THE ROLE OF GLIA IN PLASTICITY AND BEHAVIOR

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THE ROLE OF GLIA IN PLASTICITY AND BEHAVIOR

Topic Editors:
Vladimir Parpura, University of Alabama at Birmingham, USA
Tycho M. Hoogland, Erasmus MC, Netherlands

Glial cells are no longer considered passive bystanders in neuronal brain circuits. Not only are they required for housekeeping and brain metabolism, they are active participants in regulating the physiological function and plasticity of brain circuits and the online control of behavior both in invertebrate and vertebrate model systems. In invertebrates, glial cells are essential for normal function of sensory organs (*C. elegans*) and necessary for the circadian regulation of locomotor activity (*D. melanogaster*). In the mamallian brain, astrocytes are implicated in the regulation of cortical brain rhythms and sleep homeostasis. Disruption of AMPA receptor function in a subset of glial cell types in mice shows behavioral deficits. Furthermore, genetic disruption of glial cell function can directly control behavioral output. Regulation of ionic gradients by glia can underlie bistability of neurons and can modulate the fidelity of synaptic transmission. Grafting of human glial progenitor cells in mouse forebrain results in human glial chimeric mice with enhanced plasticity and improved behavioral performance, suggesting that astrocytes have evolved to cope with information processing in more complex brains. Taken together, current evidence is strongly suggestive that glial cells are essential contributors to information processing in the brain. This Research Topic compiles recent research that shows how the molecular mechanisms underlying glial cell function can be dissected, reviews their impact on plasticity and behavior across species and presents novel approaches to further probe their function.

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EDITORIAL
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Editorial: The role of glia in plasticity and behavior

Tycho M. Hoogland[1,2]* *and Vladimir Parpura*[3]

[1] Netherlands Institute for Neuroscience, Amsterdam, Netherlands, [2] Department of Neuroscience, Erasmus MC, Rotterdam, Netherlands, [3] Atomic Force Microscopy and Nanotechnology Laboratories, Department of Neurobiology, Center for Glial Biology in Medicine, Civitan International Research Center, Evelyn F. McKnight Brain Institute, University of Alabama at Birmingham, Birmingham, AL, USA

Keywords: astrocytes, glia, behavior, plasticity, transgenic mouse models

New genetic tools have not only advanced our understanding of how neurons contribute to plasticity and behavior, but also unveiled glial cells as active participants in circuit function as highlighted in this Research Topic. Glia are found in the majority of animals with a nervous system and are essential for feeding and learning behavior in a "simple" organism such as *C. elegans* (Stout et al., 2014), which has a nervous system invariantly comprising 302 neurons. Glial cells signaling via gap junctions may even have evolved convergently during evolution suggesting that spreading activity via such cellular networks is a fundamental process (Stout et al., 2014).

In more complex mammalian brains, glia respond to a variety of neurotransmitters and could be instrumental for gauging metabolic demand in times of increased vigilance -a behavioral state mediated by adrenoreceptors (Ding et al., 2013)- across brain regions (Paukert et al., 2014; De Zeeuw and Hoogland, 2015). Pankratov and Lalo (2015) present data suggesting that the activation of astroglial α1-adrenergic receptors and subsequent exocytotic release of ATP from these glial cells are important for the induction of long-term potentiation in neocortical neurons. Thus, the behavioral state of the animal, vigilance in the case of adrenergic modulation, has a big impact on the learning capacity of the brain and may rely on gliotransmission.

The role of gliotransmission is still contentious as a recent study has shown that a commonly used transgenic mouse (dnSNARE) which has been utilized to block exclusively gliotransmission (Pascual et al., 2005; Lalo et al., 2014), could lead to ectopic expression (Fujita et al., 2014); discussed in Xie et al. (2015). Bearing this in mind, Hahn et al. (2015) utilized co-cultures of neurons and astrocytes, an approach instrumental in studying astrocyte-to-neuron signaling (Parpura et al., 1994), to demonstrate that astrocytes can increase the activity of NMDA-receptor dependent synaptic transmission at the postsynaptic subunit (N2B)-specific level. Thus, astrocytes not only sense neuromodulatory state, or local circuit activity, but also influence the circuits in which they are embedded, as reviewed by Perea et al. (2014a).

Could astrocytes themselves undergo plasticity? Sibille et al. (2015) addressed this question with dual recordings of hippocampal astrocyte Ca^{2+} signaling and synaptic transmission at Schaffer collateral pathway (SC)-CA1 pyramidal neuron (excitatory) synapses. During repetitive or tetanic stimulation of SC, astrocytes showed short-term depression of cytosolic Ca^{2+} signals associated with a simultaneous short-term potentiation at SC-CA1 synapses. Moreover, chelation of Ca^{2+} in astrocytes resulted in enhanced synaptic transmission and short-term plasticity at SC-CA1 synapses. This finding adds to a growing body of evidence that astrocytes are critical for certain types of synaptic plasticity (Min and Nevian, 2012).

Although evidence is accumulating that glial cells regulate the excitability of neural circuits and could therefore contribute to behavior -at least in certain animals- there are also other controversies that need to be resolved. One of them is the role of Ca^{2+} signaling in astrocytes that has been

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Egidio D'Angelo,
University of Pavia, Italy

***Correspondence:**
Tycho M. Hoogland,
hoogland@nin.knaw.nl;
t.hoogland@erasmusmc.nl

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considered an essential feature of astrocyte function and plasticity (Nimmerjahn et al., 2009; Sibille et al., 2015).

Following up on work that proposed that Gq protein-coupled receptor Ca^{2+} mobilization in astrocytes does not affect neuronal synaptic transmission (Agulhon et al., 2010) or synaptic plasticity in hippocampal astrocytes, Petravicz et al. (2014) recently demonstrated in an inositol 1,4,5, trisphosphate receptor type 2 (otherwise providing for Ca^{2+} dynamics in astrocytes) conditional knockout mouse line that a battery of behavioral assessments (Morris water maze, elevated plus-maze, rotarod, open field activity, and acoustic startle response tests) failed to reveal any behavioral deficits. The above studies are reviewed by Xie et al. (2015), who also summarize the toolbox of currently available genetic approaches that enable the study of astrocytes *in vivo*.

Although optogenetics has proven to be clearly advantageous for studying neuronal correlates of behavior and functional connectivity in the brain (Friedman et al., 2015; Pala and Petersen, 2015), and it has been applied to selectively stimulate astrocytes (Gourine et al., 2010; Sasaki et al., 2012; Perea et al., 2014b; Natsubori et al., 2015), there are some caveats to its use in general and astrocytes in particular. These include the non-selectivity of, e.g., channelrhodopsin (ChR2) channels for cations leading to intracellular acidification (Beppu et al., 2014; also see Natsubori et al., 2015), as well as large Ca^{2+} increases that could bypass downstream signaling cascades, and which result in non-specific modulation of astrocyte function (Wang et al., 2013). Xie et al. (2015) suggest that Designer Receptors Exclusively Activated by Designer Drugs (DREADD) provide a more precise method to reversibly control astrocyte activity allowing the disentanglement of the signaling pathways that contribute to glial modulation of circuit activity.

What are the current obstacles that need to be overcome to advance the field? Jahn et al. (2015), Xie et al. (2015), and Natsubori et al. (2015) all note that there is a need for better genetic models to study astrocyte function. Such models would allow inducible knockout, expression of proteins involved in astrocyte signaling at appropriate levels, and would also make use of more specific promotors that could target various types of glial cells in different brain areas. Furthermore, Jahn et al. (2015) stress the importance of considering the lifetime of proteins. Namely, recent data obtained from Bergmann glia revealed that knockout of subtypes of AMPA receptors resulted in process retraction, which fully manifested itself only after several weeks, a time-frame matching protein degradation of these channels (Saab et al., 2012).

The goal of this Research Topic has been to summarize our most recent understanding of glial cells in the regulation of plasticity of neural circuits. It is abundantly clear that genetic approaches have been instrumental in elucidating the role of glia in plasticity and behavior and that outstanding issues are likely to be resolved in the near future with novel genetic tools. Thus, exciting times lie ahead for the study of astrocytes in the dish and the behaving brain.

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REVIEW
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Genetic control of astrocyte function in neural circuits

*Hannah M. Jahn†, Anja Scheller† and Frank Kirchhoff**

Molecular Physiology, Center for Integrative Physiology and Molecular Medicine (CIPMM), University of Saarland, Homburg, Germany

During the last two decades numerous genetic approaches affecting cell function *in vivo* have been developed. Current state-of-the-art technology permits the selective switching of gene function in distinct cell populations within the complex organization of a given tissue parenchyma. The tamoxifen-inducible Cre/loxP gene recombination and the doxycycline-dependent modulation of gene expression are probably the most popular genetic paradigms. Here, we will review applications of these two strategies while focusing on the interactions of astrocytes and neurons in the central nervous system (CNS) and their impact for the whole organism. Abolishing glial sensing of neuronal activity by selective deletion of glial transmitter receptors demonstrated the impact of astrocytes for higher cognitive functions such as learning and memory, or the more basic body control of muscle coordination. Interestingly, also interfering with glial output, i.e., the release of gliotransmitters can drastically change animal's physiology like sleeping behavior. Furthermore, such genetic approaches have also been used to restore astrocyte function. In these studies two alternatives were employed to achieve proper genetic targeting of astrocytes: transgenes using the promoter of the human glial fibrillary acidic protein (GFAP) or homologous recombination into the glutamate-aspartate transporter (GLAST) locus. We will highlight their specific properties that could be relevant for their use.

Keywords: genetic targeting of astrocytes, tamoxifen, CreERT2/loxP, Bergmann glia, human GFAP promoter, GLAST glutamate aspartate transporter

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Vladimir Parpura, University of Alabama at Birmingham, USA

Reviewed by:
Rheinallt Parri, Aston University, UK
Gertrudis Perea, Instituto Cajal, Spain
Paola Bezzi, Université de Lausanne, Switzerland

****Correspondence:***
Frank Kirchhoff, Molecular Physiology, Center for Integrative Physiology and Molecular Medicine (CIPMM), University of Saarland, CIPMM Building 48, 66421 Homburg, Germany
frank.kirchhoff@uks.eu

†These authors have contributed equally to this work.

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Introduction

Astrocytes represent an abundant, but also heterogeneous group of glial cells in all regions of the brain (**Figures 1A–F**). Their numerous interactions with capillaries and neurons are important signaling pathways for physiological brain function. Astrocytes actively control signal processing and transmission at the tripartite synapse (Perea et al., 2009). Originally, astrocytes were regarded as silent non-excitable cells, since they do not communicate via electrical signals. But now, it has become very clear that astroglial signaling is encoded in complex spatial and temporal patterns of Ca^{2+} changes within subcellular compartments as well as throughout cellular networks coupled by gap junctions. Intracellular Ca^{2+} rises indicate how they sense activity of their surroundings (Zorec et al., 2012; Araque et al., 2014; Verkhratsky and Parpura, 2014). Intracellular changes of another cation, Na^{+}, have been recognized as an additional or alternative indicator of astrocyte activation (Kirischuk et al., 2012; Verkhratsky et al., 2013; Rose and Chatton, 2015). Even over longer distances astrocytes can convey various signals, e.g., inositol-1,4,5-trisphosphate (IP3) or cyclic nucleotides that pass through gap junctions and functionally couple the astroglial syncytium (Giaume and Liu, 2012; Theis and Giaume, 2012). Within these networks information spreads with

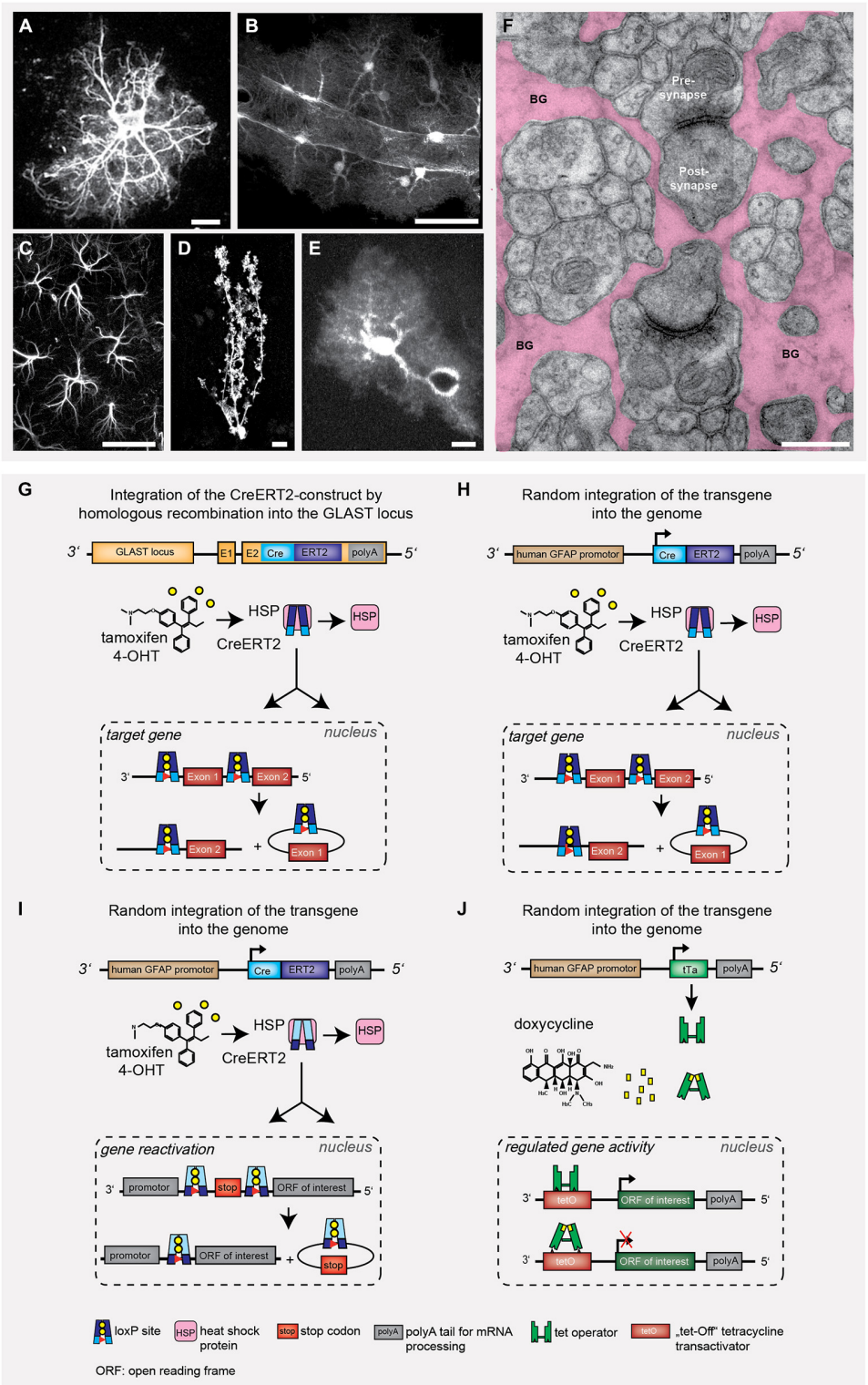

FIGURE 1 | Astrocyte heterogeneity and gene targeting strategies to influence astrocyte behavior. Throughout development and in different brain regions, the heterogeneity of astrocytes becomes rather obvious when looking at the different morphologies. It is more the cell volume with cytosol and cell membrane that helps to visualize astrocyte function rather than the cytoskeletal structure **(A–F)**. Only few genetic strategies have been used to modify astrocyte function *in vivo* **(G–J)**. **(A)** glial fibrillary acidic protein (GFAP)-stained acutely isolated astrocyte. **(B)** Cortical astrocytes expressing tdTomato in close contact to a blood vessel with their end feet, **(C)** Hippocampal astrocytes (CA1) expressing

(*Continued*)

FIGURE 1 | Continued
GFAP. **(D)** Single Bergmann glia (BG) cell with CreERT2/loxP controlled reporter expression (EGFP). **(E)** Cortical astrocyte expressing EGFP and surrounding a blood vessel. Scale bars: **A,D,E** = 10 μm, **B** = 20 μm, **C** = 50 μm. **(F)** Electron micrograph depicting the intimate enwrapping of pre- and postsynaptic terminals by astroglial processes (BG: Bergmann glial processes, scale bar: 1 μm). **(G)** Knock-in of CreERT2 into the GLAST locus leads to tamoxifen-sensitive recombination in all astrocytes with endogenous GLAST promoter activity (Mori et al., 2006). The DNA recombinase variant CreERT2 is trapped in the cytosol by heat shock proteins (HSP), after tamoxifen application the protein is released and translocated into the nucleus. **(H,I)** Transgenic GFAP-CreERT2 mice generated by non-homologous recombination can also be used to target astrocytes (Hirrlinger et al., 2006). The Cre/loxP system can either be used to selective excise gene alleles of interest (**G,H**; knockout) or to express genes of interest (e.g., reporter proteins such as GFP or genetically encoded Ca^{2+} indicators), but also to restore gene function **(I)** (Lioy et al., 2011). **(J)** Alternatively, the binary tTA/tetO system composed of (1) promoter-controlled expression of a tetracyclin transactivator protein; and (2) tetracycline/doxycycline-responsive elements driving the expression of proteins-of-interest (Pascual et al., 2005). This system allows for a certain degree of reversible gene regulation.

tens of μm/s speed, still several orders of magnitude slower than the propagation of neuronal action potentials (Haydon and Nedergaard, 2015). The interactions of astrocytes with neurons are largely based on astroglial receptors that sense neuronal communication or on the release of gliotransmitters acting back on synaptic transmission. The general importance of astrocytes for brain function is uncovered by the devastating point mutations in single genes encoding transcription factors since these factors can control extended sets of gene programs. Genetically modified mice addressing astrocyte function have been instrumental in uncovering their role in the living animal.

In general, transgenic mouse models that interfere with cell function can be categorized in three classes: (1) Modulation of information input by direct knockout of receptors or signaling molecules, thereby preventing the information to enter the cell; it becomes *insensitive*; (2) Cell *perturbation* of intracellular gene programs or metabolic pathways, e.g., by knockout of transcription factors or enzymes involved in signal processing and resulting changes of the cellular infrastructure; and (3) Inhibition of signal release to the adjacent cellular neighborhood; the astrocyte becomes *inactive* or *silent*.

Conditional gene deletions started with the use of cell-specific promoters driving the Cre DNA recombinase (Cre/loxP system) to abolish gene function in selected cell types. However, several of such regulatory elements were active in precursor cells during embryonic development, thereby affecting complete lineage trees. To achieve temporal control of cell-specific DNA recombination, tamoxifen-sensitive, i.e., inducible variants of the Cre DNA recombinase were developed (CreER, $CreER^T$, $CreER^{T2}$). Here, the DNA recombinase Cre is fused to a mutated ligand-binding domain of the human estrogen receptor (ER), which does not bind endogenous estradiol, but is highly sensitive to nanomolar concentrations of 4-hydroxytamoxifen, a metabolite of tamoxifen that can be applied intraperitoneally, but also by gavage (Feil et al., 1997; Metzger and Chambon, 2001; Weber et al., 2001; Hirrlinger et al., 2006; Mori and Zhang, 2006).

Here, we will review current genetic strategies to reveal the impact of astrocyte function in the brain by focusing on frequently used paradigmatic examples (**Figures 1G–J**): tamoxifen-sensitive cell-specific gene deletion and restoration as well as doxycycline-induced expression of functionally impaired, dominant-negative signaling molecules. The tamoxifen-sensitive CreERT2/loxP system provides temporal control of gene deletion (Hirrlinger et al., 2006; Mori et al., 2006), in contrast to conventional mouse models with Cre/loxP (Malatesta et al., 2003). The doxycycline/tTA model can be used for transient effector protein expression (Bujard, 1999; Mansuy and Bujard, 2000; Pascual et al., 2005).

In the first part we will shortly describe the mouse models and their associated astrocyte function. In the second part, we will compare the targeting of astrocytes using regulatory elements of the human glial fibrillary acidic protein (GFAP) promoter with expression from the GLAST gene locus directly, and deduce respective technical considerations and scientific limitations.

The Role of Astrocytes in Hippocampal Learning

The functional role in working memory and its molecular signaling of the hippocampal CA3-CA1 synapse have been well characterized (Neves et al., 2008; Bannerman et al., 2014). A diversity of endogenous compounds is capable of modulating its transmission and thereby affecting our memory. Interestingly, one of the most abundant G-protein coupled receptors of the brain, the cannabinoid type-1 receptor (CB_1R), has been detected on all hippocampal cell types (Marsicano and Lutz, 1999). Based on work performed on brain slices, endocannabinoids or the synthetic drug Δ9-tetrahydrocannabinol (THC), better known as marijuana, are thought to inhibit presynaptic transmitter release via CB_1R activation, thereby depressing excitatory neurotransmission, and finally, impairing spatial working memory (Misner and Sullivan, 1999; Carlson et al., 2002; Takahashi and Castillo, 2006; Bajo et al., 2009; Schoeler and Bhattacharyya, 2013). Also *in vivo* THC was found to cause long-term depression (LTD; Hampson and Deadwyler, 2000; Mato et al., 2004; Madroñal et al., 2012). However, when the impact of CB_1R was tested more selectively using genetically modified mice with cell-specific receptor deficiency, unexpectedly, it was the ablation of the astroglial (**Figure 1H**; GFAP-CreERT2 × floxed CB_1R), but not the neuronal CB_1R that completely abolished THC-dependent depression (**Figure 2A**; Han et al., 2012). For their analysis, the authors used mice with at least 4 weeks of time to allow efficient receptor protein degradation. Although 30% of CB_1R protein could still be detected using immune-EM, the receptor function in modulating synaptic efficacy, i.e., LTD, was completely gone. In parallel, the mutant mice remained unaffected after THC injection when behaviorally tested in a variant of the Morris water maze. Thereby, this study demonstrates the pivotal role of astrocytes in modulating synaptic transmission and respective circuit-associated learning behavior.

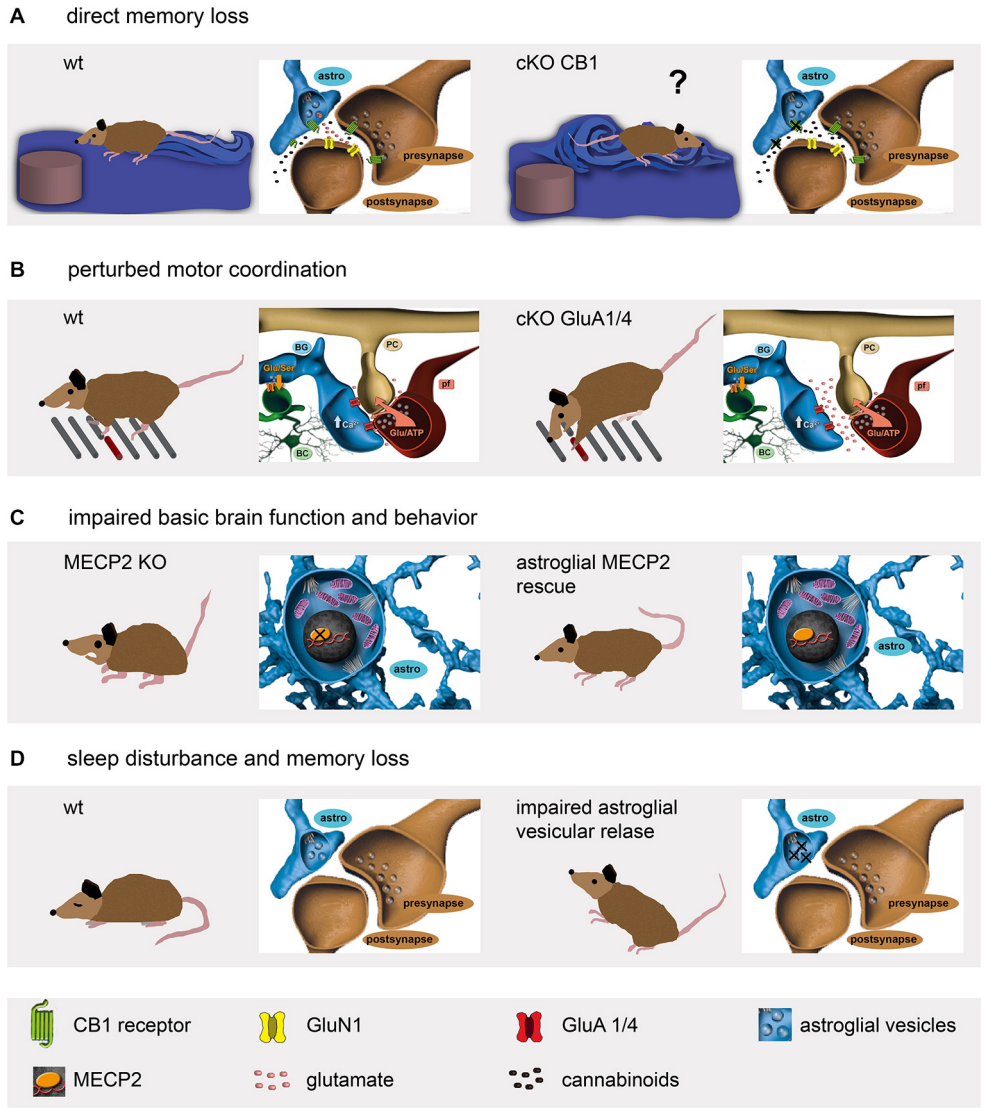

FIGURE 2 | Genetic mouse models reveal a diversity of astrocyte functions affecting mouse behavior. (A) Astroglial cannabinoid receptors are involved in spatial memory formation (Han et al., 2012). **(B)** The ionotropic glutamate receptors on BG contribute to fine motor coordination (Saab et al., 2012). **(C)** Although the lack of the widely expressed transcriptional repressor MECP2 results in synapse loss, severe mental retardation and premature death, the astrocyte-specific re-expression restores several vital functions like motor activity (Lioy et al., 2011). **(D)** Impairment of astroglial ATP release perturbs sleep behavior and induces memory loss (Pascual et al., 2005; Halassa et al., 2009).

Cerebellar Bergmann Glia Fine Tune Neural Circuits of Motor Coordination

The neural circuits of the cerebellum control the timing of motor performance by integrating sensory and motor input of climbing and mossy fibers (De Zeeuw et al., 2011; Heck et al., 2013). While climbing fibers (CF) multiply innervate the principal neurons, i.e., the Purkinje cells (PC); the information from mossy fibers is relayed via granule cells and their parallel fibers (PF). Both synapses, CF-PC and PF-PC, are characterized by their almost complete coverage by membrane appendages emanating from the main radially oriented processes of the Bergmann glia (BG; **Figures 1D,F**; Grosche et al., 2002; Lippman et al., 2008; Lippman Bell et al., 2010). The perisynaptic glial membranes are equipped with a tremendously high density of glutamate transporter to strictly control the extracellular levels of the excitatory transmitter and preventing synaptic spillover (Tong and Jahr, 1994; Marcaggi et al., 2003; Takayasu et al., 2006, 2009). Surprisingly, BG processes were found to express another glutamate sensing mechanism, ionotropic glutamate receptors very similar to neurons (Müller et al., 1992). The heteromeric ion channel complexes are formed by an assembly of the AMPA-type receptor subunits GluA1 and GluA4 (Geiger et al., 1995). However, in contrast to several neuronal AMPA

receptors, the BG receptor channels are Ca^{2+} permeable since they lack expression of the GluA2 subunit. While the importance of efficient glutamate uptake at excitatory synapses appears quite obvious, the expression of fast desensitizing AMPA receptors on BG remained enigmatic till very recently. To completely abolish AMPA receptor function in BG, another astrocyte-specific mouse, the GLAST-CreERT2 knockin (gene: *slc1a3*; Mori et al., 2006; **Figures 1G**, **2B**) was used to delete GluA1 and GluA4 simultaneously (Saab et al., 2012). In young mice (2 to 4 weeks old) GluA1/4 deletion resulted in retraction of glial appendages from PC synapses, an increased amplitude and duration of evoked postsynaptic PC currents, and a delayed formation of glutamatergic synapses. In adult (older than 2 months) mice, GluA1/4 inactivation also caused retraction of glial processes. In addition, the study provided a detailed time course for tamoxifen-evoked gene excision as well as mRNA and protein degradation. While the recombination event was almost completed after 2 days, the mRNA persisted for a week and the protein for even 3 weeks. The mutant mice showed normal behavior when observed in their cages. However, when the mice were challenged by a complex motor task running along a horizontal ladder with suddenly appearing obstacles, their fine motor coordination was significantly impaired (**Figure 2B**). Thus, AMPA receptors of BG are essential to optimize synaptic integration and cerebellar output function throughout life. AMPA receptor signaling of BG contributes to the structural and functional integrity of the cerebellar network and plays an important role in the "fine-tuning" of neuronal processing, which is crucial for a fast and precise control of complex motor behaviors (Saab et al., 2012).

Astrocyte-Specific Intracellular Gene Regulation in Signal Conversion

The above examples demonstrate how important astrocytes are in extracellular sensing of neuronal activity. Important intracellular functions such as detoxification or gene regulation can already be significantly affected by single point mutations in genes encoding enzymes or transcription factors, as observed in a variety of neurological disorders. A particularly well studied example is the methyl-CpG binding protein 2 (MECP2) that acts as a transcriptional repressor and affects the activity of broad range of downstream genes (Chahrour and Zoghbi, 2007). Mutations of the X-linked MECP2 gene cause Rett syndrome, a neurodevelopmental disorder. At the end of infancy patients suffer from progressing synaptic malfunctions associated with severe mental retardation (Chahrour and Zoghbi, 2007). Since loss-of-function mutations in mice phenocopy the human disease, genetic rescue experiments were performed to re-introduce the fully functional wild type gene. And indeed, global gene rescue restored brain development and function (Guy et al., 2007). Interestingly, neuron-specific restoration prevented several, but not all Rett-associated symptoms (Alvarez-Saavedra et al., 2007). Since MECP2 can be detected in astrocytes as well, it was selectively re-introduced in astrocytes using GFAP-CreERT2 mice crossbred to genetically modified mice with the MECP2 open reading frame encoded by the endogenous gene, but separated from the promoter region by a Cre-excisable STOP fragment (**Figures 1I**, **2C**; Lioy et al., 2011). Tamoxifen treatment of mutant mice prolonged the lifespan to 7.5 months when compared to oil-treated animals that already died at 3 months of age. Phenotype differences became evident 6 weeks after tamoxifen-induced MECP2 re-expression. Even highly symptomatic $MeCP2^{stop/y}$ mice could be rescued. While MECP2-deficient mice were hyperactive and quite anxious, behavioral testing in the open field and water maze revealed an improvement to 50% of control levels (**Figure 2C**). Similar to Rett patients, 12-week-old $MeCP2^{stop/y}$ mice displayed strong breathing problems, but 2 months after tamoxifen-induced rescue the respiration pattern recovered to a normal range. Interestingly, the astrocyte-specific deletion of MeCP2, induced at an age of 3 weeks, caused several pathological symptoms such as smaller body size, clasped hindlimb posture and irregular breathing 10 weeks later as observed in the global knockout mice (Han et al., 2012). Lifespan, locomotion and anxiety behavior, however, remained normal. Thereby, these data suggest distinct mechanisms of neuron–astrocyte interactions in different neural circuits of forebrain (anxiety, motor behavior) and hindbrain (control of respiration).

Astrocytes Modulate Synaptic Transmission

One genetic mouse model in glia research has received particular attention and is currently heavily debated, the doxycycline-switchable dnSNARE mouse (Pascual et al., 2005; Sloan and Barres, 2014; Haydon and Nedergaard, 2015; **Figures 1J**, **2D**). In this paragraph we will provide only a description of the positive findings that have been made. In the last part of this section we will shortly address the current discussion.

A very clear and evident way how astrocytes affect neural circuits became obvious when the regulated secretion of gliotransmitters such as glutamate, ATP or D-serine was investigated (Pascual et al., 2005). For this purpose a different switchable genetic mouse model was developed: the tetracycline-dependent expression of dominant-negative (dn) soluble N-ethylmaleimide-sensitive factor attached receptor (SNARE)-proteins that disrupt the vesicular release of gliotransmitters. Transgenic mice with human GFAP promoter-driven expression of the *tetoff* tetracycline transactivator (tTA; Mansuy and Bujard, 2000) were crossbred to mice with a tTA responsive element (tet operator, tetO) regulating expression of dnSNARE and reporter proteins (**Figure 1J**). In the presence of dietary doxycycline, a tetracycline derivative with improved permeability for the blood-brain barrier, the expression of dnSNAREs and reporter proteins is suppressed. Two weeks after stopping doxycycline, astrocytes efficiently express dnSNAREs and block their vesicular release of gliotransmitters (**Figure 2D**), in particular of ATP that is further degraded to adenosine. And indeed, electrophysiological analysis of hippocampal slices reveal enhanced field potentials after Schaffer collateral stimulation in dnSNARE mice (Pascual et al., 2005). Extracellular adenosine, generated from released ATP, acts on presynaptic A1 receptors and suppresses excitatory synaptic transmission. Blocking the constitutive ATP/adenosine

release from astrocytes enhances the excitatory drive that can be blocked by exogenous application of ATP (Pascual et al., 2005). By releasing activity dependent neurotransmitters, astrocytes regulate the strength of basal synaptic transmission at the circuit level. At system and behavioral level, impaired astroglial ATP release reduces the slow wave activity in the electroencephalogram (EEG), perturbs sleep homeostasis and sleep loss-associated memory deficits (**Figure 2D**; Halassa et al., 2009).

In a recent study severe concerns about the use of the transgenic dnSNARE mice have been raised (Fujita et al., 2014). The authors criticize an insufficient early characterization of the mice and present own data that suggest a predominant neuron-mediated impairment of ATP signaling rather than an astroglial one. Without joining this discussion, it is evident that far-reaching conclusions from genetically engineered animal models should be based on independent experimental systems. But this should be immanent to all types of science. Continuous generation of novel mouse models and their free sharing within the scientific community will warrant the progress in our understanding of brain function.

Technical Considerations When Using Genetically Modified Mice to Target Astrocyte Functions

Astrocytes are not only widely distributed throughout all regions of the brain, age and brain-region specific expression of genes and their distinct functions have been identified (Malatesta et al., 2003; Regan et al., 2007; Halassa et al., 2009; Robel et al.,

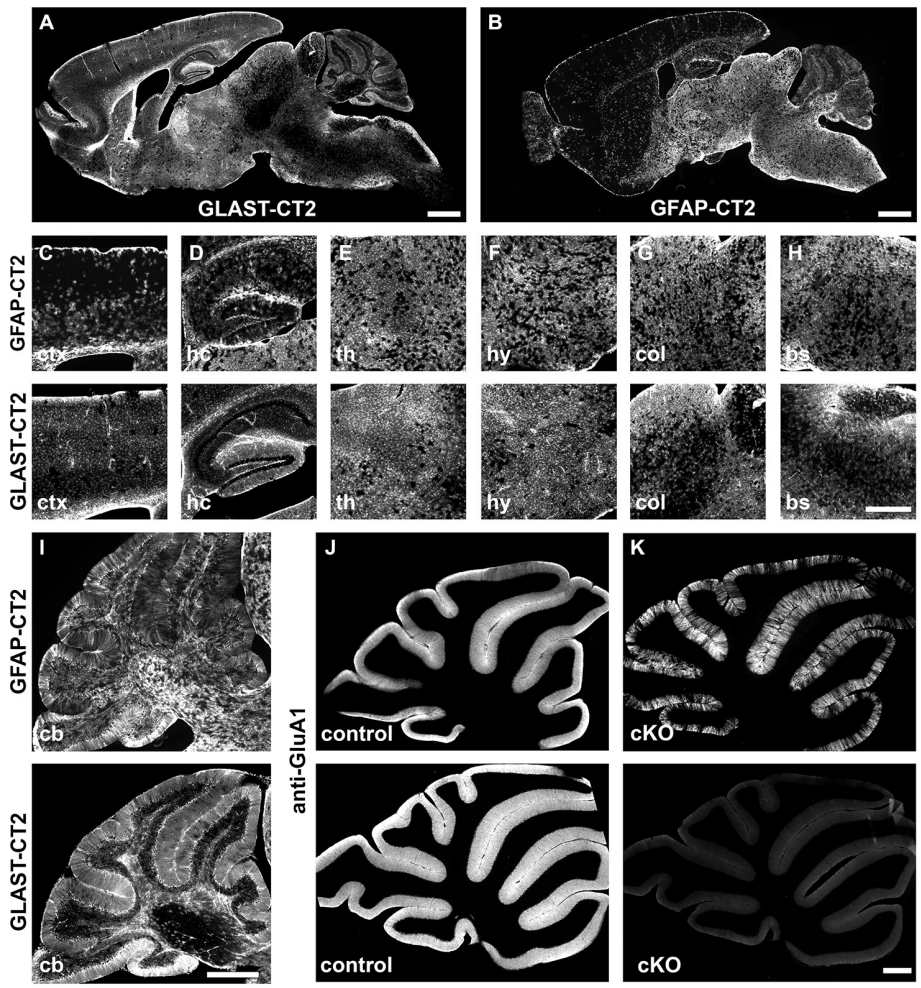

FIGURE 3 | Comparison of tamoxifen-induced DNA recombination in GLAST-CreERT2 and GFAP-CreERT2 mice. Comparison of DNA recombination in transgenic GFAP-CreERT2 and GLAST-CreERT2 knockin mice. **(A,B)** Sagittal overview of tdTomato reporter expression (Madisen et al., 2010) in the brain of GLAST-CreERT2 and GFAP-CreERT2 mice. **(C–H)** Magnified views of selected brain regions (ctx, cortex; hc, hippocampus; th, thalamus; hy, hypothalamus; col, superior colliculus; bs, brainstem). The upper panel shows reporter activation in GFAP-CreERT2 mice, the lower panel in GLAST-CreERT2. **(I–K)** In the cerebellum, tdTomato reporter activation of BG and other astrocytes is comparable in GFAP-CreERT2 and GLAST-CreERT2 mice **(I)** however, gene deletion (here GluA1) is more efficient **(K)** in BG of GLAST-CreERT2 mice (lower panel) than in GFAP-CreERT2 mice (upper panel) when compared to control mice **(J)**.

2009, 2011; Gourine et al., 2010; Lioy et al., 2011; Han et al., 2012; Saab et al., 2012). Therefore, the selection of regulatory elements of THE astrocyte-specific gene is almost impossible. Historically, the promoter of the human GFAP gene has become a widely distributed and valuable tool. Its small size of 2.2 kb offered excellent cloning properties and facilitated its use for transgenic expression of numerous proteins of current interest such as EGFP or CreERT2 (Nolte et al., 2001; Hirrlinger et al., 2006). In parallel, as an alternative genetic tool, GLAST-CreERT2 mice were generated by targeting the tamoxifen-sensitive Cre DNA recombinase CreERT2 to exon 2 of the GLAST locus using homologous recombination (Mori et al., 2006). Direct comparison of both mouse lines revealed variable differences in brain region-dependent recombination although they largely overlap (**Figure 3**). While recombination in GLAST-CreERT2 mice dominates forebrain regions, the GFAP-CreERT2 mouse displays higher recombination efficiencies in the hindbrain. Particular differences become evident when not only reporter proteins are activated, but when gene knockout experiments require the recombination of homozygous alleles as observed for the deletion of the ionotropic glutamate receptor subunit GluA1 in cerebellar BG (**Figures 3J,K**). In GLAST-CreERT2 mice the immunohistochemical signal indicating GluA1 was completely abolished to background levels 4 weeks after induction of recombination, while in GFAP-CreERT2 mice GluA1 expression still remained in numerous BG cells.

It is important to note that only heterozygous GLAST-CreERT2 mice can be used. Homozygous GLAST-CreERT2 mice will be knockouts of the GLAST-dependent glutamate uptake. Interestingly, we found that, although heterozygous mice express only 50% of GLAST mRNA and protein in comparison to wild type mice, the functional glutamate uptake current appears to be unaffected (Saab et al., 2012).

Although the GFAP-CreERT2 and GLAST-CreERT2 mice have been successfully used in a series of studies including behavioral experiments, both suffer from recombination in radial glia of the neurogenic niches (Hirrlinger et al., 2006; Mori et al., 2006; DeCarolis et al., 2013). The DNA recombination induced in the respective neuronal progeny might contribute to a phenotype in conditional knockout experiments and careful control experiments should be employed. Alternative lines such as Connexin43-CreERT mice (Eckardt et al., 2004) without neuronal recombination in stem cells have not yet been used for behavioral experiments. Similarly, behavioral experiments with astrocyte-specific AldhL1- or FGFR3-CreERT2 mice might also be difficult since recombination can also be induced in neural stem cells or interneurons (Young et al., 2010; Yang et al., 2011; Foo and Dougherty, 2013).

Tamoxifen-induced gene recombination is a very valuable tool to temporally control gene excision. But, one has to keep in mind that gene excision does not mean simultaneous disappearance of the respective protein. The half-lifes of mRNA and protein have to be considered. In case of the glutamate receptors GluA1 and GluA4, their efficient functional removal required about 2 weeks (Saab et al., 2012). In addition, the age of the mice (i.e., the level of gene activity) influenced the protein turnover as well.

In the future, genetic targeting of astrocytes has to become more sophisticated. It is now clear that astrocytes of different brain regions can fulfill different functions. Therefore, we have to identify novel regulatory elements that can be used to address the astroglial heterogeneity, either by targeting astrocytes of distinct brain regions locally or functionally, e.g., after injury or during a learning paradigm. The numerous transporter genes could be a rich source. The thyroid hormone transporter OATP1C1 is such an example. This transporter is expressed by cortical or hippocampal astrocytes, but not in the brainstem (Schnell et al., 2013). In addition, the coincident use of two different gene loci and employing the split-Cre system could provide another strategy to target subclasses of astrocytes (Hirrlinger et al., 2009a,b).

Current efforts in obtaining cell type, age and brain region-dependent gene expression profiles will facilitate the quest for suitable regulatory elements to target astrocytes. Future mouse models will then help to further highlight selective astrocyte function of distinct central nervous system (CNS) regions or learning paradigms.

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REVIEW
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Reappraisal of Bergmann glial cells as modulators of cerebellar circuit function

Chris I. De Zeeuw[1,2] *and Tycho M. Hoogland*[1,2]*

[1] Cerebellar Coordination and Cognition, Netherlands Institute for Neuroscience, Amsterdam, Netherlands, [2] Department of Neuroscience, Erasmus MC, Rotterdam, Netherlands

Just as there is a huge morphological and functional diversity of neuron types specialized for specific aspects of information processing in the brain, astrocytes have equally distinct morphologies and functions that aid optimal functioning of the circuits in which they are embedded. One type of astrocyte, the Bergmann glial cell (BG) of the cerebellum, is a prime example of a highly diversified astrocyte type, the architecture of which is adapted to the cerebellar circuit and facilitates an impressive range of functions that optimize information processing in the adult brain. In this review we expand on the function of the BG in the cerebellum to highlight the importance of astrocytes not only in housekeeping functions, but also in contributing to plasticity and information processing in the cerebellum.

Keywords: astrocytes, Bergmann glia, cerebellum, cerebellar zone, zebrin, neuron-glia interactions

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***Correspondence:**
Tycho M. Hoogland, Cerebellar Coordination and Cognition, Netherlands Institute for Neuroscience, Meibergdreef 47, 1105 BA Amsterdam, Netherlands
hoogland@nin.knaw.nl; t.hoogland@erasmusmc.nl

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Introduction

The versatile function of astrocytes is well highlighted by the Bergmann glial cell (BG) of the cerebellum, an astrocyte type that outnumbers the principle neuronal output cell of the cerebellar cortex, the Purkinje cell (PC), roughly eight-fold (Korbo et al., 1993; Reichenbach et al., 1995). BGs are essential for migration and correct layering of granule cells in early cerebellar development (Rakic, 1971), but they remain an integral part of the adult cerebellar circuit, where they subserve an important role in extracellular ion homeostasis (Wang et al., 2012), synapse stability (Iino et al., 2001; Saab et al., 2012), plasticity (Balakrishnan and Bellamy, 2009; Balakrishnan et al., 2014), metabolic function and neuroprotection (Poblete-Naredo et al., 2011; Jakoby et al., 2014). The functional relevance of BGs is also reflected by the expression of cyto-architectural markers that largely overlap with those of the cerebellar zones (Reeber et al., 2014), the canonical computational units of cerebellum (Chambers and Sprague, 1955; Groenewegen and Voogd, 1977; Groenewegen et al., 1979; Oscarsson, 1979; Zhou et al., 2014; De Zeeuw and Ten Brinke, 2015). In the intact brain, activity in BGs as measured via *in vivo* calcium imaging reveal a diverse repertoire of signals, including compartmented signaling in BG processes (Hoogland and Kuhn, 2010; Nimmerjahn et al., 2009), large scale elevations of calcium during behavior that are thought to correlate with changes in blood flow (Nimmerjahn et al., 2009), and more confined radially expanding waves that are increased in awake behaving vs. anesthetized animals (Nimmerjahn et al., 2009) and that increase in frequency with age (Mathiesen et al., 2013). In addition, homeostatic control of calcium by BGs may be important for neuroprotection and oxygen regulation (Mathiesen et al., 2013). Importantly, not only direct optogenetic manipulation of calcium levels in BGs (Sasaki et al., 2012), but also inducible

genetic deletion of AMPA receptors in BGs can affect associative motor learning and/or motor performance (Saab et al., 2012). Thus BGs are not just involved in essential housekeeping functions, but may also contribute to information processing in the cerebellum. Here, we provide more details on these diverse functions and propose the hypothesis that BGs are involved in fine-tuning activity in cyto-architecturally distinct cerebellar zones.

Cerebellar Architecture

In order to understand how BGs are integrated in the cerebellum, a brief introduction to its architecture is required. The basic circuit of the cerebellum is evolutionary conserved across vertebrates—from lampreys (Larsell, 1947) to cetaceans (Hanson et al., 2013)—and confers unique computational properties to enable sensorimotor integration and motor coordination with high temporal precision (Llinás and Sasaki, 1989; Welsh et al., 1995; Kistler and De Zeeuw, 2002). There are two main distinguishing features. One includes a rostral-caudal organization of cyto-architectural and functionally distinct sagittally oriented PC zones with further subdivisions into microzones (Groenewegen and Voogd, 1977; Zhou et al., 2014; Tsutsumi et al., 2015). The PCs provide the output of the cerebellar cortex to the cerebellar nuclei (CN) and thereby exert parcellated control over downstream effectors, which are often recruited sequentially during movements (Welsh et al., 1995; Hoogland et al., 2015). The climbing fibers (CFs), which originate from the inferior olive in the ventral medulla oblongata, project to the rostro-caudal zones of PCs (Sugihara et al., 2007; Brown et al., 2012) where they can trigger complex spikes synchronously to adjust movements (Marshall and Lang, 2009; Ozden et al., 2012; De Gruijl et al., 2014). Another integral feature of cerebellar architecture is the transverse alignment of granule cell parallel fiber axons (PFs), which cross the entire width of a cerebellar folium at right angles to the PC dendrites. The intrinsically generated simple spike firing of PCs is tuned not only by PF input, but also by CF input that can trigger short pauses of simple spike firing and determine the phase of their modulation (Schmolesky et al., 2002). Cerebellar zones are demarcated by preferential expression of select proteins in alternating parasagittal bands. The best-known example is aldolase C, or Zebrin II (Leclerc et al., 1992). Zebrin-positive PCs fire intrinsically at lower frequencies (∼60 Hz) than PCs in zebrin-negative zones (∼100 Hz) (Zhou et al., 2014) and these zones also appear to respond differentially to sensory input (Tsutsumi et al., 2015; Witter and De Zeeuw, 2015), lending support to the idea that cerebellar zones are basic operational units of cerebellar motor control. Other proteins with expression patterns similar or complementary to Zebrin are e.g., the neuronal calcium sensor protein (NCS-1; Jinno et al., 2003), the excitatory amino acid transporter 4 (EAAT4; Dehnes et al., 1998), heat shock protein HSP25 (Armstrong et al., 2001) and others (Cerminara et al., 2015). CFs projecting to zebrin-positive zones release more glutamate and generate more CS spikelets (Paukert et al., 2010). Moreover, the susceptibility of their PC targets for plasticity may be different as well (Wadiche and Jahr, 2005; Wang et al., 2011), highlighting that zones are functionally demarcated. How BGs are embedded in these demarcated zones and contribute to their function is not yet fully understood.

