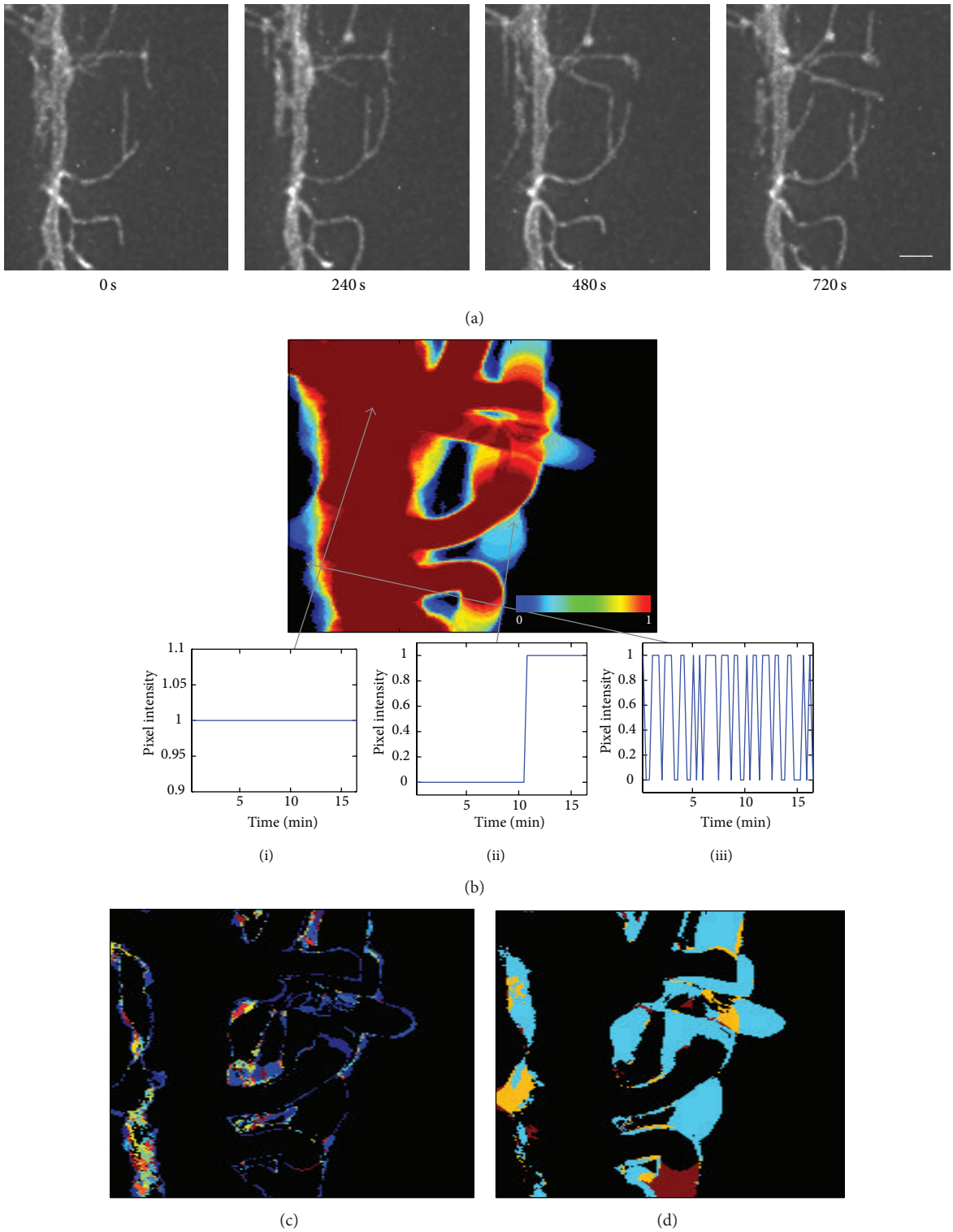

FIGURE 4: Temporal frequency filtering helps exclude artifactual pixel redistributions. (a) Time series of two-photon images of a portion of a radial glia cell. Scale bar = 2 μm. (b) Integrated intensity of a healthy cell over the course of 18 minutes, preprocessed for analysis. Red pixels have a value of 1 during most time points and blue pixels during few time points. Black pixels are negative background sites with value 0 at all time points. Time-dependent pixel intensity functions (i) for a pixel located within the main process, (ii) for a pixel into which a filopodium extended, and (iii) for a noisy pixel located along the edge of the cell. (c) Frequency map showing noisy pixels that were discarded by temporal frequency filtering. The colors represent discrete high frequencies measured in the excluded range. (d) Frequency map of the same cell, showing pixels that were accepted by the frequency filter. Each color shows a discrete frequency within the accepted range. In (c) and (d), black represents sites where pixel intensity did not change over the 18 minutes.

