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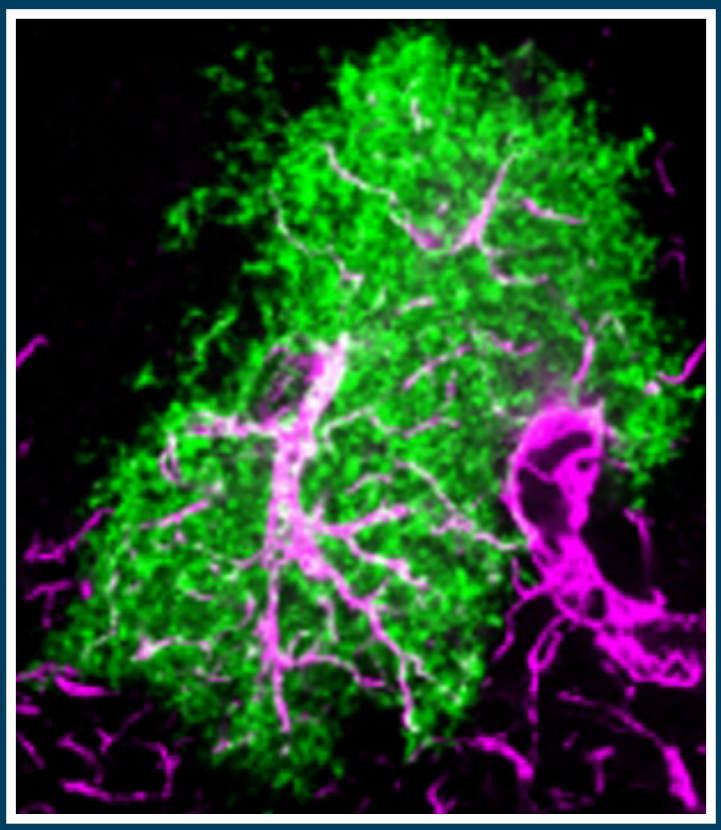

IMAGING AND MONITORING ASTROCYTES IN HEALTH AND DISEASE

Topic Editors
Carole Escartin and Keith K. Murai

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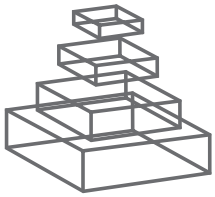

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IMAGING AND MONITORING ASTROCYTES IN HEALTH AND DISEASE

Topic Editors:
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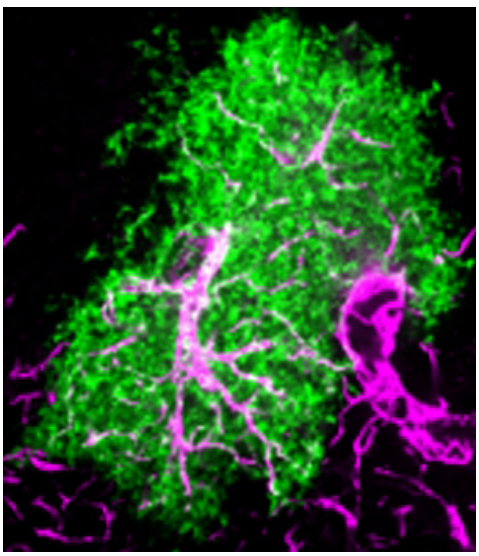

Image of a protoplasmic astrocyte expressing myristoylated enhanced green fluorescenct protein (EGFP) and glial fibrillary acidic protein (GFAP) in the adult mouse cortex. Note the fine astrocytic processes of the astrocyte which are known to associate with synapses and blood vessels.

Astrocytes are key cellular partners to neurons in the brain. They play an important role in multiple processes such as neurotransmitter recycling, trophic support, antioxidant defense, ionic homeostasis, inflammatory modulation, neurovascular and neurometabolic coupling, neurogenesis, synapse formation and synaptic plasticity. In addition to their crucial involvement in normal brain physiology, it is well known that astrocytes adopt a reactive phenotype under most acute and chronic pathological conditions such as ischemia, trauma, brain cancer, epilepsy, demyelinating and neurodegenerative diseases. However, the functional impact of astrocyte reactivity is still unclear.

During the last decades, the development of innovative approaches to study astrocytes has significantly improved our understanding of their prominent role in brain function and their contribution to disease states. In particular, new genetic tools, molecular probes, and imaging techniques that achieve high spatial and temporal resolution have revealed new insight into astrocyte functions in situ.

This Research Topic provides a collection of cutting-edge techniques, approaches and models to study astrocytes in health and disease. It also suggests new directions to achieve discoveries on these fascinating cells.

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EDITORIAL
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Imaging and monitoring astrocytes in health and disease

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Keywords: neuron-astrocyte interactions, reactive astrocytes, *in vivo* analysis, high-resolution imaging, brain imaging, electrophysiology, gene transfer, transgenesis

Astrocytes are key cellular partners to neurons in the brain. They play an important role in multiple processes such as neurotransmitter recycling, trophic support, antioxidant defence, ionic homeostasis, inflammatory modulation, neurovascular and neurometabolic coupling, neurogenesis, synapse formation, and synaptic plasticity. In addition to their crucial involvement in normal brain physiology, it is well known that astrocytes adopt a reactive phenotype under most acute and chronic pathological conditions such as ischemia, trauma, brain cancer, epilepsy, demyelinating, and neurodegenerative diseases. However, the functional impact of astrocyte reactivity is still unclear.

During the last decades, the development of innovative approaches to study astrocytes has significantly improved our understanding of their prominent role in brain function and their contribution to disease states. In particular, new genetic tools, molecular probes, and imaging techniques that achieve high spatial and temporal resolution have revealed new insight into astrocyte functions *in situ.*

This Research Topic illustrates how recent methodological advances have helped to uncover the role of astrocytes in health and disease. The articles assembled cover a range of approaches to both monitor astrocytes (high-resolution microscopy, live imaging, positron emission tomography, nuclear magnetic resonance, and electrophysiology) and manipulate their functional properties (optogenetics, mouse transgenesis, viral gene transfer, and human stem cell differentiation).

IMAGING AND MONITORING ASTROCYTES

In their Technology report, Barros et al. (2013) discuss live imaging methods based on genetically-encoded optical biosensors to quantify, at the single-cell level and with high temporal resolution, the concentration, and dynamics of intracellular metabolites. In their Methods article, Perez-Alvarez et al. (2013) present a detailed methodological procedure to make the most of standard confocal microscopy and perform real-time imaging of astrocytes in the intact mouse brain. Barcia et al. (2013) further illustrate the potency of confocal microscopy to image, in fixed tissues, the microanatomy of astrocyte interactions with immune cells during neuroinflammatory processes. In their Methods article, Haseleu et al. (2013) describe an original technique to study another fine subcellular feature of astrocytes: the peripheral astrocyte process. This method is based on the analysis by conventional microscopy of acutely-dissociated astrocytes from the mouse brain. Dallérac et al. (2013) discuss how astrocytes are not silent in the brain and how studying astrocytes by electrophysiological recordings provides insight into their complex communication with neurons at the synapse. In their Original research article, Kabaso et al. (2013) use electrophysiology, this time combined with modeling, to describe the mechanical properties of vesicular release from astrocytes.

On the larger imaging scale, two articles present brain imaging techniques applied to the study of astrocytes. In his opinion article, Gurden (2013) discusses the evidence that astrocytes have a pivotal position to translate neuronal activity into hyperemic and blood oxygenation level dependent (BOLD) signals, which are measured by functional neuroimaging techniques. O'Brien et al. (2013) present several brain imaging methods to study astrocyte interactions with cerebral tumors *in situ*, including bioluminescence, fluorescent labeling of astrocytes, single photon emission computed tomography, positron emission tomography, and magnetic resonance imaging.

MANIPULATING ASTROCYTES

Li et al. (2013) provide a detailed review of new genetic and imaging tools to study neuron-astrocyte communication at the tripartite synapse. Central to this field, is the physiological manipulation of calcium levels in astrocytes and its precise monitoring with high spatial and temporal resolution. Davila et al. (2013) review the current molecular approaches to overexpress or downregulate genes in astrocytes *in vivo* using mouse transgenesis or gene transfer. They illustrate the potency of these techniques to decipher astrocyte contribution to brain function. Merienne et al. (2013) describe the recently-developed viral vectors to achieve selective gene transfer in astrocytes *in situ*. These versatile tools can be used to model brain diseases involving astrocytes or to test astrocyte-based therapeutic strategies. Krencik and Ullian (2013) present the robustness and limits of using astrocytes derived from human pluripotent stem cells (hPSCs) to model or treat neurodevelopmental diseases. They provide a complete set of guidelines to optimize experiments with these cells.

MULTIDISCIPLINARY APPROACHES TO STUDY THE COMPLEX FEATURES OF ASTROCYTES

Finally, three Review articles extensively describe the multidisciplinary approach undertaken to understand some complex features of astrocytes. Stobart and Anderson (2013) describe our present knowledge on astrocyte contributions to neurometabolic and neurovascular coupling, and discuss how their dysfunction could participate to brain disorders. Through their historical review, Bouzier-Sore and Pellerin (2013) illustrate how

the combination of biochemical analysis, live cellular imaging, magnetic resonance spectroscopy, transcriptomics, and metabolic modeling has contributed to the characterization of the unique metabolic features of astrocytes. Last but not least, Van Horn et al. (2013) provide a historical description of the discovery of D-serine, as a crucial gliotransmitter with multiple roles in brain development and function.

Overall, this Research Topic provides a collection of cutting-edge techniques, approaches, and models to study astrocytes in health and disease. It also suggests new directions to achieve discoveries on these fascinating cells.

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TECHNOLOGY REPORT
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Small is fast: astrocytic glucose and lactate metabolism at cellular resolution

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Brain tissue is highly dynamic in terms of electrical activity and energy demand. Relevant energy metabolites have turnover times ranging from milliseconds to seconds and are rapidly exchanged between cells and within cells. Until recently these fast metabolic events were inaccessible, because standard isotopic techniques require use of populations of cells and/or involve integration times of tens of minutes. Thanks to fluorescent probes and recently available genetically-encoded optical nanosensors, this Technology Report shows how it is now possible to monitor the concentration of metabolites in real-time and in single cells. In combination with *ad hoc* inhibitor-stop protocols, these probes have revealed a key role for K^+ in the acute stimulation of astrocytic glycolysis by synaptic activity. They have also permitted detection of the Warburg effect in single cancer cells. Genetically-encoded nanosensors currently exist for glucose, lactate, NADH and ATP, and it is envisaged that other metabolite nanosensors will soon be available. These optical tools together with improved expression systems and *in vivo* imaging, herald an exciting era of single-cell metabolic analysis.

Keywords: FRET, FLII12Pglu-700δμ6, laconic, glycolysis, mitochondria, flux, cancer metabolism

INTRODUCTION

It is hard to overestimate the importance of resolution. In the absence of sufficient temporal resolution transient events go undetected. Without enough spatial resolution, opposite changes in neighboring compartments may cancel out. Classic biochemistry mapped metabolic pathways and characterized the behavior of purified enzymes in test tubes. Decades before cell sorting, enzymes had to be extracted from whole brain homogenates. Metabolites were measured, again in whole tissue extracts, and a handful of enzymes thought to catalyze far-from-equilibrium reactions were deemed to control flux. With the introduction of radioisotopes in research during the 1950s and non-invasive techniques for their detection, particularly PET and NMRS, concentrations and fluxes could be estimated in living humans, thus permitting biochemical investigation of brain disease. Meanwhile, progress in molecular biology, immunohistochemistry, and the introduction of cell cultures, revealed previously unsuspected complexities, with numerous isoforms for metabolic enzymes and transporters plus cell-specific post-translational modifications. At present, it is evident that neurons and glial cells differ metabolically as much as they differ functionally, but little is known regarding subtypes of neurons and astrocytes and their interaction with oligodendrocytes (Funfschilling et al., 2012; Lee et al., 2012). Of clinical importance are regional variations in metabolism across the brain that may help to explain susceptibility to neurodegeneration (Vaishnavi et al., 2010; Vlassenko et al., 2010; Bero et al., 2011).

Over the last decade, fluorescence microscopy-based techniques with high spatiotemporal resolution have been introduced for the study of energy metabolism in cultured cells and in brain tissue slices. Genetically-encoded sensors are becoming available and it is now possible to measure, glucose, lactate, NADH, and ATP in individual cells with sub-second resolution. The present work describes how some of these sensors may be used in combination with transport inhibitors, to quantify metabolic flux and investigate the regulation of astrocytic glycolysis in response to neuronal activity.

HOW LOCAL AND HOW FAST IS BRAIN METABOLISM?

The average rate of glucose utilization in human gray matter has been estimated at 8.8 μM/s (Huang et al., 1980; Gjedde and Diemer, 1983), ten times higher than the body's average. With this value and the known stoichiometry of the glucose oxidation ($C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$) and coupled reactions, it is possible to obtain an estimate of flux at different points in the metabolic chain. As the glucose molecule proceeds through glycolysis and the Krebs cycle, its free energy is split into smaller packets and the molar flux rises, reaching a maximum at ATP, with 31 molecules produced for each glucose molecule consumed (**Figure 1**). In addition to flux, metabolite dynamics are determined by concentration, so that the smaller the concentration, the larger the impact of a given flux on the metabolite pool. The ratio between concentration and flux is known as the *turnover time* and is a useful parameter of how dynamic a metabolite is.

The turnover time can be thought of as the time that a metabolite pool would last if production were to stop while consumption remained constant. As shown in **Table 1**, brain tissue glucose and lactate have turnover times in the order of 2 min whereas ATP and oxygen have turnover times of a few seconds, whereas for NADH it is just 7 ms. The turnover time reflect sensitivity of a given metabolite pool to flux perturbation. According to the simulation shown in **Figure 2**, a 100% increase in the rate of consumption would reduce the respective brain pools with a half time of about 1 min for glucose and lactate, 0.3 and 1.5 s for O_2 and ATP and 5 ms for NADH. Taken in combination with the diffusion coefficient, the turnover time also helps to reveal how local a metabolite may be if its diffusion were not restricted by membranes. For instance, during its turnover time, the average glucose or lactate molecule can diffuse several hundreds of micrometeres along the cytosol of a neuron, roughly the diameter of a cortical column or a cortical barrel, whereas variations in cytosolic NADH in a dendrite will not be sensed by its soma located just a few micrometers away (**Table 1**).

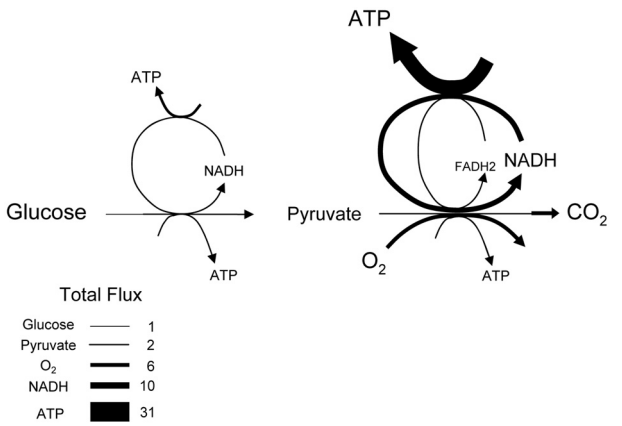

FIGURE 1 | Stoichiometry of glucose oxidation. The schematic represents the oxidation of glucose to CO_2, where the width of the arrows is proportional to flux. Cytosolic NADH is assumed to transfer its electrons to mitochondria through both the malate-aspartate shuttle (rendering 3 ATPs per NADH) and the glycerol phosphate shuttle (rendering 2 ATPs per NADH).

The above considerations help to establish *a priori* the minimum spatial and temporal resolutions required to order to characterize metabolism. Glucose and lactate dynamics have to be sampled in seconds, whereas monitoring ATP and O_2 may need techniques that resolve hundreds of milliseconds. To avoid missing NADH fluctuations, millisecond sampling will be required. In terms of size, monitoring cytosolic glucose, lactate, O_2 or ATP demand single-cell resolution, whereas cytosolic NADH and metabolites inside small membrane compartments such as mitochondria demand sub-cellular resolution. One may think that even higher resolution may be needed to characterize the immediate neighborhood of metabolic enzymes and transporters, but this is not the case. Glucose, lactate, O_2, ATP, and any other molecules present at micromolar levels or higher are not expected to form microdomains or nanodomains, because the build up or

Table 1 | Dynamics of selected metabolites in brain tissue.

Metabolite	Glucose	Lactate plus pyruvate	ATP	O_2	NADH
Stoichiometry*	1	2	31	6	2
Concentration (μM)	1000[a]	2000[b]	1250[c]	30[d]	0.13[e]
Flux[f] (μM/s)	8.8	17.6	273	53	17.6
Turnover time[g] (s)	114	114	4.6	0.6	0.007
Diffusion coefficient (D) (μm^2/s)	500[h]	130[i]	500[j]	2000[k]	500[l]
Average distance traveled over 1 and 10 turnover times[m] (μm)	585 and 1849	298 and 943	117 and 371	85 and 268	5 and 14
Generation of nanodomains	No	No	No	No	Yes

**Whole tissue stoichiometry is given for glucose, lactate/pyruvate, ATP and oxygen, while cytosolic stoichiometry is given for NADH.*

[a] *Human brain tissue (Barros et al., 2007).*

[b] *Human brain tissue (Dienel and Cruz, 2004).*

[c] *HeLa cells, MIN6 cells, and COSM6 cells (Zamaraeva et al., 2005) and references therein.*

[d] *Human brain tissue (Buxton, 2010).*

[e] *COS7 cells (Zhang et al., 2002).*

[f] *The glucose flux was calculated using non-invasive measurements in human gray matter (Huang et al., 1980) and the glucose distribution volume (Gjedde and Diemer, 1983). The flux of the other metabolites was calculated as the product of the glucose flux and the respective stoichiometry. For NADH the cytosolic flux is given.*

[g] *Turnover time is concentration divided by flux.*

[h] *Isotopic deoxyglucose in rat vagus nerve (Vega et al., 2003).*

[i] *NMRS in rat brain tissue (Pfeuffer et al., 2000).*

[j] *NMRS in rat skeletal muscle (de Graaf et al., 2000).*

[k] *Oxygen electrode measurements in rat brain tissue (Baumgartl and Lubbers, 1983).*

[l] *Assumed to be equal to that of ATP.*

[m] *Estimated assuming Brownian diffusion according to the Einstein's equation in three dimensions (distance^2 = 6 × D × turnover time).*

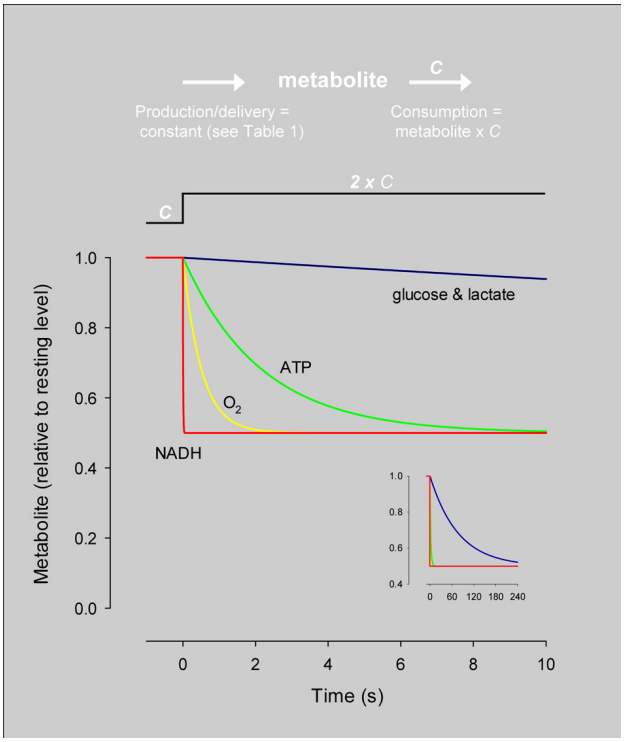

FIGURE 2 | Simulation of the response of metabolites to an instant rise in consumption. The dynamics of each metabolite were simulated independently using the concentration and steady-state flux in **Table 1** and the differential equation: d metabolite/dt = production–metabolite × *C*, where *C* is the rate constant of consumption. At time zero, *C* was increased by 100% while production was kept constant, resulting in a 50% decrease in the size of the pool. The speed at which the new steady-state is reached varies dramatically between different metabolites. The inset shows the same data over an extended timescale. The differential equation was solved by numerical simulation using Madonna software.

depletion of metabolites in the immediate vicinity of the proteins handling these molecules is negligible compared to the powerful mixing effect of diffusion in short distances (Barros and Martinez, 2007; Martinez et al., 2010). For these abundant molecules, the cytosol within an astrocyte or a neuronal soma is expected to behave as a well-mixed compartment. NADH is different, because its cytosolic concentration is very low. For example, considering a cytosolic NADH of 130 nM (u), a diffusion coefficient (D) of 500 μm^2 s^{-1} (**Table 1**), and that a single lactate dehydrogenase enzyme (LDH) produces or consumes lactate at a rate of 260 s^{-1} (q, Barros and Martinez, 2007), the relative amplitude (AMP) of the local NADH nanodomain may be estimated using the equation $AMP = 1 \pm q/(u \times D \times a)$, where a is the radius of the catalytic site (Martinez et al., 2010). According to this formula and assuming a radius of 0.5 nm (Barros and Martinez, 2007), LDH is predicted to create a local nanodomain in which the concentration of NADH is twice that of the bulk cytosolic NADH when the enzyme is consuming lactate, whereas LDH should deplete its vicinity of NADH when the enzyme is producing lactate. Thus, an accurate characterization of NADH dynamics will require nanometer resolution.

FAST GLUCOSE DYNAMICS MEASURED WITH FLUORESCENT GLUCOSE ANALOGS

2-deoxyglucose is a glucose analog that is transported into cells by the GLUT glucose transporters and then phosphorylated by hexokinase, but is not metabolized further to any significant extent. Detected in cultured cells by scintillation counting, by autoradiography in laboratory animals and non-invasively in humans with FDG-PET, radiolabeled 2-deoxyglucose has wide applications in research and clinical medicine. However, like other radioisotopes, it has limited spatiotemporal resolution, with detection requiring cell populations and typical sampling intervals in excess of 10 min.

Fluorescent analogs of glucose have been used to characterize the transport and metabolism of glucose at high resolution by means of microscopy (Kim et al., 2012). The most popular analogs are 2-NBDG and 6-NBDG. These compounds are comprised of a glucose moiety in which a fluorescent nitrobenzoxydiazoamine (NBD) group replaces the hydroxyl group at carbon 2 or 6. Both are substrates of GLUT carriers but only 2-NBDG can be phosphorylated by hexokinase. As demonstrated with 6-NBDG, the bulky hydrophobic NBD group increases the affinity of binding to GLUTs, but impairs translocation of the binding site to a larger extent (Barros et al., 2009a), making transport of 6-NBDG by GLUT1 and GLUT3, respectively 100 and 16 times slower than that of glucose (Jakoby et al., 2012). The low efficiency of translocation provides an important experimental advantage because it permits the use of confocal microscopy to monitor uptake in real time over a period of several minutes, a time window in which agonists can be applied to investigate acute modulation of glucose transporters (Loaiza et al., 2003; Porras et al., 2004, 2008). These fluorescent glucose tracers have also been used to characterize glucose uptake in many other mammalian cell types including erythrocytes, fibroblasts, smooth muscle cells, enterocytes, cardiomyocytes, endothelium, lymphocytes, pancreatic beta cells, adipocytes, and tumor cells (Barros et al., 2009a; Kim et al., 2012). Imaging of 2- and 6-NBDG by multiphoton microscopy has been used to study the transport and metabolism of glucose in cerebellar and hippocampal slices (Barros et al., 2009b; Jakoby et al., 2012) and to detect a stimulatory effect of neural activity on glucose transport in astrocytes in the somatosensorial cortex *in vivo* (Chuquet et al., 2010). Long-term (>10 min) incubation with 2-NBDG followed by a washout period to remove unphosphorylated 2-NBDG is informative about glucose consumption, but 2-NBDG cannot be used to monitor metabolism in real time, because both the phosphorylated and unphosphorylated form of the analog are fluorescent, making it impossible to differentiate between the two. However, single-cell real-time monitoring of glucose metabolism is now possible with a genetically-encoded FRET glucose nanosensor.

GLUCOSE METABOLISM MEASURED WITH A GENETICALLY-ENCODED FRET NANOSENSOR

Ten years ago Wolf Frommer and colleagues introduced the first FRET glucose nanosensor (Fehr et al., 2003), making an improved version available in 2008 (Takanaga et al., 2008). Since then, various research groups have made fluorescent nanosensors specific for ATP (Berg et al., 2009; Imamura et al., 2009) and NADH

(Hung et al., 2011; Zhao et al., 2011) and we have developed a FRET nanosensor for lactate (San Martín et al., 2013). The glucose and lactate nanosensors are of the same principle. They comprise a bacterial protein that binds the analyte, sandwiched between two fluorescent proteins with overlapping emission and excitation spectra that undergo FRET. Binding of the analyte to the bacterial protein induces a conformational change that modifies the distance between the fluorescent protein and/or its relative orientation, resulting in a change in FRET efficiency which can be calibrated. **Figures 3A**,**B** shows cultured astrocytes expressing the glucose nanosensor (FLII12Pglu-700μδ6) and the lactate nanosensor (Laconic), with the typical cytosolic distribution and exclusion of nuclei and organelles. In **Figure 3C**, Laconic has been targeted to the nucleus and FLII12Pglu-700μδ6 to the cytosol of HEK293 cells, which permits the use of confocal microscopy to simultaneously monitor glucose and lactate in the same cell.

All mammalian cells metabolize glucose but they differ in their handling of lactate. Some cells are lactate exporters, while others are lactate importers (**Figure 4A**). The concentration of a metabolite, absolute or relative, may be interesting in itself, as it informs about the balance between production and consumption (Fehr et al., 2003; Bittner et al., 2010, 2011; Takanaga and Frommer, 2010; Kovacic et al., 2011; Prebil et al., 2011). However, concentration gives no information about flux. For instance, a decrease in intracellular glucose may be due to an inhibition of GLUT-mediated transport (with flux decrease) or a stimulation of hexokinase (with flux increase). Moreover, the rate of departure from the steady-state is sensitive to the degree of GLUT or hexokinase modulation but equally sensitive to resting flux (Barros et al., 2013), with a fast cell reacting more quickly than a slow cell to the same degree of stimulation (Barros et al., 2013). However, by eliminating the contribution of transport with a GLUT blocker like cytochalasin B, the ambiguity is lifted as the glucose concentration is forced to decrease with a rate equal to that of glucose consumption (**Figure 4B**). This is a protocol that can be applied repeatedly and which has been validated in astrocytes, neurons, muscle cells, fibroblasts, adipocytes, and tumor cells (Bittner et al., 2010). Most cells express a high affinity isoform of hexokinase, with a K_D of 50 μM, and in the presence of millimolar extracellular glucose maintain steady-state intracellular glucose levels of 0.5 mM or higher, so that hexokinase runs at V_{max}. This explains the linear decrease in glucose concentration during the GLUT block. When applied to astrocytes in culture and in organotypical hippocampal slices, this GLUT-stop technique revealed that glycolysis is strongly and reversibly stimulated within seconds of exposure to elevated extracellular K^+, a cation that is released by active neurons (Bittner et al., 2011). Further work showed that K^+ activates astrocytic glycolysis by a sequence of events that begin with plasma membrane depolarization, then stimulation of the electrogenic Na^+/bicarbonate co-transporter NBCe1 and intracellular alkalinization (Ruminot et al., 2011). We also confirmed the stimulatory effect of glutamate on glycolysis that was detected two decades ago by Pellerin and Magistretti using 2-deoxyglucose (Pellerin and Magistretti, 1994; Pellerin et al., 2007) and showed that the effect of glutamate on glucose consumption develops over minutes and persists long after withdrawal of the neurotransmitter (Bittner et al., 2011). More recently, the same method detected fast changes in the rate of astrocytic glycolysis in response to variations in extracellular lactate (Sotelo-Hitschfeld et al., 2012), a phenomenon that may be relevant for the local distribution of fuel in brain tissue. A general protocol for the use of FRET nanosensors for metabolites can be found in Hou et al. (2011) and a more specific practical guide to the use of the glucose sensor for quantification of glucose consumption is given by Barros et al. (2013).

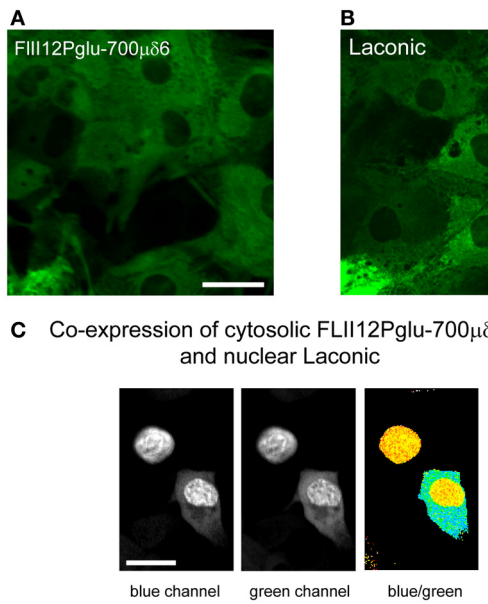

FIGURE 3 | Expression of the glucose and lactate sensors in astrocytes and HEK293 cells. (A,B) The FRET glucose nanosensor FLII12Pglu700μδ6 and the FRET lactate nanosensor Laconic were expressed in astrocytes using a custom-made adenoviral vector (Vector Biolabs). Confocal images correspond to green emission at 535 nm (Venus) as excited with a 488 nm argon laser. Bar represents 20 μm. **(C)** HEK293 cells were co-transfected with FLII12Pglu700μδ6 and nuclear-targeted Laconic. The confocal images show the CFP and mTFP emissions at 480 nm (blue channel) and the Citrine and Venus emissions at 535 nm (green channel) of a cell expressing only Laconic (top) and a cell expressing both sensors (bottom). Scale bar is 20 μm.

LACTATE DYNAMICS MEASURED WITH A GENETICALLY-ENCODED FRET NANOSENSOR

LACtate Optical Nano Indicator from Cecs (Laconic) is comprised of the *Escherichia coli* protein LldR, the teal fluorescent protein mTFP and the green fluorescent protein Venus (San Martín et al., 2013). In bacteria, LldR regulates the transcription of an operon required for the metabolism of lactate. The binding of lactate to Laconic is best described by a two-site model, having a high-affinity component with a K_D of 8 μM and a low-affinity component with a K_D of 800 μM, with each accounting for about 50% of the change in the fluorescence ratio. Expressed in mammalian cells, the maximum change in fluorescence ratio is about 40%. The sensor is insensitive to the NADH/NAD^+ ratio and physiological levels of pyruvate and other acidic metabolites. It

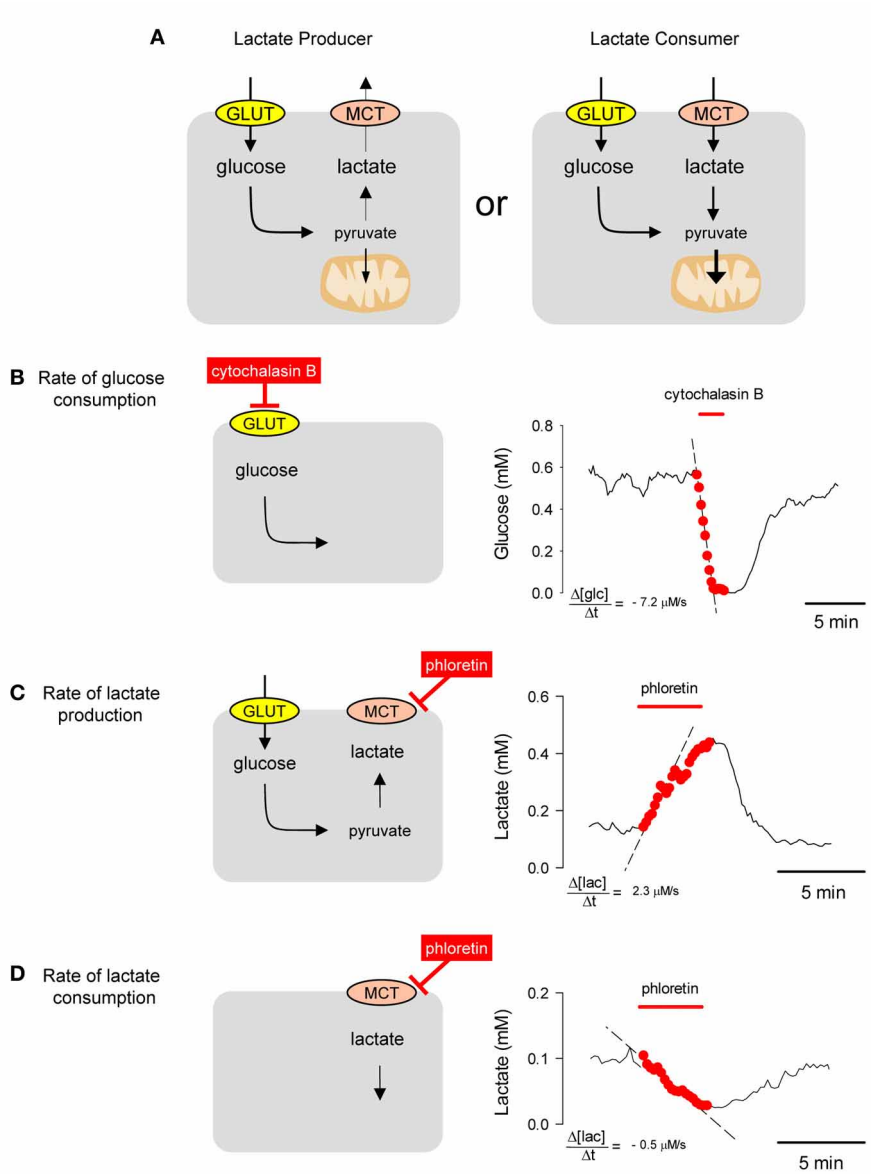

FIGURE 4 | Estimation of metabolic fluxes with transport-stop protocols. **(A)** Whereas most mammalian cells are glucose importers, some cells export lactate and some import lactate. **(B)** A single astrocyte incubated in 2 mM extracellular glucose maintained an intracellular glucose of approx. 0.6 mM. Interruption of the steady state by blocking the glucose transporter GLUT1 with 20 μM cytochalasin B caused a decrease in the cytosolic concentration of glucose at a rate of −7.2 μM/s. **(C)** A HEK293 was incubated in 25 mM glucose. Blockage of the MCT with 50 μM phloretin caused an accumulation of intracellular lactate with an initial rate of 2.3 μM/s. **(D)** Same cell as in **(B)** but incubated in 1 mM lactate in the absence of glucose. Blockage of the MCT with 50 μM phloretin caused a depletion of intracellular lactate with an initial rate of −0.5 μM/s.

shows little sensitivity to pH changes in the physiological range of the mammalian cytosol, (pH 7.0–7.4) (San Martín et al., 2013).

In mammalian cells, lactate is transported across the plasma membrane by the monocarboxylate transporter (MCT). Use of the lactate sensor to estimate the flux of lactate in HEK293 cells with an MCT-stop protocol is illustrated in **Figures 4C,D**. In the presence of glucose, exposure of cells to the MCT inhibitor phloretin caused an increase in the concentration of intracellular lactate, showing that the cell was exporting lactate. The rate of lactate accumulation reached a maximum immediately after MCT blockage and reflects the magnitude of the lactate flux in the steady-state, decreasing later because feedback inhibition of glycolysis by lactate (Sotelo-Hitschfeld et al., 2012) and/or delayed depletion of intracellular glucose caused by phloretin, also a GLUT blocker. Similar results have been obtained with the non-permeant MCT blocker pCMBS and with the more specific MCT blocker AR-C155858 (San Martín et al., 2013). Inhibition of the MCT in the same HEK293 cells incubated in the presence of lactate but with glucose absent, caused a decrease in intracellular lactate, showing that the cell was importing lactate. This type

of protocol may be used to address the question of intercellular lactate exchange, which is of great interest for neuroenergetics (Pellerin et al., 2007; Barros and Deitmer, 2010; Allaman et al., 2011; Wyss et al., 2011) and in cancer research (Yeluri et al., 2009; Tennant et al., 2010).

Normal cells oxidize most of the glucose they take up, exporting small amounts of lactate or importing lactate. Cancer cells often have deficient mitochondria and a strong glycolytic flux, exporting much more lactate than normal cells. This phenomenon is known as the Warburg effect and may be important for cancer progression (Vander Heiden et al., 2009). The Warburg effect can be detected non-invasively in humans by FGD-PET for the purposes of cancer diagnosis and staging. For basic research, the Warburg effect may be observed in cell populations or tissue

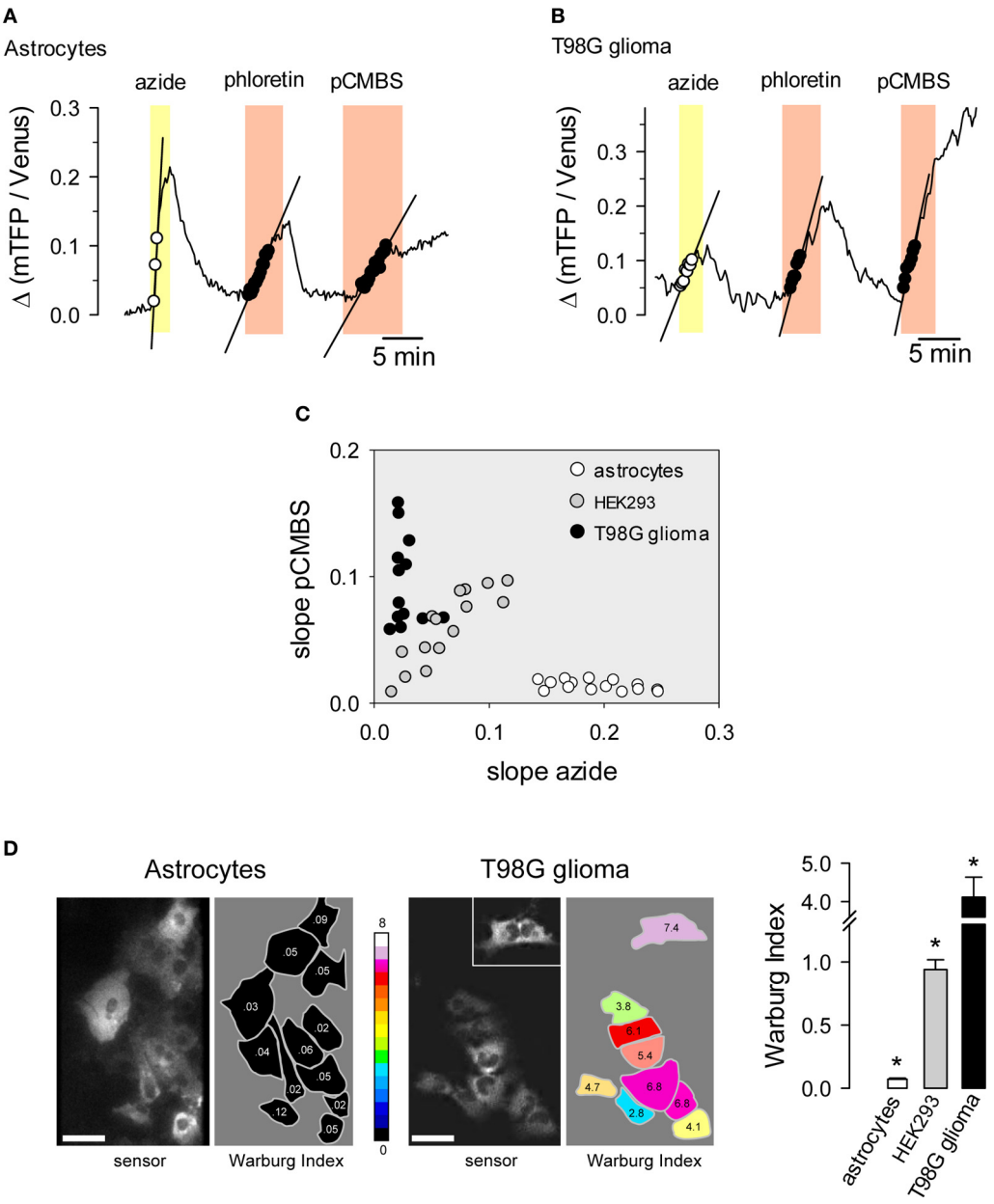

FIGURE 5 | Use of the lactate sensor to detect the Warburg effect. An astrocyte **(A)** and a T98G glioma cell **(B)** expressing Laconic were sequentially exposed to 5 mM sodium azide, 50 μM phloretin and 500 μM pCMBS. The straight lines represent initial slopes of lactate accumulation fitted by linear regression within the same range of ratio values. **(C)** Correlation plot between the rates of lactate accumulation (Δ ratio/min) in sodium azide and in pCMBS. Symbols represent single astrocytes (white), HEK293 cells (gray) or T98G cells (black). **(D)** The Warburg Index was estimated as the ratio between the rates of lactate production with pCMBS and lactate accumulation with azide, and was used to color the silhouette of each cell according to the 16-color look up table. The inset shows an isolated cell that was located about 100 μm from the cluster. The bar graph summarizes data from 3 experiments in each cell type. Scale bars are 20 μm. $^*p < 0.05$ between every cell type. Modified from San Martín et al. (2013).

explants by monitoring the rate of oxygen consumption. Taking advantage of the resolution afforded by the FRET lactate sensor, we devised a protocol that provides a quantitative estimate of the Warburg effect in single cells (San Martín et al., 2013). Cells are first exposed to sodium azide, a reversible inhibitor of oxidative phosphorylation. Normal cells respond to mitochondrial poisoning with an acute stimulation of glycolysis (Bittner et al., 2010), causing a rapid accumulation of intracellular lactate (**Figure 5A**). This response is weaker in cancer cells (**Figure 5B**). After intracellular lactate returns to baseline levels, cells are exposed to an MCT blocker in order to measure the rate of lactate production, which is higher in cancer cells. The difference between astrocytes and T98G glioma cells becomes evident when both rates are plotted for each cell as shown in **Figure 5C**. The difference can be quantified by computing the ratio between the rate of lactate production and the rate of lactate accumulation with sodium azide, termed the Warburg Index (WI), as illustrated in **Figure 5D**. Astrocytes had WI values of <0.1 whereas T98G glioma cells had WI values of >2, with intermediate values observed for the non-transformed cell line HEK293. Tumors are complex systems in which cancer cells of differing degrees of malignancy co-exist with non-cancerous cells of several lineages. The single-cell resolution provided by genetically-encoded FRET nanosensors together with multiphoton microscopy may help to investigate metabolic exchanges within tumors.

CONCLUSION AND PERSPECTIVES

Genetically-encoded sensors permit single cell estimation of metabolite concentration with sufficient temporal resolution to detect physiological fluctuations. In the case of glucose and lactate, inhibitor-stop protocols are available that measure rates of usage and production. Together with improved expression systems and imaging in tissue slices and *in vivo*, these tools open the way to a characterization of energy metabolism in identified cells in healthy and diseased tissue.

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Confocal microscopy for astrocyte *in vivo* imaging: recycle and reuse in microscopy

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In vivo imaging is one of the ultimate and fundamental approaches for the study of the brain. Two-photon laser scanning microscopy (2PLSM) constitutes the state-of-the-art technique in current neuroscience to address questions regarding brain cell structure, development and function, blood flow regulation and metabolism. This technique evolved from laser scanning confocal microscopy (LSCM), which impacted the field with a major improvement in image resolution of live tissues in the 1980s compared to widefield microscopy. While nowadays some of the unparalleled features of 2PLSM make it the tool of choice for brain studies *in vivo*, such as the possibility to image deep within a tissue, LSCM can still be useful in this matter. Here we discuss the validity and limitations of LSCM and provide a guide to perform high-resolution *in vivo* imaging of the brain of live rodents with minimal mechanical disruption employing LSCM. We describe the surgical procedure and experimental setup that allowed us to record intracellular calcium variations in astrocytes evoked by sensory stimulation, and to monitor intact neuronal dendritic spines and astrocytic processes as well as blood vessel dynamics. Therefore, in spite of certain limitations that need to be carefully considered, LSCM constitutes a useful, convenient, and affordable tool for brain studies *in vivo*.

Keywords: *in vivo*, imaging, astrocyte, two-photon, confocal microscopy, cranial window, intravital, glia

INTRODUCTION

The combination of fluorescence techniques with two-photon laser scanning microscopy (2PLSM) has become the tool of choice for *in vivo* brain imaging because high-resolution images can be obtained at relatively high depth (>500 μm) from the tissue surface (Theer et al., 2003; Helmchen and Denk, 2005). It employs ultrashort infrared laser pulses for fluorophore excitation that yield low light scattering by the tissue sample or living brain. In addition, the non-linear nature of 2PLSM excitation grants that detected photons come from fluorophore emission exclusively at the focal plane (Svoboda and Yasuda, 2006). In contrast, laser scanning confocal microscopy (LSCM) employs single-photon excitation, which is more sensitive to scattering (Centonze and White, 1998). It relies on a pinhole to reject fluorescence from out-of-focus locations to create high-resolution contrast images of relatively superficial areas (<100 μm from the surface). Interestingly, due to the use of shorter light wavelengths the resolution obtained with LSCM is better as the point spread function (e.g., pattern of diffracted light from a subresolution point-source which gives a measure of the smallest objects that can be resolved) is smaller (∼300 nm in *xy* axis; ∼900 nm in *z* axis; Abbe, 1873, 1874; Cole et al., 2011).

Although the commercial availability of two-photon laser scanning microscopes has led to their widespread use, their overall cost may still be prohibitive for some laboratories to perform brain studies *in vivo*. However, single-photon LSCM is more widely available and has also been technologically improved (e.g., being employed in neurosurgery for intraoperative diagnosis and applied for *in vivo* research in moving animals; Jung et al., 2004; Kedrin et al., 2008; Eschbacher et al., 2012; Ritsma et al., 2012).

Astrocytes and their thin processes maintain close structural and functional interactions with neurons and synapses (Ventura and Harris, 1999; Bushong et al., 2002). They respond to synaptic activity (Perea and Araque, 2005) and influence synaptic transmission (Fiacco and McCarthy, 2004; Perea and Araque, 2007; Di Castro et al., 2011; Panatier et al., 2011) and plasticity (Henneberger et al., 2010; Takata et al., 2011; Navarrete et al., 2012). Also, they enwrap blood vessels with specialized processes termed endfeet, which play relevant roles in controlling local metabolic and energetic demands through the so-called neuro-glio-vascular coupling (Zonta et al., 2003; Mulligan and MacVicar, 2004; Metea and Newman, 2006; Takano et al., 2006; Gordon et al., 2008; Attwell et al., 2010). Monitoring *in vivo* these structural and functional relationships between astrocytes and neurons may provide relevant information about their actual conditions and properties in intact or minimally perturbed preparations. While 2PLSM has been successfully applied to address these issues (Hirase et al.,

2004; Takano et al., 2006; Wang et al., 2006; Schummers et al., 2008; Takata et al., 2011), LSCM may also be useful for these purposes (Mishra et al., 2011; Navarrete et al., 2012; Srienc et al., 2012).

In this article we show that the combination of an optimized surgical procedure with intravital staining of astrocytes and LSCM represents a suitable approach for imaging *in vivo* the subcellular structure of astrocytes and neurons, monitoring calcium transients in the astrocytic soma and processes, and visualizing blood vessel dynamics. We additionally provide a detailed description of the methodology used to carry out *in vivo* imaging in the mouse brain cortex using LSCM.

MATERIALS AND METHODS

MATERIALS

Reagents

- HEPES-buffered saline (in mM: NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, EDTA 1, HEPES potassium 8.6, glucose 10)
- 0.9% (w/v) NaCl (saline)
- Urethane (Sigma, Madrid, Spain). Dissolve in saline.
- Fluo-4 AM (Life Technologies, Barcelona, Spain). Dissolve 50 μg in 4 μl pluronic [(Life Technologies, 20% in dimethyl sulfoxide (DMSO)]. Add 46 μl of HEPES-buffered saline to obtain a 1 μg/μl final concentration. Vortex to achieve dissolution.
- Sulforhodamine 101 (SR101; Sigma, Madrid, Spain). Dissolve in saline according to the weight of the animal (100 mg/kg).
- Fortex dental cement (Facident, Barcelona, Spain).
- Low melting point agarose (1% in saline; Sigma, Madrid, Spain).

Equipment

- Stereotaxic device (ASI Instruments, Warren, MI, USA)
- Mouse Adaptor (Stoelting Co, IL, USA)
- Aluminum cranial frame
- Electronic control for heat pad (Cibertec, Madrid, Spain)
- Heat pad (RS Amidata, Madrid, Spain)
- Rectal probe (Technomed Europe, Maastricht, The Netherlands, Cat No. TP/YSI402)
- Drill Volvere Vmax NE120 (Nakanishi Inc., Kanuma, Japan)
- Burrs (FST, Heidelberg, Germany, Cat No. 19007-14/07)
- Stainless Steel Mounting Screws 00-96 X 1/16 (Plastic One, VA, USA)
- Drill holder (Plastic One, VA, USA, Cat No. DH 1)
- Drill bit (Plastic One, VA, USA, Cat No. D #60)
- Screwdriver (Plastic One, VA, USA, Cat No. SD 96)
- Surgical blade
- Set of surgical forceps (FST, Heidelberg, Germany)
- Scissors [Vannas and common type; FST (Fine Science Tools), Heidelberg, Germany]
- Spatula
- Cotton
- Glass coverslips (5–6 mm diameter, 0.15 mm thickness; Menzel, Braunschweig, Germany)
- Syringe (10 ml)

Microscope

- Olympus FV300 laser scanning confocal system coupled to an Olympus BX61WI upright microscope (Olympus, Tokyo, Japan)
- Lasers: Ar 488 and HeNe 543 (2.5 and 0.5 mW, respectively at the objective back focal plane; CVI Melles Griot, Cambridge, UK)
- Fluoview software for acquisition (Olympus, Tokyo, Japan)
- Water immersion Olympus LUMPLFL 60XW/IR objective (0.9NA; Olympus, Tokyo, Japan)
- Scientifica Movable Top Plate (Scientifica, Uckfield, UK)
- PMI-100 pressure injector (Dagan, MN, USA)
- Axon Digidata 1322A (Molecular Devices, CA, USA)
- pClamp software (Molecular Devices, CA, USA)

MICE

We employed Thy-1 GFP-M transgenic mice (The Jackson Laboratory, ME, USA), which express green fluorescent protein (GFP) under the Thy1 promoter (Feng et al., 2000), to visualize dendrites projecting from layer V pyramidal neurons. All the procedures for handling and sacrificing animals followed the European Commission guidelines (86/609/CEE).

EQUIPMENT SETUP

Cranial frame

The custom-designed frames consisted of a heavy aluminum base plate (7 cm × 13 cm × 1 cm) and one light aluminum cranial frame (2 × 3.5 cm) for the cranial window. The latter has a central circular hole (10 mm diameter) and four holes in the corners to fit four M4 screws that will fix this plate to the heavy aluminum base plate (**Figure 1A**). The imaging chamber consisted of a circular plastic ring glued to the frame and centered in the cranial window. This frame provides stability for preparation and avoids the mechanical interference by respiration-induced movements caused by chest motion during breathing. The frame was attached onto the skull with two stainless steel screws and dental cement (see **Figure 1A**). The heavy aluminum base plate with the animal and the cranial frame fixed to it was attached to a Scientifica electrophysiology movable top plate and the height of the stage was adjusted in order to place it below the microscope scan head (**Figure 1B**). We removed the condenser and its holder to avoid mechanical interference with the stage when moving to search for regions of interest. Our assembly proved to be very convenient in its use during experiments due to its ample working area, stability, and smooth micromanipulator movement.

PROCEDURE

Presurgical preparation

1. Weigh the animal (4–12 weeks old).
2. Inject SR101 (100 mg/kg) intraperitoneally. Let the animal rest in the cage with food and water for 1 h.
3. Observe intense coloration of ears and paws after 30 min.
4. Anesthetize with an intraperitoneal injection of urethane (1.8 mg/kg).
5. After 5 min, check an effective anesthetized state monitoring for awareness signs such as whisker twitching, palpebral reflex, and respiration rate when pinching the tail or ears.

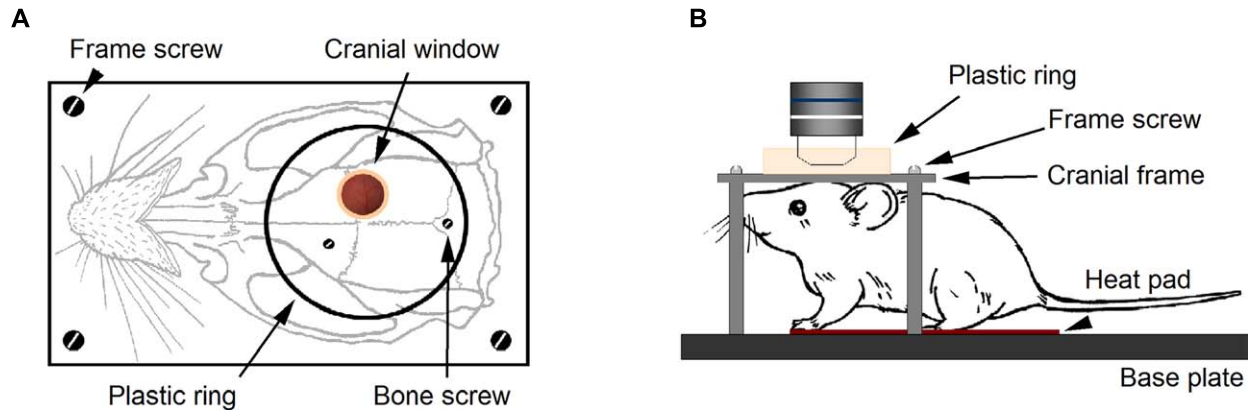

FIGURE 1 | Experimental setup for *in vivo* imaging with LSCM. After performing a cranial window on a Thy-1 GFP-M mouse, the cranial frame **(A)** is secured onto the skull with dental cement. Two cranial screws, eventually embedded in cement, provide additional grip of the frame to the skull. A plastic ring delimits the area of the skull where cement is applied. Four screws secure the cranial frame to the heavy aluminum base plate **(B)**. During imaging, the animal head remained firmly secured to the base plate under the microscope objective. The mouse rested on the heat pad for temperature control and breathing was checked to be rhythmic and effortless.