BG Structure, Circuit Embedding and Structural Plasticity

Several studies have described the cellular and subcellular structure of BGs in detail (de Blas, 1984; Siegel et al., 1991; Reichenbach et al., 1995; Castejón et al., 2002). BGs are distinct in having up to five polarized main processes (radial fibers) that extend over the full depth of the molecular layer. The BG fibers branch in the parasagittal plane (**Figure 1**), but overlap with fibers of neighboring cells to form palisades. In rodents BG fibers are regularly spaced at intervals of a few μm oriented along the parallel fiber direction with a bit wider spacing along the rostro-caudal axis of the cerebellum (de Blas, 1984; Reichenbach et al., 1995; Hoogland and Kuhn, 2010). BG radial fibers give rise to small convoluted side branches that form sites of putative neural-glial interaction and account in rat for 90% of the BG membrane surface area (Grosche et al., 1999, 2002). Two classes of protrusions from the main BG fibers have been distinguished, short thorny processes and more elaborate processes with long thin stalks several μm long. The complex BG appendages form microdomains that have surface-to-volume ratios six-fold higher than the main radial fibers, show highly complex branching patterns, and by nature of their structure can act as electrotonically and biochemically compartmentalized microdomains subserving on average five synapses (Grosche et al., 1999). Estimates on the number of PC synapses that BG microdomains encompass ranges from ∼2000–6000 (Reichenbach et al., 1995). The ensheathment of PC synapses commences near onset of synaptogenesis and could be important for regulation of synapse number, though it does not affect synapse stability in adulthood (Lippman Bell et al., 2010). What other functions do BG microdomains have? One possibility is that they restrict diffusion of neurotransmitter from the synaptic cleft (Grosche et al., 2002) and thus help to improve the fidelity of synaptic transmission. Due to the arrangement of BG palisades, side processes are ideally positioned to sample and interact with PFs (Herndon, 1964). High immuno-reactivity for glutamine synthetase was found in BG processes (Reichenbach et al., 1995) and BGs express high densities of glutamate transporters (Storck et al., 1992; Rothstein et al., 1994; Bergles et al., 1997). Together, this implicates that BGs may play a role in PF-mediated synaptic transmission. Strikingly, the first postnatal weeks show an impressive elaboration of BG appendages that parallels the development of PFs (Shiga et al., 1983; Grosche et al., 2002). Electron microscopy data have demonstrated that BG processes not only enwrap PF–PC synapses, but also appose PFs, CF collaterals, and processes of molecular layer interneurons (MLIs; Castejón et al., 2002). BG processes also express $GABA_A$ receptors in the vicinity of inhibitory synapses close to PC somata, but in a small fraction also at excitatory

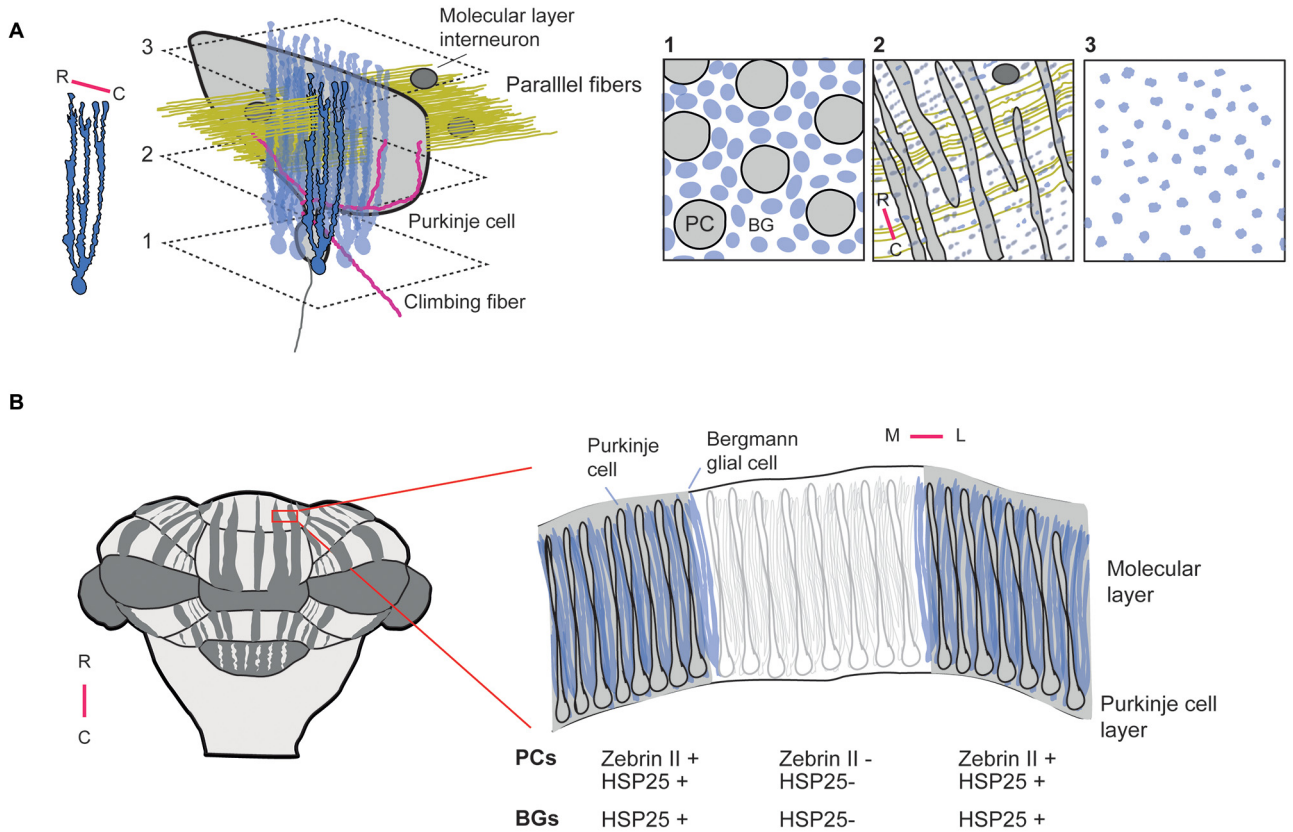

FIGURE 1 | Embedding of Bergmann glial cells in cerebellar circuits and zones (A). Arrangement of BGs relative to a single purkinje cell (PC). Left: Single BG indicating that radial fibers predominantly branch in the parasagittal plane, i.e., along the rostro-caudal axis of the cerebellum. BGs have up to 5 radial fibers that extend over the full depth of the molecular layer. In addition, BG radial fibers give off small side branches or BG microdomains (not shown) that predominantly wrap around parallel fiber- PC synapses (Grosche et al., 2002). Right: planes at different depths illustrating the PC-BG relationship. Inset 1: PC layer with arrangement of a monolayer of BG and PC somata at a density of 8 BGs (blue) to 1 PC. Inset 2: molecular layer BG processes arrange in palisades to maximize interaction with parallel fibers. Inset 3: BG fibers terminate at the surface with bulbous end-feet, the function of which has not been studied. **(B)** Zonation in the cerebellum. Left: Zebrin II (zebrin) expression in the cerebellum follows a pattern consisting of rostro-caudal parasagittal stripes that constitute functional modules with distinct input-output relationships. Right: coronal section through a cerebellar folium showing clear delineation of zebrin-positive, or negative borders. Other proteins such as the HSP25 display staining complementary to Zebrin II. BGs display a similar zonal expression of HSP25 (Reeber et al., 2014), but with less distinct boundaries due to their morphology. R: rostral, C: caudal, M: medial, L: lateral.

synapses near PC dendritic spines (Riquelme et al., 2002). Thus, BGs are equipped to sense both inhibitory and excitatory neurotransmitters. Indeed, glutamate transporters are densely expressed on BG processes and can aid glutamate uptake into BGs (Bergles et al., 1997). It has been demonstrated that GLAST and GLT-1 also affect the time course of synaptically evoked currents during repeated PF activation of a few fibers, or during single stimuli when multiple nearby fibers are activated (Marcaggi et al., 2003). By shaping the time course of postsynaptic currents glutamate transporters in BGs could regulate mGluR—mediated plasticity (Marcaggi and Attwell, 2005). Both electrical stimulation of PFs and ATP release from MLIs can trigger calcium elevations in BG processes (Beierlein and Regehr, 2006). BGs normally express calcium-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, as demonstrated by the presence of glutamate evoked AMPAR currents and immunocytochemistry (Burnashev et al., 1992; Sato et al., 1993; Bellamy and Ogden, 2006; Saab et al., 2012). They are required for maintenance of BG processes around PC dendritic spines (Iino et al., 2001). Recent work in which (calcium-permeable) AMPA receptors (GluR1 and GluR4) were conditionally knocked-out in BGs has replicated this finding and demonstrated a co-occurrence of such BG process retraction with impairments in associative motor learning during both the ErasmusLadder task and eyeblink conditioning (Saab et al., 2012). Moreover, since these phenotypes occurred in adult but not young animals, the picture emerges that BGs also serve specific and active functions in adulthood. Interestingly, AMPA receptors probably also regulate electrical coupling between BGs, as their activation results in a strong reduction of BG-BG junctional conductance (Müller et al., 1996). Even though the BG palisades are oriented along the direction of the PFs, the electrical coupling between BGs appears to be limited along the parasagittal plane matching the orthogonal orientation of PC dendritic arbors (**Figure 1A**).

Types of Calcium Signaling in BGs

Calcium dynamics in BGs have been described in detail *in vitro* and *in vivo*. Burst stimulation of PFs is sufficient to trigger local calcium increases in BG processes (Grosche et al., 1999; Beierlein and Regehr, 2006; Piet and Jahr, 2007). Such calcium elevations are sensitive to block of group I metabotropic glutamate receptors, purinergic (P2Y) receptors or AMPAR currents (Beierlein and Regehr, 2006; Piet and Jahr, 2007). Putative AMPAR-mediated calcium increases show shorter duration and smaller amplitude calcium transients upon stimulation, while larger calcium increases are sensitive to cyclopiazonic acid (CPA), blocking release from calcium stores. The slow calcium transients seen during burst stimulation can be elicited by ATP released from MLIs upon PF stimulation (Piet and Jahr, 2007). Stimulation of CFs does not elicit calcium elevations in BGs, but does evoke currents in BGs. These currents are of much lower magnitude than during PF stimulation. PF stimulation results in BG AMPAR currents that display paired pulse facilitation (PPF) as seen with synaptic PPF, but such AMPAR-mediated BG currents are of short duration as BG AMPAR currents with high-frequency stimulation cannot be sustained (Bellamy and Ogden, 2005). Taken together, it appears that BGs are more responsive to PF than to CF activity. However, synchronous CF activity could in principle boost glutamate spillover. Spillover from CF terminals has been associated with larger CF mediated AMPAR currents and could act as a cue to guide BG processes to enwrap synapses for better isolation (Matsui and Jahr, 2004). Thus, functionally BG AMPAR currents appear to be important for stabilizing BG microdomains around synaptic elements to enable effective clearance through BG glutamate transporters (Rothstein et al., 1994; Bergles et al., 1997) and thereby increase the fidelity of synaptic transmission.

Electrical stimulation of PF axonal beams in recent experiments have demonstrated clustered activation of PFs in response to sensory stimulation (Wilms and Häusser, 2015). Such PF activation could result in calcium elevations in BG processes *in vivo* similar to those that have been reported *in vitro*. In vivo, several types of BG calcium responses have been observed. These include for example single process calcium elevations as revealed through sparse viral transduction of BGs with a genetically encoded calcium indicator (GECI; Hoogland et al., 2009) or synthetic calcium indicators in combination with the astrocyte marker SR101 (Nimmerjahn et al., 2004). In addition, elevations in large fields of BG processes can occur *in vivo* during locomotion (Nimmerjahn et al., 2009; Paukert et al., 2014) and/or transglial calcium waves (Hoogland et al., 2009; Nimmerjahn et al., 2009). The frequency of BG signals of mammals in the awake state is significantly higher than those in the anesthetized state (Nimmerjahn et al., 2009; Hoogland and Kuhn, 2010) and their rate is generally sensitive to block of neural activity and glutamatergic transmission with some remaining calcium responses that might be intrinsically generated (Nimmerjahn et al., 2009). The calcium elevations in BG processes during locomotion probably reflect the increased synaptic drive observed in the cerebellum during locomotion (Ozden et al., 2012). However, it should be noted that the correlations with the onset of locomotion are weak and that BGs do not always respond with calcium increases during bouts of locomotion (Paukert et al., 2014). Transglial calcium waves in the cerebellum are triggered by ATP and rely on release of calcium from internal stores (Hoogland and Kuhn, 2010). Their frequency also increases when the animal is transferred from an anesthetized to an awake state (Nimmerjahn et al., 2009) or when it is getting older (Mathiesen et al., 2013). Indeed, the role of these waves may be metabolic and neuroprotective in that their occurrence increases with low oxygen tension (Mathiesen et al., 2013). Future studies employing selective expression of GECIs should further elucidate how calcium microdomains in BGs respond to sensorimotor stimulation in detail (Kuhn et al., 2011; Paukert et al., 2014).

BG K^+ Siphoning and Functional Implications

Astrocytes including BGs act as large sinks for redistribution of ionic gradients and could thereby have significant impact on neurotransmission and excitability of neurons (Newman et al., 1984; Reichenbach et al., 1995). This can have clear benefits for redistributing ions in regions where K^+ accumulates rapidly during periods of strong activity, ensuring that increased neuronal activity can be sustained. In fact, large 1–3 mM accumulations of extracellular K^+ have been measured *in vitro* around PCs in response to spiking activity (Hounsgaard and Nicholson, 1983). The presence of inward rectifying K^+ (KIR) channels can effectively shuttle K^+ into BGs (Butt and Kalsi, 2006), which have strongly hyperpolarized resting membrane potentials. *In situ* hybridization studies have shown that KIR4.1 has highest expression levels in hippocampus and cerebellar cortex (Poopalasundaram et al., 2000). In the cerebellum KIR4.1 channels are expressed in BGs near the PC primary branches (Poopalasundaram et al., 2000). Recent work has revealed the importance of BGs in regulating K^+ concentrations to influence PC membrane potential. Such modulation was shown to be Ca^{2+}-dependent and could trigger bistability of PCs (Wang et al., 2012), a phenomenon in which PCs show periods of firing alternated by quiescence. In the intact animal PC bistability has been shown to be strongly influenced by anesthetics and mostly absent in healthy tissue of awake animals (Schonewille et al., 2006), but it could potentially be present in the cerebellum during sleep, which currently is an active area of investigation. The function of bistability is contentious, but modeling studies suggest that it can increase the capacity of PCs to learn input-output associations (Clopath et al., 2012). Thus, since artificial elevation of calcium in BGs *in vitro* transiently reduces extracellular K^+ concentrations (Wang et al., 2012), the question emerges to what extent a particular type of calcium signal is preferably driving inward K^+ currents in BGs *in vivo* or whether other observed types of calcium conductances also contribute to regulation of K^+ in the intact cerebellum. While ATP-driven calcium elevations could trigger transient shifts of PCs to an upstate *in vitro*,

ATP-mediated transglial waves relying on calcium release from intracellular stores could in principle modulate excitability of nearby PCs *in vivo* (Hoogland et al., 2009). However, no direct role of transglial waves in physiological function has yet been demonstrated. As it stands now, these signals seem to be involved predominantly in metabolic control or are neuroprotective (Mathiesen et al., 2013). As mentioned above, the frequency of transglial waves is significantly decreased under anesthesia (∼7-fold) when bistability manifests itself most prominently (Nimmerjahn et al., 2009). This could be reconciled by the fact that increased frequency of BG calcium signals in awake mice (Nimmerjahn et al., 2009; Hoogland and Kuhn, 2010) could cause a constitutive reduction of external K^+ to drive PCs into prolonged (depolarized) up-states. It has been proposed that PCs remain in the upstate because calcium-dependent K^+ channels gradually inactivate after hyperpolarization (Wang et al., 2012), but the exact mechanisms are still unclear. Both locally and globally BGs are able to alter excitability by regulating extracellular K^+ but under which conditions this happens in the intact brain requires further investigation.

Activation of BGs during Vigilance

BGs like other astrocytes express receptors for the neurotransmitter noradrenaline and activation of such receptors can trigger calcium elevations (Salm and McCarthy, 1990; Kirischuk et al., 1996). Recent studies have revealed that noradrenaline-dependent calcium signaling in astrocytes can be triggered *in vivo* (Bekar et al., 2008; Ding et al., 2013), either after stimulating the source of noradrenergic afferents, the locus coeruleus, after strong peripheral stimulation, such as foot shocks, or even with whisker stimulation as revealed by pharmacological block with alpha-adrenergic receptor antagonists (Bekar et al., 2008; Ding et al., 2013). The release of noradrenaline is non-synaptic, diffuses throughout the neuropil volume and can thereby trigger calcium elevations and down-stream signaling in astrocytes.

Possibly, noradrenaline release also contributes to BG activation during sensorimotor stimulation. Indeed during head-fixed treadmill locomotion (Nimmerjahn et al., 2009) weak responses can be seen in BGs of the cerebellum across the field of view. These signals can be extracted from ROIs defined by the co-loading of the astrocyte marker SR101 (Nimmerjahn et al., 2004)—now known to also affect neuronal excitability (Kang et al., 2010)—and OGB-1/AM, a synthetic calcium indicator dye. Calcium signals in (purported) BG processes occur during locomotion bouts and are attenuated with short inter-movement intervals, suggesting that calcium release from internal stores underlies the calcium increases. In a recent study in which the GECI GCaMP3 was expressed selectively in astrocytes, calcium elevations were also demonstrated to encompass large fields of BG processes during locomotion bouts, but they were of low amplitude and such events had a failed rate of about one in three (Paukert et al., 2014). Strikingly, when locomotion was enforced (and thus arousal peaked) significantly larger whole field BG responses were evoked in a consistent manner. These also appeared refractory. Using pharmacological tools it was subsequently shown that such arousal-induced BG calcium elevations were dependent on noradrenaline release and activation of the α1-adrenergic receptor similar to what has been observed for cortical astrocytes (Ding et al., 2013). Calcium elevations were not restricted to BGs, but also observed simultaneously in visual cortex astrocytes—albeit with a slightly longer delay—suggesting that release of noradrenaline during a state of arousal activates astrocytes across the entire brain. Thus, the noradrenergic system can act as a gain modulator not only for neurons (Johnson et al., 1968), but also astrocytes during active behavior. Which types of natural behaviors trigger the arousal system to elicit calcium elevations in BGs remains to be elucidated.

BGs and Cerebellar Zone Patterning

The modular organization of the cerebellum into parasagittal zones as defined e.g., by Zebrin, or other markers (**Figure 1B**) has been known for decades (Voogd, 1969; Groenewegen et al., 1979), and although hypotheses were formulated on their function (Oscarsson, 1979), it was not until much later that their functional organization was investigated in awake animals (Welsh et al., 1995; Lang et al., 1999; Ozden et al., 2012; De Gruijl et al., 2014). *In vivo* two-photon microscopy recently revealed that zebrin-positive zones not only define sharp anatomical borders, but also show a sharp delineation of functional responses to sensory stimuli (Tsutsumi et al., 2015). Although it is logical to assume that BGs embedded in cerebellar zones follow the same modular organization as the neurons they interact with, it was not until recently that evidence was presented that BGs indeed show overlap with cerebellar zones (Reeber et al., 2014), as defined by marker proteins also found in PCs. Specifically, it concerned the heat shock protein, HSP25, which was shown earlier to be confined to zebrin-like parasagittal bands of PCs in mice (Armstrong et al., 2000). The function of this protein in the cerebellum is still unknown. BG processes do not have the flat topography of PC dendritic arbors and the BG borders defined by HSP25 are not as tight as those seen for PCs (**Figure 1B**). Nevertheless, there is substantial overlap. It is likely that other zone-delimiting proteins will be found to co-localize not only with cerebellar neurons, but also BGs. Interesting as this may be, the most important question remains to be answered, namely do BGs that are integrated in cerebellar zones also contribute actively to the physiology of these zones? The recent establishment of differential firing behavior of PCs in zebrin-positive vs. zebrin-negative cerebellar zones—with the latter firing at higher frequencies—(Zhou et al., 2014) has made this question quite relevant. It was found that the higher frequency of PC firing in zebrin-negative zones could be attributed to the activation of the TRPC3 channel in PCs, a channel that is under control of proteins that have expression patterns overlapping with zebrin-negative zones. Although the firing rate differences were strongly attenuated when blocking TRPC3 channel function,

alternate pathways are feasible through which PCs activity levels could be set in cerebellar zones. BGs have been shown to tonically release GABA via bestrophin 1 (Best1) channels (Lee et al., 2010; Yoon et al., 2011). The close apposition of BG processes to PFs, PCs and MLIs suggest that through tonic release of neurotransmitters BGs are capable to set activity levels in the cerebellar circuit. In granule cells tonic GABA release from astrocytes through Best1 could evoke tonic currents of up to ∼30 pA. In BGs the concentration of GABA has been estimated with the use of immunogold labeling to be between 5–10 mM and is synthesized by monoamine oxidase (Yoon et al., 2014). Thus a sufficient electrochemical gradient exists to drive currents through Best1 in BGs and sustain tonic GABA release. The GABAB receptor 2 (GABABr2) is found in PCs of zones that label positively for zebrin (Chung et al., 2008). Given the typical extrasynaptic localization of such receptors (Fritschy et al., 1999) and their known coupling to G protein-coupled inwardly-rectifying potassium (GIRK) channels that can drive membrane hyperpolarization (Lüscher and Slesinger, 2010), it is tempting to speculate that tonic GABA release in Zebrin/GABABr2-positive zones sets, or maintains lower activity levels. In addition, the exact distribution of Best1 in the cerebellum has not been reported in great detail as histology was performed on thin parasagittal sections, making it hard to assess whether perhaps Best1 itself is also expressed preferentially in a zebrin-like pattern (Lee et al., 2010; Yoon et al., 2011). Regardless, tonic GABA release from BGs (in combination with zone-delimited expression of GABA receptors) adds another layer of glial control over cerebellar circuit function over time courses that exceed the typical time scale of seconds, during which BGs modulate their activity.

BGs and Information Processing in the Cerebellum: Future Challenges

BG are versatile in their function and highly integrated in the cerebellar circuit. However, many questions remain about their exact contribution to information processing in the cerebellum. High surface-to-volume ratios and thin stalks endow BG microdomains (Grosche et al., 1999) with the ability to act as independent compartmentalized units that aid in glutamate uptake (Bergles et al., 1997), shaping of fast and slow synaptic currents (Marcaggi et al., 2003; Marcaggi and Attwell, 2005), regulation of extracellular K^+ (Wang et al., 2012) and tonic GABA release (Lee et al., 2010). Furthermore, their activity is co-modulated with neurons during increased states of vigilance (Paukert et al., 2014). Thus at a local scale BGs can modulate the efficacy of synaptic transmission of a cluster or even individual synapses, possibly facilitating memory formation associated with specific behaviorally relevant contexts. The co-expression with PCs of zone-delimiting proteins suggest that BGs in cerebellar zones could either sustain reported physiological differences in firing behavior of neurons in such zones, or set their activity levels. Concurrent electrophysiological targeting of BGs and nearby neurons and *post hoc* immunohistochemistry for zone-delimiting proteins, or transgenic mouse models that allow direct visual selection of BGs in cerebellar zones should advance our knowledge in this regard. A combination of targeted electrophysiology (Kitamura et al., 2008) and cell-selective expression of GECIs in BGs (Chen et al., 2013) should allow better assessment of the relation between calcium dynamics in BG fibers and microdomains and their temporal relation with sub- and supra-threshold activity in the neurons they interact with.

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frontiers in Cellular Neuroscience

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Role for astroglial α1-adrenoreceptors in gliotransmission and control of synaptic plasticity in the neocortex

*Yuriy Pankratov and Ulyana Lalo**

School of Life Sciences, Gibbet Hill Campus, University of Warwick, Coventry, UK

Communication between neuronal and glial cells is thought to be very important for many brain functions. Acting via release of gliotransmitters, astrocytes can modulate synaptic strength. The mechanisms underlying gliotransmission remain uncertain with exocytosis being the most intriguing and debated pathway. We demonstrate that astroglial α1-adrenoreceptors are very sensitive to noradrenaline (NA) and make a significant contribution to intracellular Ca^{2+}-signaling in layer 2/3 neocortical astrocytes. We also show that astroglial α1-adrenoreceptors are prone to desensitization upon prolonged exposure to NA. We show that within neocortical slices, α-1adrenoreceptors can activate vesicular release of ATP and D-serine from cortical astrocytes which initiate a burst of ATP receptor-mediated currents in adjacent pyramidal neurons. These purinergic currents can be inhibited by intracellular perfusion of astrocytes with Tetanus Toxin light chain, verifying their origin via astroglial exocytosis. We show that α1 adrenoreceptor-activated release of gliotransmitters is important for the induction of synaptic plasticity in the neocortex:long-term potentiation (LTP) of neocortical excitatory synaptic potentials can be abolished by the selective α1-adrenoreceptor antagonist terazosin. We show that weak sub-threshold theta-burst stimulation (TBS) can induce LTP when astrocytes are additionally activated by 1 μM NA. This facilitation is dependent on the activation of neuronal ATP receptors and is abolished in neocortical slices from dn-SNARE mice which have impaired glial exocytosis. Importantly, facilitation of LTP by NA can be significantly reduced by perfusion of individual astrocytes with Tetanus Toxin. Our results strongly support the physiological importance of astroglial adrenergic signaling and exocytosis of gliotransmitters for modulation of synaptic transmission and plasticity.

Keywords: astrocyte-neuron interactions, exocytosis, norepinephrine, ATP release, P2X receptor, desensitization, metaplasticy, D-serine

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****Correspondence:***
Ulyana Lalo, School of Life Sciences, Gibbet Hill Campus, University of Warwick, Coventry CV4 7AL, UK
U.Lalo@warwick.ac.uk

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Introduction

It is generally acknowledged that astrocytes are very important components of brain networks. The astroglial network receives and integrates signals from neurons and responds by increasing the metabolic support for neurons and by modulating neuronal activity via the release of gliotransmitters (Giaume et al., 2010; Halassa and Haydon, 2010). The variety of physiological functions of astroglia spans from "housekeeping", such as clearance and turnover of

neurotransmittes and ionic homeostasis to the control of neurovascular coupling and modulation of synaptic transmission and plasticity (Gordon et al., 2009; Attwell et al., 2010; Giaume et al., 2010; Henneberger et al., 2010; Min and Nevian, 2012). It is commonly accepted that elevation of cytosolic calcium is the major mechanism of astrocyte activation and integration of information within the glial network and underpins many functions of astrocytes.

Astrocytes are endowed with an extensive complement of Gq protein-coupled neurotransmitter receptors that trigger IP3-mediated release of Ca^{2+} from intracellular stores (Halassa and Haydon, 2010; Araque et al., 2014; Khakh and McCarthy, 2015). In addition, ionotropic Ca^{2+}-permeable receptors can contribute to astroglial signaling (Palygin et al., 2011). There is an accumulating evidence that Ca^{2+}signaling in brain astrocytes can be activated by synaptically-derived neurotransmitters (Lalo et al., 2011a, 2014a; Palygin et al., 2011; Panatier et al., 2011), by the local autocrine release of gliotransmitters such as glutamate or ATP (Araque et al., 2014) and via diffuse volume-transmitted neuromodulators such as serotonin, acetylcholine or noradrenaline (NA; Schipke et al., 2008; Ding et al., 2013; Paukert et al., 2014; Khakh and McCarthy, 2015). Although the importance of all modes of astroglial signaling is generally recognized, their specific physiological roles are yet to be fully understood (Araque et al., 2014; Khakh and McCarthy, 2015). It was shown that receptors to serotonin and acetylcholine do not bring significant contribution to calcium dynamics in cortical astrocytes (Schipke et al., 2008) whereas astrocytic α1-adrenoreceptros (α1-ARs) were recently highlighted as important participants in calcium signaling (Ding et al., 2013; Paukert et al., 2014).

Astroglial Ca^{2+}-signaling is tightly linked to the release of gliotransmitters which plays an important role in glia-neuron communication (Halassa et al., 2007; Araque et al., 2014). It has been widely reported that the release of ATP (Lalo et al., 2014a,b), glutamate and D-serine from astrocytes can modulate the activity of neuronal excitatory and inhibitory synapses (Gordon et al., 2009; Panatier et al., 2011; Lalo et al., 2014a), long-term synaptic plasticity (Pascual et al., 2005; Henneberger et al., 2010; Araque et al., 2014; Lalo et al., 2014b; Rasooli-Nejad et al., 2014) and neurovascular coupling (Attwell et al., 2010; Gourine et al., 2010). Although detailed mechanisms of gliotransmission remain uncertain, the importance of Ca^{2+}-dependent vesicular and non-vesicular pathways has been recently reported (Gourine et al., 2010; Woo et al., 2012; Araque et al., 2014; Lalo et al., 2014a).

Thus, it is conceivable that astroglial α1-ARs may be involved in glial control of synaptic activity. A number of recent studies have used NA as a tool for the activation of astrocytes and showed that NA-evoked and glia-derived ATP can modulate excitatory synapses in the hippocampus and hypothalamus (Gordon et al., 2009; Pougnet et al., 2014). However these papers do not provide direct evidence for adrenoreceptor-triggered release of gliotransmitters and report opposing effects of glia-derived ATP on AMPA receptors trafficking. As such, the functional properties of noradrenergic glial signaling and its contribution to astroglial control of synaptic plasticity require further investigation. In the present study, we demonstrate that astroglial α1-ARs can activate exocytosis of gliotransmitters, in particular ATP, and this mechanism contributes to modulation of synaptic plasticity in neocortical neurons. To verify this, we use a combination of approaches including transgenic mice with inducible astroglial expression of dominant-negative SNARE domain (dn-SNARE; Pascual et al., 2005), intracellular perfusion of astrocytes with Ca^{2+}-chelators and inhibitors of SNARE proteins.

Material and Methods

All animal work has been carried out in accordance with UK legislation and "3R" strategy; research has not involved non-human primates.

Experiments were performed on astrocytes and neurons of somato-sensory cortex of dn-SNARE transgenic mice (Pascual et al., 2005; Halassa et al., 2009), their wild-type littermates (WT) and transgenic mice expressing enhanced green fluorescent protein (EGFP) under the control of the glial fibrillary acidic protein (GFAP) promoter (Lalo et al., 2011a; Palygin et al., 2011). Data obtained in the experiments on GFAP-EGFP (GFEC) mice did not differ significantly from data obtained in the WT mice. For clarity, all data referred to here as WT are reported solely for WT littermates to dn-SNARE mice; usage of GFAP-EGFP mice was explicitly stated where appropriate.

Slice and Cell Preparation

Mice (8–12 weeks) were anesthetized by halothane and then decapitated, in accordance with UK legislation. Brains were removed rapidly after decapitation and placed into ice-cold physiological saline containing (mM): NaCl 130, KCl_3, $CaCl_2$ 0.5, $MgCl_2$ 2.5, NaH_2PO_4 1, $NaHCO_3$ 25, glucose 15, pH of 7.4 gassed with 95% O_2—5% CO_2. Transverse slices (280 μm) were cut at 4°C and then placed in physiological saline containing (mM): NaCl 130, KCl_3, $CaCl_2$ 2.5, $MgCl_2$ 1, NaH_2PO_4 1, $NaHCO_3$ 22, glucose 15, pH of 7.4 gassed with 95% O_2—5% CO_2 and kept for 1–4 h prior to cell isolation and recording. Neocortical pyramidal neurons were acutely isolated using the modified "vibrating" technique (Lalo et al., 2011a; Rasooli-Nejad et al., 2014). The glass ball (200 μm diameter) was moved slowly some 10–50 μm above the slice surface, while vibrating at 100 Hz (lateral displacements 20–30 μm). This technique mechanically isolates cells whilst preserving the function of membrane proteins and is therefore devoid of many artifacts of enzymatic cell isolation and culturing procedures. The composition of extracellular solution for all isolated cell experiments was (mM): 135 NaCl; 2.7 KCl; 2.5 $CaCl_2$; 1 $MgCl_2$; 10 HEPES, 1 NaH_2PO_4, 15 glucose, pH adjusted with NaOH to 7.3.

Astrocytes were identified by their morphology under DIC observation, EGFP fluorescence (astrocytes from dn-SNARE and GFAP-EGFP mice) or staining with sulforhodamine 101 (astrocytes from WT mice). After recording, the identification of astrocyte was confirmed via functional properties (high potassium conductance, low input resistance, strong activity

of glutamate transporters) as described previously (Lalo et al., 2011a; Palygin et al., 2011).

Electrophysiological Recordings

Whole-cell voltage clamp recordings from neocortical neurons and astrocytes were made with patch pipettes (4–5 MΩ for neurons and 6–8 MΩ for astrocytes) filled with intracellular solution (in mM): 110 KCl, 10 NaCl, 10 HEPES, 5 MgATP, 10 EGTA, 1 $CaCl_2$, pH 7.35. Currents were monitored using an AxoPatch200B patch-clamp amplifier (Axon Instruments, USA) filtered at 2 kHz and digitized at 4 kHz. Experiments were controlled by PCI-6229 data acquisition board (NI, USA) and WinFluor software (Strathclyde University, UK); data were analyzed with custom software. Liquid junction potentials were compensated with the patch-clamp amplifier. Series and input resistances were respectively 5–7 MΩ and 500–1100 MΩ in neurons and 8–12 MΩ and 50–150 MΩ in astrocytes; both series and input resistance varied by less than 20% in the cells accepted for analysis. For activation of synaptic inputs, axons originating from layer IV–VI neurons were stimulated with a bipolar coaxial electrode (WPI, USA) placed in layer V close to the layer IV border, approximately opposite the site of recording; stimulus duration was 300 μs. The stimulus magnitude was set 3–4 times higher than the minimal stimulus necessary to elicit a response in layer II pyramidal neurons (Lalo et al., 2011a; Palygin et al., 2011; Lalo et al., 2014a).

Field excitatory postsynaptic potentials (fEPSPs) were measured via a glass micropipette filled with extracellular solution (0.5–1 MΩ resistance) placed in neocortical layer II/III. In order to induce long-term plasticity of EPSPs two or five episode of theta-burst stimulation (HFS) were delivered; each HFS episode consisted of five pulses of 100 Hz stimulation, repeated 10 times with 200 ms interval (total 50 pulses per episode).

Multi-photon Fluorescent Ca^{2+}-imaging in astrocytes

To monitor the cytoplasmic free Ca^{2+}concentraton ($[Ca^{2+}]_i$) *in situ*, astrocytes of neocortical slices were loaded via 30 min incubation with 1 μM of Rhod-2AM or Calcium Green-2AM and sulphorhodamine 101 (WT mice) at 33°C. Two-photon imaging of neurons and astrocytes was performed using a Zeiss LSM-7MP multi-photon microscope coupled to a SpectraPhysics MaiTai pulsing laser; experiments were controlled by ZEN LSM software (Carl Zeiss, Germany). Images were further analyzed off-line using ZEN LSM (Carl Zeiss) and ImageJ (NIH) software. The $[Ca^{2+}]_i$ levels were expressed as ΔF/F ratio averaged over a region of interest (ROI). For analysis of spontaneous Ca^{2+}–transients in astrocytes, three ROIs located over dendrites and one ROI located over the soma were chosen. Overall Ca^{2+}-response to adrenoreceptor agonists or synaptic stimulation was quantified using an ROI covering the whole cell image.

The NA and other drugs were applied to the recording chamber using gravity-feed application system with the flow rate of 5 mL/min. The recording chamber volume was 1.2 mL, so the time of full solution exchange could be estimated as 20–30 s. In measurements of the concentration-dependent response to adrenoreceptors agonists, only two or three applications of agonist were made to each neocortical slice in order to minimize the potential effect of receptor desensitization. The reference concentration of agonist (10 μM for NA, 300 nM for A61603) was used in all experiments; the lowest concentrations were applied first. Amplitudes of all responses were normalized to the response to the reference concentration. Data were then pooled together and fitted with a sigmoidal relationship to obtain the EC_{50} and Hill coefficient. Data on the concentration-dependence for the adrenoreceptors agonists were fitted with the following equation: $F/F_{ref} = ([A]/EC_{50})^p / \left(1 + ([A]/EC_{50})^p\right)$, where F is the amplitude of response to the agonist concentration [A], F_{ref} is the response to the reference concentration, p is the Hill coefficient.

Measurement of Extracellular Concentration of ATP and D-serine in the Brain Tissue

The concentration of ATP within cortical slices was measured using microelectrode biosensors obtained from Sarissa Biomedical Ltd. (Coventry, UK). A detailed description of the properties of biosensors and recording procedure has been published previously (Frenguelli et al., 2007). Briefly, biosensors consisted of ATP or D-serine metabolizing enzymes immobilized within a matrix on thin (25 μM) Pt/Ir wire. This allowed insertion of the sensors into the cortical slice and minimized the influence of a layer of dead surface tissue. ATP and D-serine biosensors were used simultaneously. A third, null, biosensor was also used. This sensor is identical to the ATP and D-serine sensors and has a matrix, but lacks enzymes. The signal from the null sensor was subtracted from the signal obtained on the ATP and D-serine sensor. This allows the contribution of any non-specific electroactive substances that bypass the sensor screening layer to be eliminated. Biosensors show a linear response to increasing concentration of ATP and D-serine and have a rise time less than 10 s (Frenguelli et al., 2007). Biosensors were calibrated with a known concentrations (10 μM) of ATP and D-serine before the slice was present in the perfusion chamber and after the slice had been removed. This allowed compensation of any reduction in sensitivity during the experiment. The integrity of the screening layer was assessed with 10 μM 5-HT. Biosensor signals were acquired at 1 kHz with a 1400 CED interface and analyzed using Spike 6.1 software (Cambridge Electronics Design, Cambridge, UK).

Data Analysis

All data are presented as mean ± SD and the statistical significance of differences between data groups was tested by two-tailed unpaired *t*-test, unless indicated otherwise. Each neocortical slice was used only for one experiment (e.g., fluorescent recordings in single astrocyte or single long-term potentiation (LTP) induction experiment). The number of experiments/cells reported is therefore equal to the number of slices used. The experimental protocols were allocated randomly so the data in any group were drawn from at least three animals.

The spontaneous transmembrane currents recorded in neurons were analyzed off-line using methods described previously (Pankratov et al., 2007; Lalo et al., 2011a; Palygin et al., 2011). The amplitude distributions of spontaneous and evoked currents were analyzed with the aid of probability density functions and likelihood maximization techniques; all histograms shown were calculated as probability density functions. The amplitude distributions were fitted with either multi-quantal binomial model or bi-modal function consisting of two Gaussians with variable peak location, width and amplitude. The decay time distributions were fitted with bi-modal functions. Parameters of models were fit using likelihood maximization routine. To monitor and analyze the time course of changes in the amplitude and frequency of spontaneous currents, the amplitude and frequency were averaged over a 1 min time window.

Results

Functional Properties of Adrenergic Ca^{2+} Signaling in Neocortical Astrocytes *In Situ*

Under basal conditions (before application of NA), astrocytes in neocortical slices from all mice strains exhibited spontaneous Ca^{2+} transients with the average frequency varying in the range of 0.5–2.1 min^{-1} (**Figure 1A**). Bath application of 3 μM NA (60 s long) induced robust Ca^{2+} elevations both in the soma and branches and increased the amplitude and frequency of Ca^{2+} transients (**Figures 1A,B**). There was no statistically significant difference in the action of NA between astrocytes of different strains (**Figure 1B**). The action of NA was mimicked by the specific α1-AR agonist A61603 (Yoshiki et al., 2013) in all 13 cells tested (data not shown).

At the same time, astroglial α1-ARs showed evidence of desensitization. In all eight astrocytes tested (WT mice), repetitive application of NA (3 μM) or A61603 (100 nM) with intervals shorter than 5 min caused marked reduction in the response amplitude and lead to a period of non-responsiveness, typically lasting 10–15 min (**Figure 1C**). Moreover, application of NA in concentrations greater than 10 μM lead to a long-lasting period of elevated intracellular Ca^{2+} in 8 out of 10 cells tested. During this period, astrocytes did not respond further to NA and did not exhibit notable spontaneous activity (**Figure 1D**). Elevation in the baseline level of cytosolic Ca^{2+} reached 44 ± 17% (n = 8) and was statistically significant ($P < 0.01$, compared to the control level using paired t-test). The susceptibility of α1AR-mediated responses to desensitization suggests the existence of finely-tuned molecular mechanisms preventing their overstimulation, likely to avoid Ca^{2+}-overload.

The predominant contribution of astroglial α1-ARs to responses evoked by NA was confirmed by the selective α1-AR antagonist terazosin. Application of terazosin (30 nM) effectively blocked responses to NA in all 12 astrocytes tested (**Figure 1B**). Terazosin also had a considerable inhibitory effect on spontaneous Ca^{2+}-transients (**Figure 1B**). This suggests either a basal tone of NA in neocortical slices, or constitutive α1-AR activity.

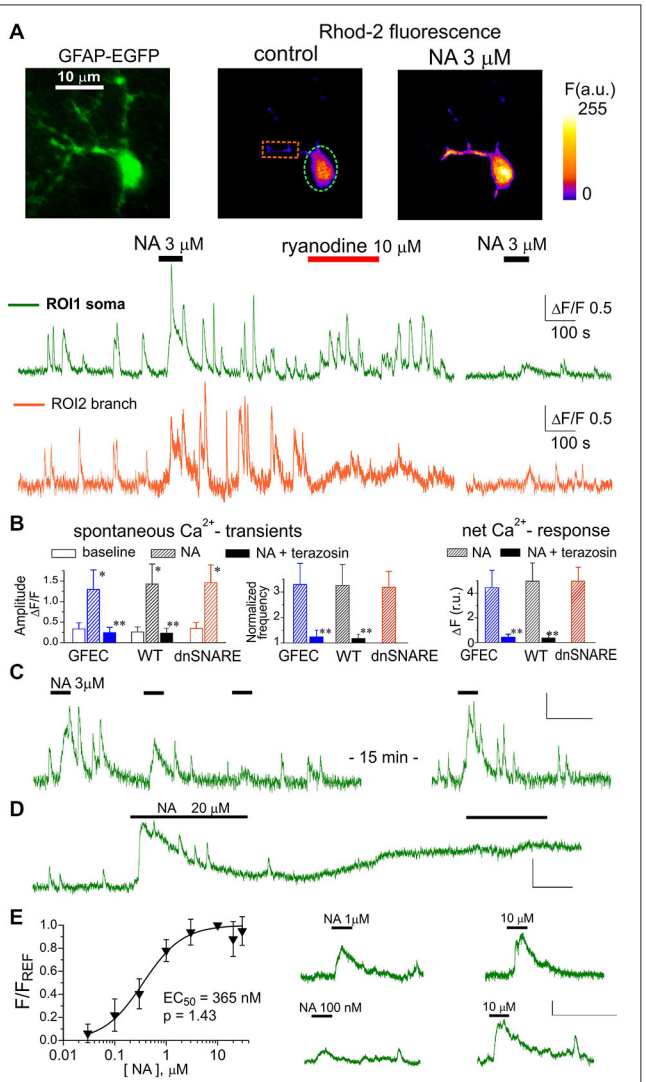

FIGURE 1 | α1-adrenoreceptors contribute to Ca^{2+} signaling in cortical astrocytes. (A) Representative multi-photon images of enhanced green fluorescent protein (EGFP) fluorescence and presudo-color images of Rhod-2 fluorescence recorded in the astrocytes from glial fibrillary acidic protein (GFAP)-EGFP (GFEC) mouse before (control) and after the application of noradrenaline (NA). Graphs below show the time course of Rhod-2 fluorescence averaged over regions indicated in fluorescence images. Note the marked spontaneous elevations in the Ca^{2+} level, which were enhanced by application of NA. Note the significant NA-activated response in the astrocytic branch and inhibition of response to NA 10 min after application of ryanodine. **(B)** The pooled data on peak amplitude and frequency of spontaneous Ca^{2+}-transients and the net response to the NA recorded in astrocytes of different mice strains in control and in the presence of α1-AR antagonist terazosin (30 nM). Frequency of spontaneous transients (middle graph) was measured within 3 min after application of NA and was normalized to baseline value. Net response was evaluated as an integral Ca^{2+}-signal measured during 3 min after NA application, averaged over the whole cell image and normalized to the integral Ca^{2+} signal measured during 3 min before NA application. Data are shown as mean ± SD for seven cells in control (for each strain) and six cells under terazosin (WT and GFEC). In the each strain, asterisks (*) indicate statistical significance of effect of NA on the peak amplitude of Ca^{2+}-transients in comparison to the corresponding baseline value, double asterisks (**) indicate significance of inhibitory effect of terazosin

(*Continued*)

FIGURE 1 | Continued
in comparison to the effect of NA alone; $P < 0.01$ given by *t*-test in both cases. **(C)** repetitive application of NA (3 μM) with 5 min interval causes the desensitization of the response; **(D)** prolonged application of 20 μM NA leads to the elevated Ca^{2+} level and non-responsiveness of neocortical astrocytes; **(E)** the concentration-dependence of net Ca^{2+}-transients evoked by NA in cortical astrocytes was assessed as described in the *Methods*; each point show mean ± SD for 4–5 cells. Fluorescent signals shown in panels **(C–E)** were integrated over the cell somata; all scale bars are ΔF 0.5 and 200 s.

Rather surprisingly, NA-evoked responses in cortical astrocytes were sensitive to ryanodine. Application of 10 μM ryanodine caused an initial augmentation of spontaneous Ca^{2+}-signaling, mainly in the soma, which lasted typically for 5–10 min and was followed by an irreversible inhibition of spontaneous transients (**Figure 1A**). Such an action is typical for ryanodine modulation of calcium-induced calcium release (CICR) mechanism (Zucchi and Ronca-Testoni, 1997). When NA was applied after cessation of spontaneous signaling (10–15 min after ryanodine) it produced a much reduced Ca^{2+} response in all seven astrocytes tested (**Figures 1A,B**). These results suggest the significant role of ryanodine receptor-mediated CICR in the amplification of astroglial Ca^{2+} signaling.

The α1-ARs of neocortical astrocytes showed rather high sensitivity; they could be activated by NA in sub-micromolar range (**Figure 1D**). The EC_{50} for NA-activated net Ca^{2+} response was 365 ± 26 nM (n = 18, WT mice) with a Hill coefficient of 1.43. The EC_{50} for the specific α1-agonist A61603 was 18.9 ± 5.1 nM (n = 13) with a Hill coefficient of 1.45. Large values for Hill coefficients are, very likely, related to the amplification of the responses to higher concentrations by CICR mechanism.

Importantly, we did not observe any significant contribution of α1-AR to neuronal signaling. The amplitude of responses evoked in pyramidal neurons by 3 μM NA was much smaller than the amplitude of glutamate-evoked response (**Figures 2A,B**). The small NA-evoked response had slower kinetics and started with considerable delay which argues against its origin from direct activation of neuronal α1-ARs. Instead, the neuronal response may have originated from some gliotransmitters released upon activation of glial α1-AR. Furthermore, neither NA nor terazosin exhibited significant effects on fEPSPs in the neocortex (**Figure 2C**). The application of NA caused a small increase in the slope and paired-pulse ratio of fEPSPs whereas terazosin decreased these parameters. In all cases, the difference from the control was not significant (paired *t*-test, n = 12 for NA and 11 for terazosin). To verify the specificity of action of NA and terazosin, we applied these drugs to acutely-isolated neocortical neurons which were devoid of the influence of glial cells (**Figure 2D**). We used a technique of non-enzymatic vibro-dissociation which allows functional synapses to be maintained on the membrane of isolated neurons, which can be verified by staining with FM1–43 and the presence of miniature spontaneous synaptic currents (Duguid et al., 2007; Rasooli-Nejad et al., 2014). We recorded whole-cell currents in acutely-dissociated neocortical pyramidal neurons at membrane potential of −80 mV. The glutamatergic miniature excitatory postsynaptic currents (mEPSCs) were recorded in the

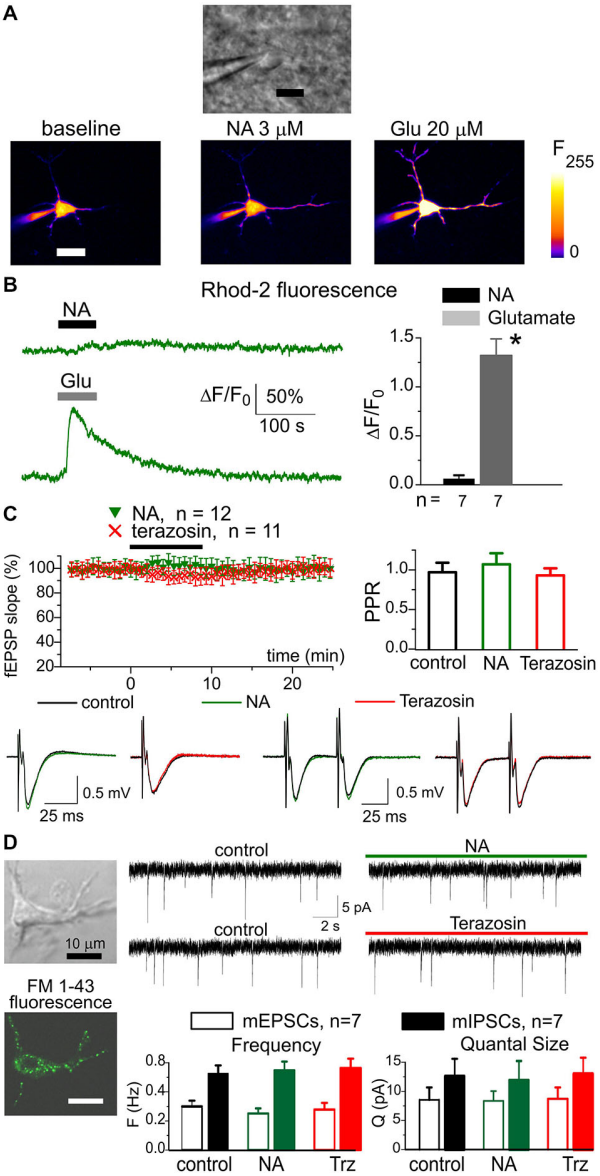

FIGURE 2 | Noradrenaline did not cause notable response in neocortical neurons. The pyramidal neurons of somatosensory cortex layer 2/3 of wild-type (WT) mice were loaded with Ca^{2+}-indicator Rhod-2 via patch-pipette. Ca^{2+}-signals were evoked in neurons by 60 s-long rapid bath application of 3 μM NA and 100 μM L-Glutamate to cortical slices. The membrane holding potential during Ca^{2+}-measurements was −40 mV **(A)**, the representative gradient-contrast image and pseudo-color fluorescent images recorded at rest and at the peak of Ca^{2+}- responses to NA **(B)**, *left* the representative Ca^{2+}-transients evoked in the neuron of dn-SNARE mouse by application of NA and glutamate. *Right*, the diagram shows the pooled data (mean ± SD for number of neurons indicated) of peak Ca^{2+}-elevation; the difference between NA and glutamate-evoked response was statistically significant with (*) $P < 0.005$ (paired *t*-test). **(C)** Application of NA and terazosin do not have notable effect on the field excitatory synaptic potentials (fEPSPs) in the neocortical layer 2/3. Upper graphs show time course of fEPSP slope during application of 3 μM NA and 30 nM terazosin and paired pulse ratio in the control and 8 min after the drugs. The graphs below show the representative fEPSPs (average of 10 waveforms). Each dot in the time course shows the average slope of fEPSPs recorded in 1 min time window and

(*Continued*)

FIGURE 2 | Continued
normalized to the control; data are presented as mean ± SD for the number of experiments indicated. **(D)** Whole-cell currents were recorded in the isolated neuron retaining functional synaptic boutons. To verify the presence of functional synapses, the cell was pre-incubated with 3 μM FM1–43 for 15 min, then dye was washed out for 15 min. The spontaneous excitatory currents (mEPSCs) were recorded at −80 mV in the presence of picrotoxin (100 μM); the inhibitory mIPSCs were recorded in the presence of NBQX (30 μM). *Left* column: representative fluorescent and gradient contrast image of neuron showing punctate staining with FM1–43. *Right*: the representative mEPSCs before and after application of NA and Terazosin as indicated in the graphs. *Below*, the diagram shows the frequency and quantal amplitude of mEPSCs and mIPSCs pooled for seven neurons. Note that application of NA and terazosin did not change the frequency and amplitude of synaptic currents. These data demonstrate specificity of NA action in the neocortex in respect to the astrocytic and neuronal signaling.

presence of 100 μM picrotoxin and 20 μM PPADS; the GABA-mediated inhibitory currents were recorded in the presence of 30 μM NBQX and 20 μM PPADS. The application of NA and terazosin did not cause notable changes in the amplitude and frequency of mEPSCs and miniature inhibitory postsynaptic currents (mIPSCs; seven isolated neurons were tested in the each case).

Combined, our results show that α1-ARs can make a substantial contribution to Ca^{2+}-signaling in neocortical astrocytes. Importantly, our data show that expression of dnSNARE protein in astrocytes did not affect adrenergic signaling.

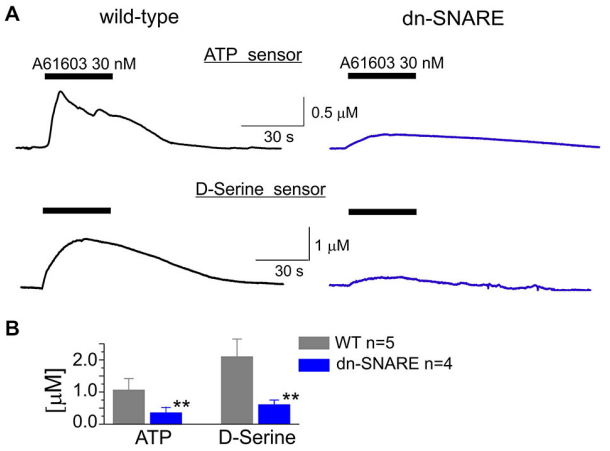

FIGURE 3 | Adrenoreceptor-activated release of ATP and D-serine in the neocortical slices. (A) The representative responses of cortical slices of WT and dn-SNARE mice to the application of selective α1-AR agonist A61603 (100 nM) were recorded using microelectrode sensors to ATP and D-serine placed in the layer II/III (see Methods). The data are shown as an elevation relative to the resting concentration. **(B)** The pooled data on the peak magnitude of ATP- and D-serine transients evoked by application of A61603; data shown as mean ± SD for number of experiments indicated. Asterisks (**) indicate statistical significance of difference in the magnitude of ATP- and D-serine responses between WT and dn-SNARE mice, $P < 0.01$ (*t*-test). The significant reduction in the NA-evoked responses in the cortical slices from dn-SNARE mice strongly supports the vesicular mechanism of ATP and D-serine release from astrocytes.

Glial Adrenoceptors Induce the Release of ATP and D-serine from Neocortical Astrocytes

To investigate the release of gliotransmitters which could plausibly follow the activation of α1Rs in astrocytes, we used microelectrode biosensors to monitor the concentration of ATP and D-serine in neocortical tissue. This technique was used previously for evaluation of transmitter release in several brain areas (Frenguelli et al., 2007; Rasooli-Nejad et al., 2014). Since NA can be directly oxidized by the sensors and thus generate a measurable sensor current, we used the specific α1-AR agonist A61603. Activation of intracellular Ca^{2+} in astrocytes by A61603 (100 nM) induced a significant increase in the levels of extracellular ATP and D-serine in the cortical tissues of WT mice (**Figure 3A**).

The α1-AR-mediated elevation of extracellular ATP and D-serine reached 1.1 ± 0.4 μM and 2.1 ± 0.7 μM, respectively (**Figure 3**). In comparison to the WT littermates, the amplitudes of ATP and D-serine transients were reduced in the dn-SNARE-expressing mice by 69 ± 19% (n = 4) and 72 ± 14% (n = 4), respectively (**Figures 3A,B**). This result suggested the astroglial origin and vesicular nature of adrenoceptor-activated release of ATP and D-serine release. It is worth noting that one could not expect a complete inhibition of astroglial exocytosis in the neocortex of dn-SNARE mice since a proportion of astrocytes do not express dn-SNARE (Pascual et al., 2005). Also, there are non-vesicular pathways of gliotransmitter release which might contribute to the adrenoreceptors-triggered response (Hamilton and Attwell, 2010; Montero and Orellana, 2015).