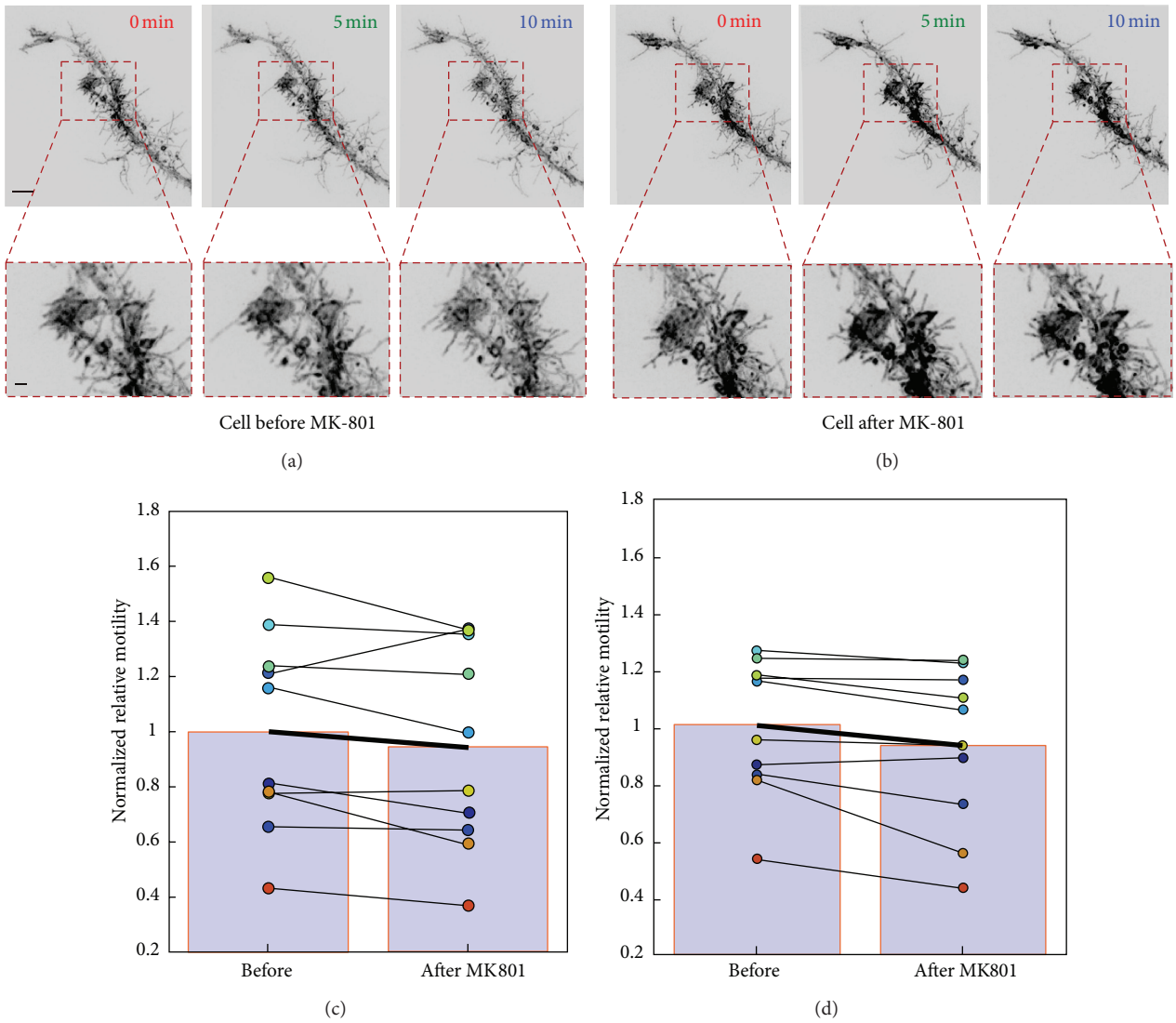

FIGURE 5: Comparing the relative sensitivity of the two motility measurement methods using images of normal radial glia cells ($N = 10$) before and after injection of 100 μM MK-801 into the ventricle of the tadpole to reduce motility. ((a), (b)) Example of glial motility before (a) and after (b) drug treatment. (c) Motility index changes measured using Method 1 from [15]. (d) Results using the boxcar-based Method 2. Each individual cell is depicted with the same color in (c) and (d). Note that Method 2 gives more consistent results. All values are normalized to the mean predrug motility index to facilitate comparison. Scale bar = 10 μm in upper panel and 2 μm in lower panel of (a).

characteristic examples of the time-dependent behavior at single pixels are shown in Figure 4(b). Pixels located at the center of the cell main process typically will have an unvarying intensity function as shown in Figure 4(b)(i). Similarly, pixels that lie within the trajectory of an elongating or withdrawing filopodium will be continuously on or off until the filopodium either moves into or out of the pixel position, after which the pixel value will flip (Figure 4(b)(ii)). Pixels that are near the edge of the cell tend to alternate between a value of 1 and 0 irregularly, due to imaging artifact and alignment-drift error (Figure 4(b)(iii)). The time-domain pixel intensity functions shown were transformed into the frequency domain by means of the discrete fast Fourier transform (DFT) algorithm of MATLAB. A large proportion of the spurious motility can be filtered based on the DFT results, as image noise is expected to have a higher frequency DFT peak than biologically relevant slower fluctuations. We therefore implemented a DFT-based image filter with a user-selectable frequency threshold as part of our analysis package. Setting the filter to separate fluctuations at a threshold of 0.0026 Hz (<6.4 min periods) appeared to effectively distinguish between image noise at high frequency (Figure 4(c)) and biologically relevant motility fluctuating at lower frequencies (Figure 4(d)).

3. Results

To assess our improved method for cell morphological dynamics measurement, we electroporated albino Xenopus laevis tadpoles with plasmid driving the expression