6. Connect the heating pad to the thermostat and set to 37°C.
7. Shave the coronal area of the head and put the animal on the heating pad.
8. Gently insert the tip of the rectal probe (use lubricant) to continuously monitor the animal temperature (37°C) and tape it to the tail.
9. Mount the animal onto the stereotaxic apparatus. Hold the animal head 1" high and slide one of the ear bars slowly into the ear canal until a little resistance is encountered and secure the screw. Then proceed with the other bar until the head rests on both (ear bar coordinates = 4 mm).
10. Lower the tooth bar and insert it gently into the animal mouth (tooth bar coordinates = 0 mm), so the head is horizontal and the animal breathes easily.

Surgical procedure

11. Clean the surgical area with a cotton pad soaked in saline.
12. With a surgical blade, make a rostrocaudal incision from the midline between the eyes to the back of the head and retract the skin to both sides using forceps.
13. After exposing the skull, use a spatula to gently scrape out the periosteum and adjoining connective tissue. In some cases, the right *temporalis* muscle was separated from the bone.
14. Locate the area of interest. In our case, the somatosensory area situated −1 mm posterior to the bregma and 3 mm lateral from midline.
15. Insert two supporting screws. Mark the skull surface where the screws are going to be located (one in the midline of occipital bone and one in the contralateral frontal bone). Start performing a hole with the electrical drill and, before complete perforation, change to a manual drill and slowly continue until the dura is reached.
16. Bottom of the hole looks pinkish. Partially screw the supporting screws.
17. Make a circular groove (4 mm diameter) on the skull surface circling the area of interest with the appropriate drill bit (FST Cat No. 19007-14) at constant speed (1300 rpm). Slowly drill in a circular fashion, stopping from time to time to wet the area with a drop of saline.
18. Gently lift up and remove the circular bone fragment with the forceps and without touching the brain surface.
19. With a Dumont 5 forceps, grab the meninges at the most caudal part and lift them 1–2 mm and employ the Vannas scissors to perform a cut following the circle of the cranial window.
20. Fluo-4 AM bulk loading. Drop 5–10 μl of Fluo-4 AM (1 μg/μl) forming a meniscus on the cranial window. If this is not possible, ensure that the exposed surface is always covered by a thin liquid layer of the mixture. Wait for 30 min. Skip this step if calcium imaging is not intended.
21. Rinse with two drops of saline and cover the surface with soaked cotton. Skip this step if calcium imaging is not intended.
22. Maintain a glass coverslip 1 cm above the cranial window in preparation for the next two steps.
23. Remove the soaked cotton and use a plastic Pasteur pipette to put a drop of agarose (1% in HEPES-buffered solution) on the cranial window. Test for adequate temperature.
24. Lower the coverslip until it touches the agarose and the borders of the cranial window. Maintain pressure for 2–5 min. After, remove agarose excess from the sides of the window and dry the skull surface.
25. Apply cement to the borders of the coverslip (1–2 mm) and the skull surface with a thin spatula. This will fix the coverslip to the skull and also prevent saline leaks into the cranial window which would result in movement during imaging.
26. Let dry (∼10 min) and check for solidness.
27. Make sure the skull is dry, specially the area where the plate is going to be cemented.

28. Put the cranial frame on the skull, placing the cranial window in the middle of the central plate aperture (see **Figure 1A**).
29. Extend cement over the skull surface and the borders of the plate aperture. Also embed the screws and the cement surrounding the coverslip.
30. Let dry (∼20 min) and check for solidness.
31. Loosen the ear bars and screw the cranial frame to the heavy aluminum base.
32. Move the base plate with the animal and the thermal blanket to the imaging stage.
33. Fix the base plate on the imaging stage and put 2 ml of saline on the coverslip.
34. Lower the objective and start imaging.

After experimentation the animal was sacrificed by cervical dislocation and the base plate carefully removed. The frame was wiped with acetone.

PROCEDURES-POINTS TO CONSIDER

Movement

Like in 2PLSM techniques, in the experimental approach with LSCM the control of movement is critical to obtain a high quality image of the brain during data acquisition. There are two well-established main sources of movement: a large amplitude respiration-induced movement caused by chest motion during breathing and regular small amplitude pulsatile movement synchronized to the heart beat. To avoid respiration-induced movements, we have used a custom-designed frame that provides stability for experimental preparation. In addition, the vascular pulsatile movement was abolished by filling the craniotomy with agarose and attaching a coverslip to the skull with dental cement. If the sealing of the cranial window with dental cement leaks, or is deteriorated by any cause, it will notably reduce the stability of the preparation.

Surgical care

The optical clarity of the cranial window depends on the quality of the surgery and the technique is highly operator-dependent. Therefore, the outcomes are sometimes unpredictable. Preventing cerebral edema and reducing inflammation during the surgery is critical for successful experimentation. It may be appropriate to use dexamethasone by an intramuscular injection to the quadriceps reducing the cortical stress response during the surgery and prevent cerebral edema. During trepanation, excessive pressure should not be applied when drilling because this might puncture the skull and damage the dura. Check the thickness of the skull during craniotomy by pushing very gently on the cranial bone with a fine forceps. If the peripheral bone moves when lightly touched, it is ready to be removed. The next critical step arrives at the time of insertion of the forceps tip into the trabecular bone. Keep the tip in a horizontal position and try to avoid direct perforation of the thinned bone with the forceps, which could damage the dura. Best results are obtained when the skull bone is gently tugged laterally until the thinned bone tears at the bottom of the groove. In our experiments, we obtained better results removing the dura. With a sharp forceps, grab the dura at the most caudal part, lift them and employ the Vannas scissors to cut following the circle of the cranial window. As the dura is attached to the inner table of the cranium, some superficial capillaries might tear during removal of the cranial bone. Small focal bleeding typically disappears spontaneously or can be controlled by gently applying cotton soaked with saline over the exposed surface and waiting 2–5 min for hemostasis.

Stimulation paradigm

The left whiskers of the animal's snout were stimulated with 100 ms puffs of air produced at 5 Hz by a pressure injector (Dagan, MN, USA) controlled by an Axon Digidata 1322A and pClamp software (Molecular Devices, CA, USA). Air was ejected at 1 bar pressure via capillary glass, attached to plastic tubing, positioned ∼1 cm lateral and anterior to the animal's nose to stimulate the whole left whisker pad. Pattern of stimulation was 5 Hz frequency (pulse width 100 ms) for 30 s. At the same time, tail pinching was performed at 2 Hz with steel forceps, providing a pairing protocol for astrocyte stimulation similar to that employed to induce cholinergic cortical plasticity (Takata et al., 2011).

RESULTS

IN VIVO IMAGING OF ASTROCYTES, NEURONS, AND BLOOD VESSELS USING LSCM

To image astrocyte morphology *in vivo*, we performed a cranial window on anesthetized mice (**Figures 1** and **2A**) and took advantage of the fluorescent dye SR101 following a slightly modified intravital method recently reported (Appaix et al., 2012; see Methods). A single intraperitoneal injection of SR101 (100 mg/kg), usually before surgery, was administered to the animal. SR101 proved to be a good contrast agent which allowed us to discern clearly the brain blood vessels, from minutes to several hours after injection. Clear astrocyte staining was observed 40–60 min after injection. Astrocytes have been shown to selectively take up SR101 *in vivo* (Nimmerjahn et al., 2004; Appaix et al., 2012). Although the mechanism of uptake is still unknown, there is evidence showing that metabolites such as glucose and sulforhodamine spread efficiently across astroglial networks through gap junctions present in the astrocytic membrane (Rouach et al., 2008). SR101 taken from blood vessels by astrocytic endfeet were observed to spread with time throughout the astrocytic syncytium, reaching a maximum staining in about 1–2 h. Astrocytic somata were clearly identified as star-like cells, forming non-overlapping domains (Bushong et al., 2002) projecting several branches into the neuropil and contacting blood vessels and neuronal dendrites (**Figure 2**).

For simultaneous visualization of neurons, we used Thy-1 GFP-M mice, in which a subset of projecting neurons selectively expresses the GFP (Feng et al., 2000; **Figures 2A,B**). Both SR101 in astrocytes and GFP expressed in dendrites mainly from layer V pyramidal neurons provided a very strong signal-to-noise ratio which allowed us to maintain confocality (pinhole Airy units = 1) at low laser power below the intact brain surface (usually ∼50 μm).

Therefore, following the methodology described in detail below, astrocytes, neurons, and blood vessels located near the brain surface can be monitored *in vivo* with high spatial resolution (∼300 nm) using LSCM.

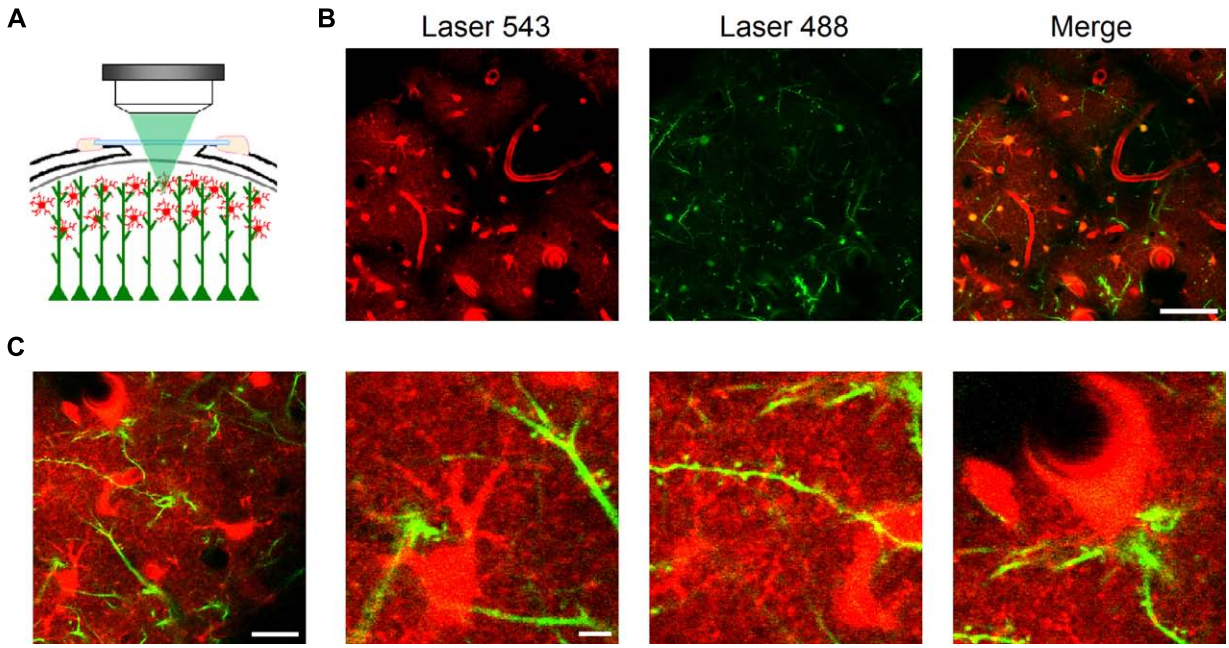

FIGURE 2 | *In vivo* imaging of astrocytes, dendritic spines, and blood vessels using LSCM. Schematic representation of an *in vivo* brain imaging experiment through a cranial window in a Thy-1 GFP-M mouse after intravital staining of astrocytes with SR101 **(A)**. At 60× magnification, astrocytic somata were clearly identified by intravital staining one hour after SR101 injection in single plane confocal images ∼50 μm below the brain surface (**B**, left panel). GFP-expressing dendrites from layer V pyramidal neurons showed also distinct staining (**B**, central panel). Astrocytes were bulk loaded employing Fluo-4 AM and thus, also show fluorescence in the 488 channel (**B**, central and right panel). Large blood vessels project black shadows on the background since they are a major source of light scattering (Haiss et al., 2009). Astrocytes were observed projecting branches into the neuropil, contacting dendrites and their spines, and blood vessels through endfeet. Images taken at higher magnification in another area **(C)** show details of astrocytic somata and processes (**C**, second panel) contacting dendritic spines (**C**, third panel) and blood vessels (**C**, fourth panel). Scale bars: 50 μm in B; 20 μm (first panel) and 5 μm (second to fourth panels) in **(C)**.

IN VIVO IMAGING OF ASTROCYTIC PROCESSES AND DENDRITIC SPINES

Using the previous configuration at higher magnifications, we were able to image with subcellular resolution astrocytic processes and dendritic spines, i.e., two partners of the Tripartite Synapse (Araque et al., 1999; Perea et al., 2009). In spite of maintaining the laser power at relatively low levels (∼0.5 mW for each laser line) to prevent photobleaching and photodamage, we were able to obtain high contrast images up to ∼100 μm below the brain surface. In confocal microscopy, images beyond that depth are seriously limited by the increased and inherent light scattering (loss of ballistic photons and rejection of scattered ones by the pinhole). We typically obtained our images ∼50 μm below the brain surface, which coincides with depths reported by many laboratories employing 2PLSM for *in vivo* recordings of astrocyte and neuronal dendrite morphology along with blood vessel integrity and dynamics (Schaffer et al., 2006; Takano et al., 2006, 2007; Mostany et al., 2010; Sigler and Murphy, 2010). Therefore, while 2PLSM allows deeper imaging, LSCM can be suitably used to monitor thin astrocytic processes and dendritic spines to extract relevant pathophysiological data (**Figures 2B,C**).

IN VIVO IMAGING OF ASTROCYTE CALCIUM DYNAMICS

Beyond obtaining structural images, we also aimed to monitor astrocyte intracellular calcium levels, which represent the basis of the astrocyte calcium excitability (Perea and Araque, 2005). Hence, we employed intravital astrocyte staining with SR101 (to identify astrocytes) along with Fluo-4-AM bulk loading (see Methods) of the barrel cortex in Thy-1 GFP-M mice (to identify dendritic spines; **Figure 3A**). Sensory stimuli of whiskers and tail (see Methods) induced intracellular calcium transients at the astrocytic somata (**Figures 3B,C**). These calcium elevations were recorded ∼50 μm below the intact brain surface, which would correspond to layer I of the primary somatosensory cortex. These results are in close agreement with previous *in vivo* studies using 2PLSM in this cortical layer that documented calcium elevations in the soma of astrocytes (Takano et al., 2006, 2007; Takata and Hirase, 2008; Takata et al., 2011) as well as changes in blood vessel diameter upon electrical and sensory stimulation (Takano et al., 2006, 2007). Sensory stimulation-evoked calcium elevations have also been recorded in the hippocampal astrocytes after decortication with LSCM (Navarrete et al., 2012).

Interestingly, we further observed sensory stimulation-evoked astrocyte calcium elevations not only in the soma but also in processes located in close apposition to identified dendritic spines (i.e., GFP-expressing dendrites projecting from layer V neurons), where most excitatory terminals establish synaptic contacts (**Figures 3D,E**).

Taken together, these results support the suitability of LSCM to study physiological calcium signaling in astrocytes *in vivo*,

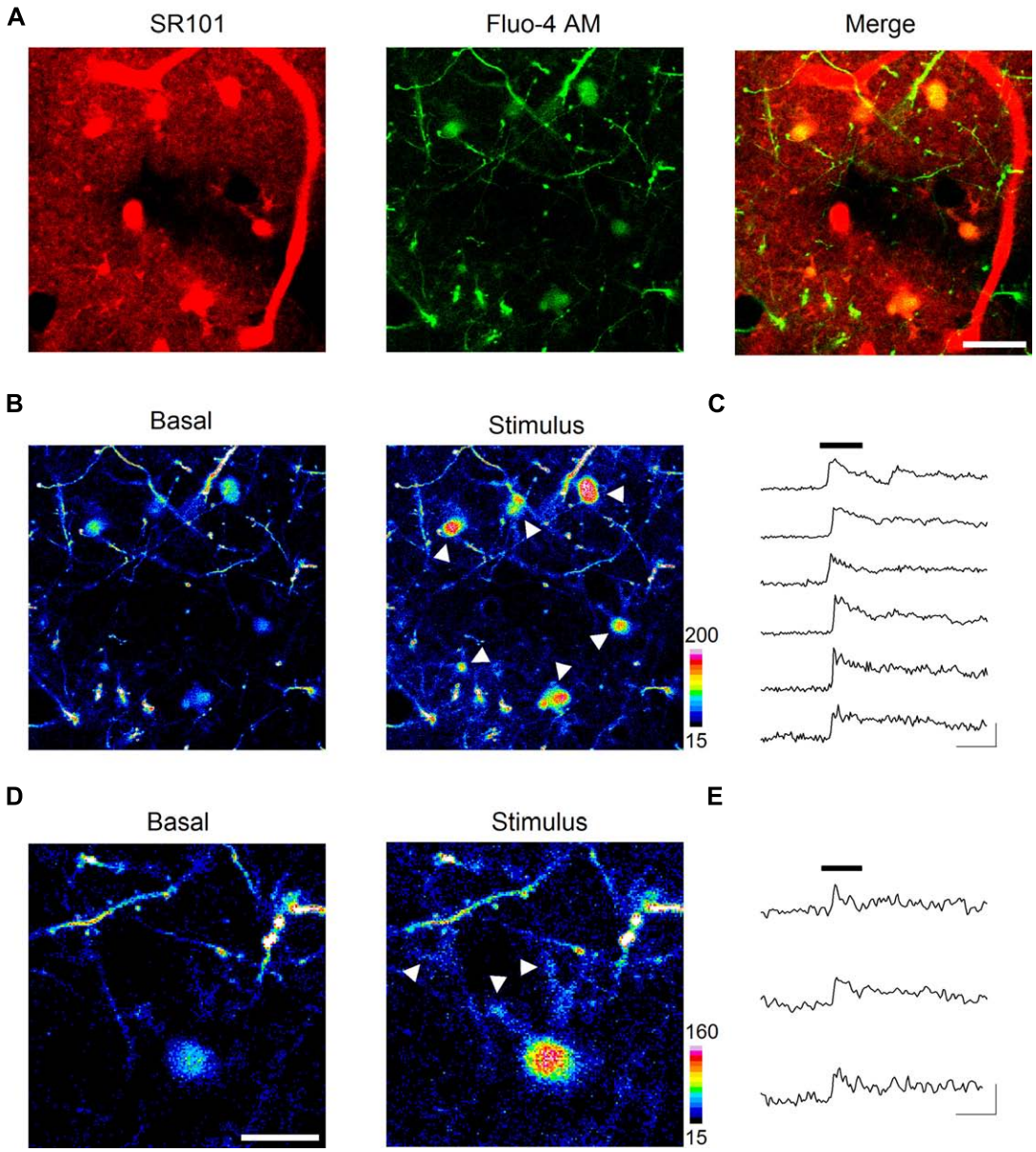

FIGURE 3 | *In vivo* imaging of astrocyte calcium dynamics. Intravitally stained astrocytes (**A**, left panel) were bulk loaded with the calcium indicator Fluo-4 AM (**A**, central panel), showed as areas of colocalization between SR101 and Fluo-4 AM in the merged image of a single confocal plane (**A**, right panel). Astrocyte basal calcium levels (**B**, left panel) increased upon sensory stimulation (see arrows in **B**, right panel). Time lapse recordings of cytosolic calcium in astrocytes reveal a strong elevation shortly after (~4 s) the onset of the 30 s sensory stimulus (black bar) and a slow recovery after cessation **(C)**. Sensory stimulation evoked calcium increases not only at astrocytic somata but also at discrete regions such as distal astrocytic processes (see arrows in **D**, right panel). Panel **(E)** shows time lapse recordings from those regions. Scale bars: 20 μm in **(A)** and **(B)**; 10 μm in **(D)**; 30 s, 100% ΔF in **(C)** and **(E)**.

and reveal the existence of localized subcellular microdomains in astrocytes that respond to sensory stimulation in the live animal.

IN VIVO IMAGING OF BLOOD VESSEL DYNAMICS

Blood flow regulation in the brain is crucial for the adequate metabolic and oxygen supply to neurons in specific brain regions, and astrocytes are recognized to be involved in the control of functional hyperemia, i.e., changes in microvessel diameter and associated blood flow. Studies in brain slices (Zonta et al., 2003; Mulligan and MacVicar, 2004; Gordon et al., 2008) as well as *in vivo* (Takano et al., 2006; Petzold et al., 2008; Mishra et al., 2011; Srienc et al., 2012) have led to the disentanglement of the complex mechanisms that rule blood microcirculation, in which astrocyte calcium signal play a prominent role mediating neuron-glia-vascular coupling (Attwell et al., 2010).

After observing that we could reliably monitor astrocytes and astrocytic-related structures along with subcellular calcium

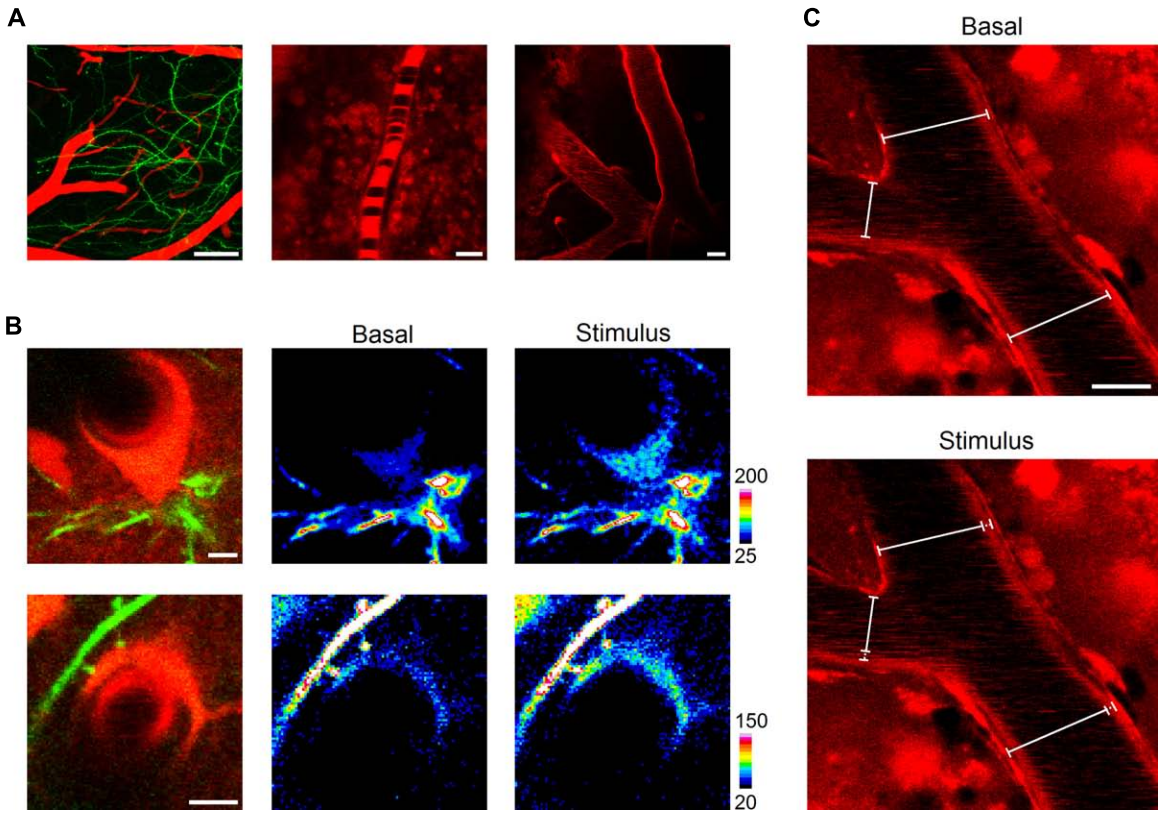

FIGURE 4 | *In vivo* imaging of blood vessel dynamics. Shortly after (~20 min) a single intraperitoneal injection, SR101 distinctly evidenced cortical blood vessels intermingled with neuronal dendrites (**A**, left panel, 50 μm z-stack projection). Erythrocytes were clearly observed circulating as dark bodies inside blood vessels of different diameters (5–50 μm; **A**, central and right panels). Astrocytic somata and endfeet were observed enwrapping blood vessels (**B**, upper and lower panel, respectively) and showed calcium elevations upon sensory stimulation (**B**, central and right panels). The distinct staining of blood vessels allowed us to monitor changes in diameter throughout the stimulation protocol **(C)**. Scale bars: 50, 5, and 20 μm for left, central, and right panels in **(A)**; 5 μm in **(B)**; 10 μm in **(C)**.

transients, we aimed to image blood vessel dynamics *in vivo* with LSCM after intraperitoneal injection of SR101. Indeed, when monitoring the barrel cortex, we were able to observe pial blood arteries penetrating into the brain accompanied by a net of dendrites arising from layer V neurons (Feng et al., 2000; **Figure 4A**). We observed blood vessels of several diameters (range 5–50 μm) and negatively contrasted erythrocytes, which were not stained with SR101 but observed as dark cell bodies over the background (**Figure 4A**). This was especially evident in the first minutes to 2–3 h after the intraperitoneal injection of SR101, when most of the dye was cleared from the blood. Also, as mentioned above, we observed characteristic astrocytic structures adjacent to and enwrapping blood vessels with their soma or endfeet which showed calcium increases upon sensory stimulation (**Figure 4B**). We then monitored blood vessel diameter in response to sensory stimulation. After imaging basal conditions (30–45 s), we observed that delivery of whisker-tail stimulus (see above) for 30 s induced a change in inner and outer diameter a few seconds (~4 s) after the onset of the stimulus (**Figure 4C**).

These results, which are in agreement with previous reports using LSCM (Villringer et al., 1989) or 2PLSM (Kleinfeld et al., 1998; Takano et al., 2006), indicate that the combination of SR101 injection and LSCM is suitable to probe functional changes in blood vessel diameters and blood flow dynamics. Furthermore, erythrocytes were observed circulating inside blood vessels and although we did not study erythrocyte velocity, this could be easily achieved employing the line scan mode of LSCM (Dirnagl et al., 1991; Kleinfeld et al., 1998).

DISCUSSION

In the present article we show the suitability of LSCM to monitor and assess important characteristics of astrocyte structure and function *in vivo*, i.e., astrocyte morphology, subcellular structural interactions between astrocytic processes and dendritic spines, calcium dynamics in astrocytic soma and processes, and changes in blood vessel diameter and blood flow dynamics. Additionally, we provide a guide that details the experimental steps used to attain these *in vivo* recordings.

While our study focused on somatosensory cortex, where cells could be directly imaged from the brain surface due to their relatively superficial location, deep imaging of the cortex without removing superficial layers can only be achieved using 2PLSM. Nevertheless, our procedure may be extended to study

different brain structures including the hippocampus, where special surgical procedures have been employed to remove the cortical overlying area and obtain *in vivo* recordings from neurons and astrocytes (Mizrahi et al., 2004; Navarrete et al., 2012).

The classical view of astrocytic function attributed to them a simple supportive role to maintain the homeostatic conditions for the proper function of neurons. However, a novel view of astrocyte function in brain physiology has emerged, i.e., the tripartite synapse concept, where astrocytes actively interact with neurons and are integral elements of synaptic physiology. This concept implies an active role of astrocytes in brain function, which would hence result from the concerted activity of astrocytes and neurons.

According to this new concept, astrocyte calcium signal is a key element because it is the second messenger that serves as substrate of astrocyte excitability underlying its responsiveness to neurotransmitter release during synaptic activity. It stimulates the release of gliotransmitters that regulate synaptic transmision and plasticity (for reviews see Araque et al., 1999; Volterra and Meldolesi, 2005; Perea et al., 2009). While most of the reports supporting this new concept originally derived from studies performed in cell cultures (Araque and Perea, 2004) and slices (Perea et al., 2009), more recent work are also based on *in vivo* studies. Indeed, astrocyte calcium elevations evoked by electrical or sensory stimulation have been reported in the hippocampus (Navarrete et al., 2012), somatosensory barrel cortex (Wang et al., 2006; Takata et al., 2011), and visual cortex (Schummers et al., 2008; Chen et al., 2012) *in vivo*. Present results show that astrocytes located in the layer I of the primary somatosensory cortex also respond to sensory stimuli, further supporting the astrocyte responsiveness to synaptic activity evoked by sensory inputs *in vivo*.

Interestingly, while previous *in vivo* astrocytic calcium transients were recorded at the soma (Hirase et al., 2004; Wang et al., 2006; Schummers et al., 2008; Takata et al., 2011; Chen et al., 2012; Navarrete et al., 2012), strong evidence obtained in slices indicate that the physiologically relevant calcium signal may occur at discrete regions -microdomains- of the astrocytic processes (Grosche et al., 1999; Perea and Araque, 2005; Di Castro et al., 2011; Panatier et al., 2011). Our present results indicate that astrocyte calcium elevations evoked by sensory stimuli *in vivo* also take place at regions of the fine processes closely associated with dendritic spines, i.e., where most synapses are established with excitatory terminals.

A recent report has questioned the validity of the tripartite synapse concept in adult animals based on the absence of mGluR5 expression in adult astrocytes and mGluR5-mediated astrocyte calcium elevations (Sun et al., 2013). However, the reported absence of mGluR5-mediated calcium responses in adults discards this particular mechanism of astrocytic activation, but the calcium responsiveness of astrocytes through different signaling mechanisms and to different synaptically released neurotransmitters [e.g., glutamate, acetylcholine (ACh), endocannabinoids, adenosine triphosphate (ATP), norepinephrine, etc.] is strongly supported by numerous evidence obtained in slices and *in vivo* (Wang et al., 2006; Navarrete and Araque, 2008, 2010; Schummers et al., 2008; Di Castro et al., 2011; Panatier et al., 2011; Takata et al., 2011; Chen et al., 2012; Min and Nevian, 2012; Navarrete et al., 2012; for a review see Perea et al., 2009). On the other hand, the tripartite synapse concept based on the ability of astrocytes to release gliotransmitters that regulate synaptic transmission and plasticity is also supported by abundant evidence obtained by numerous laboratories (Perea and Araque, 2007; Fellin et al., 2009; Henneberger et al., 2010; Navarrete and Araque, 2010; Di Castro et al., 2011; Panatier et al., 2011; Min and Nevian, 2012). Regardless the underlying molecular mechanisms, our present results obtained in adult animals *in vivo* add further support of the idea that astrocytes respond with calcium elevations to sensory stimuli.

Simultaneous imaging of astrocyte calcium and blood vessels in slices as well as *in vivo* has provided relevant information to decipher the complex mechanisms controlling cerebral blood flow microcirculation (Zonta et al., 2003; Mulligan and MacVicar, 2004; Takano et al., 2006; Gordon et al., 2008; Petzold et al., 2008; Srienc et al., 2012; for a review see Attwell et al., 2010). Notably, Eric Newman's lab has developed an intact *in vivo* preparation in which the retina of anesthetized, paralyzed rats can be directly imaged with LSCM and laser speckle flowmetry to monitor retinal glial cell responses and retinal blood flow (Mishra et al., 2011; Srienc et al., 2012). Using this intact preparation they were able to demonstrate that light stimulation evoked glial calcium waves that led to the dilation of neighboring retinal arterioles, indicating that glial cells respond to sensory stimuli and subsequently regulate blood flow *in vivo*. In agreement with these reports, our results show that sensory stimulation leads to calcium elevations in astrocytic somata and endfeet enwrapping blood vessels along with changes in blood vessel diameter in the somatosensory cortex. Furthermore, they confirm the suitability of our method to study blood flow microcirculation *in vivo*, prompting further studies regarding blood flow control in healthy as well as pathological conditions in the live animal.

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Imaging the microanatomy of astrocyte–T-cell interactions in immune-mediated inflammation

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The role of astrocytes in the immune-mediated inflammatory response in the brain is more prominent than previously thought. Astrocytes become reactive in response to neuro-inflammatory stimuli through multiple pathways, contributing significantly to the machinery that modifies the parenchymal environment. In particular, astrocytic signaling induces the establishment of critical relationships with infiltrating blood cells, such as lymphocytes, which is a fundamental process for an effective immune response. The interaction between astrocytes and T-cells involves complex modifications to both cell types, which undergo micro-anatomical changes and the redistribution of their binding and secretory domains. These modifications are critical for different immunological responses, such as for the effectiveness of the T-cell response, for the specific infiltration of these cells and their homing in the brain parenchyma, and for their correct apposition with antigen-presenting cells (APCs) to form immunological synapses (ISs). In this article, we review the current knowledge of the interactions between T-cells and astrocytes in the context of immune-mediated inflammation in the brain, based on the micro-anatomical imaging of these appositions by high-resolution confocal microscopy and three-dimensional rendering. The study of these dynamic interactions using detailed technical approaches contributes to understanding the function of astrocytes in inflammatory responses and paves the way for new therapeutic strategies.

Keywords: astrocyte, T-cell, immunological synapse, infiltration, cytokines, chemokines, glioma, immune response

INTRODUCTION

Astrocytes are sensitive to changes in the brain parenchyma and become reactive in the presence of inflammatory stimuli, changing both their protein expression and phenotype. These cellular modifications are characterized by an apparent augmentation of cell body size, an increased number of primary and secondary branches, and an intensification of various signaling cascades, such as ATP-mediated and cytokine-mediated signaling, often resulting in the overexpression of certain proteins, such as intermediate filament proteins, cytokines, and chemokines (Buffo et al., 2010; Kang and Hebert, 2011; Molofsky et al., 2012; Sun and Jakobs, 2012). The role of these responses is not well established and might be multifaceted and different for each neuro-pathological scenario and for each brain region (Zhang and Barres, 2010; Oberheim et al., 2012). Generally, when astrocytes become reactive in response to injury or inflammation, glial fibrillary acidic protein (GFAP), as well as other intermediate filament proteins such as nestin and vimentin, become overexpressed (Pekny and Nilsson, 2005; Buffo et al., 2008, 2010). Although it is a widely used and reliable marker for astrocytes, GFAP's function remains poorly understood. Furthermore, the existence of different isoforms of GFAP and different populations of astrocytes adds further complexity to understanding the role of GFAP. It is thought that GFAP may participate in many cellular processes, such as motility, proliferation, autophagy, synaptic plasticity, neural outgrowth, myelination, injury protection, and blood brain barrier (BBB) formation (Middeldorp and Hol, 2011). Importantly, some of these tasks are intimately related to immune-mediated inflammation and seem to be crucial for adequate responses in pathological conditions, particularly for protoplasmic astrocytes, which reside in the gray matter and form the neurovascular unit (Dirnagl, 2012).

The mechanism that induces the spatial reorganization of astrocytes appears to be multifaceted (Buffo et al., 2010; Kang and Hebert, 2011). One of the most important pathways that triggers this reactivity involves cAMP, which represents a crucial player in astrocyte cell-to-cell communication (Grimaldi et al., 1999; Pascual et al., 2005). Injured cells release or leak ATP into the intercellular space, thereby activating the P2 receptors of neighboring astrocytes and initiating structural modifications. Additionally, the release of cytokines into the extracellular space, either by infiltrating blood cells or by neighboring glial cells,

also contributes to astrocytic reactivity by stimulating cytokine receptors present on the astrocyte cell surface (Buffo et al., 2010; Hamby and Sofroniew, 2010).

Inflammatory GFAP immunoreactivity can be observed in brain injuries and in most prominent neurodegenerative disorders, such as Parkinson's disease (Forno et al., 1992), Alzheimer's disease (Diedrich et al., 1987), Huntington's disease (Galatioto, 1996), and multiple sclerosis (Darvesh et al., 2010), suggesting the involvement of glia-mediated inflammation in the process of degeneration. Particular attention should be given to gliomas, which are primary brain tumors that have astrocytic characteristics. Glioma cells originate from neural stem cells or oligodendrocyte progenitor cells (Sanai et al., 2005; Lindberg et al., 2009) but can also be derived from mature astrocytes or differentiated neurons that change their phenotype to a glioma cell-type (Friedmann-Morvinski et al., 2012). However, independently of their origin, glioma cells express high amounts of GFAP and vimentin (Herpers et al., 1986), much like reactive astrocytes in other inflammatory scenarios. Glioma cell morphology within the tumor is variable; it is difficult to define a particular shape for these cells. In addition, glioma cells are co-located with non-neoplastic resident astrocytes, which makes it even more difficult to distinguish between these two cell types (**Figure 1**). In experimental models, marking inoculated glioma cells with green fluorescent protein (GFP) or with similar markers makes it possible to identify glioma cells (Tatenhorst et al., 2005). In this particular model, artificially implanted GFP$^+$ glioma cells adopt a unipolar morphology, with the major processes closely aligned with blood vessels (BVs) and intercalated between endothelial cells and resident GFAP$^+$ protoplasmic astrocytes (Farin et al., 2006). This evidence suggests that similar intercellular apposition between resident astrocytes and glioma cells may occur in human glioma. In addition, glioma cells are also in constant mitosis, which entails frequent morphological changes and dramatic modifications of the intercellular space, together with the breaking of the intercellular matrix (Ziu et al., 2006). Together, these features of the astrocytic reactivity of glioma cells are important contributors to glia-mediated inflammation in neoplastic areas, sharing a common response with neurodegenerative disorders.

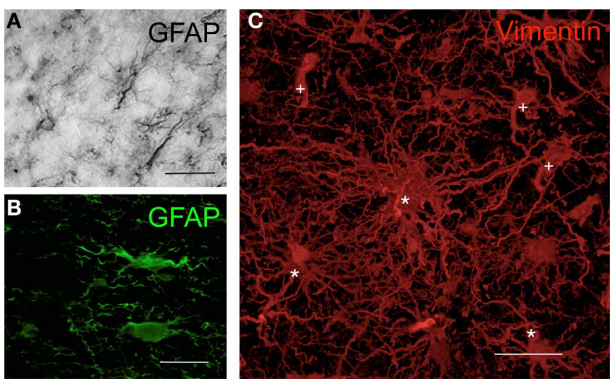

FIGURE 1 | Morphology of human astrocytes by immunohistochemistry. (A) Astrocytes in the human cortex visualized by GFAP immunohistochemistry through DAB precipitation in the peritumoral area of a glioma. **(B)** Astrocytes in human cortex visualized by GFAP immunofluorescence in the peritumoral area of a glioma. **(C)** Three-dimensional transparency of vimentin$^+$ cells in human glioblastoma. Cells in the tumor areas were marked using immunohistochemistry with antibodies against vimentin (red) and were scanned with high-resolution confocal microscopy. Some cells maintain the typical astrocytic star shape (*), whereas others show the neoplastic morphology (+) that is characteristic of astrocytomas [modified from Carrillo-de Sauvage et al. (2012)]. Scale bar: 30 μm in **(A)** and **(C)**; 25 μm in **(B)**.

In these brain diseases, astrocyte reactivity is a clear hallmark of glia-mediated inflammation and is a critical contributor to the local inflammatory environment of the brain parenchyma. Specifically, these reactive morphological changes, together with the anatomical location of protoplasmic astrocytes, are thought to be important factors for the regulation of blood cell recruitment (Voskuhl et al., 2009), particularly the infiltration of T lymphocytes, which is an essential event in immune responses in the CNS.

The frequency of T-cell entry into the brain parenchyma is low in a healthy brain. Limited subsets of T-cells patrol the CNS tissue, carrying out regular immuno-surveillance. Thus, a massive migration of immune cells to the CNS takes place only during neuro-inflammation (Engelhardt and Ransohoff, 2012). The significance of T-cell infiltration is critical in many CNS inflammatory responses. T-cells are involved in the destruction of myelin sheaths in multiple sclerosis and in experimental allergic encephalomyelitis. Additionally, brain-infiltrating T-cells are crucial to controlling brain infections, as occurs in viral or bacterial invasion and in other cases of encephalitis. However, the function of CNS-infiltrating T-cells in other brain diseases, such as in brain cancer or in neurodegenerative diseases, remains poorly understood. It is thought that certain specific subpopulations of T-cells, such as regulatory T-cells, may facilitate the progression of brain tumors (Sugihara et al., 2009); these T-cells are putative targets for immunotherapy (Von Boehmer and Daniel, 2013). Degenerative diseases, such as Alzheimer's disease and Parkinson's disease, also show infiltrating T-cells in degenerating brain regions, but the role of these infiltrating T-cells remains unclear (Togo et al., 2002; Brochard et al., 2009).

Astrocyte reactivity, and the subsequent entry of T-cells to the brain parenchyma, may be a fundamental aspect of the inflammatory response in CNS diseases. Thus, technical approaches for imaging the inter-relationship between T-cells and reactive astrocytes *in vivo* in the neuro-inflammatory environment are crucial to understanding the intricate phenomenon of T-cell infiltration and its function.

HOW TO VISUALIZE ASTROCYTES IN THE TISSUE

To visualize astrocytes in tissue, the use of GFAP-specific antibodies for immunohistochemistry techniques results in specific, feasible and reliable staining. GFAP immunohistochemistry is particularly suitable for mature fibrous astrocytes and reactive astrocytes, although the levels of GFAP are heterogeneous in astrocytes, and GFAP is also expressed in progenitor cells in the adult mouse (Garcia et al., 2004). Other markers, such as S100B, Reelin, and vimentin, have the limitation of identifying other differentiated cell types, such as oligodendrocytes or

neurons, making it difficult to distinguish astrocytes from other mature cells (Molofsky et al., 2012). Antibodies against S100B, a glia-specific calcium binding protein, provide robust astrocytic detection but also label mature oligodendrocytes. The use of antibodies against Reelin/Slit, an extracellular matrix protein, detects astrocytes in the early stages of development but may also label neurons. Antibodies against vimentin, which strongly label reactive astrocytes, may also label amoeboid microglia and active macrophages [for an extended list of astrocytic markers, see the article by Molofsky et al. (2012)]. Because GFAP-specific antibodies do not bind to other differentiated cell types, this marker is most likely the best available option for in tissue studies. By contrast, one of the disadvantages of GFAP immunohistochemical staining in tissue is that GFAP does not identify the entire cell body; additionally, some of the micro-anatomical characteristics and details of astrocytes are not easy to visualize under the microscope. Studies performed using transgenic mice with enhanced GFP (eGFP)-expressing astrocytes (Nolte et al., 2001; Suzuki et al., 2003) allowed the imaging of entire astrocytes in a living brain. High-resolution imaging of eGFP-expressing astrocytes reveals fine processes emerging from the cell body, whereas GFAP immune-reactivity remains limited to the perinuclear areas and the thick processes (Suzuki et al., 2003). This result advocates the use of eGFP as preferable, when possible, because eGFP provides detailed morphological information about the entire cell that cannot be detected with GFAP immunohistochemistry. Another option that allows a fine and detailed analysis of the entire astrocytic cell is the dye-filling method, which has the advantage of inoculating specific dyes within fixed brain tissue after extraction and fixation (Wilhelmsson et al., 2006); thus, this technique can be used in fixed tissue from human biopsies.

Currently, the two best microscopy options for visualizing brain cells within tissue are confocal and two-photon microscopy. Both techniques are complementary and can be used to answer different questions regarding the visualization of astrocytes. Two-photon microscopy allows the study of live cells *in vivo* within the brain (Theer et al., 2003; Helmchen and Denk, 2005). With this approach, live cells can be visualized several hundred microns deep within the tissue of living animals, and this approach has the advantage that the interactions of living cells can be studied in time lapse experiments (Theer et al., 2003; Helmchen and Denk, 2005). However, particularly deep brain areas, such as the basal ganglia, thalamus, and other associated structures, are difficult, if not impossible, to visualize unless micro-endoscopy is used (Jung et al., 2004). However, the resolution of two-photon microscopy is still insufficient to visualize the micro-anatomical details of intercellular interactions; furthermore, the availability of important fluorophores prevents the labeling of multiple structures or molecules simultaneously *in vivo*. Although confocal analysis of fixed cell culture preparations or of fixed tissue slides provides static images, the advantage of this analysis is the ability to observe structures at high resolution; thus, the micro-anatomical details of intercellular interactions can be appreciated. In addition, with multiple fluorochromes available, the use of confocal microscopy allows the use of many markers simultaneously, permitting the visualization of several structures with high detail in the same frame. For these reasons, confocal microscopy in fixed tissue may be preferable for analyzing the micro-anatomical details of intercellular interactions at high-resolution, whereas intra-vital two-photon microscopy is preferable for studying the dynamic view of the cellular interactions in the brain parenchyma.

To successfully study the micro-anatomical details of intercellular interactions in three-dimensional space within fixed tissue, the immunohistochemistry protocol requires the following technical specifications: (1) thick sections of tissue for recording the maximum amount of information; (2) specific antigen retrieval treatment for particular epitopes; and (3) long incubations of 24–48 h with primary antibodies. These specifications allow the antibodies to optimally penetrate to 50–60 μm within the tissue, obtaining uniform staining throughout the thickness of the sections. Therefore, the confocal analysis can be performed in depth, and the interactions between the cells can be visualized and analyzed at high detail. In addition, confocal scanning requires a large number of optical sections in order to record high-resolution detail along the z-axis, spanning entire cells and the surrounding interactions, giving the possibility of rendering stacks of images in 3-dimensional space with the appropriate blending software. When scanning is performed, 3-dimensional analyses are important for accurate interpretation of the data and quantifications. This technological approach allows the adequate study of intercellular communication at micro-anatomical levels to understand the intricacy of the glia-mediated inflammatory response in the brain. Based on these technical specifications and on the abridged literature, in the following sections, we review the micro-anatomical details of astrocyte–T-cell interactions in immune-mediated inflammation.

IMAGING ASTROCYTES AS CHEMOKINE PRODUCERS AND LYMPHOCYTE ATTRACTANTS

Astrocytes are important players in the formation of the BBB. Protoplasmic astrocytes, residents of the gray matter, are intimately associated with BVs and, together with pericytes and endothelial cells, control blood flow and BBB permeability (Attwell et al., 2010). In the perivascular compartment, astrocytes are able to produce and release certain proteins that contribute to the neuro-inflammatory response in the CNS (Croitoru-Lamoury et al., 2003). Among these proteins, chemokines are some of the most prominent factors involved in the induction of the pro-inflammatory environment in tissues (Luster, 1998). Chemokines are small proteins that are released into the parenchyma or into the blood stream, creating a gradient of chemo-attractants that other cells follow toward the chemokine source (Luster, 1998; Murdoch and Finn, 2000). Dendritic cells, neutrophils, and monocytes, among others, are typical cellular sources of chemokines in most tissues (Scapini et al., 2000; Zlotnik and Yoshie, 2000). However, in the brain, astrocytes appear to assume a significant portion of this role. *In vitro*, astrocytes respond to inflammatory stimuli, such as bacterial lipopolysaccharide (LPS) and pathogens, by expressing CCL and CXCL chemokines. After LPS treatment to simulate inflammation *in vitro*, a strong increase in the release of CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), CXCL1, and CXCL2 is observed from astrocytes (Van Neerven et al., 2010). Other inflammatory stimuli, such as the treatment of astrocytes with TNF-alpha

in vitro, also result in the increased expression of several genes in astrocytes, including those encoding the chemokines CCL2, CCL5, and CXCL8 (IL-8) (Meeuwsen et al., 2003). Importantly, in various *in vivo* models of neurodegenerative diseases, the expression of these chemokines is increased specifically in astrocytes. In an experimental model of multiple sclerosis, astrocytes are also responsible for the release of CCL2, CCL3, and CCL5 (Quinones et al., 2008). Consistent with this observation, astrocytes were also found to be the predominant source of CCL2 and CCL3 chemokines in the striatum and the substantia nigra in an experimental model of Parkinson's disease induced by MPTP (Kalkonde et al., 2007). In addition, in an experimental model of Alzheimer's disease, beta-amyloid was shown to activate astrocytes to produce CCL2 and CCL5 (Johnstone et al., 1999). Furthermore, in other scenarios, such as mechanical injury to the brain, chemokines such as CCL2 are also expressed by astrocytes (Glabinski et al., 1996).

These findings indicate that, among the chemokines, CCL2 release is a common factor that is important and predominant in areas of inflammation in the CNS. The mechanism by which astrocytes are activated and produce CCL2 in brain trauma or neuro-inflammation is not well-defined, but it is thought to be associated with stimulation by ATP released at injured areas, which activates P2X7 receptors in the astrocyte membranes (Panenka et al., 2001). After injury, CCL2 is expressed by protoplasmic reactive astrocytes under pro-inflammatory conditions. At the cellular level, CCL2 expression, detected by immunoreactivity, is associated with GFAP, although the intracellular co-localization of both proteins is not complete (Carrillo-de Sauvage et al., 2012). CCL2 immunoreactivity seems to be particularly associated with the terminal tips of GFAP filaments (**Figure 2**), which suggests a specific role of CCL2 at the edge of astrocyte filaments. In pro-inflammatory conditions in the brain, such as following LPS treatment or viral infection, $GFAP^{+}CCL2^{+}$ astrocytes have the characteristic astrocytic star shape. However, particularly in brain tumors, $GFAP^{+}CCL2^{+}$ cells adopt convoluted shapes, and CCL2 expression displays different, irregular patterns (**Figure 3**), depending on the anatomical complexity of the tumor. In all three pro-inflammatory scenarios, these CCL2-rich terminals are specifically related to the neurovascular unit, suggesting that CCL2 may facilitate the entry of blood cells into the brain parenchyma (Carrillo-de Sauvage et al., 2012).

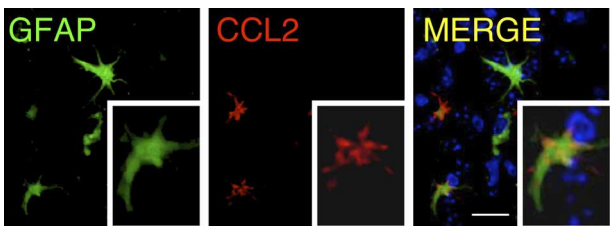

FIGURE 2 | Distribution of CCL2 in $GFAP^{+}$ astrocytes. Confocal rendering of an area of LPS-induced inflammation in the mouse brain. Astrocytes are labeled using immunohistochemistry with antibodies against GFAP (green), and the chemokine CCL2 is stained with anti-CCL2 antibodies (red). Nuclei are counterstained with DAPI (blue). CCL2 is expressed in astrocytes, and its intracellular distribution is higher in the terminal GFAP filaments [modified from Carrillo-de Sauvage et al. (2012)]. Scale bar: 30 μm.

Chemokines such as CCL3 and CCL4 mainly function as macrophage chemo-attractants (Maurer and Von Stebut, 2004), whereas CCL5 is known to predominantly function in the selective infiltration of T-cells (Schall et al., 1990; Kawai et al., 1999). However, it is not clear which mechanisms may drive the differential entry of particular subsets of cells. For example, $CD45RA^{-}$ $CD45R0^{+}$ memory T-cells migrate in response to the combination of CCL5, CCL2, and CCL3 chemokines (Qin et al., 1996), which indicates that a particular combination of chemokines may stimulate the recruitment of specific T-cell subsets. We and others have recently shown that CCL2 has a primary role in T-cell infiltration in inflamed tissues, particularly in tumors (Brown et al., 2007; Molon et al., 2011; Carrillo-de Sauvage et al., 2012). However, further research must be performed to clarify the role of chemokines in the infiltration of specific blood cell types and to determine whether this response is different in particular anatomical compartments or in specific inflammatory scenarios. Consistent with this finding, one of the crucial factors that induces the differential entry of blood cells is the presence of specific chemokine receptors on the surface of blood cells that signal the activation of diapedesis and parenchymal entry. In this context, particular chemokines, such as CCL2, are able to induce the formation of T-cell uropods, which are micro-anatomical structures that facilitate extravasation (Del Pozo et al., 1997). Importantly, the recruitment of T-cells into the brain occurs at the border of BVs where $CCL2^{+}$ astrocytes are present (Carrillo-de Sauvage et al., 2012). In the BV border, multiple physical contacts between T-cells and $CCL2^{+}$ astrocytes can be observed, particularly surrounding BVs and in areas of inflammation, which suggests the involvement of a micro-anatomical interaction with perivascular astrocytes in T-cell diapedesis (**Figure 4**). These events are observed in LPS-induced inflammation in mice, in adenoviral-injected monkeys and in astrocytomas in human brains (Carrillo-de Sauvage et al., 2012), suggesting that astrocytes located at perivascular areas may play fundamental chemical and physical roles in recruiting particular T-cell subpopulations into areas of brain inflammation.

Further studies are required to fully understand the role of astrocytes in the infiltration of T-cells and the mechanism underlying the differential entry of specific subpopulations of lymphocytes into the inflamed brain. Most likely, astrocytes release different types of chemokines depending on the inflammatory milieu, creating a route for specific lymphocyte populations to follow. Importantly, disentangling this information *in vivo* will have crucial implications for therapeutic strategies for brain diseases in the near future.