In the next series of experiments we tried to directly verify that astroglial α1-ARs contribute to triggering Ca^{2+}-dependent exocytosis. We previously demonstrated that pyramidal neocortical neurons express functional P2X receptors (Pankratov et al., 2007) and these receptors can be activated by ATP released from astrocytes (Lalo et al., 2014a). Thus, pyramidal neurons can be used as a native sensor for extracellular ATP. We recorded whole-cell currents in neocortical pyramidal neurons at a membrane potential of −80 mV in the presence of DNQX (30 μM), D-APV (30 μM) and picrotoxin (100 μM). Similar to our previous experiments (Pankratov et al., 2007; Lalo et al., 2014a), we observed residual non-glutamatergic miniature spontaneous synaptic currents (**Figure 4**). These non-glutamatergic excitatory spontaneous currents (mEPSCs) were completely abolished by application of specific P2X receptor antagonists PPADS (10 μM) and 5-BDBD (5 μM) in all seven neurons tested (data not shown). Based on these data, as well as our previous work (Pankratov et al., 2007; Lalo et al., 2014a), the phasic inward currents observed in cortical neurons in the presence of glutamatergic and GABAergic antagonists can be confidently attributed to ATP receptors. In order to inhibit astroglial exocytosis, we perfused individual astrocytes with intracellular solution containing 3 nM Tetanus neurotoxin (TeNTx) and 30 μM of the calcium indicator Calcium Green-2 (**Figure 4A**) and recorded mEPSCs in a neighboring neuron (lying within 30 μm distance from the perfused astrocyte). Perfusion of astrocytes with solution containing only Calcium Green-2

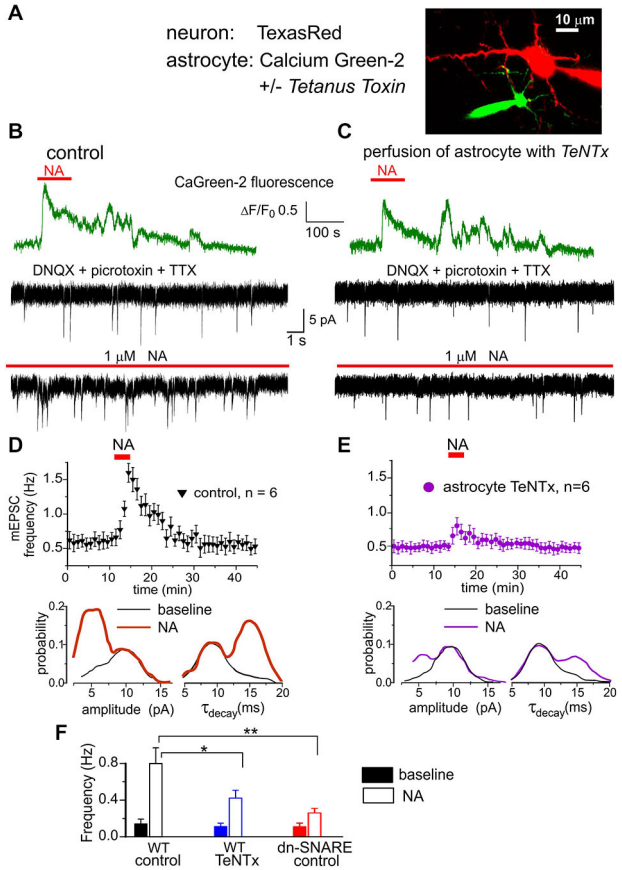

FIGURE 4 | Astrocyte-derived ATP activates purinergic receptors in the neocortical neurons. **(A)** Whole-cell currents were recorded in the pyramidal neuron of layer 2/3 simultaneously with perfusion of astrocyte with intracellular solution containing either fluorescent dye Ca-Green-2 alone or Ca-Green-2 and 3 nM of Tetanus neurotoxin light chain. **(B,D)** Activation of astrocytic Ca^{2+} signaling elicited the burst of purinergic currents in the neuron. **(B)** The astrocytic Ca^{2+} transients (green) evoked by application of 1 μM NA and neuronal whole-cell currents (black) recorded in the presence of picrotoxin, DNQX and TTX at −80 mV. Spontaneous currents recorded in these conditions are mediated by the P2X receptors. Upper and lower trace show currents recorded correspondingly before and 1 min after NA application. Note the appearance of the large number of spontaneous currents after NA. **(D)** *Upper graph*: each dot shows the average frequency of spontaneous purinergic currents recorded in 1 min time window in the pyramidal neurons; data are presented as mean ± SD for six neurons. *Lower graphs* show the amplitude and decay time distributions for the purinergic currents recoded before (baseline) and 1–3 min after application of NA. Application of NA elicited the burst of purinergic currents which had slower kinetics and smaller quantal size than currents recorded in the baseline condions. **(C,E)** Perfusion of astrocyte with TeNTx inhibited the burst of purinergic currents verifying their origin from astrocytic exocytosis. **(F)** Diagram shows the frequency of slow purinergic spontaneous currents in the neurons of WT and dn-SNARE mice averaged within 3 min time window before (baseline) and after application of NA in control and during perfusion of astrocytes with TeNTx. Data are shown as mean ± SD for the six neurons. The statistical significance of difference from the WT control values was as indicated (*) $P < 0.05$ and (**) $P < 0.01$ (unpaired *t*-test).

was used as a control. The electrophysiological recordings in neurons started 10–15 min after perfusion of astrocytes with TeNTx.

The purinergic mEPSCs recorded under control conditions had an average amplitude of 8.4 ± 2.5 pA and an average decay time of 9.6 ± 2.6 ms. Application of NA (2 μM) elicited Ca^{2+}-elevation in astrocytes and caused a dramatic increase in the frequency of purinergic mEPSCs in all seven neurons tested (**Figure 4B**). The burst of purinergic currents was accompanied by a decrease in the average amplitude to 6.7 ± 1.7 pA, and an increase in the decay time to 13.3 ± 3.5 ms (n = 7). Such behavior was similar to the previously observed burst of purinergic activity activated by astroglial PAR-1 receptors (Lalo et al., 2014a). When astrocytes were perfused with TeNTx, activation of α1-ARs caused much smaller burst of purinergic currents (**Figure 4C**).

Consistent with our previous reports (Lalo et al., 2014a), purinergic mEPSCs recorded in pyramidal neurons (**Figure 4D**) exhibited a bimodal amplitude distribution with peaks at 5.8 ± 1.5 pA and 9.8 ± 2.4 pA (n = 7). The distributions of mEPSCs decay time in these neurons had peaks at 9.1 ± 1.1 ms and 15.2 ± 2.1 ms. Previously, we demonstrated that purinergic mEPSCs of smaller amplitude and slower kinetics originated from the vesicular release of ATP from astrocytes (Lalo et al., 2014a; Rasooli-Nejad et al., 2014). Application of NA dramatically increased the proportion of these smaller and slower currents (**Figure 4E**). Perfusion of astrocytes with TeNTx selectively decreased the frequency of slower purinergic mEPSCs, both in control conditions and after application of NA (**Figure 4F**). These results strongly suggest that smaller and slower purinergic mEPSCs originated directly from vesicular release of ATP from neighboring astrocytes. Incomplete inhibition of the slower mEPSCs can be explained by release from other astrocytes not exposed to TeNTx. NA-evoked bursts of purinergic mEPSCs were significantly inhibited in the dn-SNARE mice, supporting its astroglial origin (**Figure 4F**).

Astroglial α1 Adrenoceptors Modulate Long-term Plasticity in the Neocortex

The above data provide strong evidence that astroglial α1-adrenoreceptors can trigger exocytosis of gliotransmitters, in particular ATP and D-serine (**Figures 3**, **4**). ATP and D-serine have been previously shown to regulate synaptic plasticity in the hippocampus (Pascual et al., 2005; Henneberger et al., 2010) and the neocortex (Lalo et al., 2014b; Rasooli-Nejad et al., 2014). We have shown previously that astrocyte-derived ATP can down-regulate phasic and tonic GABAergic transmission in neocortical neurons. This down-regulation, in synergy with the release of D-serine, can facilitate the induction of LTP (Lalo et al., 2014b; Rasooli-Nejad et al., 2014). Thus, astroglial α1-AR-mediated release of ATP might affect the induction of LTP in neocortical neurons.

We investigated the potentiation of fEPSPsin layer II/III of somatosensory cortex of WT and dn-SNARE mice. The fEPSPs were evoked by the stimulation of neuronal afferents descending from layers IV–V. Potentiation of fEPSPs was induced by theta-burst stimulation. In slices from WT mice, five episodes of theta-burst stimulation (5 TBS) induced robust LTP in all 20 experiments (**Figure 5A**). Application of α1-AR antagonist terazosin inhibited the induction of LTP, suggesting

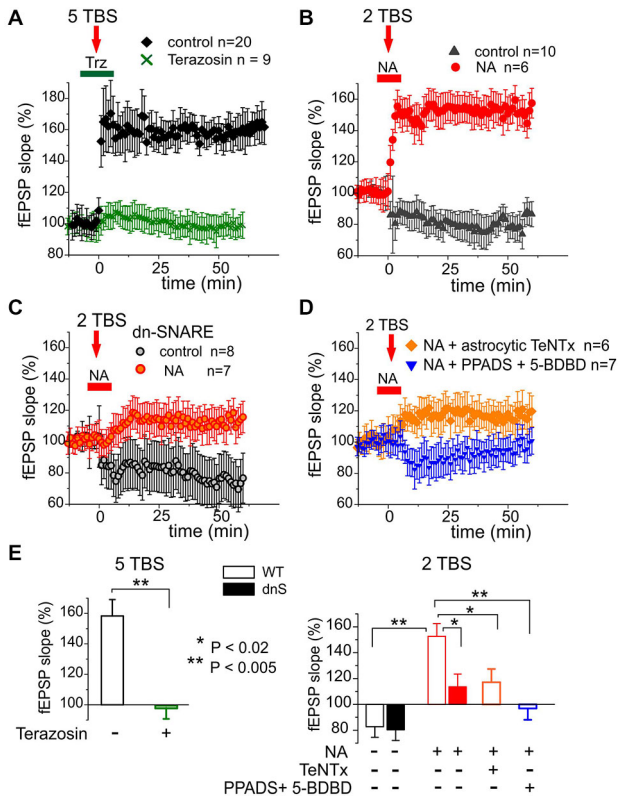

FIGURE 5 | Release of gliotransmitters and glial α1-ARs are essential for long-term potentiation (LTP) in the neocortex. (A) LTP was induced in the neocortex of the WT mice by five Θ-bursts of high-frequency stimulation. The magnitude of LTP was significantly reduced by specific antagonist α1-NRs terazosin (30 nM). **(B)** Delivering just two Θ-bursts did not induce LTP in the control but was able to induce LTP when NA (1 μM) was applied during induction as indicated in the graph. **(C)** Application of NA did not facilitate LTP induction in the dn-SNARE mice suggesting the importance of glial exocytosis for the action of NA. **(D)** Effect of NA was decreased by perfusion of astrocyte with TeNTx (similarly to the experiment shown in the **Figure 4**) or by inhibition of neuronal ATP receptors with PPADS and 5-BDBD. **(E)** Pooled data on the magnitude of LTP evaluated as relative increase in the fEPSP slope at 60th min, averaged across 10 min time window. Each data point shows mean ± SD for the number of experiments indicated in **(A–D)**. Asterisks (*, **) indicate statistical significance of difference in the LTP magnitude WT control values (unpaired *t*-test). These data suggest the importance of NA-triggered release of ATP for glial modulation of LTP in the neocortex.

the importance of glial adrenergic signaling. This notion was strongly supported by experiments on the induction of LTP by weaker stimulation (**Figures 5B–D**). In control conditions, two theta-burst episodes (2 TBS) induced a mild long-term depression rather than potentiation (**Figure 4B**). The threshold for LTP induction was three theta-bursts (n = 12, data not shown). Application of 1 μM NA enabled the induction of LTP with sub-threshold stimulation (2 TBS). Washout of NA 3 min after TBS did not affect the potentiation suggesting the importance of additional NA-mediated activation of astrocytes in the initial period of LTP induction. As mentioned above (**Figure 2C**), application of NA without TBS did not produce marked potentiation (n = 12).

Importantly, the NA-induced facilitation of LTP was significantly reduced in the dn-SNARE mice (**Figures 5C,E**). In addition, the magnitude of LTP induced by 2 TBS in presence of NA considerably decreased when fEPSPs were recorded in the vicinity of astrocytes perfused with TeNTx (similarly to the experiment with purinergic mEPSCs shown in the **Figure 4**). The facilitatory effect of NA was abolished by selective inhibition of P2X receptors with 10 μM PPADS and 5 μM 5-BDBD (**Figures 5D,E**). This result demonstrates the importance of NA-triggered ATP release and activation of ATP receptors for the facilitation of LTP. Combined, our data imply that astroglial α1-ARs contribute to the regulation of exocytosis of gliotransmitters and are important for the synaptic plasticity in the neocortex.

Discussion

Our data have shown that α1-ARs participate in Ca^{2+}-signaling in neocortical astrocytes (**Figure 1**) but do not have a strong direct action in neurons (**Figure 2**). We also demonstrate that astrocytic α1-ARs can activate the release of ATP and D-serine from astrocytes (**Figure 3**). Moreover, we have found that adrenoceptor-activated release of ATP from astrocytes can directly activate postsynaptic P2X receptors in neocortical neurons (**Figure 4**) and this cascade is involved in astroglial modulation of long-term synaptic plasticity in the neocortex (**Figure 5**).

Functional Properties of Astroglial α1-Adrenoreceptors

Our data on the substantial contribution of α1-ARs to Ca^{2+}-signaling in neocortical astrocytes agree with previous observations (Ding et al., 2013; Paukert et al., 2014). Moreover, our data reveal specific features of adrenergic signals in cortical astrocytes that have been thus far largely overlooked. Firstly, α1-ARs in cortical astrocytes are very sensitive to NA with an EC_{50} of ∼400 nM (**Figure 1**). Secondly, adrenergic signals in cortical astrocytes undergo amplification by a ryanodine-sensitive Ca^{2+}-induced Ca^{2+}-release mechanism, in particularly in astrocytic processes (**Figure 1A**). Nevertheless, application of NA at concentrations greater than 10 μM leads to a long-lasting period of elevated Ca^{2+} and non-responsiveness of astrocytes to subsequent NA (**Figure 1B**).

The vulnerability of α1AR-mediated responses to desensitization suggests the existence of finely-tuned molecular mechanisms preventing the consequences of their overstimulation, likely to avoid Ca^{2+}-overload, which can be very damaging for cells, including astrocytes. Our finding of desensitization of α1-ARs in astrocytes is in line with data obtained previously in other cell types (Akinaga et al., 2013; Jiang et al., 2013). The apparent desensitization of NA-activated responses upon prolonged exposure to an agonist usually originates from their rapid internalization (Mohan et al., 2013; Akinaga et al., 2013). The common molecular mechanisms of adrenoceptor (as well as many other GPCRs) internalization involve beta-arrestins (Mohan et al., 2013) which have been recently implicated in neurodegenerative diseases (Jiang et al., 2013). The, α1-AR-specific internalization cascade involves PKC,

which can be activated by elevations in cytosolic Ca^{2+} (Akinaga et al., 2013). Interestingly, the internalization of α1-ARs can also be influenced by their association with the cytoskeleton and lipid rafts (Morris et al., 2008; Akinaga et al., 2013), which can be linked to other astrocytic receptors, for instance P2X1 purinoceptors (Allsopp et al., 2010; Lalo et al., 2011b). The possibility of the activation of purinoceptors by autocrine release of ATP from astrocytes might provide feedback to adrenergic signaling. One might suggest the existence of a variety of mechanisms regulating desensitization and internalization of astrocytic α1-ARs thus underlying an activity-dependent plasticity of astroglial signaling. The study of the molecular mechanisms of desensitization of astrocytic α1-ARa lies beyond the scope of the present work, but this topic surely is of high interest and importance. The plasticity of adrenergic signaling in astrocytes may be very important for glia-neuron interaction and is worth exploring further.

Astroglial Adrenoreceptors and the Exocytosis of Gliotransmitters

Our data suggest that α1-ARs can trigger the exocytosis of gliotransmitters from neocortical astrocytes (**Figures 3**, **4**). The NA-triggered release of ATP exhibited the same functional properties as release activated by astroglial PAR-1 or CB1 receptors (Lalo et al., 2014a; Rasooli-Nejad et al., 2014). We would like to emphasize that the evidence for a role of vesicular gliotransmitter release in astrocyte-neuron interactions, presented in this study, were obtained mainly using intracellular perfusion of individual astrocytes with Tetanus Toxin light chain (**Figures 4**, **5**). The results obtained by this approach are in a very good agreement with our previous observations of a decrease in the release of ATP and D-serine in dnSNARE mice in which astrocytic vesicular release is compromised (Pascual et al., 2005; Lalo et al., 2014a; Rasooli-Nejad et al., 2014), our data obtained using perfusion of astrocytes with inhibitors of vesicular ATP transporters (Lalo et al., 2014a) and the results obtained using intracellular perfusion with Ca^{2+}-chelators (Henneberger et al., 2010). There is also an independent biochemical evidence of storage of D-serine in the synaptic vesicle-like structure in astrocytes (Martineau et al., 2013).

It is worth noting that most of the groups using dn-SNARE mice did not observe any evidence of neuronal expression of the dnSNARE transgene (Pascual et al., 2005; Halassa et al., 2009; Lalo et al., 2014a). Only one study suggested the possibility of neuronal expression of the dnSNARE transgene (Fujita et al., 2014), but it did not provide any evidence for an impairment of neurotransmitter release. Hypothetical neuronal expression of the dn-SNARE transgene should result in a notable deficit in synaptic transmission arising from impaired exocytosis of neurotransmitters. On contrary, there is evidence of up-regulated excitatory (Pascual et al., 2005) and inhibitory (Lalo et al., 2014a) synaptic transmission in the dnSNARE mice. The main deficits that we observed in the dnSNARE mice were in the effects caused by selective activation of Ca^{2+}-signaling in the astrocytes via PAR-1 (Lalo et al., 2014a), CB1 (Rasooli-Nejad et al., 2014) or α1-ARs (**Figures 3**, **4E**, **5C**). Combined, these results strongly support the physiological importance of vesicular exocytosis from glia and verify the validity of using dn-SNARE mice as a tool to explore astrocyte-neuronal interactions.

The capability of astrocytes to release gliotransmitters is a core element of glia-neuron communication (Halassa and Haydon, 2010; Hamilton and Attwell, 2010). We have shown previously that astrocyte-derived ATP down-regulates inhibitory transmission in pyramidal neurons via phosphorylation of $GABA_A$ receptors and thereby can facilitate the induction of long-term synaptic plasticity (LTP) in the neocortex (Lalo et al., 2014b; Rasooli-Nejad et al., 2014). As a source of Ca^{2+}-elevation to trigger exocytosis in physiological conditions, our previous data suggested a role for astrocytic NMDA, mGluR and CB1 receptors (Lalo et al., 2014a; Rasooli-Nejad et al., 2014). Our present data highlight the importance of α1-ARs for glial control of synaptic plasticity (**Figure 5**). The role for adrenergic astroglial signaling may be strengthened by the amplification via ryanodine receptor-mediated Ca^{2+}-induced Ca^{2+}-release (**Figure 1**).

Putative Role for Adrenergic Astroglial Signaling in Metaplasticity

There is growing recognition that information processing in the brain is coordinated by neuronal-glial networks (Halassa and Haydon, 2010; Hulme et al., 2013b; Araque et al., 2014). A key element of this coordination is the ability of astrocytes to integrate neuronal activity over a large spatial domain by virtue of high-affinity receptors (Araque et al., 2014). In comparison to the interaction between neurons, astroglial modulation of synaptic transmission gains many peculiar features, such as slower time-scale, but greater spatial-scale and a dependence upon the combined activity of numerous synapses (Araque et al., 2014). These features can render astrocytes particularly important in heterosynaptic metaplasticity (Panatier et al., 2011; Min and Nevian, 2012; Hulme et al., 2013b).

Contrary to the "classic" release of neurotransmitters into the synaptic cleft, the release of NA occurs from varicosities of adrenergic neurons into brain extracellular fluid, remotely from target cells, so the effective concentration of NA can be rather low. Cortical astrocytes, by virtue of highly-sensitive α1-ARs, are strategically positioned to receive and integrate diffuse adrenergic input from remote adrenergic neurons and pass the information to the local network. Indeed, adrenergic signaling has been shown to modulate the activity of astrocyte networks according to the behavioral state or sensory inputs (Ding et al., 2013; Paukert et al., 2014). Our data suggest that astroglial α1-ARs can affect the induction of long-term changes in synaptic strength (**Figure 5**). Thus, astroglial adrenoceptors may be of particular importance for brain metaplasticity induced by experience, environmental factors or neurodegenerative disease (Nithianantharajah and Hannan, 2006; Hulme et al., 2013a).

Specificity of Astroglial Adrenergic Signaling

The ability of adrenoceptors to activate Ca^{2+}-signaling in astrocytes encouraged us to use NA for triggering gliotransmission (Gordon et al., 2009; Pougnet et al., 2014).

This raises a question as to whether the action of NA is astroglia-specific. Our data show that principal neocortical neurons do not produce Ca^{2+}-transients in response to application of NA (**Figures 2A,B**). The application of NA produced a small effect on excitatory synaptic potentials (**Figure 2C**), which could possibly be attributed to the modulatory action of gliotransmitters. This notion is strongly supported by the lack of any notable effect of NA on synaptic currents in acutely-isolated neurons that are devoid of astrocyte influence (**Figure 2D**). In contrast, NA-elicited phasic purinergic currents and NA-elicited enhancement of LTP were significantly reduced by selective inhibition of astrocytic exocytosis (**Figures 4**, **5**). Thus, the observed effects of NA and terazosin on LTP can hardly be related to neuronal adrenoreceptors. Our data strongly suggest that astrocytes provide a major contribution to adrenergic modulation of synaptic transmission and plasticity in the neocortex.

Other types of glial cells, in particular microglia, can also express adrenoceptors (Gyoneva and Traynelis, 2013; Butt et al., 2014). Microglia and oligodendocytes can also release ATP and other gliotransmitters molecules, but mainly via hemichannels rather than exocytosis (Butt, 2011; Montero and Orellana, 2015). Most of the observations of microglial release of ATP were made in a pathological context, e.g., neuroinflammation or ischemia (Butt, 2011; Montero and Orellana, 2015). Hemichannel-mediated pathways of ATP release do not agree with the fast kinetics and quantal behavior of the NA-elicited purinegic currents we observe in neurons (**Figure 4**). Furthermore, microglial cells were reported to express β2-rather than α1-adrenoceptors (Gyoneva and Traynelis, 2013; Butt et al., 2014). This argues against their significant contribution to the regulation of neocortical LTP which is sensitive to the α1-AR antagonist terazosin (**Figure 5**). Still, one cannot *a priori* expect absolute astroglial selectivity of NA's action: the role for microglia and oligodendrocytes in adrenergic modulation of synaptic transmission are yet to be established.

Our observation of a lack of direct NA action on synaptic potentials contrasts with the results obtained in the hippocampus, where NA caused a depression of fEPSPs and accelerated the hypoxic depression of synaptic transmission (Pearson and Frenguelli, 2004). Most likely, the adrenergic modulation of synaptic transmission in the hippocampus occurred via α2-AR-mediated presynaptic inhibition and β1-AR- activated increase in extracellular adenosine (Pearson and Frenguelli, 2004). It remains unclear whether glial adrenoreceptors can contribute to these mechanisms, e.g., by activating the release of ATP/adenosine. It is worth noting that recent work by Pougnet et al. (2014) also reported NA-induced synaptic depression in hippocampal neurons but suggested a different mechanism, involving α1-AR-activated release of ATP from astrocytes and activation of postsynaptic purinoreceptors causing Ca^{2+}-dependent internalization of AMPA receptors. Thus, adrenergic modulation of synaptic transmission can involve multiple mechanisms and show significant regional differences. In each particular case, special care is needed to distinguish the putative involvement of glial and post- and presynaptic neuronal adrenoreceptors.

To conclude, our results strongly support the physiological importance of astroglial adrenergic signaling and the astrocytic exocytosis of gliotransmitters. This adrenoreceptor-mediated communication between astrocytes and neurons is necessary for the regulation of synaptic strength and the modulation of synaptic plasticity.

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Astroglial calcium signaling displays short-term plasticity and adjusts synaptic efficacy

Jérémie Sibille[1,2], *Jonathan Zapata*[1], *Jérémie Teillon*[1] *and Nathalie Rouach*[1]*

[1] Neuroglial Interactions in Cerebral Physiopathology, Center for Interdisciplinary Research in Biology, Collège de France, CNRS UMR 7241, INSERM U1050, Paris, France, [2] Université Paris Diderot, Sorbonne Paris Cité, Paris, France

Astrocytes are dynamic signaling brain elements able to sense neuronal inputs and to respond by complex calcium signals, which are thought to represent their excitability. Such signaling has been proposed to modulate, or not, neuronal activities ranging from basal synaptic transmission to epileptiform discharges. However, whether calcium signaling in astrocytes exhibits activity-dependent changes and acutely modulates short-term synaptic plasticity is currently unclear. We here show, using dual recordings of astroglial calcium signals and synaptic transmission, that calcium signaling in astrocytes displays, concomitantly to excitatory synapses, short-term plasticity in response to prolonged repetitive and tetanic stimulations of Schaffer collaterals. We also found that acute inhibition of calcium signaling in astrocytes by intracellular calcium chelation rapidly potentiates excitatory synaptic transmission and short-term plasticity of Shaffer collateral CA1 synapses, i.e., paired-pulse facilitation and responses to tetanic and prolonged repetitive stimulation. These data reveal that calcium signaling of astrocytes is plastic and down-regulates basal transmission and short-term plasticity of hippocampal CA1 glutamatergic synapses.

Keywords: hippocampus, glia, neurons, neuroglial interactions, calcium signals, synapses, synaptic transmission, short-term plasticity

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***Correspondence:**
Nathalie Rouach, Neuroglial Interactions in Cerebral Physiopathology, Center for Interdisciplinary Research in Biology, Collège de France, CNRS UMR 7241, INSERM U1050, 11 place Marcelin Berthelot, Paris 75005, France
nathalie.rouach@college-de-france.fr

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Introduction

Astrocytes are dynamic signaling elements able to sense, integrate and respond to synaptic activity. They are indeed equipped with a variety of channels, receptors and transporters allowing for detection of neuronal activity. They can also transmit information, in part by complex calcium signaling, to regulate in turn neurotransmission through multiple pathways (Perea et al., 2009; Pannasch et al., 2011; Rusakov et al., 2011; Dallérac et al., 2013; Bernardinelli et al., 2014).

Remarkably, astrocytes not only respond transiently to neuronal activity but can also display, like neurons, several forms of plasticity (Pirttimaki and Parri, 2013). These include a morphological plasticity of neuronal coverage during specific physiological conditions such as lactation or whisker stimulation (Iino et al., 2001; Genoud et al., 2006; Oliet and Bonfardin, 2010; Saab et al., 2012; Bernardinelli et al., 2014; Perez-Alvarez et al., 2014), as well as a functional plasticity of neuronal-induced currents or gliotransmitter release. For instance in the cerebellum, AMPAR-mediated calcium currents induced in Bergmann glia by stimulation of parallel fibers show specific activity-dependent short-term and long-term plasticity compared to adjacent Purkinje neurons (Bellamy and Ogden, 2005, 2006). Similarly in the hippocampus, astroglial potassium and glutamate uptake exhibit specific patterns of short-term plasticity (Manita et al., 2007; Sibille et al., 2014), as well as

long term potentiation (Pita-Almenar et al., 2006; Ge and Duan, 2007; Zhang et al., 2009). Finally in the thalamus, astroglial glutamate release displays long term potentiation in response to cortical or lemniscal afferent stimulation (Pirttimaki et al., 2011). Such morphological and functional plasticity of astrocytes has been shown in various physiological contexts to regulate neuronal activity (Iino et al., 2001; Oliet et al., 2001; Pirttimaki et al., 2011; Saab et al., 2012; Sibille et al., 2014).

Among the responses evoked in astrocytes by neuronal activity, calcium signaling has been extensively studied and is thought to represent their excitability, since these cells are electrically non excitable. Astroglial calcium signaling can be mediated by multiple pathways, thought to generate signals with specific temporal and spatial patterns (Verkhratsky et al., 2012, 2014; Khakh and McCarthy, 2015). Numerous studies have shown that calcium release from endoplasmic reticulum internal stores via inositol triphosphate (IP3) receptors occurs after activation of plasmalemmal G-protein coupled receptors, such as metabotropic glutamate receptors (mGluRs; Verkhratsky et al., 2012). Such pathway is thought to underlie relatively slow calcium signaling in astrocytes. Additionally, calcium can also enter from the extracellular space via activation of membrane store operated channels, sodium-calcium exchanger or ionotropic receptors, such as NMDA, P2X or TRPA1 receptors in astrocytes from specific brain regions. These pathways may rather mediate fast and local signaling, possibly in astroglial perisynaptic processes (Verkhratsky et al., 2014; Khakh and McCarthy, 2015).

Nevertheless, how astrocytes encode calcium signaling and whether such signaling also displays activity-dependent changes remains elusive. Neuronal activity has been shown to modulate the frequency of hippocampal astroglial calcium transients mediated by mGluR activation (Pasti et al., 1995, 1997). In addition, astrocytes from the hippocampus or the barrel cortex can distinguish and preferentially respond to specific excitatory inputs in a nonlinear manner (Perea and Araque, 2005; Schipke et al., 2008). Yet, although astroglial calcium signals do correlate with the number of activated synapses, they were found not to be altered shortly after induction of hippocampal long-term synaptic plasticity, when detected with Fluo-5F, a calcium indicator with low calcium binding affinity (Honsek et al., 2012). Thus unlike other glial cells types such as NG2 cells or Schwann cells, where calcium signaling plasticity has been established (Ge et al., 2006; Bélair et al., 2010), whether astrocytes also show activity-dependent plasticity of calcium signals is still currently unclear.

In addition, activity-dependent calcium transients can induce various responses in astrocytes, including release of gliotransmitters, which can in turn modulate neuronal activity (Araque et al., 2014). Astroglial calcium signaling has been reported to modulate different regimes of neuronal activity, ranging from basal synaptic transmission to epileptiform events (Perea et al., 2009; Nedergaard and Verkhratsky, 2012; Dallérac et al., 2013; Araque et al., 2014). Contradictory results have nevertheless emerged about its role in synaptic transmission and plasticity (Fiacco et al., 2007; Petravicz et al., 2008; Agulhon et al., 2010), likely due to the distinct experimental manipulations used to increase calcium in astrocytes (Nedergaard and Verkhratsky, 2012). In fact whether these manipulations relate to physiological events is still an open question. Thus the role of endogenous astrocytic calcium signaling in physiological neuronal activity is currently matter of debate.

We here show that astroglial calcium signaling displays, simultaneously to excitatory synapses, short-term plasticity in response to prolonged repetitive and tetanic stimulations of Schaffer collaterals. We also demonstrate that acute and local inhibition of astrocytic calcium signaling increases glutamatergic synaptic transmission and short-term plasticity of Shaffer collateral CA1 synapses. These results reveal that astrocytic calcium signaling is plastic and can dampen transmission and short-term plasticity of hippocampal CA1 excitatory synapses.

Material and Methods

Animals

Experiments were performed on the hippocampus of wild type mice (C57BL6/J). Experiments were carried out according to the guidelines of European Community Council Directives of January 1st 2013 (2010/63/EU) and our local animal committee (Center for Interdisciplinary Research in Biology in College de France). All efforts were made to minimize the number of used animals and their suffering. Experiments were performed on the hippocampus of wild type mice. For all analyses, mice of both genders were used (PN15-PN22).

Electrophysiology

Acute transverse hippocampal slices (400 μm) were prepared as previously described (Pannasch et al., 2012; Sibille et al., 2014) from 15 to 22 days-old mice. Slices were maintained in a storage chamber containing an artificial cerebrospinal fluid (ACSF) (containing in mM: 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$ 1.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$ and 11 glucose, saturated with 95% O_2-5% CO_2) for at least 1 h prior to recording. Slices were transferred to a submerged recording chamber mounted on an Olympus BX51WI microscope equipped for infra-red differential interference (IR-DIC) microscopy and were perfused with ACSF at a rate of 1.5 ml/min. Experiments were performed in the presence of picrotoxin (100 μM) and a cut was made between CA1 and CA3 to prevent the propagation of epileptiform activity. Extracellular field excitatory postsynaptic potentials (fEPSPs) and whole-cell patch-clamp recordings of astrocytes were performed in the CA1 *stratum radiatum* region of the hippocampus. The simultaneous recordings were carried out when intra-astroglial calcium chelation experiments (BAPTA, 10 mM) were made, and the field potential recording pipette, filled with ACSF, was placed in *stratum radiatum*, 20–50 μm away from the recorded astrocyte. Postsynaptic responses were evoked by stimulating Schaffer collaterals (0.1 Hz, stimulation intensity 10–20 μA) in CA1 *stratum radiatum* with ACSF filled glass pipettes. Paired-pulse facilitation was induced by delivery of two stimuli at an interval of 40 ms. Prolonged repetitive stimulation was performed at 10 Hz for 30 s, while post-tetanic potentiation was induced by stimulation at 100 Hz for 1 s in

the presence of 10 μM of CPP ((Rs)-3-(2-Carboxypiperazin-4-yl-)propyl-1-phosphonic acid), to prevent induction of long-term potentiation. To investigate the effect of astroglial calcium chelation on evoked synaptic transmission or on short-term synaptic plasticity (PPF, PTP or responses to prolonged repetitive stimulation), fEPSPs were recorded in the same slice before and after calcium chelation in astrocytes. To do so and minimize mechanical movements in the slice, fEPSPs were first recorded while a BAPTA-containing patch pipette was sealed on an astrocyte (control condition), and the seal was then broken to chelate calcium only once fEPSPs were stable for at least 10 min. Experiments were independently performed (on different slices) for each experimental protocol (evoked synaptic transmission, PPF, PTP or prolonged repetitive stimulation). *Stratum radiatum* astrocytes were identified by their small somata, low input resistance and resting membrane potential, passive membrane properties (linear IV relationship), lack of action potential and extensive gap junctional coupling. Somatic whole-cell recordings were obtained from visually identified *stratum radiatum* astrocytes, using 5–10 MΩ glass pipettes filled with (in mM): 105 K-gluconate, 30 KCl, 10 HEPES, 10 phosphocreatine, 4 ATP-Mg, 0.3 GTP-Tris, 0.3 EGTA (pH 7.4, 280 mOsm), supplemented or not with BAPTA (10 mM). For intercellular dye coupling experiments, the internal solution contained sulforhodamine B (1 mg/ml), which diffused passively in astrocytes patched in current-clamp mode during 20 min. Recordings were acquired with Axopatch-1D or MultiClamp 700B amplifiers (Molecular Devices) digitized at 10 kHz, filtered at 2 kHz, stored and analyzed on computer using Pclamp10, Clampfit10 (Molecular Devices) and Matlab (MathWorks) softwares. Stimulus artifacts were blanked in sample traces. After acquisition data were binned at different frequencies according to the stimulation protocol used, and in order to clearly visualize the illustrated effects in each figure. Picrotoxin was obtained from Sigma, and all other chemicals were from Tocris.

Calcium Imaging

All imaging experiments were performed simultaneously to electrophysiological field potential recordings in the same region of interest. Intracellular calcium measurements in astrocytes from hippocampal slices were made under single emission fluorescence microscopy using the fluorescent calcium indicator Fluo-4 AM (5 μM, Invitrogen), which has been demonstrated to load specifically astrocytes (Hirase et al., 2004). Loading was performed in ACSF with 0.02% Pluronic F-127 for 25 min in dark at room temperature. After recovery, slices were transferred to the recording chamber of an Olympus BX51WI microscope. Fluo-4 was excited at 488 nm through a light emitting diode (OptoLED, Cairn Research), controlled by the Axon Imaging Workbench software (Molecular Devices), triggering simultaneous acquisition of the electrophysiological recordings by Clampex10 software (Molecular Devices). Fluorescent light (>515 nm) emitted by labeled cells was detected with a long pass filter and an EM-CCD camera (Andor). Images were acquired at 4 Hz through a 20× water immersion objective (NA 0.95, Olympus) and stored on a PC. Images were processed and analyzed off-line with AIW imaging (Molecular Devices), Fiji (Image J) and Matlab (Mathworks) softwares. Background subtraction and bleaching correction were made in Fiji (Schindelin et al., 2012) and performed before fluorescence quantification. Background correction consisted in subtracting a filtered image that closely reflects the background to all of the time-serie images. This background image was obtained by applying a Gaussian blur filter with a 20-pixel radius to the first image of the serie. This filter also removed the acquisition noise on this first image, so that it doesn't appear on the subtraction result images. The processed image serie was then corrected for photobleaching using the bleaching correction function in Fiji with the « Exponential fit » method. Data are then expressed as relative changes in fluorescence over baseline (ΔF/F0). After acquisition of images at 4 Hz, data were binned at different frequencies for clear visualization of the illustrated effects in figures. In average, 4–10 astrocytes were monitored per slice and several slices (n = 6) were monitored from at least 3 different animals.

Statistics

All data are expressed as mean ± SEM and n represents the number of independent experiments. Statistical significance for within-group comparisons was determined by two-way repeated measures ANOVA, whereas two-tailed paired t-tests were used for between-group comparisons. Statistical analysis was performed in GraphPad Prism 6.

Results

Synaptically-Evoked Astroglial Calcium Signaling Displays Short-Term Plasticity

Glutamatergic synapses between Schaffer collaterals and CA1 pyramidal neurons in the hippocampus display several forms of short-term plasticity, such as post-tetanic potentiation (PTP) and facilitating and depressing responses to prolonged repetitive stimulation, which reflect alterations in presynaptic glutamate release. As calcium signaling in astrocytes is activity-dependent, we investigated whether it also exhibits some forms of short-term plasticity typical of excitatory synapses from CA1 pyramidal neurons. To do so, we recorded synchronously neuronal field excitatory postsynaptic potentials (fEPSPs) and astroglial calcium signaling in response to tetanic stimulation (100 Hz, 1 s) and prolonged repetitive stimulation (10 Hz, 30 s) of Schaffer collaterals (**Figures 1A–D**).

We found that astroglial calcium signaling displayed differential short-term plasticity patterns compared to adjacent pyramidal cells.

Tetanic stimulation of Schaffer collaterals (100 Hz, 1 s) results in PTP, a transient potentiation of excitatory transmission (**Figures 1E,G**). During the tetanus, astroglial calcium signaling continuously and gradually increased (peak increase (ΔF/F0): +28 ± 3%, n = 35 astrocytes from 6 slices), in contrast to neuronal responses, which only transiently potentiated (+61 ± 21%, n = 6) and then rapidly depressed (peak depression: −63.2 ± 8.2%, n = 6) (**Figures 1E,G**). In addition, after the tetanus astroglial calcium signaling rapidly exhibited a post-tetanic

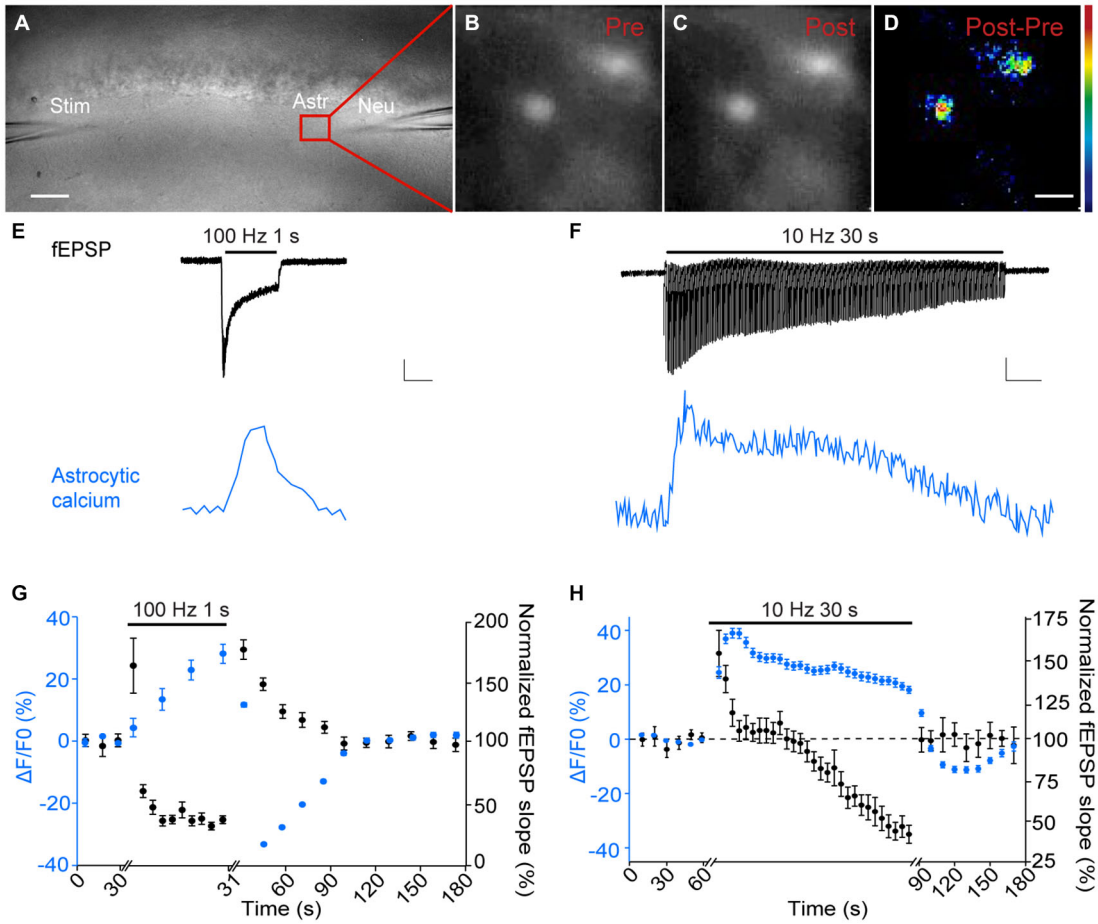

FIGURE 1 | Astroglial calcium signaling displays specific short-term plasticity patterns compared to synaptic responses. (A–D) Sample pictures depicting in a hippocampal slice **(A)** dual recordings of fEPSPs (Neu), evoked by Schaffer collaterals stimulation (Stim) and astroglial calcium levels (Astr). Sample fluorescence images recorded in the red zone shown in **(A)** illustrating stratum radiatum astrocytes loaded with Fluo-4 AM before (Pre, **B**) and after (Post, **C**) tetanic stimulation of Schaffer collaterals (100 Hz, 1 s). The color-coded image illustrates the increase in astroglial calcium levels induced by tetanic stimulation (subtraction Post-Pre, **D**). Scale bars: 50 μm **(A)** and 10 μm **(B–D)**; color bar, 0–30% (ΔF/F0) **E,F**). Representative traces of simultaneously recorded fEPSPs (black) and averaged astrocytic calcium signals (blue) evoked by tetanic (100 Hz, 1 s, **E**) or repetitive (10 Hz, 30 s, **F**) stimulations. Scale bars: 0.2 mV, 5 ΔF/F0, 0.4 s **(E)** and 0.2 mV, 5 ΔF/F0, 2.8 s **(F)**. **(G,H)** Quantification of relative changes in astroglial calcium levels (blue) and fEPSP slope (black) induced by tetanic (n = 6 independent experiments with n = 35 astrocytes) **(F)** or repetitive (n = 6 independent experiments with n = 28 astrocytes) **(H)** stimulations, over baseline levels or responses measured before the onset of stimulations.

decrease (peak decrease (ΔF/F0): −32.5 ± 0.5% 20 s after the tetanus, n = 35 astrocytes from 6 slices) in contrast to neurons, which displayed a characteristic transient potentiation (fEPSP PTP: +74 ± 7.5% 10 s after the tetanus, n = 6) with a similar time course to that of the decrease of glial responses (**Figures 1E,G**). Remarkably, both neuronal and glial responses slowly came back to baseline levels ∼1 min after the tetanus (**Figure 1G**).

Repetitive stimulation of Schaffer collaterals (10 Hz, 30 s) induces an initial facilitation of glutamatergic synaptic transmission, resulting from extensive glutamate release, followed by a depression, caused by depletion of glutamate vesicular pools (**Figures 1F,H**). As found for tetanic stimulation, astroglial calcium signaling exhibited a rapid and sustained increase during the whole repetitive stimulation (peak potentiation (ΔF/F0): +39.1 ± 1.8% after 3 s of repetitive stimulation, n = 28 astrocytes from 6 slices), in contrast to fEPSPs, which first transiently potentiated with stronger magnitude (peak potentiation: +52.8 ± 14.1% after 1 s of repetitive stimulation, n = 6) and then gradually depressed (peak depression: −57.8 ± 8% at the end of the repetitive stimulation, n = 6) (**Figures 1F,H**). Although both neuronal and glial responses reached their peak potentiation rapidly (1 s and 3 s, respectively) and then slowly decayed during the rest of the repetitive stimulation, the magnitude and time course of the responses differed, as astroglial calcium signal amplitudes reached a peak increase of ∼ +40% within ∼3–4 s of 10 Hz stimulation, and then slowly decayed to half peak responses after 30 s, while fEPSPs reached a peak potentiation of ∼ +57% within ∼1 s, fully decayed to baseline level after ∼5–13 s and

strongly depress (∼ −35 to −60%) after ∼20 s (**Figure 1H**). In addition, after the repetitive stimulation, the neuronal response returned immediately and steadily to baseline levels, while astroglial calcium signals exhibited a decrease in their amplitude below basal levels during ∼1 min (reaching −11.1 ± 1.1% 60 s after the repetitive stimulation, and returning to basal levels 90 s after the stimulation, n = 28 astrocytes from 6 slices, **Figure 1H**).

Together, these data show that astroglial calcium signals display differential short-term plasticity patterns compared to neighboring excitatory synapses.

Acute Calcium Chelation in Astrocytes Increases Evoked Excitatory Synaptic Transmission

We then investigated whether in turn astroglial calcium signaling evoked by neuronal activity regulates locally the moment-to-moment glutamatergic synaptic transmission. To do so, we acutely and selectively inhibited calcium responses in local astrocytic networks, while simultaneously recording neighboring neuronal activity with fEPSP evoked by single stimulation of Schaffer collaterals. Calcium responses were specifically inhibited in astrocytes by intracellular dialysis of BAPTA (10 mM), a low molecular weight calcium chelator (astroglial calcium response peak amplitude: −70 ± 2%, n = 4 independent experiments on 26 astrocytes). BAPTA, together with sulforhodamine-B, a gap-junction permeable dye, were initially delivered to a single astrocyte via a patch pipette, and subsequently spread within populations of astrocytes through gap junction channels (Serrano et al., 2006; Rouach et al., 2008), as revealed by the extent of astrocyte dye coupling (**Figure 2A**). In all experiments, neuronal responses were recorded in close proximity (20–50 μm) to the astrocyte patched with the BAPTA-containing pipette. fEPSPs were initially recorded while the BAPTA-containing patch pipette was sealed on an astrocyte (control condition), and the seal was then broken to chelate calcium (BAPTA condition). fEPSPs were thus analyzed in the same slice before and after calcium chelation in astrocytes. In these conditions, we found that calcium chelation in astrocytes induced a rapid and sustained increase of ∼20% in excitatory synaptic transmission (+22 ± 0.9% after 17 min of dialysis, n = 6, $p < 0.001$, **Figures 2B,C**). The increase occurred within ∼10 min and lasted for at least 30 min. This effect was not due to leakage of BAPTA from the patch pipette in the extracellular space, because sealing an astrocyte with the BAPTA-containing patch pipette for 25 min had no effect on fEPSPs recorded prior to the astroglial seal (100.5 ± 1% of baseline response from 15 to 25 min after the seal, n = 5).

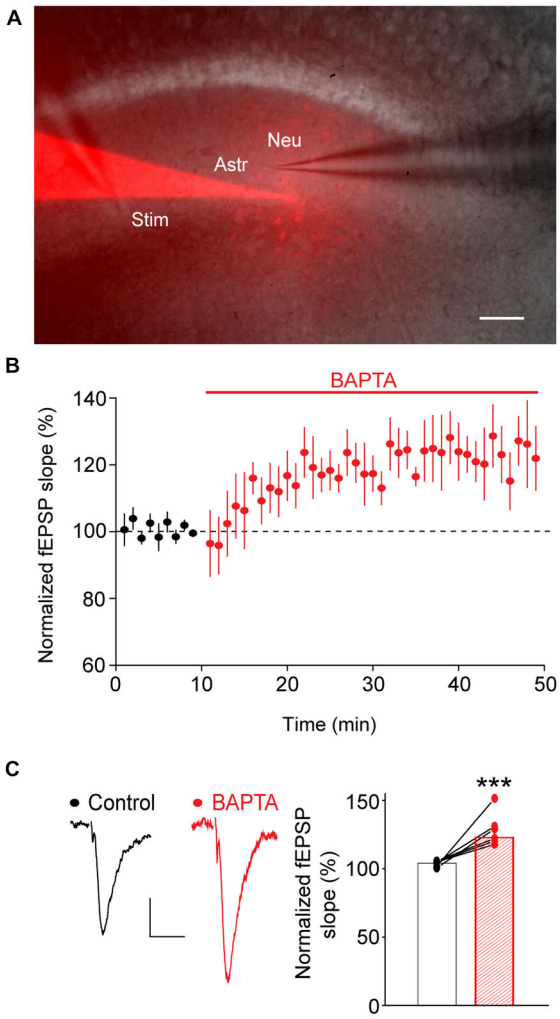

FIGURE 2 | Calcium chelation in astrocytes rapidly increases glutamatergic synaptic transmission. (A) Sample picture of a hippocampal slice depicting dual recordings of fEPSPs (Neu), evoked by Schaffer collaterals stimulation (Stim), and a neighboring astrocyte (Astro) with a pipette containing BAPTA (10 mM) and sulforhodamine-B (red, 0.1%). Note the spread of sulforhodamine-B through gap-junction-mediated astroglial networks. Scale bar: 50 μm. **(B)** Quantification over time of fEPSPs evoked by a single stimulation of Schaffer collaterals in the same slices before (black) and after astroglial calcium chelation by intracellular BAPTA delivery (red, n = 6). **(C)** Sample traces and quantification of evoked fEPSPs recorded in the same slices before (black) and after 20–40 min of astroglial BAPTA infusion (red, n = 6). Asterisks indicate statistical significance (Student's paired t-test, ***$p < 0.001$).

Astroglial Calcium Chelation Potentiates Short-Term Plasticity Of Excitatory Synapses

To test whether astroglial calcium signaling also contributes to short-term synaptic plasticity, we performed the astroglial calcium chelation experiment while stimulating Schaffer collaterals with various patterns of activity. In all experiments, short-term synaptic plasticity was assessed in the same slice before and after astrocyte calcium chelation by recording activity-dependent changes in fEPSPs while the patch pipette was sealed on an astrocyte (control condition) and after 15 min of astrocyte dialysis with BAPTA (10 mM).

Paired-pulse stimulation of Schaffer collaterals induced paired-pulse facilitation of excitatory synaptic transmission (PPF), a typical form of short-term plasticity in hippocampal CA1 pyramidal cells (**Figures 3A,B**). We found that calcium chelation in local astrocytic networks significantly increased PPF (PPF: before calcium chelation, 1.75 ± 0.13; 15 min after calcium chelation: 1.91 ± 0.18, n = 6, $p < 0.05$, **Figures 3A,B**).