of membrane-targeting farnesylated EGFP (EGFP-F). Two days after electroporation, we collected images of individual EGFP-expressing radial glia cells in the optic tectum in live tadpoles by two-photon microscopy.

We tested the sensitivity of our improved motility index (Method 2) compared to the original algorithm (Method 1) from Tremblay et al. (2009), using an established pharmacological manipulation known to reduce radial glial motility. We have previously shown that radial glia decrease their filopodial process motility *in vivo* in response to intraventricular application of the N-methyl-D-aspartate receptor (NMDAR) blocker MK-801 [15]. Here we used this glial response to MK-801 to compare the relative sensitivity of Methods 1 and 2. In the study by Tremblay et al., time-lapse images had been collected at 15 minute intervals. In order to push the limits of our detection capability, we shortened the imaging interval to 5 minutes (Figures 5(a) and 5(b)). The cumulative restructuring of the cells is considerably less during this shorter interval and we sought to determine whether either analysis method could still detect a difference between control and MK-801-treated conditions.

Tectal radial glia are highly dynamic on a timescale of minutes, continually adding and retracting many filopodial processes. However, although they remodel extensively, they do not exhibit cumulative growth over these periods. We found no significant difference between the area profiles of radial glia cells before compared to after MK-801 treatment (area mean ± SD before MK-801: 1465.25 ± 140.56 μm^2, after MK-801: 1411.36 ± 150.6 μm^2, $n = 10$, $P = 0.45$, two-tailed Student's t-test). We were unable to demonstrate a significant decrease in the motility index values of cells in response to MK-801 application when imaged at 5 min intervals and analyzed using Method 1 ($n = 10$, $P = 0.11$, paired t-test) (Figure 5(c)). In contrast, applying Method 2 to the same dataset revealed a significant decrease in the rates of dynamics of these cells after MK-801 treatment ($n = 10$, $P < 0.02$, paired t-test) (Figure 5(d)). The greater sensitivity of Method 2 was likely due to smaller variance in the motility index values resulting from the reduced contribution of artifact from nondynamic parts of the cell.