IMAGING THE MUTUAL ARRANGEMENT IN CELL-TO-CELL IMMUNOLOGICAL ENCOUNTERS

The interaction between astrocytes and lymphocytes in immune-mediated inflammation is not a static event. Both cell types undergo different changes in the process of approaching, apposing and contacting each other. As we described in the previous section, lymphocytes and astrocytes signal to each other to establish a contact that may result in the entry of lymphocytes into the

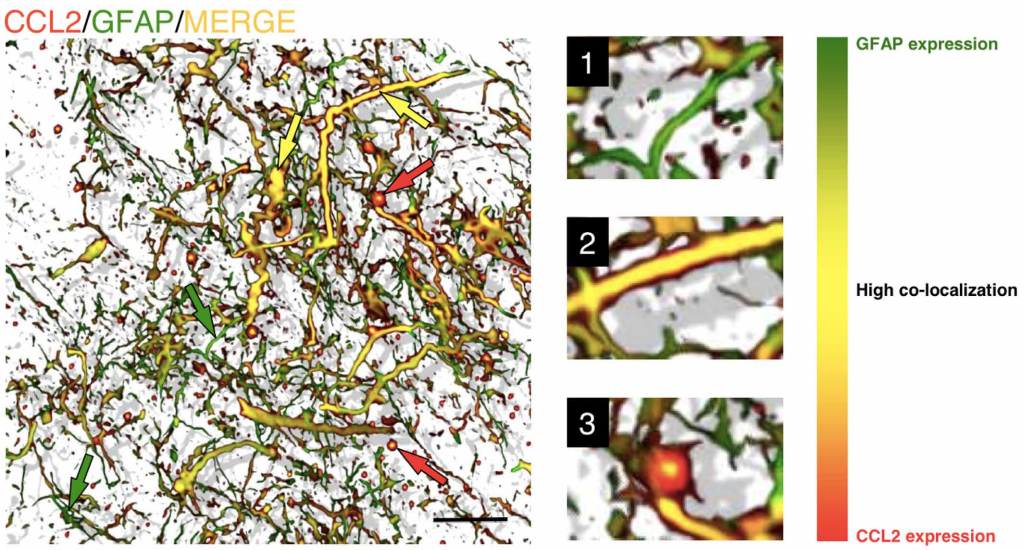

FIGURE 3 | Astrocytes in GBM are able to adopt various shapes and differentially express chemokines. Three-dimensional reconstruction, rendered from confocal images, of GFAP/CCL2^{+} astrocytes within a human brain tumor section (biopsy from glioblastoma). It is not possible to distinguish tumorigenic astrocytes from resident astrocytes, but cells adopt different shapes in the neoplastic areas, ranging from star-shaped cells to elongated cell bodies with thick and long filaments. The level of co-localization between GFAP and CCL2 can be observed as a color gradient. This gradient is shown in the scale on the right. Smaller GFAP^{+} cells and thinner filaments are shown in green, with low levels of CCL2 expression (green arrows and detail in 1). Larger cells and thick filaments are shown in yellow, which represents high co-localization of both markers (yellow arrows and detail in 2), suggesting that the expression of CCL2 is highly expressed in reactive astrocytes. Occasionally, astrocytes also show gem-like buds with large accumulations of CCL2 (red arrows and detail in 3). These accumulations, with high CCL2 expression and low GFAP expression, are related to the infiltration of T-cells into the tumorigenic areas [more information about this finding is presented in Carrillo-de Sauvage et al. (2012)]. Scale bar: 20 μm.

brain parenchyma. However, other types of lymphocyte–astrocyte contacts that occur in the tissue during immune-mediated antigen presentation may also help us to understand the features of cell-to-cell arrangements that control brain immune responses.

T-cells form a complex structure called an immunological synapse (IS) when contacting antigen-presenting cells (APCs) (Dustin, 2005). At the level of the T-cell–APC interface, this micro-anatomical structure is characterized by the specific arrangement of several adhesion molecules and receptors, such as lymphocyte function-associated antigen (LFA)-1, CD3, and the T-cell receptor (TCR), as well as the reorganization of organelles such as the microtubule organizing center (MTOC), the Golgi apparatus, and the F-actin and alpha-tubulin cytoskeleton (Kupfer et al., 1983; Monks et al., 1998; Stinchcombe et al., 2006). Most of the studies on ISs have focused on the changes that T-cells undergo, but little attention has been given to APC transformations. The first visualization of the IS *in vivo* was made in T-cells contacting adenovirus-infected astrocytes in the brain (Barcia et al., 2006). The arrangement of the T-cells *in vivo* was similar to the micro-anatomical organization previously described *in vitro* (Monks et al., 1998). The arrangement of T-cells mainly involves the clustering and polarization of the TCR/CD3 complex at the center of the intercellular interface and the segregation of LFA-1 to the outer ring of the interface (Monks et al., 1998; Dustin, 2005). The role of this arrangement at the IS remains unclear. The TCR/CD3 complex is particularly important in the detection of antigen and in the subsequent signaling that activates the T-cell. In addition, the LFA-1 arrangement is crucial in adhesion and cytoskeletal reorganization. LFA-1 is linked to TALIN and to the cytoskeleton, which guides the polarity of the T-cell after adhesion. These arrangements occur in T-cells in many immune-mediated scenarios. In the brain, T-cells rearrange their interface, forming typical mature ISs, when contacting viral-infected astrocytes or glioma cells (**Figure 5**) (Barcia et al., 2006, 2009; Yang et al., 2010). It is thought that this complex arrangement of the T-cell interface is important for a directional effector response, but further studies are required to unravel its function. T-cell–APC contact may result in the elimination of the target cell because of the release of cytotoxic granules, such as IFN-gamma, granzyme-B, or perforin, by the T-cell at the synaptic interface. In this context, targeted APCs undergo different morphological and biochemical changes that lead to the APCs final elimination. T-cell IS formation appears to be fundamental for the clearance of astrocytes infected with adenovirus from the brain (Barcia et al., 2006). In this context, IFN-gamma is polarized in T-cells at the IS established with adenovirus-infected astrocytes; thus, IFN-gamma is specifically delivered toward the synaptic interface for an effective immune response (Barcia et al., 2008b). In the case of glioma cell clearance, granzyme-B is polarized in cytolytic T-cells and delivered to the synaptic interface toward the target glioma cell. Granzyme-B induces the specific cleavage of alpha tubulin (Adrain et al., 2006), disrupting the glioma cell cytoskeleton (Barcia et al., 2009), and leading to CTL-mediated apoptosis of the target cells (Goping et al., 2006).

Notably, target astrocytes in intimate immunological contact with T-cells also have similar molecular and micro-anatomical

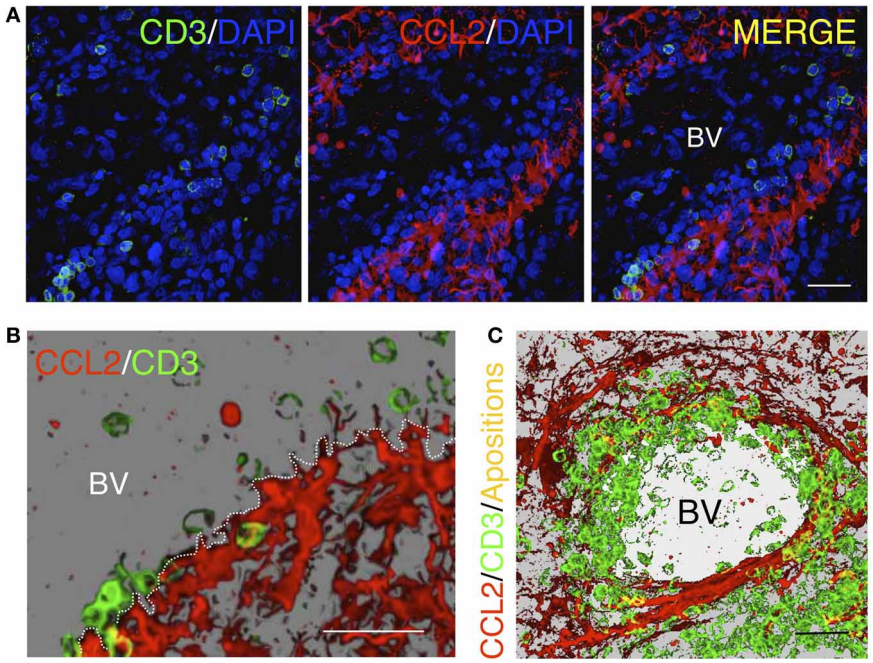

FIGURE 4 | Homing of T-cells in a tumorigenic area in the human brain. **(A)** Confocal images of immunofluorescence for $CD3^+$ T-cells (green) and the chemokine CCL2 (red). Cell nuclei are counterstained with DAPI (blue). $CCL2^+$ astrocytes delimit the outer perivascular area of the BV, whereas T-cells are localized near $CCL2^+$ perivascular astrocytes. **(B)** Three dimensional reconstruction of a detailed region obtained from the confocal images shown in **(A)**. **(C)** Three dimensional reconstruction of a blood vessel lumen in a human glioma. Note that T-cells are apposed to the perivascular $CCL2^+$ cells covering the internal wall of the BV lumen. Yellow spots represent co-localization of CD3 and CCL2, representing the areas of contact between the two cell types [Modified from Carrillo-de Sauvage et al. (2012)]. Scale bar: 40 μm in **(A)** and **(C)**; 50 μm in **(B)**.

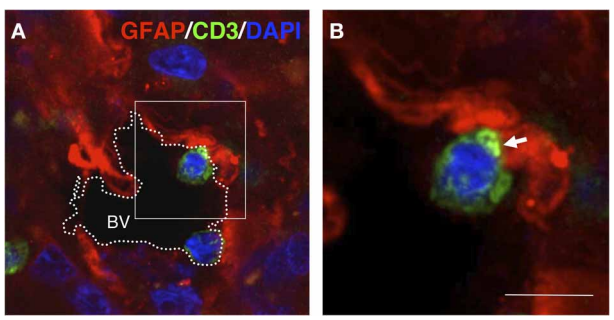

FIGURE 5 | T-cell–GFAP interactions in gliomas. Confocal section (0.5 μm) obtained from a human glioma biopsy. Immunohistochemistry was performed with anti-GFAP antibodies (red), anti-CD3 antibodies (green) for T-cells and DAPI for counterstaining (blue). **(A)** Overview of a blood vessel (BV) in the tumor area in which two T-cells are contacting $GFAP^+$ cells at the edge of the vascular lumen. **(B)** High magnification of the frame indicated in **(A)** showing details of the T-cell–GFAP interaction. Importantly, the apposed T-cell displays clustered CD3 at the interface of the intercellular contact (arrow) [original findings can be found in Barcia et al. (2009)]. Scale bar: 15 μm.

arrangements as those in lymphocytes. Astrocytes, functioning as APCs, establish ISs with T-cells and undergo massive morphological changes characterized by a reduction in cell body size, a reduction in the number of filaments, and the formation of a protrusion toward the area of contact (**Figure 6**) (Barcia et al., 2008a). Importantly, the MTOC and the Golgi apparatus also re-orient toward the interface, frequently contacting the astrocytic protrusion, suggesting that astrocytes actively respond to T-cell interactions and are not passive APCs. Similar changes are observed in primary astrocytes in response to a physical lesion; these astrocytes develop a protrusion and reorient their MTOC and Golgi apparatus toward the lesion side (Etienne-Manneville, 2006; Osmani et al., 2006). These results suggest that T-cell apposition signals to the target astrocyte, inducing changes that may result in the interchange of crucial information between the two cells (**Figure 7**). The function of this symmetric arrangement remains unknown. Astrocytes appear to prepare themselves to release signaling products toward T-cells. In particular, the orientation of the Golgi apparatus and MTOC is consistent with these observations. These changes may be related to the transmission of specific messages such as the confirmation of "kill-me" signals sent by CTLs or, by contrast, the release of "do-not-kill-me" signals to avoid elimination. In addition, because astrocytes have been described to engulf particles or entire cells, it has been hypothesized that this active rearrangement of astrocytes may also represent a physical defense of the targeted astrocyte in the process of engulfing a T-cell aggressor (Barcia et al., 2008a; Loov et al., 2012).

T-cell–astrocyte apposition is the result of the mutual approach of both cells, stimulated by the orchestrated release of cytokines or chemokines into the extra-cellular environment. As described above, particular chemokines released by

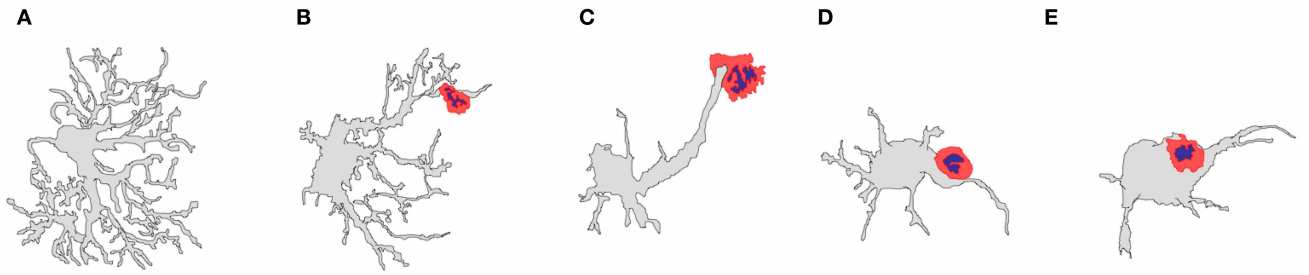

FIGURE 6 | Diagrams representing the shape variation that can be found in T-cell–astrocyte interactions in the rat striatum. Adenovirus-infected astrocytes display different shapes as the T-cell apposition become closer to the astrocyte cell body. **(A)** Typical astrocytic shape in the brain; **(B)** an astrocyte in contact with a T-cell lymphocyte (red) at its terminal processes; **(C)** an astrocyte in contact with a T-cell lymphocyte (red) distally at the astrocytic protrusion; **(D)** an astrocyte in contact with a T-cell lymphocyte (red) proximally at the astrocytic protrusion; **(E)** an astrocyte in contact with a T-cell lymphocyte (red) at the cell body [artistic sketches performed by CB, modified from Barcia et al. (2008a)].

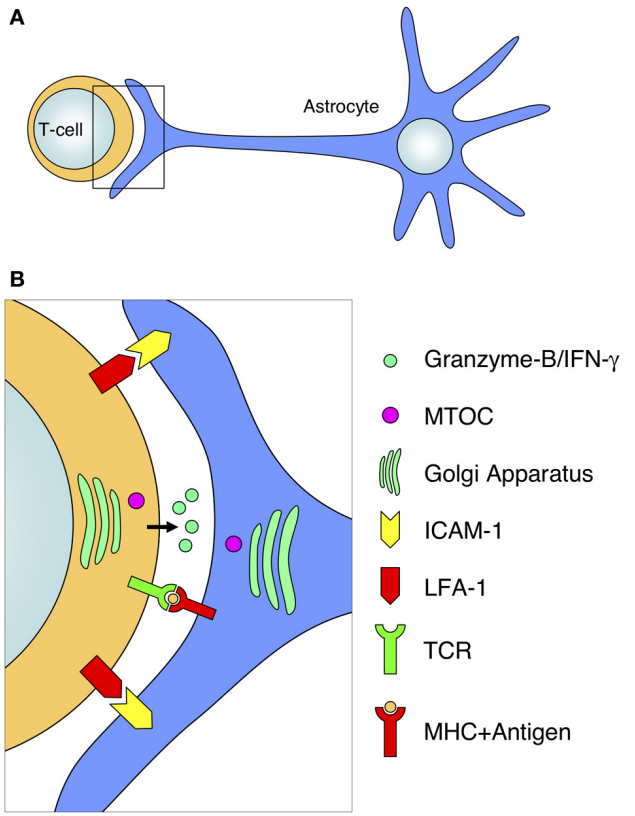

FIGURE 7 | Diagram of antigen-dependent T-cell–astrocyte interactions. The interaction of activated T-cells with MHC-expressing astrocytes involves a defined arrangement of various molecules and organelles. **(A)** Schematic representation of the interaction of a T-cell with an astrocyte at the level of the astrocytic protrusion. **(B)** Magnification of the T-cell–astrocyte interface from the frame depicted in **(A)**. The T-cell–astrocyte interface is characterized by the segregation of adhesion molecules (such as LFA-1 and ICAM-1), the interaction of the TCR with MHC molecules, the release of cytotoxic compounds (such as Granzyme-B or IFN-gamma) into the intercellular space; and the rearrangement and polarization of organelles toward the interface (for example, the relocation of the MTOC and Golgi apparatus in both cell types).

astrocytes are responsible for the recruitment of T-cell subsets into the brain parenchyma. Conversely, cytokines released by T-cells that have infiltrated into the brain parenchyma interact with local astrocytes and induce specific changes. Particular subtypes of pro-inflammatory cytokines, such as IFN-gamma, stimulate the synthesis and consequent expression of MHC molecules on the astrocytic membrane, and these molecules are required for the presentation of antigens to T-cells. Indeed, MHC-expressing astrocytes of the CNS are efficient APCs that are able to strongly activate antigen-reactive T-lymphocytes (Fierz et al., 1985; Wekerle et al., 1987). In particular, in the context of the adaptive immune response in the brain, MHC expression by astrocytes is highly upregulated by IFN-gamma, which is released by activated T-cells that infiltrate the brain parenchyma (Richt et al., 1990; Barcia et al., 2008b). Notably, a detailed micro-anatomical analysis of MHC expression in astrocytes revealed that MHC molecules accumulate at a high density in the cell body and the proximal areas, in contrast to the low levels that accumulate in the distal areas, such as in filaments or protrusions (Barcia et al., 2008a). These findings suggest that the astrocytic cell body has a gradient of affinity for T-cells. This strategy of approach and final contact suggests that astrocytes may have an active and pivotal role in the development of immune responses in the CNS, acting as APCs in response to cytokines (Fierz et al., 1985). Thus, T-cell–astrocyte interactions in brain immune responses may contribute to different outcomes depending on the released factors. A key element in this modulation is the fact that specific chemokines or cytokines induce particular T-cell subpopulation responses and may determine the type of immune response. Through different mechanisms, astrocytes are able to both prevent or promote the activation of T-cells (Wekerle et al., 1987). One of the strategies to suppress T-cell activation is the upregulation of CTLA-4, which inhibits Th1 and Th2 cell responses occurring with or without astrocyte–T-cell contact (Gimsa et al., 2004). Another strategy is the release of IL-10, which directly activates regulatory T-cells (T_{regs}). Both strategies are able to attenuate cytolytic immune responses in the brain, which may be beneficial for reducing the aggressive autoimmune responses that occur in multiple sclerosis or experimental

autoimmune encephalomyelitis. By contrast, these approaches also increase tolerance, which may be advantageous for tumor cell growth or may facilitate the expansion of infections and the development of encephalitis. Astrocytes are also able to promote the activation of T-cells through the release of certain cytokines by alternative mechanisms. In autoimmune diseases, astrocytes release IL-15, which contributes to an increase in T-cell effector function (Saikali et al., 2010), or IL-17, which activates cytolytic immune responses, facilitating cephalitogenic responses to local CNS inflammation, such as in multiple sclerosis.

Thus, the stimulation or inhibition of cytolytic or regulatory arms through the release of chemokines or cytokines by atrocytes may result in markedly different outcomes that modulate immune responses in the brain. Targeting cytokines/chemokines or their receptors can alter the course of several neurological diseases, and the effects may be beneficial or harmful, depending on the particular situation (Steinman, 2013). Therefore, the study of the mechanisms underlying T-cell–astrocyte communication will be important for the design of therapeutic strategies for neurodegenerative disorders and brain tumors that show T-cell infiltration.

CONCLUDING REMARKS

Little is known about intercellular communication in immune-mediated responses in the brain. Specifically, the role of astrocytes in adaptive and innate responses has scarcely been explored. Evidence suggests that protoplasmic astrocytes may have important functions in the regulation of immune responses in the brain, particularly regarding the infiltration of lymphocytes and their subsequent interactions with APCs once inside the brain parenchyma.

Many brain disorders, including brain cancer, involve lymphocyte infiltration and astrocyte reactivity; nevertheless, the interactions between these cell types have been largely overlooked. This large area of research remains to be explored and will produce extremely valuable information for treating CNS diseases. Astrocyte–lymphocyte communication could be targeted for therapeutic purposes in the near future.

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Studying subcellular detail in fixed astrocytes: dissociation of morphologically intact glial cells (DIMIGs)

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Studying the distribution of astrocytic antigens is particularly hard when they are localized in their fine, peripheral astrocyte processes (PAPs), since these processes often have a diameter comparable to vesicles and small organelles. The most appropriate technique is immunoelectron microscopy, which is, however, a time-consuming procedure. Even in high resolution light microscopy, antigen localization is difficult to detect due to the small dimensions of these processes, and overlay from antigen in surrounding non-glial cells. Yet, PAPs frequently display antigens related to motility and glia-synaptic interaction. Here, we describe the dissociation of morphologically intact glial cells (DIMIGs), permitting unambiguous antigen localization using epifluorescence microscopy. Astrocytes are dissociated from juvenile (p13–15) mouse cortex by applying papain treatment and cytospin centrifugation to attach the cells to a slide. The cells and their complete processes including the PAPs is thus projected in 2D. The entire procedure takes 2.5–3 h. We show by morphometry that the diameter of DIMIGs, including the PAPs is similar to that of astrocytes *in situ*. In contrast to cell culture, results derived from this procedure allow for direct conclusions relating to (1) the presence of an antigen in cortical astrocytes, (2) subcellular antigen distribution, in particular when localized in the PAPs. The detailed resolution is shown in an exemplary study of the organization of the astrocytic cytoskeleton components actin, ezrin, tubulin, and GFAP. The distribution of connexin 43 in relation to a single astrocyte's process tree is also investigated.

Keywords: actin, tubulin, ezrin, connexin 43, gap junction, synapse

INTRODUCTION

The role of astrocytes for CNS synaptic transmission, including their own active signaling has become increasingly clear over the past years (Haydon and Carmignoto, 2006). Astrocytes are also key players in the coupling of metabolism and blood flow to neuronal activity (Carmignoto and Gómez-Gonzalo, 2010; Giaume et al., 2010). In addition to their soma and GFAP-positive main processes, astrocytes—in particular in gray matter—display a high amount of extremely fine processes. The astrocyte approaches the synaptic cleft, blood capillaries, the surface of neuronal somata and neuronal compartments (Reichenbach et al., 2004) mostly through these processes. These processes have been termed peripheral astrocyte processes (PAPs; Derouiche et al., 2002), and have been shown to preferentially display proteins and organelles involved in mediating glial interactions with neurons or vessels (e.g., Danbolt et al., 1998; Derouiche and Frotscher, 2001; Rouach et al., 2008). The PAPs, although emanating from the main processes, are best regarded as sponge-like structures rather than highly branched processes, based on electron microscopic 3D reconstructions (Ventura and Harris, 1999; Witcher et al., 2007). Frequently they fully engulf or embed neuronal structures, insulating them, and creating a micro-environment (Reichenbach et al., 2010).

Light microscopical analysis of glial cells, in particular of astroglial cells has lead to misinterpretations since the beginnings of glial research. Although stained PAPs can be detected by the light microscope they cannot be resolved from small neuronal structures such as spine necks, subplasmalemmal staining in dendrites, or the extracellular matrix. At the ultrastructural level, glial antigens localized preferentially within PAPs are reliably detected based on established morphological criteria (Peters et al., 1991). However, immunoelectron microscopy is a time-consuming and complex procedure.

The present paper suggests a technique facilitating the study of these processes, in particular antigen localization, by light microscopic immunocytochemistry in astrocytes. We describe a cell dissociation procedure, which preserves the structural integrity of cortical astrocytes, including their PAPs, to a very high degree. Acutely isolated astrocytes are mainly prepared for electrophysiological analysis, sometimes combined with molecular biology (Steinhäuser et al., 1994; Seifert and Steinhäuser, 1995; Schools and Kimelberg, 2001), which requires of course live cells. Phase contrast microscopy of live cells or *post-hoc*

fixation and staining shows that those isolated astrocytes have collapsed or curled-back main processes, and reduced PAPs. Lopez et al. (1997), applying a proprietary enzyme for dissociation, focussed on structural preservation of fixed astrocytes and Müller cells, and obtained remarkable results as shown by the presence of many elongated main processes. These preparations are still not suitable to immunocytochemically investigate PAPs, which are often only 50–200 nm wide. The dissociation procedure described here allows for subcellular antigen localization even within the thinnest processes of glial cells. At the same time, many of the astrocytes dissociated and observed without surrounding cells still display their complex, elaborated morphology, which has prompted us to term both the procedure and astrocytes obtained Dissociation of/dissociated morphologically intact glial cells (DIMIGs).

MATERIALS AND METHODS

Animal handling and sacrifice was carried out so as to minimize suffering, in accordance with animal welfare legislation. The optimized dissociation procedure consists of eight main steps (**Table 1**); for details and comments see next section.

Table 1 | Principal steps of the dissociation of morphologically intact glial cells (DIMIG).

1. Cortex dissection (p13–p15)
↓
2. Cortex slicing
↓
3. Pre-incubation of cortical tissue
↓
4. Enzymatic dissociation with papain
↓
5. Mechanical dissociation with pipettes
↓
6. One-step density gradient centrifugation
↓
7. Cytospin centrifugation
↓
8. Fixation of cells on slide

CYTOCHEMISTRY

After washing with PB, fixed dissociated cells were incubated sequentially with Triton X-100 (0.2% in PB, 2 min), normal serum (10% in PB, 30 min), primary antibodies (overnight), and secondary antibodies (1 h). Antibodies and concentrations are listed in **Table 2**. For nucleus localization, cells were incubated with bisbenzimidine (Sigma-Aldrich, Deisenhofen, Germany; 1:200,000, 30 min). Specimens were documented using a fluorescence microscope (Zeiss Cell Observer, Jena, Deutschland).

MORPHOMETRY AND STATISTICAL ANALYSIS

Morphometric parameters of astrocytes were analyzed using the image analysis software ImageJ (Rasband, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011). The following morphometric parameters were determined from individual astrocytes double-stained for GFAP (main processes) and ezrin (PAPs): astrocytic diameter (based on their longest main processes, GFAP-IR positive), length of the peripheral astrocytic processes (ezrin-IR positive), and the area fraction of PAPs in relation to the overall astrocytic area. To determine the diameter of astrocytes, the distance between the most distal tips of the two longest opposing main processes was measured. The mean length of the peripheral astrocytic processes was determined by measuring the distance between the point of origin of the PAPs from the main process, and their distal tips. To account for the variability in PAP length, five PAPs emanating from different main processes of each astrocyte were measured. To obtain the area fraction of PAPs in relation to the overall astrocyte surface, the area positive for GFAP was segmented, measured, and substracted from the area occupied by either one or both of the GFAP and ezrin labelings. Statistical analysis was performed using Excel (Microsoft, Redmond, WA, USA) and SPSS 13 (SPSS Inc., Chicago, IL, USA).

HIGH RESOLUTION MICROSCOPY

For immunofluorescence, sections were photodocumented using a 63 × 1.4 lens. To increase resolution, image stacks at 50–100 nm steps were recorded (Zeiss 200 M microscope), and deconvolved using calculated PSF and iterative deconvolution

Table 2 | Summary of primary and secondary antibody combinations, and affinity labels and concentrations used.

Primary antibody (concentration)	Supplier/source	Secondary antibody (concentration)
ck anti-GFAP (1:500)	Chemicon/Millipore, Billerica, USA	AMCA-coupled to dk anti-ck (1:100)
ms anti-GFAP-CY3 (1:2000)	Sigma-Aldrich, Deisenhofen, Germany	
shp anti-GFP (1:4000)	Serotec, Düsseldorf, Germany	DyLight488-coupled to dk anti-shp (1:100)
ms anti-ezrin (1:500)	Sigma-Aldrich, Deisenhofen, Germany	CY3-coupled to dk anti-ms (1:1000)
rb anti-ezrin (1:1000)	Upstate/Millipore, Billerica, USA	CY3-coupled to dk anti-rb (1:1000)
ms anti-α-tubulin (1:500)	Sigma-Aldrich, Deisenhofen, Germany	CY3-coupled to dk anti-ms (1:1000)
ms anti-ß-actin (1:500)	Sigma-Aldrich, Deisenhofen, Germany	CY3-coupled to dk anti-ms (1:1000)
rb anti-connexin 43	Sigma-Aldrich, Deisenhofen, Germany	CY3-coupled to goat anti-rb (1:1000)
Phalloidin-Oregon Green 488 (5 units/ml)	Molecular Probes/Invitrogen	

ck, chicken; dk, donkey; shp, sheep; ms, mouse; rb, rabbit.

(Volocity Software, Perkin Elmer, Waltham, MA, USA). For colocalization studies, correction for residual chromatic aberration was applied in image stack construction (Anlauf and Derouiche, 2009).

RESULTS

DISSOCIATION PROCEDURE

The dissociation method developed here is based on previous cell isolation protocols (e.g., Steinhäuser et al., 1994; Kimelberg et al., 2000; Lu et al., 2006; Lovatt et al., 2007; Worthington Biochemical Corp., Lakewood, NJ, USA). The aim of the study was to optimize these protocols with respect to morphological preservation of the very thin glial processes.

Juvenile mice (age: postnatal day 13–15) were deeply anesthetized with isoflurane and sacrificed by decapitation; the brain was immediately removed. While floating in ice-cold sucrose-based aCSF (2.5 mM KCl, 25 mM $NaHCO_3$, 87 mM NaCl, 1.25 mM NaH_2PO_4, 25 mM D-glucose, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 75 mM sucrose; 330 mOsm; pH 7.4; pre-equilibrated with carbogen), the brain was carefully freed from meninges to reduce contaminating blood cells, and the cortices were extracted (**Table 1**, step 1). Cortical slices (300 µm) were cut in ice-cold sucrose-based aCSF (pH 7.4; 330 mOsm; pre-equilibrated with carbogen) using a vibratome (Leica VT1200S, Wetzlar, Deutschland; **Table 1**, step 2). Next, the slices were pre-incubated in aCSF for 30 min at 35°C, and subsequently in Ca^{2+}/Mg^{2+}-free EBSS (Sigma-Aldrich, Deisenhofen, Germany; equilibrated with carbogen) for 15 min at 22°C (**Table 1**, step 3). Ca^{2+}/Mg^{2+}-free EBSS is essential for preservation of cell morphology. It is used to loosen the extracellular matrix prior to enzymatic dissociation (Seifert and Steinhäuser, 1995), probably by reducing the action of the Ca^{2+}-dependent cadherins present in brain tissue. To degrade the extracellular matrix completely, the slices were incubated in a carbogenated papain solution [20 units/ml papain, 1 mM L-cysteine, 0.5 mM ethylenediaminetetraacetate (EDTA) in Ca^{2+}/Mg^{2+}-containing EBSS; Worthington Biochemical Corp., Lakewood, NJ, USA] for 15 min at 37°C (**Table 1**, step 4). During this step, Ca^{2+}/Mg^{2+}-containing EBSS was used since the cysteine protease papain is Ca^{2+}-dependent. After enzymatic treatment, the digested tissue was mechanically dissociated by gentle trituration (10 times), using a 10 ml pipette as used for cell culture media (Corning Inc., Corning, NY, USA). The resulting suspension was centrifuged for 6 min at 280 g, at 22°C (**Table 1**, step 5). Subsequently, the enzymatic reaction was stopped and extracellular DNA digested by resuspending the pellet in inhibitor solution (1 mg/ml ovomucoid, 1 mg/ml BSA, 0.0005% DNase I in Ca^{2+}/Mg^{2+}-containing EBSS; Worthington Biochemical Corp., Lakewood, NJ, USA). To remove debris and dead cells, a one-step density gradient centrifugation (70 g, 6 min, 22°C) was performed (**Table 1**, step 6). Five ml of concentrated inhibitor solution (10 mg/ml ovomucoid, 10 mg/ml BSA in Ca^{2+}/Mg^{2+}-containing EBSS) in a 15 ml plastic tube were covered by the cell suspension. After centrifugation, the supernatant was discarded, and the cells were resuspended (5–10 times trituration using a 1 ml disposable plastic micropipette tip) in 2 ml of Ca^{2+}/Mg^{2+}-containing EBSS (Worthington Biochemical Corp., Lakewood, NJ, USA) plus 1% BSA. To obtain a desired degree of cell separation on the slide, the resulting solution was further diluted to varying degrees (mostly 1:64). Finally, the cells were attached to positively charged slides (SuperFrost Plus, Thermo Fisher Scientific Inc., Waltham, MA, USA) using cytospin centrifugation (Shandon Cytospin 4 Cytocentrifuge, Thermo Fisher Scientific Inc., Waltham, MA, USA; 800 rpm, 10 min, 22°C) (**Table 1**, step 7) and immediately afterwards fixed with 4% paraformaldehyde in PB for 10 min at 22°C (**Table 1**, step 8). In order to adjust the desired cell-to-cell distance on the slide, the resuspension solution was appropriately diluted before cytospin centrifugation, with Ca^{2+}/Mg^{2+}-containing EBSS. After fixation, the slides were washed with PB and either immunostained or cryoprotected with increasing concentrations of sucrose solution (10, 20, and 30% sucrose in PB, 10 min each), snap-frozen and stored at −80°C.

The astrocytic fraction of the dissociated cells was not systematically measured. **Figure 1** shows a representative image of dissociated GFAP-IR positive astrocytes that are attached to a slide by cytospin centrifugation. A large fraction of the dissociated astrocytes appears morphologically intact while others do not satisfy the criteria of good morphology.

MORPHOMETRIC ANALYSIS OF DISSOCIATED ASTROCYTES

The dissociated astrocytes frequently retain an extensive arborization of processes. Even the very thin PAPs, which are ezrin

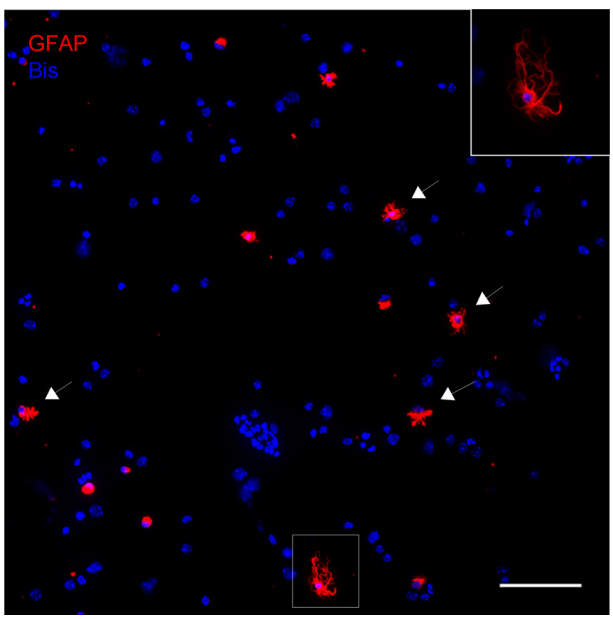

FIGURE 1 | Dissociated cells attached to a slide by cytospin centrifugation. Astroctes are stained with anti-GFAP (red) and nuclei are stained with bisbenzimide (blue). Note that the majority of the cells are not GFAP positive, including any kind of neural cells or cells present in blood and vasculature. As an indication, nuclear size and morphology are highly variable. Astrocytes can be easily identified, many of them have well-preserved main processes (arrows). Inset: Higher magnification of astrocyte in boxed area. Scale bar: 100 µm.

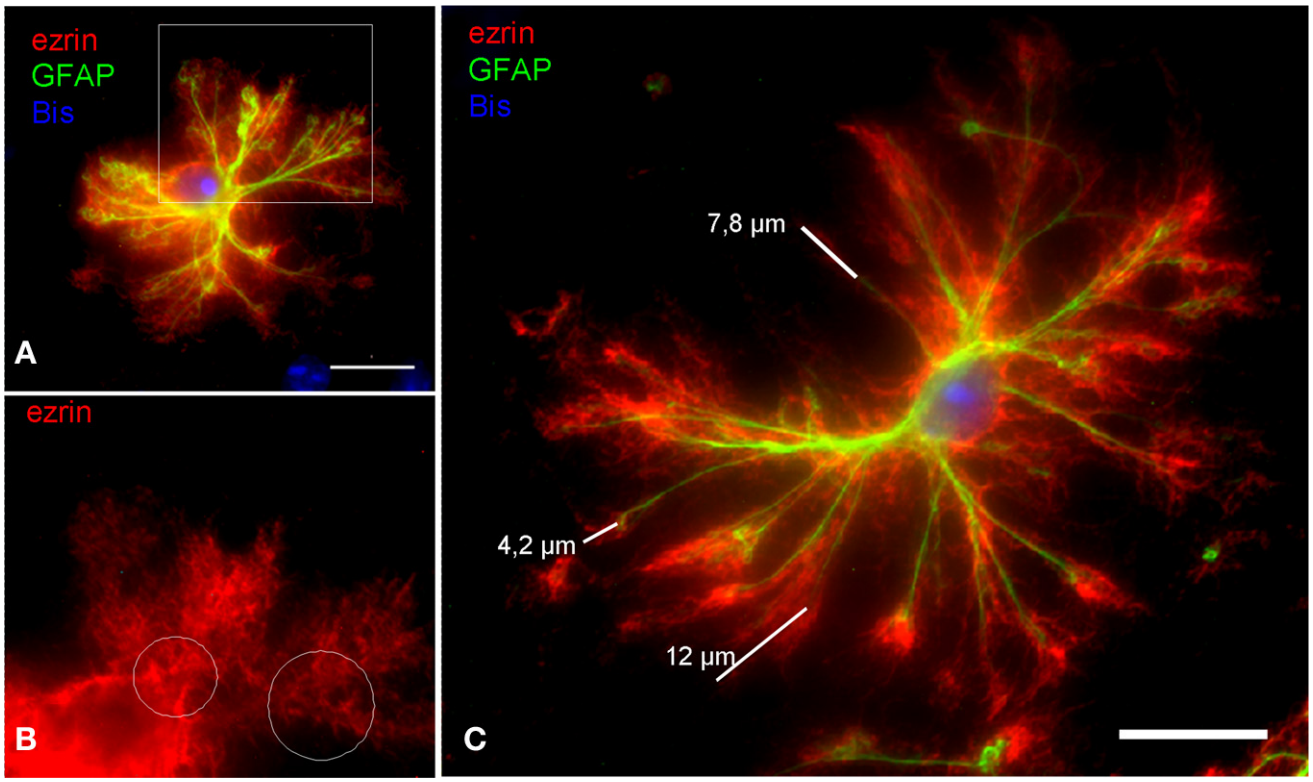

FIGURE 2 | In DIMIG astrocytes, the extensive astrocyte arborization is preserved in fine detail. (A) Ezrin-IR positive PAPs (red) cover the entire astrocytic surface. **(B)** It is evident from a magnification of the boxed area in **(A)** that ezrin (red) mainly localizes to the PAPs. White circles indicate alleys poor in ezrin, which are occupied by the GFAP positive glial filament bundles of the main processes. This further illustrates that ezrin is a preferential PAP marker. **(C)** The ezrin positive PAPs (red) increase the stretch of the GFAP positive main processes (green) by as far as 12 µm, by a mean distance, however, of 6,64 µm. Scale bars: 15 µm.

positive are preserved in fine detail (**Figure 2**). By applying morphometry we wanted to check whether the dissociation procedure yields morphologically fully intact astrocytes, or whether their processes tree is reduced in relation to astrocytes *in situ*. There are several *in situ* quantitations encompassing PAPs (e.g., Ventura and Harris, 1999; Witcher et al., 2007; for review see Reichenbach et al., 2010). We chose, however, to compare our findings to those of Chao et al. (2002), one of the very few reports selectively quantitating PAPs, i.e., in relation to glial main processes [for review see Wolff and Chao (2004)]. Chao et al. (2002) established their morphometric parameters in astrocytes *in situ* by analyzing specimens from rat cortex with electron microscopy.

The mean diameter of measured, dissociated astrocytes was 74.88 µm (SD = 27.01 µm; max = 129.96 µm; min = 35.33 µm; $n = 20$) and the reach of the main astrocytic processes was extended by PAPs on average by 4.65 µm (SD = 1.09 µm; max. = 6.64 µm; min. = 3.02 µm; $n = 100$). The corresponding diameter of rat astrocytes *in situ*, based on the main processes is 78 µm and the reach of the main processes via its PAPs is 4.85 µm (Chao et al., 2002). These values are comparable to the present measurements. We conclude that the dissociated astrocytes are preserved in nearly full detail.

THE ASTROCYTIC CYTOSKELETON IN PAPs

The cytoskeleton composition of astrocytic processes, especially that of PAPs, is not well-studied and controversially discussed in the literature (Safavi-Abbasi et al., 2001; Hatton, 2002; Potokar et al., 2007; Peng et al., 2008; Kreft et al., 2009; Ou et al., 2009). To our best knowledge, this is the first time that cytoskeletal components are unambiguously localized in the different compartments of *in situ* like astrocytes. As shown in triple stainings the cytoskeleton of main astrocytic processes is composed of microtubules (anti-a-tubulin), intermediate (glial) filaments (anti-GFAP), and microfilaments (phalloidin, anti-actin; **Figures 3**, **4**). PAPs almost exclusively and abundantly contain microfilaments (**Figures 3**, **4**) with some microtubules extending into only their proximal parts (**Figure 3**).

GAP JUNCTIONS IN PAPs

Cx43, the major astrocytic connexin, can form gap junctions and constitutes the molecular basis for interastrocytic coupling and for glial networks (Giaume et al., 2010). However, it is not clear how astroglial gap junctions are distributed within the individual astrocyte territory. Based on stainings in brain sections it cannot be concluded whether gap junctions are established only at the boundaries of the territory where the astrocyte contacts

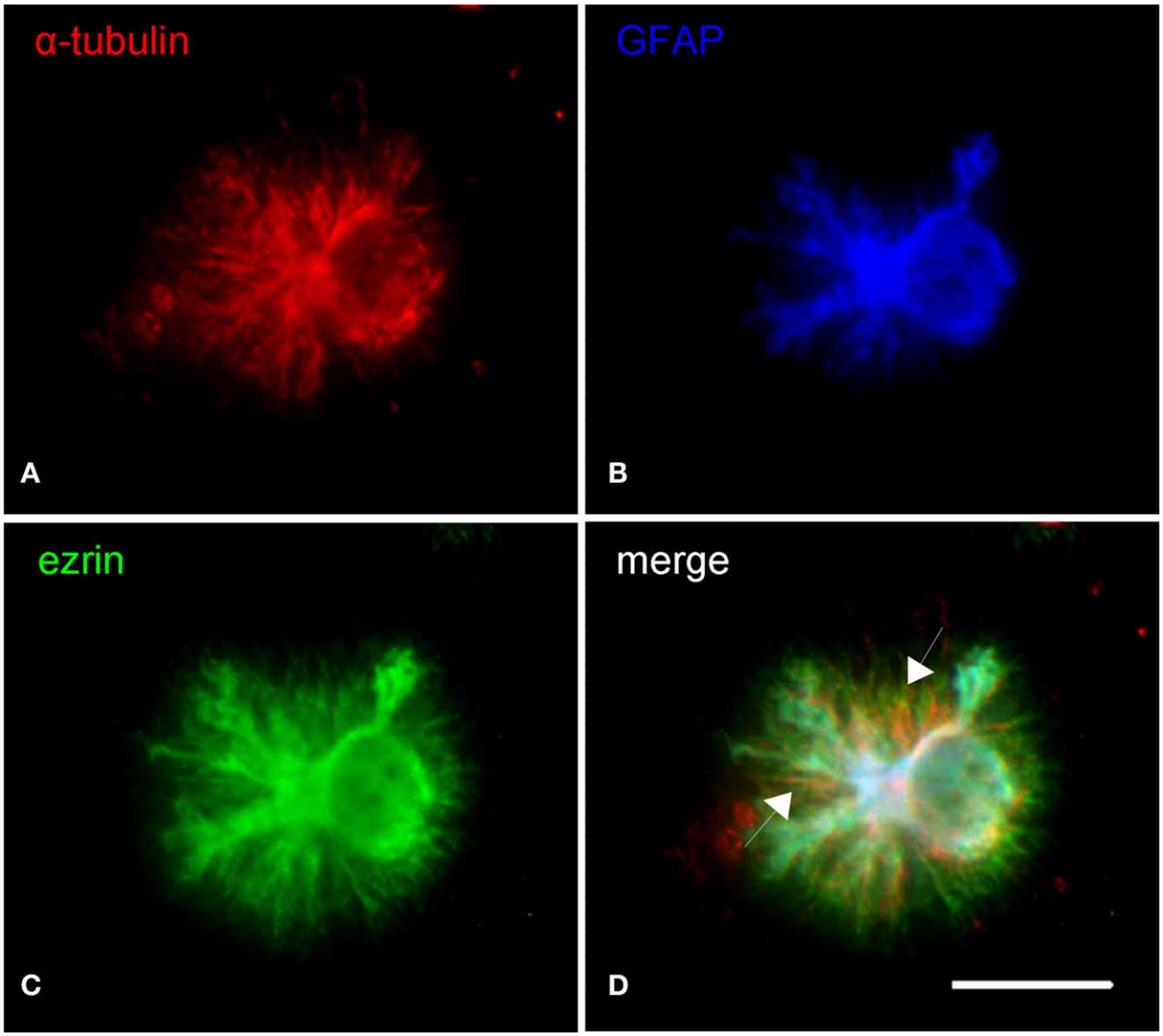

FIGURE 3 | Subcellular distribution of microtubules (α-tubulin, red) in relation to astrocytic main processes (GFAP, blue) and PAPs (ezrin, red). (A) Microtubules mainly localize to the soma and main processes of astrocytes. A minor proportion of microtubules extends beyond the tips of the main processes **(B)**. Overlaying the channels illustrates that some microtubules even stretch to within the PAPs **(C**, arrows in **D)**. Scale bar: 15 μm.

its neighbors. We thus studied the distribution of connexins in the individual astrocyte by immunostaining of DIMIGs. Cx43 was localized preferentially to the PAPs but only to a minor degree at the main processes (**Figure 5**), in the punctate manner known from tissue sections. Importantly, the Cx43 positive puncta were evenly distributed all over the astrocyte, including its more central, perinuclear portions.

DISCUSSION

DISSOCIATION OF MORPHOLOGICALLY INTACT GLIAL CELLS

We describe here a protocol optimized for the acute dissociation of morphologically well-preserved cortical astrocytes from juvenile (p13–15) mouse brain. Several parameters like (1) incubation time and (2) temperature, (3) ionic composition and (4) osmolarity of media, (5) the way of mechanical dissociation and (6) centrifugation had to be optimized. In particular, tight control of parameters (1)–(6) appears essential for obtaining a relatively high yield of sufficiently well-preserved astrocytes suitable for subcellular antigen localization. The proportion of well-preserved cells varies from slide to slide, and is roughly estimated 10–20% (cf. **Figure 1**) depending on which degree of morphology is considered acceptable for immunocytochemistry. Amongst the criteria for good, acceptable morphology are the presence of a few or several long main processes, which are also non-collapsed. In addition, PAPs are a clear indication of intact morphology, in particular PAPs extending from the distal parts of main processes,

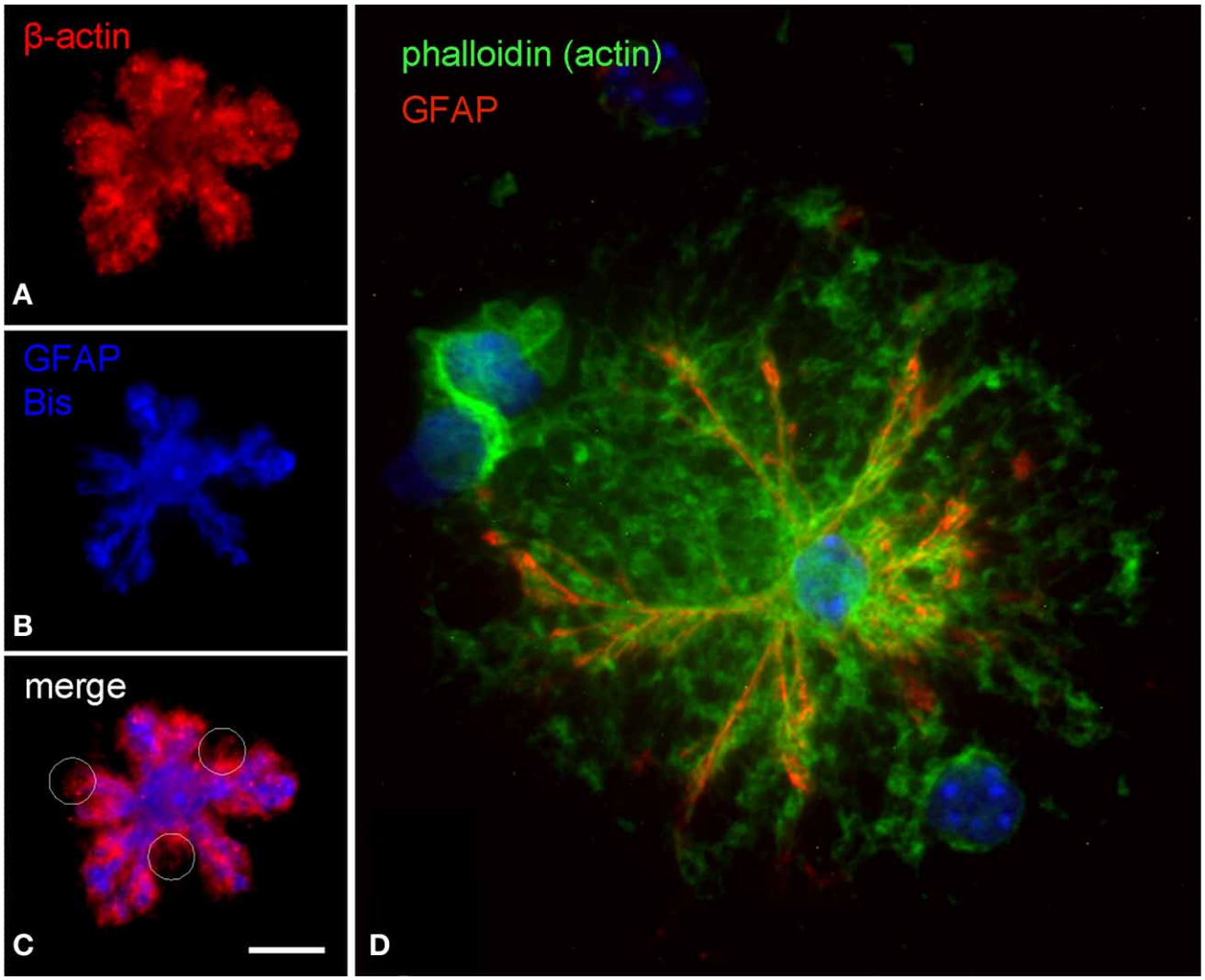

FIGURE 4 | Subcellular distribution of actin filaments in DIMIG astrocytes. Actin was labeled by anti-actin (red in **A,C**) or by phalloidin coupled to Oregon-green **(D)**. Astrocytes display a dense meshwork of actin filaments, which distributes evenly all over the cell **(A,C,D)**. The actin meshwork clearly extends beyond the main processes (GFAP) and is strongly labeled also in the PAPs **(C,D)**. Scale bars: 10 μm **(A–C)**, 15 μm **(D)**.

where they would be assumed to be torn first. When appreciating the shape of well-preserved astrocytes, one has to bear in mind that cells from all cortical layers are expected including various sizes of gray matter astrocytes, and fibrous white matter astrocytes.

A routine method in medical cytology, the cytospin centrifuge (Shandon Cytospin 4 Cytocentrifuge, Thermo Fisher Scientific Inc., Waltham, MA, USA) is definitely a key feature of the DIMIG—preparation. It greatly contributes to the outstretching of the astrocytes with their main and peripheral processes intact. This is a prerequisite for the visualization of the PAPs (**Figure 2**), which would otherwise be collapsed together with the main processes. Importantly, the DIMIG—procedure described here yields many other cell types present in live brain tissue, including those of blood and vessel wall, which are spun down together with astrocytes (see **Figure 1**). Although this remains to be demonstrated, we expect that structurally well-preserved cells of all other glial types can be found as well, since with this method antigens have been localized even in the fine and long processes of NG2 cells (Haberlandt et al., 2011). There is, thus, no isolation, purification, or enrichment of astrocytes, and consequently astrocyte-directed biochemistry or molecular biology cannot be performed.

DIMIG astrocytes appear as a valuable tool, since they combine several major advantages. The astrocytes are preserved in nearly full detail, as suggested by qualitative assessment of their main and peripheral processes (e.g., **Figure 2**), and morphometric validation (see below). Although DIMIG astrocytes frequently display long PAPs extending even from the distal end of the main processes (see **Figure 2**), it would not be realistic to assume that these astrocytes are 100% spared by the DIMIG procedure. They should not be taken as a 1:1 morphological

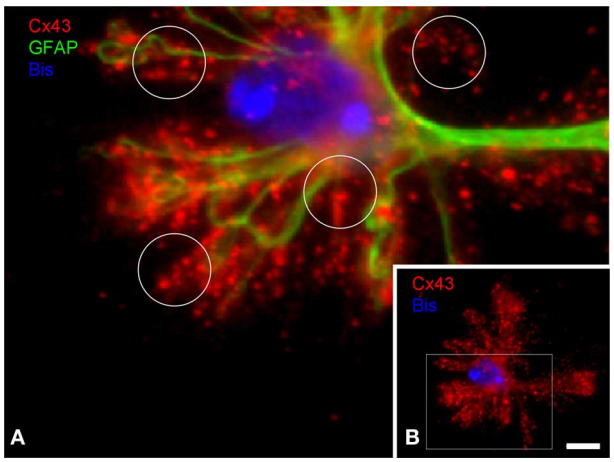

FIGURE 5 | Subcellular distribution of connexin 43 (C×43) in DIMIG astrocytes. (A) Higher magnification of boxed area in **(B)**. C×43 is present in the perinuclear region (bisbenzimidine, blue for nucleus), as well as in the GFAP positive main processes and the PAPs (circles in **A**). C×43 distributes evenly to the three compartments soma, main processes, and PAPs **(B)**. Scale bar: 10 μm.

representation of astrocytes *in situ*. The morphology of DIMIG astrocytes can be astonishing, but they should also not be used for morphometric characterization of *in situ* astrocytes, in particular since the dissociated cells are projected onto one plane. No three dimensional information can be obtained from DIMIG prepared astrocytes. Using DIMIGs, glial process architecture may only be analyzed with a limited set of parameters, such as process length, number, and distance of branching points. More detailed description encompassing e.g., process angles, relative density of PAPs, and volumenometric parameters clearly will have to be based on astrocytes *in situ*. Similarly, the glial relation to specific neighboring structures, such as synapses, neuronal compartments, and blood vessels cannot be analyzed in DIMIGs, since disconnecting them is the primary aim of dissociation.