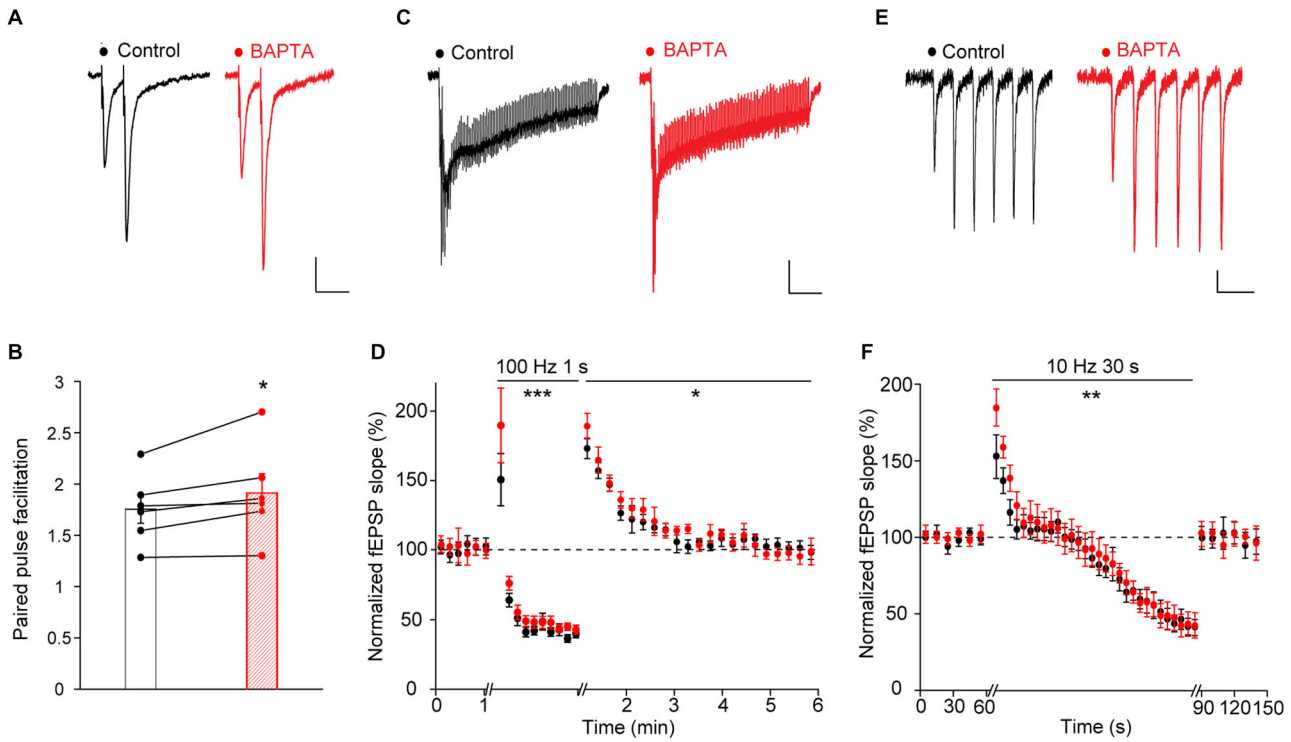

FIGURE 3 | Acute astroglial calcium chelation increases short-term synaptic plasticity. (A) Sample traces and quantification of fEPSP paired-pulse facilitation (**A,B**, $n = 6$), post-tetanic potentiation (**C,D**, $n = 10$) and responses to repetitive stimulation (**E,F**, $n = 6$) recorded independently before (black) and after astroglial calcium chelation by BAPTA (10 mM, 20 min, red) in the same slices. Asterisks indicate statistical significance (**B**, Student's paired *t*-test; **(D,F)**, two-way repeated measures ANOVA; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Tetanic stimulation of Schaffer collaterals (100 Hz, 1 s) resulted in PTP, a transient potentiation of glutamatergic synaptic transmission (**Figure 3D**). Similarly to the effect observed on PPF, we found that the astroglial calcium chelation enhanced PTP (peak potentiation 10 s after the tetanus: before calcium chelation, +74 ± 7.5%; after calcium chelation: +90 ± 9.5%; normalized fEPSP 110 s after the tetanus: before calcium chelation, 105 ± 8%; after calcium chelation, 103 ± 4.4%; $n = 10$, $p < 0.05$, **Figure 3D**). Moreover, inhibition of calcium responses in astrocytes also potentiated the neuronal responses recorded during the tetanic stimulation (peak potentiation after 100 ms of stimulation: before calcium chelation, +50 ± 19%; after calcium chelation: +90 ± 27%; peak depression at the end of the tetanus: before calcium chelation, −65 ± 7.2%; after calcium chelation: −61 ± 6.8%; $n = 10$, $p < 0.001$), **Figures 3C,D**).

Prolonged repetitive stimulation of Schaffer collaterals (10 Hz, 30 s) induced a transient facilitation of excitatory synaptic transmission, followed by a depression (**Figures 3E,F**). As found for paired-pulse and tetanic stimulations, acute chelation of astroglial calcium increased the magnitude of the transient potentiation (peak potentiation after 1 s of repetitive stimulation: before calcium chelation, 52 ± 14.1%; after calcium chelation: 84 ± 11.8%, $n = 6$, $p < 0.05$, **Figures 3E,F**). However, amplitude of the maximal depression was unchanged (maximal depression at the end of the stimulation: before calcium chelation, −59 ± 6.5%; after calcium chelation, −57 ± 7.2%, $n = 6$, **Figure 3F**).

The effects of astroglial calcium chelation on the three forms of short-term synaptic plasticity described above were specific, as performing two consecutive times with a 15 min interval either paired-pulse, tetanic or prolonged repetitive stimulation of Schaffer collaterals did not alter by itself the induced short-term plasticities (PPF: 1.725 ± 0.09; 15 min after: 1.655 ± 0.006, $n = 4$, $p > 0.05$, Student's paired *t*-test; PTP, peak potentiation 10 s after the tetanus: +75.50 ± 20.93; 15 min after: +72.29 ± 6.73, $n = 5$, $p > 0.05$, two-way repeated measures ANOVA; repetitive stimulation: peak potentiation after 1 s of repetitive stimulation: +59.1 ± 11; 15 min after: +60.4 ± 2.1; maximal depression at the end of the stimulation: −36.3 ± 4.4; 15 min after: −36.9 ± 5.7, $n = 5$, $p > 0.05$, two-way repeated measures ANOVA).

Discussion

We here show that calcium signaling in hippocampal astrocytes displays several forms of activity-dependent short-term plasticity, with differential patterns compared to adjacent excitatory synapses. We also show that endogenous calcium signaling in astrocytes can down-regulate evoked glutamatergic synaptic transmission and short-term synaptic plasticity. These data provide novel insights into the dynamics of astroglial calcium signaling and its role in synaptic efficacy.

Short-Term Plasticity of Astroglial Calcium Signaling

Activity-dependent plasticity refers to the strengthening or weakening of a response as a result of an increase or decrease in activity. Although this term is traditionally used for synapses, it is also currently used for glial cells, in particular for currents and calcium signaling from NG2 cells (Ge et al., 2006; Bélair et al., 2010), as well as for currents from astrocytes (Linden, 1997; Bellamy and Ogden, 2005, 2006; Pita-Almenar et al., 2006; Ge and Duan, 2007; Zhang et al., 2009; Sibille et al., 2014). Consistent with these findings, we here identified a novel type of astrocytic plasticity, i.e., an activity-dependent short-term plasticity of calcium signaling. We found two forms of calcium signaling short-term plasticity, which describe changes over time of astroglial calcium responses evoked by Schaffer collateral tetanic (100 Hz, 1 s) or prolonged repetitive stimulation (10 Hz, 30 s), which are stimulation patterns commonly used to induce specific forms of short-term synaptic plasticity (PTP or facilitating and depressing responses to prolonged repetitive stimulation, respectively).

Remarkably the two forms of plasticity were evoked by relatively low amplitude stimulation of Schaffer collaterals (10–20 μA), indicating that astrocytes display adaptive responses to moderate physiological synaptic activity. Interestingly, astrocytes were able to integrate relatively rapidly fast neuronal activity induced by either tetanic or repetitive stimulations, since astroglial calcium responses were evoked within hundred(s) of milliseconds. Moreover, both the short tetanic (1 s) and repetitive (30 s) stimulations evoked over time similar magnitude of intracellular calcium level increase in astrocytes, although the timing of the peak responses slightly differed (1 s vs. 3 s, respectively). This difference most likely reflects summation over time of evoked calcium elevations to reach peak amplitude. These data suggest that both types of stimulation may recruit similar intracellular pathway within slightly different timeframes.

In addition, both types of astrocytic plasticity displayed differential patterns compared to the short-term plasticity of synaptic responses. Although it is well admitted that astrocytes integrate neuronal activity through their calcium signaling, our data suggest that such signaling does not simply reflect neuronal responses, but exhibits specific behavior with proper dynamics (Volterra et al., 2014). Our results also suggest that during tetanic stimulation, astrocytes can integrate graded levels of presynaptic activity, through their calcium signaling, which likely does not saturate. In contrast, calcium signals may saturate in astrocytes after a few seconds of the 10 Hz stimulation, as they slowly decrease after 3 s.

The pathway underlying astroglial calcium signaling short-term plasticity was not identified in this study, but likely relies on activation of the canonical calcium release from internal stores induced by activation of glutamate metabotropic receptors. Indeed, both tetanic and prolonged repetitive stimulation of Schaffer collateral induce significant presynaptic release of glutamate, which spill over to likely activate the abundantly expressed mGluR5 in neighboring astrocytes from juvenile mice (Sun et al., 2013; Verkhratsky et al., 2012). This pathway has already been shown in astrocytes to be recruited in a graded manner during Schaffer collateral stimulation, correlating with the number of activated synapses (Honsek et al., 2012). In addition, the time needed to reach maximum amplitude of mGluR-mediated calcium responses in astrocytes is relatively slow, i.e., in the range of seconds (1–3 s) (Honsek et al., 2012), as reported in our study. Finally, the slow decrease in calcium signaling that occurs after a few seconds of the 10 Hz stimulation may reflect desensitization of mGluRs as well as gradual depletion of internal calcium stores during the course of the stimulation. We cannot however exclude the involvement of other molecular pathways mediating extracellular calcium entry in astrocytes through store operated channels, sodium-calcium exchanger or ionotropic receptors, such as P2X or TRPA1 receptors, which may underlie fast signaling initiated in astroglial perisynaptic processes that slowly propagate to cell soma (Verkhratsky et al., 2012, 2014; Khakh and McCarthy, 2015).

Remarkably, astrocytes did not only show a differential pattern compared to neurons during the tetanic and repetitive stimulations, but also after. Indeed, while postsynaptic neuronal responses displayed a typical post-tetanic potentiation, astroglial calcium signals showed a mirror response with a post-tetanic decrease, slowly coming back to baseline levels within a minute, akin to neuronal responses. Similarly astroglial calcium signals showed a decrease after the repetitive stimulation, in contrast to neuronal responses which returned immediately to baseline levels. Such post-tetanic and post-repetitive decreases in astroglial calcium signaling resemble the depression of the synaptically-evoked potassium and residual currents (I_K and I_{res}) that we recently identified in hippocampal astrocytes after tetanic and repetitive stimulations (Sibille et al., 2014). They are also reminiscent of typical undershoot responses (such as extracellular potassium levels and space volume) occurring after similar high frequency stimulations (D'Ambrosio et al., 2002; Chever et al., 2010; Haj-Yasein et al., 2011; Pannasch et al., 2011; Bay and Butt, 2012). These short-term plasticities may represent an adaptive response to prevent prolonged activation of astroglial calcium signaling, thus enabling local and transient increase in astroglial signaling during the stimulations. They may also serve to increase the signal-to-noise ratio by contrasting the astroglial calcium responses. In addition, the short-term decreases in astroglial calcium signaling might also code for downstream intracellular pathways, which would regulate in turn neuronal activity. The mechanism underlying the transient depression of astroglial calcium signaling after tetanic and repetitive stimulations still needs to be investigated. It could result from the combination of mGluRs desentization, intracellular calcium store depletion and astroglial calcium release through calcium ATPases, sodium-calcium exchangers or connexin and pannexin hemichannels, which can be activated by increase in cytosolic calcium (Verkhratsky et al., 2012; Cheung et al., 2014).

Regulation of Neurotransmission by Endogenous Calcium Signaling

Astroglial calcium signaling is generally thought to modulate neuronal activity through multiple and complex pathways (Volterra et al., 2014). Although many studies have shown that calcium signaling in astrocytes regulates various regimes

of neuronal activity (Perea et al., 2009; Nedergaard and Verkhratsky, 2012; Dallérac et al., 2013; Araque et al., 2014; Volterra et al., 2014), some failed to detect any effect on synaptic transmission or plasticity (Fiacco et al., 2007; Petravicz et al., 2008; Agulhon et al., 2010). These discrepancies led to the view that calcium signaling in astrocytes is more complex than initially thought, and that different effects may occur according to the manipulations used to alter astroglial calcium. Indeed to interfere with astroglial calcium signaling, several approaches have been used, including acute downregulation using intracellular calcium chelation with BAPTA or calcium-clamp, chronic and targeted inhibition using transgenic mice with impaired astroglial store-mediated calcium signaling ($IP3R2^{-/-}$ mice), as well as acute activation using pharmacogenetic approach to activate specifically calcium signaling in astrocytes in a transgenic mice expressing in astrocytes in an inducible manner a Gq-G protein coupled receptor (MrgA1) not expressed in the brain and activated by a brain exogenous ligand (Fiacco et al., 2007; Petravicz et al., 2008; Agulhon et al., 2010; Henneberger et al., 2010; Nedergaard and Verkhratsky, 2012; Volterra et al., 2014). Identifying the manipulations that relate to physiological events or interfere with endogenous signaling is a current effort to unravel the actual role of astroglial calcium in physiological processes.

We here aimed at clarifying such role on hippocampal physiological synaptic transmission and short-term plasticity. To do so, we used calcium chelator infusion into populations of astrocytes through patch pipettes to downregulate acutely calcium signals in astrocytes. Remarkably, we found that such manipulation relatively rapidly unleashed evoked excitatory synaptic transmission and several forms of its short-term plasticity. Using similar approaches, several studies have shown *ex vivo* or *in vivo* during alternative paradigms that acute downregulation of endogenous calcium signaling, via calcium chelation with BAPTA or calcium-clamp (Henneberger et al., 2010), regulates physiological synaptic activity such as basal synaptic transmission (Panatier et al., 2011), spontaneous postsynaptic currents and evoked bursts (Benedetti et al., 2011), LTP (Henneberger et al., 2010), heterosynaptic depression (Serrano et al., 2006), short-term synaptic depression (Andersson and Hanse, 2010), as well as cholinergic-induced LTP (Navarrete et al., 2012). Noteworthy in most cases, astroglial calcium signaling was found to increase neuronal activity through gliotransmitter release (Serrano et al., 2006; Henneberger et al., 2010; Panatier et al., 2011; Navarrete et al., 2012). However, each regulation was reported to be mediated by a different gliotransmitter or targeted receptor, such as D-serine (Henneberger et al., 2010), glutamate (Navarrete et al., 2012) or ATP, subsequently metabolized into adenosine and activating either presynaptic A1 (Serrano et al., 2006) or A2 receptors (Panatier et al., 2011). We here found that calcium chelation in astrocytes increased evoked excitatory synaptic transmission, paired pulse facilitation, post-tetanic potentiation and responses to repetitive stimulation in the hippocampus, implying that in these cases astroglial calcium signals downregulate synaptic transmission and short-term plasticity.

However, the underlying mechanism still remains to be determined. These astroglial regulations of synaptic efficacy may be directly mediated by calcium-dependent release of inhibitory gliotransmitters, such as ATP-derived adenosine activating A1 receptors, cannabinoids activating CB1 receptors (Navarrete and Araque, 2008), or GABA (Angulo et al., 2008) acting on $GABA_A$ or $GABA_B$ receptors, pathways all well known to decrease presynaptic glutamate release. However other mechanisms may be at play, such as calcium-dependent potassium uptake through astroglial Na/K ATPase, leading to decreased extracellular potassium levels and thereby reduced excitatory synaptic transmission, as recently reported in the hippocampus (Wang et al., 2012). Alternatively calcium-dependent astroglial synapse coverage, promoting at the synapse tight morphological interactions (Iino et al., 2001; Saab et al., 2012; Tanaka et al., 2013; Bernardinelli et al., 2014) and efficient astroglial clearance of glutamate (Pannasch et al., 2014) or likely potassium, as well as availability of astrocyte-derived neuroactive factors, might also dampen glutamatergic transmission. Interestingly our results are comparable to the ones obtained in two recent studies, showing that calcium chelation in the barrel cortex increased the frequency of spontaneous excitatory postsynaptic currents, as well as the amplitude and duration of evoked bursts (Benedetti et al., 2011), while in the hippocampus it inhibited a post-burst short-term depression of glutamatergic transmission (Andersson and Hanse, 2010). In both studies the underlying mechanism was not identified, although D-serine, adenosine or glutamatergic transmission were not found to be involved in regulation of neuronal activity from the barrel cortex (Benedetti et al., 2011). It is thus tempting to speculate that the differential effects of astroglial calcium chelation on neuronal activity may rely on the type of neuronal activity, the brain area and the developmental stage studied, which are likely associated with different forms of astroglial calcium signals and downstream intracellular pathways. Such multiple effects illustrate the complexity of the astroglial calcium signaling code, which remains to be further unraveled.

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frontiers in Cellular Neuroscience

REVIEW
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Observation and manipulation of glial cell function by virtue of sufficient probe expression

*Akiyo Natsubori, Norio Takata and Kenji F. Tanaka**

Department of Neuropsychiatry, School of Medicine, Keio University, Tokyo, Japan

The development of gene-encoded indicators and actuators to observe and manipulate cellular functions is being advanced and investigated. Expressing these probe molecules in glial cells is expected to enable observation and manipulation of glial cell activity, leading to elucidate the behaviors and causal roles of glial cells. The first step toward understanding glial cell functions is to express the probes in sufficient amounts, and the Knockin-mediated ENhanced Gene Expression (KENGE)-tet system provides a strategy for achieving this. In the present article, three examples of KENGE-tet system application are reviewed: depolarization of oligodendrocytes, intracellular acidification of astrocytes, and observation of intracellular calcium levels in the fine processes of astrocytes.

Keywords: knockin-mediated enhanced gene expression, tet system, tTA, tetO, optogenetic actuator, calcium indicator, genetically modified mice

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Shinghua Ding, University of Missouri, USA

****Correspondence:***
Kenji F. Tanaka, Department of Neuropsychiatry, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan
kftanaka@keio.jp

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Expressing Functional Genes to Observe and Manipulate Glial Cells

To observe and manipulate glial cell functions, probes needed for selective observation and manipulation of cells should be expressed in advance. These functional probes include the intracellular calcium sensor molecules such as GCaMP (Nakai et al., 2001) for observation, and light-sensitive membrane potential converting molecules, such as channelrhodopsin for depolarization (Nagel et al., 2003) and halorhodopsin (HaloR; Zhang et al., 2007) or archaerhodopsin (Arch)/ArchT (Chow et al., 2010; Han et al., 2011) for hyperpolarization. Mouse Genetics can be used for glial cell-specific expression of these molecules.

Glial cells are present in every brain region and show complex interactions with neurons. Therefore, when observing or manipulating glial cell functions, glial cell-specific expression of functional molecules is vital. These functional molecules should be fully expressed in transfected glial cells in a cell type-specific manner to exert their activities. In general, however, it is not easy to simultaneously achieve both a high level and a cell type-specific expression. The worst-case scenario would be a failure to express these molecules at a level sufficient to exert their functions, as a result of too much focus placed on the cell type specificity. For example, channelrhodopsin 2 (ChR2) molecules were successfully expressed specifically in astrocytes, but failed to trigger photocurrent because of low expression. To continue with glial cell research, cell type-specific expression of functional probes need to be pursued, while simultaneously avoiding such undesirable scenarios.

To accomplish cell type-specific expression of functional probes in the brain, many researchers have employed local viral injections as a means to introduce those genes. However, viral injection via a needle causes cerebral parenchyma injury and will induce substantial alterations in the nature of glial cells. Injury-induced augmentation of glial cell activity and subsequent cross-interactions between glia and neurons are inevitable to some degree.

Therefore, any research projects that cannot disregard the effects of external injury need alternative methods. If mouse genetics were adapted to this purpose, functional molecules could be expressed without any problems caused by external injuries.

Making History of KENGE-tet

What are the possibilities for reasonable expression of functional probes specifically in glial cells using mouse genetics? We have tried glial cell-specific expression of ChR2 using transgenic mouse technology. We attempted not only to achieve glial cell-specific expression of ChR2, but also to fulfill high enough expression to function, in this case to induce photocurrent.

ChR2 conductance was reported to be 50–250 fS (Lin et al., 2009), indicating a smaller conductance compared to those of most ionic channels reported until then. This fact suggested that a "more than a moderate level of expression" of ChR2 would be needed to control the membrane potential. Although this information had been recognized early, scientists did not know how much ChR2 should be expressed on the cell membrane to successfully control the membrane potential. Higher level of expression was considered to be better than less expression. However, aggregation of ChR2 molecules was a concern for having too much expression. Scientists were confused by the variety of information. Furthermore, it was suggested that ChR2 molecules stayed in the endoplasmic reticulum and did not reach the cell membrane causing failure of detection of photocurrents. To evaluate this possibility, expression of a ChR2-green fluorescent protein (GFP; or mCherry) fusion protein was attempted. This made the already difficult situation more difficult. Expression of a ChR2-GFP (or mCherry) fusion protein was a challenging subject when compared to expression of ChR2 molecules alone.

The tet system (tetracycline-controllable gene expression system) is a gene expression system (Gossen and Bujard, 1992) allowing enhanced expression of an exogenous gene in a cell type-specific manner. The tet system is a bipartite system (**Figure 1**). The first mouse line expresses the gene encoding tetracycline transactivator (tTA) protein under the control of cell-type specific promoter. In the second mouse line, ChR2 complementary DNA (cDNA) is connected downstream of the tet operator (tetO) promoter, which is activated only by the presence of tTA protein. Cell-type specific expression of ChR2 can be expected only in tTA::tetO double transgenic mice. If any undesirable events, such as toxicity, are caused by the overexpression, the amount of expression can be lowered using doxycycline, a tetracycline analog.

We first generated tetO-ChR2-mCherry plasmid transgenic lines (actually tetO-ChR2-mCherry, HaloR-GFP lines) and established 6 lines (Chuhma et al., 2011). When we crossed them with neuronal tTA line (*Camk2a*-tTA, Jax stock number 003010), we succeeded in functional ChR2-mCherry expression with all combination, indicating that tet system worked. However, when we crossed them with astrocytic tTA line (*Mlc1*-tTA; Tanaka et al., 2010), Riken BioResource Center (BRC) number 05450), none of combination yielded a red fluorescence in astrocytes. We observed a few HaloR-GFP expressing Bergmann glia in line 6

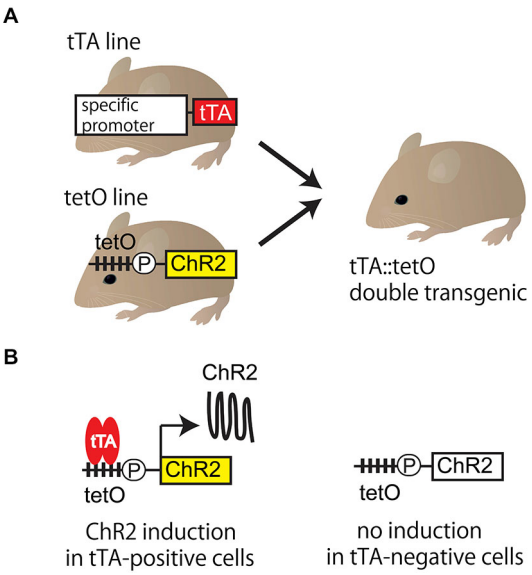

FIGURE 1 | Tet system. **(A)** Tet system requires two mouse lines. The first is a mouse line expressing gene encoding tetracycline transactivator (tTA) protein under the control of cell-type specific promoter. The second is one expressing the functional probe (such as ChR2) connected downstream of the tetO promoter. Targeted tTA::tetO double transgenic mouse is generated by mating. **(B)** The functional probe is expressed in tTA-positive cells, but not in tTA-negative cells in tTA::tetO double transgenic mouse. In tTA-positive cells, tTA binding to the tetO promoter triggers the transcription, which results in the cell-type specific expression of ChR2. TetO is the cassette containing the tetracycline operon site (multiple closed squares) and CMV minimal promoter (circle P). ChR2: channelrhodopsin 2, tetO: tet operator, tet system: tetracycline-controllable gene expression system, tTA: tetracycline transactivator.

(Tanaka et al., 2012, Jax stock number 017906), which was the best line among them. The chromosomal positional effect on the tetO transgene by the random insertion, the copy number of tetO transgene, or the DNA methylation of tetO promoter may account for the cell-type dependent difference of gene induction.

We then attempted to improve the tet system. What was the reason why the tetO promoter failed to induce ChR2 expression despite the fact that expression of tTA was obtained? For this question, we got ideas from our own experience (Tanaka et al., 2010). In past experiments, we established tetO mice by microinjection of transgenic plasmid DNA into the fertilized eggs, resulting in poor tTA-mediated gene induction. However, we found that the efficiency of tTA-mediated gene induction was improved dramatically when tetO mice were generated by homologous recombination technique through embryonic stem cells. From this, we generated the tetO-ChR2 mouse not by transgenic but by knocking-in approach, called the Knockin-mediated ENhanced Gene Expression (KENGE)-tet (Tanaka et al., 2012). The house-keeping β-actin gene locus was used as the knock-in site and the tetO-ChR2 cassette was inserted (**Figure 2**). This strategy was a major success and tremendously high levels of ChR2 expression was achieved compared to the previous tetO lines. Importantly, the levels of ChR2 in astrocytes, oligodendrocytes, and microglia were high enough to trigger

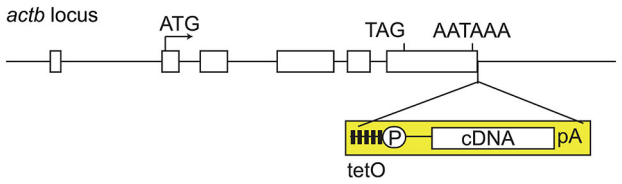

FIGURE 2 | KENGE-tet system requires tetO cassete knockin. *Actb* gene structure and insertion site of tetO cassette. Mouse *Actb* gene consists of 6 exons (rectangles), and the tetO cassette (yellow) is inserted downstream of the polyadenylation signal of *Actb* gene. TetO is the cassette containing the tetracycline operon site (closed squares) and CMV minimal promoter (circle P). AATAAA is a polyadenylation signal. Knocking-in of tetO cassette into the euchromatic locus is a key point for the achievement of higher expression with tTA. *Actb*: β-actin, cDNA: complementary DNA, pA: ploydenilation signal, tetO: tet operator, tTA: tetracycline transactivator.

photocurrents with *Mlc1*-tTA, *Plp1*-tTA (Inamura et al., 2012, Riken BRC number 05446), and *Aif1*-tTA (also known as *Iba1*-tTA; Tanaka et al., 2012, Riken BRC number 05769) lines, respectively.

KENGE-tet is not limited in the usage of β-actin gene locus. The usage of euchromatic locus, such as collagen type 1 alpha 1 gene (*Col1a1*) locus (Beard et al., 2006; Egli et al., 2007; Carey et al., 2010; Premsrirut et al., 2011) or TIGRE (tightly regulated) locus (Zeng et al., 2008; Madisen et al., 2015) shares the same idea of KENGE-tet and yields high levels of tTA-mediated gene expression.

Expression of Functional Probes using Genetically Modified Mice

In addition to the KENGE-tet sysetm, an improved Cre-loxP system can be used to satisfy the requirements of both a high level and cell type-specific expression. The combination of the euchromatic locus with the stronger promoter greatly enhanced the expression levels of functional probes. The Ai (Allen Institute) mouse (Madisen et al., 2010, 2012), with a CAG promoter inserted in the ROSA26 locus, and the PC (*Polr2a*-CAG) mouse (Gee et al., 2014), with a CAG promoter inserted in the polymerase (RNA) II (DNA directed) polypeptide A (*Polr2a*) gene locus, have been developed as loxP lines.

Both the tet and Cre-loxP systems use double-transgenic mice obtained by mating two mouse lines. One mouse line is aimed at ensuring a cell type-specific expression (Cre or tTA mice), and the other to obtain a high expression level (ROSA-CAG/Polra2-CAG or KENGE-tet mice). LoxP or tetO line has been further improved, providing a wider repertoire of functional probes to be expressed.

Table 1 shows the repertoire of mouse lines for the expression of functional molecules in a glial cell-specific manner (tTA mice), and those for the expression of sufficient amounts of functional molecules (KENGE-tet mice). tTA and tetO mice can be mated in any combination. Although not all the theoretically obtainable mouse lines have been tested, we have confirmed that sufficient expression was obtained for photocurrent induction in astrocyte-specifically expressed ChR2 and ArchT, oligodendrocyte-specifically expressed ChR2 and ArchT, and microglia-specifically expressed ChR2. Yellow cameleon nano50 (YC; Horikawa et al., 2010) was also expressed in an astrocyte-specific manner to a level sufficient for the observation of intracellular calcium level *in vivo*. Most of these mouse lines can be obtained from Riken BioResource Center.[1] Ai mice are available from the Jackson Laboratory.[2]

Manipulation and Observation of Glial Cells Avoiding Injury

Using genetically modified mice, functional molecules can be expressed specifically in glial cells without injury. However, it is meaningless if the brain injury occurs in the process of observation and manipulation (Xu et al., 2007). What are the possibilities of carrying out observations and manipulations without any injuries? It was demonstrated that mice expressing ChR2(C128S) in astrocytes responded to blue light illumination over the skull (Tanaka et al., 2012), since ChR2(C128S) variant had higher photosensitivity (Berndt et al., 2009). These data indicated that cortical astrocytes could be manipulated without an insertion of optical fiber, but the optic fiber insertion was still required when astrocytes in the deep brain were targeted.

To visualize the effects of optogenetic manipulation, the laser speckle method can be used to observe blood stream changes through the cranial bone (Zakharov et al., 2009), and functional magnetic resonance imaging (MRI) can be used to observe the blood oxygenation level-dependent (BOLD) signal changes from the intact brain (Ogawa et al., 1990). Intracellular calcium concentrations in glial cells *in vivo* can be observed using two-photon microscopy (Hirase et al., 2004; Wang et al., 2006; Schummers et al., 2008; Takata et al., 2011; Nimmerjahn and Bergles, 2015), in which brain parenchyma injuries can be minimized. The process of combining these manipulation and observation methods does not cause injuries to the brain parenchyma, avoiding unwanted glial cell activation.

Glial cells respond to injuries. If you compare this response to a "scream," and responses during normal interactions between glia and neurons to a "whisper," then the most intriguing scientific phenomenon for glia researchers is the "whisper" being washed out by the "scream". To date, there is no definitive evidence indicating responses to injury are extensive and normal responses are imperceptible. However, glia researchers need to implant functional molecules into glial cells like a "spy" to extract information and to alert them to any tiny response in glial cells. We believe that only through these types of efforts we can understand the glial cell function.

Functional Analysis of Glial Cells after Optogenetic Manipulation

What are the purposes of ChR2-mediated manipulation in glial cells? The purposes of optogenetic manipulation

[1]http://mus.brc.riken.jp/en/
[2]http://jaxmice.jax.org/list/ra2600.html

TABLE 1 | The repertories of glia-specific tTA lines and KENGE-tetO lines.

Glial tTA lines				
Target cell	**Line name**	**Ref**	**RIKEN BRCstock number**	**Crossing with KENGE-tet mouse**
Astrocyte	Mlc1-tTA	Tanaka et al. (2010)	05450	Okada et al. (2012), Sasaki et al. (2012), Tanaka et al. (2012), and Beppu et al. (2014)
	Slc1a2-tTA (GLT1-tTA)	Tanaka et al. (2013)	–	–
	Gfap-tTA	Pascual et al. (2005)	–	–
Oligodendrocyte	Plp1-tTA	Inamura et al. (2012)	05446	Inamura et al. (2012), Tanaka et al. (2012), and Yamazaki et al. (2014)
Oligodendrocyteprogenitor cell	Sox10-rtTA	Ludwig et al. (2004)	–	no induction with tetO-ChR2 (C128S) (Inamura et al., 2012)
Microglia	Aif1-tTA (Iba1-tTA)	Tanaka et al. (2012)	05769	Tanaka et al. (2012)
KENGE-tetO lines				
Probe	**Line name**	**Ref**	**RIKEN BRC stock number**	
ChR2 (C128S)	tetO-ChR2 (C128S)	Tanaka et al. (2012)	05454	
ChR2 (E123T/T159C)	tetO-ChR2 (E123T/T159C)	Tsunematsu et al. (2014)	05843	
ArchT	tetO-ArchT	Tsunematsu et al. (2013)	05842	
YC nano50	tetO-YC	Kanemaru et al. (2014)	–	

(–): not available.tTA: tetracycline transactivator, KENGE: Knockin-mediated ENhanced Gene Expression, GLT-1: glial glutamate transporter-1, Iba1: Ionized calcium-binding adapter molecule 1, ChR2: channelrhodopsin 2, ArchT: archaerhodopsin from Halorubrum strain TP009, YC: Yellow cameleon nano50.

are clear in the case of neurons. In neurons, an inward current generated by light opens voltage-dependent sodium channels at the axon initial segment and induces an action potential. Therefore, ChR2 expressing neurons can generate action potentials on demand. In glial cells, however, action potentials cannot be generated by depolarization. Apart from action potential generation, expected approaches to glial cells using ChR2 are indicated in the following sections:

Depolarization of Oligodendrocytes by ChR2

Hippocampal slices are often used in experiments for induction of long-term potentiation (LTP) of synaptic transmission. When high-frequency stimulation was delivered to the slice to induce LTP, the depolarization of oligodendrocytes at about 15–20 mV was observed (Yamazaki et al., 2007). The effects of oligodendrocytic depolarization on neuronal activity were investigated from the viewpoint of the oligodendrocyte-neuron interaction. To directly demonstrate the physiological implication of oligodendrocytic depolarization, Yamazaki et al. depolarized a single oligodendrocyte at the alveus of the hippocampus using a glass electrode (Yamazaki et al., 2007). The response was recorded from a single CA1 pyramidal neuron around which clamped-oligodendrocyte wrapped. An action potential was generated by electrostimulation of the distal axon, which traveled retrogradely, and was recorded in the soma of CA1 neuron. The clamped-oligodendrocyte was located between the sites of stimulus and observation. The latency between electrostimulation of the distal axon and detection of the action potential reflects the axonal conduction velocity. Normally the latency, or conduction velocity, is constant, but oligodendrocytic depolarization decreased the latency and increased the conduction velocity of action potentials. These findings suggested that oligodendrocytes regulated the neuronal conduction velocity through the depolarization of oligodendrocytes themselves.

Multiple oligodendrocytes wrap the single axon, therefore, the single axonal activity should affect multiple oligodendrocytes. However, it is technically difficult to mimic such situation by using multiple glass electrodes. To achieve this, Yamazaki et al. constructed an experimental system for simultaneous multiple oligodendrocytic depolarizations by optogenetical approach (Yamazaki et al., 2014). That system using *Plp1*-tTA::tetO-ChR2(C128S) double transgenic animals enabled −20 mV oligodencrocytic depolarization without high-frequency electrical stimulation. They investigated if multi-cellular optogenetic manipulation could induce an increase in the conduction velocity similar to single-cellular glass electrode-mediated depolarization. The results showed a transient (∼10 min) but increased conduction velocity. The disappearance of this effect coincided with the termination of oligodendrocytic depolarization.

Yamazaki et al. (2007) also investigated axon excitability using the extracellular recordings. In these recordings, axon excitability was measured as a compound action potential (CAP). Immediately after the illumination of oligodendrocytes, CAPs significantly increased and this effect continued for 3 h. These results suggested either an increased number of excited axons, or increased action potentials from each axon. Interestingly, transient oligodendrocytic depolarization

resulted in the plastic change of the nerve conduction for several hours. The oligodendrocytic depolarization by an optogenetic approach recapitulated the response to high-frequent neuronal firing and these data demonstrated that the oligodendrocytic reaction resulted in short- and long-term plasticity of axons.

Astrocytic Intracellular pH Control by Opsins

Since astrocytes have small membrane resistance, their membrane potential changes very little, even when a photocurrent is delivered. Despite this fact, it was demonstrated that photostimulation of ChR2 expressing astrocytes resulted in the excitation of adjacent neurons (Okada et al., 2012). Using ChR2 expressing Bergmann glial cells of the cerebellum, Sasaki et al. revealed that (1) glutamate was released from Bergmann glia during ChR2 photoactivation; and (2) glutamate was released through 4, 4′-Diisothiocyanostilbene-2, 2′-disulfonic acid (DIDS)-sensitive anion channels (Sasaki et al., 2012). However, the trigger of glutamate release after ChR2 activation remained unclear.

Beppu et al. (2014) focused on the fact that ChR2 shows greater permeability for H^+ compared to Na^+ (Nagel et al., 2003). They suspected that glutamate was released secondary to intracellular acidification induced by H^+ influx into Bergmann glial cells through ChR2 (Beppu et al., 2014). As expected, they demonstrated that ChR2 opening induced the intracellular acidification and triggered the release of glutamate. Furthermore, Beppu et al. (2014) found the intracellular acidification of Bergmann glial cells during the cerebral ischemia. They hypothesized that the intracellular acidification of Bergmann glial cells triggered glutamate release, which exacerbated neuronal damage by ischemia. They carried out a series of experiments in an attempt to counteract this pathologic condition. They showed that the illumination on ArchT-expressing Bergmann glial cells induced H^+ efflux. Furthermore, the illumination on these cells mitigated intracellular acidification of Bergmann glial cells during the ischemia and suppressed glutamate release. Subsequently, neuronal damage was reduced.

These experiments suggested that ChR2 and ArchT could be used as tools to manipulate intracellular pH, and that intracellular acidification of astrocytes induced glutamate release.

Analysis of Glial Cell Intracellular Calcium Level

In studies of glial cells, especially astrocytes, the fluctuation of the intracellular calcium level has been analyzed to monitor the cellular response to the external stimulus (Wang et al., 2006; Schummers et al., 2008). Studies have been focused on the temporal relationships between intracellular calcium responses and other physiological or pathological events, such as changes in blood flow (Takano et al., 2006) or synaptic activity (Panatier et al., 2011; Min and Nevian, 2012), and if calcium transients occur before or after such events. It was found that the timing of calcium transient in the soma of astrocytes differed from that in their fine processes (Shigetomi et al., 2010; Otsu et al., 2015). These observations enhanced opportunities for *in vivo* research of calcium transients in astrocyte fine processes, and for re-investigation of the temporal relationships between those changes and other events (Volterra et al., 2014).

An essential requirement to visualize calcium transients in astrocytic fine processes is the expression of sufficient amounts of calcium indicators. To satisfy this requirement, Yellow Cameleon nano50, a ratiometric calcium indicator, was expressed sufficiently and specifically in astrocytes using the KENGE-tet system. Calcium indicator amount was high enough to monitor astrocytic calcium dynamics *in vivo* using two-photon microscopy (Kanemaru et al., 2014). The results demonstrated calcium transients in resting state astrocytes under anesthesia and that most (80%) transients appeared in the fine processes. The half-life of calcium transients was about 40 s. In response to sensory stimulation, intracellular calcium level increased in both processes and soma. Meticulous observation revealed that the calcium transient initially occurred at the distal end of fine processes and spread toward the soma. This suggested that astrocytes detected extracellular environmental changes, such as neural activity, via fine processes.

Conclusion

The continuous improvement of observation and manipulation probes in glial cell research is of great importance. One of the important points is the expression of sufficient amounts of these probes specifically in glial cells. The KENGE-tet system is a useful tool for this purpose.

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Molecular approaches for manipulating astrocytic signaling *in vivo*

Alison X. Xie[1], *Jeremy Petravicz*[2] *and Ken D. McCarthy*[1]*

[1] Department of Pharmacology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA,
[2] Department of Brain and Cognitive Sciences, Picower Institute for Learning and Memory, Massachusetts Institute of Technology, Cambridge, MA, USA

Astrocytes are the predominant glial type in the central nervous system and play important roles in assisting neuronal function and network activity. Astrocytes exhibit complex signaling systems that are essential for their normal function and the homeostasis of the neural network. Altered signaling in astrocytes is closely associated with neurological and psychiatric diseases, suggesting tremendous therapeutic potential of these cells. To further understand astrocyte function in health and disease, it is important to study astrocytic signaling *in vivo*. In this review, we discuss molecular tools that enable the selective manipulation of astrocytic signaling, including the tools to selectively activate and inactivate astrocyte signaling *in vivo*. Lastly, we highlight a few tools in development that present strong potential for advancing our understanding of the role of astrocytes in physiology, behavior, and pathology.

Keywords: astrocyte, *in vivo*, GPCR signaling, DREADD, IP3R2 KO

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***Correspondence:**
Ken D. McCarthy, Department of Pharmacology, School of Medicine, UNC-Chapel Hill, 120 Mason Farm Road, Chapel Hill, NC 27599-7365, USA
ken_mccarthy@med.unc.edu

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Introduction

G protein coupled receptors (GPCRs) are the primary molecules through which non-excitable cells transduce information from external cues to biological responses. There are four major families of GPCRs that are distinguished by their composition, ability to activate intracellular signaling cascades, and the functional consequences associated with their activation. The importance of GPCRs is underscored by the fact that these molecules are the most common targeted class of proteins of therapeutic agents. Astrocytes express each of the major classes of GPCRs (Porter and McCarthy, 1997) clearly demonstrating that these cells are dynamically coupled to the activity of their surrounding cellular and chemical milieu. It is likely that astrocyte GPCRs are activated by neurotransmitters released from neurons synaptically as well as through volume transmission. It is also likely that neighboring non-neuronal cells including microglia, vascular endothelial cells, astrocytes, and other resident CNS cells release molecules that activate astrocyte GPCRs and modulate astrocyte activity. In certain situations, low levels of ambient neurotransmitters might tonically activate astrocyte GPCRs. However, it is likely that in most cases astrocyte GPCRs are spatially restricted to discrete signaling domains that are activated with different temporal characteristics dependent on the source of the signal and biological response being affected. Layered onto this signaling complexity is the morphological complexity of astrocytes; the fine processes of an individual astrocyte within the CA1 region of the hippocampus can associate with ~100,000 synapses (Bushong et al., 2002). This being the case, different regions of an individual astrocyte are likely responding simultaneously

to local signals (from neurons or other cell types) with different functional outcomes. Collectively, this information underscores how difficult it is to replicate *in vitro* or *in situ* the complicated pattern of GPCR activation that normally occurs *in vivo*.

By far, the emphasis in astrocyte GPCR signaling activity has been on the regulation of Ca^{2+}. This is not surprising given that Ca^{2+} fluxes play a very important role in regulating biological processes and Ca^{2+} is the only signaling molecule that we can readily monitor selectively in astrocytes in complex tissue such as brain. Consequently, we know a lot about astrocyte Ca^{2+} responses following the activation of Gq-GPCRs and the consequences of increasing astrocyte Ca^{2+} by a number of different approaches. Two important points need to be kept in mind when considering findings in this area. First, most investigations linking increases in astrocyte Ca^{2+} with functional responses use pharmacological methods to increase astrocyte Ca^{2+} and consequently findings may not reflect physiological responses. Second, while the field has focused on the role of astrocyte Ca^{2+} in functional responses, the activation of Gq-coupled GPCRs leads to the modulation of a broad set of signaling cascades beyond changes in Ca^{2+}; the variety of effector proteins in Gαq "interactome" may affect astrocyte Ca^{2+} responses as well as play important roles in physiological responses to Gq-GPCR activation (**Figure 1**; Sanchez-Fernandez et al., 2014).

Molecular Tools for Selective Activation of Astrocytes *In Vivo*

Temporal Control of Astrocyte Activation *In Vivo* Using Optogenetics

Optogenetics is an extremely powerful tool for activating and inactivating neuronal circuits *in vivo* with high temporal resolution. Optogenetic regulation of neuronal activity generally occurs through the flux of ions that either depolarize or hyperpolarize neurons with high temporal and spatial resolution (Schoenenberger et al., 2011; Lin, 2012). More recently, a number of investigators have used this technology to activate astrocyte signaling *in situ* and *in vivo* (Gourine et al., 2010; Perea et al., 2014; Yamashita et al., 2014). It is important to understand that while optogenetic activation of astrocytes can lead to changes in ion fluxes across plasma and intracellular compartment membranes, these fluxes do not remotely reflect the changes in signaling that occur following the activation of astrocyte GPCRs, the primary mode for activating astrocyte signaling (Agulhon et al., 2012; Sanchez-Fernandez et al., 2014).

Multiple variants of channelrhodopsin (ChR2) have been expressed in astrocytes, primarily to elicit Ca^{2+} responses (**Table 1**). *In vivo*, activation of the ChR2 variant ChR2 (H134R) (Nagel et al., 2005) in astrocytes in the ventral surface of the medulla oblongata was shown to lead to increases in intracellular Ca^{2+} and alteration in respiratory activity of rats (Gourine et al., 2010). This same variant of ChR2 was employed to illustrate the ability of astrocyte Ca^{2+} elevations to alter the firing and orientation responses of mouse primary visual cortex excitatory and inhibitory neurons *in vivo* (Perea et al., 2014). Most recently, Beppu et al. (2014) created mice expressing the ChR variant ChR2 (C128S) or an optogenetic proton pump (ArchT). Activation of these optogenetic tools lead to, respectively, acidification or alkalization of astrocytes which modulated glutamate release and ischemic damage *in vivo*. The remainder of studies using ChR2 or its variants have been performed using culture or slice preparations, but provide valuable insights into the mechanisms of their ability to activate astrocytes signaling (Figueiredo et al., 2014). A second variant, CatCh (Ca^{2+} translocating ChR, which has improved Ca^{2+} permeability) (Kleinlogel et al., 2011) when expressed in cultured astrocytes, was found to also increase Ca^{2+} but with varying reliability compared to ChR2 (H134R) (Li et al., 2012; Figueiredo et al., 2014). A non-ChR2 light activated channel, the light-gated Ca^{2+}-permeable ionotropic GluR6 glutamate receptor (LiGluR), has also been used in cultured astrocytes, with again varying results (Li et al., 2012).

The advantages of using optogenetics to activate astrocytes are the ability to temporally and spatially control the extent of activation. However, there are a number of important caveats involved in the use of optogenetics to activate astrocyte signaling cascades. First, it is unresolved as to the source of Ca^{2+} when using these optogenetic actuators in astrocytes. Extracellular Ca^{2+} entry through ChR2 variants seems the most logical mechanism, but a recent comparative study found that intracellular stores are the primary source (Figueiredo et al., 2014). These finding are at odds with studies from other laboratories (Li et al., 2012), and previous research from the same authors (Gourine et al., 2010). Current evidence indicates that both external and internal Ca^{2+} sources are involved in the ChR2 induced Ca^{2+} signal. Second, ChR2 and its variants are non-selective cation channels, allowing the entry of Na^+ K^+, H^+, and Ca^{2+} upon activation (Nagel et al., 2003). ChR2 expressed in the cultured astroglia cell line GL261 display significant influx of Na^+ and Ca^{2+} across the cell membrane based upon fluorescent indicator imaging (Ono et al., 2014). Additionally, significant alterations in intracellular pH were also observed in the same study, which has been corroborated in the recent study by Beppu et al. (2014). Interestingly, the ChR2 (H134R) used in the two current *in vivo* studies of astrocyte function was engineered to increase its ability to depolarize cultured HEK293 and neuronal cells over wild type ChR2 (Nagel et al., 2005; Beppu et al., 2014). Astrocytes process intracellular changes in Na^+, Ca^{2+}, and protons in different ways that may affect several different astrocyte functions. For example, increases in intracellular sodium and membrane depolarization, which is known to occur with ChR2-stimulation (Gourine et al., 2010), could alter glutamate reuptake efficacy (Djukic et al., 2007; Unichenko et al., 2012; Verkhratsky et al., 2013). Alterations to intracellular pH in astrocytes are important in the regulation of ischemic damage *in vivo* (Benesova et al., 2009; Beppu et al., 2014) and gap junction connectivity (Duffy et al., 2004). It is not unreasonable to hypothesize that ChR2 activation leads to membrane depolarization of astrocytes and interferes with a number of transport systems, most importantly glutamate, which may lead to effects on neuronal activity. In addition, to use optogenetics *in vivo* it is necessary to use a viral vector to deliver the optogenetic construct and may require the insertion of a light probe to activate the optogenetic channel in deeper brain regions. It seems very likely that these insults will lead to pathological

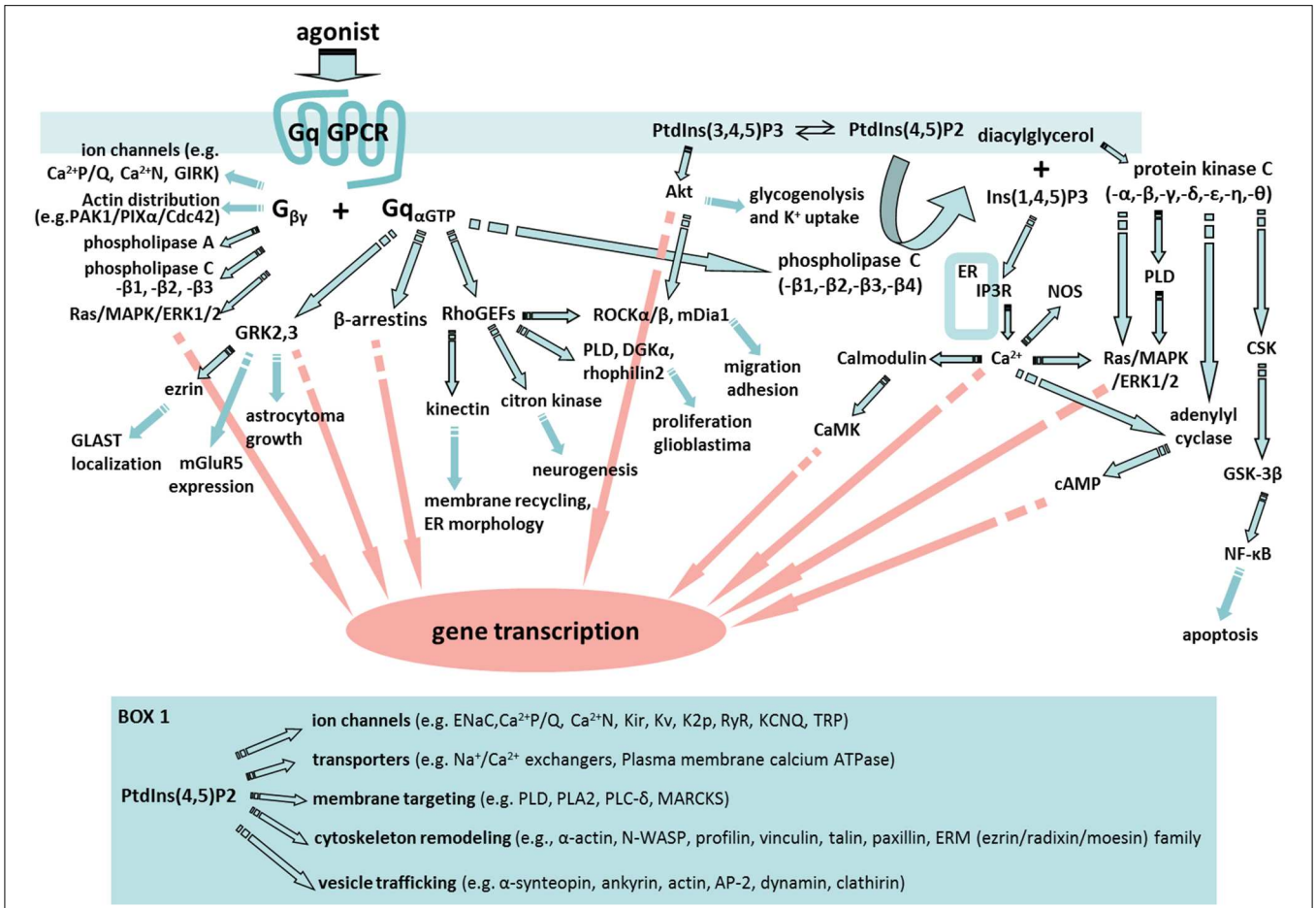

FIGURE 1 | A fabric of signaling cascades can be activated by astrocyte Gq-GPCRs. Upon Gq-GPCR activation, Gαq subunits are known to interact with G protein-coupled receptor kinases (GRKs), β-arrestins, Rho family of guanine exchange factors (RhoGEFs) and phospholipase C (PLC) in astrocytes or astroglia. In addition, Gβγ subunits are able to regulate ion channel properties, as well as to interact with signaling molecules including cdc42, PAK-PIXα, phospholipase A (PLA), PLC, and Ras/MAPK/ERK1/2. Many of the key signaling molecules regulated by Gβγ are expressed in astrocytes or astroglia. Therefore, Gq-GPCR activation in astrocytes is likely to activate an entire fabric of downstream signaling pathways that may include (1) GRKs-mediated glutamate transporter (GLAST) localization (Nijboer et al., 2013), gene transcription (Atkinson et al., 2009), metabotropic glutamate receptor 5 (mGluR5) expression (Sorensen and Conn, 2003), and astrocytoma growth (Woerner et al., 2012); (2) β-arrestin-mediated gene silencing (McLennan et al., 2008; Miyatake et al., 2009; Zhu and Reiser, 2014); (3) RhoGEFs-mediated activation of Kinectin (Santama et al., 2004), citron kinase (Ackman et al., 2007), phospholipase D (PLD) (Burkhardt et al., 2014), diacylglycerol kinase (DGKa) (Kefas et al., 2013), Rhophilin2 (Vedrenne and Hauri, 2006; Danussi et al., 2013), Rock (Holtje et al., 2005; Lau et al., 2011), and mDia1 (Shinohara et al., 2012); (4) PLC regulation of PtdIns(3,4,5)P3 (PIP3)/protein kinase B (PKB or Akt) pathway (DiNuzzo et al., 2013; Kong et al., 2013), PtdIns(4,5)P2(PIP2)/diacylglycerol (DAG)/protein kinase C (PKC) pathway, and PIP2/Ins(1,4,5)P3(IP3) pathway. Moreover, PKC activation in astrocytes (Wang et al., 2002) engages PLD (Servitja et al., 2003), c-src tyrosine kinase (CSK) (Jo et al., 2014), glycogen synthase kinase (GSK) (Sanchez et al., 2003), and cAMP signaling. Many of these signaling pathways are known to trigger cellular responses that are important for astrocyte function including gene transcription and cell migration. Box 1. Selected PIP2-induced signaling pathways in which key molecules are expressed by astrocytes. These signaling molecules include: Epithelial sodium channel (ENaC) (Miller and Loewy, 2013), PLA2 (Ha et al., 2014), Myristoylated alanine-rich C-kinase substrate (MARCKS) (Vitkovic et al., 2005), Wiskott–Aldrich syndrome protein (WASP) (Murk et al., 2013), profilin (Molotkov et al., 2013), vinculin, talin, and paxillin (Kalman and Szabo, 2001), ERM protein family (Persson et al., 2010), α1-syntrophin (Masaki et al., 2010), ankyrin (Lee et al., 2012), adipocyte protein 2 (AP2) (Rossello et al., 2012), and clathirin (Pascual-Lucas et al., 2014).

changes that alter astrocyte responses. Overall, while optogenetic tools are great tools to activate or silence neurons, using optogenetics to increase astrocyte Ca^{2+} bypasses the majority of signaling pathways that are activated by GPCRs, as well as result in aberrant ion fluxes that likely never occur under physiological conditions (Fiacco et al., 2007; Agulhon et al., 2012; Wang et al., 2013).