To assess the relative contributions of spurious motility caused by imaging artifact, we compared motility index values of control EGFP-F-expressing radial glial cells in living tadpoles ($n = 4$) with cells from the animals that had been euthanized and fixed with 4% PFA ($n = 5$) (Figures 6(a) and 6(b)). Fixation abolishes all cellular dynamics, leaving only imaging artifact, photobleaching, and Brownian motion of the fluorophore molecules. Not surprisingly, a large difference in cell motility scores between the control and fixed groups was obtained using both the old and new analysis methods. Somewhat unexpectedly, motility index scores obtained using Method 1 (Figure 6(c)) were on average lower, after normalization to the matched control group, than those produced by Method 2 (Figure 6(d)), probably reflecting a greater sensitivity of the new method to systematic artifact due to photobleaching or specimen drift. However, Method 1 exhibited larger variance in the motility indices obtained and thus gave a wider 95% confidence interval for the difference between means (CI = [0.3589, 0.7854]). Analysis by Method 2 resulted in a smaller two-sided 95% confidence interval for the difference between means (CI = [0.2607, 0.5342]) and consequently a lower P value by the two-tailed Student's t-test (Method 1, $P = 0.00039$; Method 2, $P = 0.00024$).

We also applied the DFT-based temporal frequency filter to a different set of control and PFA-fixed cell image sequences collected at higher temporal resolution to further distinguish actual biological motility from artifact. Filtering cells with a 0.0026 Hz temporal frequency (period < 6.4 min) threshold decreased the already low motility index values of PFA-fixed cells (as measured using Method 2) by 66% on average (Figure 6(e)), confirming the value of the temporal frequency filter for reducing spurious "motility" from measurements. In contrast, the motility index value of the control live cells was only reduced by an average of 23%, consistent with most of the signal being biological in origin. Thus, the temporal frequency filter in conjunction with our improved motility index provides a set of useful and sensitive tools for the detection and quantification of biologically relevant morphological remodeling of cells from 2-photon time-series images.

4. Discussion

We present a novel method for motility analysis of a wide variety of morphologically complex cells. This versatile approach offers increased sensitivity compared to the earlier version due to its superior discrimination of real movement from noise. This is achieved by boxcar averaging of pixel redistribution images, which results in the relatively greater weighting of areas in the images where large groups of adjacent pixels exhibit coordinated changes from one time point to another, while reducing the contribution of spurious pixel fluctuations. When the size of the boxcar window is set to roughly match that of the most relevant motile structure for the phenomenon under study (e.g., dendritic spines, axonal growth cones, and glial filopodia), our algorithm returns a single motility index value that effectively reflects the degree of motility of the specific individual structures of interest without requiring painstaking and time-consuming reconstruction of each of these cellular protuberances. In addition, we demonstrate that further improvement can be obtained by temporal frequency filtering of the time series.

Real biologically relevant movements like filopodial extension typically involve changes of larger groups of neighboring pixels. Changes entailing the redistribution of a smaller number of adjacent pixels from one time point to another often arise from artifacts including photobleaching, detector noise, and imperfect alignment of time series images. This is most evident in the faint outlining of the whole perimeter of the cell in pixel redistribution images (Figure 3(b)). This perimeter outlining is unlikely to be related to cellular metabolism or function, as it is also present in the PFA-fixed cells. The use of boxcar averaging dramatically reduces the contribution of this artifactual component to our motility measurements by weakening the influence of isolated pixel values while enhancing the weighting of clusters of pixels that change together.