An advantage of dissociation, however, this procedure leads to very high signal-to-noise ratio after labeling. Prior to cytospin centrifugation, the cell suspension is diluted to yield clearly separated cells, without surrounding cellular structures, or other background, which further aids unequivocal antigen detection, and subcellular colocalization studies in subresolution structures such as PAPs. Subcellular (co)localization has been carried out in primary culture, where vesicular antigens can be localized at the single orgenelle level (Coco et al., 2003; Anlauf and Derouiche, 2005) or in membrane sheets (Lang et al., 2002). These labeling and microscopy techniques, unsuitable for the tissue section, can now be applied to DIMIGs, an astrocyte preparation much closer reflecting the *in vivo* situation than primary astrocytes.

As another advantage over studying tissue sections by light or electron microscopy, DIMIGs also permit regarding the individual astroglial cell as a unity of observation. To study differential antigen localization in astrocytic compartments—soma, main processes, PAPs—the method of choice has been electron microscopic examination of astrocytes *in situ*, which is very laborious and time-consuming. The method described here greatly facilitates investigations on antigen distribution in relation to the individual astrocyte as a whole cell. Even using electron microscopy, this is possible only by statistical conclusion and within demanding projects (Wolff and Chao, 2004). For example, in DIMIGs, main and peripheral processes can easily be segmented by using multiple labeling and image processing, thus enabling object or compartment-oriented morphometry and densitometry (**Figure 2**).

Based on the maturation of the extracellular matrix and cell shapes, the structural preservation after dissociation highly depends on the animal age and to a lesser extent on the CNS region. Enzymatic digestion times and further incubation parameters have to be optimized for each postnatal age and region (cf. Kaneda et al., 1988; Lopez et al., 1997), implying that the protocol presented here specifically relates to dissociation of p13–15 mouse cortex. Within this age range, no obvious, systematic variability was observed. Although the animal age at which the cells are prepared (p13–15) is not fully adult, it can be classified as juvenile. Like for many electro-physiological investigations on astrocytes, this age has been chosen as a compromise between CNS maturation and cell viability; the resulting morphology can thus be compared to extant physiological data (Verkhratsky and Steinhäuser, 2000). The protocol given has been systematically developed by optimizing the parameters and checking for consistency. We found that deviating from the protocol substantially decreases the quality of the dissociated cells. In particular, severe cell swelling or cell lysis were found to result from variations in osmolarity, which needs to be tightly checked. For the trituration step, the amount of pipetting and the pipette have to be standardized. This step is an important variable in the actual process of cell separation, an optimum between minimizing both, the amount of persisting cell clusters and cell damage. Temperature control is a less rigid parameter, which further increases the quality of both cell separation and cell integrity.

By applying the DIMIG procedure described here, morphologically intact astrocytes can be obtained within less than 3 h, from anesthetizing the animal until fixation of the cytospinned cells. It is assumed that major changes in protein expression levels, antigen localization, or organelle distribution are unlikely to occur within this time period. For example, it is hard to conceive a short-term redistribution of gap junctions at the scale of a cell's territory. Other, more rapid processes such as membrane internalization, vesicle fusion, or rapid process motility (Cornell-Bell et al., 1990; Lavialle et al., 2011) might induce differences with regard to *in vivo* astrocytes. However, the DIMIG astrocytes are considered to be *in situ* like and to represent the morphology as well as the immunocytochemical phenotype of astrocytes *in situ*. As seen even at low magnification the dissociated astrocytes display many different shapes. Some GFAP positive astrocytes appear round or clumped (see **Figure 1**), with even the main processes collapsed or torn-off, obvious signs of compromised structural preservation. Also those astrocytes classified structurally well-preserved are morphologically heterogeneous.

Considering that cortex was dissociated with all regions and layers including white matter, this may reflect the spectrum of astrocytic morphologies observed in the cortex *in situ*. Thus, GFAP positive DIMIGs were reminiscent of the classical, star-shaped astrocyte, but also of astrocytes in white matter, the multiform or molecular layers, or those apposed to blood vessels, which all differ in size, process pattern, and/or territorial shape. As a logical consequence of the dissociation method, the putative laminar origin of the individual DIMIG astrocyte cannot be verified.

Previous dissociation and isolation procedures for astrocytes mainly focus on purity and viability rather than morphology of cells to enable biochemical, single-cell RT-PCR, and electrophysiological experiments (Steinhäuser et al., 1994; Seifert and Steinhäuser, 1995; Schools and Kimelberg, 2001). These previously described astrocyte dissociations are based on the method described by Kimelberg et al. (2000). It is not optimized for morphological integrity, since shearing-off of processes at 15 days postnatal (in rat) was consistently noted (Kimelberg et al., 2000). In particular, in combination with cytoplasmic GFP fluorescence in GFP-GFAP astrocytes, that method could still be used to verify astrocyte-specific localization of putatively neuronal proteins e.g., synaptotagmin IV (Zhang et al., 2004a) or several other vesicular proteins implicated in astrocytic glutamate release (Zhang et al., 2004b). Working on retinal (Müller) glial cells, Reichenbach et al. (1990) succeeded in preparing dissociated cells with preserved resting membrane potential, by reducing papain digestion to 20 min. Inspite of apparently torn-off lateral processes (corresponding to PAPs), these Müller cells had good ultrastructure. Because of differing solutions and concentrations, the papain digestion parameters of Reichenbach et al. (1990) and ours cannot be directly compared. In agreement with the findings of Reichenbach et al. (1990), we were able to minimize papain digestion time to 15 min, at the same time permitting a sufficient degree of dissociation. To our knowledge the only dissociation method aiming at morphological integrity of astrocytes (and Müller cells) is that described by Lopez et al. (1997). Unfortunately, their enzyme used for dissociation has not been available, and the main focus of that study was the preservation of glial main processes. Primary astrocyte culture, a standard model in glial research, serves as a good tool to obtain first hints concerning astrocytes *in situ*, but may display marked differences in relation to them. For example, cultured astrocytes abound with organelles positive for the vesicular glutamate transporters 1 and 2 (Anlauf and Derouiche, 2005), whereas they are hard to detect and have long been overlooked in astrocytes *in situ* (Bergersen and Gundersen, 2009).

CYTOSKELETON AND CONNEXIN 43 IN PAPs

The GFAP positive processes of DIMIGs also contain microtubules and actin microfilaments, which was to be expected. The PAPs are particularly rich in microfilaments, which is also seen in the filopodia of cultured astrocytes (Lavialle et al., 2011). Only occasionally, PAPs have microtubules, which when present reach only into their proximal parts but not into their tips. Such compartment specific findings, again, would be hard to assess using electron microscopy, which has much higher resolution but a more limited scope of observation relating to the individual cell. Reflecting the multiple functions of PAPs in glia-neuronal and glia-vascular communication, many of the corresponding proteins are preferentially targeted to the PAP, although the underlying mechanisms are unknown. With the presently observed high content in actin and the occasional presence of microtubules in PAPs, it might be speculated that transport organelles or protein complexes move along the microtubule to at least the base of the PAP, where the cargo is transferred to actin-based transport mechanisms. Ezrin, an ERM protein first associated with tethering the cell membrane to the actin cytoskeleton (Tsukita et al., 1997; Gautreau et al., 2002), and known to be present in PAPs (Derouiche and Frotscher, 2001), might also contribute in this process.

Orthogonal arrays of particles (OAPs) in glial cells, which are known to correspond to connexins, disappear after long papain treatment but remain in a localization comparable to *in situ* specimens after mild papain treatment (Reichenbach et al., 1990). The minimized papain digestion applied in the DIMIG procedure, and the finding of the well-known punctate pattern in Cx43 immunostained DIMIGs strongly indicate that the subcellular connexin localization is maintained after dissociation. Cx43 immunostaining in DIMIGs displays the well-known punctate pattern. It is interesting to note, however, that the puncta do not directly delineate the main processes of the individual astrocyte but rather form "clouds" of puncta, representing gap junctions established by the PAPs. It has been calculated from ultrastructural material that an average (rat cortical) astrocyte carries 30,000 gap junctions, and that 4000 of them are engaged in intra-astrocytic coupling, i.e., autocoupling (Wolff and Chao, 2004), a phenomenon also seen in primary astrocyte culture (Wolff et al., 1998). The present DIMIG preparation would be the first way to visualize the great extent of autocellular coupling, since CX43 positive puncta are dense also in the more central and perinuclear part of the territory.

In summary, the DIMIG procedure described here combines the microscopic advantages of primary astrocytes (high resolution, very high signal-to-noise ratio) with the properties of *in situ* like dissociated astrocytes. It can be used to overcome several disadvantages of the tissue section, primary astrocyte culture, and previously described dissociation procedures. Various antigens can be unambiguously localized, even within the thinnest, subresolution processes of astrocytes by applying conventional light microscopy. Maybe this preparation can be further exploited and developed at the stage prior to fixation to study, in viable astrocytes, the physiology of processes.

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How do astrocytes shape synaptic transmission? Insights from electrophysiology

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A major breakthrough in neuroscience has been the realization in the last decades that the dogmatic view of astroglial cells as being merely fostering and buffering elements of the nervous system is simplistic. A wealth of investigations now shows that astrocytes actually participate in the control of synaptic transmission in an active manner. This was first hinted by the intimate contacts glial processes make with neurons, particularly at the synaptic level, and evidenced using electrophysiological and calcium imaging techniques. Calcium imaging has provided critical evidence demonstrating that astrocytic regulation of synaptic efficacy is not a passive phenomenon. However, given that cellular activation is not only represented by calcium signaling, it is also crucial to assess concomitant mechanisms. We and others have used electrophysiological techniques to simultaneously record neuronal and astrocytic activity, thus enabling the study of multiple ionic currents and in depth investigation of neuro-glial dialogues. In the current review, we focus on the input such approach has provided in the understanding of astrocyte-neuron interactions underlying control of synaptic efficacy.

Keywords: glia, neurons, neuroglial interactions, synapses, ionic channels, plasticity, dual recordings, electrophysiology

INTRODUCTION

Dynamic bidirectional communication between astrocytes and neurons is now thought to contribute to brain information processing. Indeed, astrocytes are equipped to sense and integrate neuronal information through ionic channels, neurotransmitter receptors and transporters and intracellular signaling pathways. Such machinery is reported to endow astrocytes with the ability to modulate neurotransmission through a variety of mechanisms involving morphological plasticity and uptake or release of numerous neuroactive factors. For instance, astrocytes regulate the formation and stability of synapses, receptor trafficking and the moment-to-moment synaptic activity by releasing various molecules such as proteoglycans, cytokines, energy metabolites or neuromediators, as described in several recent comprehensive reviews (Perea et al., 2009; Giaume et al., 2010; Hamilton and Attwell, 2010; Ben Achour and Pascual, 2012; Nedergaard and Verkhratsky, 2012; Santello and Volterra, 2012). They can also modify the efficacy of synapses by controlling extracellular glutamate concentration via transporters mediating clearance of neurotransmitters (Oliet et al., 2001) or by changing the extracellular space volume (Piet et al., 2004) through a plastic physical coverage of neurons. Although the repertoire of such astroglial regulations of neurotransmission continuously expands, their detailed cellular and molecular mechanisms, as well as their occurrence at certain developmental stages during physiological or pathological conditions remain unclear. Indeed, as neurons and astrocytes use many similar transmitters, receptors and transporters, thereby limiting utilization of a gliotransmission selective pharmacology, the specific contribution of astrocytes to synaptic transmission and plasticity has been difficult to demonstrate directly. Several approaches have been used to tackle the involvement of astrocytes in neuronal activity, ranging from molecular biology, pharmacology, imaging, electrophysiology to behavioral studies. The main difficulties in the field of glia research lie in developing experimental tools allowing to selectively perturb astroglial functions, as well as choosing the best readout to assign specific neuromodulatory functions to astrocytes.

Astrocytes are considered to be electrically non-excitable cells, as they do not fire action potentials. Thus, in the last decades, most of the attention in the glial community has focused on imaging and altering dynamic calcium (Ca^{2+}) signaling of astrocytes, proposed to represent their excitability and to mediate neuromodulatory actions of astrocytes. However, astrocytes are not electrically silent cells, and pioneer *in vivo* intra-glial electrophysiological recordings in the sixties revealed their peculiar cellular dynamic profile (Phillips, 1956; Sugaya et al., 1964; Karahashi and Goldring, 1966; Castellucci and Goldring, 1970; Ransom and Goldring, 1973). Astrocytes indeed exhibit unique biophysical and functional electrical properties, sensitive to neuronal activity and capable of modulating neurotransmission. Thus, electrophysiological recordings of activity-dependent astroglial and neuronal responses have unexpectedly turned out to be a powerful method to unravel online the dynamics of neuroglial ionic signaling. In the current review, we focus on how electrophysiological recordings have provided unique quantitative information about the membrane properties of astrocytes, and the active ionic neuroglial dialog involved in information processing. Limitations

and future directions on the use of such technique in the field of neuroglial research are also discussed.

ASTROCYTIC PROPERTIES DETERMINED BY ELECTROPHYSIOLOGICAL RECORDINGS

BIOPHYSICAL MEMBRANE PROPERTIES OF ASTROCYTES

Astroglial membrane properties in vivo

Mature glia recorded *in vivo* with sharp electrodes, mainly identified as astrocytes in the gray matter (Mishima et al., 2007), display homogeneous, specific and easily identifiable properties: a hyperpolarized resting membrane potential (∼–80 mV) and low input resistance (∼4–20 MΩ) and capacitance (∼10–25 pF) compared to neurons (Amzica and Neckelmann, 1999; Amzica, 2002; Amzica and Massimini, 2002; Amzica et al., 2002; Seigneur et al., 2006; Mishima et al., 2007; Mishima and Hirase, 2010). The astroglial membrane potential is close to the nernstian equilibrium for potassium ions (E_K), thus reflecting the presence of high resting conductances for potassium ions (K^+) (Somjen, 1975). Furthermore, although no action potential or synaptic event can be recorded or induced by depolarizing pulses, astrocytic membranes are animated by very slow fluctuations that are intimately related to changes in neuronal activities. Indeed, due to their strong K^+ conductances, astrocytes are highly sensitive, with a quasi-nernstian relationship, to changes in extracellular K^+ levels associated with neuronal activity (Amzica, 2002; Amzica and Massimini, 2002) (**Figure 3B**).

Such properties are also used to identify mature astrocytes in slices (Zhou, 2005). Classically, membrane resistances of astrocytes is determined by quantifying the current/voltage (IV) relationship, i.e., the current readout in response to voltage incremental impositions or voltage response to current injections through whole cell recording pipettes. Because of the linear, quasi-ohmic profile of the IV curve usually recorded in astrocytes, these cells are conventionally considered as passive (**Figure 1**). This same electrophysiological profile can also be found in juvenile tissues, irrespective of brain structure and animal species (Chvátal et al., 1995; Matthias et al., 2003; Grass et al., 2004; Wallraff et al., 2004; Isokawa and McKhann, 2005; Djukic et al., 2007; Adermark and Lovinger, 2008; Kafitz et al., 2008; Même et al., 2009; Pannasch et al., 2011).

Major resting conductances sustaining hyperpolarized membrane potential

High K^+ permeability of glial cell membranes is a major characteristic discovered in the late sixties by Orkand and Kuffler (Kuffler et al., 1966; Orkand et al., 1966). For decades, the nature of such conductance remained unknown. Major insights in this field have been obtained by pioneer work on retinal Müller cells (Newman and Reichenbach, 1996). Since then, numerous studies based on pharmacological approaches or using transgenic animals have now provided strong evidences that the inward rectifier $K_{ir}4.1$ channel, the main K_{ir} channel expressed in astrocytes (Olsen et al., 2006), is responsible for the main astroglial K^+ conductances. The latter were first observed *ex vivo* (Neusch et al., 2006; Olsen et al., 2006; Djukic et al., 2007; Kucheryavykh et al., 2007; Bay and Butt, 2012) and subsequently confirmed *in vivo* (Chever et al., 2010). Also, two-pore K^+ channels (K2P) participate to astroglial K^+ conductances (Zhou et al., 2009), yet secondarily to $K_{ir}4.1$ channels (Seifert et al., 2009; Zhou et al., 2009).

High membrane permeability for K^+ is tightly linked to the degree of hyperpolarization of mature astrocytes. Indeed during development, hyperpolarization of the astrocytic membrane, progressively approaching a resting potential close to E_K, and the establishment of highly passive conductances occurs in parallel. Such property has been attributed to a progressive increase in $K_{ir}4.1$ channel expression (Bordey and Sontheimer, 1997; Kalsi et al., 2004). Furthermore, specific deletion of $K_{ir}4.1$ or pharmacological blockade of K_{ir} channels leads to a pronounced depolarization of astrocytes (Neusch et al., 2006; Olsen et al., 2006; Djukic et al., 2007; Chever et al., 2010) and to membrane potential fluctuations that fail to reflect changes in extracellular K^+ levels (**Figure 3C**) (Chever et al., 2010), indicating that $K_{ir}4.1$ are responsible for the main resting K^+ conductance of astroglial membranes.

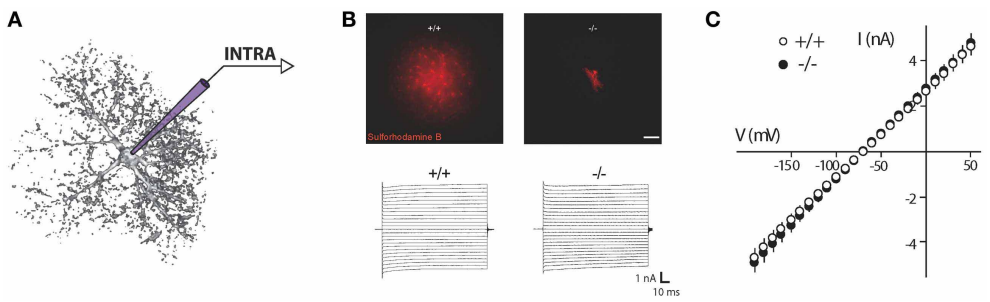

FIGURE 1 | Recording of astroglial membrane properties. (A) Schematic representation of intracellular (whole cell patch clamp recording or sharp electrode) recording of an astrocyte. **(B)** Upper panel: dye coupling experiments show tens of coupled cells after patching of a single astrocyte with an intra-pipette solution containing sulforhodamine-B (red). Knockout mice for astroglial connexins ($Cx30^{-/-}Cx43^{fl/fl}$ hGFAP-cre) exhibit a total absence of astrocytic gap junctional coupling. Lower panel: to determine astroglial membrane resistance in a whole cell patch clamp configuration, short incremental voltage pulses are imposed to the astroglial membrane clamped at −80 mV, and evoked currents are recorded. **(C)** Quantification of the current/voltage relationship (IV curve). Illustration depicting current responses recorded in a hippocampal astrocyte from wild type and astroglial connexins knockout ($Cx30^{-/-}Cx43^{fl/fl}$ hGFAP-cre) animals. Both groups display a quasi-ohmic profile of the IV curve, with a similar slope, indicating a comparable membrane resistance. Adapted, with permission, from Pannasch et al. (2011) **(B,C)**.

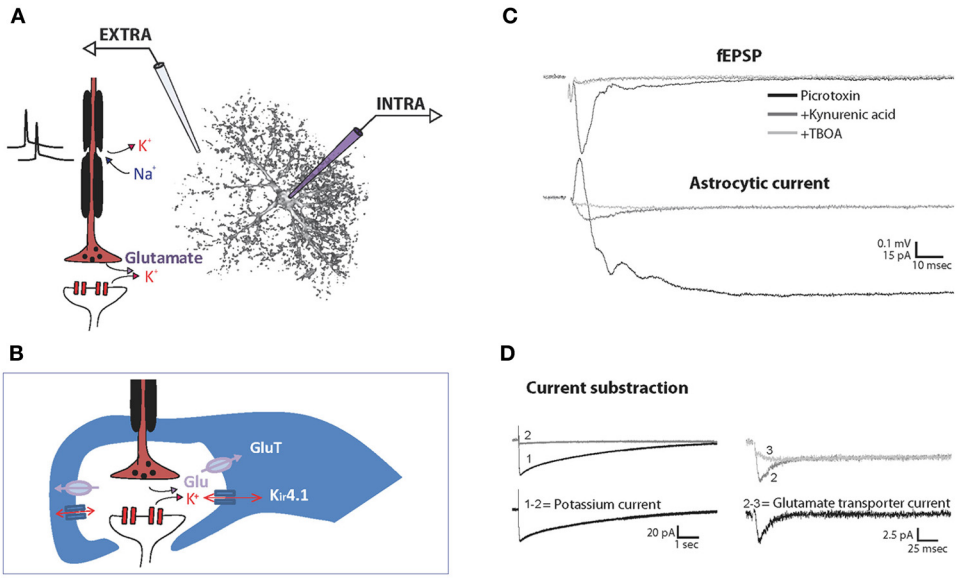

FIGURE 2 | Recording of synaptically evoked GLT and K^+ currents in astrocytes. Schematic representation of the experimental setting for simultaneous recording of extracellular fEPSP (EXTRA) and intracellular astrocytic currents (INTRA) **(A)** and of the main channels and transporters involved in the synaptically evoked astroglial currents at the tripartite synapse **(B)**. Simultaneous recordings of hippocampal fEPSP and astroglial currents evoked synaptically by single stimulation in basal conditions, after application of the ionotropic glutamate receptor blocker kynurenic acid, and further application of the GLT antagonist TBOA. To isolate excitatory currents, the $GABA_A$ receptor blocker picrotoxin is present throughout the experiment **(C)**. Note that the initial fast outward current component in the presence of picrotoxin reflects fEPSP generated by adjacent pyramidal cells. Astroglial K^+ currents are isolated by subtracting currents remaining after kynurenic acid application (2) to total evoked currents (1). Astroglial GLT currents are then isolated by further subtraction of the currents remaining after TBOA application (3) **(D)**. Adapted, with permission from Pannasch et al. (2012b) **(C,D)**.

Given the major impact these channels have on astroglial membrane K^+ permeability, $K_{ir}4.1$ invalidation is expected to result in a severe reduction of passive conductances; this has been observed, but to a much lower extent than anticipated (Neusch et al., 2006; Olsen et al., 2006; Djukic et al., 2007), thus pointing out the limits of IV protocols for analysis of intrinsic membrane properties from mature astrocytes *in situ*. Indeed, conductances revealed by IV protocols are not necessarily those involved in the resting state of astrocytes. The general viewpoint is that $K_{ir}4.1$ channels are enriched in distal processes of astrocytes, and therefore do not contribute to the major conductances measured from the cell body. Considering the reduced space clamp of astrocytic membranes (Zhou et al., 2009), patch clamp of astroglial processes would be more adapted to specifically assess $K_{ir}4.1$ functions.

Implication of gap junctional conductances in membrane properties

Astrocytes are interconnected through pores called gap junctions that enable passage of various small molecules (<1.5 kDa), hence implying ionic and metabolic coupling between cells. Biochemical intercellular transfer (such as glucose or ATP) has been extensively studied using passive dyes loaded in the astrocytic network through a patch pipette (Giaume et al., 2012). Complementary information has also been obtained in culture using paired recording of connected astrocytes, allowing assessment of gap junctional conductances; in weakly coupled pairs, even single gap junctional channel events could be detected (Giaume et al., 1991). Such approach has proven difficult to perform in acute slices, as in these conditions, astrocytes present larger passive properties and exhibit substantially more intercellular connections (Même et al., 2009; Xu et al., 2010). Traditionally, the low passive resistance of mature astrocytes has been attributed to their extensive gap junction mediated coupling. However, such conclusion is mostly based on pharmacological inhibition of gap junctional communication, using agents that are well-known for their poor selectivity (e.g., acidification, anesthesics, alcohols, endothelins, carbenoxolone). Their application on slices has been shown to decrease astrocytic conductances without affecting the general passive pattern (Wallraff et al., 2004; Schools et al., 2006; Adermark and Lovinger, 2008). Furthermore, it has been recently shown that treatment of slices with gap junction blockers would facilitate the analysis of non-linear profiles (Seifert et al., 2009; Olsen, 2012), placing astrocytic gap junctional coupling as a major current leak. However, whilst gap junctional communication mainly occurs between astrocytic processes, excised outside-out patch recordings from soma show intact passive current profile, suggesting that the major conductances subtending the linear profile of IV relationships are not located in processes. This result is further supported by double patch clamp recordings performed on the same astrocytic soma, showing that 80% of the current injected through one electrode is lost when recorded *via* the second electrode (~5 μm away) (Zhou et al., 2009). Finally, selective deletion of the main connexins expressed in astrocytes, connexin 43 and 30, leading to a complete loss of intercellular dye coupling, only weakly reduces (Wallraff et al., 2006) or leaves intact (Pannasch et al., 2011) the membrane resistance

of astrocytes (**Figures 1B,C**). Altogether, these studies indicate that astroglial gap junctional coupling plays a minor role, if any, in astroglial low membrane resistance and somatic passive IV relationship.

ASTROGLIAL FUNCTIONAL CHANNELS

Considering astrocytes as passive cells that mainly express leak K^+ channels and only undergo passive membrane potential fluctuations is an old fashioned simplistic view. Astrocyte membranes are composed of a large variety of ion channels, transmitter receptors and transporters (Barres, 1991; Sontheimer, 1994; Verkhratsky and Steinhäuser, 2000). Intensive characterization of astroglial electrophysiological properties using patch clamp recordings has revealed in the past decades diverse profiles, depending on the brain region and developmental stage investigated, unraveling the complexity of astrocytic populations. Moreover, and above all, a variety of functional channels with dynamic expression patterns has been reported.

Dynamic and evolution of expression patterns

Astrocytes express at high density a whole range of channels, including K^+, Na^+, Cl^-, or Ca^{2+} permeable channels (Steinhäuser et al., 2013). The ionic channel expression pattern of astrocytes is dominated by K^+ channels, of which the relative contribution to the general conductances evolves with cell maturation (Sontheimer and Waxman, 1993; Kressin et al., 1995; Bordey and Sontheimer, 1997; Zhou, 2005). It is generally assumed that differentiation into mature astrocytes takes place within the first 2–3 weeks after birth (Nixdorf-Bergweiler et al., 1994; Zhou, 2005). In immature tissues, the majority of astrocytes displays prominent delayed K^+ outwardly rectifiers (K_D) and transient "A" type currents (K_A), which dominate whole cell electrophysiological pattern. With time, more astrocytes progressively express K_{ir} channels and the large inward rectifier currents overwhelm all other K^+ conductances. This change in expression profile is accompanied by a hyperpolarization of the membrane potential, a large increase in the general cellular conductance and a quasi-linear IV curve. Such change in electrophysiological pattern is characteristic of a mature, differentiated astrocyte, and has been suggested to be at the origin of cell proliferation arrest (Olsen and Sontheimer, 2008). Na^+ channels have also been detected in astrocytes (Kressin et al., 1995; Bordey and Sontheimer, 1997), although their relative conductance is too weak, compared to K^+ channels, to trigger action potentials (Sontheimer and Waxman, 1992; Bordey and Sontheimer, 1997). Thus, astroglial Na^+ likely serves other functions (Sontheimer et al., 1996).

Implication in pathology

Characterization of channel expression developmental patterns in astrocytes has been particularly helpful to understand modifications affecting astrocytes in various pathologies. Indeed, the electrophysiological phenotypes of astrocytes are severely altered in gliomas (Ransom et al., 2001), reactive gliosis associated with brain insult (MacFarlane and Sontheimer, 1997), cortical dysplasia (Bordey et al., 2001) or epilepsy (Bordey and Sontheimer, 1998; O'Connor et al., 1998; Schröder et al., 2000), and curiously indicate reprogramming into an immature current pattern. Some common characteristics are a decrease or abolishment of K_{ir} conductances, in some cases an up-regulation of Na^+ conductances, and overall a drastic dominance of K^+ outward conductances, directly linked to cellular proliferation (Olsen and Sontheimer, 2008; Molenaar, 2011). Numerous questions, however, remain open, notably how these changes in channel expression affect cell functions.

Assessment of electrophysiological cellular profiles in slices or in cultures allows unraveling functional properties of channels expressed at astrocytic membranes, appreciating their weight with regard to the general cell conductance, and exploring their specific properties in terms of kinetics and modulations. However, deciphering the physiological implications of channels that do not prevail in mature astrocytes is more complicated, especially in slices, because of the predominance of K_{ir} conductances and technical limitations such as low space clamp.

Numerous channels, such as K_D or K_A are probably not open at resting membrane potential, because their activation voltage thresholds are far from resting membrane potential (Bordey and Sontheimer, 1997), and the latter only changes by a few millivolts upon neuronal activation in a physiological context. Thus, these voltage gated ion channels are likely to be primarily active in pathological conditions where astrocytes reach aberrant depolarized states during paroxysmal events such as seizures or following trauma, hypoxia or ischemia (Somjen, 1975). This presumption is however extrapolated from whole cell recordings, where astroglial membrane isopotentiality is assumed. Recordings from soma are most certainly not adequate to investigate fine electrical compartments where particular sets of channels might be functional in specific microdomains.

Are they all astrocytes?

Caution is needed when considering past developmental studies characterizing astroglial channel pattern, because some of them were performed at a time where the complexity of the glial lineage was not yet clearly set. An attempt to develop a classification of astrocytes according to their pattern of protein expression and electrophysiological profiles has enabled, in the last years, to define a new class of glial cells. First called "complex cells" by opposition to "passive cells," they were initially identified as astrocytes in patch clamp investigations on slices from immature or juvenile animals (Steinhäuser et al., 1994; Kressin et al., 1995; Bordey and Sontheimer, 1997). Later renamed NG2 cells, in reference to the NG2 proteoglycan expressed on their surface, these peculiar cells exhibit numerous distinct morphological and functional properties compared to astrocytes. This includes in particular their lack of interconnection through gap junction channels and their functional expression of voltage dependent K^+, Na^+, and Ca^{2+} conductances, as well as ionotopic GABA and glutamate receptors (Lin and Bergles, 2002; Matthias et al., 2003; Wallraff et al., 2004). Interestingly, this new class of cells is also detectable in the adult brain (Zhou, 2005). NG2 cells acquire with maturation a linear electrophysiological profile, which closely parallels myelinization (Maldonado et al., 2013). They are oligodendrocyte precursor cells, i.e. a class of progenitors that generate myelinating oligodendrocytes during development and after demyelinating injury in the mature brain (Richardson et al., 2011). A new field

of investigation is now emerging to decipher specific properties and functions of NG2 cells in developing and mature brain. The specific interactions of this novel neuroglia with astrocytes and neurons should be, in the forthcoming years, of particular interest (Bergles et al., 2010).

MONITORING ASTROCYTE FUNCTIONS WITH ELECTROPHYSIOLOGY

Astrocytes are part of the tripartite synapse and endorse important functions, among which supporting adequate neurotransmission. They undertake several functions at the level of excitatory synapses: they buffer K^+ released by neurons, efficiently uptake glutamate and release gliotransmitters. Neuromodulation by astrocytes has been investigated using different approaches, such as recordings of neurons whilst activating or inhibiting specific astroglial pathways. Interestingly, glutamate transporter (GLT, comprising GLT-1 and/or GLAST subtypes) uptake and K^+ buffering can be monitored directly by intra-astroglial recordings as these are the major currents that can be elicited by neuronal stimulations (**Figure 2**). They have been described in astrocytes from many structures, including the hippocampus (Bergles and Jahr, 1997; Araque et al., 2002; Ge and Duan, 2007; Meeks and Mennerick, 2007), the spinal cord (Zhang et al., 2009), the olfactory bulb (De Saint Jan, 2005), the cortex (Bernardinelli and Chatton, 2008; Unichenko et al., 2012), the optic nerve (Kuffler et al., 1966), the striatum (Goubard et al., 2011), the thalamus (Parri et al., 2010) and in more specialized glia such as Bergmann cells of the cerebellum (Clark and Barbour, 1997; Bellamy and Ogden, 2005). The profile and magnitude of these currents vary among studies, mainly because of protocol heterogeneity and glial diversity between brain regions. In the following section, we highlight the input electrophysiological approaches have provided in our understanding of astrocytic functions.

POTASSIUM FLOW THROUGH ASTROCYTIC MEMBRANES

Major contribution of K_{ir}4.1 channels

Astrocyte networks ensure K^+ homeostasis, uptaking excess extracellular K^+ following neuronal activity, and thereby controlling neuronal excitability. Several co-transporters and K^+ permeable channels are involved in this function, mainly Na^+/K^+ ATPases pumps, K_{ir} channels and $Na^+/K^+/2Cl^-$ transporters (Amedee et al., 1997; Somjen, 2002; Kofuji and Newman, 2004; Butt and Kalsi, 2006). The implication of K2P channels has also been reported (Päsler et al., 2007; Zhou et al., 2009). Astroglial K_{ir} channels, and especially K_{ir}4.1 channels, are believed to extensively participate to K^+ buffering, allowing excess of extracellular K^+ to flow into astrocytes. This has been suggested by several lines of evidence: (1) developmental up-regulation of K_{ir}4.1 channels is correlated with maturation of K^+ regulation mechanisms (Connors et al., 1982; Gabriel et al., 1998); (2) K_{ir}4.1 are the main K_{ir} channels expressed in astrocytes (Olsen et al., 2006) and are highly expressed in astroglial perisynaptic processes (Higashi et al., 2001; Hibino, 2004), presumably in regard to hotspots of K^+ release; (3) K_{ir}4.1 channels underlie the major component of K^+ astrocytic currents (De Saint Jan, 2005; Djukic et al., 2007); (4) the regulation of extracellular K^+ excess is less efficient in tissues from K_{ir}4.1 knockout than wild type mice (Neusch et al., 2006; Chever et al., 2010; Haj-Yasein et al., 2011; Bay and Butt, 2012).

Synapse as a major source of K^+ for astrocytes

Astrocytic currents evoked in slices are mainly mediated by K^+ conductances. Astrocytes display large and long lasting currents elicited in response to neuronal activities, which largely exceed the timescale of neuronal events. As illustrated in **Figure 2C**, whilst the postsynaptic response to single stimulation occurs within 20–40 ms following presynaptic activation, the astrocytic response is more than 200 times longer. In voltage clamp mode, this inward current is almost totally abolished by extracellular application of barium or by knocking out the K_{ir}4.1 gene (Djukic et al., 2007), thus indicating a major contribution of K^+ conductances. Astrocytic currents are induced by neuronal activity, as they are totally abolished by tetrodotoxin (Bergles and Jahr, 1997), and more precisely, depend on synaptic activation since they are abrogated by Cd^{2+} or total depletion of extracellular Ca^{2+}, both of which prevent presynaptic release of vesicles (Bergles and Jahr, 1997; De Saint Jan, 2005). Furthermore, postsynaptic blockers of excitatory transmission are commonly used to block K^+ currents evoked in astrocytes (De Saint Jan, 2005; Ge and Duan, 2007; Pannasch et al., 2011). Altogether, these studies indicate that astrocytes, through K_{ir}4.1 channels densely expressed on processes at the vicinity of synapses, sense extracellular K^+ mostly released from postsynaptic receptors. This is probably the case in areas enriched in synaptic contacts such as the *stratum radiatum*, in which most of the studies have been performed and where excitatory synapses have been determined to be the major source of K^+ release (Pumain and Heinemann, 1985). However, non-synaptic release of K^+ also occurs during firing of action potentials (D'Ambrosio et al., 2002; Bay and Butt, 2012). As a result, the contribution of axons and synapses to the general increase of K^+ (Aitken and Somjen, 1986) and the associated astrocytic buffering varies between brain areas.

Are we recording K^+ buffering?

One of the important role of astrocytes is to uptake K^+ released by active neurons. However, considering that the K^+ current recorded in astrocytes in voltage-clamp mode strictly corresponds to the buffered K^+ through K_{ir}4.1 channels would be a clear overestimation: as astrocytes depolarize in response to neuronal activity, a proportion of the evoked inward current usually attributed to K^+ in astrocytes actually corresponds to the holding current needed to maintain the membrane potential at imposed voltage. Similarly, astroglial membrane potential depolarizations recorded in current clamp mode with $I = 0$ do not fully reflect K^+ buffering, because they fluctuate according to the K^+ equilibrium potential (E_k) (**Figure 3B**), which is dynamic during neuronal activity and depends on both, intracellular and extracellular K^+ concentrations. Consequently, to adequately assess K^+ buffering, estimations of astroglial K^+ intracellular concentration increase should be performed, and require simultaneous recordings of extracellular K^+ levels and astroglial membrane potential fluctuations (**Figure 3A**)

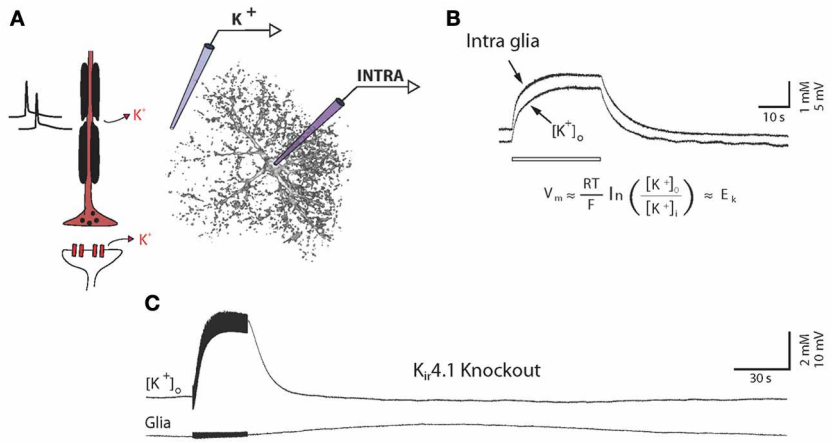

FIGURE 3 | High potassium permeability of glial cells is mediated by K_{ir}4.1 channels. (A) Schematic illustration of simultaneous recordings of extracellular K^+ concentration (K^+) and glial membrane potential (INTRA). **(B)** *In vivo* simultaneous recordings of extracellular K^+ concentrations and glial membrane potential fluctuations during long lasting stimulations (10 Hz, 30 s). Glial membranes behave as K^+ electrodes, reflecting their high K^+ permeability. Abbreviation for Nernst equation: R, universal gas constant; T, temperature; F, Faraday constant; E_K, K^+ equilibrium potential; Vm, membrane potential; $[K^+]_o$, extracellular K^+ concentration; $[K^+]_i$, intracellular K^+ concentration. **(C)** Similar protocol applied in a K_{ir}4.1 knockout animal. Glial membrane potential does not follow K^+ fluctuations, indicating a strong loss of K^+ conductances. A small and delayed depolarization, which is not associated with K^+ increase, could, however, be observed in response to long lasting stimulations only. Adapted, with permission, from Chever et al. (2010) **(B,C)**.

(Amzica et al., 2002). Alternatively, K^+ fluorescent indicators can be used to monitor intracellular K^+ increase in tissues (Dufour et al., 2011). Although such imaging approach can provide important spatial information about the cellular dynamics of K^+, electrophysiology offers a considerably better temporal resolution.

Physiological relevance of membrane fluctuations

The high K^+ permeability of astroglial membranes may represent a way to indirectly sense instantly changes in neuronal activity states, without energy (ATP) consumption. Whether these depolarizations are physiologically relevant *per se* has yet to be demonstrated and does represent a challenge. K^+-mediated depolarizations have been proposed to modulate gap junction coupling (Enkvist and McCarthy, 1994; De Pina-Benabou et al., 2001; Roux et al., 2011), extracellular pH homeostasis (Chesler and Kraig, 1987, 1989) and recently rate of glycolysis (Ruminot et al., 2011). Indeed, astrocytes express a variety of channels and transporters that are sensitive to voltage changes (e.g., GLTs, Na^+/K^+ ATPases, channels, $Na^+/HCO3^-$ transporters), suggesting that astroglial depolarizations could favor adaptative responses to neuronal activity through changes in the functionality of these transporters and channels. Supporting this notion, small fluctuations in glial membrane potentials can indeed be recorded between UP and DOWN states of cortical slow wave sleep (Amzica and Massimini, 2002; Mishima et al., 2007). Astrocyte membrane potential also varies according to physiological (REM sleep/wakefulness vs. slow wave sleep) or pathological (coma, spreading depression, epilepsy) neuronal activity states (Amzica et al., 2002; Seigneur et al., 2006; Kroeger and Amzica, 2007). It is therefore tempting to suggest that astroglial functions and efficiency may be finely adapted to arousal and behavioral states.

GLUTAMATE TRANSPORTERS CURRENTS

Blocking the K^+ component of evoked astroglial currents by application of ionotropic receptor antagonists unmasks a small transient current (**Figure 2D**). In current clamp mode, a depolarization is recorded and corresponds to an inward current in voltage clamp mode (Bergles and Jahr, 1997; Bernardinelli and Chatton, 2008). Such response has been attributed to activation of astroglial GLTs, since it is blocked by specific inhibitors such as DL-threo-β-benzyloxyaspartate (TBOA) and dihydrokainate (DHK) (Anderson and Swanson, 2000).

Among the five high affinity Excitatory Amino Acid Transporters (EAAT1-5) identified in the mammalian CNS, the two most abundant, EAAT1 (human homologue of L-glutamate/L-aspartate transporter GLAST) and EAAT2 (human homologue of glutamate transporter 1 GLT-1), are highly expressed in astrocytes. They are enriched on astrocytic processes (Rothstein et al., 1994; Chaudhry et al., 1995; Minelli et al., 2001), and are activated by an increase in extracellular glutamate concentration mainly originating from synaptic vesicular release (Mennerick and Zorumski, 1994; Bergles and Jahr, 1997). Astrocytic transporters are responsible for most of the glutamate uptake (Danbolt, 2001). Astroglial GLT currents have relatively low peak amplitude (∼5–15 pA) during basal synaptic activity. Indeed, although GLTs are highly expressed on astroglial membranes (∼10,000/μm^2), show a high affinity for glutamate (EC_{50}: 4–30 μM), and can bind glutamate in less than a millisecond, acting as glutamate buffers, their cycling time is low (∼70 ms), and their access to synaptic glutamate is reduced, because of the marginal enwrapping of synapses by astrocytes in the CA1 hippocampal region (Tzingounis and Wadiche, 2007). Hippocampal astroglial GLTs are nevertheless responsible for most of extracellular glutamate clearance, which is otherwise performed by passive diffusion. They prevent spillover and activation

of extrasynaptic receptors and neighboring synapses, as shown in numerous studies (Tzingounis and Wadiche, 2007).

The transport of one glutamate molecule is coupled to co-transport of 3 Na^+ and 1 H^+ ions and counter-transport of one K^+ ion (Amato et al., 1994; Levy et al., 1998; Owe et al., 2006). According to this stoichiometry, glutamate transport is electrogenic as for each transport cycle, two net positive charges are translocated into the cell. One full cycle is composed of sequential steps that imply two conformation states: while binding of extracellular Na^+/H^+ ions and glutamate lead first to the translocation of glutamate from outside to inside, intracellular binding of K^+ is associated with the reorientation of the glutamate binding site toward the outside (Tzingounis and Wadiche, 2007).

Yet, description of GLT functionality and of factors limiting transport of glutamate has enabled to unravel their key role in brain homeostasis. GLT currents have been found to be highly voltage dependent; their magnitude decreases with depolarization, becomes negligible at extreme values, but do not reverse (Barbour et al., 1991; Kirischuk et al., 2007). In addition, rate of glutamate uptake is also highly dependent on ionic environment, being primarily affected by Na^+ (Levy et al., 1998; Kirischuk et al., 2007; Unichenko et al., 2012), K^+ and glutamate concentrations (Brew and Attwell, 1987; Barbour et al., 1988). These limiting factors become crucially relevant with regard to pathological states. Indeed, in several pathological conditions such as ischemia and anoxia, extreme increase in extracellular K^+ concentrations concomitant with neuronal and glial depolarizations are reported (Müller and Somjen, 2000). Because both increases in extracellular K^+ concentration and glial depolarization reduce electrogenic glutamate uptake (Barbour et al., 1988), impaired glutamate uptake is suggested to participate to the excitotoxic rise of ambient glutamate concentrations (Jabaudon et al., 2000).

DECIPHERING NEUROGLIAL INTERACTIONS USING ASTROCYTE-NEURON DUAL ELECTROPHYSIOLOGICAL RECORDINGS

The aforementioned astrocytic properties and activities may be intrinsic features of these cells or, on the contrary, attributable to surrounding neuronal activities. Accurate assessment of astrocytic functions therefore implies simultaneous monitoring of astrocytic and neuronal activity. This can be achieved *in vitro* or *ex vivo* with fluorescent techniques allowing detection of cellular Ca^{2+} signals or with the powerful approach of using whole cell patch clamp of astrocytes coupled to neuronal recordings, allowing examination of multiple currents of different origins, as we recently described (Pannasch et al., 2012b). Such investigations have enabled to gain insights into the role and physiology of neuroglial interactions by interfering positively or negatively with astrocytic and/or neuronal function, as well as investigating the neuron-astrocyte interplay that occurs during particular activities, such as short- and long-term plasticity or in pathological conditions such as epilepsy. We shall discuss in this section how dual monitoring of neurons and astrocytes can decipher mechanisms of astroglial control of neurotransmission.

ASTROGLIAL RESPONSES ARE SHAPED BY NEURONAL ACTIVITY

Entry of ions released during neuronal activation into astrocytes induces measurable currents of different origins. The main protagonists involved are K^+ channels (Orkand et al., 1966; Karwoski et al., 1989; Meeks and Mennerick, 2007), glutamate and GABA transporters (Bergles and Jahr, 1997; Diamond et al., 1998; Lüscher et al., 1998; Goubard et al., 2011), as well as AMPA receptor currents in Bergmann glial cells (Clark and Barbour, 1997; Bellamy and Ogden, 2006). As discussed above, K^+ currents are crucial to maintain the astrocyte at a markedly negative resting membrane potential, as well as for K^+ clearance during and after neuronal activity. Glutamate and GABA transporters are essential for maintaining low concentration of extracellular neurotransmitters, thereby avoiding spillover and activation of extrasynaptic receptors and neighboring synapses (Oliet et al., 2001; Huang et al., 2004). In addition, they participate to the recycling of neurotransmitters, the glutamate-glutamine cycle and neurometabolic coupling (Anderson and Swanson, 2000; Danbolt, 2001; Huang and Bergles, 2004; Tzingounis and Wadiche, 2007).

Functional plasticity of neuroglial interactions

Most interestingly, triggering and recording neuronal plasticity while monitoring glial activity through a patch clamp electrode has enabled to observe short- and long-term changes of astrocytic currents that are associated to, but do not always match modifications in neuronal activity. In the cerebellum, intracellular monitoring of Bergmann cell currents, referred to as extrasynaptic currents (ESCs), revealed that glial short-term plasticity assessed using paired pulse stimulation protocols is 4 fold larger in magnitude than the well-known associated neuronal facilitation. Interestingly, such facilitation was found to be mainly mediated by AMPA receptors and GLT activation (Bellamy and Ogden, 2005). These investigators later showed using the same preparation that plasticity of cerebellar glial cells is not a simple reflection of surrounding neuronal activity. Indeed, long-term depression of GLT and AMPA receptor currents could be induced in Bergmann cells using 0.2 Hz stimulation that do not generate any change in evoked excitatory postsynaptic current (EPSC) amplitude from Purkinje neurons (Bellamy and Ogden, 2006). Using paired neuronal-glial recordings in cerebellar cultures, long-term potentiation (LTP) of Ca^{2+} permeable AMPA receptor glial currents has also been demonstrated to be inducible by prolonged 4 Hz stimulation of the granule cell. In this case, however, plasticity appeared to purely reflect changes in probability of glutamate release from the presynaptic terminal (Linden, 1997).

In the hippocampus, we and others found that single stimulation of Schaffer collaterals evoking typical fEPSP recorded in the *stratum radiatum* elicits a complex astroglial response, composed of at least two components, i.e. a long lasting K^+ current and a transient GLT current (Bergles and Jahr, 1997; Diamond et al., 1998; Lüscher et al., 1998; Meeks and Mennerick, 2007; Bernardinelli and Chatton, 2008; Pannasch et al., 2011, 2012b). The complex astroglial current shows paired pulse facilitation (Pannasch et al., 2012b) and, noticeably, the GLT component presents a neuronal like short-term plasticity, as

it reflects synaptic neurotransmitter release (Bergles and Jahr, 1997; Diamond et al., 1998; Lüscher et al., 1998; Bernardinelli and Chatton, 2008). Interestingly, such property was actually exploited to ascertain, using astrocytic patch clamp coupled to extracellular field recording, whether hippocampal long-term potentiation (LTP) is associated with a sustained increase in the probability of glutamate release. It was found that although astrocytic glutamate currents do display post-tetanic potentiation, they return to baseline within minutes, implying a postsynaptic expression of the CA1 hippocampal LTP (Diamond et al., 1998; Lüscher et al., 1998). Notwithstanding, Ge and colleagues were able to show, by maintaining stable astrocytic recordings for more than an hour and simultaneously monitoring field excitatory postsynaptic potentials (fEPSPs), a LTP like persistent facilitation of evoked currents in hippocampal astrocytes, which is abolished by an inhibitor of K^+ channels and thus presumably mediated by K^+ conductances (Ge and Duan, 2007). Based on our observation that most of the astroglial response to Schaffer collateral stimulation is mediated by K^+ conductances (Pannasch et al., 2011, 2012b), we may speculate that these channels underlie the effect reported in the latter study.

Altogether, the few investigations focusing on the plasticity of neuronal induced glial currents highlight different mechanisms depending on the plasticity considered (i.e., short- vs. long-term; potentiation vs. depression) and on the brain structure studied (e.g., cerebellum vs. hippocampus). Yet, more research taking advantage of dual electrophysiological recordings of neuronal and astroglial activities is clearly needed to fully decipher the mechanisms at play in this peculiar form of plasticity. Noteworthy, this approach being often challenging as it requires the use of multiple electrodes in a confined space, such investigations may be facilitated by the use of simultaneous monitoring of fEPSPs and astrocytic conductances through a single patch pipette as described in voltage clamp mode (Bergles and Jahr, 1997; Diamond et al., 1998; Lüscher et al., 1998) (**Figures 4A,B**) and recently re-validated in the current clamp configuration (Henneberger and Rusakov, 2012) (**Figures 4C–F**).

Morphological plasticity of neuroglial interactions

Astrocytes are also prone to a different form of plasticity: morphological changes in response to neuronal activity. Morphological plasticity of neuronal coverage has been demonstrated in various brain regions, including: (1) the somatosensory cortex, where stimulation of mouse whiskers resulted in significant increase in the astrocytic envelopment of excitatory

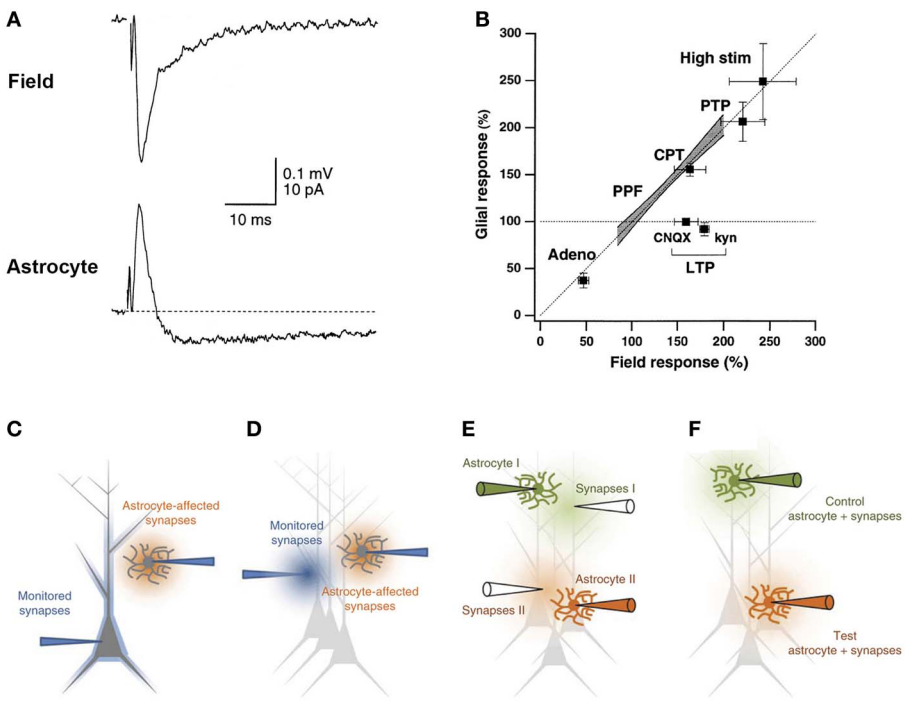

FIGURE 4 | Dual electrophysiological recording of neuronal and astroglial activities. **(A)** Example showing that the extracellular field response corresponds to the initial outward signal recorded through an astrocytic patch pipette in the voltage clamp configuration. **(B)** Correlation between field and glial responses to various manipulations shows a linear relationship that falls in the line of identity. Adeno, adenosine application; PPF, paired pulse facilitation; CPT, A1 receptor antagonist CPT, High stim, high stimulation intensity. **(C–F)** Possible experimental arrangements for simultaneously monitoring neuronal and astroglial activities. **(C)** Double whole cell patch clamp experiments give access to intracellular currents for both astrocytes and neurons. **(D)** Typical electrode placement for monitoring fEPSP in parallel to astrocytic currents. **(E)** Such electrode placement can be extended to include an internal control pathway in the experimental set-up. **(F)** Monitoring the fEPSP through the astrocytic glass pipette greatly simplifies such arrangements. Adapted, with permission, from Diamond et al. (1998) **(A)**, Lüscher et al. (1998) **(B)**, and Henneberger and Rusakov (2012) **(C–F)**.

synapses on dendritic spines (Genoud et al., 2006), (2) the supraoptic nuclei, which undergo pronounced reduction of the astrocytic coverage of oxytocin neurons during particular conditions such as lactation or chronic dehydration (Oliet and Bonfardin, 2010), (3) the hippocampus after LTP (Wenzel et al., 1991; Lushnikova et al., 2009), and (4) the visual cortex of rats raised in enriched environment (Jones and Greenough, 1996). Other investigations have also reported a glial swelling following electrical stimulation (MacVicar and Hochman, 1991; Hawrylak et al., 1993; MacVicar et al., 2002), most probably attributable to K^+ buffering (Ballanyi et al., 1987). Such swelling is however meant to be transitory, as it modifies extracellular space volume and astrocytic properties (Ransom et al., 1985). Yet, in pathological condition such as epilepsy, where neuronal activity is abnormally strong, astrocytes become reactive and are swollen, so that impaired K^+ buffering and glutamate uptake support neuronal hyperexcitability and seizure activity (Proper et al., 2002; Schröder et al., 2002). Further, using simultaneous patch clamp recording and Ca^{2+} imaging techniques, it was recently shown that initiation and maintenance of focal seizure like discharges correlate with astroglial Ca^{2+} activation, suggesting that neuronal hyperactivity engages astrocytes in a recurrent excitatory loop that promotes seizure ignition and sustains ictal events (Gómez-Gonzalo et al., 2010).