Selective Activation of Astrocyte Gq-GPCR Signaling with MrgA-1

The first experimental system that enabled the specific activation of endogenous astrocyte signaling cascades in complex tissue was the Mas-related gene A1 (MrgA-1) transgenic mouse line (Fiacco et al., 2007) (**Table 1**). MrgA-1 is a Gq-GPCR that is normally expressed in a subsets of nociceptive sensory neurons

TABLE 1 | Tools for selectively activation of astrocyte signaling *in vivo*.

Class	Tools	Molecule	Application method	Comments	Selected references
Optogenetics	Photo-activated cation channel, ChR2 variant	ChR2 (H134R)	Lenti-viral vectors	Localized, high temporal control of glial activation, yet bring in non-physiological sources of Ca^{2+} and significantly alter intracellular pH; Non-selective to all cations; Require viral injections and light-source	Gourine et al., 2010; Figueiredo et al., 2014; Perea et al., 2014
		ChR2 (L132C); CatCh	AAV vector	Improved Ca^{2+} permeability compared to ChR2 (H134R) with varying reliability; Tested in cultured astroglia and no *in vivo* data	Figueiredo et al., 2014; Li et al., 2012
		ChR2 (C128S)	tetO-ChR2(C128S)-EYFP β-actin locus knockin mice (crossed to Mlc1-tTA mice)	Does not require virus injection but requires light activation. Highly sensitive to light activation. Provide high temporal control of glial acidification	Tanaka et al., 2012, 2010; Beppu et al., 2014
	Photo-activated outward proton pump	Archaerhodopsin (ArchT)	tetO-ArchT-EGFP β-actin locus knockin mice (crossed to Mlc1-tTA mice)	Does not require virus injection but requires light activation; High temporal control; Only available tool for glial alkalization	Tanaka et al., 2010; Tsunematsu et al., 2013; Beppu et al., 2014
	Photo-activated Ca^{2+}-permeable iGluR6	LiGluR	Lipofectamine 2000 transfection	Varying reliability; Tested in cultured astroglia and no *in vivo* data	Li et al., 2012
Pharmacogenetics	Endogenous Gq GPCR expressed outside of the brain	MrgA-1	tetO-MrgA-1 transgenic mouse line (crossing to GFAP-tTA*)	Stable expression in brain astrocytes; Peptide ligands do not cross BBB, therefore requires brain infusion, injection or implantation for *in vivo* activation; Low temporal control; Potential over-activation	Fiacco et al., 2007; Agulhon et al., 2010; Cao et al., 2013
	Engineered Gq GPCR	Gq-DREADD (hM3Dq)	GFAP-hM3Dq transgenic mouse line**; AAV-GFAP***	Stable expression in brain astrocytes; ligand crosses BBB and bio-inert; Long-lasting physiological and behavioral phenotypes; Also expressed in PNS $GFAP^+$ glial cells and certain non-neural cells; Low temporal control; Potential over-activation	Armbruster et al., 2007; Agulhon et al., 2013; Bonder and McCarthy, 2014
	Engineered Gi GPCR	Gi-DREADD (hM4Di)	Transgenic mouse line	Under characterization	Armbruster et al., 2007
	Engineered Gs GPCR	Gs-DREADD	Transgenic mouse line; AAV-GFAP***	Under characterization	Guettier et al., 2009

**Mouse line available in the Jackson Laboratory, stock No. 005964; **For various DREADD constructs and transgenic mouse lines currently available, see http://pdspit3.mml.unc.edu/projects/dreadd/wiki/WikiStart; ***AAV-GFAP minimal promotor available via UNC Vector core.*

(Dong et al., 2001) but not in the brain. The ligand used to activate MrgA-1, the peptide FMRF, does not activate endogenous brain Gq-GPCRs (Fiacco et al., 2007). To achieve cell specificity in the brain, MrgA-1 expression was controlled by using a tetracycline-controlled inducible expression system (tetO system), which requires a second transgene, the transcription transactivator (tTA). By crossing tetO-MrgA-1 transgenic mouse to GFAP-tTA transgenic mouse, $GFAP^+$ glia become the only cells in the brain that express both tTA and tetO-MrgA-1, therefore the only CNS cells to express MrgA-1 receptor (Fiacco et al., 2007). As a native GPCR, MrgA-1 activation triggers the entire fabric of signaling cascades normally activated by endogenous astrocyte Gq-GPCRs; an important component that is absent when selectively increasing specific signaling molecules such as IP3 and Ca^{2+}. With respect to Ca^{2+}, MrgA-1 activation leads to a similar spatial and temporal response as endogenous Gq-GPCRs in hippocampal astrocytes (Fiacco et al., 2007).

MrgA-1 transgenic mice were used to prepare brain slices to test the gliotransmission hypothesis at hippocampal CA3-CA1 synapses (Fiacco et al., 2007; Agulhon et al., 2010; Wang et al., 2012, 2013; Devaraju et al., 2013). Initial reports showed that bath application of FMRFa induced widespread Ca^{2+} elevations in stratum radiatum astrocytes from MrgA-1 mice, while CA1 neuronal Ca^{2+} activity, excitatory synaptic transmission and short- or long-term excitatory synaptic plasticity in CA3-CA1 synapses were not affected (Fiacco et al., 2007; Agulhon et al., 2010). Later Wang et al. reported that although MrgA-1 mediated astrocyte activation did not change neuronal excitability and miniature excitatory synaptic currents (mEPSCs) in neurons near the surface of hippocampal slices, both bath and microinjection of FMRFa led to a transient hyperpolarization and decreased mEPSC frequency in neurons below 80 μm depth in the slices (Wang et al., 2012, 2013). These studies suggested that selective activation of Gq-GPCR signaling in astrocytes increased activity

of the Na^+/K^+ ATPase, resulting in a reduction of extracellular K^+ which consequently hyperpolarized neurons and suppressed excitatory transmission (Wang et al., 2012). The Gq-GPCR activated change in $[K^+]$ was hard to detect in the superficial layer of the slices, where the constant bath perfusion buffered the effects (Wang et al., 2012). The K^+ removal hypothesis was supported by an independent study from Devaraju et al. who found that both Schaffer Collaterals stimulation and selective stimulation of astrocytic MrgA-1s potentiated inward K^+ current and glutamate uptake in hippocampal astrocytes (Devaraju et al., 2013). These data suggest that astrocytic Gq-GPCR activation may regulate neuronal excitability and modulate neuronal network activity indirectly rather than inducing the release of gliotransmitters.

The MrgA-1 mouse line is rarely used for studying astrocyte function *in vivo* because the peptide agonists do not effectively cross blood brain barrier. Recently, Cao et al. used MrgA-1 mouse line to study the role of astrocytic activation in behavior by implanting infusion cannula or osmotic pumps into the brain of MrgA-1 mouse (Cao et al., 2013). Brain infusion of the peptide agonist of MrgA-1 mice induced antidepressant-like effect in forced swimming test and reversed depression-like behavior in MrgA-1 mice suggesting that astrocytic Gq-GPCR signaling is capable of modulating depressive-like behaviors (Cao et al., 2013).

Pharmacogenetic Activation of Astrocytic Signaling *In Vivo* Using DREADDs

In 2007, a new family of engineered GPCRs, Designer Receptor Exclusively Activated by Designer Drugs (DREADD) were developed (Armbruster et al., 2007) and have become the best option for activating GPCR signaling in specific cell populations *in vivo* (Rogan and Roth, 2011) (**Table 1**). A significant advantage of DREADDs compared to MrgA-1 is that the ligand of DREADDs, clozapine N-oxide (CNO), crosses BBB (Bender et al., 1994), therefore enabling non-invasive manipulation of receptor activity via peripheral injections (e.g., intraperitoneal or intravenous injections) and even via drinking water (Jain et al., 2013). The M3 muscarinic cholinergic receptor (M_3AChR) was engineered through directed molecular evolution (Dong et al., 2010) that led to a striking decrease the affinity of this receptor for its native agonist (acetylcholine) as well as to a large increase in affinity for CNO. In addition, DREADDs do not exhibit constitutive activity and CNO is pharmacologically inert in the absence of DREADDs (Armbruster et al., 2007; Nichols and Roth, 2009; Dong et al., 2010). Consequently, mice expressing DREADDs do not exhibit a phenotype in the absence of CNO and CNO does not lead to a phenotype in wild type mice (Alexander et al., 2009; Guettier et al., 2009; Agulhon et al., 2013). Since their development, DREADDs have been extensively used to chronically and acutely activate (Gq-DREADDs) and silence (Gi-DREADDs) specific subsets of neurons *in vivo* (Wess et al., 2013).

Gq-DREADD was introduced into astrocyte research studies with the development of GFAP-Gq-DREADD mice for specifically activating $GFAP^+$ glial Gq-GPCR signaling *in vivo* (Agulhon et al., 2013). Gq-DREADD expression was regulated by the 2.2 Kb human GFAP promotor fragment; a hemagglutinin (HA) tag was added to the N-terminus of the Gq-DREADD for highly specific antibody staining. Extensive immunostaining studies demonstrated that the expression of Gq-DREADD was restricted to $GFAP^+$ glia in the CNS and PNS (Agulhon et al., 2013). Bath application of CNO *in situ* or i.p. injection of CNO *in vivo* led to Ca^{2+} increases in brain astrocytes, without affecting Ca^{2+} in nearby neurons; CNO induced Ca^{2+} increases occurred throughout astrocytes including their fine processes within the neuropil. The development of this model enabled, for the first time, examination of the behavioral and physiological consequences of specifically activating Gq-GPCR signaling in $GFAP^+$ glia. CNO administration to GFAP-Gq-DREADD transgenic mice revealed robust and unexpected behavioral and physiological phenotypes that were absent in litter mate controls; phenotypic changes include robust increases in heart rate and blood pressure, saliva formation, a decrease in body temperature, and increased sedation in the presence of a GABA receptor agonist (Agulhon et al., 2013). These findings suggest that $GFAP^+$ glia have the potential for modulating a number of important physiological processes.

In addition to GFAP-Gq-DREADD mice, transgenic mouse lines expressing Gs- and Gi-DREADD specifically in $GFAP^+$ glia were developed and are currently under characterization in the McCarthy laboratory. GFAP-DREADD transgenic mice offer the best system to non-invasively and simultaneously activate widely distributed astrocyte populations. Other systems for manipulating astrocytic activity *in vivo*, including MrgA-1 transgenic mice and optogenetics, requires direct application of ligand/light to brain tissue and thus have spatial limitations with regard to cells being activated at a given time. Region-specific expression DREADD can be achieved via viral delivery (Bull et al., 2014). Once the expression pattern is established, one can activate a subset of astrocytes acutely or chronically in dose-dependent manner, and behavior and physiological outcome can be measured from free-moving, awake mice. For the first time in astrocyte research, we can now test the contribution of astrocytes GPCR signaling in physiology and behavior, as well as verify the previously known astrocytic function in intact animals.

There are caveats associated with using pharmacogenetic systems to study the role of astrocyte Gq-GPCR signaling *in vivo*. First, engineered GPCRs are driven by an exogenous promoter system and consequently the levels of expression are likely to be different than that of endogenous astrocytic GPCRs. While this does not appear to lead to markedly different Ca^{2+} responses compared to endogenous receptors, it has not been confirmed that the engineered GPCR signaling cascades are regulated in a similar manner. Second, it is impossible to mimic the temporal and spatial characteristics of *in vivo* GPCR activation, a caveat associated with all pharmacological stimulation. Following an i.p. injection of CNO, most of the physiological phenotypes were observed in 5 min and peak in 30 $\sim$ 45 min (Agulhon et al., 2013). Increased temporal and spatial resolution can be obtained by either microinjecting CNO into the region of interest or uncaging CNO with laser pulse activation; the latter approach has temporal and spatial resolution similar to optogenetic activation; caged CNO has recently been prepared (Brian Roth, personal communication). Overall, DREADD technology enables activation of the entire fabric of endogenous signaling cascades in specific cell types that are generally stimulated by GPCRs; this

is a striking advantage over most other methods used to activate glial signaling *in situ* or *in vivo*.

Selective Astrocyte Gene Rescue in Mice with Global Gene Deletion

The optogenetic and pharmacogenetic approaches for activating astrocyte *in vivo* share a common pitfall of potential over-activation. Recently, a conditional endogenous gene repair approach was used to isolate the role of astrocyte-specific endogenous signaling *in vivo* in a mouse model of Rett's syndrome (RTT).

RTT is an X-chromosome-linked autism spectrum disorder due to the loss of function of the transcriptional regulator methyl-CpG-binding protein 2 (MeCP2) in the brain (Amir et al., 1999; Guy et al., 2007). Because MeCP2 is expressed in all CNS cell types (Ballas et al., 2009), a conditional knock-in mice, $MeCP2^{loxp}$ mice, was developed to study cell-specific disease mechanisms. In this model, the endogenous *Mecp2* gene is silenced by insertion of a *lox*P-*Stop* cassette, but can be activated when combined with Cre- or Cre-ER system (Guy et al., 2007). When $MeCP2^{loxp}$ mice were crossed to hGFAP-$CreER^{T2}$ mice (Hirrlinger et al., 2006), the expression of MeCP2 was selectively restored in $GFAP^{+}$ astrocytes when mice were treated with tamoxifen (Lioy et al., 2011). The specific re-expression of MeCP2 in astrocytes significantly improved RTT phenotype possibly by restoring normal dendritic morphology and levels of the excitatory glutamate transporter VGLUT1 (Lioy et al., 2011). This model illustrates the potential of using conditional astrocyte-specific rescue model to isolate the function of astrocyte signaling *in vivo* and in disease.

Spatial Control of Astrocytic Signaling via Viral Delivery—Advantages and Disadvantages

At this time, there are no astrocyte transcriptional units that can be used to target specific populations of astrocytes in mature brain. Consequently, a large number of investigators have used viral vectors to perturb signaling in subpopulations of astrocytes using both adeno-associated viruses (AAV) and lentiviral vectors (Figueiredo et al., 2014). Several AAV serotypes show tropism toward astrocytes, including AAV 2/5 and AAV 8 (Koerber et al., 2009; Aschauer et al., 2013; Petrosyan et al., 2014). In combination with an astrocytic selective promoter (Lee et al., 2008; Pfrieger and Slezak, 2012), these AAVs are expected to express the gene of interest selectively in astrocytes in specific brain regions.

The disadvantages of using viral vectors to express genes in astrocytes should not be overlooked. The most obvious concerns are tissue damage and reactive gliosis induced by viral injection. Reactive astrocytes display more robust, frequent and widely-spread intracellular Ca^{2+} activity, and intercellular coupling and Ca^{2+} waves are exaggerated among reactive astrocytes (Agulhon et al., 2012). A recent study showed that AAV 2/5 vector can be used to induce astrogliosis and disrupt the glutamate-glutamine cycle in astrocytes, which led to glutamate-reversible hyperactivity of nearby neurons (Ortinski et al., 2010). The primary methods for assessing whether or not astrocytes are pathologically transformed by viral infection are through morphological studies and GFAP expression levels (Xie et al., 2010; Shigetomi et al., 2013a). However, it is likely that more subtle undetected changes in astrocytes occur that could influence their functional interactions with surrounding cells.

Another challenge of using viral injection to express constructs in astrocytes is cell-specificity. AAVs show tropism to all cell types in the CNS (Aschauer et al., 2013; Gholizadeh et al., 2013; Petrosyan et al., 2014; Yang et al., 2014). Even with an astrocytic-specific promoter, it is important to carefully verify astrocyte specific transduction using the most sensitive methods available. A low level of GPCR expression in non-astrocyte cell types could lead to significant downstream signaling and confound the interpretation of findings.

One final consideration to keep in mind is that it is currently impossible to transduce functionally distinct populations of astrocytes in a manner analogous to transducing a subpopulation of functionally distinct neurons; this will only be solved as new subpopulation specific astrocyte transcriptional units are identified.

Current Knock-Out Models for Selective Inactivation of Astrocytic Signaling

Specific GPCR Knockout Models

To date, most investigators have used optogenetic, pharmacogenetic or pharmacological tools to determine the consequences of activating astrocyte signaling cascades (Agulhon et al., 2008). None of these approaches recapitulate the complex regulation of signaling cascades occurring *in vivo*. Findings from these studies (see review by Agulhon et al., 2008) provide insight into the potential outcome(s) of astrocyte signaling. However, one has to keep in mind that these highly artificial types of stimulation may lead to outcomes that rarely, or perhaps never, occur in physiology. To determine the functional significance of astrocytic signaling, a more powerful approach is to demonstrate that the loss-of-function of a particular pathway affects physiological processes such as synaptic transmission or behavior. Here we review current knockout (KO) and conditional KO (cKO) models for deleting specific astrocytic signaling pathways (**Table 2**).

GFAP-CB1R-KO

One of the first GPCRs targeted for a cKO in astrocytes was the cannabinoid type-1 receptor (CB1R), a GPCR predominately coupled to Gi signaling (Han et al., 2012). GFAP-CB1R cKO mouse line was accomplished by crossing floxed CB1R mouse line with an inducible Cre system driven by the GFAP promoter (GFAP-CreERT2) (Hirrlinger et al., 2006). Following tamoxifen administration, CB1R expression was reduced in $GFAP^{+}$ astrocytes in the brain, providing the first model to study the contribution of astrocytes in cannabinoid induced working memory impairment *in vivo*. Han et al. found that conditional knockout of astrocytic CB1R abolished cannabinoid-induced impairment of spatial working memory and *in vivo* long-term depression at hippocampal CA3-CA1 synapses. In contrast, a cKO of CB1R in glutamatergic or GABAergic neurons did not rescue cannabinoid induced deficit (Han et al., 2012). This study revealed significant impact of a single astrocyte GPCR signaling system on synaptic

TABLE 2 | Current molecular tools for perturbing astrocyte signaling *in vivo*.

Class	Mouse line	Confirmed expression profile	*In vivo* phenotype	Comments	Selected references
GPCR KO	Floxed CB1R mouse line (crossed to GFAP-CreERT2)	79% reduction in the number of CB1R^{+} CA1 astrocytes by immunohistochemistry	Cannabinoid-induced impairment of spatial working memory and *in vivo* long-term depression at hippocampal CA3-CA1 synapses were abolished	For GFAP-CreERT2 please see Hirrlinger et al., 2006	Marsicano et al., 2003
	Floxed DrD2 mouse line (crossed to hGFAP-Cre)	No marked excision detected in striatal tissue by qPCR analysis	Mild increase in pro-inflammatory mediators in the striatum was detected by qPCR; Neuronal development, neuronal numbers and astrogliosis in advanced age appear normal in the substantia nigra (SNC) and the ventral tegmental area (VTA)	Compared to global DrD2 KO, the pro-inflammatory phenotypes of astrocytes in the SNC and in the striatum are very mild. Further characterization in other brain regions needed	Shao et al., 2013 (created by Shanghai Research Center for Model Organisms)
Perturbing Ca^{2+} signaling	Global IP3R2 KO	Global knockout	Loss of astrocyte GPCR mediated Ca^{2+} increase; conflicting evidence for alterations in synaptic transmission; minor behavioral alterations; no evidence for altered vascular control; alterations in astrocyte processes mobility in response to LTP-inducing sensory stimuli	There are two sources for the global IP3R2 KO: Ju Chen lab at UCSD (used by McCarthy lab) and Katsuhiko Mikoshiba lab at RIKEN Brain Science Institute. There has been no direct comparison of these two lines for differences	Petravicz et al., 2008; Agulhon et al., 2010; Navarrete et al., 2012; Bonder and McCarthy, 2014
	Floxed IP3R2 (crossed to hGFAP-Cre)	Conditional knockout, with 80–95% reduction in the number of IP3R2^{+} astrocytes in brain by immuno-histochemistry and Gq GPCR agonist-evoked Ca^{2+} imaging	Loss of astrocyte GPCR mediated Ca^{2+} increases; no reported behavioral phenotypes; reduced cholinergic modulation of visual responses	For hGFAP-Cre, please see Casper and McCarthy, 2006	Chen et al., 2012; Petravicz et al., 2014
	AAV2/5-gfaABC1D-p130PH		Reduced ATP-evoked Ca^{2+} signaling *in vitro* and *in vivo*	No effect on spontaneous Ca^{2+} increase	Xie et al., 2010
	tetO-GST-"IP3 sponge" transgenic mice (crossed to GLT-1-tTA mice)	80–90% S100β+ cells are lacZ+ in many brain regions including CA1; Each astrocyte on average express 3800 "IP3 sponge" molecules	Reduced spontaneous and GPCR evoked Ca^{2+} responses; reduced synaptic coverage by astrocyte processes; impaired glutamate uptake; impaired spatial reference memory and contextual fear responses	While alterations to the listed behaviors and impaired glutamate uptake were observed, there we no significant alterations to any synaptic measures such as LTP and LTD	Tanaka et al., 2013
Blocking vesicular release	tetO-dnSNARE (crossed to GFAP-tTA)	Global astrocyte expression; recently discovered cortical neuronal expression	Changes in sleep-wake cycle, sleep loss related cognitive functions, cortical slow waves, depressive-like behavior, LTP induction threshold in hippocampus, neuronal survival after stroke, development of temporal lobe epilepsy, and baseline mechanical nociception	Recent in-depth analysis of dnSNARE expression in large population of neurons suggest re-evaluation of thought-to-be astrocyte-driven *in vivo* phenotypes	Pascual et al., 2005; Nam et al., 2012; Fujita et al., 2014
	TRE-loxP-STOP-loxP-TeNT$^{\Delta 1}$-GFP (crossed to GFAP-tTA and GFAP-CreERt2 lines)	No direct data on the percentage of astrocytes expressing TeNT in different brain regions from the transgenic animals	Unaltered sleep patterns, only the EEG power in low-gamma range during wakefulness was reduced; Unaltered basal synaptic transmission as well as normal short- and long-term plasticity in hippocampus	Characterization of TeNT expression profile is needed at least in the region-of-interest in future studies. The expression is expected to be variable among users due to differences in the induction protocols	Lee et al., 2014

(Continued)

TABLE 2 | Continued

Class	Mouse line	Confirmed expression profile	*In vivo* phenotype	Comments	Selected references
Glutamate transporter KO	Global Glt-1 KO	Global knockout	Reduced body weight, increased morbidity, excitotoxic neuronal death		Tanaka et al., 1997
		Heterozygous global knockout	Behavioral alterations in sensorimotor function, locomotor activity, anxiety, contextual and cue-based fear conditioning		Kiryk et al., 2008
	Floxed Glt-1 (crossed to GLAST-CreERT2)	Conditional knockout	Excessive repetitive and injurious self-grooming, bodily tics, increased excitatory transmission at corticostriatal synapses		Aida et al., 2015
	Global GLAST KO	Global knockout	Altered glutamate/glucose uptake and sensory evoked neuronal oscillations in olfactory bulbs; impaired vestibular activity in aged mice; reduced sociability, reduced acoustic startle response; impaired visual discrimination; hyperactive locomotor activity		Karlsson et al., 2008, 2009; Martin et al., 2012; Schraven et al., 2012
NT synthesis enzyme KO	Nitrous oxide synthase 2 KO	Global knockout	Increased NO production *in vivo*; stress and anxiety-related behavior alterations		Buskila et al., 2007; Abu-Ghanem et al., 2008
	Serine-racemase cKO	Conditional knockout; Analyses of astrocyte specific D-serine KO mice show only a modest reduction in serine racemase activity		No alteration to hippocampal LTP *in situ*; No *in vivo* phenotype to date	Benneyworth et al., 2012
Ion channel KO	Kir4.1 cKO	Conditional knockout; Complete loss of Kir4.1 expression from S100β+ and GLAST+ astrocytes as well as CNP+ oligodendrocytes throughout brain and spinal cord	Increased morbidity, ataxia, stress-induced seizures, brain anatomical changes, white-matter vacuolization; reduced potassium buffering *in vivo*	Disorganized and fragmented processes of cerebellar Bergman glia, spinal cord white matter astrocytes, and motor neurons	Djukic et al., 2007; Chever et al., 2010
Connexin KOs	Cx30 and Cx43 KO and cKO	Multiple global and conditional knockout models	Alterations to spatial working memory, motor coordination, sensorimotor adaptation, anxiety, and pain perception	There exist multiple global, conditional and combinatorial knockouts (for example global Cx30 KO with astrocyte specific Cx43 knockout)	For comprehensive reviews of connexin genetic models, please see reviews by Giaume and Theis (2010) and Pannasch and Rouach (2013)

modulation and behavior, as well as presented an elegant *in vivo* system for temporal control of astrocytic signaling.

GFAP-DRD2-cKO

In 2013, an astrocyte dopamine D2 receptor (DRD2) conditional knockout mouse was generated to study the role of astrocytic GPCR signaling in aging-related neuroinflammation (Shao et al., 2013). DRD2 couples to Gi (Missale et al., 1998) and is expressed in astrocytes *in vivo* (Khan et al., 2001). During aging, DRD2 expression is downregulated in the brain (Kaasinen et al., 2000), suggesting potential involvement of DRD2 in aging related neuroinflammation. In this study DRD2-deficient astrocytes were found to produce more proinflammatory mediators compared to wild-type astrocytes. Further, this effect appears to be mediated through a decrease in αB-crystallin (CRYAB) signaling, a small heat-shock protein known to negatively regulate

pro-inflammatory mediator production and to display neuroprotective effect (Ousman et al., 2007). Interestingly, DRD2-deficient astrocytes also show robust GFAP upregulation, and a reactive morphology in the substantia nigra and the striatum of aged mice (Shao et al., 2013), suggesting the possible link between astrocytic GPCR signaling and age-related impairments in cognitive and motor function.

Models for Removing or Partially Removing Astrocytic Ca^{2+} Fluxes *In Vivo*

IP3R2 Germline and Conditional IP3R2 Knockout

Astrocytic Gq-GPCR/PLC/IP3 signaling is the most intensely studied pathway in the proposed modulation of neuronal activity and cerebral blood flow by astrocytes and has been the subject of numerous reviews (Haydon and Carmignoto, 2006; Agulhon et al., 2008, 2012; Fiacco et al., 2009; Halassa and Haydon, 2010; Hamilton and Attwell, 2010). The activation of this pathway results in the release of Ca^{2+} from IP3 receptor (IP3R) regulated intracellular stores in the endoplasmic reticulum. Astrocytes express only one of three subtypes of this receptor, IP3R type 2 (IP3R2) (Sharp et al., 1999; Holtzclaw et al., 2002; Foskett et al., 2007; Hertle and Yeckel, 2007). The first functional report to confirm this was published by Petravicz et al. (2008), in which the germline IP3R2 knockout mouse (generated by the Ju Chen lab; Li et al., 2005) was found to lack somatic increases in intracellular Ca^{2+} upon activation of multiple subtypes of Gq-GPCRs known to exist on astrocytes. Further experiments using this model have confirmed that astrocytes lack Gq-GCPR elicited, IP3R-dependent Ca^{2+} signals in their processes and soma (Agulhon et al., 2010; Di Castro et al., 2011; Panatier et al., 2011; Takata et al., 2011; Navarrete et al., 2012; Tamamushi et al., 2012; Nizar et al., 2013). The IP3R2 KO mouse is fertile, displays no overt alterations in brain development, and displays no obvious behavioral alterations (in contrast to the IP3R1 KO, the primary neuronal IP3R Matsumoto and Nagata, 1999). Due to these features, this mouse model has become one the most utilized mouse models in astrocyte research. Use of this model has led to novel findings (Panatier et al., 2011; Navarrete et al., 2012; Haustein et al., 2014; Perez-Alvarez et al., 2014), several of which have been contradictory with previously held theories concerning astrocyte-neuron communication and vascular control (Fiacco et al., 2007; Petravicz et al., 2008; Agulhon et al., 2010; Nizar et al., 2013; Takata et al., 2013; Bonder and McCarthy, 2014). Recently, evidence of residual Ca^{2+} signaling of a non-IP3R origin has been published using genetically encoded Ca^{2+} indicators (GECIs) in the IP3R2 KO model (Haustein et al., 2014; Kanemaru et al., 2014), further illustrating the model's usefulness for discovery of novel signaling events in astrocytes; importantly, there is no evidence suggesting that this residual Ca^{2+} signaling is regulated by neuronal activity.

Due to its restricted expression pattern in the CNS, a germline knockout of IP3R2 provides a clean and reliable model to block the release of intracellular Ca^{2+} in astrocytes elicited by Gq-GPCR activity *in vivo*. However, this model suffers in that it lacks tissue specificity, as do all germline knockout models. IP3R2 is expressed in multiple tissues outside the CNS including the heart (Li et al., 2005), pancreas (Orabi et al., 2012), lungs, liver, and kidneys (Fujino et al., 1995); this leads to potential confounding issues in the use of this model *in vivo* when assessing the role of astrocyte GPCR-dependent Ca^{2+} fluxes in behavior. Additionally, concerns over compensation due to the role of intracellular Ca^{2+} signaling during development have been raised regarding this model; however to date no evidence for altered development leading to compensation has been reported.

In an attempt to address some of these issues, a conditional IP3R2 knockout mouse model was developed by our laboratory. This model was generated by crossing the original floxed IP3R2 mouse developed by Dr. Ju Chen at UCSF (Li et al., 2005) to a GFAP-Cre recombinase mouse (Stehlik et al., 2006) to restrict the deletion of IP3R2 to $GFAP^+$ cells in the CNS. This model recombines the floxed IP3R2 allele at a high rate (>80–85% $GFAP^+$ cells lack IP3R2), significantly reducing the number of astrocytes responding to Gq-GPCR activation or neuronal activity in multiple brain regions (Petravicz et al., 2008; Chen et al., 2012). The use of the GFAP-Cre system spatially and temporally restricts the IP3R2 deletion to $GFAP^+$ glia, thereby making it more appropriate for *in vivo* analysis such as behavioral characterization and reduces potential developmental compensation. Our lab recently published a behavioral analysis of the IP3R2 cKO mice and found no significant alteration to behavior (Petravicz et al., 2014). Most importantly, no alterations to learning and memory as assessed by the Morris Water Maze test were observed in these mice, despite previous literature proposing an important role for astrocyte IP3R-mediated Ca^{2+} signaling in hippocampal LTP. It is unlikely that developmental compensation occurs such that alternative ions substitute for Ca^{2+} in physiological processes or that global rewiring of neuronal circuits occurs to compensate for the loss of astrocyte Ca^{2+} fluxes. Nevertheless, to completely rule out developmental compensation, it will be necessary to prepare inducible IP3R2 cKO mice using mice expressing floxed IP3R2 and an astrocyte specific inducible Cre system.

IP3 Sponges

Activation of Phospholipase C beta (PLCβ) and sequential release of IP3 are the key steps in Gq-GPCR mediated intracellular Ca^{2+} elevations. Traditional approaches to abolish Gq-GPCR mediated Ca^{2+} elevation in astrocytes include chelating intracellular Ca^{2+} with BAPTA, or preventing IP3-mediated release of Ca^{2+} from ER using IP3R2 KO mice. Recently, the Pleckstrin Homology domain of PLC-like protein (p130PH), which binds cytosolic IP3 molecules, was used to suppress astrocytic Ca^{2+} signaling *in vitro* and ATP-induced astrocytic Ca^{2+} responses *in vivo* (Xie et al., 2010). In this study, p130PH was selectively expressed in cortical astrocytes *in vivo* using rAAV2/5 vector in combination with a specific astrocyte promoter, gfaABC(1)D (Lee et al., 2008; Xie et al., 2010). p130PH transduced astrocytes in the somatosensory cortex exhibited reduced amplitude and frequency of Ca^{2+} activity in response to direct ATP application on cortex compared to non-transduced astrocytes, whereas the characteristics of spontaneous Ca^{2+} activity in p130PH-transduced astrocytes remained unchanged (Xie et al., 2010). Therefore, p130PH serves as a more selective tool to suppress Gq-GPCR induced Ca^{2+} elevations without chelating Ca^{2+} activity in astrocytes completely. This system serves as a nice addition to IP3R2KO and IP3R2 cKO

mouse lines to distinguish astrocyte functions regulated by ER released Ca^{2+} vs. channel mediated Ca^{2+} *in vivo*.

The expression of a fragment of the IP3 binding domain of IP3R1, an "IP3 sponge" (Iwasaki et al., 2002) can also be used to suppress IP3 induced Ca^{2+} release in astrocytes *in vivo* (Tanaka et al., 2013). These investigators found that suppression of astrocyte Ca^{2+} responses affected several behavioral responses, but no underlying evidence for alterations to synaptic transmission was found. Surprisingly, astrocytes expressing the "IP3 sponge" exhibited process retraction surrounding synapses, which was attributed to underlie the behavioral phenotypes. Recently, evidence that astrocyte processes retract in response to LTP-inducing stimuli *in vivo* was reported, and that this feature was lacking in IP3R2 KO mice (Perez-Alvarez et al., 2014). These findings appear to contradict those reported by Tanaka et al. (2013). Further comparison between these two methods of blocking astrocyte Ca^{2+} increases will be required to clarify this contradiction.

Blocking Vesicular Release from Astrocytes with dnSNARE or Tetanus Toxin

Ca^{2+} dependent release of neurotransmitters from astrocytes, termed "gliotransmission," is one of the most important concepts presented in glial biology over the past several decades (Araque et al., 2014). While several mechanisms have been suggested to underlie gliotransmission, most studies support a process dependent on a vesicular release system (i.e., a SNARE dependent process) similar to that found in neurons (Zorec et al., 2012; Sahlender et al., 2014). To test the significance of SNARE-mediated gliotransmission to synaptic function, a mouse line that expresses a dominant negative form of SNARE (dnSNARE) in astrocytes was developed in our laboratory in 2005 (Pascual et al., 2005). This line was prepared by coinjecting three independent constructs (tetO-lacZ, tetO-dnSNARE, and tetO-eGFP) into fertilized zygotes. In dnSNARE transgenic mice, the expression of the cytosolic portion of the SNARE domain of synaptobrevin 2, lacZ, and eGFP are controlled by tetracycline regulatory system. When crossed with GFAP-tTA mice, the expression of dnSNARE, lacZ, and eGFP are independently controlled by doxycycline. In the absence of doxycycline, dnSNARE is expressed and interferes with SNARE-dependent vesicular release. A limitation of this model is that since dnSNARE was not directly tagged, there is no way *in situ* to verify that it is not expressed in cells other than astrocytes. Nevertheless, the dnSNARE mice have been used in a large number of studies to demonstrate a role for gliotransmission in synaptic transmission, synaptic plasticity, as well as behavior. However, a recent paper performed an in-depth analysis of dnSNARE mice and found that that the expression of dnSNARE was also expressed by a large population of neurons (Fujita et al., 2014). Given the critical role of the SNARE complex in neurotransmitter release, these findings bring into question the validity of this model and the findings obtained using this system.

Studies using dnSNARE mice suggest that SNARE-mediated astrocytic release of ATP and subsequent adenosine receptor activation regulates neuronal excitability and synaptic plasticity in many brain regions as well as modulate certain behaviors including sleep (Nam et al., 2012). The dnSNARE mice exhibit a weak sleep phenotype under basal conditions, as well as an attenuated "rebound" response to sleep deprivation (Halassa et al., 2009). Cortical slow oscillations, a rhythm characterizing non-rapid eye movement (non-REM) sleep was also found impaired in dnSNARE mice (Fellin et al., 2009). Further, the hippocampal dependent memory deficits produced by sleep deprivation were rescued in dnSNARE mice (Florian et al., 2011). These studies suggest that astrocytes release ATP in vesicular manner and that this plays an important role in sleep patterns and cortical oscillations.

Tenanus toxins (TeNTs) are known to interfere with synaptic vesicular release as well as other processes dependent on vesicular protein trafficking (Galli et al., 1994). Recently, a transgenic model system was developed using TeNT to block vesicular release in astrocytes *in vivo* (Lee et al., 2014). This transgenic model took advantage of both the tetracycline inducible regulatory system and the Cre-dependent inducible system to block vesicular release from astrocytes. A transgenic line was prepared that contained the tetracycline response element (TRE) followed by a floxed stop cassette and a cassette that when expressed led to the expression of eGFP tagged TeNT (TRE-loxP-STOP-loxP-$TeNT^{\Delta 1}$-GFP). In this system, the expression of TeNT required Cre expression to remove the floxed stop cassette and tetracycline transactivator (tTA) to activate the TRE. TRE-loxP-STOP-loxP-$TeNT^{\Delta 1}$-GFP mice were crossed with GFAP-tTA and GFAP-CreERt2 lines to create a triple transgenic mouse line. In the triple transgenic mice TeNT can be expressed in astrocytes following tamoxifen treatment; the expression of TeNT is suppressed in the presence of doxycycline. In this model, astrocyte expression of TeNT requires that two different transgenes driven by the GFAP promoter be expressed in the same astrocyte markedly increasing the probability of astrocyte specific expression. Further, as TeNT was directly tagged with eGFP, it is possible to identify all cells expressing TeNT. In contrast to the impaired sleep pattern in dnSNARE mice, astrocytic TeNT-expressing mice showed unaltered sleep patterns compared to triple transgenic mice without tamoxifen or double transgenic mice with tamoxifin (Lee et al., 2014). Studies using this mouse line indicate that basal synaptic transmission as well as normal short- and long-term plasticity in hippocampus *in situ* is not altered by the expression of TeNT (Lee et al., 2014); these findings bring into question the concept that astrocytes release gliotransmitters that modulate synaptic transmission and plasticity via a vesicular dependent process. Interestingly, the EEG power in low-gamma range during wakefulness was reduced, whereas the EEG power during but not non-REM sleep remain unchanged (Lee et al., 2014). These observations in sleep regulation from astrocytic TeNT-expressing mice do not match with those from dnSNARE mice.

Memory deficit was also detected in the mice expressing TeNT in astrocytes using novel object recognition test (Lee et al., 2014). Fast local field potential oscillations in the gamma frequency are closely correlated with many cognitive functions, including learning, memory storage and retrieval and attention (Basar-Eroglu et al., 1996). Astrocytic TeNT-expressing mice did not show deficit in other behavior tests that involves simpler form of memory processing or are less dependent on cortical processing. The reduction of gamma oscillation power and significant deficit

in novel object recognition suggest that the fast neural circuit oscillations are regulated by astrocytes (Lee et al., 2014).

Mouse Models for Assessing Astrocyte Neurotransmitter Regulation

Glutamate Transporter Knockout Mouse Models

Astrocytes are responsible for 80–90% of glutamate reuptake in the brain (Tzingounis and Wadiche, 2007), and two of the five glutamate transporters (GluTs) are primarily expressed in astrocytes: Glt-1/EAAT2 and GLAST/EAAT1 (Danbolt, 2001; Huang and Bergles, 2004). There are currently germline knockout mouse models for both Glt-1 (Tanaka et al., 1997) and GLAST (Harada et al., 1998); however their use *in vivo* has been limited. The Glt-1 germline knockout mouse model suffers from reduced body weight, increased morbidity, and progressive neuronal death due to excitotoxocity (Tanaka et al., 1997). This limits the ability to conduct *in vivo* experiments, which typically require older mice, to examine the role of astrocytic glutamate reuptake via Glt-1. Recently, the heterozygous Glt-1 (Glt-1 Het) knockout model has become an attractive model for studying the role of Glt-1 *in vivo*. The Glt-1 Het model does not suffer from the more obvious adverse effects of the full Glt-1 knockout, and displays several interesting behavioral phenotypes and has allowed for the study of Glt-1 in several brain pathologies (Kiryk et al., 2008). The GLAST knockout mice are viable, enabling *in vivo* studies. GLAST is primarily expressed in the cerebellum and olfactory bulb (Regan et al., 2007), and this mouse model has led to interesting findings concerning the physiological (Martin et al., 2012; Schraven et al., 2012) and pathological (Karlsson et al., 2008, 2009) functions of GLAST. Recently, a Glt-1 floxed mouse model has been developed (Aida et al., 2015). Induced knockout of Glt-1 in adult animals resulted in development of repetitive behaviors and alterations to excitatory transmission due to reduced glutamate uptake. The floxed Glt-1 model when combined with inducible Cre systems will open up new avenues of research into the role of Glt-1 *in vivo* that were not possible due to the lethality of the germline Glt-1 KO.

Nitrous Oxide Synthase 2 Knockout

In the central nervous system nitric oxide serves a number of roles, and has been shown to act at glutamatergic synapses to enhance glutamate release (Garthwaite, 2008). While neurons in many brain regions are known to produce and release NO via nitric oxide synthase (nNOS), this does not account fully for the activity of NO in several brain regions where excitatory neurons lack nNOS expression. Astrocytes are known to express all three isoforms of NOS, and are the sole expressers of an inducible form of NOS (iNOS or NOS2) that is activated in response to physiological stress in a Ca^{2+} dependent manner (Murphy, 2000; Buskila et al., 2005; Amitai, 2010). Astrocyte-derived NO has been shown to enhance LTP of presynaptic afferents in the spinal cord, as well as enhance synaptic transmission in the neocortex in acute slice preparations (Ikeda and Murase, 2004; Buskila and Amitai, 2010). *In vivo* evidence for astrocytic-derived NO being a modulator of neuronal transmission has primarily come from the use of a NOS2 knockout mouse, in which it was discovered that deletion of the calmodulin-binding domain of NOS2 led to a net increase in overall NO concentrations in the brains of mutant mice. Interesting, the increase in NO originated from astrocytes through an alternate mechanism without alterations in the relative levels of NOS isoforms (Buskila et al., 2007). Further, these mice display stress and anxiety-related alterations to behavior suggesting a role for astrocyte-derived NO in the modulation of neural circuits (Abu-Ghanem et al., 2008). Currently, the role of astrocyte-derived NO in modulation of neuronal circuit activity remains an understudied area of glial research.

Astrocytic Serine-Racemase Conditional Knockout

D-serine has long been considered one of the three primary gliotransmitters along with ATP/adenosine and glutamate, with putative astrocyte derived D-serine reported to modulate neuronal NMDA receptors (Panatier et al., 2006; Billard, 2008; Oliet and Mothet, 2009; Martineau, 2013; Shigetomi et al., 2013b; Sild and Van Horn, 2013). The enzyme responsible for D-serine production (serine racemase, SR) was initially found to be primarily expressed by astrocytes with some modest expression in neurons (Schell et al., 1995; Mothet et al., 2005). However, more recent studies have called this expression pattern into question (Miya et al., 2008; Ding et al., 2011; Ehmsen et al., 2013). The most recent study of SR localization in mice and human brains finds that nearly all immunostaining for SR is found in neurons and not astrocytes (Balu et al., 2014). Recently, cell type specific knockouts of SR were generated to examine the relative contributions of astrocytes and neurons in the forebrain of mice (Benneyworth et al., 2012). The astrocyte specific knockout of SR led to a modest (∼15%) reduction in SR expression, while the neuronal knockout in forebrain neurons reduced SR by much larger amounts (∼65%). Further, the neuronal specific SR knockout displayed alterations to LTP at hippocampal synapses that were not found in the astrocyte SR knockout. These findings raise new questions in how astrocytes may be regulating D-serine availability in the brain, which the astrocyte specific SR knockout will be crucial to resolving.

Knockout Models for Astrocyte Membrane Channels

Kir4.1

One of the major roles in the CNS for astrocytes is the buffering of potassium ions in response to neuronal activity. Astrocytes express a variety of potassium channels, but among them Kir4.1 plays a predominant role in their K^+ buffering capacity (Takumi et al., 1995; Higashi et al., 2001; Djukic et al., 2007). A germline full Kir4.1 knockout model has provided insight into the role of potassium buffering in response to hyperammonemic conditions (Stephan et al., 2012) and the channel's role in regulating astrocyte membrane potential during development (Seifert et al., 2009). An astrocyte conditional knockout model for Kir4.1 was generated by the McCarthy lab to provide a cleaner animal model alternative to the germline KO (Djukic et al., 2007). The conditional knockout astrocytes display reduced glutamate clearance and decreased resting membrane voltage, while neuronal plasticity was enhanced implicating Kir4.1 as an important mediator of extracellular potassium regulation. These finding have been confirmed in an *in vivo* study utilizing the Kir4.1 cKO, with the

cKO mice found to have reduced capacity to regulate extracellular potassium levels compared to controls (Chever et al., 2010). Usage of this model in awake mice to examine the effect of altered potassium homeostasis may provide unique insights into how astrocytes regulate neuronal networks.

Connexins

Astrocytes in the brain exist not only as single units occupying a discrete domain, but also as a network of cells connected by connexin (Cx) gap junctions. Astrocytes express two major connexin proteins (Cx43 and Cx30) with germline and conditional knockout mouse models existing (Dermietzel et al., 2000; Teubner et al., 2003; Wiencken-Barger et al., 2007). These mouse models display a variety of alterations to behavior, synaptic transmission, metabolic support, and ion homeostasis (Giaume and Theis, 2010; Pannasch and Rouach, 2013). However, there are a large number of open questions concerning the role of gap junction communication and astrocyte networks *in vivo*. Currently, the only *in vivo* experiments in these models have involved behavioral studies, indicating a significant impact on neuronal circuit function (for an excellent review please see Pannasch and Rouach, 2013). Further exploration of neuronal activity *in vivo* utilizing these models, as well as generation of inducible knockout systems, represents novel avenues of research for understanding astrocyte network function.

Current Limitations in Genetically Targeting Astrocytes

At this time, there is not a single gene delivery system that can be used to exclusively express transgenes or recombine naïve genes in astrocytes. The common astrocyte marker proteins (GFAP, S100b, glutamine synthetase, aquaporin 4, connexin43, GLAST, GLT1, ALDH1L1) are either expressed in alternate subsets of mature CNS cells (Dunham et al., 1992; Zhuo et al., 2001; Su et al., 2004; Hachem et al., 2005; Regan et al., 2007; Donato et al., 2013), in progenitor CNS cells that give rise to multiple CNS cell types (Hartfuss et al., 2001; Casper and McCarthy, 2006), or are expressed outside the CNS (Jessen et al., 1990; Rinholm et al., 2007; Darlot et al., 2008; Meabon et al., 2012; Rutkovskiy et al., 2012; Jesus et al., 2014; Kato et al., 2014). The GFAP transcriptional regulatory unit (TRU) is probably the best characterized and most frequently used TRU for regulating gene expression in astrocytes and serves as a good example to illustrate the difficulties in targeting genes to astrocytes. The GFAP TRU is active in progenitor cells that give rise to astrocytes, neurons, and oligodendrocytes (Casper and McCarthy, 2006). Even in the mature CNS, certain populations of neurons express GFAP (Zhuo et al., 2001; Su et al., 2004; Regan et al., 2007). Further, in the periphery, non-myelinating peripheral glia (Jessen et al., 1990) as well as certain populations of non-neural cells (e.g., stellate cells in the liver) are $GFAP^+$ (Lim et al., 2008) and will express transgenes driven by the GFAP TRU. To avoid transgene expression in progenitor cells, many laboratories have developed inducible gene regulatory systems (Casper and McCarthy, 2006; Hirrlinger et al., 2006; Mori et al., 2006). This approach circumvents gene expression in progenitors during development but does not affect the expression of transgenes in peripheral glia, adult stem cells or small populations of neurons that normally express GFAP. How much of a problem this presents depends on the question being asked. When studying a transgene in the mature CNS that can be assumed not to affect developmental processes nor lead to a peripheral phenotype it is reasonable to use many of the available astrocyte TRU to drive transgenes to astrocytes. For example, the GFAP TRU can be used to express eGFP, GCaMP, or DREADD receptors that must be activated by an exogenous ligand without preventative concern about developmental expression. However, when using the GFAP TRU to drive bioactive molecules such as DREADD receptors or inducible Cre recombinase, it is important to remain cognizant that in addition to astrocytes, peripheral $GFAP^+$ cells, adult stem cells, and certain neurons will also be affected. Alternatively, when expressing molecules that innately affect biological processes (e.g., a dominant negative mutation, constitutively active signaling molecule or Cre recombinase), it is very important to consider the consequences of expression during development.

The above discussion assumes that the astrocyte TRU is acting with the fidelity of the endogenous TRU. Unfortunately, this is often not the case and is largely dependent on the genomic construct used to prepare the TRU. Most typically, investigators use a fragment of the TRU to drive transgene expression. As transgenes generally integrate somewhat randomly at active sites in the genome, the activity of surrounding genomic regulatory units can markedly affect transgene expression levels as well as the cells the transgene is expressed. This problem is markedly reduced using a BAC approach where very large genomic segments containing the TRU and inserted transgene are used to prepare transgenic lines. One final difficulty is that there are no astrocyte TRU systems that can be used to target subpopulations of astrocytes. Currently, the only way to genetically-manipulate subpopulations of astrocytes is to transduce these cells using viral vectors. Unfortunately, this generally restricts the size of the TRU used to target astrocytes and requires viral injection that may lead to subtle or striking changes in astrocyte function.

In summary, genetic manipulation of astrocytes is a very powerful tool for assessing the role of these cells in physiology, disease, and behavior. However, just as one has to verify the specificity of pharmacological reagents, great care must be used to insure the specificity of genetic manipulations.

Emerging Technologies for Manipulating Astrocytic Signaling *In Vivo*

Temporal Control of GPCR Signaling Using Optogenetic GPCRs

Optogenetically activated GPCR signaling is a reasonable alternative to ChR2 stimulation in astrocytes. There are two systems based on adrenergic receptors: Opto-alpha-1 (Gq linked) and opto-beta-1 (Gs linked) (Airan et al., 2009). Activation of these systems use similar experimental methods as optogenetics, but have the advantage of activating endogenous signaling cascades that exist in astrocytes. To date, these systems have not been tested for astrocytes *in vivo*, only in astroglia in culture (Figueiredo et al., 2014). While the Gq-linked opto-alpha-1

predictably elicited Ca^{2+} increases via release from intracellular stores, the Gs-linked opto-beta-1 was found to also trigger Ca^{2+} increases in a cyclic AMP-dependent manner. In culture systems, it has been shown that the Gβγ subunit of Gs-coupled GPCRs is capable of directly gating IP3Rs (Zeng et al., 2003). Recently, experiments in HEK293 cells discovered that IP3R2 complexes with Gαs and type 6 adenyl cyclase (AC6), and facilitates crosstalk between the two signaling pathways (Tovey et al., 2008, 2010). Further study into the mechanism behind increases in Ca^{2+} in astrocytes may lead to the identification of novel pathways regulating Ca^{2+} signaling.

Recently, the concept of using opsin based-pigments to develop optogenetic tools for modulating GPCR signaling was suggested (Koyanagi and Terakita, 2014). One of the candidates is melanopsin (OPN4), a Gq-coupled opsin that is originally found in a subtype of retinal ganglion cells (Hatori and Panda, 2010; Sexton et al., 2012). Several groups have used ectopic expression of OPN4 to control intracellular Ca^{2+} dynamics in neurons (Koizumi et al., 2013). In 2013, Karunarathne and colleagues used non-rhodopsin opsins to activate native Gq, Gi/o, and Gs signaling in localized regions of a single cell, and were able to gain spatial-temporal control over immune cell migration (Karunarathne et al., 2013b) as well as neurite initiation and extension (Karunarathne et al., 2013a). These studies suggest high potential of opsins as optogenetic GPCRs for *in vivo* astrocyte research.