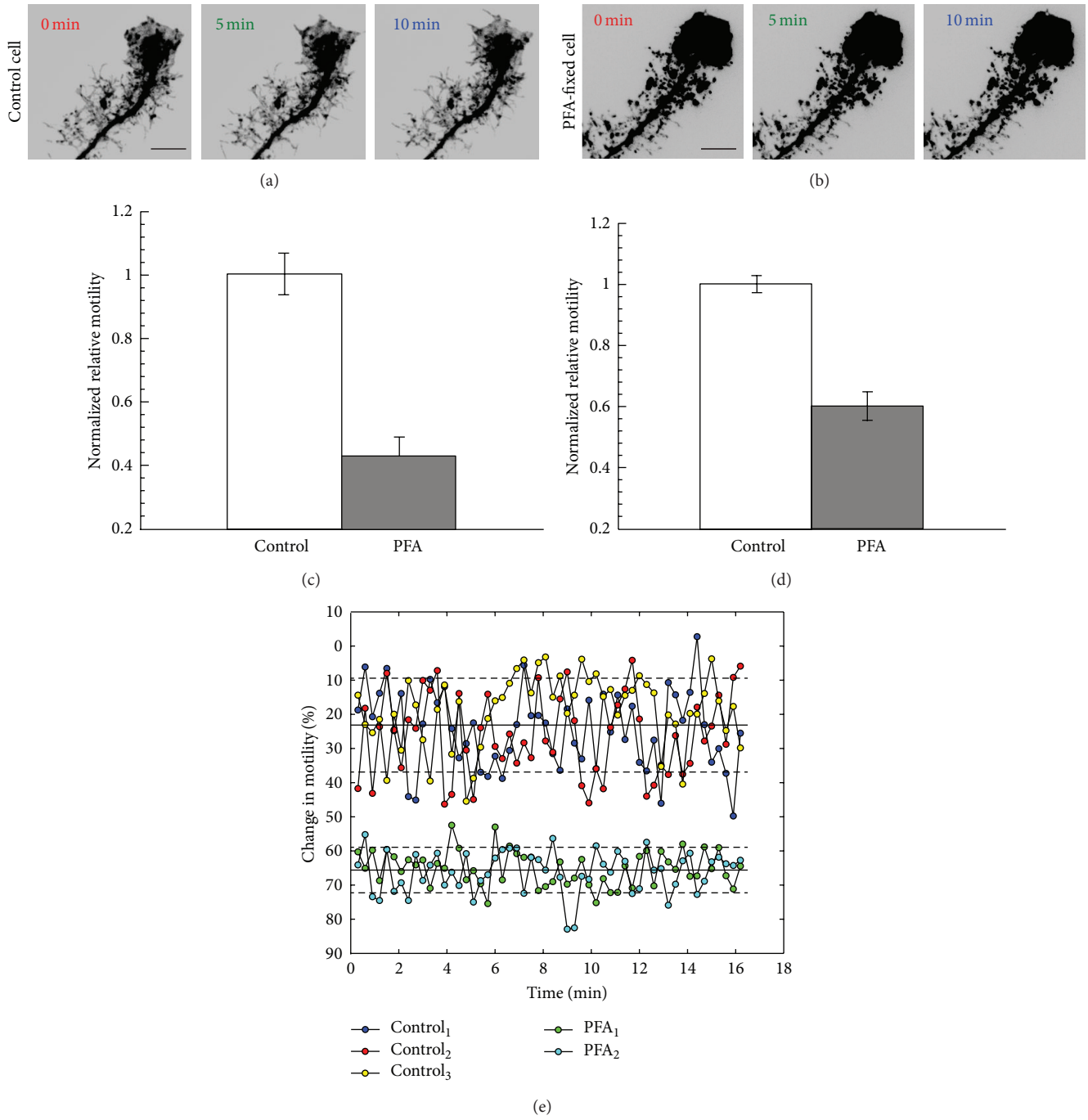

Figure 6: Use of paraformaldehyde- (PFA-) fixed cells to reveal nonbiological artifacts that contribute to the motility index scores. (a) The motility of control living cells ($N = 4$) was compared with (b) dead cells fixed in paraformaldehyde ($N = 5$). ((c), (d)) Bars indicate the motility values of the two groups normalized to the mean of control values using Method 1 in (c) and Method 2 in (d). Error bars are SEM. (e) Applying a low-pass temporal frequency filter with a threshold of 0.0026 Hz (6.4 min period) reduces the motility index scores (measured using Method 2) of the PFA-fixed cell group by substantially more than the control group, indicating that the filter is effective at eliminating nonbiological noise. Dashed lines indicate full width at half maximum. Scale bars = 10 μm.