In all, analysis of morphological and functional astrocytic responses to neuronal activity have revealed that instead of being passive, these cells are, akin to neurons, able to display several forms of plasticity, of which the role remains to be identified. As exampled herein for the case of epilepsy, determining the precise function of such astrocytic adaptation to neuronal activity shall most certainly allow identifying new therapeutic targets in the numerous brain pathologies involving astrocytic inflammation and dysfunction.

NEURONAL ACTIVITY IS TUNED BY ASTROCYTES

Calcium signaling

Ca^{2+} elevation represents a hallmark of cellular activation and has been shown to occur in astrocytes in response to neuronal activity (Verkhratsky and Kettenmann, 1996; Newman, 2003; Deitmer and Rose, 2010). Electrophysiology has been extensively used to impair Ca^{2+} signaling locally in populations of astrocytes through selective intracellular delivery of Ca^{2+} chelators contained in a patch pipette during whole cell recording of a single astrocyte. Such manipulation relies on the extensive connectivity of astrocytes by gap junction channels, which are permeable to Ca^{2+} chelators such as BAPTA and EGTA, enabling their diffusion in astroglial networks. This approach confirmed the importance of astrocytic Ca^{2+} rises, which trigger numerous downstream events, in the regulation of neuronal activity.

In the hippocampus, astrocytic Ca^{2+} elevation has indeed been shown to up-regulate synaptic activity through several mechanisms. Di Castro and collaborators recently evidenced in the *dentate gyrus* that BAPTA infusion in the astroglial network *via* a patch pipette (**Figure 5A**) results in a diminished efficacy of granule cells synapses as measured intracellularly using whole cell patch clamp recording in minimal stimulation conditions (**Figure 5B**) (Di Castro et al., 2011). The authors attribute the Ca^{2+} rise blocked by BAPTA to activation of purinergic receptors P2Y1R, as antagonizing these leads to the same decrease in synaptic efficacy, and the presence of BAPTA in the astrocytic network occludes such effect. P2Y1Rs have recently been shown in this same hippocampal region to result in glutamate gliotransmission, activating presynaptic NMDA receptors and facilitating neurotransmitter release (Santello et al., 2011). In the CA1 area of the hippocampus, preventing astrocytic Ca^{2+} signaling using BAPTA, also reduces basal synaptic transmission efficacy, as recorded in whole cell patched pyramidal neurons subject to minimal stimulations (Panatier et al., 2011).

In contrast to the mechanism shown at perforant path-dentate gyrus synapses, Ca^{2+} rises in CA1 astrocytes during basal synaptic activity appear to mainly rely on the activation of metabotropic glutamate receptor (mGluR) mGluR5, given that application of the selective antagonist 6-methyl-2-(phenylethynyl)-pyridine (MPEP) recapitulates the increase in synaptic failures induced by BAPTA infusion in the astrocytic network, and that such chelation of astrocytic Ca^{2+} precisely occludes the MPEP effect (Panatier et al., 2011). Whether such regulation occurs in the adult brain remains to be determined, as these data were obtained from young rats (15–21 days old) and a recent investigation reports, albeit in mice, that mGluR5 signaling is barely detectable in the brain passed 3 weeks of age (Sun et al., 2013). Panatier and colleagues further demonstrate that the downstream events following astrocytic Ca^{2+} elevation include activation of presynaptic A_{2A} adenosine receptors, suggesting that in this case, gliotransmission of purines up-regulates basal synaptic activity. These important findings provide great details on the activation of hippocampal astrocytes through glutamatergic signaling.

Previous studies have also shown that astrocytes can release glutamate in response to activation of several pathways, including activation of mGluRs, but also endocannabinoid CB1 receptors or protease activated receptor PAR-1, as well as mechanical stimulation or intracellular application of inositol 1,4,5-trisphosphate (IP3), which up-regulate the frequency of slow transient NMDA currents in pyramidal neurons (Angulo et al., 2004; Fellin et al., 2004; Kang et al., 2005; Navarrete and Araque, 2008; Shigetomi et al., 2008). In line with this, work on hippocampal neurons/astrocytes cultures also reported that Ca^{2+} uncaging in astrocytes or mechanical, as well as electrical stimulation trigger glutamate gliotransmission that evokes slow inward excitatory currents in adjacent neurons (Araque et al., 1998a,b; Parpura and Haydon, 2000; Fellin et al., 2004; Navarrete et al., 2012).

CA1 fast neuronal transmission has also been found to be influenced by astroglial glutamate release subsequent to astrocytic Ca^{2+} elevation (Fiacco and McCarthy, 2004; Navarrete and Araque, 2010). In addition, the study by Henneberger and colleagues interestingly shows that clamping astrocytic free Ca^{2+} concentration at 50–80 nM, by adding 0.45 mM EGTA and 0.14 mM Ca^{2+} to the intra-pipette solution for patch clamp, impairs isolated NMDA receptor fEPSP and that such effect is due to inhibition of the NMDA receptor co-agonist D-serine gliotransmission. Remarkably, the dependence of NMDA receptor efficacy on astroglial release of D-serine was further shown to

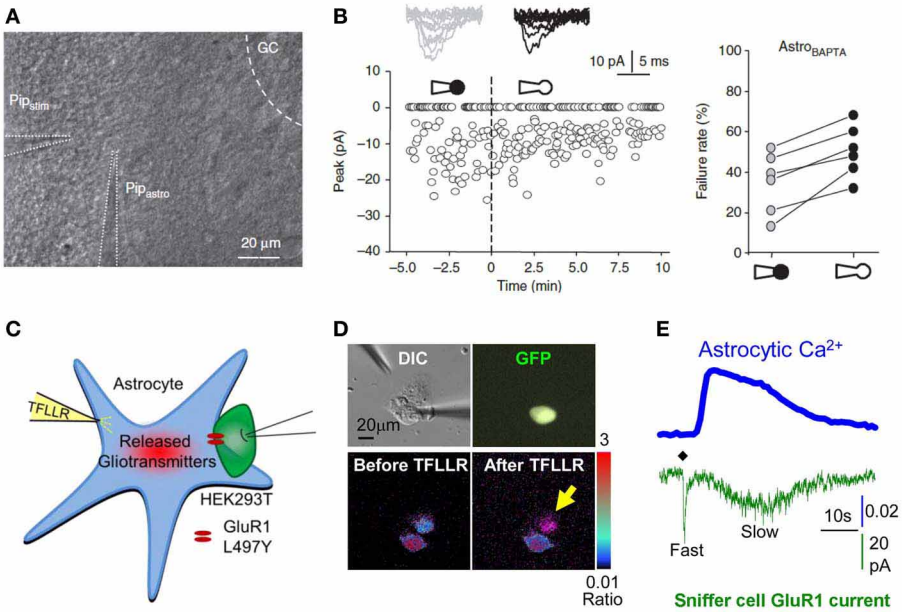

FIGURE 5 | Interfering with astrocytes influences neuronal activity. (A,B) Infusion of BAPTA in the astrocytic network decreases synaptic efficacy in minimal stimulation conditions. **(A)** Experimental arrangement showing whole cell patch clamp of an astrocyte (Pip_{Astro}) present in the dendritic tree of the recorded granule cell and 20–30 µm away from the stimulating pipette (Pip_{stim}). The intra-pipette solution for patch clamp contains the Ca^{2+} chelator BAPTA. **(B)** Failure rate changes in granule cells after breaking the cell membrane and dialyzing the astrocyte with the intra-pipette solution containing BAPTA. **(C–E)** Measure of astrocytic glutamate release using the sniffer patch technique. **(C)** Schematic illustration of the sniffer-patch. The left pipette (yellow) is used for pressure application of the PAR-1 agonist TFLLR. This stimulation results in glutamate release (red cloud) producing a measureable inward current in the HEK293T sensor cell (green) expressing GluR1-L497Y containing AMPA receptors (red). **(D)** Images for sniffer patch. DIC image (upper left): two cells with two glass pipettes. GFP image (upper right): sensor cell expressing GluR1-L497Y and GFP. Pseudocolor images: Fura-2 loaded astrocyte (source cell) and sensor cells before (lower left) and after stimulation (lower right). Yellow arrow: increased Ca^{2+} in astrocyte. **(E)** Representative traces recorded from the sniffer-patch technique. Blue trace: Ca^{2+} transient recorded from astrocyte. Green trace: whole cell current recorded from sensor cell (voltage clamped at −70 mV) upon TFLLR pressure application. Diamond: TFLLR application (10 psi, 100 ms, 500 µM). Adapted with permission from Di Castro et al. (2011) **(A,B)** and Woo et al. (2012) **(C–E)**.

be required for synaptic plasticity, as assessed by induction of LTP (Henneberger et al., 2010). Clamping intracellular free Ca^{2+} at baseline levels is theoretically a more accurate way to assess the relevance of astrocytic Ca^{2+} activation by neuronal activities, as classical Ca^{2+} buffering most probably impairs signaling and metabolic pathways that depend on free Ca^{2+} availability. This implies that the impact of intracellular Ca^{2+} chelators on neurotransmission should be considered with great caution, and often requires confirmation of the results by using blockers of downstream pathways, such as the light chain of tetanus toxin, which blocks vesicular transmitter release.

Interestingly, to our knowledge although baseline astrocytic Ca^{2+} concentrations have been shown to lie around 50–100 nM using ratiometric Ca^{2+} indicators (Grimaldi et al., 2001; Floyd et al., 2005), no investigation as yet quantitatively reported the extent of Ca^{2+} rise following evoked synaptic transmission. Such issue is important to tackle as an intriguing recent investigation demonstrates that chimeric mice grafted with human astrocytes, which exhibit 3 fold larger and faster Ca^{2+} signals, display greatly enhanced plasticity and learning (Han et al., 2013), supporting the notion that in contrast with the "all or nothing" action potential system utilized by neurons, for Ca^{2+} astroglial signaling, size matters. One study reported in culture that bath application for 5 min of 100 µM and 1 mM of L-glutamate increases astrocytic Ca^{2+} concentration by ∼400 and ∼800 nM, respectively (Floyd et al., 2005). These uniform and long lasting elevated concentrations of glutamate are likely to be non-physiological, although other work on cultured astrocytes has shown that Ca^{2+} wave propagation results in accumulation of glutamate in the range of 1–100 µM (Innocenti et al., 2000). Further, Parpura and Haydon reported that more physiological increases in Ca^{2+} levels, from 84 to 140 nM, induced by flash photolysis, is sufficient to trigger SICs, proposed to be mediated by astroglial glutamate release (Parpura and Haydon, 2000). These investigations were, however, performed on cultured cells with artificial astroglial stimulations. Astroglial intracellular Ca^{2+} levels that actually occur in response to basal synaptic transmission and plasticity *in situ* therefore remain unknown.

Corroborating hippocampal investigations, slow neuronal depolarizations recorded in pyramidal neurons from mouse visual cortex slices and induced by brief (0.2–1 s) pulse application of acetylcholine through a glass pipette is also drastically reduced by prior BAPTA infusion *via* a patch pipette in the astrocytic network, as acetylcholine evokes a Ca^{2+} rise in astrocytes of this brain region. Since such neuronal depolarization was shown to rely on NMDA receptor activation, the authors suggest that

astrocytic activation may lead to glutamate or D-serine gliotransmission, or to Ca^{2+} dependent regulation of extracellular K^+ and/or glutamate (Chen et al., 2012). With regard to the astroglial signaling subtending these regulations involving gliotransmission, an outstanding investigation aiming at deciphering the mechanism of astrocytic glutamate release used dual patch clamp to elegantly measure the actual release of glutamate from cultured astrocytes. The trick used in this study was to patch a HEK293T "sniffer cell" expressing a non-desensitizing form of AMPA receptors and placed in direct apposition to an astrocyte (**Figures 5C–E**). The latter is then activated either by application of TFLLR, a selective peptide agonist of the PAR-1 G-protein coupled receptor, or by patching the cell using an intra-pipette solution containing high Ca^{2+} concentrations. After screening the different pathways that might be involved in glutamate release, the authors conclude that a fast and slow mode of astrocytic glutamate release co-exist and are mediated by glutamate permeable two-pore domain K^+ channel TREK-1 and glutamate permeable Ca^{2+} activated anion channel Best1, respectively (Woo et al., 2012).

In sharp contrast to the aforementioned up-regulatory action of astrocytic glutamate on neuronal activity, Ca^{2+} chelation in barrel cortex astrocytes has been found to increase the frequency of spontaneous excitatory postsynaptic potentials, as well as evoked neuronal depolarization and excitability recorded in patch clamp conditions, suggesting in this case a down-regulatory role of neuroglial signaling. Although the precise mechanism could not be completely unraveled, it appeared to involve GABAergic signaling suggesting that, in this brain region, GABAergic gliotransmission could bridle neuronal activity (Benedetti et al., 2011). This finding is in line with the demonstration, in the mouse olfactory bulb, that both slow inward (SIC, excitatory) and outward (SOC, inhibitory) currents are observed spontaneously, can be evoked by mechanical stimulation of astrocytes and are attributable to the glial release of glutamate and GABA, respectively (Kozlov et al., 2006). Down-regulation of synaptic activity as a result of glial cell stimulation has also been demonstrated in the cerebellum, where directly applied depolarizing steps on patch clamped astrocytes in the imposed voltage configuration resulted in a marked decrease in the frequency of Purkinje neurons spontaneous postsynaptic potentials (Brockhaus and Deitmer, 2002). Finally, the inhibitory action of astrocytes on neuronal activation has also been shown in CA1 neurons to include the participation of local circuit interneurons, since an astrocytic Ca^{2+} rise dependent on $GABA_B$ receptor activation was found to increase the frequency of inhibitory postsynaptic currents, an effect blocked by intra-astrocytic application of BAPTA (Kang et al., 1998). Altogether, this body of investigations indicates that astrocytic Ca^{2+} activation leads to the release of neuroactive molecules able to up- as well as down-regulate synaptic and neuronal activity.

The importance of such form of "astrocytic excitability" is empowered by the fact that extracellular pathways involving gliotransmitters such as ATP, and potentially amplification of signaling through gap junction mediated astroglial networks, likely contribute to the propagation of intercellular Ca^{2+} waves or glissandi, as recently reported *in vivo* (Kuga et al., 2011). This strongly suggests an important role for astrocytes, at the individual cell or network level, in the synchronization of neuronal activities (Cornell-Bell et al., 1990; Dani et al., 1992; Angulo et al., 2004; Fellin et al., 2004; Wang et al., 2006). Yet, this is subject to controversies as recent data suggest, on the contrary, that specific Ca^{2+} activation does not influence neurotransmission (Fiacco et al., 2007; Agulhon et al., 2010). In these investigations, specific Ca^{2+} activation was achieved through the use of transgenic mice in which astrocytes express MrgA1 Gq-coupled receptors that respond to a specific agonist and are normally not found in the CNS. Limitations of this approach, however, include the fact that uniform Ca^{2+} increase induced by activation of a receptor that is exotic to the CNS may not be linked to intracellular signaling that is relevant in terms of gliotransmission. Besides, expression of receptors leading to the release of neuroactive molecules is expected to be targeted to specific cellular locations, which is not the case of this non-physiological expression MrgA1 receptors. The same research group also demonstrates that obliterating Ca^{2+} activation in a mouse line in which astrocytic inositol triphosphate receptors (i.e., InsP3R2) are knocked out also results in unaltered basal transmission, short-, and long-term plasticity, indicating that Ca^{2+} release from glial intracellular stores is not a *sine qua non* condition to normal neuronal activity (Petravicz et al., 2008; Agulhon et al., 2010). However, InsP3-dependent source of astroglial Ca^{2+} may not be at play in the regulation of synaptic transmission and plasticity.

Sodium signaling and energy metabolism

In addition, despite overwhelming evidence pointing toward the key function of Ca^{2+} elevation in astrocytes, one should bear in mind that the latter is to be considered as one facet of the multiple mechanisms at play in the astroglial control of synaptic transmission. Accordingly, using patch clamp recording of neurons and astrocytes coupled to Ca^{2+} imaging, an early investigation performed on cortical primary culture unraveled rapid inward glial currents that coincided with bursts of electrical activity in neighboring neurons, and which were not associated with Ca^{2+} signal; the authors further stressed the fact that the temporal scale of the slow Ca^{2+} waves does not match the fast astroglial current observed, more reminiscent of a depolarization due to AMPA receptor activation or glutamate transport (Murphy and Wier, 1993). Recent investigations combining intracellular recordings with sodium (Na^+) imaging, utilizing the fluorescent Na^+ indicator SBFI, showed in cerebellar Bergmann glial cells that bursts of activity rapidly triggers Na^+ signals attributable to AMPA receptor activation, as well as Na^+ dependent glutamate uptake (Kirischuk et al., 2007; Bennay et al., 2008). Furthermore, Na^+ rise in astroglia in response to neuronal activity has been found in cultures and in hippocampal slices to result in propagating Na^+ waves (Bernardinelli et al., 2004; Langer et al., 2012). Interestingly, although the work performed on cultured astrocytes suggested that Ca^{2+} and Na^+ waves are intrinsically linked, as chelation of Ca^{2+} also resulted in Na^+ wave abolition, the recent investigation performed *in situ* demonstrates that Ca^{2+} signaling up-regulates, but is not a prerequisite for the dispersion of Na^+ through the astroglial network (Langer et al., 2012). The impact of interfering with this phenomenon on neuronal activity has been

difficult to investigate, as no way of specifically chelating Na^+ in astrocytes is currently available. Developing such tool would thus be greatly beneficial to our understanding of neuroglial interactions.

Yet, such increases in Na^+ concentrations have been shown to activate the Na^+/K^+ ATPase, resulting in a high energy demand and consequently to an increased glucose uptake though the glucose transporter GLUT1, thereby initiating the astrocyte-neuron lactate shuttle consisting in the activation of phosphoglycerate kinases that triggers glycolysis and thus results in the production of lactate, released in the extracellular space and taken up by neurons (Pellerin and Magistretti, 2012). Na^+ waves are therefore thought to translate into metabolic waves (Pellerin and Magistretti, 1994; Chatton et al., 2000, 2003; Bernardinelli et al., 2004). Thus, through the generation of ionic waves in the astrocytic net, local neuronal activity increases astrocyte to neuron lactate supply toward *loci* of high energy demand. Strikingly, we were able to directly show such activity-dependent preferential supply of energy metabolites mediated by gap junctional inter-astrocytic communication, using the fluorescent glucose analog 2-NBDG (Rouach et al., 2008). Indeed, by experimentally creating a high local energy demand in the *stratum radiatum* (using 1 Hz electrical stimulation) and observing the 2-NBDG diffusion from a single astrocyte patched in the *stratum oriens*, it was found that the fluorescent metabolite spread through the astroglial network is extended and directed toward the site of high neuronal activity. Further, the efficiency of such astrocytic metabolic supply was demonstrated by recovering the loss of neurotransmission normally observed in glucose deprivation conditions, *via* selective application of glucose in the astroglial network (Rouach et al., 2008).

Potassium signaling

Activation of Na^+/K^+ ATPase by intracellular Na^+ rise also implies a concomitant K^+ entry occurring as a result of extruding Na^+. Uptake of K^+ in astrocytes is also known to strongly depend on inwardly rectifying K^+ channels, notably $K_{ir}4.1$ (Olsen and Sontheimer, 2008). K_{ir} channels can, however, transport K^+ out or inside the cell depending on the K^+ electrochemical gradient, and have been reported to mainly undertake maintenance of the pronounced astrocytic resting membrane potential, and to be only moderately involved in extracellular K^+ clearance as compared to the Na^+/K^+ ATPase (Ransom et al., 2000; Xiong and Stringer, 2000; D'Ambrosio et al., 2002). D'Ambrosio and colleagues claimed that pharmacological blockade of K_{ir} channels with bath application of barium does increase basal concentration of extracellular K^+, but leaves intact its clearance after high frequency neuronal stimulation; instead, such clearance is heavily disturbed by inhibition of the Na^+/K^+ ATPase using ouabain (D'Ambrosio et al., 2002). While barium impact on K^+ uptake is still controversial (Xiong and Stringer, 2000; Jauch et al., 2002; Meeks and Mennerick, 2007), the effect of ouabain is consistent among studies (Förstl et al., 1982; Ransom et al., 2000). However, these findings suffer from the pharmacological tools used, which lack astrocytic specificity, and most likely disturb general ionic homeostasis as the main role of Na^+/K^+ ATPase is to maintain transmembrane ionic gradients. Further, these results have been contradicted by a recent *ex vivo* study on hippocampal slices from $K_{ir}4.1$ knockout mice (Haj-Yasein et al., 2011). This also reinforces previous investigations showing that $K_{ir}4.1$ channels promote K^+ uptake after moderate extracellular rises (Neusch et al., 2006; Chever et al., 2010). Together, these studies indicate that both Na^+/K^+ ATPases and astroglial K_{ir} channels are implicated in K^+ excess uptake. These mechanisms therefore work synergically in maintaining K^+ homeostasis. In particular Na^+/K^+ ATPase activity could lead to membrane hyperpolarization, thereby favoring K^+ entry through K^+ channels. The relative contribution of Na^+/K^+ ATPases and K_{ir} channels most probably vary according to the regime of activity (Somjen et al., 2008). Na^+/K^+ ATPases efficiency is indeed prominent in case of strong neuronal activity. In this context, the main current view actually proposes that $K_{ir}4.1$ channels could also be responsible for counterbalancing K^+ uptake through Na^+/K^+ ATPase activity (D'Ambrosio et al., 2002; Haj-Yasein et al., 2011; Bay and Butt, 2012; Bay and Butt, but see Chever et al., 2010).

Noteworthy, the pharmacological and genetic tools used to study Na^+/K^+ ATPases and K_{ir} functions in astrocytes lack specificity and target different cell types, such as neurons or oligodendrocytes. One acute way of accurately invalidating K_{ir} function in astrocytes would be to apply barium intracellularly in the astroglial network through a patch pipette, as this ion is a potent K_{ir} channel blocker inside, as well as outside the cell (Solessio et al., 2000). Alternatively, delivery of K_{ir} antibodies through the patch electrode has been shown to be an efficient blocker in bipolar cells of the retina (Raz-prag et al., 2010), and might therefore represent an interesting way of specifically assess K_{ir} functions in astrocytes. However, such approach might not be efficient in all cell types, as in our hands, intrapipette application of K_{ir} antibodies in hippocampal astrocytes resulted in no alteration of neither glial nor neuronal currents (Sibille and Rouach, unpublished observations).

In sum, blocking particular sets of astrocytic functions and recording the consequences on neuronal activity and/or on regulation of extracellular medium composition has proven to be a very informative approach to explore the relevance of neuroglial interactions and has enabled to unravel the importance of gliotransmission, metabolic coupling and ionic wave propagation in the regulation of neuronal activity. Yet, the relatively limited number of tools allowing disruption of specific functions in comparison with the tremendous number of elements involved in neuroglial signaling has, to some extent, biased this field of research toward mechanisms that are possible to assess, thereby preventing a clear discernment of the whole picture sketching out. In particular, one blatant example is the direct correspondence often used between astrocytic activation and Ca^{2+} signaling, which is most certainly attributable to the availability of chelators that are specific to Ca^{2+} as opposed, for instance, to Na^+ or K^+.

SUMMARY AND CONCLUSIONS: WHAT ELECTROPHYSIOLOGY CAN AND CANNOT ASSESS IN NEUROGLIAL INTERACTIONS

Excitability of astrocytes has been proposed to lie primarily in their dynamic Ca^{2+} signaling, rather than their electrical

responses. Thus, in the last decades, to unravel the neuroglial dialog engaged in processing brain information, the main focus of physiologists from the glia field has been Ca^{2+} signaling using imaging at the levels of astroglial microdomain, single cells and networks. This body of work has provided a wealth of valuable information which has tremendously advanced our understanding of neuroglial interactions. However, signaling in astrocytes also includes other ionic players. Indeed, membrane depolarization induced by neuronal activity was the first activity dependent signal identified in glia (Orkand et al., 1966). Since then, astroglial ionic responses other than Ca^{2+} received less attention because of their slow time scale, the passive membrane properties of glia, and the lack of selective tools to assess their functional consequences. However, recent data have revealed that astrocytes express on their membrane a variety of ion channels, transmitter receptors and transporters, which mediate alternative signaling pathways, via for instance Na^{+}. In addition, molecular tools targeting specific glial ionic channels have been developed. Thus, the simplistic view of astrocytes as passive cells that express only leak K^{+} channels undergoing passive membrane potential fluctuations needs to be updated. The information reviewed herein show that electrophysiology is a valuable online technique, which has provided major insights on the dynamic neuroglial ionic dialogue mediating information processing at the cellular and molecular level. In particular, dual recordings of synaptically evoked neuronal and astroglial responses have generated information about concomitant alterations in the activity of pre- or postsynaptic elements and associated astrocytes. Thanks to their ionic signaling, astrocytes are now promoted to both, good electrophysiological readouts and important regulators of synaptic activity (Diamond et al., 1998; Lüscher et al., 1998; Djukic et al., 2007; Henneberger and Rusakov, 2012; Pannasch et al., 2012b). The astroglial depolarization evoked synaptically is a direct measure of the increase in extracellular K^{+} levels (Amzica, 2002), occurring as a result of presynaptic action potential firing and subsequent postsynaptic depolarization (Poolos et al., 1987). Thus, astroglial membrane potential dynamics is a good sensor for changes in presynaptic excitability, postsynaptic activity, extracellular space volume and K^{+} buffering capacities (Amzica, 2002; Pannasch et al., 2012a). Alternatively, the GLT current from astrocytes is a reliable detector of glutamate release from presynaptic terminals, and can thus monitors short-term changes in release probability (Bergles and Jahr, 1997; Diamond et al., 1998; Lüscher et al., 1998). Such transporter currents may also reflect the level of astroglial synapse coverage, which is known to be plastic during various physiopathological conditions (Wenzel et al., 1991; Hawrylak et al., 1993; Genoud et al., 2006; Lushnikova et al., 2009; Oliet and Bonfardin, 2010), and are thus good indicators of morphological and functional neuroglial interactions. But astroglial membrane potential dynamics and GLT currents do not only reflect synaptic activity. They are also regulated during development and in numbers of physiopathological conditions, including by neuronal activity or epilepsy, *via* changes in the expression, localization and function of K_{ir} channels and GLT, respectively, and can thus directly affect neighboring synaptic activity and plasticity (Djukic et al., 2007; Tzingounis and Wadiche, 2007; Jabs et al., 2008; Benediktsson et al., 2012). Therefore, combining electron microscopy, biochemistry or imaging to electrophysiolology is now crucial to decipher whether ionic changes detected on astroglial membranes just reflect, or rather cause, alterations in neuronal activity.

LIMITS AND PERSPECTIVES

Up to now, electrophysiological whole cell recordings from astrocytes are primarily performed at the level of the soma. Such recordings allow detection of currents which mostly originate from the cell soma or proximal processes, and whose identity is still unclear. Somatic passive conductances are thought to be responsible for the major leak of currents occurring in response to somatic current injections applied to perform IV curves. These passive currents likely subtend the typical low membrane resistance of astrocytes. Determining the components of somatic passive conductances is therefore a major issue to reliably assess the electrophysiology of mature astrocytes, especially in tissues. K2P (Seifert et al., 2009; Zhou et al., 2009) and gap junction channels (Seifert et al., 2009; Olsen, 2012) have been proposed to contribute to passive currents, although the involvement of connexin channels is controversial (Schools et al., 2006; Wallraff et al., 2006; Pannasch et al., 2011). Identifying the nature of somatic passive currents would open the possibility of deterring such major conductances, hence increasing the astroglial membrane resistance, and thereby unmask small non-passive activity dependent conductances that may be involved in sensing and modulating synaptic activity. In addition, novel pharmacological or genetic tools are also needed to unravel the role of non-passive conductances in astroglial physiopathology and neuronal activity.

Thus, basal activity of channels and receptors in fine distal perisynaptic astrocyte processes (PAPs) is currently hardly detectable, due in part to the low spatial and temporal control of membrane currents and potentials by patch clamp recordings of astrocytes *in situ* (Zhou et al., 2009). Such limit is unfortunate, because the surface of the tiny astroglial processes exceeds by far the membrane area of the soma and main processes, and the PAPs are the most interesting *loci* with regard to astroglial regulation of neurotransmission as they contain the functionally relevant channels, transporters and receptors, such as $K_{ir}4.1$ channels and GLT, which are likely the crucial players in neuroglial interactions and synaptic modulation. Therefore, dual patch clamp recording of astrocytes and neurons cannot be used to study the dialog between individual synapses and neighboring fine astroglial processes occurring during basal spontaneous activity. Instead, such recordings may be useful to investigate the integration by astrocytes of coordinated activity from neuronal assemblies occurring particularly during afference stimulation. It is thus no wonder that isolation of synaptically activated currents in astrocytes, such as GLT currents, is delicate; it requires the use of pharmacology, to inhibit the main K^{+} conductances and it is also often necessary to boost synaptic activity in order to increase the observable astroglial response. However, such manipulations results in experimental conditions drifting away from the physiological situation and may thus not relate to the native neuroglial dialog occurring in basal conditions. In addition, the actual time course of astroglial glutamate clearance derived from

the recorded GLT current can be partially obscured by current filtering, which distorts their kinetics, due to diverse factors such as astroglial electrotonic properties and asynchronous transmitter release. Nevertheless, methods utilized to extract the temporal features of the filtering mechanisms can be used to derive the actual glutamate clearance time course in physiological or pathological situations, as recently performed (Diamond, 2005; Scimemi et al., 2009; Pannasch et al., 2011).

Ideally, to investigate local astroglial ionic currents triggered by basal synaptic transmission, patch clamp recordings from fine astroglial processes should be developed, in a similar fashion to what is now currently performed on dendrites (Davie et al., 2006). Although patching fine perisynaptic astroglial processes will most likely be challenging because of their tiny size, it would permit to decipher the intimate communication ongoing between astroglial microdomains and individual synapses. However, to be detected, electrophysiological responses recorded from individual astroglial processes would need to display an amplitude above threshold detection (∼5 pA), because electrical noise can reach ∼2–4 pA in patch clamp recordings. Alternatively, the use of voltage sensitive dyes could reveal the heterogeneity of membrane potentials in astrocytes, and help defining whether fine perisynaptic astroglial processes play active functions mediated by K^+ channels, enriched in such processes. Yet, dual electrophysiological recording from astrocytes and neurons offers quantitative information about all ionic currents, and thus strikes as being a unique and efficient method to dissect online the dynamics of neuroglial ionic signaling and its role in information processing.

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Comparison of unitary exocytic events in pituitary lactotrophs and in astrocytes: modeling the discrete open fusion-pore states

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In regulated exocytosis the merger between the vesicle and the plasma membranes leads to the formation of an aqueous channel (a fusion-pore), through which vesicular secretions exit into the extracellular space. A fusion pore was thought to be a short-lived intermediate preceding full-fusion of the vesicle and the plasma membranes (full-fusion exocytosis). However, transient exocytic events were also observed, where the fusion-pore opens and closes, repetitively. Here we asked whether there are different discrete states of the open fusion-pore. Unitary exocytic events were recorded by the high-resolution cell-attached patch-clamp method in pituitary lactotrophs and brain astrocytes. We monitored reversible unitary exocytic events, characterized by an on-step, which is followed by an off-step in membrane capacitance (C_m), a parameter linearly related to the membrane area. The results revealed three categories of reversible exocytic events (transient fusion-pore openings), which do not end with the complete integration of the vesicle membrane into the plasma membrane. These were categorized according to the observed differences in the amplitude and sign of the change in the real (*Re*) parts of the admittance signals: in case I events ($Re \approx 0$) fusion pores are relatively wide; in case II ($Re > 0$) and case III ($Re < 0$) events fusion pores are relatively narrow. We show that case III events are more likely to occur for small vesicles, whereas, case II events are more likely to occur for larger vesicles. Case III events were considerably more frequent in astrocytes than in lactotrophs.

Keywords: capacitance measurements, equivalent circuit, transient fusion-pore, modeling, astrocytes

INTRODUCTION

In regulated exocytosis the fusion between the vesicle and the plasma membrane is important not only for the secretion of signaling molecules, such as hormones and transmitters, but is also key for the membrane recycling and for the translocation of membrane receptors and other proteins to the plasma membrane (White, 1992; Jahn et al., 2003; Vardjan et al., 2009). The membrane fusion process is an energetically unfavorable event, since it consists of bringing close to each other negatively charged opposing membranes, and involves the bending of a lipid-bilayer into a highly curved structure, which is assembled by different proteins and lipids (Kozlov and Markin, 1983). Following the merger of the two membranes, an important step in regulated exocytosis is the formation of a fusion-pore, an aqueous channel connecting the vesicle lumen and the exterior of the cell (reviewed in Chernomordik and Kozlov, 2008).

The nature of this structure can be studied by the electrophysiological patch-clamp membrane capacitance (C_m) technique, which allows monitoring interactions of a single vesicle with the plasma membrane (Neher and Marty, 1982; Lindau and Neher, 1988; Lollike et al., 1995), and the dynamics of an individual fusion-pore. In addition to simple discrete step increases in C_m, which were considered to represent full-fusion exocytic events (Neher and Marty, 1982), some of the unitary exocytic events are transient in nature, characterized by repetitive discrete on-steps (increases) followed by an equal amplitude off-steps (decreases) in C_m. The increment and the ensuing decrement step in C_m are considered to be due to a transient fusion-pore opening of a fused vesicle (Heuser and Reese, 1973; Alvarez de Toledo et al., 1993; Vardjan et al., 2007). These events were also termed transient or reversible exocytic events, also ≫kiss-and-run≪ exocytosis. Transient fusion-pore openings are considered physiologically relevant, since the fusion-pore is not formed upon each round of exocytosis (Ceccarelli et al., 1972; Kozlov and Markin, 1983). Moreover, a narrow fusion-pore, once it is established, appears to be energetically relatively favorable (Jorgačevski et al., 2010, 2011).

These conclusions were made on the basis of monitoring repetitive unitary exocytic events in which the narrowness of the fusion-pore can be determined from optical

and electrophysiological measurements (Vardjan et al., 2007; Jorgačevski et al., 2008). In electrophysiological measurements specifically, by determining fusion-pore conductance (G_p) and vesicle capacitance (C_v) from admittance measurements (Lindau, 1991; Lollike and Lindau, 1999). In these recordings, changes observed in the imaginary (ΔIm) and in the real (ΔRe) parts of admittance signals reflect changes in C_v and G_p, which can be used to determine vesicle diameter and fusion pore diameter (Lindau, 1991; Rosenboom and Lindau, 1994). Occasionally, the admittance traces associated with a transient fusion-pore opening exhibit an incremental or a decremental cross-talk in the *Re* signal (Breckenridge and Almers, 1987; Lindau, 1991; Henkel et al., 2000). However, the underlying mechanisms responsible for these non-zero projections in the *Re* signal are not fully understood.

In previous studies, the equivalent circuit of the fusion pore was reported (Lindau and Neher, 1988; Scepek and Lindau, 1993; Lollike et al., 1995). It was shown analytically that the incremental cross-talk projection on the *Re* signal could be due to the fusion-pore opening devoid of complete vesicle membrane integration into the plasma membrane (Lindau, 1991; Henkel et al., 2000). By studying pituitary lactotrophs, an ideal cell preparation to study secretory activity at the single vesicle level (Stenovec et al., 2004; Vardjan et al., 2007), and astrocytes which release gliotransmitters (Parpura et al., 1994) by likely employing regulated exocytosis (Parpura and Zorec, 2010), we compared the properties of unitary exocytic events. Using equivalent circuit analysis, we here demonstrate that the decremental cross-talk projection on the *Re* signal depends on the G_p as well as on the size of the fused vesicle. Moreover, these results indicate the existence of a very narrow, nearly closed, open fusion-pore state in pituitary lactotrophs and in astrocytes.

MATERIALS AND METHODS

CELL CULTURES

Primary lactotroph and astrocyte cultures were prepared from adult male (lactotrophs) and 2–3 days old female Wistar rats as described previously (Schwartz and Wilson, 1992; Ben-Tabou et al., 1994; Jorgačevski et al., 2008). After the isolation we plated cells on poly-L-lysine-coated coverslips and maintained them in high-glucose DMEM (Invitrogen) medium, supplemented with 10% newborn calf serum and 2 mM L-glutamine in an atmosphere of humidified air (95%) and CO_2 (5%). We cared for the experimental animals in accordance with the International Guiding Principles for Biomedical Research Involving animals, developed by the Council for International Organizations of Medical Sciences, and the Directive on Conditions for Issue of License for Animal Experiments for Scientific Research Purposes (Official Gazette of the Republic of Slovenia 40/85 and 22/87). The procedures using animals were approved by the Veterinary Administration of the Republic of Slovenia (approval no. 34401-29/2009/2).

ELECTROPHYSIOLOGY

Cell-attached capacitance measurements on isolated rat lactotrophs and astrocytes were performed with a dual-phase lock-in patch-clamp amplifier (SWAM IIC and SWAM CELL, Celica, Ljubljana, Slovenia) as described (Kreft and Zorec, 1997; Vardjan et al., 2007; Jorgačevski et al., 2010). Briefly, a sine wave voltage (1591 or 6400 Hz, 111 mV) was applied to the pipette, while holding the pipette potential at 0 mV. The phase of the lock-in amplifier was adjusted to nullify the changes in *Re*. A 10 fF calibration pulse was manually generated every 10 s to ensure correct phase angle settings. We used thick-walled, fire polished glass pipettes, which were heavily coated with a resin (Sylgard®184) and had a resistance of 2–5 MΩ.

THE EQUIVALENT CIRCUIT OF A TRANSIENT FUSION PORE

A patch-clamp configuration could be approximated by the series combination of the membrane and the access resistance (R_A) of the pipette tip through the patch (**Figure 1A**). The membrane included a parallel setup of the whole-cell C_m and the membrane conductance (G_M). When a small vesicle fused with the patch region, the patch-clamp system could detect the fusion event by the observed changes in the admittance measurement. It has been previously confirmed that the fusion of a vesicle was accompanied by an increase in the measured C_m (Neher and Marty, 1982). The equivalent circuit of the patch-configuration with a fused vesicle is shown in **Figure 1B**. The vesicle C_m is denoted by C_v, and the fusion-pore conductance is denoted by G_p.

In the present analysis, all the parameters except G_p were held constant. It was assumed that $C_v << C_m$ or $\omega C_v << 1/R_A$, which are reasonable assumptions, since the vesicle C_m (vesicle surface area) is considerably smaller than the cell C_m (cell surface area), and the patch resistance is considerably greater than the capacitor load of the vesicle (ωC_v). The admittance change (ΔY) describes the change in the admittance between the open and nearly closed state of the fusion pore. According to the equivalent circuit, the admittance difference is (Lindau, 1991):

$$\Delta Y = T^2(\omega)\left(\frac{(\omega C_v)^2/G_p}{1+(\omega C_v/G_p)^2} + i\frac{\omega C_v}{1+(\omega C_v/G_p)^2}\right), \quad (1)$$

where $T^2(\omega)$ stands for the factor $T^2(\omega) = 1/(1 + R_A G_M + i\omega C_m R_A)^2 = |T(\omega)|^2 \cdot e^{i\theta}$, and i is $\sqrt{-1}$. The admittance change

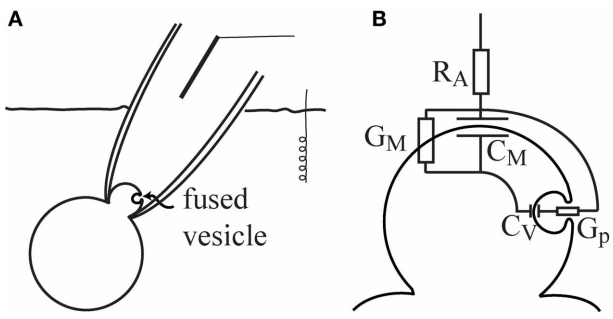

FIGURE 1 | The equivalent circuit of a patched membrane with a fused vesicle. The cell-attached patch-clamp configuration enables the detection of vesicle fusion in the patched membrane region **(A)**. In the equivalent circuit, the fusion of a vesicle is considered in parallel to the patch membrane **(B)**. Note that R_A is the access resistance, C_m is the whole-cell membrane capacitance, G_M is the membrane conductance, C_v is the vesicle membrane capacitance, and G_p is the fusion-pore conductance.

(ΔY) is an imaginary number, in which the first term in the parenthesis is the ΔRe, and the second term in the parenthesis is the ΔIm. For the sake of simplicity, the fusion pore state is denoted as fully open or incompletely open. In the fully open state, the G_p is infinite ($G_p \rightarrow \infty$), and the ΔRe in Equation (1) vanishes, which gives $\Delta Y = iT^2(\omega)\omega C_v$. On the other hand, when there is incomplete fusion, the G_p can be on the same scale as ωC_v. As a result, both the ΔRe and ΔIm have a finite value, which can be used for the calculation of the unknown G_p (Breckenridge and Almers, 1987). The C_v and the G_p can be obtained from the real and imaginary parts (Lindau, 1991; Lollike and Lindau, 1999), as follows:

$$C_v = \frac{\Delta Re^2 + \Delta Im^2}{\Delta Im}/\omega$$
$$G_p = \frac{\Delta Re^2 + \Delta Im^2}{\Delta Re}. \quad (2)$$

RESULTS

ADMITTANCE MEASUREMENTS OF THE THREE CASES OF TRANSIENT EXOCYTIC EVENTS

The admittance measurements of repetitive opening and closure of fusion-pores were obtained in lactotrophs (**Figures 2Ai,ii**) and in astrocytes (**Figures 2Biii,iv**), where representative transient exocytic events are shown, denoted as cases I, II, and III on **Figure 2**. The asterisks in these recordings indicate calibration pulses which were used to adjust the phase of the lock-in amplifier. The events in panels **Figures 2A,B** were recorded in the same membrane patch of a lactotroph and an astrocyte, respectively. Using Equation (2), the corresponding vesicle surface area in fF is calculated by incorporating ΔIm and ΔRe estimated for each event from the admittance records. On **Figure 2A** (recorded in lactotrophs) the C_v in case I, case II, and case III, was 0.9, 4, and 1.2 fF, respectively. Whereas, on **Figure 2B**, where representative recordings in astrocytes are shown, the C_v was in case I (0.3 fF), case II (1.6 fF), and case III (0.6 fF). One can note that the amplitude in *Im*, reflecting C_v, appears larger in events of case II in comparison with case I events. Case I events exhibit an incremental change in *Im* trace and are devoid of projection on the *Re* trace. In case II events and increment in *Im* trace is associated with an incremental projection on the *Re* trace, whereas, in case III events an increment in *Im* trace is associated with a decremental projection on the *Re* trace. In **Figure 2C** the three cases of transient exocytic events are diagrammatically presented as pre-fused vesicles with an initial narrow fusion-pore (narrower than the detection limit of the recording system), which can reversibly widen to a larger diameter—a state that can be detected electrophysiologically.

The experimental datasets used in our analysis, include ΔRe and ΔIm of cases I–III obtained in lactotrophs (**Figures 3A–D**) and astrocytes (**Figures 3E–H**). The G_p of each event is calculated from ΔRe and ΔIm of cases II (ΔRe incremental) and III (ΔRe decremental). **Figures 3A–H** shows scatter plots of ΔRe and ΔIm as a function of *Gp*. The relationships between ΔIm versus ΔRe are plotted for the datasets of incremental and decremental ΔRe projections. A strong linear relationship ($r = 0.79$ in **Figure 3C**; $r = 0.75$ in **Figure 3G**) was revealed between ΔIm and ΔRe in

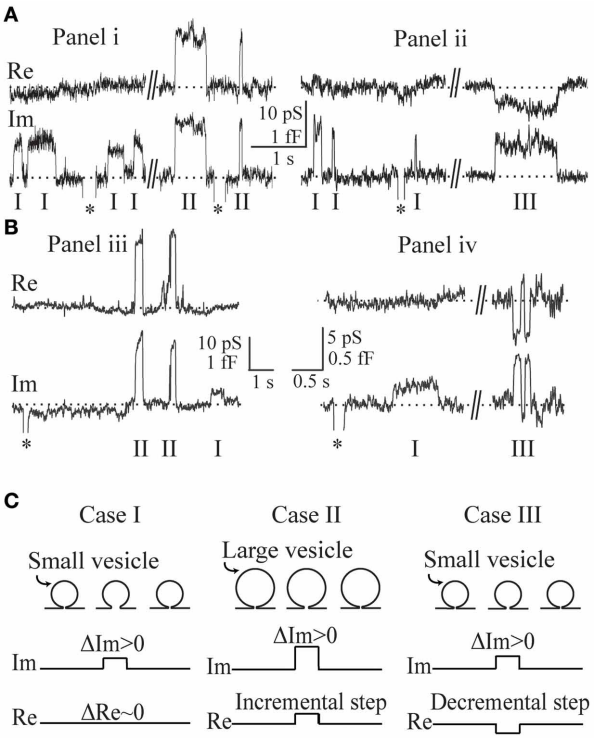

FIGURE 2 | Representative admittance measurements recorded in lactotrophs (Ai,ii) and astrocytes (Biii,iv) revealing a zero projection (case I), an incremental projection (case II), and a decremental projection (case III), on the *Re* part of the admittance signal. The asterisks in these recordings indicate calibration pulses. The recordings in **(Ai,ii)** and **(Biii,iv)** are recorded in the same patch of a lactotroph and an astrocyte, respectively. By incorporating the estimated ΔIm and ΔRe into the Equation (2), the corresponding vesicle capacitance amplitudes are determined for each event from the admittance records. For the recording in lactotrophs **(Ai,ii)**, the vesicle capacitance of case I, case II, and case III, is 0.9 fF, 4.0 fF, and 1.2 fF, and for the astrocytes **(Biii,iv)**, it is 0.3 fF, 1.6 fF, and 0.6 fF, respectively. Note the larger vesicle capacitance of case II when compared to case I. A schematic representation of the three cases as pre-fused vesicles with an initial narrow fusion-pore **(C)**.

the incremental ΔRe projection group (**Figures 3C,G**). The slope of this relationship in astrocytes is smaller than one (**Figure 3G**), however that in lactotrophs is close to unity (**Figure 3C**), suggesting that ΔRe and ΔIm are of similar size. The non-zero ΔRe can be attributed to the incomplete opening of the fusion-pore, which is accompanied by the increased fusion-pore resistance, whereas, the fully open fusion-pore exhibit a negligible resistance. On the other hand, in the decremental ΔRe projection datasets, a weak correlation between ΔIm and ΔRe was revealed ($r = 0.49$ in **Figure 3D**; $r = 0.08$ in **Figure 3H**). We noted that the datasets of decremental *Re* projections are clustered more in the range of small G_p (**Figures 3B,F**).

REPRODUCING THE THREE CASES OF ADMITTANCE MEASUREMENTS

Next, we considered the mechanisms responsible for the incremental and decremental ΔRe projections in the admittance measurements of the two cell types. According to the equivalent electrical circuit, the change in the real (ΔRe) and imaginary

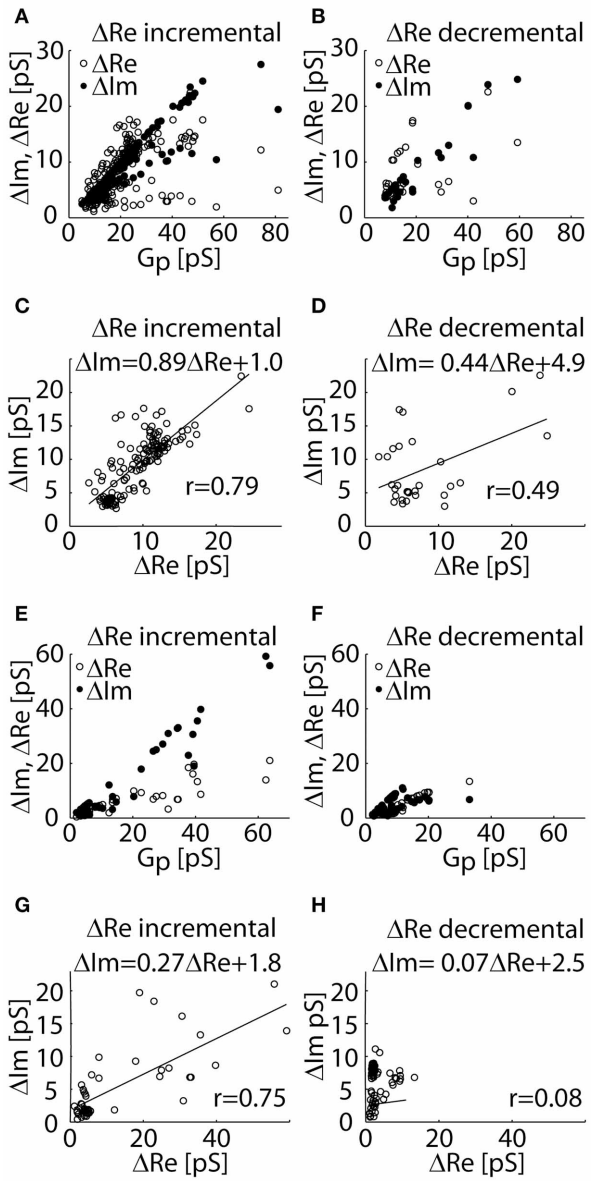

FIGURE 3 | The experimental datasets of Δ*Re* and Δ*Im* of admittance measurements recorded in lactotrophs (A–D) and astrocytes (E–H) incremental (case II) and decremental (case III) Δ*Re* projections. The distributions of Δ*Re* and Δ*Im* as a function of G_p for the datasets of incremental Δ*Re* **(A,E)** and for the decremental Δ*Re* **(B,F)** datasets. A scatter plot of Δ*Im* against Δ*Re* reveals a strong linear relationship in the incremental Δ*Re* datasets of projections **(C,E)**, and a weak linear correlation in the dataset of decremental Δ*Re* projections **(D,H)**.

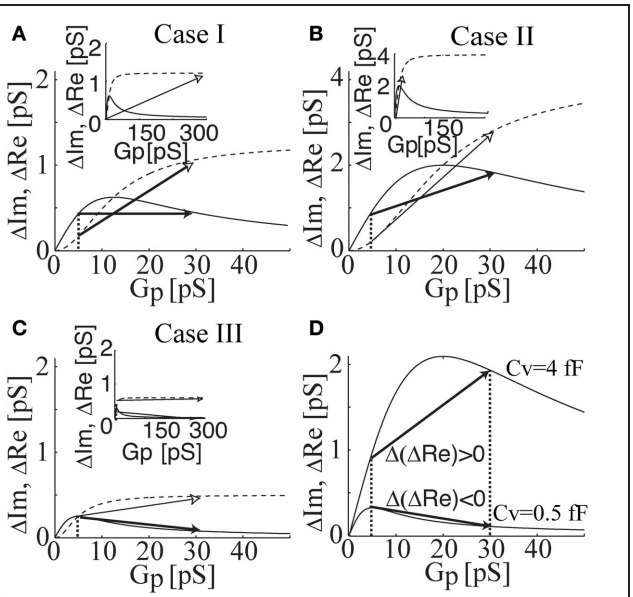

FIGURE 4 | The relationships of Δ*Re* (solid lines) and Δ*Im* (dahsed lines) as a function of G_p calculated for intermediate (C_v = 1.2 fF; case I), large (C_v = 4 fF; case II), and small (C_v = 0.5 fF; case III) vesicle size. Note that the change in Δ*Re* and Δ*Im* are drawn by a full and an open arrow, respectively. The zero projection on the *Re* part is reproduced by a change from a fully closed state to a fully open fusion-pore state (see inset) or by a change from a nearly closed state to an incompletely open fusion-pore state **(A)**. The incremental projection in case II is reproduced by a change from nearly closed to incompletely open state **(B)**. The decremental projection in case III can be reproduced, only when the pre-fused state is nearly closed **(C)**. In addition, the change in Δ*Re* can be considerably larger than the change in Δ*Im* (see inset). Overlay of Δ*Re* (*Gp*) of cases I and III reveals the effect of vesicle size on the projection type **(D)**. By having the same pre-fused state (i.e., the same fusion pore conductance), the incomplete fusion-pore opening of a large vesicle size leads to an incremental projection, whereas, the same incomplete opening of a small vesicle causes a decremental projection in *Re*.