Studying G-Protein Independent Signaling Using Biased DREADDs

One important aspect of GPCR signaling in astrocytes that has rarely been explored is the role of G-protein independent signaling. Endogenous GPCR activation not only initiates signaling via heterotrimeric G proteins, but also recruits proteins of the arrestin family, which act as scaffolding proteins and promote G protein-independent signaling (Pierce et al., 2002; Rajagopal et al., 2010; Shukla et al., 2011). Research has shown that arrestin 3 (β-arrestin 2) is expressed in astrocytes *ex vivo* (Bruchas et al., 2006; McLennan et al., 2008), and it is involved in kappa opioid receptor (KOR)-induced proliferation (McLennan et al., 2008; Miyatake et al., 2009), reduction of chemical-induced apoptosis (Zhu and Reiser, 2014), CXCR7 mediated inflammatory response (Odemis et al., 2012; Lipfert et al., 2013) and beta 2-adrenergic receptor (β2AR)-mediated glycogenolysis (Dong et al., 2001; Du et al., 2010) in astrocytes. As a scaffolding protein, β-arrestins also mediate internalization and ubiquitylation for many ion channels and transporters expressed in astrocytes (Shukla et al., 2011). As the list of signaling pathways that β-arrestins can regulate in astrocytes grows, it is important to dissect the relative contribution of G-protein independent signaling pathway to known functions of astrocytic GPCR signaling.

Recently, a modified Gq-DREADD that has strong biases toward arrestin-signaling was developed (Nakajima and Wess, 2012). This receptor was generated by introducing a point mutation within the highly conserved DRY motif [Rq(R165L)] of Gq-DREADD, which results in lack of ability to activate heterotrimetic G proteins. Therefore, CNO-induced Rq(R165L) activation has no effect on the levels of conventional second messages, but can promote CNO-dependent and arrestin-dependent signaling in biological systems (Nakajima and Wess, 2012). This novel GPCR represents an excellent tool to study the relative contribution of G protein-dependent and independent pathways in the known function of astrocytes, as well as reveal the physiological roles of astrocytic-arrestin signaling *in vivo*.

Conditional Gαq/Gα11 KO

GPCR KO mouse lines for all the known Gα subunits have been developed to analyze the physiological function of GPCR signaling *in vivo* (Offermanns, 1999), many of which show deficiencies in CNS-related physiology (Offermanns, 2001). Although these KO mouse lines are not astrocyte specific, inducible and conditional Gαq/Gα11 KO mice are available (Wettschureck et al., 2001) and can be combined with astrocytic specific Cre mouse line to selectively knock out Gq-GPCR signaling in astrocytes.

The conditional Gαq/Gα11 KO system was developed to study the role of Gαq/Gα11 signaling in specific tissues without developmental problems exhibited in the constitutive KO mouse line for both genes. This mouse line was developed using the Cre/LoxP system where the Gαq gene (*gnaq*) is conditionally inactivated in Gα11 KO (*gna11*–/–) mice, which do not have obvious behavior defect (Stanislaus et al., 1998). Therefore, mice homozygous for $gnaq^{flox}$ gene appear normal until Cre recombinase is introduced. The conditional Gαq/Gα11 KO mouse was first used with *MLC2a*-Cre to obtain cardiomyocyte-specific Gαq/Gα11 deficiency, resulting in a nearly complete recombination of $gnaq^{flox}$ in cardiomyocytes (Wettschureck et al., 2001).

In 2006, the Gαq/Gα11 cKO mice was crossed to mice that express Cre under the control of the promoter of the Ca^{2+}/calmodulin-dependent protein kinase IIα gene (*Camkcre4* mice) to generate forebrain specific and neuronal Gαq/Gα11 double KO mice (Wettschureck et al., 2004, 2006; Broicher et al., 2008), resulting in impaired endocannabinoid levels, increased seizure susceptibility (Wettschureck et al., 2006), and lack of maternal behavior in females (Wettschureck et al., 2004). The Gαq/Gα11 cKO line was also used to disrupt glial Gq/11 signaling in combination with Nestin-Cre mouse (Wettschureck et al., 2005), resulting in loss of Gq/11 in the neural stem cells that gives rise to both neurons and astrocytes. Although the Gq/11 signaling deficiency did not cause gross morphological changes in the developing nervous system, pups with insufficient Gq/11 signaling suffers from hypothalamic growth hormone deficiency and somatotroph hypoplasia, dwarfism, and anorexia (Wettschureck et al., 2005). Given the availability of astrocytic-specific Cre mice, it will be possible to isolate the contribution of astrocytic Gq/11 signaling *in vivo*.

Summary and Future Directions

There is no doubt that genetic tools will play an important role in understanding the role of astrocytes in physiology, behavior, and neurological disorders. Studies cited above provide strong evidence that astrocytes are doing much more than simply insulating synapses and providing nutrients to neurons. It is not

surprising that findings using genetic tools may conflict with previous findings using pharmacological methods to perturb astrocyte function. It is also not surprising that different genetic approaches (e.g., dnSNARE and tetanus toxin expression) may yield different results. These conflicts require additional studies using multiple tools (pharmacological and genetic) to refine our understanding of astrocyte function. Currently, there are several important limitations in the genetic tools available for perturbing astrocyte function *in vivo*. First, we are extremely limited in the transcriptional units (promoters) available for expressing molecules in astrocytes. Currently, all available transcriptional units drive transgene expression in cells other than astrocytes. Further, during development, most astrocyte promoters drive gene expression in progenitor cells that give rise to neurons, oligodendrocytes, and astrocytes (Casper and McCarthy, 2006). Consequently, it is necessary to use inducible systems linked to astrocyte promoters to avoid transgene expression in progenitors during development. Second, when expressing transgenes that drive function, it is important to remember that the transgene may be overexpressed and targeted to cellular compartments not normally found. While this can be partially overcome using an inducible gene regulatory system, it is likely that this will remain a caveat until we know a great deal more about the cell machinery in astrocytes that target molecules to specific cellular compartments. Third, we currently lack promoters that can be used to drive transgene expression in subtypes of astrocytes. Subtype specific astrocyte promoters will enable important advances with respect to the heterogeneity of astrocytes as well as their different functional roles.

In summary, while genetic manipulation of astrocytes is in its early stages of development, genetic models have already provided important insight into the role of astrocytes in physiology, behavior, and neurological diseases. It seems very likely that future advances in this field will depend largely on genetic approaches currently available as well as those under development.

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Astrocytes increase the activity of synaptic GluN2B NMDA receptors

*Junghyun Hahn, Xianhong Wang and Marta Margeta**

Department of Pathology, University of California San Francisco, San Francisco, CA, USA

Astrocytes regulate excitatory synapse formation and surface expression of glutamate AMPA receptors (AMPARs) during development. Less is known about glial modulation of glutamate NMDA receptors (NMDARs), which mediate synaptic plasticity and regulate neuronal survival in a subunit- and subcellular localization-dependent manner. Using primary hippocampal cultures with mature synapses, we found that the density of NMDA-evoked whole-cell currents was approximately twice as large in neurons cultured in the presence of glia compared to neurons cultured alone. The glial effect was mediated by (an) astrocyte-secreted soluble factor(s), was Mg^{2+} and voltage independent, and could not be explained by a significant change in the synaptic density. Instead, we found that the peak amplitudes of total and NMDAR miniature excitatory postsynaptic currents (mEPSCs), but not AMPAR mEPSCs, were significantly larger in mixed than neuronal cultures, resulting in a decreased synaptic AMPAR/NMDAR ratio. Astrocytic modulation was restricted to synaptic NMDARs that contain the GluN2B subunit, did not involve an increase in the cell surface expression of NMDAR subunits, and was mediated by protein kinase C (PKC). Taken together, our findings indicate that astrocyte-secreted soluble factor(s) can fine-tune synaptic NMDAR activity through the PKC-mediated regulation of GluN2B NMDAR channels already localized at postsynaptic sites, presumably on a rapid time scale. Given that physiologic activation of synaptic NMDARs is neuroprotective and that an increase in the synaptic GluN2B current is associated with improved learning and memory, the astrocyte-induced potentiation of synaptic GluN2B receptor activity is likely to enhance cognitive function while simultaneously strengthening neuroprotective signaling pathways.

Keywords: NMDA receptor, GluN2B subunit, astrocytes, synapses, protein kinase C

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Vladimir Parpura, University of Alabama, USA

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Takumi Takizawa, Gunma University, Japan

****Correspondence:***
Marta Margeta, Department of Pathology, University of California San Francisco, UCSF Pathology, Box 0511, 513 Parnassus Ave., HSW-514, San Francisco, CA 94143, USA
Tel: 415-514-0228,
Fax: 415-514-3165
Marta.Margeta@ucsf.edu

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Introduction

Glial cells play a critical role in the formation and function of excitatory synapses. In particular, it is well established that during development astrocyte-secreted soluble messengers promote synapse formation and surface expression of glutamate AMPA receptors (AMPARs) in both retinal ganglion cells and hippocampal neurons (Ullian et al., 2001; Christopherson et al., 2005; Xu et al., 2010; Allen et al., 2012). Much less is known about glial regulation of NMDA glutamate receptors (NMDARs), which together with AMPARs mediate excitatory postsynaptic signaling.

NMDARs are crucial for a wide range of brain functions, including synaptic plasticity and neural development (Cull-Candy and Leszkiewicz, 2004; Zito et al., 2009). NMDAR dysfunction

is implicated in many neurologic and psychiatric disorders (Cull-Candy et al., 2001; Hardingham and Bading, 2003; Schwartz et al., 2012), highlighting the importance of the precise regulation of NMDAR activity. NMDAR functional diversity is controlled through two distinct mechanisms: subunit composition and subcellular localization (Paoletti et al., 2013; Sanz-Clemente et al., 2013). NMDAR channels consist of two GluN1 and two GluN2 subunits, with GluN2 subunits conferring distinct functional properties on the assembled heterotetrameric channel complexes. Hippocampal neurons primarily express GluN1/GluN2A and GluN1/GluN2B NMDARs; in comparison with GluN1/GluN2A channels, GluN1/GluN2B channels have higher glutamate and glycine sensitivity, lower open probability, and slower deactivation kinetics (Paoletti et al., 2013). In addition, GluN2A and GluN2B subunits differ in their binding affinities for various intracellular scaffolding and signaling proteins (Paoletti et al., 2013). Irrespectively of the subunit composition, NMDAR coupling to intracellular signaling cascades also depends on the receptor subcellular localization. Although non-physiologic (tonic) activation of synaptic NMDARs can lead to excitotoxicity (Parsons and Raymond, 2014), these receptors preferentially couple to neuroprotective signaling pathways when activated by physiologic (phasic) stimuli (Hardingham and Bading, 2010; Martel et al., 2012). In contrast, activation of extrasynaptic NMDARs is generally neurotoxic and has been implicated in the pathogenesis of several neurodegenerative disorders (Parsons and Raymond, 2014).

Astrocytes can activate NMDARs by secreting the NMDAR ligands glutamate and D-serine, thus directly modulating synaptic efficacy and plasticity (Haydon and Carmignoto, 2006; Lee et al., 2007; Perea and Araque, 2010; Han et al., 2013). Astrocytes were also shown to regulate the surface expression of GluN2A and GluN2B NMDARs (Fellin et al., 2009; Deng et al., 2011), but a recent study raised the possibility that these findings were an artifact of the transgenic model system used for experiments (Fujita et al., 2014). Here, we use wild-type hippocampal cultures with mature synapses to show that astrocytes secrete a soluble factor or factors that increase the activity, but not the cell surface expression, of synaptic GluN2B-containing NMDARs through a protein kinase C (PKC)-mediated mechanism. Thus, astrocytes can regulate NMDAR function in both a subunit- and localization-specific manner, without altering the trafficking of NMDAR channel subunits.

Materials and Methods

Cell Cultures

Mixed, neuronal, and astrocytic primary hippocampal cultures were prepared from E18–E19 embryos obtained from Sprague-Dawley rats (Charles River), as described previously (Habas et al., 2013). All procedures involving animals were approved by the UCSF Animal Care and Use Committee. Mixed cell suspension was plated at a density of 600 cells/mm^2, either in tissue culture dishes coated with 0.1 mg/mL poly-L-lysine or on 12-mm glass cover slips (Bellco) coated with the mixture of poly-L-lysine and laminin. To obtain predominantly neuronal cultures, a subset of cultures was treated with 1 μM AraC (Sigma) to suppress glial cell growth; mixed cultures were left untreated. Mixed and neuronal cultures were grown in Neurobasal/B27 culture medium for 2–3 weeks before experiments. For astrocytic cultures, hippocampal cell suspension was plated in 75 cm^2 tissue culture flasks and grown in DMEM/fetal bovine serum culture medium. One week after plating (when the astrocyte layer became confluent), cultures were vigorously shaken (300 rpm at 37°C) overnight to remove microglia and oligodendroglia; astrocytes were then trypsinized, re-plated into 175 cm^2 tissue culture flasks, and maintained for another 3–4 days before being used for direct or indirect neuron-astrocyte co-cultures (Habas et al., 2013). For direct co-cultures, astrocytes were plated into DIV10 AraC-treated neuronal cultures at a density of ∼120 cells/mm^2; for indirect co-cultures, astrocytes were plated at the same density on cell culture inserts, which were then placed on top of DIV10 neuronal cultures. (The insert membrane has a pore size of 1 μm and is separated from the well bottom by 800 μm; this culture arrangement allows neurons and astrocytes to exchange soluble factors while preventing a direct cell-cell contact.). In both co-culture arrangements, the two cell types were cultured together for 4–7 days prior to experiments.

Electrophysiology

DIV13–17 neurons were used for patch-clamp recordings. NMDA-evoked currents were recorded at −60 mV (except in I/V analysis experiments) in the external solution containing (in mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, and 10 glucose, with 1 μM glycine, 10 μM bicuculline, and 1 μM TTX (∼305 mOsm, pH 7.4); for 0 Mg^{2+} external solution, $MgCl_2$ was replaced with glucose. The pipette solution contained (in mM) 120 CsCl, 20 TEA-Cl, 1 $MgCl_2$, 1 $CaCl_2$, 2.25 EGTA, 10 HEPES, 2.5 Mg_2ATP, 1 Na_3GTP (∼298 mOsm, pH 7.4; calculated free Ca^{2+} concentration, 70 nM). The liquid junction potential, measured with 3 M KCl, was −8.8 mV and was not adjusted except for I/V and unitary conductance analyses. mEPSC recording solutions were adapted from previously published studies (Watt et al., 2000; Fu et al., 2005; Lee et al., 2007). For total excitatory mEPSC recordings, 0 Mg^{2+} external solution was supplemented with 10 μM glycine, 1 μM TTX, and 20 μM bicuculline; AP5 (100 μM) was added to isolate AMPAR-mediated mEPSCs, while 1 μM strychnine and 10 μM NBQX were added to isolate NMDAR-mediated mEPSCs. The pipette solution contained (in mM): 125 K-gluconate, 10 KCl, 10 Na-phosphocreatine, 2.25 EGTA, 2.5 Mg_2ATP, 0.5 Na_3GTP, 10 HEPES (∼297 mOsm, pH 7.4); this pipette solution was also used for whole-cell recordings in MK-801 experiments. Drugs were purchased from Tocris Bioscience [except for bicuculline (Sigma-Aldrich) and thrombospondin (Haematologic Technologies)] and were applied through an electronically controlled, gravity-fed perfusion system with a response time of 15–20 ms (ALA Scientific); after placing a cell in the center of the visual field under 10× objective, the perfusion tip with an opening diameter of 500 μm was placed at the field edge. The pipette electrodes were pulled

from borosilicate glass tubes (Sutter Instruments) and had resistances of 3.5–4.2 MΩ. Recordings were obtained using a Multiclamp 700B amplifier (Molecular Devices), with signals filtered at 1 kHz and sampled at 10 kHz using a Digidata 1440A analog-to-digital converter (Molecular Devices). All data were recorded and analyzed with pClamp 10 software (Molecular Devices).

mEPSC Analysis

mEPSC data were analyzed using the Mini Analysis Program (Synaptosoft). Total and AMPAR mEPSC amplitudes were calculated by averaging the peak amplitudes of all individual mEPSC events recorded from a single neuron in the absence (total mEPSCs) or presence of AP5 (AMPAR mEPSCs). To obtain the average NMDAR-mediated mEPSC amplitude from the same set of recordings, the average AMPAR mEPSC trace (obtained by aligning and averaging all mEPSC events recorded from one neuron in the presence of AP5) was subtracted from the average total mEPSC trace (obtained by aligning and averaging all events recorded from the same neuron in the absence of AP5). In a separate set of recordings, NMDAR mEPSCs were recorded directly after blocking AMPAR mEPSCs with NBQX; for these experiments, NMDAR mEPSC amplitude was calculated by averaging the peak amplitudes of all individual NMDAR mEPSCs (amplitude threshold, 7 pA) recorded from a single neuron. To perform kinetic separation of AMPAR and NMDAR mEPSC components, we followed the general procedure described by Watt et al. (2000), except that the specific time points used for measurement of AMPAR and NMDAR mEPSC components were determined based on pharmacologic experiments performed in our cultures. Specifically, AMPA mEPSCs recorded in the presence of AP5 peaked close to 1 ms in both neuronal and mixed cultures, while NMDA mEPSCs recorded in the presence of NBQX, bicuculline, and strychnine peaked at 7.2 ± 0.6 ms in neuronal cultures and at 14.2 ± 1.0 ms in mixed cultures (n = 50–60). The residual AMPA mEPSCs were less than 1 pA when measured 7 ms after the peak in neuronal cultures or 15 ms after the peak in mixed cultures, indicating that total mEPSCs were not contaminated by AMPAR components around those time points. For kinetic analysis of the NMDAR/AMPAR ratio, we therefore measured the NMDA mEPSC peak (averaged over 1 ms) from total mEPSCs in a window of 6–10 ms after the AMPA mEPSC peak in neuronal cultures and in a window of 13–17 ms after the AMPA mEPSC peak in mixed cultures. To exclude synapses containing only AMPA components, we selected mEPSC events in which the NMDA mEPSC peak was larger than 2 pA.

Immunofluorescence

Cells were fixed for 30 min on ice in 4% paraformaldehyde in PBS, washed three times in ice-cold PBS, and blocked for 30 min in a blocking buffer (2% goat serum and 0.25% Triton X-100 in PBS). Cells were then incubated overnight at 4°C with primary antibodies [guinea pig polyclonal anti-synaptophysin (Synaptic System, 1:1000) and mouse monoclonal anti-PSD-95 (6G6-1C9, Abcam, 1:300)], washed three times with PBS at room temperature (RT), and then incubated for an hour at RT with secondary antibodies [Alexa-594-conjugated goat anti-guinea pig (Molecular Probes, 1:1000) and Alexa-488-conjugated goat anti-mouse (Molecular Probes, 1:1000)]. Following 5 min RT incubation with DAPI nuclear dye, coverslips were washed three times in PBS and mounted in Fluoromount-G (Cell Lab, Beckman Coulter).

Imaging

Images were acquired with a spinning disk confocal microscope (Yokogawa). A healthy-appearing neuron that was about two cell diameters away from its neighbors was placed in the center of the camera field to capture digital images of fluorescence emissions at 488 nm and 594 nm using MetaMorph image capture software. The selected cell was imaged in serial optical sections at 0.33 μm intervals over a total depth of 5 μm, for a total of 15 optical sections. Maximum intensity projections (MIPs) were generated from these sections, yielding 5 MIPs representing 1 μm of depth each; fluorescence intensity in each MIP was quantified as described previously (Ippolito and Eroglu, 2010). Synaptic puncta, defined by co-localization of synaptophysin and PSD-95 labeling, were quantified in the selected regions of interest (ROI; 89 × 89 μm square) using Puncta Analyzer program written by Bary Wark for ImageJ 1.26.[1] A total of 9 cells from 3 coverslips (each coverslip from a different culture batch) were analyzed for each experimental condition.

Western Blotting

Cells were lysed on ice with modified RIPA buffer (0.5% SOD, 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 50 mM TrisCl, 1 mM EDTA) supplemented with a protease inhibitor cocktail (Roche). Crude lysates were cleared by centrifugation at 6000 rpm for 10 min at 4°C, solubilized with LDS sample buffer (Invitrogen) supplemented with TCEP reducing reagent (Pierce; final concentration 12.5 mM), and heated for 10 min at 70°C prior to loading; given different cellular compositions of the cultures, samples were loaded on an equal volume basis. Samples were electrophoretically resolved with 4–12% Bis-Tris NuPAGE gels (Invitrogen) and electroblotted to nitrocellulose membranes. Membranes were blocked for 1 h at RT in TBS (150 mM NaCl, 20 mM TrisCl; pH = 7.4) containing 3% nonfat dried milk, incubated with primary antibodies for 2–3 h at RT or overnight at 4°C, washed 4 times at RT, incubated with the corresponding secondary antibody for 1 h at RT, and washed for at least 30 min at RT. Following a final wash in TBS with 0.1% Tween for 10 min at RT, protein-antibody complexes were detected using an ECL chemiluminescent kit (Pierce Biotechnology) and CL-XPosure Film (Thermo Scientific) with a Konica SRX-101A film developer. In a subset of experiments (**Figure 10**), the Odyssey Fc infrared imaging system (LI-COR Biosciences) was used for detection of the protein-antibody complexes. In these experiments, electrophoresis was performed using 3–8%

[1] http://rsbweb.nih.gov/ij/

Tris-Acetate NuPAGE gels (Invitrogen). The membranes were dried at RT for at least 1 h and then blocked with Odyssey Blocking Buffer (LI-COR Biosciences) for 1 h at RT. Odyssey Blocking Buffer supplemented with 0.1% Tween 20 was used for dilution of primary and secondary antibodies, while PBS supplemented with 0.1% Tween 20 was used for washing. Membranes were incubated with primary antibodies for 2 h at RT or overnight at 4°C, washed 4 times (5 min/wash) at RT, incubated with secondary antibodies for 1 h at RT, washed 4 times (5 min/wash) at RT, and then imaged using Odyssey Fc (LI-COR Biosciences) at 700 nm and 800 nm; images were analyzed using Image Studio Imaging Software (LI-COR Biosciences). Primary antibodies included: mouse monoclonal anti-NR1 (BD Biosciences, 1:1000), rabbit monoclonal anti-GluN2A (Millipore, 1:1000), mouse monoclonal anti-GluN2B (BD Biosciences, 1:100 [film] or 1:500 [Odyssey Fc]), rabbit polyclonal anti-phospho-GluN2B (Ser1303) (Millipore, 1:1000), mouse monoclonal anti-GluR1-NT (Millipore, 1:500), mouse monoclonal anti-PSD-95 (NeuroMab, 1:1000), rabbit polyclonal anti-synaptophysin (Santa Cruz Biotechnology, 1:1000) and mouse monoclonal anti-actin (Sigma, 1:5000). Secondary antibodies included goat horseradish peroxidase (HRP)-conjugated anti-rabbit secondary H + L IgG antibody and goat HRP-conjugated anti-mouse secondary H + L IgG antibody (Jackson ImmunoResearch, 1:5000 for both) or goat IRDye® 800CW-conjugated anti-rabbit secondary H + L IgG antibody and goat IRDye® 680RD-conjugated anti-mouse secondary H + L IgG antibody (LI-COR Biosciences, 1:15000 for both).

Surface Protein Biotinylation

After 2 washes with ice-cold PBS, live cultures were incubated with 1 mg/mL sulfo-NHS-S-S-Biotin (Pierce) for 20 min on ice. Following 2 washes with ice-cold PBS and 2 washes with ice-cold TBS (to remove and quench unbound reagent, respectively), cells were lysed in a modified RIPA buffer. Crude lysates were cleared by centrifugation at 6000 rpm for 10 min at 4°C; 10% of cleared lysate was reserved, while the rest was incubated with 50% NeutraAvidin agarose beads (Pierce) for 2–3 h or overnight at 4°C. After 5 washes with RIPA buffer, bound proteins were eluted with DTT- and TCEP-containing LDS sample buffer by heating for 30 min at 75°C; cleared whole cell lysate (input) and biotinylated protein fractions were then analyzed by Western blotting, as described above.

Data Fitting and Statistical Methods

Using a nonlinear least-squares algorithm, the dose-response data were fitted to Eq. 1 (where Top and Bottom are maximal and minimal current density values respectively, y is the current density obtained in response to a concentration of NMDA x, and EC_{50} is the concentration of NMDA that yields the half-maximal current density).

$$y = Bottom + \frac{Top - Bottom}{1 + 10^{LogEC_{50} - x}} \quad (1)$$

The standard slope (Hill slope = 1) was adopted because (i) estimated Hill coefficients obtained from all groups were similar to 1 [0.9–1.4, as reported previously (Verdoorn and Dingledine, 1988; Patneau and Mayer, 1990; Linsenbardt et al., 2013)]; and (ii) curves were fitted better without a slope parameter.

To determine weighted mean unitary conductance, MATLAB (MathWorks) was used to perform fluctuation analysis on the slowly decaying whole-cell currents evoked by 5 μM NMDA in the presence of 1 μM glycine. (To achieve the sufficiently slow current decay, the wash in these experiments was performed through a whole bath solution exchange rather than through the fast perfusion system used in other experiments.) The mean current and noise variance were calculated from adjacent 500 ms intervals during the portion of the current decay phase that was well fitted with a single exponential; to minimize the effect of changing current amplitude, the current variance was determined after subtracting this single exponential fit from the raw current trace. The mean current-variance plots showed a linear relationship, indicating that only a small fraction of the available channels were open; the weighted mean unitary current was determined from the slope of the linear fit (Watt et al., 2000). The weighted mean single channel conductance was obtained by dividing the mean unitary current by the driving force (−68.8 mV).

Statistical analyses were performed with GraphPad Prism statistical software; the specific test used for each experiment was chosen based on the experimental design and is specified in the main text and figure legends. The data are shown as mean ± SEM, unless indicated otherwise; $p < 0.05$ was considered statistically significant.

Results

In Hippocampal Cultures with Mature Synapses, Astrocytes Increase Postsynaptic NMDAR Current Density Through Secretion of a Soluble Factor (or Factors)

To investigate the effect of glia on neuronal NMDARs, we compared NMDAR activity between mixed and predominantly neuronal primary hippocampal cultures by recording whole-cell currents evoked by bath application of NMDA in the presence of glycine, with or without extracellular Mg^{2+}. [In our culture system, the neuron-glia ratio is approximately 50%–50% in mixed cultures and 95%–5% in AraC-treated, neuron-enriched cultures; for images of each culture condition, see Habas et al. (2013)]. NMDA-evoked currents were larger in mixed than in neuronal cultures; representative traces from recordings in 0 mM Mg^{2+} are shown in **Figure 1A**. To examine NMDA current differences in a systematic way, we constructed dose-response curves (Eq. 1) for steady-state current density (plateau current normalized by cell capacitance; **Figures 1B,C**). NMDA EC_{50} values did not significantly differ between the two culture conditions (0 Mg^{2+}: 13 μM in mixed vs. 10 μM in neuronal cultures; 1.2 mM Mg^{2+}: 44 μM in mixed vs. 39 μM in neuronal cultures; $p > 0.05$, F-test) and were comparable to values previously reported in the literature [7–16 μM in Mg^{2+}-free and 25–40 μM in Mg^{2+}-containing

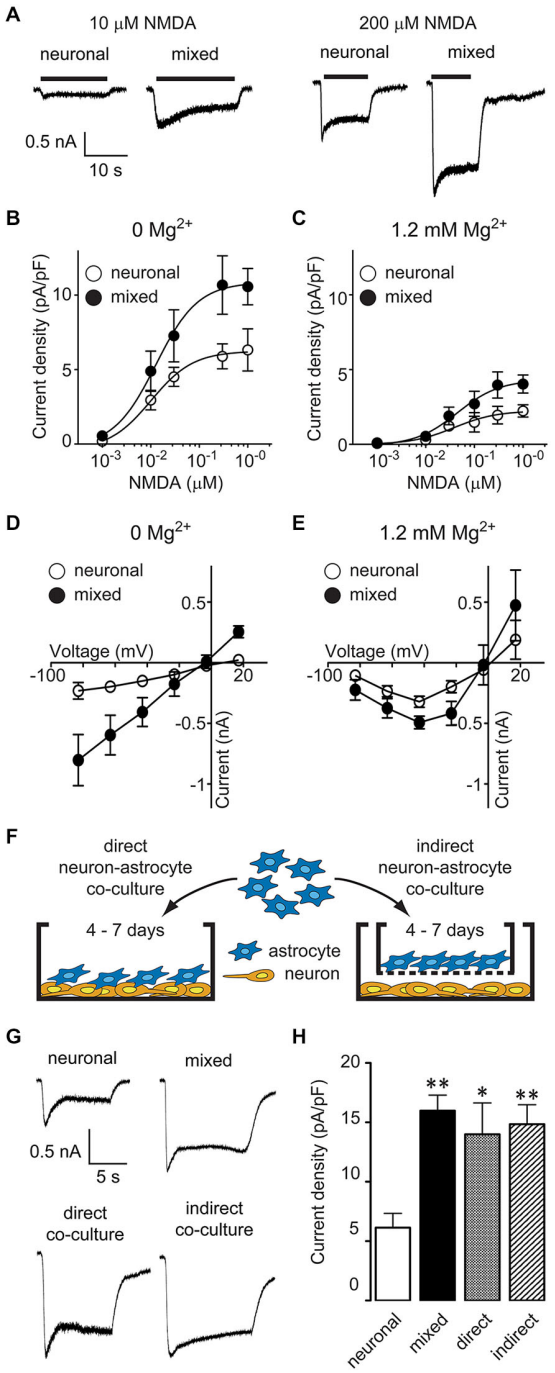

FIGURE 1 | Astrocyte-secreted factor(s) increase neuronal NMDA-evoked currents in a Mg^{2+}- and voltage-independent manner. **(A)** Representative current traces evoked by 10 and 200 μM NMDA in the presence of 1 μM glycine in neuronal and mixed cultures (0 Mg^{2+} external solution). **(B,C)** NMDA dose-response curves showed an increase in the maximal NMDA current density, both in the absence **(B)** and presence **(C)** of extracellular Mg^{2+}. The plateau current amplitudes were obtained by averaging current values during 1 s at the end of NMDA application; each data point is an average obtained from 2–15 neurons. **(D,E)** Current-voltage relationship of steady state (plateau) NMDA-evoked currents recorded in the absence **(D)** or presence **(E)** of extracellular Mg^{2+}. Each data point is an average obtained from 3–8 neurons. **(F)** A schematic diagram of the two neuron-astrocyte co-culture arrangements used for experiments. In direct co-cultures (left), astrocytes were plated directly onto AraC-treated neuronal cultures. In indirect co-cultures, astrocytes were plated on culture inserts that were then placed into wells with AraC-treated neuronal cultures; the insert membrane (1 μm pore size) allowed free exchange of soluble factors but prevented a direct contact between the two cell types. **(G)** Representative current traces evoked by 100 μM NMDA in the presence of 1 μM glycine in neuronal cultures, mixed cultures, direct neuron-astrocyte co-cultures, and indirect neuron-astrocyte co-cultures. **(H)** The average NMDA-evoked current density was significantly larger in mixed cultures and astrocyte co-cultures compared to neuronal cultures (*$p < 0.05$, **$p < 0.01$; one-way ANOVA with Dunnett post-test).

recording solutions (Verdoorn and Dingledine, 1988; Patneau and Mayer, 1990; Dildy-Mayfield and Leslie, 1991; Jaekel et al., 2006; Linsenbardt et al., 2013)]. In contrast, the maximal NMDAR current density was two-fold greater in mixed than neuronal cultures regardless of the Mg^{2+} concentration used (0 Mg^{2+}: 10.6 ± 1.2 pA/pF vs. 6.3 ± 1.4 pA/pF; 1.2 mM Mg^{2+}: 4.0 ± 0.6 pA/pF vs. 2.2 ± 0.4 pA/pF, $p < 0.05$, *F*-test). Since the voltage dependence of Mg^{2+} block shapes the voltage dependence of NMDAR currents (Nowak et al., 1984), this finding suggested that glial modulation of NMDARs was voltage independent; to investigate this further, we directly assessed the current-voltage (I-V) relationship of NMDA-evoked currents (**Figures 1D,E**). In Mg^{2+}-free recording solution (**Figure 1D**), I-V curves were linear with a reversal potential of ∼0 mV in both culture conditions, as described previously (Nowak et al., 1984); however, the current amplitudes were significantly greater in mixed than neuronal cultures ($p < 0.0001$; two-way ANOVA). In the presence of 1.2 mM Mg^{2+} (**Figure 1E**), I-V curves exhibited a typical block at negative voltages while maintaining the significant amplitude difference between the two culture conditions ($p < 0.05$; two-way ANOVA).

Mixed hippocampal cultures used in these experiments contain a heterogeneous population of glia. To determine whether the increase in neuronal NMDAR current density was mediated by astrocyte-secreted factor(s), we used two different modes of neuron-astrocyte co-culture, as described previously (Habas et al., 2013): in direct co-cultures, there was a physical contact between neurons and astrocytes, while in indirect co-cultures, the two cell types shared the same medium but were not in a direct contact (**Figure 1F**). The density of NMDAR currents evoked by 100 μM NMDA and 1 μM glycine (in the absence of Mg^{2+}) was comparable between mixed cultures (16.0 ± 1.3 pA/pF), direct neuron-astrocyte co-cultures (14.0 ± 2.6 pA/pF), and indirect neuron-astrocyte co-cultures (14.8 ± 1.6 pA/pF) and was approximately twice as large as the density of NMDAR currents in neuronal cultures (6.1 ± 1.2 pA/pF) (**Figures 1G,H**; one-way ANOVA with *post hoc* Dunnett test, $p < 0.01$, $p < 0.05$, and $p < 0.01$, respectively; $n = 8$). Taken together, these results demonstrate that astrocytes increase neuronal NMDAR current density in a voltage-independent fashion through a secretion of a soluble factor (or factors).

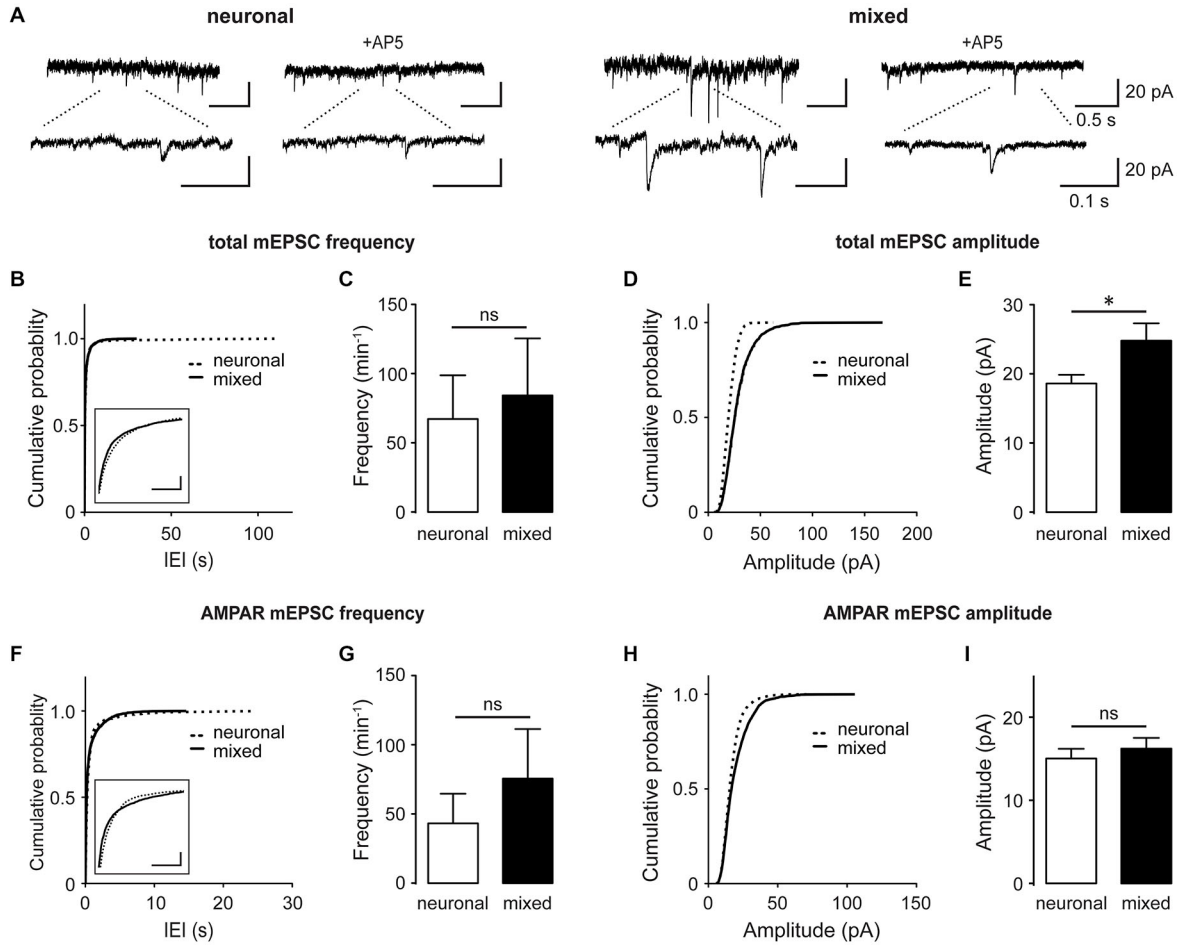

FIGURE 2 | Culturing neurons with glia increases the total mEPSC amplitude without significantly affecting AMPAR mEPSCs. (A) Spontaneous mEPSCs recorded at −60 mV in neuronal and mixed cultures. Total mEPSCs (left) contain both AMPAR and NMDAR-mediated components; AMPAR-mediated mEPSCs were isolated using the NMDAR blocker AP5 (100 μM, right). Bottom traces are an expanded view of top traces. **(B–E)** Cumulative probability and mean plots of total mEPSC frequency **(B,C)** and peak amplitude **(D,E)**. **(F–I)** Cumulative probability and mean plots of AMPAR mEPSC frequency **(F,G)** and peak amplitude **(H,I)**. For **(B–I)**, cumulative probability plots were generated using all events from 7 cells for each culture condition; summary bar graphs show values averaged from 7 cells for each group. (*$p < 0.05$, *t*-test). In **(B)** and **(F)**, the segments of cumulative probability curves with the largest difference between neuronal and mixed cultures are shown in insets (horizontal scale bars, 1 s; vertical scale bars, cumulative probability of 0.1); in both plots, the differences between the two curves were statistically significant but very small and thus likely not biologically significant.

In the Presence of Astrocytes, there is an Increase in the Size of NMDAR-Mediated Synaptic Currents but No Significant Change in the Overall Synaptic Density

Several studies have shown that astrocyte-secreted factors regulate synapse formation and function during development of retinal ganglion cells (Pfrieger and Barres, 1997; Ullian et al., 2001; Christopherson et al., 2005; Allen et al., 2012), raising the possibility that the astrocyte-induced increase in the whole-cell NMDAR current density was a result of the increase in the overall synapse density. However, glial influence on excitatory synapses varies with neuronal cell type (Steinmetz et al., 2006) and developmental stage (Xu et al., 2010). To directly examine glial effect(s) on excitatory synapses in our hippocampal culture system, we recorded spontaneous synaptic activity at DIV13–17, when synaptogenesis is known to reach a plateau (Grabrucker et al., 2009; Xu et al., 2010). Total miniature excitatory postsynaptic excitatory currents (mEPSCs), which contain both AMPAR- and NMDAR-mediated components, were recorded in the presence of 1 μM tetrodotoxin (TTX) to block action potentials and 20 μM bicuculline to block inhibitory synaptic transmission (**Figures 2A–E**). Cumulative probability plots of the mEPSC inter-event-interval (IEI; **Figure 2B**) and amplitude (**Figure 2D**) showed small but statistically significant changes in the overall event distribution (total mEPSC IEI: $p < 0.005$, total mEPSC amplitude: $p < 0.001$, Kolmogorov-Smirnov [KS] test); however, the mean mEPSC frequency did not significantly differ between the two culture conditions (84.3 ± 41.2 min^{-1} for mixed vs. 67.2 ± 31.6 min^{-1} for neuronal cultures, $n = 7$, $p = 0.6$, *t*-test; **Figure 2C**). In contrast,

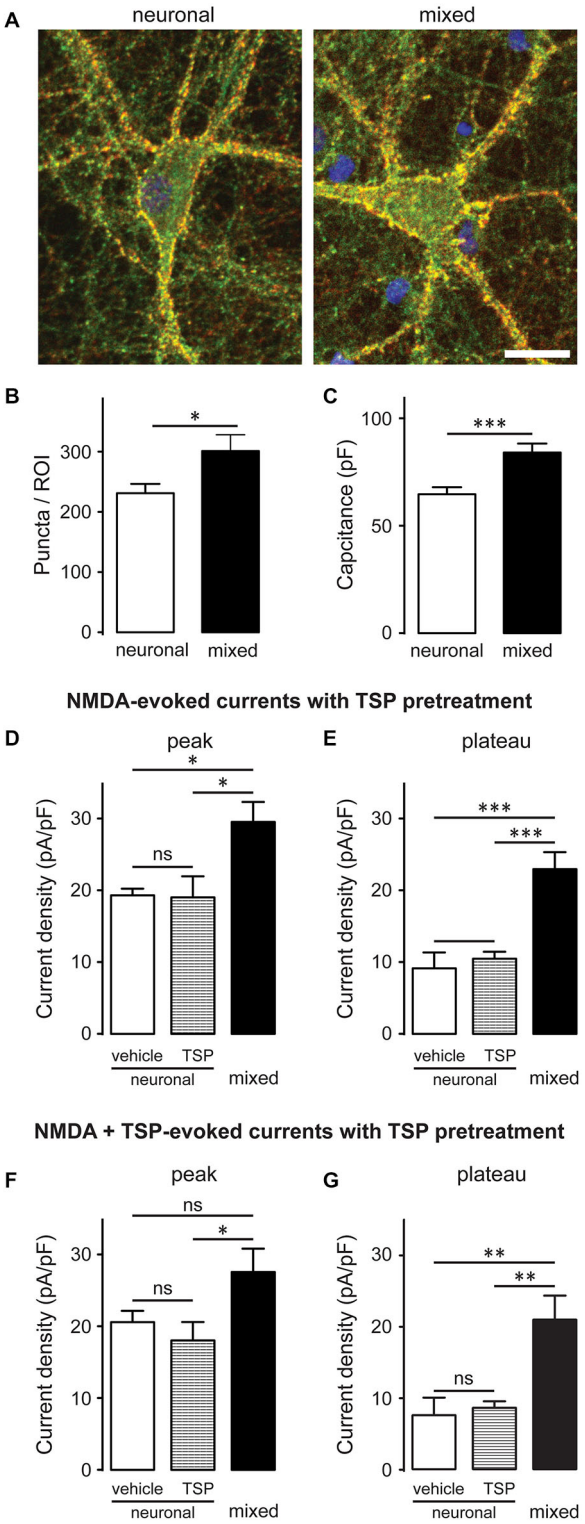

FIGURE 3 | Glia-induced increase in the NMDAR current density is not due to an appreciable change in synaptic density and cannot be mimicked by thrombospondin (TSP) treatment of neuronal cultures. **(A)** Synaptic puncta were visualized by co-localization of presynaptic marker synaptophysin (red) and the postsynaptic marker PSD-95 (green). Scale bar, (*Continued*)

FIGURE 3 | Continued
20 μm. **(B,C)** The number of synaptic puncta per ROI (which included the soma and proximal dendrites of each imaged neuron) was higher in mixed than neuronal cultures **(B)**; however, this increase was proportional to the increase in the neuronal membrane capacitance (a proxy for the neuronal surface area) observed in mixed cultures **(C)**. (**B,C**: $^*p < 0.05$, $^{***}p < 0.001$; *t*-test) **(D,E)** 10-day pretreatment of neuronal cultures with 5 μg/ml TSP had no effect on NMDAR current density measured at either peak or plateau time points; for comparison, NMDAR current density in mixed sister cultures was significantly larger than the current density in neuronal cultures after either pretreatment. Control neuronal cultures were pretreated with vehicle (0.07% glycerol); NMDA currents were evoked by application of 200 μM NMDA in the presence of glycine. **(F,G)** Acute TSP treatment (5 μg/ml) had no effect on NMDAR current density in neuronal cultures. In these experiments, neuronal cultures were first pretreated with 5 μg/ml TSP or vehicle, as described for **(D,E)**; TSP or vehicle were then co-applied with NMDA in the presence of glycine. There was no difference in the mean NMDAR current density between TSP- and vehicle-treated cultures at either time point (peak and plateau); as expected, NMDAR current density was significantly larger in mixed sister cultures. (**D–G**: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA with Bonferroni post-test).

the peak total mEPSC amplitude was 33% larger in mixed than neuronal cultures (24.8 ± 2.5 pA vs. 18.6 ± 1.3 pA respectively, $n = 7$, $p < 0.05$, *t*-test; **Figure 2E**). When recordings were performed in the presence of 100 μM AP5 to block the NMDAR mEPSC component (**Figures 2A,F–I**), we again observed very small but statistically significant changes in the distribution of AMPAR mEPSC IEI (**Figure 2F**) and amplitude (**Figure 2H**) ($p < 0.001$, KS test). However, there was no statistical significance in the mean frequency (76.4 ± 35.6 min^{-1} in mixed vs. 43.2 ± 22.0 min^{-1} in neuronal cultures, $n = 7$, $p > 0.05$, *t*-test; **Figure 2G**) or the mean peak amplitude of AMPAR mEPSCs between the two culture conditions (16.2 ± 1.3 pA in mixed vs. 15.0 ± 1.2 pA in neuronal cultures; $n = 7$, $p > 0.05$, *t*-test; **Figure 2I**).

A subset of glutamatergic synapses contains only NMDARs (Hanse et al., 2013), rendering them AMPAR-silent and thus undetectable in standard mEPSC recordings; therefore, we also compared the overall number of synapses between the two culture conditions. Neurons were immunostained with antibodies against synaptophysin (a presynaptic marker; red) and PSD-95 (a postsynaptic marker; green), as described previously (Ullian et al., 2001; Christopherson et al., 2005; Ippolito and Eroglu, 2010); synapses were detected as yellow puncta, indicating co-localization of the two markers (**Figure 3A**). The number of synaptic puncta per ROI (which included the soma and proximal dendrites of imaged neurons) was 30% greater in mixed than neuronal cultures, a statistically significant difference (301.1 ± 27.2 vs. 231.2 ± 15.4 puncta/ROI; $n = 9$, $p < 0.05$, *t*-test; **Figure 3B**). However, neuronal surface area (measured as membrane capacitance) was also 30% greater in mixed than neuronal cultures (84.1 ± 4.2 pF vs. 64.7 ± 3.3 pF respectively, $n = 25$, $p < 0.001$, *t*-test; **Figure 3C**). These measurements were performed on different cell populations and thus could not be used for direct calculation of the number of synapses per unit of the membrane capacitance; nonetheless, together they strongly

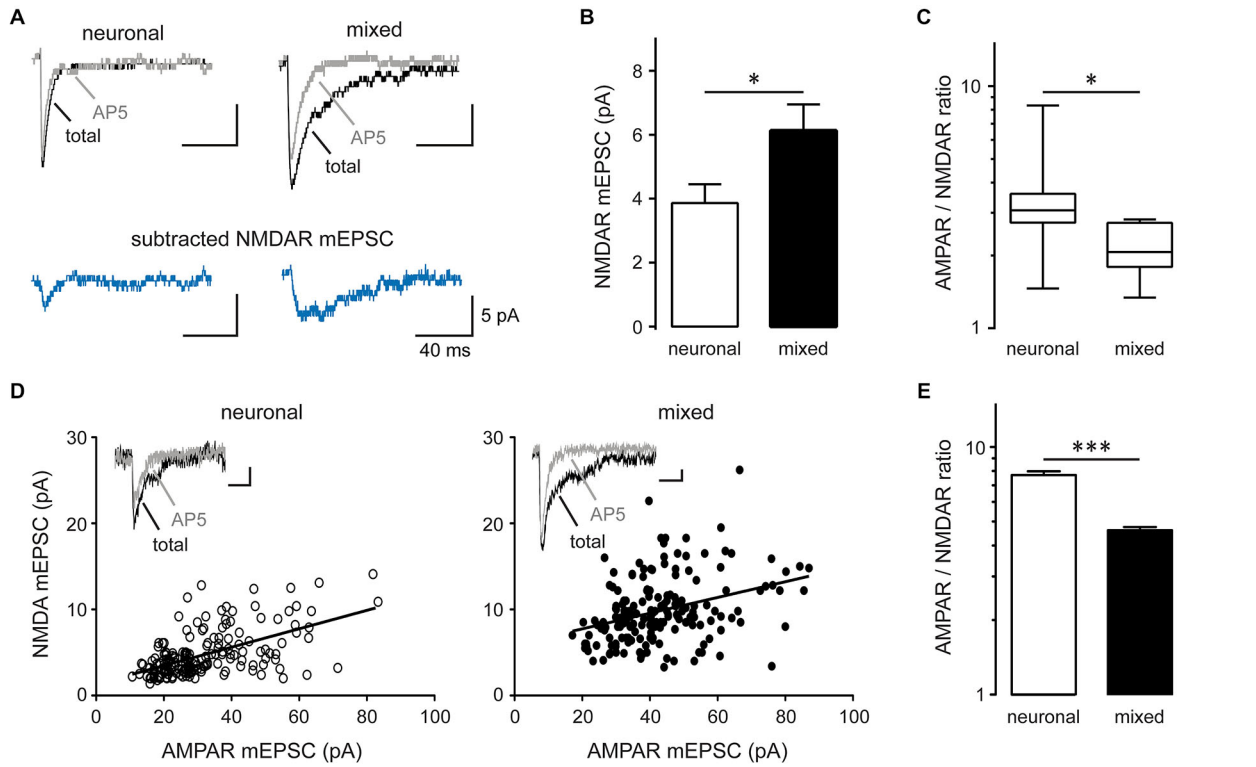

FIGURE 4 | Synaptic AMPAR/NMDAR ratio is decreased in mixed compared to neuronal cultures. (A) Representative average mEPSC traces recorded before (total mEPSC) and after AP5 treatment (AMPAR mEPSC); the NMDAR mEPSC trace was obtained by subtracting the average AMPAR mEPSC trace from the average total mEPSC trace. **(B)** The peak NMDAR mEPSC amplitude (obtained from averaged NMDAR mEPSC traces) was significantly larger in mixed than neuronal cultures (*$p < 0.05$). **(C)** The AMPAR/NMDAR mEPSC ratio (calculated from the peak amplitudes of averaged AMPAR and NMDAR traces) was significantly smaller in mixed cultures. The data were not normally distributed and are shown as box-and-whiskers plots (box edges, 25th and 75th percentiles; whiskers, 10th and 90th percentiles; *$p < 0.05$, Mann-Whitney nonparametric test on ranks). **(D)** Peak amplitudes of AMPAR and NMDAR mEPSC components obtained from individual total mEPSCs (see Materials and Methods for details) were plotted across neurons (180 events from $n = 7$ for each culture condition); the solid line is the best straight line fit (neuronal, $r^2 = 0.32$; mixed, $r^2 = 0.12$). The correlation coefficient decreased from 0.57 in neuronal to 0.34 in mixed cultures, indicating differential regulation of AMPAR and NMDAR mEPSCs by glia. Insets: representative single mEPSC traces before and after AP5. Horizontal scale bars, 10 ms; vertical scale bars, 5 pA. **(E)** The AMPAR/NMDAR mEPSC ratio obtained from analysis of individual mEPSC events was significantly smaller in mixed cultures (***$p < 0.001$, *t*-test).

suggest that synaptic density did not appreciably differ among the two culture conditions. In agreement with this finding, thrombospondin [astrocyte-secreted factor that promotes retinal ganglion cell synaptogenesis (Christopherson et al., 2005)] had no effect on the NMDAR current density in neuronal cultures (**Figures 3D–G**). Collectively, these data indicate that (i) the presence of glial cells does not have a major effect on the density of excitatory synapses in mature hippocampal cultures; and (ii) that the two-fold difference in whole-cell NMDAR current density between mixed and neuronal cultures cannot be explained by the difference in synaptic density between the two culture conditions.