The new Method 2 consistently yields lower coefficients of variance than the original Method 1. The principal reason is the above-mentioned reduction of noise at the cell perimeter. In addition, eliminating the need for normalization by cell area removes another source of variability that is not relevant to actual motility. Radial glia cells appear to come in an endless variety of shapes and sizes, and the number of processes does not necessarily linearly scale with the area. Because Method 1 relies on counting the total number of redistributed pixels between time points, it is necessary to

normalize these values in order to correct for different cell sizes to prevent larger, less motile cells from being scored higher than highly motile cells that are just very small. However, normalizing to total cell area can result in two cells with identical motility of their processes but different 2D areas being reported as having different motility rates. The use of boxcar averaging in Method 2, in contrast, gives a value that reflects process movement within local domains that is independent of the total size of the cell.

To further eliminate spurious pixels, we have designed a filter based on temporal frequency of the pixel fluctuations. Some of the most rapid and stochastic changes in pixel values occur along the perimeter of imaged cells where out-of-focus light, photon scattering, as well as fine image drift enhance the inherent fluctuations in signal detection at photomultiplier tubes or other detectors. By thresholding out the highest temporal frequency pixels, mainly biologically relevant pixels remain. A surprisingly large proportion (23%) of the prefiltered motility measures of normally active cells appeared to be derived from spurious fluctuations. This also helps explain why the motility index scores of PFA-fixed glial cells were not simply zero, as a considerable fraction of the motility index appears to be derived from nonbiological artifact. Thus, the application of temporal frequency domain filtering has tremendous potential for improving the dynamic range and sensitivity of the motility index. However, temporal frequency filtering can be properly applied only when numerous rapid interval images have been collected. The optimal acquisition rate will depend upon the velocity of the remodeling processes of the cell and on the pixel size of the imaging system.

The question of what constitutes the remaining "motility" in fixed cells, after the spurious edge pixels have been removed, is intriguing. The images may gradually change due to photobleaching, photochemistry releasing reactive species, and perhaps even thermal effects from the imaging laser. The finding that fixed material exhibits considerable spurious motility raises potential questions about the correct interpretation of the numerous studies reporting continuous subtle movements of processes like dendritic spines or astrocytic filopodia under the microscope. This argues that appropriate fixative-treated controls should be tested as a first step whenever measuring cellular dynamics in order to define the noise floor of the imaging system used.

In certain cases it would be useful to measure motility of only selected parts of a cell. We tested choosing user-defined smaller parts of the images for motility analysis during the course of our algorithm development. However, we ultimately settled on the automatic boxcar-filtered approach for the majority of our analyses because it allows for truly objective quantification of motility without introducing investigator bias, for example, due to selecting specific regions of interest. One interesting future possibility for comparing defined subregions of cells without bias might be to have the software create an internal standard coordinate system for all cells, as was done in a study analyzing bacterial division [20]. Another important future direction would be to implement a similar unbiased motility analysis for the full original three-dimensional data sets, rather than maximum intensity projections. In light of the large amount of noise introduced by image drift and registration over time, this problem will be compounded greatly by adding a three-dimensional registration step, especially complicated given the anisotropy of axial resolution compared with lateral image resolution on most optical microscopes. New high-resolution light-sheet microscopy techniques such as selective plane illumination microscopy (SPIM) and Bessel beam microscopy should facilitate extending these analyses into 3D [21, 22].

5. Conclusions

We have created a robust and sensitive method to measure structural motility of any cell type and generate a quantitative motility index. This method is especially useful for cells with complex morphologies that are complicated to analyze by other means. The programs implementing this method with a user-friendly interface are freely available for download at http://ruthazerlab.mcgill.ca/downloads/motility_GUIv2.zip.

Conflict of Interests

The authors declare no conflict of interests.

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References

[1] J. W. Lichtman and S. E. Fraser, "The neuronal naturalist: watching neurons in their native habitat," *Nature Neuroscience*, vol. 4, supplement 1, pp. 1215–1220, 2001.

[2] C. M. Niell and S. J. Smith, "Live optical imaging of nervous system development," *Annual Review of Physiology*, vol. 66, pp. 771–798, 2004.

[3] J. Y. Hua and S. J. Smith, "Neural activity and the dynamics of central nervous system development," *Nature Neuroscience*, vol. 7, no. 4, pp. 327–332, 2004.