(Δ*Im*) parts of admittance signals can be employed for the calculation of C_v and G_p (Lindau, 1991; Lollike and Lindau, 1999).

In **Figure 4**, Δ*Re* and Δ*Im* are plotted as a function of G_p calculated for intermediate ($C_v = 1.2$ fF), large ($C_v = 4$ fF), and small ($C_v = 0.5$ fF) vesicles, representing events of case I, II, and III, respectively. The fact that in case I there is no projection on the *Re* trace can be reproduced by a change from a fully closed fusion-pore state ($G_p = 0$ pS) to a fully open fusion-pore state (e.g., $G_p > 500$ pS in lactotrophs and $G_p > 70$ pS in astrocytes) or by a change from a nearly closed fusion-pore state (e.g., $G_p = 5$ pS in lactotrophs and $G_p = 2$ pS in astrocytes) to an incompletely open fusion-pore state (e.g., $G_p = 30$ pS) (**Figure 4A**). The positive projection in case II can be due to fusion-pore opening from a fully closed state, or a nearly closed state, to an incompletely open state (**Figure 4B**). On the other hand, the negative projection in case III can be reproduced only when the pre-fused state is nearly closed (**Figure 4C**), which suggests the possible existence of a nearly closed state of the fusion-pore. The underlying hypothesis for the different transient exocytic event cases is that the incremental and decremental projections to the *Re* trace are due to differences in the fused vesicle size and its nearly closed fusion-pore state. According to the presented equivalent circuit, small vesicles will tend to exhibit decremental Δ*Re* projections when their initial fused state is nearly closed. In **Figure 4D**, the relationships of Δ*Re* as a function of G_p of a small and a large vesicle are demonstrated. The overlay of the two relationships reveals that the maximum of Δ*Re* is at a lower G_p ($G_p = 5$ pS) for the small vesicle than the counterpart ($G_p = 20$ pS) in the case of the large vesicle. Assuming that initial vesicle fusion status arises from the

same nearly closed state (e.g., $G_p = 5$ pS), the incomplete opening of the fusion pore would lead to a positive projection in the case of the large vesicle and a decremental projection in the ΔRe in the case of the small vesicle (**Figure 4D**).

DISCUSSION

In the present paper, we analyzed the discrete open fusion-pore states as well as the conditions under which these states existed. Three different cases are categorized, in which the transitions between discrete states of the fusion-pore do not end with the complete fusion (i.e., exocytosis) of a vesicle and the plasma membrane.

These cases are evident from the changes in the real (ΔRe) and imaginary (ΔIm) parts of admittance measurements (**Figure 2**). In the first case (denoted as case I), the event is characterized by a step increase in ΔIm and an approximately zero ΔRe. In the second case (denoted as case II), both ΔIm and ΔRe are exhibiting a step increase. The third case (denoted as case III) has an incremental ΔIm and a decremental ΔRe. The underlying assumption of our model is that the non-zero ΔRe is due to an incomplete vesicle fusion-pore opening, and that the initial status of the fused vesicle may exhibit a non-zero G_p (**Figure 2**). The equivalent circuit of a patch-clamp configuration was constructed, in which the fused vesicle is considered in parallel to the cell plasma membrane (**Figure 1**). The fused vesicle is assumed to be connected to the cell membrane via an aqueous channel (the fusion-pore). The C_v and the G_p are derived from the imaginary (ΔIm) and real (ΔRe) parts of the admittance measurements. The experimental data of ΔRe and ΔIm are obtained from admittance measurements in lactotrophs and astrocytes (**Figure 2**). In case II, a strong linear relationship between ΔRe and ΔIm suggests that the fusion-pore opening of a vesicle is incomplete (**Figures 3C,G**). In case III, there is a weak correlation between ΔRe and ΔIm (**Figures 3D,H**). According to the relationships of ΔRe and ΔIm as a function of G_p, it is demonstrated that the decremental *Re* projection is more likely to occur for small vesicles (**Figure 4C**). Finally, the present calculations reveal that while the incomplete opening of the fusion-pore may be accompanied by the same change in fusion-pore conductance, the resulted projection is predicted to be decremental for the relatively small vesicle and incremental for the large vesicle (**Figure 4D**).

The high bending energy during the formation of a fusion-pore can be overcome by the assembly of curvature membrane constituents (proteins and lipids) (Kozlov and Markin, 1983; Jorgačevski et al., 2010; Kabaso et al., 2012; Jesenek et al., 2012). The stability of the fusion-pore of a fused vesicle can be due to anionic lipids of negative spontaneous curvature, modulating the formation of the fusion pore (Coorssen and Rand, 1990; Chen and Rand, 1997; Churchward et al., 2008; Rituper et al., 2012). It is then possible that the density of these curvature membrane constituents can affect the G_p of the nearly closed fusion-pore state as well as the projection type and amplitude. The possible incomplete fusion-pore state opens a communication venue, in which the passage of small molecules such as ions may be facilitated continuously through the narrow pore. However, larger molecular weight molecules are unable to exit the narrow fusion pore.

What appears interesting is that in both cell types, electrically excitable (pituitary lactotrophs) and electrically non-excitable (brain astrocytes), fusion-pore properties appear to be shared. While vesicles in the lactotrophs exhibit larger diameters and are therefore more accessible to experimentation (Stenovec et al., 2004; Vardjan et al., 2007), secretory vesicles in astrocytes appear to exhibit relatively large and relatively small diameters. The latter ones can be revealed by the higher-resolution cell-attached patch-clamp measurements (Kreft and Zorec, 1997). Interestingly, it is the smaller ones that exhibit fusion-pores with extremely narrow fusion-pore diameters (**Figure 4**). The probability of observing an increment in *Im* trace, associated with a decremental change in *Re* trace, indicates that a relatively large fraction of vesicles, which are already fused with the plasma membrane, exhibit a very narrow fusion pore. These may pass protons as has been reported previously by using a pH-sensitive vesicle luminal fluorophore in lactotrophs (Vardjan et al., 2007) and in astrocytes (Malarkey and Parpura, 2011). However the relatively large abundance of these events recorded in astrocytes, may not mean that fusion-pore openings mediate a productive release of gliotransmitters. A G_p of less than 5 pS means that the fusion pore diameter is less than 0.2 nm, too narrow to pass even glutamate or acetylcholine (Vardjan et al., 2007). These results are consistent with the view that fusion-pores, when they are established, are relatively stable structures (Jorgačevski et al., 2010). The regulation of exocytotic release of hormones and transmitters, thus involves also the regulation at the fusion-pore level, at the level, when the fusion-pore has been already established, but is too narrow to functionally contribute to the exit of secretions form the vesicle lumen. In the present paper we have revealed that fusion-pores may exhibit distinct fusion-pore diameters and the future work will have to address question of how these open fusion-states transit to a release productive state.

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Astrocytes: can they be the missing stars linking neuronal activity to neurofunctional imaging signals?

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Functional neuroimaging techniques are currently used in fundamental neuroscience as well as in cognitive neuroscience and clinics to study brain function at large spatial scales. They include Blood Oxygenation Level Dependent-functional Magnetic Resonance Imaging (BOLD-fMRI, Kim and Ogawa, 2012), the gold standard of functional neuroimaging techniques, and many optical techniques including functional Near InfraRed Spectroscopy (fNIRS) in human research or intrinsic/spectroscopic optical signal (IOS) imaging in animal research (Devor et al., 2012). These techniques rely on vascular signals [cerebral blood flow (CBF) and volume and metabolic rate of oxygen consumption] that constitute a proxy for neuronal activity because of the existence of functional hyperemia, a mechanism defined as the matching of vascular changes to the activity level in a given brain area (Iadecola and Nedergaard, 2007). To date, some crucial open questions remain concerning these functional signals, including: what kind of brain activity are they related to? What are their cellular/molecular sources? Can astrocytes, in addition to roles in translating neuronal into vascular activity, also be capable of generating functional neuroimaging signals on their own?

THE DISPUTED SOURCES OF BOLD: CORRELATION WITH SYNAPTIC vs. SPIKING ACTIVITY

Are BOLD signals correlated to local synaptic activity and/or to spiking activity? Answering this question will bring us a step forward in the understanding of functional connectivity within BOLD maps. Since Logothetis et al. (2001), who combined electrophysiological recordings with fMRI, BOLD signals were thought to be tightly correlated to Local Field Potentials (an electrophysiological signal related to changes in the input and local activities within a given brain structure) and "less correlated" with the output spiking activity. Viswanathan and Freeman (2007) have further shown that BOLD signals are correlated with synaptic activity. However, recent data by Lee et al. (2010) who combined optogenetics with fMRI have revealed that spiking activity can be a predictor of BOLD as efficiently as LFPs. These discrepancies can be due to the study of different species (primate in Logothetis et al. vs. rodent in Lee et al.), different brain structures (visual vs. motor area), and brain states (awake monkey vs. anesthetized mouse). Alternatively, since astrocytes bridge anatomically and functionally neurons with blood vessels (Iadecola and Nedergaard, 2007), they can be considered as an explanation for the coupling of neuronal activity with vascular signals. Indeed, astrocytic processes ensheath synapses as well as blood vessels. In addition, in response to neuronal activity, astrocytes are capable of gliotransmitting active molecules regulating synaptic, metabolic, and hemodynamic activities (Iadecola and Nedergaard, 2007).

Schulz et al. (2012) have recently evaluated how neuronal and astrocytic activities correlate with the BOLD signal using fiber-optic recordings of calcium dyes in the rat somatosensory cortex. They have recorded slow BOLD signals which are systematically accompanied by slow astrocytic calcium signals. This correlation at long time scales (peak activity >3 s following sensory stimulation onset) fits with the *in vivo* recordings of astrocytic calcium dynamics in anesthetized preparations from the ferret visual cortex (>3 s, Schummers et al., 2008) and the mouse olfactory bulb (>1 s, Petzold et al., 2008). Still, astrocytes are capable of fast calcium activity (peak activity 0.5–1 s following sensory stimulation onset) in the somatosensory cortex of anesthetized (Winship et al., 2007) or awake (Dombeck et al., 2007) mice. This faster time scale is in the range of functional hyperemia and related signals such as IOS.

This piece of literature concerning the cellular sources of BOLD shows that further work is needed to identify the local synaptic vs. the spiking activity as being the best correlate/predictor of BOLD as well as the neuronal and/or astrocytic components of these signals. But one thing is sure: BOLD is arising from the vascular compartment. Thus detailed anatomo-functional studies of the neurovascular unit have been undertaken to explore mechanisms making functional hyperemia possible, in order to find some clues about BOLD sources. These studies have led to many conflicting results and conclusions, especially concerning the glutamate signaling pathways which may contribute to activity-dependent regulation of local CBF (**Table 1**).

WHAT MOLECULAR KEYS FROM ASTROCYTES ARE NEEDED TO START THE VASCULAR ENGINE IN RESPONSE TO NEURONAL ACTIVITY?

Among the candidates responsible for functional hyperemia (Attwell et al., 2010; Petzold and Murthy, 2011) much attention has been focused on astrocytic metabotropic glutamate receptors (especially mGluR5) but the importance of calcium dynamics triggered by these receptors in astrocytic physiology is a matter of some debate (Agulhon et al., 2008; Calcinaghi et al., 2011). Astrocytic glutamate transporters (GluTs) constitute another exciting candidate for the function of the neurovascular unit. GluTs activity is required for: (1) glutamate

Table 1 | Examples of receptor candidates for the feed-forward triggering of CBF increase *in vivo*.

	Support a role in functional hyperemia	Argue against a role in functional hyperemia
Molecular candidates		
Neuronal and astrocytic metabotropic glutamate receptors	Takano et al. (2006, SC)	Calcinaghi et al. (2011, SC)
Neuronal and astrocytic ionic glutamate receptors	Offenhauser et al. (2005, Cb); Chaigneau et al. (2007, OB)	Petzold et al. (2008, OB); Scott and Murphy (2012, SC)

I have selected some significant references in the literature that support or argue against a role for each family of molecules in contributing to activity-dependent functional hyperemia in vivo. Cb, cerebellum; OB, olfactory bulb; SC, somatosensory cortex.

clearance and regulation of activity at excitatory synapses (2) glucose uptake to build up glycogen stores, synthetize glutamate, and produce lactate (3) glutamine recycling to restore presynaptic glutamate vesicles (Danbolt, 2001). GluTs are glutamate/sodium co-transporters. Intra-astrocytic sodium increases glucose uptake and subsequent transformation into lactate which is released in the extracellular medium. Lactate could serve both as an energetic substrate (Pellerin et al., 2007), a regulator of CBF (Gordon et al., 2008), and also as a mediator of metabolic information for synapses (Bergersen and Gjedde, 2012). Thus GluTs constitute a cross road for the coordinated control of information and energy processing (Martin et al., 2012). Can GluTs lead to functional signals? This question was addressed by two groups who impaired GluTs activity and followed changes in complex IOS signals. IOS were measured as red light absorption changes due to vascular changes in CBF and volume as well as light scattering. In both cases, the authors mapped sensory activity (rat olfactory bulb, Gurden et al., 2006; ferret visual cortex, Schummers et al., 2008) and demonstrated that ionotropic glutamate receptors inhibition did not affect IOS, whereas TBOA, a blocker of GluTs, did. In addition, Schummers et al. showed that astrocytic calcium signals were reduced by TBOA. This molecule also inhibits functional hyperemia in the olfactory bulb (Petzold et al., 2008). How GluTs activity is mechanistically linked to functional hyperemia is an open question. GluT-induced IOS (Gurden et al., 2006) and CBF changes (Petzold et al., 2008) were not mediated indirectly via glutamate receptors nor presynaptic GABA-B receptors. Sodium waves in the astrocytic network are triggered by GluTs (Bernardinelli et al., 2004) and it seems very likely that some interaction with calcium dynamics (Schummers et al., 2008) may be responsible for GluT-induced CBF changes. Interestingly, plasma level changes in lactate and pyruvate levels were found to be reflected in BOLD signals (von Pföstl et al., 2012) but these results will have to be confirmed during the activation of local brain networks. Finally it is not known whether any of the candidates responsible for functional hyperemia and/or for functional optical signals (including GluTs) are indeed related to BOLD signals. For example, no activation or impairment of a specific neurotransmitter system was undertaken during BOLD recordings.

Since astrocytes (1) express receptors for neuromodulators such as noradrenaline, which is known to regulate both the vascular tone (Hamel, 2006; Bekar et al., 2012) and astrocytic activity (Bekar et al., 2008) and (2) have their own specific calcium dynamics, which do not match in space and time the neuronal activity response to visual stimuli (Schummers et al., 2008), can they generate functional signals (Figley and Stroman, 2011)?

SOLVING THE CELLULAR BASIS OF FUNCTIONAL NEUROIMAGING SIGNALS: SPECIFIC NEURONAL AND ASTROCYTIC OPTOGENETICS COUPLED TO BOLD AND OPTICAL IMAGING

To further understand the roles of astrocytes vs. neurons in BOLD signals, the following approaches could be combined: (1) *in vivo* imaging of awake mice to avoid any anesthetic effects on neuronal and astrocytic activity (Thrane et al., 2012), (2) specific optogenetic targeting and stimulation of either astrocytes or excitatory neurons and interneurons and manipulate the temporal sequence of activation (3) fiber optics to stimulate and monitor activity dynamics in each of these cellular populations during BOLD recordings. All of these approaches exist in the literature separately. For example, Desai et al. (2011) have run experiments on awake mice in fMRI and Dombeck et al. (2007) have performed optical imaging of astrocytic activity in awake mice. Schulz et al. (2012) have recorded simultaneous BOLD and calcium signals in neurons and astrocytes using fiber optics. In addition, optogenetic stimulation of parvalbumin interneurons in the mouse barrel cortex (Kahn et al., 2011) or pyramidal cells in the motor cortex (Lee et al., 2010), with both type of cells expressing Channelrhodopsin-2 (ChR2), has been shown to produce BOLD signals. These studies establish a causal relationship between the activation of defined neuronal populations and BOLD signals. ChR2 optogenetics was also used in astrocytes by Gourine et al. (2010) who studied respiratory regulation in the brainstem. The next step is thus to express ChR2 specifically in cortical astrocytes and stimulate them to test whether they can lead to significant BOLD signals. New tools such as genetically engineered calcium sensors (GCaMPs) and effector proteins (ChRs) expressed in astrocytes (Figueiredo et al., 2011) will also make possible the accurate monitoring and the specific stimulation/inhibition of astrocytic activity during BOLD recordings. For the spatiotemporal control of cellular activation, electrical microstimulation (Tolias et al., 2005) and optogenetic (Lee et al., 2010; Kahn et al., 2011) stimulations were used. In addition to these techniques, DREADD ("Designer Receptor Exclusively Activated by a Designer Drug") would help to pinpoint specific transmitter systems involved in BOLD generation (Nawaratne et al., 2008). The prototype DREADD is a variant of a human muscarinic receptor that is no longer activated by its endogenous ligand, acetylcholine, but by exogenous clozapine-N-oxide, an inert metabolite of the antipsychotic drug clozapine (Dong et al., 2010). A combined chemical–genetic approach, in which mouse genetics limit the spatial expression of the DREADD to a specific cellular type and the designer

drug (intraperitoneal injection) provides temporal control over G protein–coupled receptors activity could help describing the links between activity of a particular receptor and BOLD signals.

In conclusion, determining the relative role of astrocytes vs. neurons in functional hyperemia and BOLD signals is still an exciting challenge. Since technical tools are now available, experiments should follow soon to lighten up the cellular dark side of BOLD.

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The role of astrocytes in CNS tumors: pre-clinical models and novel imaging approaches

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Brain metastasis is a significant clinical problem, yet the mechanisms governing tumor cell extravasation across the blood-brain barrier (BBB) and CNS colonization are unclear. Astrocytes are increasingly implicated in the pathogenesis of brain metastasis but *in vitro* work suggests both tumoricidal and tumor-promoting roles for astrocyte-derived molecules. Also, the involvement of astrogliosis in primary brain tumor progression is under much investigation. However, translation of *in vitro* findings into *in vivo* and clinical settings has not been realized. Increasingly sophisticated resources, such as transgenic models and imaging technologies aimed at astrocyte-specific markers, will enable better characterization of astrocyte function in CNS tumors. Techniques such as bioluminescence and *in vivo* fluorescent cell labeling have potential for understanding the real-time responses of astrocytes to tumor burden. Transgenic models targeting signaling pathways involved in the astrocytic response also hold great promise, allowing translation of *in vitro* mechanistic findings into pre-clinical models. The challenging nature of *in vivo* CNS work has slowed progress in this area. Nonetheless, there has been a surge of interest in generating pre-clinical models, yielding insights into cell extravasation across the BBB, as well as immune cell recruitment to the parenchyma. While the function of astrocytes in the tumor microenvironment is still unknown, the relationship between astrogliosis and tumor growth is evident. Here, we review the role of astrogliosis in both primary and secondary brain tumors and outline the potential for the use of novel imaging modalities in research and clinical settings. These imaging approaches have the potential to enhance our understanding of the local host response to tumor progression in the brain, as well as providing new, more sensitive diagnostic imaging methods.

Keywords: astrogliosis, brain metastases, glioma, nuclear imaging, MRI

Metastasis, the spread of cancer from the primary tumor site to distant organs, is the leading cause of cancer morbidity and mortality and 10–40% of all cancer patients will develop metastatic spread to the brain (Nussbaum et al., 1996). However, our understanding of brain metastasis is still incomplete, and the unique microenvironment of the CNS, both on a cellular and metabolic basis, means mechanistic insights from peripheral organs cannot be readily translated. The processes underlying the extravasation of neoplastic cells across the blood-brain barrier (BBB), their subsequent colonization of the perivascular space (Carbonell et al., 2009) and later the parenchyma, are yet to be fully characterized. Progress in this area is limited by the lack of robust *in vitro* assays that truly reflect the complex nature of the CNS. It is necessary, therefore, to develop better pre-clinical models, in tandem with sophisticated imaging modalities, to better allow the investigation of the pathogenic mechanisms underlying metastasis progression. In turn, imaging may identify novel biomarkers for early tumor detection and new therapeutic avenues.

Astrocytes are the most abundant member of the glial family and have multiple roles in the central nervous system. As well as providing structural support for neurons and the BBB, as outlined below, they play an integral role in maintaining CNS function, participating in synaptic activity, mediating ionic and transmitter homeostasis, and regulating blood flow. At the same time, astrocytes actively respond to challenges such as infection, injury, ischemia, and neurodegeneration, by changing their transcriptional profile and morphology in the process of reactive astrogliosis, which has been extensively reviewed (Pekny and Nilsson, 2005; Sofroniew, 2009; Middeldorp and Hol, 2011) and is characterized by up-regulation of glial fibrillary acidic protein (GFAP).

This review aims to detail the role of astrocytes in both primary and secondary CNS tumors, as determined from both *in vitro* studies and *in vivo* pre-clinical models. Secondly, novel imaging techniques, many of which have been successfully used in other neuropathologies, will be discussed in the context of investigating astrogliosis in CNS tumors, both *in vivo* and potentially in the clinic.

PRE-CLINICAL MODELS; MECHANISTIC INSIGHTS INTO THE ROLE OF ASTROCYTES IN CNS TUMORS

PRIMARY BRAIN TUMORS

Besides the malignant astrocytes that comprise many primary brain tumor sub-types, a role for *stromal* astrocytes in tumor

progression has been constructed through human biopsy samples, co-culture *in vitro* experiments and transgenic *in vivo* models. Broadly speaking, two main forms of malignant brain tumor exist, as classified according to cellular origin; oligodendrocytomas, comprised of oligodendrocytes, and astrocytic neoplasms, also known as gliomas, which can be further stratified into diffuse astrocytoma, anaplastic astrocytoma, and glioblastoma multiforme (GBM) (Louis et al., 2007). GBM consists of neoplastic astrocytes that are poorly differentiated, and is characterized by high invasive potential and angiogenesis. It is the most commonly diagnosed and aggressive glioma in adults and, hence, will be the focus of work described here.

Immunohistochemistry reveals reactive astrocytes surrounding glioma in both human biopsies (Nagashima et al., 2002) and murine models, as shown in **Figure 1**, and have been proposed to have pro-immunogenic roles. For instance, glioma associated astrocytes have a markedly different mRNA expression profile to normal astrocytes, primarily displaying components of the antigen presentation pathway, such as MHC Class II proteins (Katz et al., 2012). This observation suggests that astrocytes interact with "helper" T cells, leading to localized inflammation. Astrocytes surrounding human glioma biopsies secrete CCL2, a macrophage and T cell recruiting chemokine, with a strong positive correlation between CCL2 expression and T cell infiltration (Carrillo-De Sauvage et al., 2012). However, as with all aspects of the immune response to tumor growth, it does not necessarily follow that the presence of T cells leads to tumor cell clearance. Indeed, Barcia et al. demonstrate that although T cells infiltrate the GBM microenvironment, very few cytotoxic T cells (CTLs) make immunological synapses with tumor cells, whilst there is a greater population of potential regulatory T cells (Barcia et al., 2009), which attenuate immune responses.

Further evidence that this interaction downregulates immune function comes from co-culture experiments. Co-culture of either GBM astrocytes or normal human astrocytes with T cells, leads to a downregulation of IFN-γ production (Kostianovsky et al., 2008), hence inhibiting effector function. Additionally, astrocytes have been shown to directly induce T cell apoptosis via cell-cell contacts; astrocytoma derived astrocytes express Fas ligand (FasL) which interacts with Fas expressing cells, such as T cells, to induce cytolysis (Saas et al., 1997). This mechanism is not restricted to malignant astrocytes, but has been demonstrated in normal astrocytes (Bechmann et al., 1999, 2002), suggesting that astrocytes in the glioma periphery could also be involved in repressing anti-tumor immune function.

Interactions with microglia, as well as infiltrating monocytes, also indicate that astrocytes could play a role in modulating immune function in the tumor periphery. Both GBM derived malignant astrocytes and normal human astrocytes suppressed TNF secretion by microglia and monocytes, and also inhibited the ability of these cell types to activate T cells owing to downregulation of co-stimulatory molecules (Kostianovsky et al., 2008).

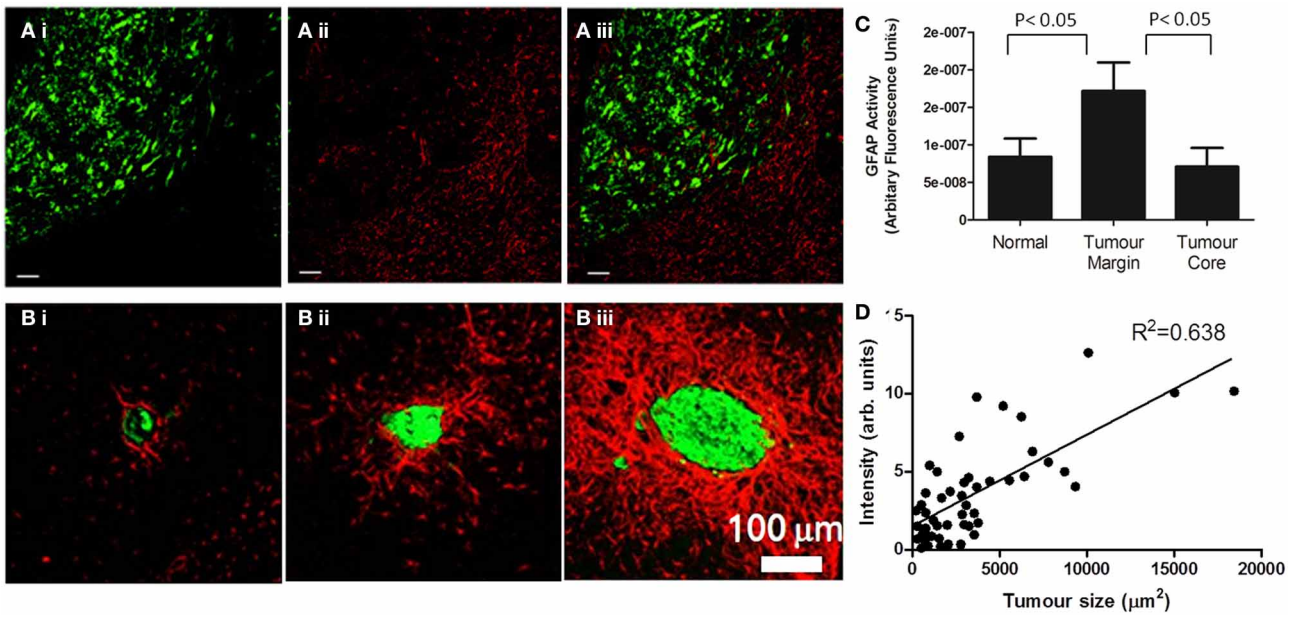

FIGURE 1 | Astrocyte activation, as determined by GFAP staining, is present in the peri-tumoral area of both primary (A) and secondary (B) tumors. (A) (i) In a mouse model of glioma, the growth of DBRTG glioma cells can be seen in green (GFP labeled), adjacent to a wall of astrocyte activation, seen in red (Alexa555 probe) (ii). The merged image (iii) indicates little infiltration of activated astrocytes into the tumor mass, as quantified by arbitrary fluorescence units of GFAP activity **(C)**. Figure adapted from Lee et al. (2011). **(B)** In a mouse model of lung derived brain metastasis, in which HARA-B cells were inoculated intra-cardially, astrocyte activation (Cy3 probe, red) was observed surrounding metastatic growth (Alexa488 probegreen). As with the glioma model, astrocytes are present in the tumor periphery, rather than the core. The extent of astrocyte activation increased with tumor size **(D)**. Figure adapted from Seike et al. (2011).

Interleukin-1β (IL-1β) has been identified in reactive astrocytes in the glioma periphery, suggesting an anti-tumor immune response (Nagashima et al., 2002). Conversely, *in vitro*, IL-1β has been implicated in inducing glioma invasion (Bryan et al., 2008; Huang et al., 2009), as well as promoting tumor growth via autocrine induction of TNF signaling (Chung and Benveniste, 1990; Adachi et al., 1992). Other astrocyte-derived cytokines, such as stromal derived factor-1(SDF-1), have also been identified *in vitro* as enhancing glioma growth (Barbero et al., 2003) and invasiveness (Zhang et al., 2005). Furthermore, this invasive phenotype has been demonstrated *in vivo* using a transgenic mouse model in which glioma is induced by platelet derived growth factor (PDGF) over-expression. In this study, stromal astrocytes in the peri-tumoral region were shown to express elevated levels of Tenascin-C (TN-C) (Katz et al., 2012), an extra-cellular matrix glycoprotein, previously shown to promote GBM invasion (Sarkar et al., 2006).

Matrix metalloproteinases (MMPs) have been proposed to play a key role in mediating glioma invasion through the parenchyma, and peri-tumoral reactive astrocytes have been shown, immunohistochemically, to express MMP2 in post-mortem samples (Nagashima et al., 2002). At the same time, *in vitro* co-culture experiments between human fetal astrocytes and human glioma cell lines (U251N and U87), indicate that soluble factors released by glioma cells cleave the MMP2 pro-enzyme to its active form (Le et al., 2003), demonstrating the dynamic interaction between GBM and the stroma. Angiogenesis is another key feature of GBM, and vascular endothelial growth factor (VEGF), a mediator of *de novo* vessel formation in glioma (Plate et al., 1993), has been observed in reactive stromal astrocytes (Nagashima et al., 1999, 2002). These findings suggest another route by which astrocytes drive tumorigenicity.

SECONDARY BRAIN TUMORS

As mentioned, the dissemination of cancer cells from primary tumors to the brain is a common end-point for cancer patients with metastatic disease and is associated with poor prognosis. Most frequently, metastatic cells that colonize the CNS are derived from lung tumors, followed by melanoma, breast and renal malignancies (Barnholtz-Sloan et al., 2004). The molecular mechanisms governing such pathologies are beyond the scope of this article, and are yet to be fully characterized, although specific gene signatures have been shown to be predictive of brain metastasis (Bos et al., 2009; Harrell et al., 2012; Lee et al., 2012). To understand the complexities of the brain metastatic process, and the involvement of astrocytes, we must first consider the BBB.

The BBB serves as both a physical and metabolic barrier, separating the interstitial fluid of the brain parenchyma from peripheral blood flow, and is critical for maintenance of the neural environment in the CNS. It is comprised of multiple cell types, but is primarily regulated by the endothelium and astrocytes, with specialized tight junctions between endothelial cells serving to exclude metabolites. The unique composition of the BBB also serves as an obstacle to circulating cancer cell extravasation. Colonization of the brain parenchyma is not just reliant on traversing the endothelium, but also the glia limitans, the layer of astrocytic endfoot processes that provide structural and metabolic support to the endothelium.

Astrocytes contribute not just to BBB structure, by contacting the vascular endothelium, but also to its genesis, inducing tight junction formation between endothelial cells (Goldstein, 1988), and up-regulating expression of transporters such as the brain specific glucose transporter, GLUT1 (Boado and Pardridge, 1990; McAllister et al., 2001). Astrocytes also subserve a metabolic barrier function, for instance, inducing expression of endothelial cell enzymes such as manganese superoxide dismutase, the inducible metabolizer of oxygen free radicals (Schroeter et al., 2001). Calcium signaling between the endothelium and astrocytes (Leybaert et al., 1998; Braet et al., 2001) suggests that endothelial changes may also have a dynamic effect on astrocyte function. Hence, one could hypothesize that astrocytes will be activated as early as the initial steps of adhesion of metastasizing cells to the vascular endothelium.

Sophisticated imaging of fluorescent metastatic cells, using multi-photon laser scanning microscopy (MPLSM), has allowed the stages of brain metastasis to be visualized *in vivo* (Kienast et al., 2010). The tortuous nature of the cerebral microvasculature enforces a reduction in the speed of circulating cells and arrest at vascular branch points is observed. Such arrested cells can reside on the luminal side of the brain endothelium for up to 5 days, in contrast to elsewhere in the body where metastasizing cells rapidly extravasate within 24 h (Lorger and Felding-Habermann, 2010). Using MPLSM, extravasation into the perivascular space appeared to occur via active transmigration through mechanically induced pores in the endothelial membrane, as previously hypothesized (Kawaguchi et al., 1982). In addition, it was demonstrated that growth within the perivascular space is dependent on contact with the abluminal endothelial membrane (Carbonell et al., 2009), as has also been demonstrated in glioma (Winkler et al., 2009). Only upon growth of macrometastases, will the glia limitans be breached, allowing spread of metastases into the parenchyma (Saito et al., 2007).

The characteristic response of astrocytes to injury, seen in numerous neuropathologies, including glioma, has also been identified as a feature of the brain metastatic microenvironment. A wall of reactive astrocytes has been reported around haematogenous brain metastases in human post mortem tissue (Zhang and Olsson, 1995; He et al., 2006), and recently there has been great interest in establishing murine mouse models of brain metastasis to determine astrocyte reactivity *in vivo* (Mendes et al., 2005, 2007; Fitzgerald et al., 2008; Lorger and Felding-Habermann, 2010; Seike et al., 2011), as demonstrated in **Figure 1**. Astrocytic responses have been demonstrated in BALB/c mice in response to both the syngeneic 4T1 mammary carcinoma cell line, as well as in SCID mice in the response to the human MDA-MB-435 cell line, as early as 3 days post metastatic induction via intra-carotid inoculation (Lorger and Felding-Habermann, 2010). Astrocyte reactivity was induced whilst MDA-MB-435 cells were still intra-vascular, in contact with the luminal endothelial membrane, and continued throughout extravasation and growth over a 50 days time course.

Likewise, Mendes et al. utilized a mammary carcinoma cell line, ENU1564, injected intra-cardially, to demonstrate astrogliosis in response to brain metastases in a rat model (Mendes et al., 2007).

Gliosis is not only found in response to metastases of mammary origin; Izraely et al. demonstrated astrocyte activation in nude mice in response to metastatic melanoma (Izraely et al., 2012) and Seike et al. demonstrated a wall of astrogliosis in nude mice in response to the human lung cancer cell, HARA-B. In this study, a positive correlation was found between tumor size and the extent of astrocyte activation and, interestingly, astrocyte activation was more robust in hippocampal metastases as compared to cortical metastases (Seike et al., 2011). Such differential astrocytic responses have also been observed in other disease models, such as intracerebral lipopolysaccharide (LPS) challenge (Espinosa-Oliva et al., 2011). These differential reactions may reflect the regional heterogeneity of astrocytes, both in terms of astrocytic density (which is greater in the hippocampus than the cortex), proliferation rates (which are higher in the hippocampal dentate gyrus than the cortex) (Emsley and Macklis, 2006) and molecular signature with regards to immune function. *In vitro,* hippocampal astrocytes have been shown to express higher levels of MHC Class II protein, Il-6 and ICAM-1 compared to cortical astrocytes, as well as displaying increased nitric oxide (NO) production (Morga et al., 1998), all of which one would hypothesize to modulate astrocyte response to metastatic growth. Indeed, studies of metastatic distribution in human patients, suggest that the hippocampus is a rare locale for tumor growth, as compared to the cerebellum or frontal lobe (Delattre et al., 1988; Ghia et al., 2007; Bender and Tome, 2011). Potentially, the more robust responses of astrocytes in the hippocampus, both on a cellular and immunological basis, could hamper metastatic growth.

As in glioma, the "double edged sword" of inflammation that is so often reviewed in the literature, not just in cancer (Hagemann et al., 2007; Lin, 2010; Rizzo et al., 2011) but in other diseases such as asthma (Balhara and Gounni, 2012), stroke (Doyle and Buckwalter, 2012), and neurodegeneration (Wyss-Coray and Mucke, 2002), appears to also be a feature of the astrocytic response to brain metastasis. *In vitro* studies have shown that astrocytes produce NO, via inducible nitric oxide synthase (Simmons and Murphy, 1992), and that this has tumoricidal effects in both primary and secondary cancer cell lines (Samdani et al., 2004). Evidence that NO induces GFAP upregulation (Brahmachari et al., 2006) suggests that this may result in increased detection of reactive astrocytes with disease progression.

However, a greater burden of evidence suggests a tumor promoting role for astrocytes. Immunohistochemical analysis of human biopsies has demonstrated up-regulation of endothelin [a cancer cell mitogen (Bagnato et al., 1997)] expression by tumor associated astrocytes (Zhang and Olsson, 1995). Moreover, co-culture experiments between astrocytes and various tumor cell lines indicate that astrocytes release soluble factors that can enhance tumor cell growth. For instance, endothelin-1, besides its role in vasoconstriction, has also been proposed to be mitogenic (Kasuya et al., 1994; Bagnato et al., 1997, 2002), and analysis of biopsy samples suggests that endothelin-1 is expressed by astrocytes in 85% of patient cases (Zhang and Olsson, 1995). Additionally, the receptor for endothelin-1, ET_B, is upregulated in a brain-metastatic melanoma cell line over 3 fold, as compared to a non-metastatic cell line (Boukerche et al., 2004). This finding suggests that astrocytes may drive the molecular determinants of metastatic potential. Furthermore, incubation of the lung adenocarcinoma cell line PC14-PE6 with an immortalized astrocytic cell line induced cancer cell ERK1/2 phosphorylation (Langley et al., 2009), part of the MAP kinase signaling pathway heavily implicated in tumor progression. ERK1/2 phosphorylation has also been demonstrated in a metastatic mammary carcinoma cell line, ENU1564, in response to astrocyte conditioned media (Mendes et al., 2007). In this case, ERK1/2 activation was shown to increase tumor cell invasiveness *in vitro* via induction of MMP2 expression, as was seen in astrocyte-glioma co-culture models (Le et al., 2003).

Metastatic invasion may also be facilitated by astrocyte-derived heparanase, which degrades heparin sulphate proteoglycans, a major component of the extracellular matrix. Astrocyte heparanase expression has been demonstrated in the peri-infarct regions of *in vivo* stroke models (Takahashi et al., 2007; Li et al., 2012a) and in rat astrocyte-tumor cell culture models. In the latter case, it was shown that co-culture of astrocytes with brain-metastatic melanoma cell lines led to a super-additive increase in enzyme activity, potentially through neurotrophin signaling (Marchetti et al., 2000). Treatment of melanoma cell lines with astrocyte conditioned media led to increased cell invasion, an effect which was abrogated with antibody-mediated neutralization of heparanase. Further co-culture experiments between astrocytes and several lung cancer derived cell lines indicate that astrocytes secrete IL-6, TNF, and IL-1β, which stimulate tumor cell growth (Seike et al., 2011). It has also been suggested that astrocytes increase the anchorage-independent growth of cancer cell lines and that this correlates with metastatic ability *in vivo* (Fitzgerald et al., 2008), although the mechanism has yet to be identified.

Astrocytes have also been shown to induce transcriptional changes in co-cultured tumor cells that reflect the transcriptional changes seen *in vivo* (Park et al., 2011). Here, a Competitive Hybridization of Microarray Experiment (CHME) was used to tease apart genes upregulated in metastases as compared to the tumor micro-environment. In this case, human cancer cells were introduced into immuno-compromised mice, and differing gene signatures and methylation statuses between the cell populations were demonstrated. Subsequently, astrocytes were cultured with a breast carcinoma cell line, MDA-MB-231, and were shown to induce a similar genomic signature in the tumor cells to that seen *in vivo*, suggesting that astrocytes are mediators of tumor cell transcriptional reprogramming. For instance, brain metastatic cells were shown to upregulate genes involved in neuronal processes such as glutamate receptor signaling, axonal guidance, and neurotransmission. This novel approach to probing astrocytic function in brain metastasis demonstrates the key role astrocytes play in tumor progression, but also highlights the need to determine both the mechanism and the growth advantage conferred.

On balance, the studies detailed above suggest a pro-tumorigenic role for astrocytes, however, further *in vivo* studies are required to elucidate this. Besides these proposed roles in tumor pathogenesis, astrocytes have also been implicated in protecting tumor cells from chemotherapeutic agents. Co-culture of astrocytes with breast and lung cancer cell lines (MDA-MB-231 and PC14Br$_4$, respectively) leads to up-regulation of survival genes such as *BCL2L1*, an anti-apoptotic member of the BCL-2 protein family (Kim et al., 2011). Such genes confer resistance to a range of chemotherapeutics, and are absent in cell lines at secondary sites other than the brain, highlighting a novel role for astrocytes. Further work has shown that the mechanism of cell protection is cell contact dependent and mediated via gap junction facilitated sequestration of calcium from tumor cells (Lin et al., 2010).

IMAGING ASTROCYTES *in vivo*

The advent of molecular imaging, both as a stand-alone modality and combined with transgenic mouse models, has enabled the dynamic responses of astrocytes in numerous CNS pathologies to be visualized. Such techniques, in addition to yielding mechanistic insights, have potential for diagnostic imaging of neuroinflammation. *In vivo* research into the role of astrocytes in the tumor microenvironment is still in its infancy; however, using the numerous methodologies outlined below, there is much potential for better understanding the contribution of this most abundant CNS cell type to the pathogenesis of primary tumors and metastases.

In vivo LABELING OF ASTROCYTES

Astrocytes are primarily identified, *in vitro, in vivo,* and *ex vivo*, by their specific expression of GFAP, a cytoskeletal protein upregulated in astrogliosis, via antibody mediated detection methods, or transgenic systems, as outlined below. Alternatively, the propensity of astrocytes to specifically take up dyes such as sulforhodamine 101 (SR101), and facilitate their spread via gap-junctions (Nimmerjahn et al., 2004; Appaix et al., 2012), has enabled the study of astrocytic function, predominantly in slice models. Recently, such studies have been translated into the *in vivo* setting, with SR101 applied to the exposed cortex or injected intraperitoneally, immediately prior to imaging (Nimmerjahn and Helmchen, 2012). Using this technique, the intimate relationship between astrocytes and the endothelium in healthy tissue has been demonstrated, with gap-junction signaling between the two populations proposed (McCaslin et al., 2011).

Dyes, however, only enable transient labeling over a matter of hours and, therefore, techniques that allow long-term visualization are more useful for studying astrocytes in pathology. Viral vectors can be used to transfect cell types with fluorescent dyes. For example, a recombinant adenovirus-associated vector (AAV) has been used to stably and chronically transduce neurons and astrocytes, inducing green fluorescent protein (GFP) expression and allowing *in vivo* two photon microscopy of the visual cortex via cranial windows (Lowery et al., 2009). Additionally, BBB permeable, GFP-labeled anti-GFAP antibodies have very recently been used to image astrocytes, both *in vivo* and *ex vivo* (Li et al., 2012b). The potential for longitudinal imaging of astrogliosis by these means in response to CNS disease or injury will enable the temporal and spatial profile of reactivity to be mapped.

Transgenic models offer an even more stable approach. The specific expression of GFAP by astrocytes allows its promoter to be manipulated to drive the expression of reporter genes solely in astrocytes, as first described in a transgenic mouse model in which bacterial LacZ was placed under the GFAP promoter (Brenner et al., 1994). Since then, transgenic models have been engineered in which astrocytes express GFP (Zhuo et al., 1997; Nolte et al., 2001), allowing for the observation of astrogliosis in brain slices with fluorescent microscopy, and real-time *in vivo* detection of astrogliosis using multi-photon microscopy. This technique has been employed by several groups investigating systems such as retinal gliosis in diabetes (Kumar and Zhuo, 2010), retinal neurotoxicity (Ho et al., 2009) and NMDA-induced astrocyte activation (Serrano et al., 2008). This transgenic model can also facilitate sorting of astrocytes from surrounding brain tissue, allowing mRNA expression profiling in glioma (Katz et al., 2012), as described above.

Bioluminescence is another technique that allows visualization of reactive astrocytes *in vivo*, by placing the luciferase gene under the control of the GFAP promoter, as first described by Zhu et al. (2004). This transgenic mouse line has enabled astrocyte activation, in response to both glioma and metastatic cell lines implanted directly into the brain, to be studied dynamically and non-invasively (Lee et al., 2011). Using this approach, Lee et al. showed that astrogliosis peaks 3 days after tumor implantation and showed a biphasic time course over a 28 days experimental period in both glioma and metastatic models. An extended astrocyte response to brain metastasis has also been observed in other groups (Lorger and Felding-Habermann, 2010; Seike et al., 2011), including our own (unpublished observations), using conventional immunohistochemistry.

It is evident that labeling of astrocytes is highly dependent on GFAP. It should be noted however that not all astrocytes express detectable levels of GFAP and its expression in disease states can be variable (Wang and Walz, 2003). S100β, a calcium binding protein (Baudier et al., 1986), is often used as a astrocyte marker, however there is cross-reactivity with oligodendrocytes (Deloulme et al., 2004; Hachem et al., 2005). In addition, a number of other proteins are selectively expressed by astrocytes, such as the enzyme glutamine synthetase and aquaporin-4, but their sub-cellular distribution renders them impractical for astrocyte labeling (Yang et al., 2011). One should also note the differential expression of astrocytic markers throughout development. For instance, the glutamate transporter GLT-1 has been shown, *in vitro*, to be expressed at high levels in astrocyte cultures from embryonic stages, but not post-natal time points, whereas the glutamate transporter GLAST is highly expressed in astrocytes cultured from early post-natal time points, with a decline seen from p10 (Stanimirovic et al., 1999). *In vivo*, the spatial expression of these two transporters appears to change during post-natal development (Voutsinos-Porche et al., 2003).

Rather than pursuing global markers for astrocyte identification, attempts to identify markers for different phenotypes of astrocyte reactivity would allow greater understanding of the functional impact of gliosis at different spatial and temporal locales. For instance, the cytoskeletal protein vimentin, can be used to define astrocytes proximal to ischemic lesions, where neuronal damage is present, but is absent in distal gliosis (Petito et al., 1990; Wang and Walz, 2003). A recent comprehensive transcriptional profile of astrocytes in response to two different *in vivo* challenges in mice, LPS and middle cerebral artery occlusion (MCAO), demonstrates differential molecular profiles in response to the nature of the insult and time from lesion induction (Zamanian et al., 2012). For example, nestin and tenascin-C expression were observed in the astrocyte response to stroke, but not LPS-induced inflammation. Studies such as these demonstrate the considerable heterogeneity in astrocytic phenotypes, within and between disease states, and highlight the need for imaging agents that reflect this diversity of responses.

FUNCTIONAL IMAGING

Astrocyte reactivity is just one facet of the contribution of glia to the healthy and diseased brain. Astrocyte *excitability* [which is based on oscillations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)] elicits effects in the healthy and diseased brain (Halassa et al., 2007; Kuchibhotla et al., 2009), and requires sophisticated imaging modalities to elucidate its functional impact. Neurotransmitter release by neurons leads to elevation of astrocyte $[Ca^{2+}]_i$ upon binding to receptors on peri-synaptic astrocytic membranes, and consequent activation of Phospholipase C. Spontaneous excitation can also occur. Such elevations in $[Ca^{2+}]_i$ result in astrocytic signaling to neurons, due to the release of gliotransmitters such as glutamate and D-serine (Araque et al., 2001; Fellin et al., 2004; Henneberger et al., 2010), and to other astrocytes.

Calcium oscillations and waves are typically investigated using chemical calcium indicators. Upon calcium chelation, the spectral properties of such dyes are altered such that they provide a fluorescent read-out for $[Ca^{2+}]_i$ (Tsien, 1988). A cell permeable acetoxymethyl (AM) ester form of the indicator dye is often used. Once inside the cell, the AM bond is cleaved by endogenous esterases and the dye is trapped within the cell. Using bulk loading techniques all cells will take up the dye. However, by using astrocyte marker dyes, such as SR101, astrocytes can be identified (Nimmerjahn et al., 2004). More recently, genetically encoded molecules have been developed (Miyawaki et al., 1997), which confer the advantage of being targetable to a specific cell type or subcellular compartment (Shigetomi et al., 2010). This approach is particularly exciting for *in vivo* imaging, as the stable expression of the calcium reporter allows for long term imaging of calcium signals (Mank et al., 2008). Generally these indicators comprise of a calcium binding protein fused to a variant of GFP. Upon calcium binding, a conformational change of the indicator results in either a change in the fluorescence resonance energy transfer (FRET) between the flanking GFPs (Miyawaki et al., 1997; Pologruto et al., 2004) or a fluorescence change in the molecule itself (Nagai et al., 2001; Nakai et al., 2001; Shigetomi et al., 2010).

Owing to the optical scattering properties of brain tissue, fluorescence imaging is degraded with increasing depth below the brain surface. Light scattering can be decreased by using infrared-shifted light, compared to the use of visible wavelengths. As light of a longer wavelength has less energy per photon, the near-simultaneous absorption of multiple photons is required in order to excite a fluorophore, as occurs in multi-photon microscopy. Although most chemical calcium indicator dyes were originally developed for single photon microscopy, some (e.g., rhod-2, Fluo-4) also have sufficient two-photon cross-section.

Historically, two-photon imaging of calcium oscillations and waves in astrocytes has been limited to slice preparations (Mulligan and MacVicar, 2004; Tian et al., 2005; Di Castro et al., 2011). However, translation of the technique into *in vivo* systems has recently become possible. For example, Cirillo et al. have demonstrated calcium signaling in spinal cord astrocytes in response to sensory stimulation (Cirillo et al., 2012). Similarly, whisker stimulation was shown to elicit astrocyte responses in the barrel cortex (Tian et al., 2006; Wang et al., 2006). With regards to astrocyte calcium signaling in the diseased brain, elevated signaling has been observed in status epilepticus (Ding et al., 2007) and has also been demonstrated in the ischemic penumbra following stroke, where such increased signaling has been linked to increased neuronal damage (Ding et al., 2009). To date, application of calcium signaling imaging in CNS tumors, has been restricted to *in vitro* preparations of glioma cells (Charles et al., 1992; Yamasaki et al., 1994) or astrocyte and glioma cell co-cultures (Zhang et al., 1999). The latter, gap-junction dependent, interaction has been shown to be necessary for glioma invasion (Oliveira et al., 2005). Mediators of inflammation have also been shown to modulate astrocyte calcium signaling *in vitro* (Hamby et al., 2012) and, hence, one would anticipate alterations in signaling in the tumor microenvironment that could be probed *in vivo* using the novel methodologies outlined above.

MAGNETIC RESONANCE IMAGING

Magnetic resonance imaging (MRI) provides a powerful tool for imaging soft tissue contrast in the brain. As discussed, changes in astrocytic morphology accompany astrogliosis, and there have been attempts to image these changes with MRI. In both an Endothelin-1 induced ischemia rat model and an NMDA-induced model of cytotoxicity, T_1 relaxation times have been shown to increase independent of changes in cerebral blood flow. The observed T_1 hypointensities were seen in areas correlating to astrocyte activation and were reduced upon administration of arundic acid (Sibson et al., 2008), a selective inhibitor of astrocyte activation (Tateishi et al., 2002).

Other MRI parameters have been proposed to visualize astrocyte activation. Diffusion tensor imaging (DTI) facilitates observation of tissue microstructure, by both the rate and direction of water proton diffusion through a given area. By modeling diffusion information in a different manner, accounting for diffusion kurtosis (DK), it has been proposed that subtle heterogeneity of tissue can be further characterized. The work of Zhuo et al. suggests that changes in DK can be correlated with astrocyte activation and, thus, DK imaging could be used as a tool for

investigating traumatic brain injury (Zhuo et al., 2012). Both this modality and the changes in T_1 relaxation described above are still under review with regards to imaging tumor-induced gliosis. The resolution of MRI may not allow gliotic regions to be distinguished from the neoplasm itself, however, both of these techniques could hold potential as surrogate markers for *clinical* tumor detection.

Astrocytes are highly metabolically active, and multiple metabolic changes also accompany astrogliosis. For example, increased antioxidant activity is observed upon CNS insult; IL-1β signaling leads to the expression of cerruloplasmin, which buffers free copper ions and oxidizes ferrous iron (Kuhlow et al., 2003). Altered metabolic activity is a further feature of astrogliosis; in a ciliary neurotrophic factor (CNTF) induced model of astrogliosis, fatty acid oxidation and ketone body metabolism is increased, alongside decreased glycolysis (Escartin et al., 2007), conferring resistance to metabolic insults.

Under normal conditions, it has been proposed that the ATP requirements of astrocytes are primarily served by glycolysis (Pellerin and Magistretti, 1994, 1997), with the end-product, lactate, extruded into the extracellular space either for uptake by neurons (Aubert et al., 2005) or clearance from the brain. However, the TCA cycle and subsequent oxidative phosphorylation are also a source of ATP (Serres et al., 2008), and recent transcriptome analyses of acutely isolated mouse cortical astrocytes have demonstrated elevated levels of TCA cycle enzymes as compared to neurons, combined with high mitochondrial numbers in astrocyte foot processes (Lovatt et al., 2007). Whilst, to date, metabolic changes in reactive astrocytes surrounding CNS tumors have not been investigated, studies demonstrating increased glucose utilization by astrocytes in response to TNF, IL-1β, IL-6, and IFN-γ (Yu et al., 1995; Gavillet et al., 2008; Belanger et al., 2011), and the synergic activity of TNF and IL-1β on astrocyte metabolic activity (Gavillet et al., 2008), suggest that astrocytes in the pro-inflammatory microenvironment will be in a metabolically hyperactive state. Additionally, the ability of astrocytes to protect tissue during oxidative stress via the secretion of thiols and enhanced glutamate uptake can be modulated by the cytokine context (Garg et al., 2009). If this is indeed the case, a metabolic marker of astrocyte activity, *in vivo,* could be of considerable use.