The mean amplitude of total mEPSCs (**Figure 2E**), but not AMPAR mEPSCs (**Figure 2I**), was significantly larger in mixed than neuronal cultures, suggesting an increase in the NMDAR-mediated mEPSC component. Indeed, the mean amplitude of average NMDAR mEPSCs (obtained by subtraction of the average AMPAR mEPSC trace from the average total mEPSC trace for each neuron; **Figure 4A**) was significantly larger in mixed than neuronal cultures (6.1 ± 0.8 vs. 3.9 ± 0.6 pA, respectively; $n = 7$, $p < 0.05$, *t*-test; **Figure 4B**), resulting in a decreased AMPAR/NMDAR ratio in mixed cultures (median of 2.1 in mixed vs. 3.1 in neuronal cultures, $n = 7$, $p < 0.05$, Mann-Whitney test; **Figure 4C**). To determine whether this increase in the average NMDAR mEPSC reflects a population of new synapses or an increase in the NMDAR mEPSC component of existing synapses, we examined the AMPAR/NMDAR ratio at individual synapses using kinetics analysis that is based on distinctive inactivation properties of these two types of glutamate receptors, as described previously (Edmonds et al., 1995; Watt et al., 2000). When NMDAR and AMPAR mEPSC amplitudes from each event were plotted against each other, the correlation coefficient became smaller in mixed cultures (0.34) than in neuronal cultures (0.57) (**Figure 4D**), indicating that AMPAR and NMDAR components of mEPSC were not proportionally scaled at individual synapses. In agreement with this observation and with the results from the average mEPSC analysis (**Figures 4A–C**),

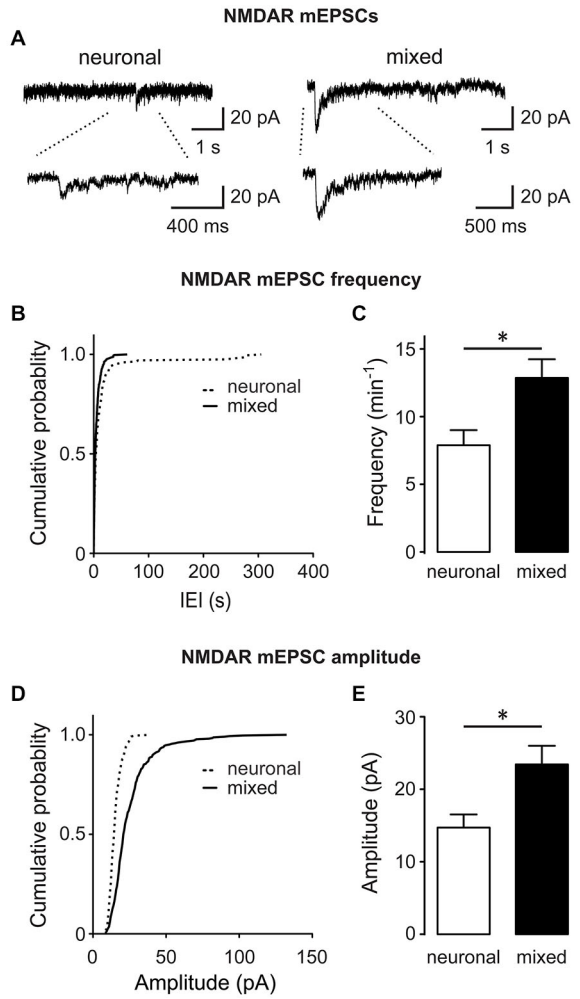

FIGURE 5 | The frequency and amplitude of directly recorded NMDAR mEPSCs are increased in the presence of glia. **(A)** Representative NMDAR mEPSC traces recorded at −60 mV from neuronal and mixed cultures in the presence of TTX, NBQX, bicuculline and strychnine; bottom traces show recordings on an expanded time scale. **(B–E)** Cumulative probability, mean frequency, and mean amplitude plots of NMDAR mEPSCs are shown; both frequency and amplitude of NMDAR mEPSCs were significantly larger in mixed cultures (*$p < 0.05$, *t*-test). Of note, the peak NMDAR mEPSC amplitudes obtained from this analysis were larger than the peak NMDAR mEPSC amplitudes obtained by subtracting the average AMPAR mEPSC traces from the average total mEPSC traces (**Figure 4B**); the most likely explanation for this discrepancy is our inability to resolve small and medium-sized (<7 pA) NMDAR mEPSC events from the background noise in direct recordings, biasing the overall analysis to the largest mEPSCs.

the AMPAR/NMDAR ratio obtained from individual mEPSCs was significantly smaller in mixed than neuronal cultures (4.6 ± 0.1 vs. 7.7 ± 0.3, $n = 7$; $p < 0.001$, *t*-test; **Figure 4E**). To directly measure differences in NMDAR mEPSCs between mixed and neuronal cultures, we performed recordings in the presence of the AMPAR blocker NBQX (10 μM) in addition to TTX, bicuculline, and strychnine (1 μM) (**Figure 5A**). Cumulative plots of the NMDAR mEPSC IEI and amplitude showed a large and statistically significant change in the overall event distribution (total mEPSC IEI: $p < 0.005$, total mEPSC amplitude: $p < 0.001$, KS test; **Figures 5B,D**). In addition, there was a significant increase in the NMDAR mEPSC frequency and peak amplitude in mixed compared to neuronal cultures (12.9 ± 1.4 vs. 7.9 ± 1.1 min^{-1} and 23.4 ± 2.6 vs. 14.0 ± 1.4 pA, $n = 6–7$, $p < 0.05$, *t*-test; **Figures 5C,E**). Taken together, these results demonstrate that (i) NMDAR-mediated synaptic currents in mature hippocampal neurons are potentiated in the presence of astrocytes and (ii) astrocytes differentially affect NMDARs and AMPARs at individual synapses, resulting in a lower AMPAR/NMDAR ratio in mixed cultures.

Astrocytes Selectively Regulate Synaptic GluN2B NMDARs

Synaptic and extrasynaptic NMDARs have a similar subunit composition, but different cellular functions (Hardingham and Bading, 2010). To determine whether glia differentially modulate these two NMDAR populations, we used the activity-dependent open NMDAR channel blocker MK-801 to isolate synaptic from extrasynaptic NMDAR currents, as described previously (Papadia et al., 2008): after synaptic NMDA currents were irreversibly blocked by MK-801 application during a period of spontaneous neuronal activity, residual extrasynaptic NMDAR currents were evoked by bath application of NMDA (**Figure 6A**; representative recordings from neuronal and mixed cultures are shown in **Figures 6B,C**). In agreement with the data from our mEPSC experiments, the density of the synaptic (MK-801-sensitive) NMDAR current was significantly greater in mixed cultures at both peak and plateau time points [peak: 18.4 ± 2.0 vs. 9.8 ± 1.9 pA/pF, $p < 0.01$; plateau: 13.5 ± 1.6 vs. 7.0 ± 2.0 pA/pF, $p < 0.05$; $n = 12–13$, *t*-test (**Figure 6D**)]. In contrast, the density of the extrasynaptic (MK-801-insensitive) NMDAR current did not significantly differ between the two culture conditions [peak: 5.2 ± 1.4 pA/pF in mixed vs. 2.9 ± 1.0 pA/pF in neuronal cultures, $p > 0.05$; plateau: 2.1 ± 0.4 in mixed vs. 1.6 ± 0.3 pA/pF in neuronal cultures, $p > 0.05$; $n = 12–13$, *t*-test (**Figure 6E**)].

A significant fraction of the synaptic NMDAR current is carried by channels that incorporate slowly-inactivating GluN2B subunit, although these channels are found at both subcellular locations (Harris and Pettit, 2007). To examine the subunit specificity of glial NMDAR potentiation, we recorded NMDA-evoked currents in the presence of ifenprodil (3 μM), a GluN2B-selective inhibitor (Williams, 1993; **Figure 7A**). The density of ifenprodil-insensitive, largely GluN2A-mediated NMDAR currents was not significantly different between the two culture conditions [peak: 12.6 ± 1.8 pA/pF in mixed vs. 10.0 ± 0.9 pA/pF in neuronal cultures, $p > 0.05$; plateau: 3.5 ± 1.0 pA/pF in mixed vs. 1.8 ± 0.7 pA/pF in neuronal cultures, $p > 0.05$; $n = 8–10$, *t*-test (**Figure 7B**)]. In contrast, ifenprodil-sensitive, GluN2B-mediated currents were significantly larger in mixed cultures when measured at the plateau, but not at the peak time point [peak: 9.6 ± 3.0 vs. 8.0 ± 1.5 pA/pF, $p > 0.05$; plateau: 9.8 ± 1.4 vs. 4.2 ± 1.1 pA/pF, $p < 0.001$; $n = 8–10$, *t*-test

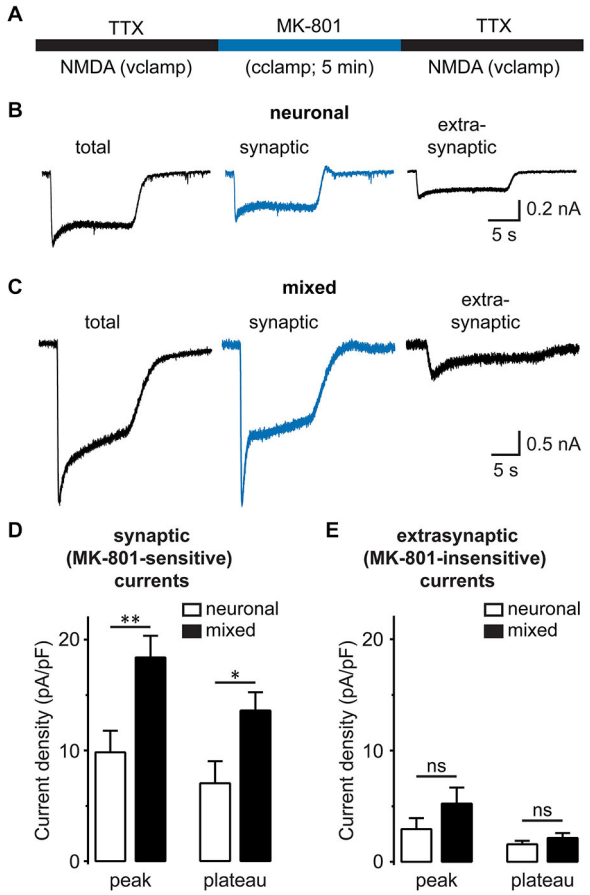

FIGURE 6 | Synaptic, but not extrasynaptic, NMDA-evoked currents are increased in the presence of glia. **(A)** A schematic diagram of the drug application protocol used to separate synaptic from extrasynaptic NMDAR currents. The total whole-cell NMDAR currents were first recorded in a Mg^{2+}-free external solution containing TTX. To block synaptic NMDA currents, neurons were then switched for 5 min to the current-clamp configuration and a TTX-free solution containing Mg^{2+} and 10 μM MK-801; under these conditions, the spontaneous neuronal firing activates synaptic (but not extrasynaptic) NMDAR channels, which are then irreversibly blocked by the activity-dependent, irreversible NMDAR channel blocker MK-801. Finally, neurons were switched back to the regular recording solution and voltage clamp configuration to record the residual extrasynaptic NMDA currents. Between patch-clamp recording configuration switches, neurons were perfused for 2 min. **(B,C)** Representative current traces evoked by 200 μM NMDA in the presence of glycine in neuronal **(B)** and mixed cultures **(C)**. Total NMDAR currents (left) were recorded before MK-801 treatment, while residual/extrasynaptic NMDAR currents (right) were recorded after MK-801 treatment, as described in **(A)**. Synaptic NMDA currents (center) were obtained by subtracting MK-801-insensitive from total NMDA currents. **(D,E)** Mean peak and steady-state (plateau) current densities are plotted for synaptic **(D)** and extrasynaptic NMDA currents **(E)**. The peak current amplitude (averaged over 5 ms) was determined within 1 s of NMDA application; the plateau current amplitude was obtained by averaging during the last 1 s of 10–15 s NMDA application. Both peak and plateau synaptic NMDAR currents were greater in mixed cultures, but extrasynaptic currents did not significantly differ between the two culture conditions (*$p < 0.05$, **$p < 0.01$, *t*-test).

(**Figure 7C**)]. Taken together, these data indicate that synaptic GluN2B-containing NMDAR channels are the target of glial modulation.

Astrocytes Regulate NMDAR Channel Activity Through a PKC-Mediated Mechanism

Increased whole-cell current density can be due to an increase in the number of ion channels on the cell surface or to an increase in the activity of ion channels already localized to the plasma membrane. To differentiate between these two possibilities, we examined the total and cell surface expression of the major hippocampal NMDAR subunits, GluN1, GluN2A and GluN2B. In contrast to glial modulation of AMPAR [which involves increased cell surface expression and clustering of AMPAR GluA1 subunits (Allen et al., 2012)], neither total protein level nor cell surface expression of GluN1, GluN2A and GluN2B subunits was significantly higher in mixed compared to neuronal cultures (**Figures 8A–D**), indicating that the increase in the whole-cell current density was due to a change in the activity of the surface-expressed NMDAR channels. Ion channel activity is a function of two different aspects of single channel behavior: single channel (unitary) conductance and single channel kinetics. While evaluation of single channel kinetics requires patch recordings not suitable for analysis of physically inaccessible synaptic channels (Traynelis and Jaramillo, 1998),

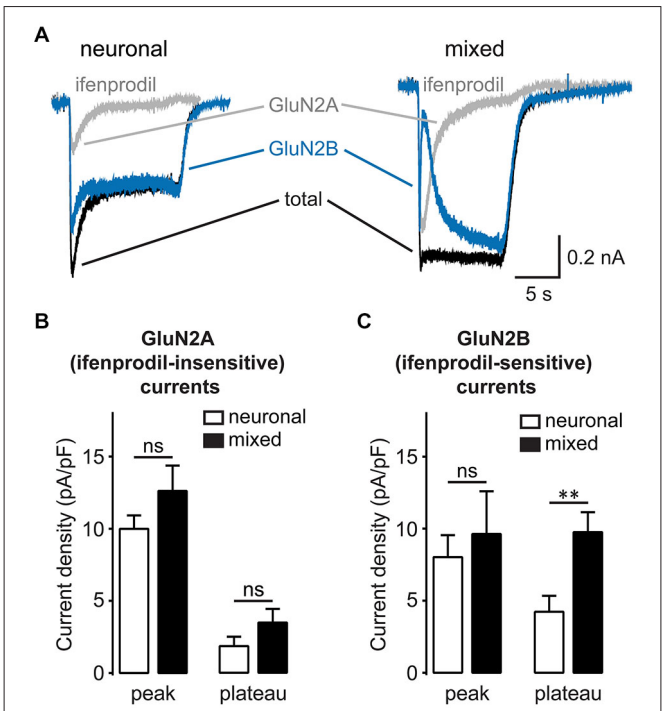

FIGURE 7 | Glia-induced enhancement of NMDA-evoked currents is GluN2B subunit specific. **(A)** Representative current traces evoked by 200 μM NMDA in the presence of glycine recorded before and after treatment with the GluN2B-specific antagonist ifenprodil (3 μM). Ifenprodil-sensitive GluN2B NMDAR currents (blue traces) were obtained by subtracting the ifenprodil-insensitive GluN2A currents (gray traces) from the total NMDAR currents (black traces). **(B,C)** Mean peak and steady-state (plateau) current densities are plotted for GluN2A **(B)** and GluN2B currents **(C)**. The plateau, but not peak, GluN2B currents were significantly larger in mixed cultures, while GluN2A currents did not significantly differ between the two culture conditions at either time point (**$p < 0.01$, *t*-test).

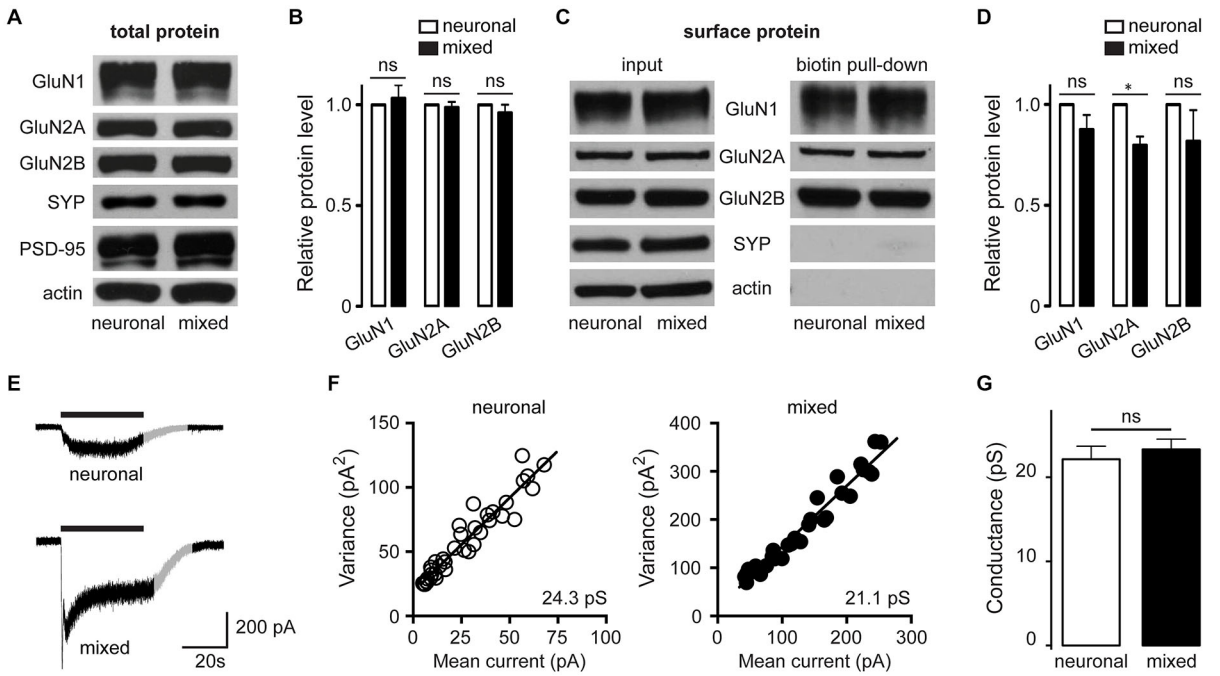

FIGURE 8 | In the presence of glia, there is no increase in the neuronal expression of NMDAR channel subunits or the size of the unitary NMDAR channel conductance. **(A,B)** Expression of GluN1, GluN2A and GluN2B proteins in the whole cell lysates from neuronal and mixed cultures; a representative Western blot **(A)** and quantification of data from 3 independent experiments **(B)** are shown. Synaptophysin (SYP), PSD-95, and actin were used as loading controls; for quantification, NMDAR subunit levels were normalized to SYP level. **(C,D)** Expression of biotinylated (surface-expressed) GluN1, GluN2A and GluN2B proteins in neuronal and mixed cultures; a representative Western blot **(C)** and quantification of data from 3 independent experiments **(D)** are shown. Note that intracellular proteins (SYP and actin) are not detectable in this preparation; for quantification, NMDAR subunit expression was normalized to SYP levels in the input (whole cell lysate) portion of the same samples. In the presence of glia, there was a small but statistically significant decrease in the GluN2A subunit surface expression, while the GluN2B subunit surface expression did not differ between the two culture conditions (*$p < 0.05$, *t*-test). **(E-G)** Fluctuation analysis was used to determine the weighted mean unitary NMDAR channel conductance for each culture condition. **(E)** Representative recordings (evoked by application of 5 μM NMDA in the presence of 1 μM glycine); the gray portion of each trace highlights the section used for fluctuation analysis **(F)** Mean current-variance plots from the two traces showed in **(E)**; weighted mean unitary conductance values were derived from the slope of the linear fit. **(G)** The weighted mean unitary NMDAR channel conductance did not significantly differ between the two culture conditions ($p > 0.05$, *t*-test).

unitary conductance can be determined from fluctuation analysis of macroscopic currents (Sigworth, 1981). To further elucidate the mechanism mediating astrocytic enhancement of NMDAR channel activity, we therefore performed fluctuation analysis of the slowly decaying whole-cell currents evoked by 5 μM NMDA (Mayer et al., 1988; Watt et al., 2000). **Figures 8E,F** show representative current traces and mean current-variance plots for one neuron from each culture condition, with the slope of the linear fit yielding the weighted mean unitary conductances of 24.3 pS (neuron without glia) and 21.1 pS (neuron with glia) for the two neurons shown; these values are smaller than the NMDAR conductance values obtained from outside-out patch recordings [40–50 pS (Jahr and Stevens, 1987; Ascher et al., 1988; Robinson et al., 1991)], but are in line with a broad range of NMDAR single channel conductance values obtained from fluctuation analysis [9–41 pS (Ascher et al., 1988; Sah et al., 1989; Watt et al., 2000)]. Importantly, the mean weighted unitary NMDAR channel conductance did not significantly differ between the two culture conditions (22.2 ± 1.5 pS in neuronal vs. 23.3 ± 1.2 pS in mixed cultures, $n = 6–7$, $p > 0.05$, *t*-test; **Figure 8G**), suggesting that a change in the single channel kinetics rather than a change in the single channel conductance is the key biophysical mechanism mediating the astrocytic modulation of synaptic GluN2B NMDAR channels.

PKC was previously shown to increase the whole-cell NMDAR current by phosphorylation of Ser1303 and/or Ser1323 in the C-terminus of GluN2B (Liao et al., 2001). To investigate whether PKC is involved in the astrocyte-induced potentiation of NMDAR currents, neuronal and mixed cultures were pretreated with PKC inhibitor GF 109203X (GFX; 5 μM) for 40 min prior to recording (**Figures 9A,B**). [GFX inhibits PKC with IC_{50} of ∼10 nM (Toullec et al., 1991), but in physiological experiments it is typically used at concentrations between 1–10 μM.] GFX pretreatment significantly attenuated NMDAR current density in mixed cultures (16.1 ± 1.2 vs. 8.6 ± 1.1 pA/pF; $n = 6–10$, $p < 0.01$, two-way ANOVA with Bonferroni multiple comparison post-test) but had no effect in neuronal cultures (10.1 ± 0.6 vs. 11.5 ± 1.4 pA/pF; $p > 0.05$); as a result, the difference in NMDAR current density between the two culture types, which was significant in vehicle-pretreated cultures ($p < 0.01$), was completely abolished following GFX

pretreatment ($p > 0.05$). In agreement with these results, PKC activator phorbol-12-myristate-13-acetate (PMA) had the opposite effect (**Figures 9C,D**): pretreatment with 100 nM PMA significantly increased NMDAR current density in neuronal cultures (17.4 ± 2.3 vs. 4.6 ± 1.1 pA/pF; n = 8–12, $p < 0.001$, two-way ANOVA with Bonferroni multiple comparison post-test) but had no effect in mixed cultures (15.0 ± 1.8 vs. 11.0 ± 2.3 pA/pF; $p > 0.05$); consequently, the difference in NMDAR current density between the two culture types (which was significant in vehicle-pretreated cultures; $p < 0.001$) was not significant following PMA pretreatment ($p > 0.05$). Interestingly, however, there was no significant difference in the phosphorylation status of GluN2B Ser1303 between the two culture conditions in either vehicle- or GFX-treated cultures (**Figure 10**; phosphorylation of Ser1323 could not be evaluated due to the lack of phospho-specific antibodies targeting that site). Taken together, these data indicate that PKC mediates the astrocyte-induced increase in the synaptic GluN2B channel activity, but that this effect does not involve phosphorylation of GluN2B-Ser1303.

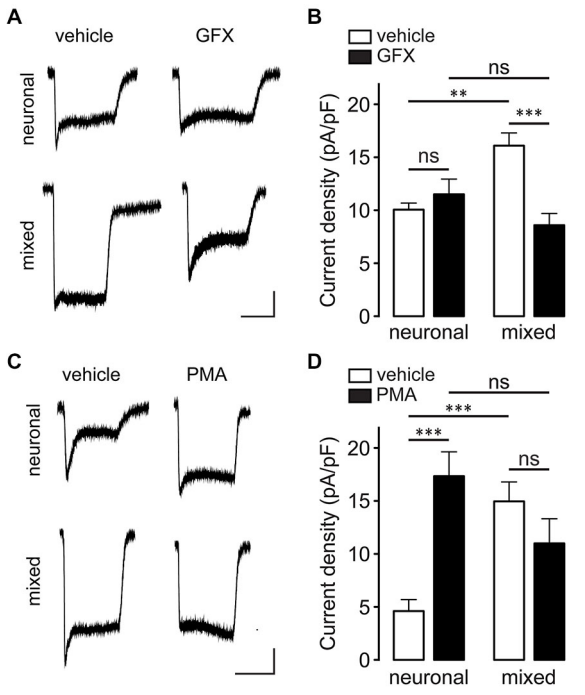

FIGURE 9 | Glia-induced NMDAR current enhancement is mediated by PKC. **(A)** Representative current traces evoked by 100 μM NMDA in the presence of 1 μM glycine recorded after 40 min pretreatment with either vehicle (0.1% DMSO) or GFX (5 μM). Horizontal scale bar, 10 s; vertical scale bar, 250 pA. **(B)** GFX pretreatment blocked the glia-induced potentiation of NMDA-evoked currents in mixed cultures but had no effect in neuronal cultures (**$p < 0.01$, ***$p < 0.001$, two-way ANOVA with Bonferroni post-test). **(C)** Representative current traces evoked by 100 μM NMDA in the presence of 1 μM glycine recorded after 10 min pretreatment with either vehicle (0.1% DMSO) or PMA (100 nM). Horizontal scale bar, 10 s; vertical scale bar, 250 pA. **(D)** PMA pretreatment significantly increased NMDAR current density in neuronal cultures but had no effect in mixed cultures (***$p < 0.001$, two-way ANOVA with Bonferroni post-test).

Discussion

Neuronal NMDARs play a critical role in synaptic plasticity, neuroprotection, and neurotoxicity, but relatively little is known about their regulation by glial cells. Our study identified a novel form of astrocytic NMDAR modulation that occurs in a subunit- and subcellular localization-dependent manner. The density of NMDA-evoked whole-cell current was approximately doubled in neurons cultured in the presence of a mixed population of glia compared to neurons cultured alone (**Figure 1**), indicating a change in either expression or function of postsynaptic NMDAR channels. The glial effect was mediated by (an) astrocyte-secreted soluble factor(s) (**Figure 1**), was Mg^{2+} and voltage independent (**Figure 1**), and could not be explained by an appreciable change in the synaptic density (**Figure 3**). Instead, we found that the peak amplitude of total and NMDAR mEPSCs, but not AMPAR mEPSCs, was significantly larger in mixed cultures, resulting in a decreased synaptic AMPAR/NMDAR ratio (**Figures 2**, **4**, **5**). Astrocytic modulation was restricted to synaptic NMDARs that contain the GluN2B subunit (**Figures 6**, **7**), did not involve an increase in the cell surface expression of NMDAR subunits (**Figure 8**), and was mediated by PKC (**Figure 9**). Collectively, these data show that astrocyte-secreted soluble factor(s) can fine-tune synaptic NMDAR activity through the PKC-mediated regulation of GluN2B NMDAR channels already present at postsynaptic sites, presumably on a rapid time scale (**Figure 11**).

Based on studies of transgenic mice that express dominant negative SNARE (dnSNARE) construct under the control of a glial fibrillary acidic protein (GFAP) promoter, it was previously suggested that astrocytes regulate the surface expression of GluN2A and GluN2B subunits by increasing the extracellular concentration of adenosine, which activates A1 receptors and leads to tyrosine phosphorylation of Src kinase and the GluN2B subunit (Deng et al., 2011). However, these findings have been called in question by a recent study by Fujita et al., which showed that GFAP-dnSNARE mice (i) express dnSNARE not just in astrocytes but also in a subset of cortical and hippocampal neurons and (ii) do not exhibit increased extracellular concentration of adenosine *in vivo* (Fujita et al., 2014). We have not directly tested whether adenosine plays a role in the astrocytic modulation of synaptic GluN2B currents; however, using wild-type neurons and astrocytes, we have not observed a significant increase in the normalized surface expression of GluN2A and GluN2B subunits in mixed relative to neuronal cultures (**Figures 8**, **10**). Our findings thus confirm that astrocytes regulate neuronal NMDAR channels, but suggest that the underlying mechanism does not involve changes in NMDAR subunit trafficking.

NMDAR currents are regulated by PKC in a complex manner; PKC-mediated NMDAR potentiation has been observed most often (Xiong et al., 1998; Lu et al., 1999; Zheng et al., 1999; Liao et al., 2001), but PKC-mediated suppression has also been reported (Markram and Segal, 1992). In the best studied form of PKC-mediated NMDAR current regulation, activation of G_q G protein-coupled (i.e., muscarinic M1 and glutamate mGluR5) receptors leads to activation

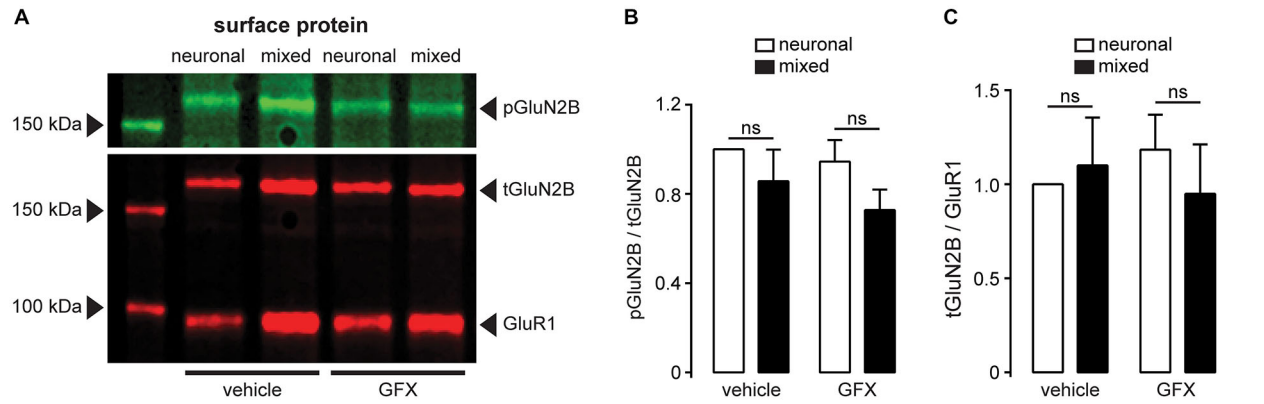

FIGURE 10 | The degree of GluN2B-Ser1303 phosphorylation is not affected by the presence of glia or PKC inhibition. **(A)** A representative immunoblot of surface-expressed proteins showing GluN2B (tGluN2B), phospho-GluN2B-Ser1303 (pGluN2B), and AMPAR subunit GluR1 (loading control); cultures were treated for 3.5 h with either vehicle (0.1% DMSO) or GFX (5 μM). **(B)** Quantification of pGluN2B surface expression normalized to tGluN2B; neither the presence of glia nor the inhibition of PKC affected the phosphorylation status of Ser1303 in the C terminus of GluN2B ($n = 6$; two-way ANOVA with Sidak post-test). **(C)** Quantification of tGluN2B surface expression normalized to GluR1. In agreement with the data shown in **Figure 8**, neither the culture condition nor the PKC inhibition affected the cell surface expression of GluN2B subunit ($n = 6$; two-way ANOVA with Sidak post-test).

of the phospholipase C/PKC/Src kinase signaling cascade and enhancement of NMDA-evoked whole-cell currents that is greater at the peak than at the plateau (Lu et al., 1999, 2000). The preferential enhancement of peak NMDAR currents was originally interpreted as PKC regulation of Ca^{2+}-dependent inactivation (Lu et al., 1999), but is now understood as selective potentiation of GluN2A-containing NMDARs, which preferentially contribute to the peak NMDA-evoked currents (Yang et al., 2014). G_q/PKC/Src-mediated regulation of GluN2A NMDARs is thus distinct from the astrocyte-induced potentiation of GluN2B NMDARs reported in the current study, although both events seem to be mediated by a change in the single channel kinetics rather than a change in the single channel conductance [(Lu et al., 1999) and **Figure 8**]. It is not entirely clear why the PKC-mediated modulation of GluN2B NMDARs has not been observed in the earlier studies; however, it is worth noting that the previous work was performed in the absence of glia, largely using heterologous expression systems or acutely dissociated neurons. It is possible that astrocyte-secreted factors responsible for GluN2B NMDAR regulation activate PKC through a non-canonical, G_q G protein-independent mechanism that is yet to be elucidated.

What is the molecular target of PKC phosphorylation? Although both GluN1 and GluN2 subunits contain multiple PKC phosphorylation consensus sites (Chen and Roche, 2007), mutation or deletion of these sites does not completely eliminate PKC-mediated NMDAR current enhancement, suggesting that PKC effects are at least partly indirect (i.e., mediated by phosphorylation of other neuronal proteins). The C terminus of the GluN2B subunit contains two adjacent Ser residues (Ser1303 and Ser1323) that can be phosphorylated by PKC (Chen and Roche, 2007); Ser1303 is also phosphorylated by calcium/calmodulin-dependent protein kinase II (CaMKII) (Omkumar et al., 1996). In *Xenopus* oocytes, mutations of Ser1303 and Ser1323 strongly attenuate, but do not completely block, the PKC modulation of recombinant GluN1/2B channels (Liao et al., 2001). We have not observed any difference in the phosphorylation status of Ser1303 between the two culture conditions either at baseline or following treatment with the PKC inhibitor GFX (**Figure 10**), indicating (i) that this residue is not involved in the astrocyte- and PKC-mediated modulation of synaptic GluN2B receptors and (ii) that in the intact neuronal milieu a kinase other than PKC (presumably CaMKII) has a dominant effect on Ser1303 phosphorylation. Additional work will be required to establish whether astrocyte-induced PKC activation leads to phosphorylation of a different residue within the GluN2B subunit or acts through an ancillary protein that is part of the NMDAR signaling complex (**Figure 11**). Notably, astrocytic regulation is restricted to synaptic GluN2B receptors (**Figures 6**, **7**); given that GluN2B receptors can move between synaptic and extrasynaptic sites (Groc et al., 2006), it is not currently clear how this specificity is achieved. Most likely, kinases, phosphatases, and other signaling proteins that make up the supramolecular NMDA receptor complex differ between synaptic and extrasynaptic sites, and the differences in this subcellular environment play a role in the regulation of NMDAR channel activity.

What is the functional significance of synaptic GluN2B receptor regulation by astrocytes? At the early stages of postnatal development, there is a change in the neuronal NMDAR composition: GluN2B-containing NMDARs, which are the main NMDAR subtype expressed at birth, are partially replaced by GluN2A-containing NMDARs, which have a lower glutamate affinity and exhibit faster inactivation kinetics (Paoletti et al., 2013). This GluN2A-2B switch coincides with synaptic maturation; however, synapses with high GluN2A to GluN2B ratio require stronger stimulation to undergo long term potentiation (LTP; Paoletti et al., 2013). Thus, the GluN2A-2B switch is likely to have a negative effect on learning and

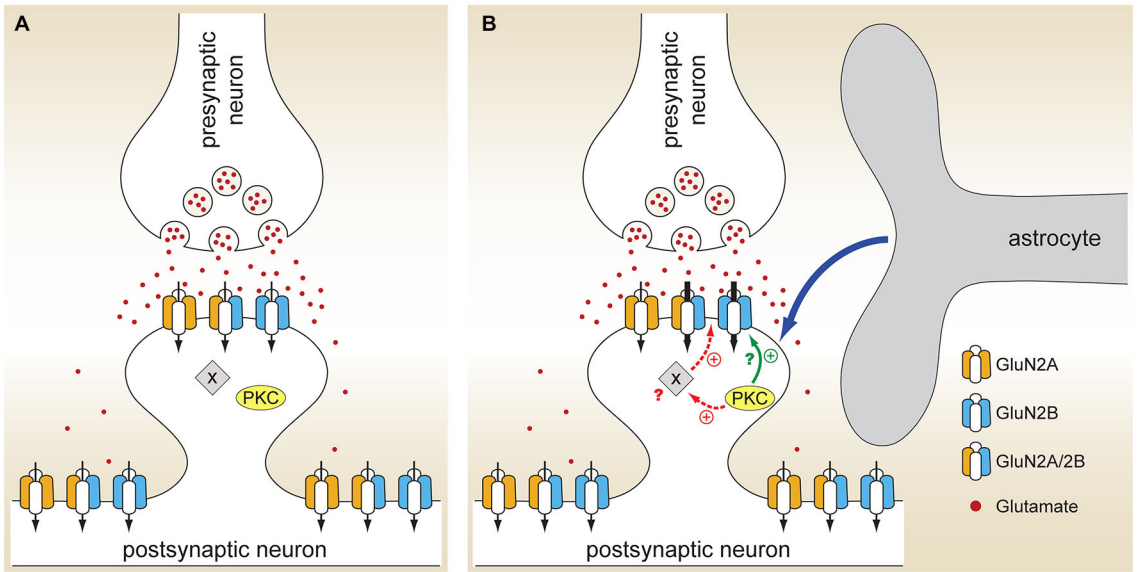

FIGURE 11 | Model for astrocyte-mediated regulation of neuronal NMDARs. (A) When hippocampal neurons are cultured in the absence of glia, glutamate released from the presynaptic terminal activates both GluN2A and GluN2B NMDARs localized at synapses. **(B)** In the presence of glia, astrocytes secrete a currently unidentified soluble factor or factors (blue arrow) that activate PKC. Through phosphorylation of either the GluN2B subunit itself (solid green arrow) or, more likely, an ancillary protein (X) associated with the NMDAR signaling complex (dashed red arrows), PKC activation leads to an increase in the activity of synaptic GluN2B NMDARs without affecting the activity of either synaptic GluN2A NMDARs or extrasynaptic GluN2B NMDARs; it remains to be seen whether astrocytic modulation is specific for GluN1/2B heterodimers or it also affects GluN1/2A/2B heterotrimers (as shown).

memory. In agreement with this hypothesis, overexpression of the GluN2B subunit in the adult forebrain leads to more robust LTP and enhanced performance on various learning and memory tasks in both mice (Tang et al., 1999) and rats (Wang et al., 2009), even at an old age (Cao et al., 2007). Of greater physiological relevance, transgenic mice with high serum levels of the anti-aging factor klotho show an increase in the GluN2B portion of synaptic NMDAR currents, which is accompanied by enhanced LTP and better performance on tests of learning and memory; in humans, heterozygosity for a lifespan-extending variant of the *KLOTHO* gene is associated with increased klotho serum levels and better cognition (Dubal et al., 2014). While klotho appears to regulate the number of GluN2B receptors expressed at synapses (Dubal et al., 2014), a similar learning enhancement would be expected to result from an astrocyte-mediated increase in the activity of GluN2B NMDARs that are already present at postsynaptic sites. We therefore propose that astrocytes can dynamically regulate the synaptic GluN2A/2B current ratio and the threshold for LTP induction; identification of the astrocyte-secreted factor(s) that mediate(s) NMDAR regulation will enable the direct testing of this hypothesis.

Ca^{2+} influx through synaptic NMDARs leads to activation of FOXO, C/EBPβ, and AP-1 signaling pathways that together have a neuroprotective effect (Papadia et al., 2008). In contrast, activation of extrasynaptic NMDARs is generally neurotoxic (Hardingham et al., 2002). GluN2A- and GluN2B-containing NMDARs were originally hypothesized to preferentially signal to cell survival and cell death cascades, respectively (Liu et al., 2007); however, later studies have demonstrated that either receptor subtype can activate both neuroprotective and neurotoxic signaling pathways (von Engelhardt et al., 2007; Martel et al., 2009). More recently, it has been shown that the C terminus of GluN2B is more effective than the C terminus of GluN2A in promoting neuronal death; however, this effect was observed when both synaptic and extrasynaptic NMDARs were chronically activated (Martel et al., 2012). In contrast, trans-synaptic (physiologic) activation of GluN2B receptors was shown to be neuroprotective (Papadia et al., 2008; Martel et al., 2009). Interestingly, we have found that astrocyte-secreted factor(s) selectively increase the current flow through synaptic, but not extrasynaptic, GluN2B-containing NMDARs. In the setting of physiologic synaptic activity, the astrocyte-mediated increase in the synaptic GluN2B receptor activity would thus be expected to have a net neuroprotective effect.

In summary, we have shown that astrocyte-secreted soluble factor(s) can fine-tune the activity of synaptic GluN2B NMDAR channels. Through this presumably rapid and reversible PKC-mediated molecular mechanism, astrocytes can regulate synaptic AMPAR/NMDAR and GluN2A/2B ratios without altering the expression or trafficking of NMDAR channel subunits. Elucidating the details of the signal transduction pathway that mediates astrocytic regulation of synaptic GluN2B channels thus has a potential to foster the development of pharmacologic treatments that will enhance cognitive performance while strengthening neuroprotective signaling.

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Neuron-glia networks: integral gear of brain function

Gertrudis Perea[1]*, Mriganka Sur[2] and Alfonso Araque[3]*

[1] *Functional and System Neurobiology, Instituto Cajal, Consejo Superior de Investigaciones Científicas, Madrid, Spain*
[2] *Department of Brain and Cognitive Sciences, Picower Institute for Learning and Memory, Massachusetts Institute of Technology, Cambridge, MA, USA*
[3] *Department of Neuroscience, University of Minnesota, Minneapolis, MN, USA*

Edited by:
Vladimir Parpura, University of Alabama, USA

Reviewed by:
Dmitri A. Rusakov, University College London, UK
Jean-pierre Mothet, CNRS - Aix Marseille University, France

****Correspondence:***
Gertrudis Perea, Functional and System Neurobiology, Instituto Cajal, Consejo Superior de Investigaciones Científicas, Av. Doctor Arce, 37, Madrid 28002, Spain
e-mail: gperea@cajal.csic.es; Alfonso Araque, Department Neuroscience, University of Minnesota, 4-110 Wallin Medical Biosciences Building, 2101 6th Street, Minneapolis, 55455 MN, USA
e-mail: araque@umn.edu

Astrocytes, the most abundant glial cell in the brain, play critical roles in metabolic and homeostatic functions of the Nervous System; however, their participation in coding information and cognitive processes has been largely ignored. The strategic position of astrocyte processes facing synapses and the astrocyte ability to uptake neurotransmitters and release neuroactive substances, so-called "gliotransmitters", provide the scenario for prolific neuron-astrocyte signaling. From studies at single-cell level to animal behavior, recent advances in technology and genetics have revealed the impact of astrocyte activity in brain function from cellular and synaptic physiology, neuronal circuits to behavior. The present review critically discusses the consequences of astrocyte signaling on synapses and networks, as well as its impact on neuronal information processing, showing that some crucial brain functions arise from the coordinated activity of neuron-glia networks.

Keywords: astrocytes, neuron-glia network, synaptic plasticity, gliotransmission, information coding

INTRODUCTION

Brain information processing is conventionally recognized as derived from neuronal activity, with neurons and their dynamic signaling responsible for the transfer and processing of information (Majewska and Sur, 2006). However, the brain also contains other non-neuronal cells, glial cells, which exceed the number of neurons and have been largely ignored as being involved in the processes related with information coding handling by neural networks and underlying brain function. Nonetheless, decisive advances in the characterization of the molecular and physiological properties of astrocytes, a particular type of glial cells have revealed that they may play active roles in neurotransmission and neuronal physiology (Araque et al., 1999; Perea et al., 2009). Accordingly, novel concepts in brain physiology have been coined, such as "tripartite synapse", to highlight the direct involvement of astrocytes in synaptic function, gliotransmitters, to generically name neuroactive substances released by astrocytes, or gliotransmission, to define the active signaling between astrocytes and neurons (Volterra and Bezzi, 2002).

There is common agreement regarding the crucial roles of astrocytes in controlling the homeostasis of surrounding synapses, with a fundamental role in energy metabolite supply (Hassel et al., 1995; Westergaard et al., 1995; Allaman et al., 2011), clearance of extracellular potassium (Sontheimer, 1994; Kressin et al., 1995; Butt and Kalsi, 2006), and glutamate (Hansson et al., 1985; Bergles et al., 1997; Coulter and Eid, 2012). Moreover, astrocytes enwrapping synapses also control extracellular space volume, and hence the extracellular levels and diffusion of neuroactive substances (Nagelhus and Ottersen, 2013). Besides these homeostatic functions, astrocytes display dynamic signaling with neurons and synapses. They sense neuronal and synaptic activity through activation of ion channels, neurotransmitter transporters and receptors. Activation of these molecules may results in elaborate Ca^{2+} signals into astrocytes. In turn, astrocytes, via uptake or release of gliotransmitters, such as glutamate, ATP, and D-serine, (Santello et al., 2012), can modulate neighboring pre- and postsynaptic neuronal elements inducing functional as well as morphological changes in synapses. However, whether astrocytes are active elements in neural network function and whether and how they play active roles in brain information processing is an open debate (Agulhon et al., 2008; Hamilton and Attwell, 2010; Araque et al., 2014).

In this review, we will present a brief summary of the currently available data that has challenged the classical idea that brain function results exclusively from neuronal activity, suggesting that astrocytes are integral units of neural circuits playing a role in the coding information by neural networks. We will also discuss the available as well as the still required evidence that suggests brain function actually arises from the coherent coordinated activity of Neuron–Glia networks.

ASTROCYTES PROCESS SYNAPTIC INFORMATION

For a cell to be considered as an active element in the brain coding network, it should be able to: (1) receive incoming information; (2) integrate and code that information; and (3) transfer the information to other cells. Neurons have the ability to perform these actions due to their anatomical characteristics and intrinsic electrical properties. Can astrocytes, which do not display electrical excitability to elicit significant active signals (Sontheimer, 1994), behave as transfer and/or processors of synaptic inputs in a neural network?

While astrocytes lack electrical excitability they show intrinsic cellular excitability based on intracellular Ca^{2+} variations (Charles et al., 1991). More importantly, astrocytes are able to respond to different neurotransmitters that trigger intracellular Ca^{2+} signals (Duffy and MacVicar, 1995; Bezzi et al., 1998; Shelton and Mccarthy, 2000; Araque et al., 2002, 2014). Indeed, astrocytes are able to sense the synaptic activity in different regions induced by several neurotransmitters, such as glutamate, GABA, acetylcholine, ATP or nor-epinephrine (Zorec et al., 2012). A remarkable ability shown by astrocytes is that they sense different neurotransmitters and discriminate between different synaptic inputs. Astrocytes located in stratum oriens of hippocampus differentially sense glutamate released from distinct synapses, i.e., they respond to glutamate released from Schaffer collateral axons of hippocampal CA3 neurons but not to glutamate released from other extrinsic afferences (Perea and Araque, 2005). Likewise, astrocytes located in layer 2/3 barrel cortex selectively respond to the activity of glutamatergic inputs from layer 4 of the same column but not to glutamatergic projections from layer 2/3 of adjacent columns (Schipke et al., 2008), showing preference for sensory incoming inputs rather than lateral cortical signaling. Another example of the response selectivity is provided by astrocytes of the ventrobasal thalamus, which show preferential responsiveness to corticothalamic inputs vs. sensory pathways, in spite that both inputs release the same neurotransmitter glutamate (Parri et al., 2010). Therefore, since astrocytes are able to sense incoming information, and also show selectivity in their responses and discrimination of specific synapse activity, they fulfill the first requirement postulated.

The second requirement postulates that a processor must have the ability to integrate different inputs and elaborate specific responses; that is, perform a nonlinear input–output readout. Do astrocytes display integrative properties of the incoming synaptic information? It has been demonstrated that astrocytes are able to discriminate between the activity of different synaptic inputs releasing different neurotransmitters, i.e., cholinergic and glutamatergic hippocampal synapses belonging to different axon pathways, alveus and Schaffer collaterals pathways, respectively; showing selective Ca^{2+} responses to those inputs (Perea and Araque, 2005). Furthermore, as consequence of simultaneous activity of these synapses astrocyte can integrate these inputs and modulate their Ca^{2+} signal producing a nonlinear input–output response. The astrocyte Ca^{2+} signaling is bidirectionally regulated by synaptic activity, showing an enhancement or depression by low and high synaptic activity, respectively (Perea and Araque, 2005). The integration of different signals also arises in the absence of neural network activity indicating that this modulation is due to cellular intrinsic properties. Thus astrocytes, rather than being simple elements performing a linear readout of synaptic activity are active elements endowed with integrative properties, hence satisfying the second requirement to participate in the information coding by the neuronal networks.

BRAIN INFORMATION PROCESSING BY NEURON–GLIA NETWORKS

The third postulate to consider astrocytes as unit processors in the coding of information by neuronal networks requires that they transfer the information to other elements, i.e., influencing neuronal activity and synaptic transmission. Astrocytes can control the activity of neurons and synapses through the uptake of neurotransmitters or the release of gliotransmitters, thus contributing to neuronal network function.

The ability of astrocytes to release gliotransmitters in response to neuronal activity has been reported in several brain areas (Zorec et al., 2012). Those gliotransmitters activate receptors located at presynaptic and postsynaptic sites in neuronal membranes leading the regulation of neuronal excitability and synaptic transmission and plasticity (Kang et al., 1998; Fellin et al., 2004; Pascual et al., 2005; Perea and Araque, 2005, 2007; Panatier et al., 2006, 2011; Stellwagen and Malenka, 2006; Henneberger et al., 2010; Navarrete and Araque, 2010; Di Castro et al., 2011; Santello et al., 2011; Fossat et al., 2012). These findings indicate that astrocytes signal to neurons, transferring information to other elements in the network, thus satisfying the third postulated requirement.

Moreover, this astrocyte-to-neuron signaling has been revealed to present interesting and sophisticated properties. For example, a single gliotransmitter may exert multiple effects on the neuronal network depending on the receptor subtype and its subcellular membrane location. Indeed, glutamate released by astrocytes increases neuronal excitability by inducing slow inward currents (SICs) in excitatory neurons through activation of postsynaptic NMDA receptors (Parri et al., 2001; Angulo et al., 2004; Fellin et al., 2004; Perea and Araque, 2005; Navarrete and Araque, 2008; Shigetomi et al., 2008; Chen et al., 2012); but it also enhances synaptic transmission through activation of presynaptic metabotropic glutamate receptors (mGluRs) group I (Fiacco and McCarthy, 2004; Perea and Araque, 2007; Navarrete and Araque, 2010; Bonansco et al., 2011; Perea et al., 2014); and stimulates synaptic transmission by activation of presynaptic NMDA receptors in dentate granule cells (Jourdain et al., 2007). In addition, astrocytes release different gliotransmitters with different neuromodulatory effects. The concurrent or differential weight of these effects at individual neurons may strongly affect the degrees of freedom of the system and hence the neuronal output and circuit function. Indeed, considering that a single hippocampal astrocyte has been estimated to contact ∼100.000 synapses (Bushong et al., 2002), and that a single gliotransmitter may have different effects depending on the target neurons and neuronal elements (pre- or postsynaptic), as well as the activated receptor subtypes, the variability and complexity of the potential

impact of a single astrocyte on neural network operations can be significant.

Additionally, evidence from hippocampal slices has shown that gliotransmission is a regulated process controlled by activation of particular membrane receptors, i.e., protease-activated receptor 1 (PAR-1), but not purinergic G protein-coupled receptors (P2Y1) stimulates astrocytic glutamate release that evokes SICs (Shigetomi et al., 2008), which add further complexity to astrocytic output signaling. The physiological and molecular conditions that control the richness of this signaling, such as the precise release of each gliotransmitter, and the co-release of different gliotransmistters by single astrocytes (Bergersen et al., 2012; Martineau et al., 2013) or by defined astrocyte subpopulations are still unresolved.

Thus, the existence of different gliotransmitters, the different mechanisms of action of a single gliotransmitter, and the cellular selectivity of the effects on particular neurons, such as in the olfactory bulb where astrocytes releasing GABA and glutamate lead to cell-specific modulation of neuronal activity (Kozlov et al., 2006), provide an enormous amount of degrees of freedom to the possible network states.

The above discussed evidence indicates that astrocytes can be considered as information processors in neural networks. These findings may expand the possible functional consequences of a single neurotransmitter in the network; thus, the existence of emergent properties in network activity provided by astrocyte signaling has been recently reported in the hippocampus and cortex. The well-known phenomenon observed in hippocampus called heterosynaptic depression of excitatory synaptic transmission (Lynch et al., 1977) results from the coordinated activity of neurons and astrocytes; that is, the glutamate released by Schaffer collaterals, with excitatory effects in the network, excites postsynaptic CA1 is a well known acronym for that specific region of the hippocampus. To simplify the reading I recommend to keep like it is. pyramidal neurons as well as inhibitory interneurons that in turn activate astrocytes through GABA release. Then, GABA-stimulated astrocytes release ATP that after being degraded to adenosine leads to the synaptic depression of adjacent excitatory synapses (Zhang et al., 2003; Serrano et al., 2006). Another example is provided by the effects of endocannabinoid (eCB) signaling to astrocytes. eCBs are known to retrogradely depress synaptic transmission (Chevaleyre et al., 2006), but they can also trigger intracellular calcium signaling in astrocytes (Navarrete and Araque, 2008). eCB-activation of astrocytes stimulates the release of glutamate, which leads to an heterosynaptic enhancement of excitatory synaptic transmission by activation of mGluRs in the hippocampus (Navarrete and Araque, 2010), and the spike timing-dependent depression by activation of NMDA receptors in neocortex (Min and Nevian, 2012).