[4] J. Lippman and A. Dunaevsky, "Dendritic spine morphogenesis and plasticity," *Journal of Neurobiology*, vol. 64, no. 1, pp. 47–57, 2005.

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

[6] D. Dormann, T. Libotte, C. J. Weijer, and T. Bretschneider, "Simultaneous quantification of cell motility and protein-membrane-association using active contours," *Cell Motility and the Cytoskeleton*, vol. 52, no. 4, pp. 221–230, 2002.

[7] M. Machacek and G. Danuser, "Morphodynamic profiling of protrusion phenotypes," *Biophysical Journal*, vol. 90, no. 4, pp. 1439–1452, 2006.

[8] L. Bosgraaf and P. J. M. van Haastert, "Quimp3, an automated pseudopod-tracking algorithm," *Cell Adhesion and Migration*, vol. 4, no. 1, pp. 46–55, 2010.

[9] S. Costantino, C. B. Kent, A. G. Godin, T. E. Kennedy, P. W. Wiseman, and A. E. Fournier, "Semi-automated quantification of filopodial dynamics," *Journal of Neuroscience Methods*, vol. 171, no. 1, pp. 165–173, 2008.

[10] M. Lavialle, G. Aumann, E. Anlauf, F. Pröls, M. Arpin, and A. Derouiche, "Structural plasticity of perisynaptic astrocyte processes involves ezrin and metabotropic glutamate receptors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 31, pp. 12915–12919, 2011.

[11] A. Dunaevsky, A. Tashiro, A. Majewska, C. Mason, and R. Yuste, "Developmental regulation of spine motility in the mammalian central nervous system," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 23, pp. 13438–13443, 1999.

[12] M. Fischer, S. Kaech, U. Wagner, H. Brinkhaus, and A. Matus, "Glutamate receptors regulate actin-based plasticity in dendritic spines," *Nature Neuroscience*, vol. 3, no. 9, pp. 887–894, 2000.

[13] M. Iino, K. Goto, W. Kakegawa et al., "Glia-synapse interaction through Ca^{2+}-permeable AMPA receptors in Bergmann glia," *Science*, vol. 292, no. 5518, pp. 926–929, 2001.

[14] H. Nishida and S. Okabe, "Direct astrocytic contacts regulate local maturation of dendritic spines," *Journal of Neuroscience*, vol. 27, no. 2, pp. 331–340, 2007.

[15] M. Tremblay, V. Fugère, J. Tsui et al., "Regulation of radial glial motility by visual experience," *Journal of Neuroscience*, vol. 29, no. 45, pp. 14066–14076, 2009.

[16] P. D. Nieuwkoop and J. Faber, *Normal Table of Xenopus Laevis (Daudin)*, North-Holland Publishing, Amsterdam, The Netherlands, 1956.

[17] E. S. Ruthazer, A. Schohl, N. Schwartz, A. Tavakoli, M. Tremblay, and H. T. Cline, "Bulk electroporation of retinal ganglion cells in live Xenopus tadpoles," *Cold Spring Harbor Protocols*, 2013.

[18] C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, "NIH image to imageJ: 25 years of image analysis," *Nature Methods*, vol. 9, pp. 671–675, 2012.

[19] P. Thévenaz, U. E. Ruttimann, and M. Unser, "A pyramid approach to subpixel registration based on intensity," *IEEE Transactions on Image Processing*, vol. 7, no. 1, pp. 27–41, 1998.

[20] J. M. Guberman, A. Fay, J. Dworkin, N. S. Wingreen, and Z. Gitai, "PSICIC: noise and asymmetry in bacterial division revealed by computational image analysis at sub-pixel resolution," *PLoS Computational Biology*, vol. 4, no. 11, Article ID e1000233, 2008.

[21] J. Huisken, J. Swoger, F. del Bene, J. Wittbrodt, and E. H. K. Stelzer, "Optical sectioning deep inside live embryos by selective plane illumination microscopy," *Science*, vol. 305, no. 5686, pp. 1007–1009, 2004.

[22] T. A. Planchon, L. Gao, D. E. Milkie et al., "Rapid three-dimensional isotropic imaging of living cells using Bessel beam plane illumination," *Nature Methods*, vol. 8, no. 5, pp. 417–423, 2011.