Specificity for astrocyte metabolism, as opposed to neuronal activity, can be achieved through the differential uptake of certain metabolic substrates. In particular, acetate is preferentially taken up by astrocytes over neurons (Waniewski and Martin, 1998) and incorporated into the TCA cycle. ^{13}C magnetic resonance spectroscopy (MRS) studies have demonstrated that it is possible to detect the transfer of ^{13}C label from [2-^{13}C]acetate into glutamine within astrocytes. The ^{13}C label is first transferred to glutamate via the TCA cycle and exchange between the TCA cycle intermediate α-ketoglutarate (α-KG) and glutamate (Kanamatsu and Tsukada, 1999; Sibson et al., 2001). Both the α-KG and glutamate pools in astrocytes are at an extremely low concentration and, thus, are undetectable by the relatively low sensitivity ^{13}C MRS. Subsequently, however, the labeled glutamate is converted to glutamine, which is a much larger metabolic pool (*ca*. 5–6 mM) and label flux into this pool is detectable by ^{13}C MRS (Sibson et al., 1997). The flux of ^{13}C label from acetate to glutamine can be converted into absolute rates of astrocytic TCA cycle (oxidative metabolism) through the use of metabolic modeling (for review see de Graaf et al., 2011). This ^{13}C MRS approach has been used in transgenic mouse models of Alexander disease to show astrocyte dysfunction, with regards to reduced utilization of acetate for the synthesis of glutamate and glutamine (Meisingset et al., 2010), as well as in human studies. For instance, human studies in patients with Alzheimer's disease indicate that [2-^{13}C]acetate can be used to detect gliosis (Sailasuta et al., 2011), with enhanced metabolism of acetate to ^{13}C bicarbonate detected. While MRS has yet to be used to probe stromal astrocyte metabolism, studies of neoplastic astrocytes in glioma suggest that intermediates detected with spectroscopy (Doblas et al., 2012), could be translated into useful imaging tools in PET. Of relevance here is the use of the PET tracer ^{18}F-acetate which has been used to demonstrate enhanced glial metabolism in both a rat stroke model and a glioblastoma model (Marik et al., 2009). In humans, the PET tracer ^{11}C-acetate has been used for the detection of gliomas (Liu et al., 2006; Tsuchida et al., 2008). The use of nuclear imaging to detect gliosis will be further discussed below.

SPECT AND PET IMAGING

Nuclear imaging, with single photon emission computed tomography (SPECT) and positron emission tomography (PET), is a tool routinely used in the diagnosis of cancer. Radiolabeled tracers, primarily ^{18}F-deoxyglucose (FDG), are used to detect tumor growth based on their hyper-metabolism of glucose as compared to healthy tissue. If astrocytes in and around tumors are truly hyper-metabolic (see above) then it is likely that FDG tumor detection may reflect, in part, reactive astrocytosis. However, imaging the tumors themselves is not the only possibility for the detection of neoplasms. The use of radiolabeled compounds to bind to astrocyte specific markers, or indirect measures of astrocyte activity, are avenues that have been pursued in many CNS diseases, with some agents being translated into a clinical setting. Whilst SPECT and PET cannot provide sufficient spatial resolution for probing individual cells, or subpopulations of cells, these approaches do allow detection of neuroinflammation, which will facilitate disease diagnosis and monitoring.

^{99}Tc-HMPAO is a BBB-permeable SPECT imaging agent, with non-specific uptake by the brain proportional to blood flow. Retention of the compound is dependent on its intracellular reduction and, thus, the high glutathione concentration present in astrocytes leads to increased and sustained uptake of ^{99}Tc-HMPAO by astrocytes as compared to neurons (Slosman et al., 2001). Although not specific for astrocyte activation, retention of this compound is enhanced in gliotic lesions in disease states such as dementia and Alzheimer's disease (Slosman et al., 2001) and herpes encephalitis (Launes et al., 1995). Alternatively, ^{11}C-deuterium-L-deprenyl (^{11}C-DED) is an irreversible monoamine oxidase B inhibitor. Activated astrocytes express elevated levels of monoamine oxidase and, consequently, this agent can be used to detect astrogliosis with PET imaging, as has been demonstrated to date in pathologies such as

Alzheimer's (Carter et al., 2012), amyloid lateral sclerosis (ALS) (Johansson et al., 2007), and Creutzfield–Jakob disease (Engler et al., 2012).

Probing the increased metabolic activity evident in gliosis at the mitochondrial level has enabled *in vivo* imaging of astrocytes in multiple CNS pathologies, and is a promising tool for monitoring primary and secondary tumor growth. Translocator protein (TSPO—also known as the peripheral benzodiazepine receptor) is an outer mitochondrial membrane protein (Squires and Brastrup, 1977), and has multiple functions, including cholesterol import for steroid synthesis (Hauet et al., 2005), regulation of mitochondrial metabolism (Hirsch et al., 1989), and apoptosis (Hirsch et al., 1998). These diverse functions render knockout models embryonically lethal (Papadopoulos et al., 1997). Although the TSPO was originally thought to be exclusively upregulated on microglia in disease states, recent studies have also demonstrated increased expression on astrocytes in humans, as well as in rodent models (Cosenza-Nashat et al., 2009; Lavisse et al., 2012), as shown in **Figure 2**. Consequently, interest has grown in the development and use of radiolabeled compounds against TSPO for imaging neuroinflammation in diverse CNS pathologies, such as multiple sclerosis (Harberts et al., 2012), dementia (Cagnin et al., 2001), Alzheimer's disease (Versijpt et al., 2003) and stroke (Gulyas et al., 2012a,b), as reviewed in (Ching et al., 2012).

In the context of imaging astrocytes in primary brain tumors (**Figure 2**), numerous glioma cell lines have been shown to highly express TSPO (Winkeler et al., 2012), and recent studies in rat models of glioma (Buck et al., 2011; Tang et al., 2012; Winkeler et al., 2012) suggest that this approach could be used as a clinical tool to detect brain tumors. Work in our own group suggests that radiolabeled anti-TSPO agents can also be used to detect brain metastases, on the basis of the large area of astrogliosis associated with these tumors (unpublished work). As discussed with regard to MRI, these techniques are unlikely to allow differentiation of tumor growth from associated astrogliosis, especially as TSPO upregulation has been observed in metastatic breast tumors (Zheng et al., 2011; Batarseh et al., 2012). However, this approach does hold potential as a surrogate biomarker for the clinical detection of CNS tumors.

CONCLUSIONS

The role of the microenvironment in tumor progression has been extensively reported, and an inflammatory response to both primary and secondary brain tumors is becoming increasingly apparent. As detailed above, astrocytes are key players in the

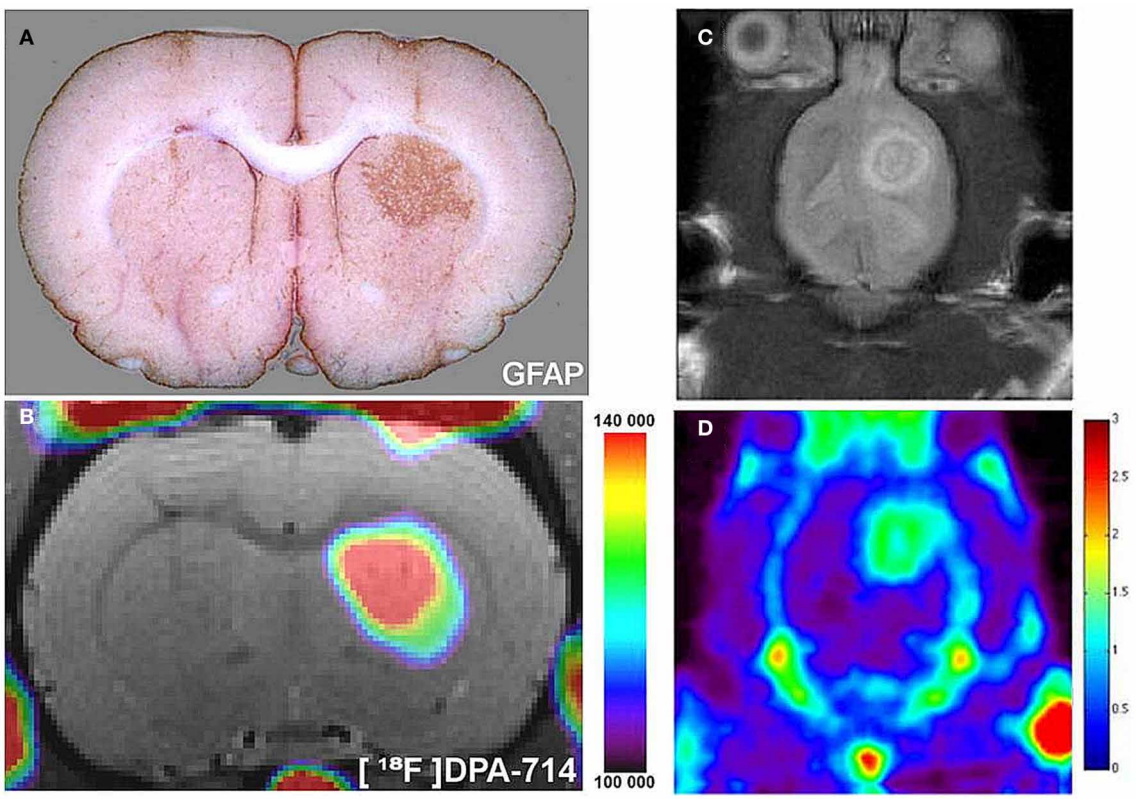

FIGURE 2 | Radiolabeled TSPO ligands can be used to image astrocyte activation *in vivo*. (A) In a rat model, astrocytes were chronically activated (GFAP staining, brown) by lentiviral gene transfer of the cytokine ciliary neurotrophic factor (CNTF). **(B)** ^{18}F-labeled DPA-714, a TSPO ligand, binding spatially correlates with areas of astrocyte activation, as observed with *in vivo* PET imaging. As adapted from Lavisse et al. (2012). Such TSPO targeted agents can be used to image TSPO expressing gliomas in pre-clinical models. **(C)** A pre-clinical rat model of glioma detected with MRI **(D)** ^{18}F-PBR06, a TSPO ligand, binding spatially correlates with glioma growth, as observed *in vivo* with PET. As adapted from Buck et al. (2011).

CNS response to tumor growth, and there have been a flurry of studies attempting to uncover the role of these glial cells in disease progression. Many of these studies were conducted *in vitro,* and suggest both pro- and anti-tumor effects. However, the balance of evidence seems to point toward a pro-tumorigenic role for astrogliosis, with the release of growth factors and matrix remodeling enzymes. Now that more sophisticated animal models have been developed, we can begin to understand the complex role of these cells *in vivo*. Importantly, functional imaging measurements, via methods such as calcium signaling, will allow a greater understanding of how astrocytes communicate in the tumor microenvironment. Multinuclear MRS will yield insights into tumor metabolism, and potentially elucidate the metabolic interactions between astrocytes and tumor cells. To elucidate astrogliotic functions in response to tumor growth, numerous imaging modalities can be utilized. Not only do these techniques allow the spatial and temporal profile of gliosis to be visualized and quantified, but they may potentially yield mechanistic insights through functional imaging and new clinically-relevant diagnostic approaches.

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New tools for investigating astrocyte-to-neuron communication

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Gray matter protoplasmic astrocytes extend very thin processes and establish close contacts with synapses. It has been suggested that the release of neuroactive gliotransmitters at the tripartite synapse contributes to information processing. However, the concept of calcium (Ca^{2+})-dependent gliotransmitter release from astrocytes, and the release mechanisms are being debated. Studying astrocytes in their natural environment is challenging because: (i) astrocytes are electrically silent; (ii) astrocytes and neurons express an overlapping repertoire of transmembrane receptors; (iii) the size of astrocyte processes in contact with synapses are below the resolution of confocal and two-photon microscopes (iv) bulk-loading techniques using fluorescent Ca^{2+} indicators lack cellular specificity. In this review, we will discuss some limitations of conventional methodologies and highlight the interest of novel tools and approaches for studying gliotransmission. Genetically encoded Ca^{2+} indicators (GECIs), light-gated channels, and exogenous receptors are being developed to selectively read out and stimulate astrocyte activity. Our review discusses emerging perspectives on: (i) the complexity of astrocyte Ca^{2+} signaling revealed by GECIs; (ii) new pharmacogenetic and optogenetic approaches to activate specific Ca^{2+} signaling pathways in astrocytes; (iii) classical and new techniques to monitor vesicle fusion in cultured astrocytes; (iv) possible strategies to express specifically reporter genes in astrocytes.

Keywords: photoactivation, pharmacogenetics, optogenetics, gliotransmission, GCaMP, LiGluR, CatCh, ChR2

INTRODUCTION

The concept of gliotransmission at the tripartite synapse developed more than 10 years ago (Araque et al., 1999; Perea and Araque, 2010) is very attractive: it suggests that cerebral gray matter protoplasmic astrocytes are not only supportive cells with homeostatic functions, but that they also play a role in information processing by responding to neuronal synaptic activity with Ca^{2+} elevations that induce the subsequent release of gliotransmitters and modulate neuronal excitability and synaptic plasticity [reviewed in (Angulo et al., 2008; Bergersen and Gundersen, 2009; Cali et al., 2009; Perea et al., 2009; Santello and Volterra, 2009; Halassa and Haydon, 2010; Perea and Araque, 2010; Parpura et al., 2011; Gucek et al., 2012; Zorec et al., 2012)]. However, the concept of gliotransmission is debated. First, the ability of astrocytes to release neuroactive compounds in a Ca^{2+}-dependent manner has been questioned (Agulhon et al., 2008; Fiacco et al., 2009). Second, there is no consensus concerning the mechanisms of gliotransmitter release. In fact, studying astrocytes *in situ* is very challenging and an agreement is emerging that new methods are needed to selectively activate (Fiacco et al., 2009; Hamilton and Attwell, 2010) and read out Ca^{2+} signals in astrocytes *in situ* (Shigetomi et al., 2013b).

By gliotransmission, we mean Ca^{2+}-dependant release of fast-acting neuroactive compounds, the gliotransmitters. Astrocytes can also release other molecules acting not only on neighboring neurons but also on nearby glial cells such as microglia, NG2 cells, and on cellular constituents of the blood brain barrier. Our review will focus on the fast acting gliotransmitter candidates, glutamate mostly, as well as D-serine, ATP, and GABA. Several pathways have been suggested for glutamate release: exocytosis, hemichannels, sodium-dependent transporters, volume-regulated anion channels, purine P2X7 receptor channel [reviewed in (Hamilton and Attwell, 2010)], and more recently the bestrophin 1 (Best1) chloride channel and the two-pore domain potassium TREK1 channel (Woo

Abbreviations: AAV, adeno-associated virus; ACR, astrocyte Cre reporter; ALDH1L1, aldehyde dehydrogenase 1 L1; AM, acetoxymethyl; AV, adenovirus; CatCh calcium-translocating channelrhodopsin 2; ChR, channelrhodopsin; CMV, cytomegalovirus; Cx30, connexin 30; DHPG, dihydroxyphenylglycine; DsRed, discosoma red protein; EGFP, enhanced green fluorescent protein; GCaMP, green fluorescent protein-based Ca^{2+} sensor; GECI, genetically encoded calcium indicator; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; GLAST, glutamate-aspartate transporter; GLT-1, glutamate transporter 1; hGFAP, human glial fibrillary acidic protein; IP_3, inositol triphosphate; LiGluR, light-gated glutamate receptor; LimGluR, light-gated metabotropic glutamate receptor; LV, lentivirus; mGluR, metabotropic glutamate receptor; Mlc1, megalencephalic leukoencephalopathy with subcortical cysts 1; MrgA1, Mas-related gene A1; OGB1, oregon green BAPTA1; STED, stimulated emission depletion; TIRF(M), total internal reflection fluorescence (microscopy); TA, tetracycline transactivator; TetO, tetracycline operator; Tg, transgenic; YFP, yellow fluorescent protein

et al., 2012). The release of glutamate by Ca^{2+}-regulated vesicular fusion is considered as an important pathway for gliotransmitter release because, by analogy with neuronal exocytosis, it appears to be the most suitable pathway for rapid information processing by astrocytes. Experimental evidence in favor of glutamate exocytosis has been provided using (i) dihydroxyphenylglycine (DHPG), mechanical stimulation, inositol triphosphate (IP_3) and Ca^{2+} uncaging to activate astrocytes; (ii) Ca^{2+} buffering with BAPTA, VAMP2/3 cleaving with tetanus toxin (TeNT) or botulinum toxin (BoNT), and generating a dominant negative SNARE (dnSNARE)-expressing mouse line to inactivate vesicular release in astrocytes; (iii) fluoroacetate to inactivate astrocyte metabolism [reviewed in Bergersen and Gundersen, 2009; Cali et al., 2009; Gucek et al., 2012; Zorec et al., 2012], but contrasts with the relative absence on electron micrographs of small vesicles in astrocyte processes, when compared to the neuronal presynaptic terminal.

In this review, we discuss several new genetically encoded tools to read out astrocytic Ca^{2+} activity, to activate Ca^{2+} signals in astrocytes, and to monitor gliotransmitter release from astrocytes. Optical methods for astrocyte photoactivation and imaging, and strategies to selectively target the genes in astrocytes in their native environment are also reviewed.

IMAGING ASTROCYTE ACTIVITY

ORGANIC vs. GENETICALLY ENCODED CA^{2+} INDICATORS

Protoplasmic astrocytes are electrically silent. However, they may be considered as excitable cells in the sense that they show Ca^{2+} signals, both spontaneously and in response to neuronal activity. In spite of the evidence suggesting that Ca^{2+} signals are necessary and sufficient to induce gliotransmitter release, many questions remain, concerning both the role and sources of Ca^{2+} signals in astrocytes (Agulhon et al., 2008; Fiacco et al., 2009; Parpura et al., 2011). One limiting factor to study Ca^{2+} signaling has been methodological. So far, most studies in acute brain slices and *in vivo* have been based on bulk-loaded membrane-permeable chemical Ca^{2+} indicators. There exist many organic Ca^{2+} indicators with different spectral properties and affinity for Ca^{2+} which can monitor either Ca^{2+}-related fluorescence changes or, for the ratiometric probes, can be calibrated to provide absolute Ca^{2+} concentration [reviewed in (Paredes et al., 2008)]. The membrane-permeable acetoxymethyl (AM) esters, Fluo4-AM and Oregon Green BAPTA1-AM (OGB1-AM), the most popular dyes used to image Ca^{2+} activity in populations of astrocytes, allow imaging the somatic region and the larger proximal processes but leave the very thin distant processes that participate to the tripartite synapse unsampled (Reeves et al., 2011). At the laser powers, dye concentrations and integration times typically used and with the spatial resolution available, fluorescence changes in fine processes are generally not resolved (see below for a detailed discussion).

Another limiting factor has been the lack of specificity of these membrane-permeable Ca^{2+} indicators, which label both neurons and astrocytes (Garaschuk et al., 2006) with cell-type preferences, depending on the indicator, the protocol of application, and the age of the animal. Therefore, sulforhodamine 101 (SR101) that is specifically taken up by astrocytes has been generally used as a secondary fluorescent marker for astrocyte identification. The deep-red emission of SR101 can be detected with negligible spectral overlap with GFP or green fluorescein-based membrane-permeable Ca^{2+} indicators (Nimmerjahn et al., 2004). However, SR101 uptake is age-dependent (Kafitz et al., 2008) and it does not work in all brain regions (Schnell et al., 2012). Also, at concentrations needed for astrocyte labeling, SR101 leads to increases of neuronal excitability and long-term potentiation (Kang et al., 2010; Garaschuk, 2013), and thus might affect functional studies. As an alternative, transgenic (Tg) mouse lines (Nolte et al., 2001; Vives et al., 2003; Heintz, 2004; Zuo et al., 2004; Regan et al., 2007) expressing a green/yellow fluorescent protein (GFP/YFP) or *Discosoma* red protein (DsRed) under astrocyte-specific promoters (GFAP, S100β, GLT-1, ALDH1L1) can be used to identify astrocytes.

In this context, the recent genetically encoded Ca^{2+} indicators (GECI) provide a new alternative for non-invasive imaging of Ca^{2+} activity *in vivo* and in brain slices [reviewed in (Knopfel, 2012; Looger and Griesbeck, 2012)]. Their level of expression can be stable for months in the absence of apparent adverse effect (Zariwala et al., 2012). GECIs can be targeted to the plasma membrane of astrocytes (Shigetomi et al., 2010) and their specific expression by astrocytes *in vivo* is being developed using viral constructs and Tg mouse lines (see below). Finally new GECI variants are being generated having greater signal to noise ratio, different Ca^{2+}-binding affinities, and different spectral properties (Horikawa et al., 2010; Zhao et al., 2011; Ohkura et al., 2012; Akerboom et al., 2013; Chen et al., 2013b) which further enlarge their utility for studying the role of astrocytes on synaptic transmission (Tong et al., 2013).

Among the most recent GECIs, several variants of the original GFP-based Ca^{2+} sensor GCaMP1 (Nakai et al., 2001) have been tested in astrocytes: GCaMP2 (Hoogland et al., 2009), GCaMP3 (Shigetomi et al., 2010, 2013b; Tong et al., 2013), GCaMP5 (Akerboom et al., 2012), and red GECIs (Akerboom et al., 2013), as well as Case12 (Souslova et al., 2007; Gourine et al., 2010), and yellow Cameleon YC3.60 (Atkin et al., 2009). In neurons GCaMP5G and GCaMP6 variants have been shown to produce a higher signal-to-noise ratio than GCaMP3 and can detect Ca^{2+} changes evoked by single action potential (Akerboom et al., 2012). GCaMP3, GCaMP5G, and GCaMP6 are all compatible with two-photon excitation at 910-930 nm (Akerboom et al., 2012; Mutze et al., 2012). Recently, Khakh's group (Tian et al., 2009; Shigetomi et al., 2010, 2013b) compared Ca^{2+} changes in astrocytes using a membrane-permeable Ca^{2+} indicator (Fluo4-AM) with those detected with two GECIs, the cytosolic GCaMP3 and a membrane-targeted Lck-GCaMP3 (**Figure 1**). Ca^{2+} signals were recorded with a confocal microscope at the surface of acute hippocampal slices from adult mice. Specific astrocytic targeting of GCaMP3 and Lck-GCaMP3 was obtained using a short version (gfaABC1D) of the human glial fibrillary acidic (hGFAP) promoter. Unlike Fluo4-AM which diffuses poorly to the thin astrocyte processes (Reeves et al., 2011), both GCaMP3 and Lck-GCaMP3 reported a wealth of Ca^{2+} signals in distant thin astrocytic processes with relatively less activity in the

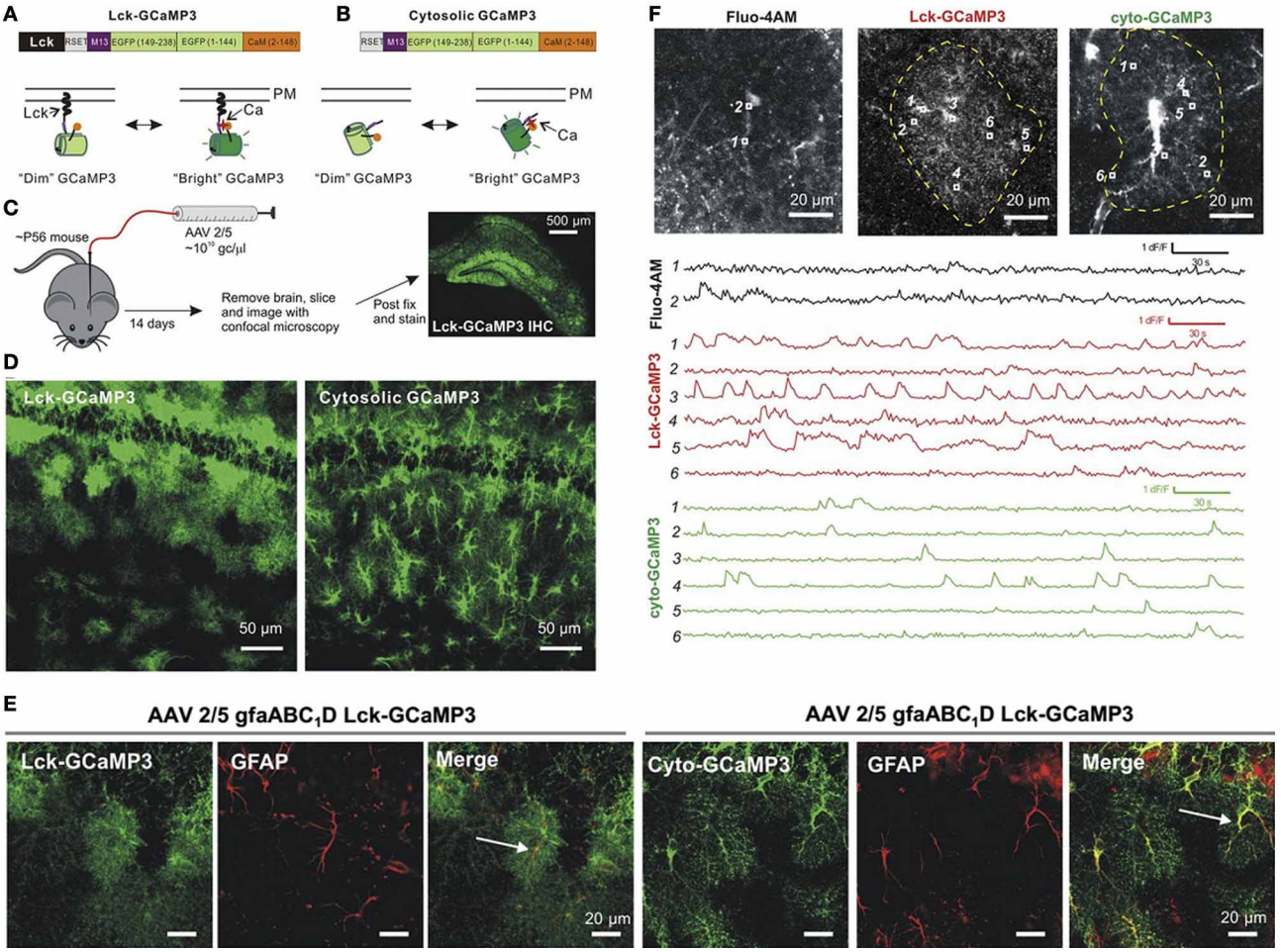

FIGURE 1 | Expression of GCAMP3 in astrocytes and comparison of Ca^{2+} signals detected with Fluo-4, cyto-GCAMP3, and Lck-GCAMP3. (A,B) Illustration of the membrane-targeted Lck-GCAMP3 and cytosolic non-targeted Cyto-GCAMP3. **(C)** Protocol of AAV2/5 injections into a mouse hippocampus. **(D)** Confocal images in CA1 stratum radiatum for Lck-GCaMP3 and cyto-GCaMP3. **(E)** Colocalization between Lck-GCaMP3 and cyto-GCaMP3 with the astrocytic marker GFAP. **(F)** Ca^{2+} signals imaged with Fluo-4 (black traces), cyto-GCaMP3 (green traces), and Lck-GCaMP3 (red traces). Top, representative images of astrocytes loaded with Fluo-4AM, Lck-GCaMP3 or cyto-GCaMP3. ROIs are shown in each image, and their time-lapse intensities are shown below. Adapted from (Shigetomi et al., 2013b).

soma and proximal processes. Interestingly, (i) spontaneous Ca^{2+} rises recorded with the GCaMP3 are highly localized and desynchronized, as suggested previously from whole-cell dye loading single astrocytes with higher dye concentrations through the patch pipette (Nett et al., 2002; Di Castro et al., 2011; Panatier et al., 2011); (ii) spontaneous somatic activity does not appear to integrate the signals generated locally in the thin processes; (iii) using Lck-GCaMP3, a new Ca^{2+} signaling pathway has been suggested in astrocytes involving the A1 transient receptor potential (TRPA1) channel (Shigetomi et al., 2011) that has been proposed to contribute to D-serine release (Shigetomi et al., 2013a). In summary, earlier studies using membrane-permeable Ca^{2+} indicators may have underestimated the variety of Ca^{2+} signaling mechanisms, and missed local interactions between astrocytes and neurones.

GECIs have been introduced only recently compared to chemical Ca^{2+} indicators that have been used since the 80s. Therefore, their photophysical properties and impact on intracellular Ca^{2+} homeostasis have not yet been characterized to the same extent as their chemical counterparts (Perez Koldenkova and Nagai, 2013). For example, the Ca^{2+}affinity of many GECIs has not been yet determined in the complex intracellular milieu; their binding kinetics (on- and off-rates for Ca^{2+} binding), as well as their aggregation and bleaching rates are not well established. Open questions concern their precise mobility, local concentration, Ca^{2+} buffer capacity and subcellular localization, which can be engineered by adding genetically encoded targeting sequences, as done with Lck-GCaMP3 (Shigetomi et al., 2010). However, the capacity of GECIs to specifically detect local Ca^{2+} signals in a population of astrocytes together with their low photobleaching and high signal-to-noise ratio seem to outweigh these limitations. Indeed experiments become feasible that were simply not possible with earlier small-molecule chemical indicators. The new Ca^{2+} data with the GECIs provide

intriguing clues to explore astrocyte functions but also present new challenges. New tools will be needed to reliably detect and quantify the wealth of rapid asynchronous and local fluorescence changes. The mechanisms and functional significance of these Ca^{2+} signals are far from being understood. Given the striking difference between fluorimetric Ca^{2+} signals detected with chemical Ca^{2+} indicators and GECIs, the relation between neuronal activity and astrocytic Ca^{2+} signals needs to be re-investigated under physiological and pathological conditions using acute brain slices, and anesthetized or non-anesthetized mouse preparations.

OPTICAL METHODS FOR IMAGING ASTROCYTE ACTIVITY

Imaging GECIs with two-photon microscopy holds important potential for monitoring astrocytic Ca^{2+} signals *in situ*. However, major challenges remain when imaging astrocyte signals in the neuropil. The limited spatial resolution is an issue when it comes to tell apart morphological changes and Ca^{2+} signals. An astrocyte process occupies only a small fraction of the two-photon excitation volume and, this fraction will become smaller with increasing imaging depth, for which two-photon resolution degrades due to scattering and wave front aberrations (Chaigneau et al., 2011). Thus, subtle morphological changes expected to modulate synapse coverage and modulate astrocyte-neuron interactions are unlikely to be resolved in two-photon microscopy and will be confounded with Ca^{2+} changes.

Two-photon microscopy combined with two-photon stimulated emission depletion (STED) (Ding et al., 2009; Li et al., 2009b; Moneron and Hell, 2009; Nägerl and Bonhoeffer, 2010) increases lateral resolution and super-resolution imaging of dendritic spines in live mouse brain *in vivo* has been recently reported (Berning et al., 2012). Two-photon-STED is attractive because it brings two-photon resolution closer to typical dimensions of astrocyte processes but it aggravates the temporal resolution problem inherent to scanning microscopies where temporal resolution depends on the number of pixels (i.e., the sampling rate imposed by the spatial resolution) and the dwell time per pixel (i.e., the number of photons available). As spatial resolution is privileged in 2PE-STED, the number of image pixels increases as the resolution gain squared, and the temporal resolution drops accordingly. Simultaneous multi-spot detection (Cheng et al., 2011; Grosberg et al., 2012; Ducros et al., 2013) speeds up image acquisition by an order of magnitude compared to conventional raster scanning, but does not fundamentally address the temporal undersampling problem. Finally, STED resolution scales with the square-root of power of the depletion beam so that STED increases the already high light burden of two-photon imaging (Koester et al., 1999). Faster imaging, i.e., shorter pixel dwell times will thus require better fluorophores with higher fluorescence quantum yield, greater depletion efficiency, and higher photostability, all at the same time. Also, while these arguments already hold for imaging a single focal plane, their weight increases when it comes to imaging Ca^{2+} changes in three dimensions along a branch or an entire astrocyte. In summary, major technological advances are still needed to image small signals in fine astrocyte processes and track their subtle morphological changes.

ACTIVATION OF ASTROCYTES

Following neuronal activity, the activation of astrocytes is mediated by neurotransmitter released from synaptic terminals (Porter and McCarthy, 1996; Wang et al., 2006). The subsequent release of gliotransmitters from mature protoplasmic astrocytes has been reported to depend upon G_q GPCR activation leading to astrocytic type-2 IP_3 receptor (IP_3R2) activation and Ca^{2+} release from the endoplasmic reticulum [reviewed in (Halassa et al., 2007)]. While this pathway has been implicated in gliotransmitter release, the mechanisms and the concept of gliotransmission remains debated (Agulhon et al., 2008; Fiacco et al., 2009; Hamilton and Attwell, 2010) in part because of our inability to selectively activate Ca^{2+} signals in astrocytes. The exogenous generation of Ca^{2+} signals that mimic those evoked by neuronal stimuli should clarify the interactions between neurons and astrocytes.

PHARMACOGENETICS

Since most cell types in the brain express an overlapping array of GPCRs, conventional pharmacological approaches consisting of bath application or local pipette perfusion of G_q GPCR agonists to evoke Ca^{2+} elevations in astrocytes *in situ* lack cellular selectivity, For instance, one of the agonists most frequently used to stimulate astrocytes, dihydroxyphenylglycine (DHPG), a group I metabotropic receptor (mGluR) agonist, has direct effects on neurons, and elicits neuronal Ca^{2+} elevations, long-term depolarization (Mannaioni et al., 2001; Rae and Irving, 2004), and potentiation of N-methyl-D-aspartate (NMDA) receptor-mediated currents (Benquet et al., 2002). Therefore, the use of G_q GPCR agonists, not only DHPG but also many other agonists, will lead to direct activation of neuronal receptors.

To overcome these limitations, a novel Tg mouse model was created (Fiacco et al., 2007) using the G_q GPCR MrgA1 receptor normally expressed by nociceptive sensory neurons (Dong et al., 2001). In the MrgA1 mouse model: (i) the GFP-tagged MrgA1 receptor is expressed selectively by astrocytes using an inducible tet-off system transcribed from a tet (tetO) minimal promoter; (ii) it is not activated by endogenous ligands found in brain; (iii) its ligand, the FMRF peptide, does not activate any endogenous brain G_q GPCRs. These mice were crossed with mice in which the tetracycline transactivator (tTA) is targeted to astrocytes using the hGFAP promoter. In the absence of doxycycline, tTA binds to tetO and drives expression of the MrgA1-GFP construct selectively in astrocytes and the MrgA1 receptor is functional in most astrocytes (Fiacco et al., 2007).

MrgA1-receptor-mediated Ca^{2+} release from astrocytic internal Ca^{2+} stores did not affect synaptic transmission and plasticity (Fiacco et al., 2007; Agulhon et al., 2010), raising questions about the ability of astrocytes to undergo Ca^{2+}-dependent gliotransmitter release. Using the same MrgA1 mouse model, it was later shown that instead, astrocytic MrgA1R-mediated Ca^{2+} elevations potentiate glutamate and K^+ uptake (Wang et al., 2012; Devaraju et al., 2013). The MrgA1 Tg mouse model demonstrates the interest of a pharmacogenetic approach to investigate the role of astrocytic Ca^{2+} in acute slices and *in vivo*. However, astrocytes are heterogeneous (Zhang and Barres, 2010) and they

are likely to exhibit different functions depending on the brain area. Consequently, continued improvements are needed to activate discrete populations of astrocytes in specific brain areas. The combination of pharmacogenetic with the use of adeno-associated viral technology to deliver the expression of genetically engineered new G_q GPCRs (Wess et al., 2013) is a promising approach (see below). Additionally, two-photon uncaging of caged FMRF at the vicinity of thin astrocyte processes should better mimic synaptically-induced G_q GPCR activation and therefore help addressing further the role of this signaling pathway in astrocyte physiology.

OPTOGENETICS

Over the last decade, the development of new photoswitchable genetically encoded channels and receptors to activate and inactivate specific neuronal subtypes had a significant impact on Neuroscience. The simultaneous methodological advances in several fields: (i) optics for photoactivation and imaging *in situ*, (ii) molecular engineering for developing new photoswitchable proteins, (iii) molecular biology for specific targeting of the light sensitive proteins, have been instrumental for the success of optogenetics in elucidating the function of neuronal circuits (Szobota and Isacoff, 2010; Fenno et al., 2011; Miesenbock, 2011). The most popular photoswitchable channel to activate neurons is the H314R channelrhodopsin 2 [ChR2(H314R)], a variant of the wild type ChR2 with reduced desensitization (Nagel et al., 2005). ChR2 is a cationic channel highly permeable to proton ($P^+_H/P^+_{Na} \sim 10^6$) but weakly permeable to Ca^{2+} ($P^{2+}_{Ca}/P^+_{Na} \sim 0.117$) (Nagel et al., 2003; Lin et al., 2009). In neurons, its photoactivation triggers Ca^{2+} elevations which depend mainly on the secondary activation of voltage-gated Ca^{2+} channels (VGCC) (Nagel et al., 2003; Zhang and Oertner, 2007; Li et al., 2012).

Attempts have been made to photoactivate protoplasmic astrocytes. *In situ* experiments suggest that the photoactivation of ChR2-expressing astrocytes can trigger gliotransmitter release (Gradinaru et al., 2009; Gourine et al., 2010; Sasaki et al., 2012; Chen et al., 2013a). In the rat brain stem retrotapezoid nucleus, ChR2-expressing astrocytes responded to long lasting (20–60 s) illumination by slow Ca^{2+} rises that lasted for minutes (Gourine et al., 2010). In the hippocampal CA1 region, blue light pulses induce rapid time-locked Ca^{2+} signals in astrocytes (Chen et al., 2013a). However, our own experiments using mouse cortical astrocytes in culture, show that ChR2 activation induces variable and weak Ca^{2+} elevations (Li et al., 2012). Instead we found that the activation of the Ca^{2+}-permeable light-gated glutamate receptor (LiGluR) [reviewed in (Szobota and Isacoff, 2010)], and the Ca^{2+}-translocating ChR2 (CatCh) (Kleinlogel et al., 2011) evokes reliable and robust Ca^{2+} signals in astrocytes (**Figure 2**). We attributed the low efficacy of ChR2 in astrocytes to its relatively weak Ca^{2+} permeability (Nagel et al., 2003; Lin et al., 2009), and to the absence of VGCC in protoplasmic astrocytes (Carmignoto et al., 1998; Parpura and Verkhratsky, 2012). Interestingly, LiGluR can be rapidly switched ON and OFF to mimic endogenous Ca^{2+} signals recorded with the GCaMP3 (Shigetomi et al., 2013b). Finally, LiGluR activation induces a large Ca^{2+} influx that is further shaped by internal stores, while CatCh activation generates a Ca^{2+} influx insensitive to internal Ca^{2+} store depletion, indicating that LiGluR and CatCh are interesting tools to activate differentially selective Ca^{2+} signaling pathways and to study their downstream effects.

Astrocytes express a rich repertoire of metabotropic G_q, $G_{i/o}$, and G_s GPCRs (Porter and McCarthy, 1997). New light-gated proteins that mimic these GPCR-mediated pathways have been developed (Schroder-Lang et al., 2007; Airan et al., 2009; Ryu et al., 2010; Gutierrez et al., 2011; Stierl et al., 2011; Levitz et al., 2013), but they have not yet been tested on astrocytes. Since astrocytes express store-operated Ca^{2+} channels Orai1 (Akita and Okada, 2011; Linde et al., 2011; Moreno et al., 2012), it should also be of interest to activate them with the new photosensitive synthetic protein LOVS1K that reversibly translocates to Orai1 channels and generates either local Ca^{2+} signals at the plasma membrane or global Ca^{2+} signals upon repeated photoactivation (Pham et al., 2011).

OPTICAL METHODS TO PHOTOACTIVATE ASTROCYTES

While imaging morphological dynamics of astrocytic fine processes may not yet be possible, stimulating astrocytes *locally* with light is more promising because the astrocyte-specific expression of light-sensitive Ca^{2+}-permeable ion channels circumvents the optical resolution problem, and therefore even one-photon whole-field illumination is sufficient to stimulate specifically the astrocytes. A more specific photoactivation of a subset of cells, and the local subcellular stimulation of a single astrocyte can be achieved using spatial light modulators [reviewed in (Maurer et al., 2011)] to shape the light (Shoham, 2010; Vaziri and Emiliani, 2012; Papagiakoumou, 2013). One-photon digital holography allows photoactivation within precisely shaped regions of interest at or near the tissue surface.

Combining digital holography and two-photon excitation with temporal focusing to modulate the temporal width of the pulsed laser, several groups reported shaped two-photon excitation deep inside scattering tissue (Andrasfalvy et al., 2010; Papagiakoumou et al., 2010). The spatial patterns thus generated are robust against scattering and remained confined at depths of 100 µm (Papagiakoumou, 2013). Combining optogenetics, shaped photoactivation and two-photon imaging for the optical readout of astrocytes (combined with electrophysiology for recording neuronal signals) holds important promises for interrogating interactions between neurons and astrocytes in intact brain tissue. To probe specific signaling pathways, wave-front light shaping can be combined with uncaging of classical IP_3 and Ca^{2+} cages (Ellis-Davies, 2011), and new endothelin cage (Bourgault et al., 2007).

MONITORING GLIOTRANSMITTER RELEASE

Among the mechanisms of gliotransmitter release, Ca^{2+}-regulated exocytosis of synaptic-like small vesicles has been proposed as a major pathway (Cali et al., 2009). Total internal reflection fluorescence microscopy (TIRFM) is a powerful technique to monitor single-vesicle behavior and to study the mechanisms of vesicular docking and fusion in cultured cells (Holz and Axelrod, 2008). Since cultured astrocytes may differ from their *in situ* counterparts,

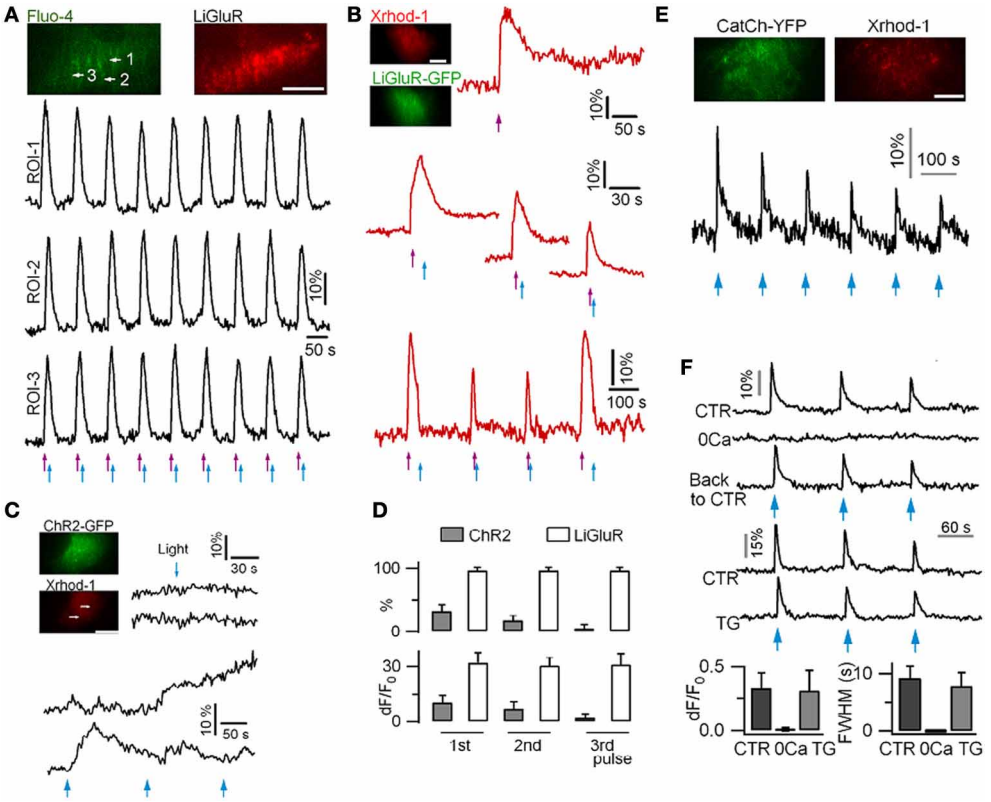

FIGURE 2 | Ca^{2+} signals recorded in mouse cortical astrocytes in culture using LiGluR, ChR2(H134R) and CatCh photoactivation. (A) Light-gated Ca^{2+} rises in an astrocyte expressing LiGluR-mRFP. Ca^{2+} rises were imaged with dual-color TIRFM and repetitively switched on and off by 385-nm (violet arrows, 0.3 mW/mm^2, 50 ms) and 488-nm (blue arrows, 39.1 mW/mm^2, 200 ms) light pulses, respectively. **(B)** LiGluR(GFP)-gated astrocytic Ca^{2+} elevations monitored with the red-fluorescent Ca^{2+} dye Xrhod-1. **(C)** In astrocytes expressing ChR2(H134R), short photoactivation (458-nm, 27.3 mW/mm^2, 500 ms) of ChR2 failed to evoke near-membrane Ca^{2+} elevation (top). Longer light pulses (458-nm, 1 s) evoked variable Ca^{2+} signals (bottom). **(D)** Comparison of the percentage of astrocytes showing light-gated Ca^{2+} rises, and of the amplitude of Ca^{2+} responses in LiGluR- and ChR2-expressing astrocytes. LiGluR evokes more reliable and reproducible Ca^{2+} rises in astrocytes. **(E)** CatCh-evoked astrocyte Ca^{2+} elevations following blue light photoactivation (1 s, 458-nm). **(F)** CatCh-induced Ca^{2+} signaling was abolished in the absence of extracellular Ca^{2+}, but unaffected when ER Ca^{2+} store is perturbed by thapsigargin (TG). Bars, 10 μm. Adapted from (Li et al., 2012).

the physiological relevance of the findings made in culture with TIRFM need to be validated *in situ* using other approaches.

TIRFM has been used to visualize near membrane single vesicles and monitor single vesicle fusion in cultured astrocytes (Bezzi et al., 2004; Zhang et al., 2004; Bowser and Khakh, 2007; Li et al., 2008; Malarkey and Parpura, 2011; Potokar et al., 2013). In early experiments (Bezzi et al., 2004), the fluorescent weak base acridine orange (AO) was used to report exocytosis, and the vesicular glutamate transporter (VGLUT) tagged with the enhanced GFP (EGFP) was overexpressed to identify the AO-positive vesicles. Following DHPG application, rapid millisecond Ca^{2+}-dependent flashes of AO-labeled vesicles were detected and interpreted as the exocytosis of glutamatergic vesicles (Bezzi et al., 2004; Domercq et al., 2006). This interpretation was soon complicated by studies showing that AO metachromasy results in its simultaneous emission of green and red fluorescence, which invalidates the identification of the AO-positive vesicles with EGFP labeling (Nadrigny et al., 2006, 2007). It has also been shown that the flash events of AO-loaded astrocyte vesicles are not solely due to exocytosis but also reflect intracellular vesicle photolysis (Jaiswal et al., 2007; Li et al., 2008), due to the action of AO as a photosensitizer. Styryl pyridinium FM dyes, established markers of vesicular release in neurons (Rizzoli and Betz, 2005), were also used to label the astrocytic vesicular compartments and report exocytosis. However, FM dyes are handled differently by neurons and astrocytes (Li et al., 2009a) and they label mainly lysosomes (Zhang et al., 2007; Li et al., 2008; Liu et al., 2011).

Later, the genetically encoded exocytotic reporter, pHluorin, has emerged as a valuable tool to monitor astrocyte vesicle exocytosis. As a pH-sensitive GFP mutant, pHluorin fluorescence is quenched in the acidic vesicle lumen and becomes bright upon vesicle fusion when the fluorescent protein is exposed to external neutral pH (Miesenbock et al., 1998). Since there was evidence that astrocytes release glutamate via Ca^{2+}-regulated exocytosis (Cali et al., 2009), pHluorin was targeted to the lumen of putative glutamatergic vesicles in astrocytes by using the fusion protein VGLUT1-pHluorin (Marchaland et al., 2008). TIRFM imaging of single vesicles in cultured astrocytes labeled with

VGLUT1-pHluorin revealed fusion events occurring within hundreds of milliseconds after Ca^{2+} rise evoked by either mGluR (Marchaland et al., 2008) or purinergic P2Y1 receptor activation (Santello et al., 2011). These results were consistent with those obtained by the same lab using AO-labeled (Bezzi et al., 2004; Domercq et al., 2006), and FM-labelled astrocytes (Cali et al., 2008).

Different kinetics have been reported for the exocytosis of the putative glutamatergic vesicles in astrocytes when using another pHluorin-based exocytotic reporter synaptopHluorin (spH), a chimeric construct tagging the luminal side of synaptobrevin 2 (Burrone et al., 2006). As synaptobrevin 2 appears to colocalize with VGLUT1 on the same vesicles in astrocytes (Montana et al., 2004; Zhang et al., 2004; Liu et al., 2011), expressing spH in astrocytes leads to the labeling of VGLUT1-positive vesicles (Bowser and Khakh, 2007; Liu et al., 2011). However, unlike VGLUT1-phluorin that reports fast millisecond kinetics of exocytosis (Cali et al., 2008; Santello et al., 2011), spH-labeled vesicles undergo slow exocytosis that is loosely coupled to stimulation, with most events occurring ∼2 min after P2 receptor-mediated Ca^{2+} rise (Malarkey and Parpura, 2011), and within hundreds of milliseconds following Ca^{2+} increase evoked by mechanical stimulation (Liu et al., 2011; Malarkey and Parpura, 2011).

Genetically encoded reporters of exocytosis set the stage for investigating the mechanisms of astrocyte exocytosis and for addressing several remaining questions. First, the reasons for the variable fusion kinetics of putative VGLUT-positive vesicles remain to be elucidated. Second, several studies failed to detect the presence of VGLUT expression in astrocytes (Cahoy et al., 2008; Juge et al., 2010; Li et al., 2013), therefore, new experiments are needed to clarify the molecular identity of the VGLUT-positive vesicles. Recently, a new genetically encoded red pH-sensitive probe, pHTomato, has been introduced to image single vesicle exocytosis (Li and Tsien, 2012). It should allow monitoring exocytosis, and, simultaneously, activating Ca^{2+} signal with optogenetic tools that typically require blue light illumination (Li et al., 2012). Finally, the new genetically encoded glutamate sensor, iGluSnFR, shows fast kinetics (Marvin et al., 2013) and is potentially suitable for fast real-time recording of glutamate release from astrocytes. Combining it with TIRFM detection of single vesicle exocytosis would help to clarify the relative contribution of vesicular vs. non-vesicular release pathways to glutamate release (Kimelberg et al., 2006; Li et al., 2012; Woo et al., 2012). Fourth, a combination of fast two-photon imaging, local photoactivation and genetically targeted expression of pHluorins in slice and *in vivo* must validate earlier findings from cell-culture studies.

TARGETING GENETICALLY ENCODED PROTEINS TO ASTROCYTES

Most optical techniques available today lack the spatial resolution for imaging thin astrocyte processes. Bulk-loaded Ca^{2+} indicators that label both astrocytes and neurons, report mixed signals. However, using GECIs targeted selectively to astrocytes, it becomes possible to record astrocyte-specific Ca^{2+} signals with standard imaging techniques, like confocal, spinning-disk confocal, and two-photon microscopies. Similarly by targeting the light-gated proteins, selective photoactivation of astrocytes can be achieved (see below). Therefore, targeting the GECIs and the light-gated channels/receptors to astrocytes *in situ* is a critical step, and several strategies have been used. First, plasmids can be electroporated *in utero* (Yoshida et al., 2010) but the yield of electroporation is variable, therefore, protocols using intracerebral and intravenous injections of viral constructs and Tg mouse lines are being favored.

Viral constructs are relatively easy to generate and adeno-associated virus (AAV) are the most widely used for astrocyte infection (**Table 1**). Intracerebral injections of lentivirus (LV) and adenovirus (AV) (Liu et al., 2008; Colin et al., 2009; Gourine et al., 2010) have been limited due to their possible toxic effects. Among the AAV variants, the serotype 5 with a high tropism for astrocytes (Ortinski et al., 2010), is commonly used for packaging the DNA constructs. Following intracerebral viral injections, the area of infection extends well beyond the injection site and the expression of the reporter genes is stable for months. In adult mice, intravenous injections of AAV serotype 9 labels mostly astrocytes rather than neurons (Foust et al., 2009). Intraventricular injection of AAV serotype 8 at postnatal day 3 (P3) labels preferentially astrocytes (Kim et al., 2013). New serotypes have been developed using a directed evolution approach with higher transduction level for astrocytes leading to 94% specific expression in retinal Müller glial cells after intravitreal injection (Klimczak et al., 2009). This seems to be promising to reduce the virus titer needed for expression. More specific targeting can be achieved by inserting an astrocyte-specific promoter. The 2.2 kb hGFAP promoter targets selectively ChR2 to astrocytes using LV constructs (Gradinaru et al., 2009). However, since AAVs cannot carry constructs larger than 4.7 kb, a shorter 681 bp hGFAP promoter ($gfaABC_1D$ also called gfa104) (Lee et al., 2008) has been used to target selectively EGFP, ChR2, GECIs (case12, GCaMP3, Lck-GCaMP3), and a pleckstrin homology (PH) domain of phospholipase C-like protein p130 (p130PH) to astrocytes (Ortinski et al., 2010; Xie et al., 2010; Shigetomi et al., 2013b).

Gene targeting of viral constructs can also be achieved by the Cre-Lox or tetO-tTA strategies injecting floxed (or flexed) viral constructs in Cre mouse lines, or tetO virus in the tTA mouse lines (Pfrieger and Slezak, 2012). Several astrocyte-specific Cre- and tTA-expressing mouse lines have been generated (**Table 2**). When a Cre-dependent ChR2-expressing virus was injected in hippocampus (Chen et al., 2013a) of a P14 hGFAP mouse line (Casper and McCarthy, 2006), selective expression of ChR2 was obtained in the astrocytes.