ASTROCYTE ROLE IN COGNITIVE FUNCTIONS, FROM *IN VIVO* DATA TO ARTIFICIAL NETWORKS

The current knowledge of the astrocyte neuromodulatory roles in neuronal function mainly derives from studies performed in slices, which have great accessibility to explore the particular properties as well as the cellular and molecular mechanisms of neuron-glia signaling. Hence, the impact of astrocytic function on brain activity and animal behavior are still largely undefined. Novel genetic tools have made possible to specifically manipulate astrocyte signaling *in vivo* while maintaining neuronal signaling intact. Recent studies have focused on the role of ATP/Adenosine as well D-serine released by astrocytes (Fossat et al., 2012) in the slow oscillations brain waves, which are related to sleep (Fellin et al., 2009; Halassa et al., 2009). Inhibiting gliotransmission attenuates both the slow cortical oscillations and accumulation of sleep pressure, which caused by prolonged wakefulness periods can impair cognitive function (Yoo et al., 2007); thus, mice with downregulated purinergic gliotransmission do not show cognitive deficits associated with sleep loss (Halassa et al., 2009). It has been recently suggested that one important role of sleep would be the removal of waste products from the brain (Xie et al., 2013). Astrocytes control brain microcirculation (Iadecola and Nedergaard, 2007), and through aquaporin four water channels located in the vascular endfeet facilitate convective flow out of the para-arterial space and into the interstitial space, which is related with removal of waste products made during neuronal activity. The exchange rate of fluids between those spaces is more effective during sleep than during awakening periods, suggesting an important role of astrocytes in sleep function (Xie et al., 2013). In addition, a proper astroglial network signaling is essential for precise synaptic information transferring between neurons as it has been shown in the gap junction proteins connexin 30 (Cx30) and connexin 43 (Cx43) knock out mice, where altered rate of extracellular glutamate and potassium removal during synaptic activity induce impairments in short and long-term synaptic plasticity (Pannasch et al., 2011, 2014), as well as deficits in sensorimotor and spatial memory tasks (Theis et al., 2003; Lutz et al., 2009). Another important role of the astrocyte-neuron signaling relies on the metabolic coupling (Magistretti et al., 1999) and the appropriate energy supports for neuronal activity demand. In this context, cognitive processes and their cellular and molecular substrates (i.e., long-term memory formation), that are high metabolically demanding have been shown to be related with an intercellular trafficking of glucose through astroglial networks (Rouach et al., 2008), and astrocytic lactate transporters (monocarboxylate transporter 4 (MCT4) or MCT1) (Suzuki et al., 2011), where dysfunctions of these lactate transporters causes amnesia and long-term potentiation (LTP) impairments (Suzuki et al., 2011).

Astrocytic role in other critical brain functions such as control of breathing and locomotion have also been reported (Baudoux and Parker, 2008; Gourine et al., 2010), showing that astrocyte–neuron interaction occurs *in vivo* and providing insights into the astrocytic influence to the output of complex neuronal networks and behavior.

Besides the contribution of astrocytes to those homeostatic functions, a challenging question refers to their role in higher brain functions, such as coding information in network activity, and in cognitive processes. Astrocytes immersed in local circuits can sense and respond with Ca^{2+} signals to different sensory stimuli and neurotransmitters; i.e., visual stimuli and somatosensory whisker stimulation (Schummers et al., 2008;

Takata et al., 2011; Chen et al., 2012; Navarrete et al., 2012; Perea et al., 2014), indicating that astrocytes are able to perceive incoming sensory inputs. But do they have a role in the coding of sensory information? If they were actively involved in this task, they should locally impact synapses of the sensory system, which would be translated into a modulation of global brain state dynamics. Recent studies focused on the cholinergic system function have revealed the active and crucial roles of astrocytes in information processing by neuronal networks (Takata et al., 2011; Chen et al., 2012; Navarrete et al., 2012). Cholinergic activity is related with changes in brain states, such as during attention and vigilance states (Everitt and Robbins, 1997; Sarter et al., 2005), contributes to hippocampal theta rhythm oscillations (Hasselmo, 2006), and induces synaptic plasticity processes (Picciotto et al., 2012). Thus, these novel studies have shown that astrocytes and gliotransmission are the cellular and molecular mechanisms underlying certain cholinergic effects (Takata et al., 2011; Chen et al., 2012; Navarrete et al., 2012). In the hippocampus, astrocytes respond with Ca^{2+} elevations to cholinergic activity triggered by either sensory stimuli or direct stimulation of cholinergic nuclei and axons (Araque et al., 2002; Perea and Araque, 2005; Navarrete et al., 2012). This astrocyte Ca^{2+} signal is necessary to trigger the synaptic LTP associated with the cholinergic activity through a mechanism that involves the release of the gliotransmitter glutamate and the subsequent activation of mGluRs (Navarrete et al., 2012). In somatosensory and visual cortex, cholinergic activation of astrocytes stimulates the release of D-serine or glutamate, which, in association with whisker (Takata et al., 2011) or visual stimuli (Chen et al., 2012), mediate long-term plasticity changes in cortical sensory responses mediated by NMDA receptors. Notably, the astrocyte-mediated potentiation of visual inputs is indeed stimulus-specific, because only synapses active during cholinergic stimulation display such modulation, indicating that the influence of astrocyte-mediated plasticity is synapse-specific and suggesting the existence of intimate organization of astrocytes and synapses that convey and generate visual-specific responses (Schummers et al., 2008; Chen et al., 2012).

New useful tools have been developed in the last few years to decipher the contribution of specific cellular types to the brain information processing. Specifically, the optical control of cellular activity using optogenetics represents a major technical breakthrough in the field of Neurosciences (Fenno et al., 2011). Exploiting the advantages of optogenetics, astrocytes can be selectively stimulated with light to evaluate their consequences either at the local circuits and global network (Gourine et al., 2010; Sasaki et al., 2012; Chen et al., 2013; Perea et al., 2014; Tang et al., 2014). Astrocytes placed in visual cortex, in addition to sensing visual information, when stimulated with the opsin channelrhodopsin-2 lead to an increase in synaptic transmission by releasing glutamate, which regulates excitatory and inhibitory transmission through activation of presynaptic mGluRs (Perea et al., 2014). Furthermore, astrocytes impact visual responsiveness of both excitatory and inhibitory cortical neurons, showing specific modulation of key neuronal response features (Perea et al., 2014). Thus, astrocytes through the dual control of excitatory and inhibitory drive, influence neuronal integration critical for sensory information processing. As a consequence of differential astrocytic neuronal subtype modulation, changes in the excitatory-inhibitory balance in the local network might impact the final output of the circuit, indicating that astrocytes would be involved in multiple aspects of information coding by cortical networks.

Other accumulating evidence indicates the active role of astrocytes in brain cognitive functions (Parpura et al., 2012). Indeed, behavioral studies have demonstrated the correlation between astrocyte signaling dysfunction and cognitive deficits associated with neurobiological diseases, such as major depressive disorder (Cao et al., 2013; Lima et al., 2014), and Huntington's disease (Tong et al., 2014). Abnormalities of astrocyte-neuron signaling due to the reduced glial release of ATP in prefrontal cortex, a brain region implicated in attentional processes, decision-making, working memory and processing of emotional stimuli, among others (Goldman-Rakic, 1995), are related with depression-like behaviors (Cao et al., 2013), affecting important functions of this cortical area, such as attentional context, working memory and reversal learning functions (Lima et al., 2014). Furthermore, through glial activation of cannabinoid receptor type 1, astrocytes are shown to be responsible for the impairment in working memory performance induced by marijuana (Han et al., 2012), one of the most common effects of cannabinoid intoxication in humans. Likewise, the dysfunctions in astrocyte-mediated potassium homeostasis in striatal astrocytes (Tong et al., 2014), and AMPA receptors expression Bergmann glial cells (Saab et al., 2012), have strong impact on the fine-tuning control of complex motor behaviors, revealing astrocytes as therapeutic targets for motor phenotype disorders (Tong et al., 2014). Despite these compelling findings, many questions remain open and more effort is still needed to fully understand the molecular mechanisms and the physiological conditions underlying neuron-glia signaling and their relevance for other brain functions.

Taken together, this growing body of evidence illustrates the existence of Neuron-Glia networks in brain circuits, and demonstrates the ability and influence of astrocytes in neuronal network operations. They show that astrocytes are a structurally and functionally indivisible part of such networks (**Figure 1**), and reveal how the coordinated signaling pathways established between neurons and astrocytes enables a wide range of, and perhaps all, brain functions.

Finally, in order to fill the gap between the molecular and cellular mechanisms identified by *in situ* studies and *in vivo* behavioral outcomes, the use of mathematical models that include Artificial Neuron-Glia networks appears as a promising approach to better understand the potential emergent properties provided by astrocytes to neural network operation. A pioneering computational study showed that dynamic interaction between astrocytes and presynaptic terminals optimizes synaptic transmission of information (Nadkarni et al., 2008). A more recent study modeling artificial astrocytes in artificial neuronal networks has demonstrated that the presence of astrocytes enhanced the learning potential efficacy of neural networks, i.e., the performance of artificial neuron-glia networks that include artificial astrocytes is improved when compared with similar purely neuronal networks

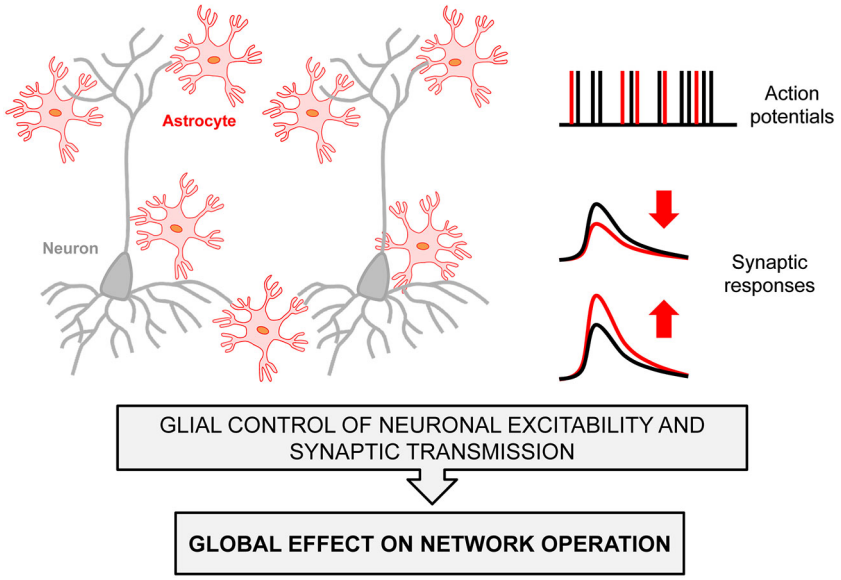

FIGURE 1 | Neuron-Glia Network scheme. Astrocytes (red) interact with neurons (gray) and impact their cellular excitability (*right-top* panel) and synaptic responses (*right-bottom* panel), which influence important features of information coding by local circuits and hence neural networks operation. Black and red labels in the right panels denote activity signals of neuron networks versus neuron-glia networks.

lacking astrocytes (Porto-Pazos et al., 2011). Indeed, including astrocytes into a multilayer feed forward artificial neural network model designed to solve different classification tasks improve the network performance. This relative improvement depends on the intrinsic properties of astrocytes and the strength of the neuron-glia connections and varies with the complexity of the problem tested. Interestingly, the relative efficacy of artificial neuron-glia networks vs. pure neuronal networks increases as the complexity of the network increases (Porto-Pazos et al., 2011), which is in agreement with the gradual increase of the astrocyte-neuron ratio of cells observed in the phylogeny as the nervous system complexity increases. In addition, astrocytes are able to change the threshold value controlling the transition of synchronous to asynchronous behavior among neurons (Amiri et al., 2013). In this way, changes in the interaction properties of astrocyte-neuron signaling lead to the emergence of synchronous/asynchronous patterns in neural responses, showing how astrocytes play a primary role in neuronal firing synchronicity and synaptic coordination (Amiri et al., 2013). Artificial astrocytes through the activation of SICs in neurons participate directly in synaptic plasticity processes, synchronizing postsynaptic activity in neuron clusters and subsequently allow Spike-Timing-Dependent Plasticity based learning to occur at the associated synapses (Wade et al., 2011).

Therefore, the computational evidence of the impact of astrocyte-neuron interactions in neural networks suggests that the richness of biological interactions and brain cognitive functions might emerge from the coordinated activity of Neuron-Glia Networks. Because these computational studies generate in many cases new questions to the field rather than answers, refined future models of Artificial Neuron-Glia Networks implementing more realistic bioinspired network models are needed, which incorporating more features of this complex bidirectional signaling would help to better understand the emergent properties provided by astroytes to network operations. In conclusion, our current knowledge indicates the existence of a rich and complex array of mechanisms underlying astrocyte-neuron interactions that involve different signaling properties determined by multiple elements, e.g., neurotransmitters, gliotransmitters, membrane channels and receptors, intracellular pathways, structural arrangements, etc., and which exert powerful effects on the synaptic function and network operation that underlie brain states and enable behavior.

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Caenorhabditis elegans glia modulate neuronal activity and behavior

Randy F. Stout Jr.[1]*, Alexei Verkhratsky[2,3,4] and Vladimir Parpura[5,6]*

[1] Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY, USA
[2] Faculty of Life Sciences, The University of Manchester, Manchester, UK
[3] IKERBASQUE, Basque Foundation for Science, Bilbao, Spain
[4] Department of Neurosciences, University of the Basque Country UPV/EHU, Leioa, Spain
[5] Department of Neurobiology, Center for Glial Biology in Medicine, Civitan International Research Center, Atomic Force Microscopy and Nanotechnology Laboratories, and Evelyn F. McKnight Brain Institute, University of Alabama, Birmingham, AL, USA
[6] Department of Biotechnology, University of Rijeka, Rijeka, Croatia

Edited by:
Tycho M. Hoogland, Netherlands Institute for Neuroscience, Netherlands

Reviewed by:
Bernd Kuhn, Okinawa Institute of Science and Technology Graduate University, Japan
Ping Liu, University of Connecticut Health Center, USA

***Correspondence:**
Randy F. Stout Jr., The Dominick P. Purpura Department of Neuroscience, Rose F. Kennedy Center, Albert Einstein College of Medicine, 1410 Pelham Parkway South, Room 840, Bronx, NY 10461, USA
e-mail: randy.stout@einstein.yu.edu
Vladimir Parpura, Department of Neurobiology, University of Alabama, 1719 6th Avenue South, CIRC 429, Birmingham, AL 35242, USA
e-mail: vlad@uab.edu

Glial cells of *Caenorhabditis elegans* can modulate neuronal activity and behavior, which is the focus of this review. Initially, we provide an overview of neuroglial evolution, making a comparison between *C. elegans* glia and their genealogical counterparts. What follows is a brief discussion on *C. elegans* glia characteristics in terms of their exact numbers, germ layers origin, their necessity for proper development of sensory organs, and lack of their need for neuronal survival. The more specific roles that various glial cells have on neuron-based activity/behavior are succinctly presented. The cephalic sheath glia are important for development, maintenance and activity of central synapses, whereas the amphid glia seem to set the tone of sensory synapses; these glial cell types are ectoderm-derived. Mesoderm-derived Glial-Like cells in the nerve Ring (GLRs) appear to be a part of the circuit for production of motor movement of the worm anterior. Finally, we discuss tools and approaches utilized in studying *C. elegans* glia, which are assets available for this animal, making it an appealing model, not only in neurosciences, but in biology in general.

Keywords: glia, evolution, behavior, invertebrate, *Caenorhabditis elegans*

INTRODUCTION: A BRIEF ON EVOLUTION OF NEUROGLIA

"Nothing in biology makes sense except in the light of evolution".
Theodosius Dobzhansky (1900–1975)

Evolution of the nervous system proceeded through an increase in number and complexity of the nervous elements and through their specialization into electrically excitable neurons connected through defined synaptic contacts and electrically non-excitable neuroglia forming networks through intercellular gap junctions. Intercellular chemical neurotransmission is, however, characteristic for both forms of the neural cells that express appropriate receptors and are capable of secreting neurotransmitters. The evolution of the nervous system was not a straight journey from less complex and accomplished networks to the more refined ones; at the turning point between invertebrates and vertebrates, a fundamental metamorphosis occurred that changed the overall structure of the central nervous system (CNS). This change is associated with an appearance of radial glia, that in the vertebrates, serve as a universal neural precursor and a guide for neural cells to migrate through the thickness of the neural tube thus creating a layered organization of the CNS (Kriegstein and Alvarez-Buylla, 2009). This layered organization is at odds with the CNS of invertebrates that essentially appears as fused neural ganglia. This major metamorphosis in the CNS organization also coincided with an extinction of the whole class of cells highly elaborated in the invertebrates—the parenchymal neuroglia. Indeed, in hemichordates (that barely have any CNS) glial cells seem to be completely absent, whereas in the primitive vertebrates (such as, for example, zebra fish or certain sharks and rays) the CNS contains essentially one functional layer that could be completely penetrated by radial glial cells, which perform major homeostatic functions. Increase in the thickness and size of the CNS strained the radial glia, and a new wave of evolution of parenchymal neuroglia began in some Elasmobranchi with "elaborated" brain (Reichenbach et al., 1987; Ari and Kálmán, 2008). This evolution rapidly resulted in high diversification of neuroglia, which assumed all major homeostatic and many defensive responsibilities in the mammalian brain. However, the direction of neuroglial evolution in Chordata is rather similar to that in the invertebrates, in which neuroglia similarly underwent remarkable morphological and

function diversification while climbing the phylogenetic ladder from the most primitive bilateralia to the arthropods with their well-developed nervous system (for detailed account on glial evolution and references see Hartline, 2011; Verkhratsky and Butt, 2013).

The early evolutionary history of neuroglia is complex and is far from being characterized in detail. There is a general agreement that supportive neural cells are absent in the diffuse nervous system of Cnidaria and Ctenophora, although there are unconfirmed reports about the existence of glia-like cells in the ganglia of scyphomedusae (Bullock and Horridge, 1965). Supportive neural (glia-like) cells are present in the nervous system of Acoelomorpha that are generally considered as the first bilateralia (Bery et al., 2010). Glial cells are found in Nematoda (Heiman and Shaham, 2007), but are absent in phylogenetically more advanced Bryozoa and Gnathifera/Rotifera, even though the Rorifera have a proper CNS, in which neuronal structures are surrounded by either epithelial or muscle cells (Wallace and Smith, 2013). Nonetheless, in Annelida and Arthropoda the neuroglia are well defined and diversified; glial cells become responsible for homeostasis of the nervous system, they provide the hemolymph-brain barrier, they are capable of mounting astrogliotic response to insult and they create ancestral myelin-like sheath around axons (Deitmer et al., 1999; Edwards and Meinertzhagen, 2010). Here, we focus on recent research on the neuroglia of the nematode, *Caenorhabditis elegans*, with particular attention to reports of glia modifying neuronal activity and behavior of this round worm, along with discussion of advantages and limitations of studying glia in the worm, particularly for neuron-glial interactions outside of development.

CHARACTERISTICS OF *C. ELEGANS* GLIA

We provide only a brief overview of the general properties of *C. elegans* glia, as the detailed information can be found in recent reviews in respect to roles in development (Shaham, 2005, 2006; Oikonomou and Shaham, 2011) and evolutionary aspects of worm glia (Heiman and Shaham, 2007).

THE EXACT NUMBER OF GLIA IN THE WORM

Early studies of the nervous system of *C. elegans* produced extremely detailed and meticulously categorized structural information allowing identification of each neural cell (Ward et al., 1975; White et al., 1986; Hall and Russell, 1991). This makes *C. elegans* one of the few animals whose full complement of individual cells has been mapped throughout development and the only such animal widely used as a model in neuroscience. The neuroglia of *C. elegans* were described based on light and electron microscopy, i.e., (ultra)structural characteristics of these cells; they appear to be a part of the nervous system, but did not have morphological characteristics of neurons, i.e., lack pre-synaptic structures (Ward et al., 1975; Thomas, 1994). At that time, a set of 56 cells were classified as glia-like support cells in hermaphrodites (Altun and Hall, 2010). Developmental lineage maps further supported this classification; 50 of the 56 glia-like cells were shown to be of the ectodermal lineage. Of note, the "Handbook of Worm Anatomy" section of the website www.wormatlas.org provides an overview of the anatomic and developmental characteristics of the glia of *C. elegans* in the "support cells" sub-section on the hermaphrodite nervous system.

AN EPHEMERAL COMPARISON OF WORM AND MAMMALIAN GLIA

It is tempting to compare the glia of the worm to those of animals possessing more complex nervous systems. For these comparisons to be accurate, it is important to keep in mind that the nervous system of the worm likely contains fewer cells and connections that are optimal for its ecological niche, and not because it did not have enough evolutionary time or flexibility to attain greater complexity. Worms are not in possession of genes homologous to encoding glial fibrillary acidic protein (GFAP), a marker of astrocytes in mammals (although many other invertebrates express GFAP). Clearly, the functions that mammalian astrocytes perform in controlling blood flow and in contributing to the blood-brain barrier will not be possible for worm glia. Indeed, these functions are not required in the worms due to the small body size and characteristics of the environment in which they live. It is currently unknown if other functions of mammalian glia such as K^+ clearance, and vesicular release and re-uptake of neurotransmitters are performed by glial cells of the roundworm.

Some genetic pathways for glial specification and development do seem to be shared between glia of mammals and those of *C. elegans.* The transcription factor LIN-26 was found to be required for glial cell development and ablation of the *lin-26* gene may cause cells that would become sheath glia to take on some characteristics of neurons (Labouesse et al., 1994). This was the first in a series of genetic/developmental findings that should be considered when we think about how glia evolved in different species. For example, the *hlh-17* gene promoter has been used as a marker for the cephalic (CEP) sensilla sheath (CEPsh) glia (McMiller and Johnson, 2005). The *hlh-17* gene seems to be important for development, but not initial specification of the CEPsh glia (Yoshimura et al., 2008). The *hlh-17* gene has homology to the mammalian regulator of glial development *Olig2*. Genes that regulate the dorsal/ventral patterning of OLIGodendrocyte lineage transrctiption factor 2 (OLIG2) expression in mammals share homology to those required for normal HLH-17 expression specifically in the dorsal CEPsh glia of the worm (Yoshimura et al., 2008 and as reviewed in Oikonomou and Shaham, 2011).

WORM GLIA ARE UNNECESSARY FOR NEURONAL SURVIVAL

Based on experiments using gene deletions and/or cell ablations of glial precursors during development, we do know that a major difference between nervous system of *C. elegans* and nervous system of more advanced animals is that glia are mostly not required for survival of neurons within the mature nervous system of the worm and also the worms are able to survive and reproduce without glia. Except for the notable exceptions discussed later in this review, the glia of the worm seem to function mainly in guiding development of sensory structures in the worm and then act as a barrier by ensheathing the sensory structures in the adult worm. We know a great deal about how genes modulate the development and activity of *C. elegans* neurons, and also the neural molecular components that are shared (and not shared) between the neurons of *C. elegans, Drosophila*, mice, and human.

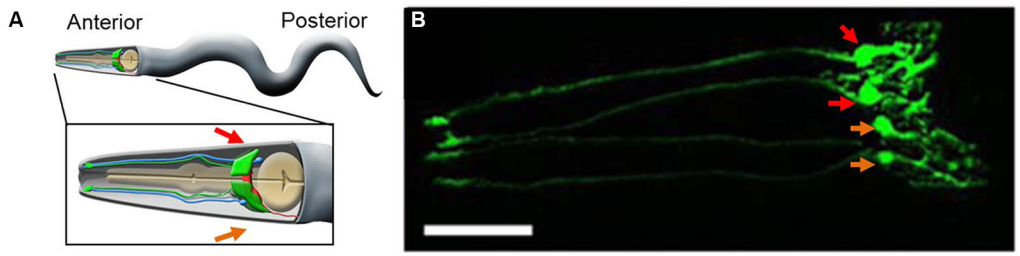

FIGURE 1 | The CEPsh glia. (A) A cartoon of an adult worm showing the four CEPsh glial cells (green) positioned in the anterior of the worm (inset). The CEPsh cell bodies with their velate extensions are positioned around the central nerve ring (red) which they enwrap along with the proximal section of the ventral nerve cord. Additionally, each CEPsh glial cell possesses a long anterior process, emanating to the anterior sensory tip, which closely interacts with the dendritic extension of a nearby CEP neuron (blue). Arrows indicate the dorsal (red arrows) and ventral (orange arrows) side of the worm. **(B)** A confocal image showing green fluorescent protein (GFP) expression driven by the *hlh-17* promoter to visualize the four CEPsh glial cells (worm strain VPR839). The anterior (head) of a juvenile (larval stage 4) worm is shown; the worm is turned ∼45° from "upright" such that all four CEP sheath cells are visible. The sheath portion of the cells that form a tube around the dendritic endings of the CEP neurons are seen at the left of the image. The dorsal (red arrows) and ventral (orange arrows) CEPsh cell bodies are seen. The thin sheet-like extensions that surround and invade the nerve ring are seen in the rightmost part of the image. Scale bar, 20 μm. Image adapted from Stout et al. (2013).

Examining the role of *C. elegans* glia in neural function may help us to understand of how these cells modulate neuronal activity or behavior in order to compare roles that glia of *C. elegans* and mammals play in information processing (see below).

WORM GLIA PLAY A ROLE IN PROPER DEVELOPMENT OF SENSORY ORGANS

Early ablation studies indicated that the sheath and socket glia played a role in the development of the ciliated sensory ending (Bargmann et al., 1990; Vowels and Thomas, 1994). The tractable genetics and other advantages of the worm were used to show that the glia of *C. elegans* affected sensory activity by controlling the development of cellular compartments surrounding sensory cilia (reviewed in Procko and Shaham, 2010). All of the ectoderm-derived glial cells of the worm are associated with the endings of these sensory neurons; neuronal dendritic endings together with glia form sensory organs of the worm known as sensilla. These specialized structures are prominent aspects of the nematode nervous system, and they fail to develop correctly without normal neuron-glia interactions. Neuronal development and maintenance of sensory structures require not only a set of genes expressed in the neurons, but also glial specific genes (reviewed in Oikonomou and Shaham, 2012). Attachments for sensory dendrites during migration of neurons during development also require factors released by the glia of *C. elegans* (Heiman and Shaham, 2009). Furthermore, it was recently suggested that the sensory synapse of the worm could be used as a model to study neuron-glia interactions in the human CNS (Shaham, 2010), albeit the usefulness of this model in this context remains to be seen.

Neuroglia in *C. elegans* perform at least four broad roles in the nervous system: (1) establishment of the location of neuronal structures; (2) regulation of sensory ending size and morphology; (3) a barrier that bundles and separates neuronal elements from other cells; and (4) modulation of neuronal activity. In a certain way this quartet resembles general roles that vertebrate glia are thought to have in the central and peripheral nervous systems. The latter two roles, interrelated with some worm cells and behaviors, are further discussed below.

THE CEP SHEATH GLIA IN SYNAPSE MAINTENANCE AND DOPAMINE-LINKED BEHAVIOR

As already implicated, there appears to be a special class of cells among worm glia. We start our discussion by describing the four CEPsh glial cells (**Figure 1**). These ensheathing cells form a tubular structure surrounding the anterior tip of the sensory ending of CEP neurons and are therefore categorized into the group of 24 sheath glia found in the anterior of the worm. The CEPsh cells are unique in this glial pack in that they also extend thin sheet-like processes which ensheath the nerve ring, i.e., the worm "brain". Thin CEPsh cell processes also extend into the neuropil (White et al., 1986; Durbin, 1987; Oikonomou and Shaham, 2011).

There is morpho-functional heterogeneity between worm glia as in other animals. The morphology of the four CEPsh glia differs substantially from that of other sheath and socket glia (see below). The two ventral CEPsh cells express netrin (uncoordinated-6) but expression of this neuroligand involved in axon guidance is not detected or required in the dorsal pair of CEPsh glia (Hedgecock et al., 1990; Wadsworth et al., 1996; Yoshimura et al., 2008). Thin velate protrusions of the membrane of the CEPsh cells into the nerve ring appear to have some specificity as they are seen in proximity of the same neuronal synapses across different individual worms examined by electron microscopy (Ward et al., 1975; Durbin, 1987). Indeed, the CEPsh cells regulate synapse location through expression of the worm homolog of mammalian netrin (Colón-Ramos et al., 2007). More generally, the CEPsh glia are required for the maintenance of synaptic connectivity within the nerve ring (Shao et al., 2013, and reviewed in Yates, 2013).

Exciting, although circumstantial, evidence indicates that the CEPsh cells modulate dopamine-dependent behaviors in the worm, including feeding and a form of learning (Felton and Johnson, 2011). The *hlh-17* gene encodes the basic Helix-Loop-Helix transcription factor HLH-17 that is expressed almost

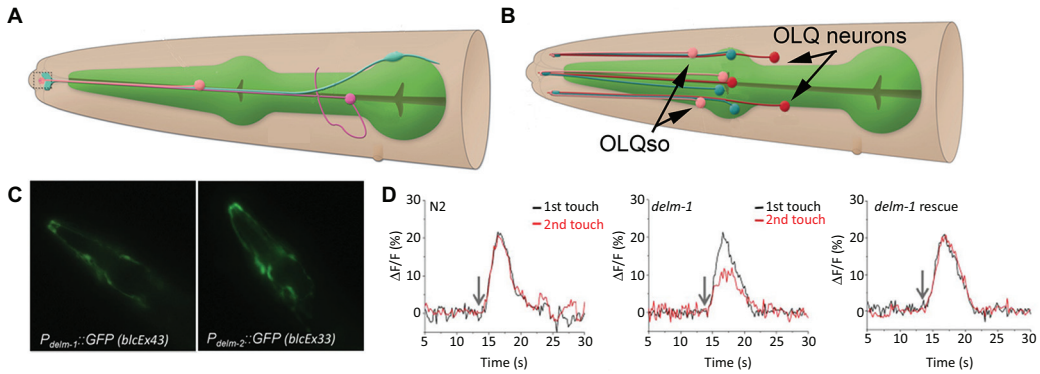

FIGURE 2 | The amphid sheath and outer labial sensilla socket cells. **(A)** The amphid sheath glia (blue) cell body is positioned near the nerve ring (not shown) and sends a long, thin process, along with a neuronal dendrite (magenta) and the amphid socket cell process (pink) to the anterior tip of the worm (left side, dashed box); adapted from *wormatlas.org*. **(B)** A cartoon showing the outer labial sensilla sheath (OLLsh and OLQsh, blue) and socket (OLLso and OLQso, pink) cells and their extensions to the anterior of the worm where they ensheath the ciliated dendrite of the neurons (OLL and OLQ, red); note that inner labial sensilla is not shown here; adapted from *wormatlas.org*. **(C)** The promoters for the *delm-1* and *delm-2* drive reporter GFP expression in the outer and inner labial sensilla socket glial cells (OLQso and ILso, respectively) of worms (strains blcEx43 and blcEx33, respectively). **(D)** Knock out of *delm-1* leads to reduced OLQ neuron calcium response to mechanical stimulation of the worm (middle), while re-expression of the channel in OLQso glia (using the glial promoter *itx-1*) rescued the neuronal responsiveness (right); N2, background strain (left). **C** and **D** adapted from Han et al. (2013).

exclusively in the CEPsh cells (McMiller and Johnson, 2005; Yoshimura et al., 2008). Disruption of the *hlh-17* gene led to changes in egg-laying behavior, feeding behavior-plasticity deficits, and impaired a form of gustatory associative learning. The four CEPsh glia are closely associated with the four CEP neurons, which help mediate the aforementioned behaviors through release and up-take of dopamine (other neurons and neurotransmitters are mediators as well). Although the *hlh-17* gene is not required for development/survival of the CEP neurons and sheath cells, the gene is required for dopamine-dependent behaviors as the loss of *hlh-17* somehow affects dopamine signaling between the CEP neurons. These data represent an exciting hint that CEPsh glia modulate dopamine signaling and future research into this area of worm neurobiology is highly anticipated, especially in light of the role for dopaminergic signaling and dopamine transporters in human neurological diseases.

AMPHID SHEATH AND SOCKET GLIA TUNE SENSORY NEURON ACTIVITY AND SENSORY BEHAVIOR

There is strong evidence that channel activity within other (than CEPsh) sensilla-associated glia modulates neuronal activity, which in turn, affects behavioral responses to environmental stimuli (Wang et al., 2008, 2012; Han et al., 2013). These glia form sheaths around bundles of ciliated sensory dendrites (known collectively as the amphid inner and outer labial sensory organs) at the anterior tip of the worm. Promoter-reporter approaches mapped expression of several DEGenerin/Epithelial Na^+ Channels (DEG/ENaC) class channels to the sheath and socket glia. Through glial specific re-expression of DEG/ENaC channels ACid-sensitive Degenerin (ACD)-1 and DEgenerin Linked to Mechanosensation (DELM)-1,2 it was shown that expression of these channels in sheath and/or socket glia modulates the activity of sensory neurons (Wang et al., 2008, 2012; Han et al., 2013). The ACD-1 channel is required in the amphid sheath glia (depicted in **Figure 2A**), while the DELM-1 and 2 channels act in the inner and outer labial socket glia (depicted in **Figure 2B**). The DELM-1 and -2 are required in the glia but not in neurons in order for the worm to perform a set of foraging related behaviors. In the cells expressing the DELM-1 and 2 channels only re-expression under a glial socket cell promoter rescued sensory behavior and neuronal calcium signaling deficits (**Figures 2C, D**). Similarly the effects of *acd-1* knockout were only rescued by re-expressing the gene in the amphid sheath glia.

So, how could expression of an ion channel in glia modulate sensory neuron activity and mechanosensory behavioral responses? It has been postulated that activity of glial DEG/ENaC channels leads to an increase in extracellular K^+ and thereby to an increase in excitability of the nearby neuronal processes. There is some semblance of this mechanism to specialized regulation of K^+ concentration at sensory endings of vertebrates (Pacinian corpuscle), which modulates sensory neuron activity (Hyinsky et al., 1976). In another study, calcium responses of chemosensory neurons (Amphid Wing Cell (AWC) neurons) to chemical (isoamyl alcohol) stimulations near the detection threshold were used to show that loss of the glial specific ACD-1 channel is required for normal AWC neuron calcium response, but that ACD-1 is not directly affected by such stimulation. Artificial manipulation of the baseline activity of the AWC neurons by expressing a human capsaicin-sensitive TRPV1 channel in the worm's AWC neurons masked the effect of the loss of glial ACD-1. Finally, it was demonstrated that the ACD-1 channels were localized to the anterior end of the worm where the AWC sensory dendrites interacted with chemical stimulant. While all aspects of this neuron-glia interaction are not clear, available evidence supports the idea that the glia

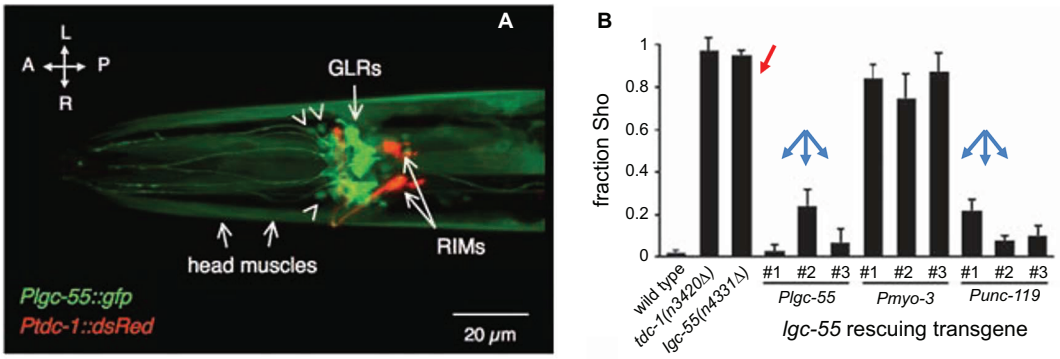

FIGURE 3 | LGC-55 expressed in the GLRs is required for normal tyraminergic modulation of head movements by *C. elegans*. (A) The promoter for the tyramine receptor LGC-55 drives expression of GFP in the six GLRs and head muscle cells of *C. elegans*; RIMs, tyraminergic interneurons expressing red fluorescent protein. Orientation arrows, L-R, left-right, A-P, anterior-posterior. Arrowheads indicate some of the unidentified head neurons that express the *gfp* transgene. **(B)** Worms carrying a mutation in the *lgc-55* gene (or in the *tdc-1* gene, encoding tyrosine decarboxylase, an enzyme that converts L-tyrosine to tyramine) do not suppress foraging movements when crawling backward (Sho phenotype; red arrow). Re-expression of the LGC-55 driven by either the *lgc-55* or *unc-119* (pan-neural) promoters (blue arrows), but not by *myo-3* (muscle) promoter, rescues the behavioral phenotype, indicating that glia or neurons are responsible for the behavior. Adapted from Ringstad et al. (2009).

modulate the threshold for neuronal excitability. Furthermore, it seems that two different sets of glial cells modulate the activity of sensory synapses using different ion channels and that this action is specific to a subset of synapses within the same glial "cradle" structure, this latter being a concept put forward to characterize the role of astrocytes at the vertebrate synapse (Nedergaard and Verkhratsky, 2012). In conclusion, the CEPsh glia are important for development, maintenance and activity of central synapses, whereas the amphid glia seem to set the tone of sensory synapses.

THE GLRS: AN UNORTHODOX GLIAL TYPE

We next discuss the evidence for a signaling pathway between neurons to muscle cells that may pass through an unusual type of *C. elegans* glial cells. Namely, the six Glial-Like cells in the nerve Ring (GLRs) were named based on their morphology and location; however, unlike other worm glia the GLRs are of mesodermal origin (reviewed in www.wormatlas.org (Altun and Hall, 2009)). It is somewhat surprising that a cell type of different origin can so closely morphologically resemble all other glial cells of the worm. Because of the lineage, a comparison to mammalian microglia is tempting, especially in light of a GLR cell having been observed (by electron microscopy) apparently engulfing a dying CEP neuron (Nass et al., 2002). The GLRs are integrated into the nervous system of the worm and contribute to the development of the nerve ring and pharynx (muscle-based feeding organ of the worm). GLRs are connected to both neurons and muscle cells in the head by gap junctions and may be part of the circuit for producing specialized fine motor movements of anterior of the worm during foraging that are analogous to neck movements of vertebrates (White et al., 1986; Ringstad et al., 2009). Two reports examining the role of Ligand-Gated ion Channel (LGC)-55, a tyramine receptor, pointed to its expression in the GLRs. One report indicates that LGC-55 could function in GLRs or neurons to suppress foraging activity during backward movement (Ringstad et al., 2009; **Figure 3**). Another report points to the function of the LGC-55 in muscle and to a lesser extent in neurons (Pirri et al., 2009).

Potential involvement of GLRs in a circuit relevant for behavior is intriguing and warrants further research. This research, into the function of GLRs, however, has been hampered by the lack of a cell specific promoter. This challenge could potentially be circumvented with combinatorial-conditional promoter systems to limit genetically based markers, activity indicators, or functional modulators to the GLRs (Voutev and Hubbard, 2008). The finding that there are gap junctions between the GLRs and neurons is intriguing especially in light of recent discoveries that gap junctions seem to connect the neurons to glia of the sensory ganglia (Suadicani et al., 2010) and as reviewed by Hanani (2012) and Huang et al. (2013) and in the developing CNS (Pakhotin and Verkhratsky, 2005) of mice. The protein subunits that make up the gap junction channels of invertebrates (innexins) and vertebrates (connexins) share no protein sequence homology, but form similar macromolecular structures that connect the cytoplasm of adjacent cells, as reviewed in Scemes et al. (2007) and Simonsen et al. (2014). Additionally, the GLRs of the worm have taken on morphological characteristics of glia although they follow different developmental path, and express a different set of genes than other *C. elegans* glia. This may represent a case of compound convergent evolution and points to a role of gap junction based neuron-glia interaction as a rare but fundamental process, since it seems to have arisen independently in highly divergent nervous systems. Therefore, this may be a particularly important area for future study. The genome of *C. elegans* contains 25 genes for innexins (Starich et al., 2001); a promoter-reporter based survey indicated that the sheath and socket glia also express several innexin genes (Altun et al., 2009). This is another common feature shared between the CEPsh glia and vertebrate astrocytes—prominent expression of gap junctions. However, gap junctions

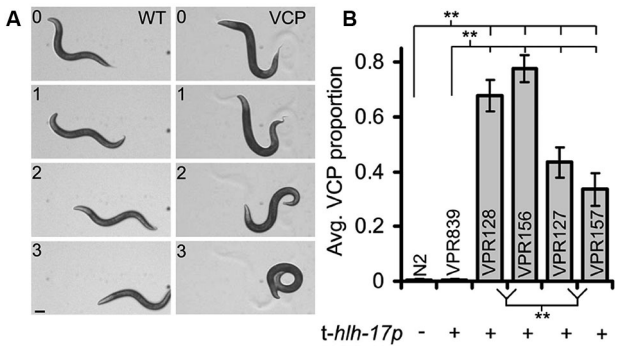

FIGURE 4 | A subset of *C. elegans* strains carrying transgenic arrays containing the trans-*hlh-17* promoter (t-*hlh-17p*) to drive expression of reporters in the CEPsh glial cells display a ventral coiler phenotype (VCP) during backward movement. (A) A time-lapse montage of worms showing a normal/sinusoidal pattern during backwards locomotion (WT, left column). Worms with many copies of the t-*hlh-17p* coil when they attempt to crawl in reverse (VCP, right column; strain VPR156). Numbers indicate time in seconds. Scale bar, 100 μm. **(B)** Non-transgenic worms (WT, N2) do not display the VCP, but different strains of worms (VPR) containing the t-*hlh-17p*, except the VPR839 strain, display varying degrees of abnormal locomotion. ** Indicates a significant difference. Adapted from Stout et al. (2013).

connecting CEPsh glia to other cells have not been investigated and this is currently a woefully understudied aspect of worm glia.

The recently discovered/renamed cell type known as telocytes may be the mammalian equivalent of the worm GLRs (Gherghiceanu and Popescu, 2011; Cretoiu et al., 2012; Smythies and Edelstein, 2014). If the set of genes expressed by the GLRs turns out to share similarity to the set expressed in telocytes, perhaps the GLR-type should be reclassified from glia to ancestral telocytes. Although this is currently purely speculative, such classification would establish the GLRs as a model to study the biology of this exciting new vertebrate cell type.

Finally, it is worth mentioning that the GLRs are connected by gap junctions to ring motor neurons which themselves receive synaptic input from cells that make up a gap junction-mediated circuit with coincidence detection features (Rabinowitch et al., 2013). It will be important to test if the activity of the GLRs influence this or associated circuits and if gap junctions are involved in GLR physiology.

APPROACHES IN STUDYING GLIA IN *C. ELEGANS*

Several themes emerge in the approaches used in the research highlighted above. Rescue of genetic ablation-induced changes to neuronal function or behavior through use of glia-specific promoters is widely used in both invertebrates and vertebrates. This approach is particularly powerful for *C. elegans* since an enormous number of mutant strains are available. The determined cell lineage and ease of specific cell identification is an advantage. Additionally, *C. elegans* is probably the easiest and cheapest animal model organism in which to produce and maintain transgenic animal lines. Cell-type specific promoters are available and, in combination with the phenomenon of transgene mosaicism, genetic rescue or other manipulations can be targeted to individual glial cells (Colón-Ramos et al., 2007; Yoshimura et al., 2008). Transgenes can affect neuronal and behavioral phenotypes in unexpected ways, but if such effects are detected they can be controlled for by using alternative strategies for transgene introduction (Kage-Nakadai et al., 2012; Stout et al., 2013). For example, worms carrying many (more than ∼200) copies of the transgenic *hlh-17* promoter display a ventral coiling behavior during backward locomotion (**Figure 4**); coiling occurrences correlated to the transgene promoter copy number (Stout et al., 2013). This behavior is variable across different transgenic worm strains and can range from subtle to near complete inability for worms to crawl in the backward direction. If worm strains used in studies on worm behavior exhibit even a very mild form of the ventral coiling behavior it would be expected to have a major impact on sensitive behavioral tests such as salt-food associative learning and measures of reversal frequency or on measures of shape of body-bending during locomotion. Future studies where behavioral alterations are attributed to glia will need to control for the possibility that promoters used to target glial cells may cause (in)direct behavioral effects (Stout et al., 2013).

Optical methods for recording neuronal activity are currently more widely used than electrophysiological methods due to difficulty in accessing neurons with patch electrodes which is hampered by the worm cuticle and internal pressure (Kerr et al., 2000; Kerr and Schafer, 2006). Thus far, glial cell activity has only been assessed by genetically encoded optical indicators (Stout and Parpura, 2011; Wang et al., 2012). The ability to culture embryonic and adult stage *C. elegans* cells (Christensen et al., 2002; Frøkjaer-Jensen et al., 2006; Strange et al., 2007), including glia (Stout and Parpura, 2012; Sangaletti and Bianchi, 2013), should ease electrophysiological access to glial cells and has allowed acute application of pharmacological manipulations (Stout and Parpura, 2011; **Figure 5**). For instance, the combination of genetically encoded indicators, mutant strains carrying deletions of voltage-gated Ca^{2+} channels (VGCCs), and pharmacological treatments showed that cultured CEPsh glia respond to membrane depolarization with increases in intracellular Ca^{2+} mediated by various types of VGCCs (e.g., the role of L-type is shown in **Figure 5**). Optogenetic manipulations, i.e., use of channelrhodopsin, have been very useful in the study of *C. elegans* neurons (Nagel et al., 2005), but behavioral effects of light-activated channels have not been hitherto reported for *C. elegans* glia.

It may seem surprising that no reports of laser or genetic ablation of *all* CEPsh glia in *adult* worms have been published, particularly since post-embryonic, larval-stage ablation of a subset of the CEPsh cells led to interesting phenotypes. Briefly, when the precursor cells of the CEPsh glia are ablated during embryonic development the CEP neuron dendrites are shortened, axons in the nerve ring are disrupted, and the entire nerve ring is disrupted in some animals that lack the CEPsh glia. Some worms even failed to develop past the larval L1 stage when CEPsh glia were ablated (Yoshimura et al., 2008). Heat shock-inducible expression of a cell-killing caspase (Chelur and Chalfie, 2007) in adult CEPsh cells led to defects in synapse maintenance in the Amphid

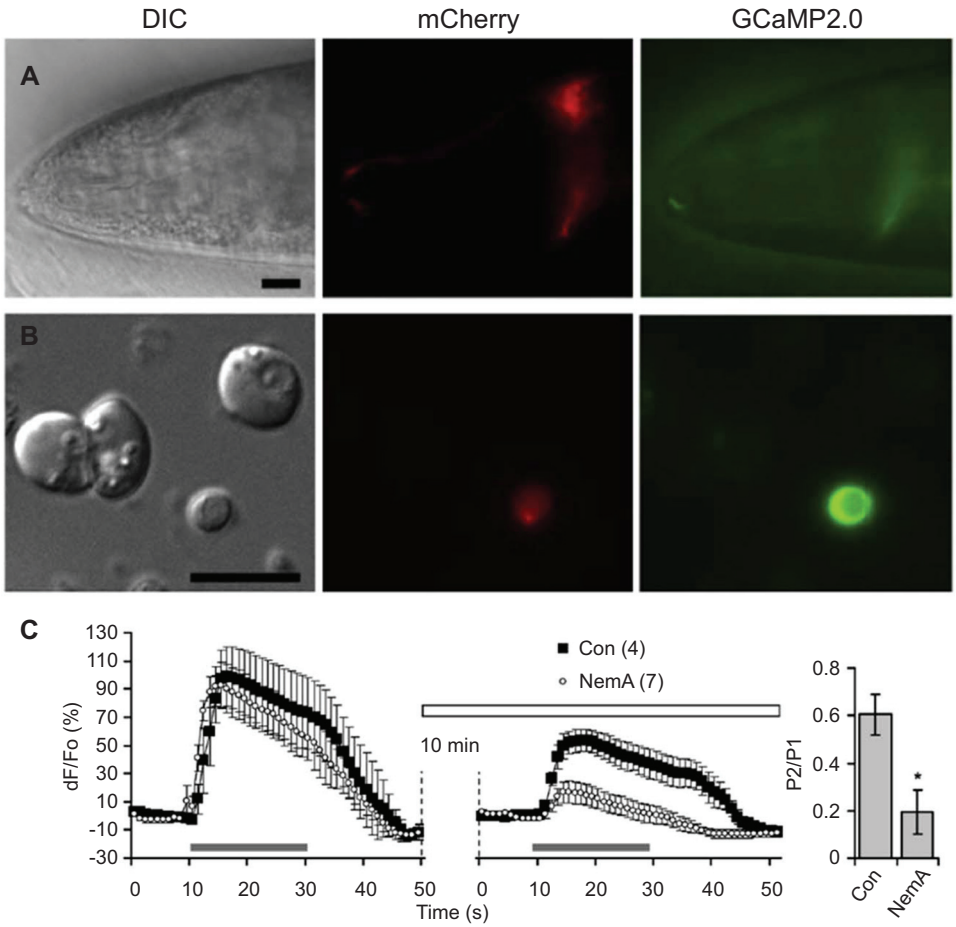

FIGURE 5 | L-type voltage-gated Ca^{2+} channels (VGCCs) play a role in depolarization-induced intracellular Ca^{2+} elevations in CEPsh glial cells. (A) The *hlh-17* promoter can be used to drive expression of a red fluorescent protein marker (red, mCherry) in the CEPsh glia along with a fluorescent protein-based Ca^{2+} sensor (green, GCaMP2,0). Differential interference contrast (DIC). An anterior portion of an L4 stage worm (VPR108 strain) is shown. **(B)** CEPsh glial cells in mixed culture prepared from embryos can be identified based on their mCherry/GCaMP2.0 expression. **(C)** Time-lapse of GCaMP2.0 fluorescence emission from CEPsh glial cells. Paired-pulse application of a depolarization stimulus, high extracellular potassium (HiK^+, 100 mM; horizontal grey bar) to CEPsh glial cells results in an elevation of intracellular Ca^{2+} levels (black squares). Nemadipine-A (NemA), a pharmacological L-type VGCC blocker, can be used to test the channels present in glial cells in culture (horizontal open bar); Con, sham stimulated control (right, bar graph). Ratio of the peak Ca^{2+} level in response to the second HiK^+ application (P2) over the first application (P1). * Indicates a significant difference. Adapted from Stout and Parpura (2011).

Interneuron Y (AIY), but incomplete ablation of all CEPsh glia across individual worms may have occurred (Shao et al., 2013). These data highlight the need for future research to further assess the effect of acute loss of CEPsh glia in adulthood. The present lack of experiments ablating the all CEPsh glia in adult worms, however, may be due to the unusual nature of the CEPsh glia in that they have a large cellular surface area that is spread over a large portion of the anterior nervous system.

In general, many of the methods that worked so well for the study of worm neurons have been harder to implement for glial biology. The development of new optogenetic probes and channels along with our rapidly increasing knowledge of gene expression in worm glia (Spencer et al., 2011) can be expected to make the discoveries we described in this review a start to an exciting time in research on glia-neuron interactions in the adult worm.

ENVOI

The intent of this focus review was to summarize current evidence indicating that the glia of *C. elegans* have an important role in modulation of neuronal activity and behavior. Indeed, future studies are needed to understand details of glial roles in the nervous system of this nematode. Meanwhile, *C. elegans* has proven enormously helpful in unveiling mysteries surrounding the operation of not only the nervous system, but also of many other basic biological functions. Consequently, it shall not come as a surprise that the National Institutes of Health list *C. elegans* as one of the model organisms for biomedical research.[1] This "feather in the cap" has been earned as a consequence of *C. elegans* being one of the animals whose full complement of individual cells has been mapped throughout development and

[1]http://www.nih.gov/science/models/

due to the ease of genetic manipulations, with rapid outcomes, which this nematode offers. Moreover, there are publicly available information resources (e.g., WormAtlas, WormBase and WormBook; http://www.wormatlas.org, http://www.wormbase.org, and http://www.wormbook.org, respectively), and consortia providing various mutation/knockout/transgenic strains on a thrifty budget (e.g., Caenorhabditis Genetics Center at University of Minnesota, Minneapolis, MN; http://www.cbs.umn.edu/research/resources/cgc), making *C. elegans* an appealing model in neurosciences and biomedicine.

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