Yet an important limitation of the viral delivery strategies needs to be taken into account. Following intracerebral injection of AAV2/5-gfa104-EGFP construct, a significant dose-dependent reactive gliosis has been observed (Reimsnider et al., 2007; Klein et al., 2008; Ortinski et al., 2010). Since gliosis is associated with changes of several signaling pathways in astrocytes (Hamby et al., 2012), it will be important to develop alternative approaches to study the role of astrocytes in physiological conditions. Introducing a sequence encoding the VIVIT peptide that interferes with the calcineurin/nuclear factor of activated T-cells signaling pathway and down regulates GFAP overexpression (Furman et al., 2012), may reduce AAV-induced

Table 1 | Viral constructs for specific targeting mouse astrocytes.

Viral construct[a]	Applications[b]	Brain regions	Specificity	References
AAV1/2-GFAP-GFP AAV1/2-CBA-GFP[c]	IC at P0, P90	Cortical and subcortical	AAV1/2-GFAP-GFP expressed mostly ALDH1L1(+) cells, AAV1/2-CBA-GFP specific for subcortical neurons at P90	von Jonquieres et al., 2013
AAV2/5-gfaABC$_1$D-Lck-GCaMP3[d] AAV2/5-gfaABC$_1$D-GCaMP3	IC at P49 to P63	CA1 region	Labels GFAP(+) cells	Shigetomi et al., 2013b
AAV8-CBA[e]-eYFP-2A-tTA(2S)	IVE at P3	Whole brain	Labels S100β (+) cells	Kim et al., 2013
AAV2/5-, AAV2/8-, AAV2/9-CBA-EGFP	IVE at P0 to P3	Whole brain	AAV2/5 at P0 to P3 transduces mostly GFAP(+) cells, AAV2/8, AAV2/9 at P3 transduce mostly GFAP(+) cells	Chakrabarty et al., 2013
AV-CMV-Flox-ChR2-mCherry (injected in hGFAP-Cre line)	IC at P14	CA1 region	Labels GFAP(+) cells, but not NeuN(+) cells	Chen et al., 2013a
AAV1-, AAV8-, AAV9, AAVrh10-CAG-GFP	IV at P0, P5, P14, P42	Whole brain	Both neurons and astrocytes were labeled	Miyake et al., 2011
scAAV7-, AAV9-, rh10-rh39-, rh43-CB-EGFP[f]	IV at P1	Whole brain	Both neurons and astrocytes were labeled	Zhang et al., 2011
AV-mCMV-gfaABC$_1$D-Case12 AV-mCMV-gfaABC$_1$D-ChR2(H134R)-Katushka1.3	IC	Hypoglossal motor nucleus	All labeled cells are GFAP(+)	Gourine et al., 2010
AAV2/5-, AAV2/9-CMV-EGFP AAV2/5-gfa104-EGFP	IC	CA1 region	AAV5-CMV shows tropism for astrocytes AAV5-gfa104 >99% selectivity for astrocytes	Ortinski et al., 2010
AAV2/5-gfaABC$_1$D-mRFP-p130PH	IC	Cortex Hippocampus	Selective expression in GFAP(+) cells	Xie et al., 2010
LV-GFAP-hChR2(H134R)-mCherry	IC	Subthalamic nucleus	Labelled cells are GFAP(+)	Gradinaru et al., 2009
LV-PGK-nlsLacZ -miR124T/ LV-PGK-GLAST-miR124T Mokola pseudotyped	IC	Hippocampus, Cerbellum, Striatum	6% neuronal expression in striatum	Colin et al., 2009
scAAV9-CB-GFP	IV, tail injection at P70	Whole brain	Labels mostly astrocytes	Foust et al., 2009
LV-mCMV-gfaABC$_1$D-EGFP	IC	Hypoglossal motor nucleus	All labeled cells are GFAP positive	Liu et al., 2008

[a] We keep the names given in the quoted papers. Notation AAV2/x (also called AAVx) stands for pseudotyped AAV. ITR of coding plasmid derived from AAV serotype 2 and capsid sequence from serotype x.

[b] IC, intracerebral; IV, intravenous; IVE, intraventricular; P0 day of birth.

[c] AAV1/2 chimeric AAV with capsid proteins from AAV1 and AAV2.

[d] gfaABC$_1$D (also called gfa104) is a short (681 bp) version of the hGFAP promoter.

[e] CBA, CB, CAG, hybrid promoter composed of CMV early enhancer element and chicken β-actin promoter.

[f] Sc, self complementary, see McCarty (2008) Self-complementary AAV vectors; advances and applications. Mol. Ther. 16, 1648–1656.

Table 2 | Transgenic (Tg) mouse lines for specifically targeting mouse astrocytes.

Tg mouse lines	Brain regions studied	Specificity	References
Mlc1-tTA & TetO-ChR2(C128S)-YFP	Cerebellum	Light-evoked current in the Bergmann glia	Sasaki et al., 2012; Tanaka et al., 2012
hGFAP-CreER^{T2} & Mecp2$^{+/Stop}$	Whole brain	<5% expression in neurons	Lioy et al., 2011
hGFAP-tTA & tetO-MrgA1-GFP	Hippocampus	Labels GFAP(+) cells	Fiacco et al., 2007; Agulhon et al., 2010
S100β-YC3.60 cameleon	Whole brain	Labels astrocytes, NG2 cells and oligodendrocytes	Atkin et al., 2009
S100β-Cre & Cx43(fl/fl)	Cerebellum	Specific Cx43 deletion in Bergmann glia and molecular layer astrocytes	Tanaka et al., 2008
hGFAP-CreER^{T2} hGFAP-MerCreMer	Hippocampus Cortex Cerebellum	Specific recombination in cortical astrocytes and Bergmann glia in hGFAP-CreER^{T2} and hGFAP-MerCreMer lines	Casper et al., 2007
GLAST-CreER^{T2} Cx30-CreER^{T2} ApoE-CreER^{T2} AQ4-CreER^{T2}	Whole brain	Specific labeling of GFAP- and S100β-positive astrocytes in cortex, hippocampal CA1 region and cerebellum in Cx30-CreER^{T2} and GLAST-CreER^{T2} lines. Density of labeled astrocytes is higher in Cx30-CreER^{T2} line. ApoE-CreER^{T2} and AQ4-CreER^{T2} lines do not label specifically the astrocytes.	Slezak et al., 2007
GLAST-CreER^{T2}	Cortex Hippocampus Cerebellum	Tamoxifen injection in adult mice induce specific labeling of S100β-positive astrocytes in cortex; olfactory bulb, dentate gyrus and cerebellum. Adult-born doublecortin-positive neurons were labeled. Tamoxifen injection at E18 induces recombination both in neurons and astrocytes	Mori et al., 2006
human or mouse GFAP-Cre	Whole brain	Recombination occurs both in astrocytes and neurons	Casper and McCarthy, 2006
hGFAP-CreER^{T2}	Whole brain	Recombination is specific in GFAP- and S100β-positive astrocytes in hippocampus and cerebellum. Recombination is weak in cortex.	Hirrlinger et al., 2006

gliosis. Replacing viral constructs by Tg floxed/tetO mouse lines is a very promising approach to control gliosis. Several floxed (Slezak et al., 2012; Zariwala et al., 2012) and tetO (Fiacco et al., 2007; Agulhon et al., 2010) mouse lines of interest have been generated. With a hGFAP-CreER^{T2} mouse line in which the recombination can be induced in juvenile or adult mice by tamoxifen injections, astrocyte-specific targeting has been obtained in cortex, hippocampal CA1 region, cerebellum, diencephalon and brain stem, with weaker levels of recombination in cortex (Hirrlinger et al., 2006; Lioy et al., 2011). In glutamate-aspartate transporter (GLAST)- and connexin 30 (Cx30)-CreER^{T2} mouse lines, astrocyte-specific recombination occurs in cortex, hippocampal CA1 region, and cerebellum. But in GLAST-CreER^{T2} and hGFAP-CreER^{T2} mouse lines, the recombination is not astrocyte-specific in brain regions including the olfactory bulb and hippocampal dentate gyrus, where neurons are also labeled (Mori et al., 2006; Slezak et al., 2007). The floxed/tetO strategy is advantageous since it does not require surgery for viral injections. A Ai38 floxed GCaMP3 reporter mouse line was generated by a knockin strategy to insert GCaMP3 into the ROSA26 locus (Zariwala et al., 2012). When the Ai38 mouse was crossed with an inducible Wfs1-Tg2-CreER^{T2} mouse

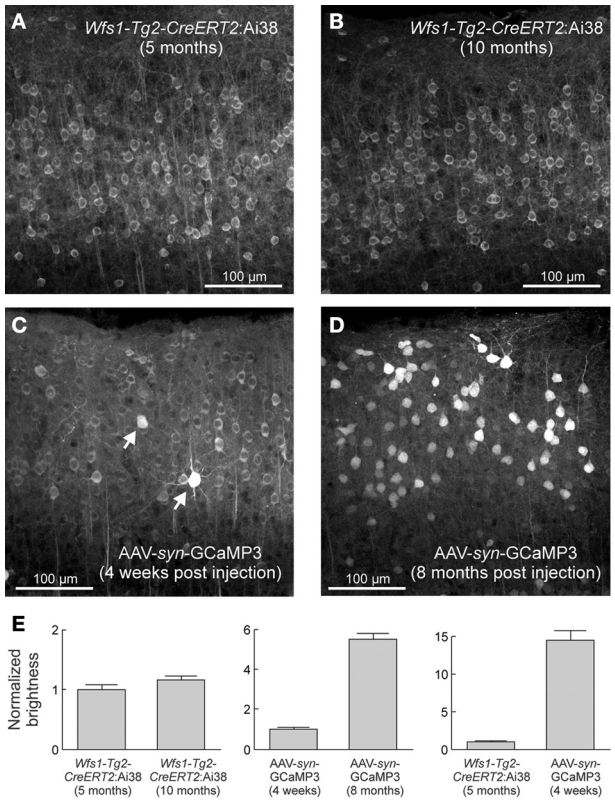

FIGURE 3 | Stable expression levels in the Ai38 ROSA26-GCaMP3 mouse line over months. Native GCaMP3 fluorescence in layer2/3 excitatory neurons of visual cortex from Wfs1-Tg2-CreERT2:Ai38 mice **(A,B)** and adult wild-type mice injected with AAV-syn-GCaMP3 **(C,D)**. **(E)** Quantification of neuronal brightness. Error bars correspond to SEM. From (Zariwala et al., 2012), with permission.

line, a uniform expression of the reporter genes was obtained in cortical excitatory neurons without over expression of GCaMP3 in the nucleus as observed after cortical injection of an AAV-syn-GCaMP3 construct (**Figure 3**). Importantly, it appears that various Cre- and tTA-dependent mouse lines differ in their ability to induce recombination, and also the specificity of recombination can vary with the brain region, and with the age. Therefore, in order to ascertain specificity of astrocytic signal measurement and photoactivation, it will be critical to carefully validate astrocyte-specific expression, for example, by using cell type-specific antibodies and confocal microscopy.

PERSPECTIVES

In conclusion, new genetically targeted optical and pharmacological tools allow the selective measurement and activation of astrocytic Ca^{2+} signals. These tools should be of value for studying the mechanisms of gliotransmitter release, the role of astrocytes, and more specifically the bidirectional communication between astrocytes and neurons.

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Recent molecular approaches to understanding astrocyte function *in vivo*

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Astrocytes are a predominant glial cell type in the nervous systems, and are becoming recognized as important mediators of normal brain function as well as neurodevelopmental, neurological, and neurodegenerative brain diseases. Although numerous potential mechanisms have been proposed to explain the role of astrocytes in the normal and diseased brain, research into the physiological relevance of these mechanisms *in vivo* is just beginning. In this review, we will summarize recent developments in innovative and powerful molecular approaches, including knockout mouse models, transgenic mouse models, and astrocyte-targeted gene transfer/expression, which have led to advances in understanding astrocyte biology *in vivo* that were heretofore inaccessible to experimentation. We will examine the recently improved understanding of the roles of astrocytes – with an emphasis on astrocyte signaling – in the context of both the healthy and diseased brain, discuss areas where the role of astrocytes remains debated, and suggest new research directions.

Keywords: astrocytes, knockout mice, transgenic mice, chemogenetics, viral gene transduction, glial cell progenitors, GPCR, neuron–glia interactions

INTRODUCTION

Although astrocytes are the most abundant glial cell type in the mammalian nervous system and have emerged as crucial regulators of nervous system development, function, and health, our understanding of the physiology of astrocytes remains limited. How do astrocytes interact with neurons and other cell types of the nervous system? What are the primary functions of astrocytes in brain development, health, and disease? One challenge to address these questions is identifying the function of individual astrocytic molecules that regulate brain function. A unique approach to investigate the molecular basis of astrocyte activity consists of manipulating the genome of higher organisms. The mouse represents a great animal model that has been used extensively for genetic manipulation in neuroscience, leading to understanding of neuronal functions in unprecedented detail. A concerted effort in recent years to develop genetic approaches to study astrocytes is guiding the field into new territory and improving our understanding of interactions between neurons and astrocytes. Here we will review techniques for genetic manipulation of astrocytes and highlight the most recent innovative and elegant approaches that are providing insight into fundamental roles of astrocytes in pathophysiology *in vivo*. In particular, we will describe the main molecular approaches used in this field, including knockout (KO) mouse models, transgenic mouse models, and astrocyte-targeted gene transfer and expression using adeno-associated viral (AAV) or *in utero* electroporation (IUE) approaches. We will also touch on a remarkable recent study involving engraftment of genetically modified human glial progenitors into the mouse brain, providing insight into the role of human astrocytes in the unique cognitive abilities of the human brain. The present review does not attempt to be comprehensive; rather it highlights certain major themes and areas of recent progress on the roles of astrocytes in brain function, with an emphasis on astrocyte signaling and *in vivo* studies. For complementary reviews on astrocyte function in health and disease with an emphasis on molecular approaches, readers are directed to (Fiacco et al., 2009b; Figueiredo et al., 2011; Agulhon et al., 2012; Nedergaard and Verkhratsky, 2012; Clarke and Barres, 2013; Freeman and Rowitch, 2013; Tong et al., 2013), as well as the other contributions to this special topic.

STUDYING ASTROCYTE FUNCTION THROUGH GENE DISRUPTION

The elimination of one or more specific genes in an animal model is a reliable and widespread approach to discovering the function of specific proteins and the cell types expressing them. Many genes have been identified, isolated, and subsequently manipulated to fully or conditionally suppress their expression (Sikorski and Peters, 1997; Grimm, 2006; Vogel, 2007). These molecular

developments represent powerful tools that can be used *in vivo* to aid in discovering the role of astrocytes in the healthy and diseased brain.

FULL (CONSTITUTIVE) KNOCKOUT MOUSE MODELS

As research into astroglia physiology *in vivo* is still a newly developing field, much information can be gleaned from the elimination of a gene or the deletion of a functional domain of a protein in astrocytes to shed light on the function of both the targeted gene and astrocytes in general. The process of generating a new line of KO mice is laborious, but has been refined to maximize efficiency (reviewed in Hall et al., 2009; Limaye et al., 2009). Once the desired gene is identified, gene targeting can be used to generate a KO mouse. A targeting vector containing a neomycin-resistant marker is inserted into embryonic stem (ES) cells via electroporation and is introduced into the DNA through homologous recombination, allowing complete removal of one or more exons from the gene of interest (**Figure 1A**). This results in the production of a mutated or truncated protein or, more often, no protein at all. ES cells that do not take up the foreign construct are killed through exposure to neomycin, and those that have successfully replaced the gene or the exons of this gene survive and are subsequently microinjected into mouse blastocysts, which are then grown in surrogate mouse uteri. Strategic mating of the chimeric mice will ultimately result in a mouse with the gene globally eliminated (Capecchi, 1989; Hall et al., 2009; Limaye et al., 2009; **Figure 1A**).

Role of GFAP in the healthy and diseased brain

One of the first genes that was removed in astrocytes is the gene encoding glial fibrillary acidic protein (GFAP; Lewis et al., 1984; Reeves et al., 1989; Masood et al., 1993; McCall et al., 1996; Eng et al., 2000). GFAP is a member of the family of intermediate filament structural proteins, which, in the mature nervous system, is found predominantly in protoplasmic and specialized astrocytes of the central nervous system (CNS) as well as in satellite cells, non-myelinating Schwann cells, and enteric glia in the peripheral nervous system (PNS; Jessen et al., 1984; Eng, 1985; Kato et al., 1990). Outside the nervous system, GFAP has also been detected in some rare non-glial cells of the salivary glands (Achstatter et al., 1986; Gustafsson et al., 1989), fibroblasts (Hainfellner et al., 2001), myoepithelial cells (Viale et al., 1991), liver stellate cells (Gard et al., 1985), and lymphocytes (Riol et al., 1997). This protein is one of the key elements of the cytoskeleton that contributes to the morphology and motility of astrocyte processes (Fuchs and Weber, 1994; Pekny and Pekna, 2004; Gomi et al., 2010; Middeldorp and Hol, 2011) and is upregulated in reactive astrocytes (astrogliosis) in essentially any CNS pathology (Eng and Ghirnikar, 1994; Eng et al., 2000; Pekny and Nilsson, 2005; Sofroniew, 2009; Sosunov et al., 2013). During development, GFAP is expressed widely in a number of progenitor cell types giving rise to both neurons and glia. For example, GFAP-expressing radial glia in the ventricular zone (VZ) give rise to mature astrocytes, oligodendrocytes and neurons, as well as guiding subsequent migration of neurons (Gotz et al., 2002; Malatesta et al., 2003; Anthony et al., 2004; Merkle et al., 2004).

The seminal studies reporting the findings obtained with the GFAP KO mouse models were made by targeted deletion of the GFAP gene in ES cells (Pekny et al., 1995; Liedtke et al., 1996; McCall et al., 1996) or by targeted disruption of the GFAP gene by insertion of a *LacZ* cassette (Gomi et al., 1995). These GFAP KO mouse lines have been used to investigate whether changes in astrocyte processes and their morphological structure can influence brain morphogenesis and function, the physiology of adjacent synapses, and functional recovery after CNS insults. The overall appearance of the GFAP KO mice is indistinguishable from wild-type mice; they develop normally and display no gross alterations in behavior or CNS morphology. This observation suggests at first that GFAP is not essential for normal brain morphogenesis and function. However, closer analyses have indicated the involvement of GFAP in a wide variety of processes. First, these mice display enhanced hippocampal long-term potentiation (LTP) and deficient cerebellar long-term depression (LTD) in acute brain slices from adult mice, suggesting that GFAP intermediate filament protein is important for astrocyte–neuronal interactions and that astrocyte processes play a vital role in modulating synaptic efficacy in the CNS (McCall et al., 1996; Shibuki et al., 1996). In agreement with the cerebellar *ex vivo* findings, a significant impairment of eye blink conditioning was found in the GFAP KO mice, suggesting that GFAP is required for normal communication between Bergmann glia (specialized astrocytes) and Purkinje cells during induction and maintenance of cerebellar LTD *in vivo* (Shibuki et al., 1996). Second, morphological and functional alterations in the blood–brain barrier (BBB), disorganization of white matter architecture and vascularization, as well as hydrocephalus were reported in 18–24 month GFAP KO mice, suggesting an involvement of GFAP in the long-term maintenance of normal BBB and CNS myelination (Liedtke et al., 1996). Third, when challenged by diverse brain injuries *in vivo*, the GFAP KO mice were more vulnerable to: (i) CNS mechanical trauma (Nawashiro et al., 1998; Otani et al., 2006), (ii) cerebral ischemia (Nawashiro et al., 2000; Tanaka et al., 2002), (iii) kainic acid-induced neurotoxicity (Otani et al., 2006), and (iv) autoimmune encephalomyelitis (Liedtke et al., 1998). This was indicated by: (i) increased brain hemorrhage and mortality of mice; (ii) larger cortical infarct volume and profound decrease in cerebral blood flow; (iii) neurodegeneration; and (iv) enhanced clinical course of autoimmune encephalomyelitis and lesions, respectively. Conversely, other investigators have found that suppressing astrocytic GFAP expression in reactive astrocytes increases their basal levels of glial cell derived neurotrophic factor (GDNF), leading to improvement in neuronal survival from metabolic and excitotoxic insults (Hanbury et al., 2003). Beneficial neuroprotective and regenerative effects have also been reported after hippocampal and spinal cord injuries in mice lacking both GFAP and vimentin, another astrocyte intermediate filament protein (Kinouchi et al., 2003; Menet et al., 2003; Wilhelmsson et al., 2004). Collectively, these findings suggest that the GFAP component of the astrocyte cytoskeleton plays an important role in the physiology and pathology of the nervous system. However, because GFAP is expressed in progenitor cells during development giving rise to neurons, oligodendrocytes and astrocytes, these findings have to be viewed carefully. Inducible GFAP KO strategies can be used to circumvent this problem by removing GFAP only from astrocytes after this developmental window has closed (see below).

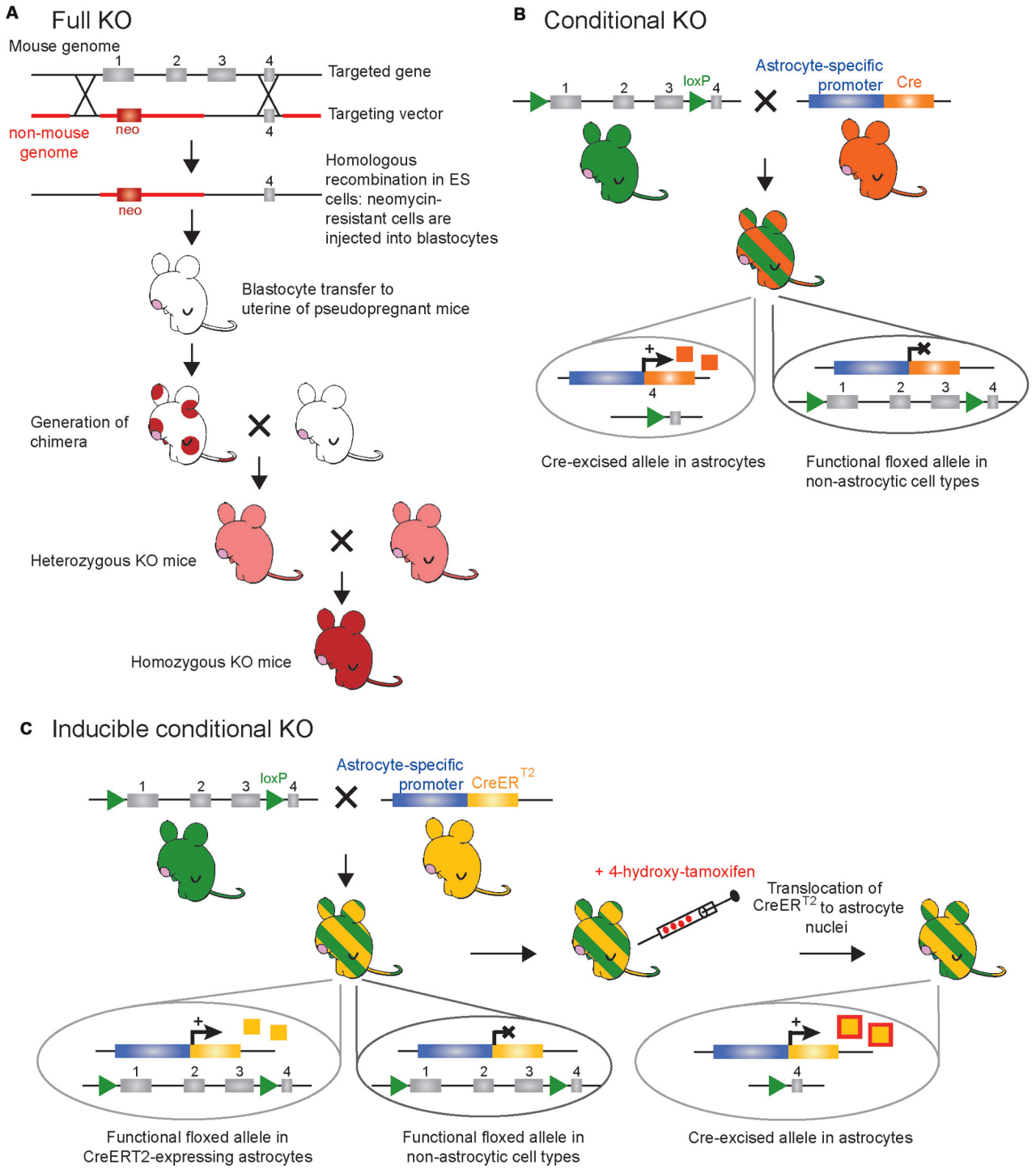

FIGURE 1 | Schematic representation of the main genetic manipulations to make knockout (KO) mice. (A) Conventional strategy for full (constitutive) gene knockout. The targeted gene is inactivated following insertion of an antibiotic (neomycin or neo) resistance gene within, or in place, of one of several essential exons(s) through electroporation of a targeting vector in murine embryonic stem (ES) cells and subsequent homologous recombination. Totipotent embryonic ES cells with successful recombination will survive neomycin treatment to be selectively chosen and injected in mouse blastocysts. Blastocytes are then transferred to the uterus of pseudopregnant females where they can differentiate into all cell types of a chimeric mouse (red circles). After breeding the chimeric mice, the resulting offspring will derive from the ES cells – as seen with the transmission of coat color (red) – if the introduced ES cells become established into the germline of the chimeric mouse. In the full KO, the gene of interest is knocked out in all cell types (not only astrocytes) of the offspring. **(B)** Classical Cre-*LoxP* strategy for conditional gene knockout. The creation of two mouse lines is necessary. First, a floxed mouse line (green) is obtained by homologous

(Continued)

FIGURE 1 | Continued
recombination in ES cells. Two *LoxP* sites are positioned in intronic regions that flank one or several essential exons of the targeted gene. In this mouse line the targeted gene is normally expressed. Second, a Cre mouse line (orange) created by classical transgenesis, following pronuclear injection of the cDNA encoding the Cre-recombinase under the control of an astrocyte-specific promoter. This line gives astrocyte selectivity to the system. Breeding of the floxed mouse with the Cre mouse leads to the generation of a new mouse line (green/orange-striped) in which the floxed exon(s) are excised only in astrocyte-expressing Cre, while the targeted gene remains functional in other cell types. In this case the Cre-recombinase (orange squares) is constitutively expressed, i.e., inactivation of the targeted gene occurs as soon as the astrocyte-specific promoter driving Cre-recombinase is active. **(C)** Inducible $CreER^{T2}$-*LoxP* strategy for temporal control of gene knockout. As in **(B)**, a floxed mouse line (green) and a $CreER^{T2}$ mouse line (yellow) are necessary to obtain a third mouse line (green/yellow-striped) in which the floxed exon(s) are excised only in astrocyte-expressing $CreER^{T2}$. In this mouse line, the $CreER^{T2}$-recombinase (yellow squares) is expressed in astrocytes but is kept in the cytoplasmic compartment and is inactive. To achieve temporal control of the gene knockout, $CreER^{T2}$ activity (red/yellow squares) is induced by synthetic steroid ligand (tamoxifen or 4-hydroxy-tamoxifen capable of crossing the blood brain barrier) administrated systemically at any chosen time. Binding of steroid to $CreER^{T2}$ allows translocation of $CreER^{T2}$ to the nucleus where recombination of floxed genes can occur selectively in astrocytes while the targeted gene remains functional in other cell types.

In light of more recent evidence suggesting that astrogliosis is not a uniform process but rather a multifaceted response with context-dependent reactions (reviewed in Sofroniew, 2009; Sofroniew and Vinters, 2010), the GFAP KO mouse models will continue to be valuable tools in future studies seeking to further unravel the function of GFAP and astrocytes in the variety of human brain challenges or diseases *in vivo*. These mouse models will assist in understanding the stages when astrocytes are engaged in beneficial or detrimental functions. Furthermore, a number of considerations can be taken into account which may lead to both a re-evaluation of earlier findings using these mice as well as open up new research directions. These include the following: (i) the heterogeneity of astrocyte morphology and physiology (Aley et al., 2006; Emsley and Macklis, 2006; Lee et al., 2006; Matyash and Kettenmann, 2010; Zhang and Barres, 2010; Oberheim et al., 2012; Freeman and Rowitch, 2013; Schreiner et al., 2013); (ii) the variability of GFAP expression levels among this heterogeneous cell population (Bernal and Peterson, 2011; Daniel-Christoph et al., 2013); (iii) the fluctuation of GFAP expression during circadian light–dark cycles, hormonal cycles, developmental, or pathological stages (Hajos, 2008); (iv) the existence of about eight alternatively spliced GFAP isoforms that may execute distinct functions in specific subsets of astrocytes (Middeldorp and Hol, 2011); and (v) the post-translational modification of these different isoforms such as phosphorylation and glycosylation that may influence GFAP assembly into intermediate filaments (Middeldorp and Hol, 2011). Suitable GFAP isoform-specific KO models will be required in future studies to address these issues. One way to investigate the impact of alternative splicing of the GFAP gene in a mouse model would be to selectively delete a single isoform. This can be accomplished by deleting a coding exon from the genome, introducing a stop codon, inactivating the splice sites responsible for generating a specific isoform, or overexpressing a dominant negative version of a certain isoform (Moroy and Heyd, 2007). An alternative way to investigate the impact of alternative GFAP splicing would be to use antisense oligonucleotides to prevent the inclusion of a particular exon in the mature mRNA (Gebski et al., 2003; Dori et al., 2005). Isoform-specific KO mouse models provide a compelling approach to study how alternative splicing of the GFAP gene may contribute to the regulation of pathophysiological CNS processes *in vivo*.

Role of IP_3R2 in normal synaptic transmission and plasticity

Another global KO of a gene in astrocytes is the gene encoding the inositol-1,4,5-trisphosphate type 2 receptor (IP_3R2). Two constitutive IP_3R2-deficient mouse models have been obtained through a variation of the gene targeting strategy. In one, the targeting construct was generated by flanking exon 3 of IP_3R2 with two *LoxP* sites and flanking the neo-cassette by FRT sites (Li et al., 2005). In the other, exon 1 of the gene was fused with a *LacZ* cassette (Futatsugi et al., 2005). Deletion of the IP_3R2 subtype is a valuable tool in astrocyte research, as it is the only functional IP_3R subtype expressed by astrocytes and the main mechanism by which astrocytes elevate intracellular Ca^{2+} levels (Petravicz et al., 2008; Agulhon et al., 2010; Di Castro et al., 2011; Takata et al., 2011). This mouse line has been critical to address the concept of gliotransmission, which stipulates that neuroactive molecules including neurotransmitters (called gliotransmitters) are released from mature passive astrocytes in a neuronally-induced and Ca^{2+}-dependent manner downstream of G_q protein-coupled receptors (G_q GPCRs) to quickly modulate synaptic transmission and plasticity (illustrated and comprehensively discussed in Agulhon et al., 2008, 2012; Hamilton and Attwell, 2010). Upon G_q GPCR activation, IP_3 is produced intracellularly, leading to astrocytic IP_3R2 activation and Ca^{2+} release from the endoplasmic reticulum. *Ex vivo* and *in vivo* studies demonstrate that Ca^{2+} transients in mature passive astrocytes are driven by metabotropic G_q GPCRs, which can be activated by the spillover of neurotransmitters released from presynaptic terminals (Porter and McCarthy, 1996; Wang et al., 2006). Therefore, astrocytic G_q GPCRs are considered to be the physiological link between neuronal activity and detectable Ca^{2+} increases in mature astrocytes. However, it is important to keep in mind that future studies using improved imaging methods may reveal alternate sources of activity-driven Ca^{2+} transients in astrocytes. For example, new membrane-tethered genetically encoded Ca^{2+} indicators have allowed for detection of previously unreported constitutive Ca^{2+} transients (Shigetomi et al., 2013b; Tong et al., 2013).

The use of the IP_3R2 KO mouse model was done to selectively obliterate the endogenous G_q GPCR/IP_3R2-mediated Ca^{2+} elevations. The initial studies using this mouse model reported that removing astrocytic G_q GPCR/IP_3R2-mediated Ca^{2+} fluxes did not affect basal and evoked excitatory synaptic transmission (EPSCs), or short- and long-term plasticity (NMDA receptor-dependent LTP) in the hippocampus *ex vivo* (Fiacco et al., 2007; Petravicz et al., 2008; Agulhon et al., 2010). The implications of these findings have already been well documented and discussed (Agulhon et al., 2008, 2010, 2012; Petravicz et al., 2008; Fiacco et al., 2009a; Hamilton and Attwell, 2010; Kirchhoff, 2010; Nedergaard and Verkhratsky, 2012). Recently, an *ex vivo* study

replicated some of the above-described data by showing that hippocampal NMDA receptor-mediated LTP is normal in IP_3R2 KO mice, supporting the initial findings that the IP_3R2-mediated internal Ca^{2+} store pathway is not involved in activity-evoked gliotransmitter release at the hippocampal CA1–CA3 synapses (Shigetomi et al., 2013b). Moreover, the authors of this study discovered a novel mechanism based on transient receptor potential A1 (TRPA1)-mediated transmembrane Ca^{2+} fluxes, through which astrocytes can modulate LTP. They found that pharmacological blockade or genetic deletion of a recently described TRPA1 channel in astrocytes alters free basal Ca^{2+} levels, leading to a decrease in Ca^{2+}-dependent constitutive/homeostatic release of D-serine, and thus LTP reduction – in support of a previous study (Henneberger et al., 2010). Such Ca^{2+} rises are not the type of neuronally-induced astrocyte Ca^{2+} elevations suggested to drive fast release of gliotransmitters by mature astrocytes. Overall these findings emphasize how different astrocyte Ca^{2+} sources (i.e., activity-evoked G_q GPCR/IP_3R2-dependent Ca^{2+} elevations vs. constitutive TRPA1-dependent basal Ca^{2+} dynamics) have distinct effects on LTP (Shigetomi et al., 2013b), and potentially other physiological effects previously attributed to IP_3R2-driven astrocyte Ca^{2+} elevations.

Recent studies using IP_3R2 KO mice *in vivo* reported that hippocampal muscarinic or cortical NMDA receptor-mediated LTP is diminished or abolished, suggesting a role for IP_3R2-driven Ca^{2+} elevations in synaptic plasticity (Takata et al., 2011; Navarrete et al., 2012). Two exciting new reports are providing a potential explanation for the differences observed between the early and later studies using the IP_3R2 KO. By using a broad range of G_qGPCR antagonists, Wang and coauthors excluded the occurrence of gliotransmission both *ex vivo* and *in vivo* (Wang et al., 2012a,b), consistent with the prior *ex vivo* studies using the IP_3R2 KO mice. Rather, they identified a mechanism by which astrocytic G_q GPCR/IP_3R2-mediated Ca^{2+} elevations stimulate the Na^+, K^+-ATPase, leading to a transient K^+ uptake by astrocytes, a decrease in the extracellular K^+ concentration, and a subsequent modulation of excitatory postsynaptic currents. This modulation of synaptic transmission was not observed in the IP_3R2 KO mice, implying that astrocyte G_q GPCR/IP_3R2/Ca^{2+}-induced decrease of extracellular K^+ concentration endows astrocytes with a simple and powerful mechanism for rapid modulation of neuronal activity (Wang et al., 2012a,b). Modulation of K^+ uptake by astrocytic G_q GPCRs was also observed *ex vivo* (Devaraju et al., 2013; Wang et al., 2013). Rapid modification of K^+ uptake provides a mechanism which may be responsible for effects on neuronal activity that hitherto have been ascribed to gliotransmission. Nevertheless, synaptic properties are variable within the nervous system, and astrocytes represent a genetically and functionally heterogeneous group of cells (Zhang and Barres, 2010; Oberheim et al., 2012); these cells are likely to exhibit different functions in different areas of the CNS or even within the same area. Therefore, the different effects of knocking out IP_3R2 on neuronal activity support the need to consider heterogeneity of astrocytes between different brain regions when comparing data across studies. Further investigation is necessary to determine the mechanisms involved in activity-induced astrocytic G_q GPCR/IP_3R2/Ca^{2+}-mediated modulation of neuronal excitability and LTP *in vivo*.

In our view, a fundamental and still open question remains to be answered in order to address this issue thoroughly: do neurons express IP_3R2? If so, does this neuronal receptor subtype play a role in specific types of synaptic transmission and plasticity? Fully deleting IP_3R2 abolishes spontaneous and activity- or agonist-dependent G_q GPCR/IP_3R2-mediated Ca^{2+} increases in astrocytes but leaves intact neuronal Ca^{2+} signaling (Petravicz et al., 2008; Di Castro et al., 2011; Takata et al., 2011; Chen et al., 2012; Navarrete et al., 2012; Wang et al., 2012a,b). However, caution should be exercised in the interpretation of the positive physiological data using this mouse model. Indeed, past immunohistochemical studies aimed at identifying the expression of IP_3R2 in neurons were inconclusive (Sharp et al., 1999; Holtzclaw et al., 2002; Hertle and Yeckel, 2007), and most recent antibodies against this IP_3R subtype are of poor quality (Takata et al., 2011; Chen et al., 2012). Additionally, functionally testing whether neurons display IP_3R2-mediated Ca^{2+} signaling would require the use of double KOs for the two other IP_3R subtypes (IP_3R1 and IP_3R3), which is complicated because most of the IP_3R1-deficient mice die *in utero* (Matsumoto et al., 1996). We are then left with the possibility that some neurons, which also have heterogeneous subtypes throughout the brain, may express IP_3R2 in addition to IP_3R1 and/or IP_3R3. Such a hypothesis, if validated, could help address some of the discrepancies between positive and negative findings using the IP_3R2 KO mice. The use of inducible conditional IP_3R2 KO mice to selectively knock out IP_3R2 in neurons vs. astrocytes in adult mice appears to be the next obvious step to help differentiate between neuronal vs. astrocytic G_q GPCR effects on neuronal excitability and LTP *in vivo*. Finally, the currently available and future inducible IP_3R2 KO mouse models may help provide insight into brain pathology; recent studies suggest that abnormal (increased) astrocytic Ca^{2+} dynamics are linked to the symptoms of Alzheimer's disease (Takano et al., 2007; Kuchibhotla et al., 2009; Grolla et al., 2013), suggesting that controlling astrocytic Ca^{2+} homeostasis might be a potential form of therapy for neurodegenerative disease.

CONDITIONAL AND INDUCIBLE CONDITIONAL KNOCKOUT MOUSE MODELS

The complete knock out of genes in mice has allowed researchers to investigate the role of specific genes *in vivo*. When a gene is selectively expressed in a specific cell type and/or tissue, a constitutive KO is thus cell-specific and/or tissue-specific. However, it is rare that genes are expressed by a single cell type. In such instances, the phenotypes of KO mice can be very complex to interpret and it is not uncommon for a KO mouse to display embryonic lethality (Matsumoto et al., 1996), show no phenotype at all, or affect other gene products (Iacobas et al., 2004). To overcome these obstacles, genes have been knocked out in a cell-specific manner with the use of Cre recombinase/*LoxP* technology. Such gene KOs are referred to as conditional gene KOs (cKO; **Figure 1B**). The site-specific recombination system of the P1 bacteriophage using Cre and *LoxP*-flanked genes is well documented (Sternberg and Hamilton, 1981), although it was only much later that it was used in mouse lines to induce cell-specific gene cKOs in non-nervous and/or nervous tissues (Gu et al., 1994;

Morozov et al., 2003). In order to accomplish this, two mouse lines are required: one that has been genetically engineered to express the Cre site-specific DNA recombinase of bacteriophage P1 under a cell-specific promoter (transgenic mice; **Figure 2A**), and a second that has been made via homologous recombination in ES cells expressing the 34-base-pair *LoxP* site (recognition site for Cre recombinase) flanking the gene of interest or one or more exons of this gene. When these two mouse lines are crossed, the gene or exons of interest are excised to obtain the desired cKO mouse line (reviewed in Sauer, 1998; **Figure 1B**). While cKO mice are very valuable tools, their use has been limited in the astrocyte field because the promoters that are known to be astrocyte specific in the adult are also expressed in progenitor cells of the developing brain. As a consequence, not only astrocytes, but also a large percentage of neurons and oligodendrocytes, exhibit recombination when using astrocyte-specific promoters to drive the expression of Cre recombinase (Gotz et al., 2002; Malatesta et al., 2003; Anthony et al., 2004; Merkle et al., 2004; Casper and McCarthy, 2006). This limitation prompted investigators in the field to develop transgenic mice that enable *inducible* cell-specific gene KOs in order to recombine astrocytic genes postdevelopmentally (referred to as inducible cKO mice; **Figure 1C**). To this end, mice have been developed which express Cre recombinase fused to a mutated form of the human estrogen receptor (ER^{T2}) that restricts the fusion protein to the cytoplasm unless exposed to the estrogen analog tamoxifen (or 4-hydroxy-tamoxifen; Feil et al., 1997). This form of the Cre recombinase, called $CreER^{T2}$, enters the nucleus to cause cell-specific recombination only when tamoxifen is given to mice

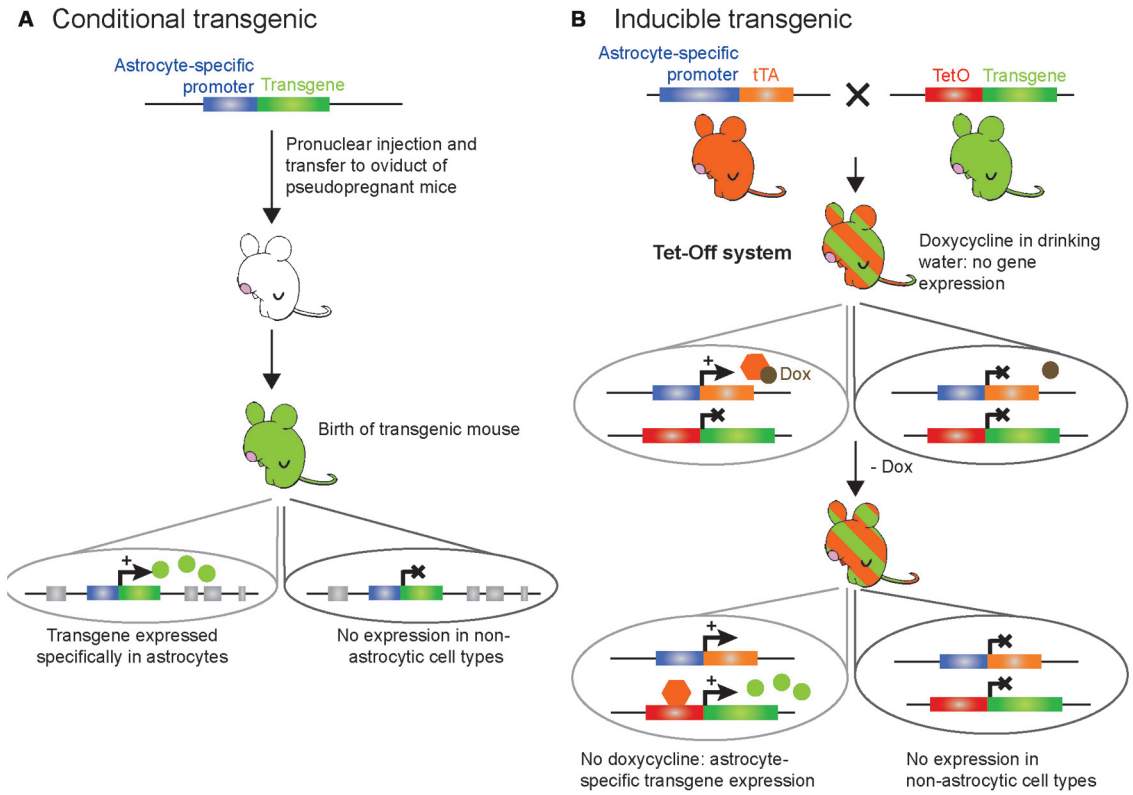

FIGURE 2 | Schematic representation of the genetic modification systems to make astrocyte-specific transgenic mice. (A) Conventional strategy for conditional expression of a transgene selectively in astrocytes. A transgene of interest is placed under the control of an astrocyte-specific promoter; this cDNA construct is purified and microinjected into the male pronucleus of fertilized murine eggs. Several copies of the DNA will be randomly inserted in the mouse genome. Then, the eggs are transferred to the oviduct of pseudopregnant females. The resulting offspring (green) deriving from the eggs will display constitutive transgene expression selectively in astrocytes as soon as the astrocyte-specific promoter is active, while it will not be expressed in other cell types. **(B)** Tet-Off strategy for inducible and temporal control of transgene expression selectively in astrocytes. The TeT-Off system has been used more frequently than the Tet-On system, and is based on the tetracycline (tet) bacterial resistance gene operon. The creation of two transgenic lines is required. The first transgenic line (orange) contains the tTA under the control of an astrocyte-specific promoter: this line provides astrocyte selectivity to the system. The second transgenic line (green) contains the transgene of interest under the control of the tet operon (TetO) DNA-binding element fused to a minimal promoter from cytomegalovirus. The TetO promoter element can be activated upon binding of the tetracycline transactivator (tTA) to drive ubiquitous expression. When a tetracycline derivative, such as doxycycline, is given to the bigenic mice (green/orange-striped – obtained from breeding the tTA line with the TetO line) in the drinking water, doxycycline binds to tTA to prevent its interaction with the TetO minimal promoter and thereby turn off transgene expression selectively in astrocytes. Removing doxycycline from the drinking water of the bigenic mice leads to binding of tTA to the TetO minimal promoter and transcription of the transgene of interest within a week. This allows desired temporal expression of the transgene selectively in astrocytes while it is not expressed in other cell types. Additionally, this system allows testing whether potential physiological or behavioral effects of the transgene are reversible by putting the bigenic mice back on doxycycline.

(Hayashi and McMahon, 2002). Thus, genetically engineered mice expressing CreER^{T2} under the control of an astrocyte-specific promoter (e.g., GFAP, glutamate transporter GLAST, Aldh1l1 or Cx43) allows study of the effect of gene deletion specifically in astrocytes if tamoxifen is given to mice at later development stages (Eckardt et al., 2004; Hirrlinger et al., 2006; Mori et al., 2006; Casper et al., 2007; **Figure 1C**). One limitation of the CreER^{T2} inducible system and inducible systems in general is that recombination efficiency may be low and therefore the phenotypes generated may be more subtle, as has been reported for Cx43 compared to floxed reporter genes (Casper et al., 2007).

Role of CB$_1$R in working memory

Multiple inducible cKO mice have been recently developed to study astrocytic function *in vivo*. One example is a tamoxifen-inducible cKO mouse specifically lacking cannabinoid type-1 receptor (CB$_1$R) expression in astrocytes (Han et al., 2012). A mouse line carrying a *LoxP*-flanked CB$_1$R (Marsicano et al., 2003) was crossed with a line expressing CreER^{T2} under the control of the human GFAP promoter (Hirrlinger et al., 2006). The resulting new line of mice (GFAP-CB$_1$R-KO) has been used to investigate the mechanisms underlying impairment of working memory, which is one of the most important deleterious effects of marijuana intoxication in humans (Ranganathan and D'Souza, 2006) and animals (Lichtman and Martin, 1996; Wise et al., 2009). Understanding the side-effects associated with the use of marijuana has important clinical implications because the derivatives of marijuana or synthetic cannabinoids represent promising therapeutic molecules for several human conditions, including pain, nausea, seizures, ischemia, cerebral trauma, and tumors (Lemberger, 1980; Carlini, 2004; Hall et al., 2005). The endocannabinoid system has recently emerged as an important neuromodulatory system, and the CB$_1$R (GPCR predominantly coupling to G$_i$ proteins) is highly abundant in the CNS (Herkenham et al., 1990; Matsuda et al., 1990). In particular, the CB$_1$R is expressed in glutamatergic and GABAergic neurons (Herkenham et al., 1990; Kawamura et al., 2006) as well as in astrocytes (Navarrete and Araque, 2008; Han et al., 2012) of the hippocampal CA1 area, an area that contributes to spatial working memory (SWM). Because past studies have established that CB$_1$Rs mediate retrograde inhibition of neurotransmitter release, control neuronal excitability, and regulate short- and long-term plasticity (Di Marzo et al., 1994; Kreitzer and Regehr, 2001; Alger, 2002; Wilson and Nicoll, 2002; Freund et al., 2003; Chevaleyre et al., 2006; Maldonado et al., 2006; Heifets and Castillo, 2009; Kano et al., 2009), research on the function of CB$_1$R signaling in pain (Kato et al., 2012), aversive memory (Marsicano et al., 2002), epilepsy (Monory et al., 2006), food intake (Bellocchio et al., 2010), analgesia (Zimmer et al., 1999), or development (Jin et al., 2004) has focused mainly on neuronal CB$_1$Rs using neuronal-specific CB$_1$R KO mouse models. However, Han et al. (2012) report an unappreciated, yet major, role of astrocytic CB$_1$Rs in SWM. Their results show that acute cannabinoid exposure *in vivo* elicits a previously unreported form of LTD at CA3–CA1 hippocampal synapses, which is associated with an impairment of SWM; both effects are abolished in tamoxifen-treated GFAP-CB$_1$R-KO, but conserved in mice lacking CB$_1$R in glutamatergic or GABAergic neurons (Han et al., 2012). These findings strongly suggest that astrocytic CB$_1$R is a primary mediator of cannabinoid-induced LTD. Based on further *in vivo* pharmacological studies, the authors speculated that their findings are consistent with the possibility that acute exogenous cannabinoid exposure leads to glutamate release from astrocytes, which in turn could activate extrasynaptic NR2B-containing NMDA receptors to trigger AMPA receptor internalization at CA3–CA1 synapses. These events could eventually induce cannabinoid-mediated LTD at these synapses, altering hippocampal SWM. However, several points should to be kept in mind when considering this interpretation of the data, which might imply a substantially more complex mechanism at the CA1–CA3 synapses: (i) the apparent mechanistic discrepancy between this *in vivo* cannabinoid-induced LTD and previous *ex vivo* "gliotransmission" studies suggesting that astrocytic CB$_1$R-mediated Ca^{2+} increases trigger the release of glutamate to activate pre-synaptic mGluRs that leads to glutamate presynaptic release and subsequent potentiation of postsynaptic NMDA receptor-mediated currents (Navarrete and Araque, 2008, 2010); (ii) the current debate as to whether astrocytes actually release glutamate in a GPCR/Ca^{2+}-dependent manner *in vivo* (Wang et al., 2012a compared to Navarrete et al., 2012); (iii) the emerging evidence that astrocyte GPCR/Ca^{2+}-dependent release of glutamate occurs in the early steps of inflammatory processes rather than during normal physiology (Agulhon et al., 2012; Pascual et al., 2012); (iv) the recent findings suggesting that astrocytic GPCR/Ca^{2+} triggers an increase of K^+ uptake (Wang et al., 2012a,b; Devaraju et al., 2013); and (v) a past study reporting that LTD is modulated by astrocytic K^+ uptake (Janigro et al., 1997). Further investigation should help determine whether cannabinoid-induced LTD is due to an increase of extracellular glutamate, a decrease of extracellular K^+, a combination of both, or some other mechanism.

Role of DRD2 in neuroinflammation

Astrocytes become reactive in nearly all brain pathologies, and play important roles in neuroinflammation, a common feature of the aging brain and most neurological disorders and neurodegenerative diseases (Sofroniew, 2009; Sofroniew and Vinters, 2010). Changes in reactive astrocytes include upregulation of GFAP expression (Pekny and Nilsson, 2005), secretion of inflammatory mediators from astrocytes (Lucas et al., 2006; Peng et al., 2006; Farina et al., 2007; Brambilla et al., 2009; Steele and Robinson, 2012), and altered expression of astrocytic GPCRs (Aronica et al., 2003; Hamby et al., 2012). One such GPCR is the dopamine D2 receptor (DRD2) that couples to G$_i$ (Missale et al., 1998) and is expressed not only in neurons, but also in astrocytes (Bal et al., 1994; Khan et al., 2001; Luo et al., 2009). Downregulation of DRD2 expression has been reported in the brain of the elderly (Kaasinen et al., 2000), suggesting that astrocytic DRD2 signaling may be involved in neuroinflammation that occurs in the CNS during aging and disease. A recent study has investigated this question using both astrocyte-specific cKO and tamoxifen-inducible cKO mouse models (Shao et al., 2013). Several complementary methodological approaches have been used, including immunohistochemistry, biochemistry, and molecular biology to show that DRD2-deficient astrocytes show robust GFAP upregulation in the substantia nigra of aged

mice. Moreover, DRD2-deficient astrocytes were found to produce more proinflammatory mediators than their wild-type counterparts, suggesting that astrocytic DRD2 is a key negative regulator of neuroinflammation. To identify the downstream effectors of DRD2 that might be involved in the regulation of inflammatory mediator production, microarray analysis was carried out and showed a pronounced decrease of the small heat-shock protein αB-crystallin (CRYAB) in DRD2-deficient astrocytes, a protein known to display anti-inflammatory and neuroprotective activities (Ousman et al., 2007; Bhat and Steinman, 2009). These findings suggest that activation of astrocytic DRD2 controls CRYAB expression to suppress inflammatory responses in astrocytes and thus contribute to the maintenance of the immune state balance under normal conditions (Shao et al., 2013). The implications of these findings are profound in age-related diseases, where a decrease of DRD2 signaling in astrocytes may lead to an abnormal increase of proinflammatory mediator release, and underlie the progression of cognitive and motor function impairments. Therefore, the astrocyte DRD2 signaling pathway could be a potential target for therapy of several neurological, neuroinflammatory, and neurodegenerative disorders, in which production of inflammatory mediators is a common element (Lucas et al., 2006).

It is interesting, however, to note that activation of another G_i GPCR, called CXCR4 in cultured cortical astrocytes has previously been reported to increase release of the proinflammatory cytokine tumor necrosis factor α (TNFα; Bezzi et al., 2001), and not to negatively control the release of proinflammatory molecules as reported by Shao et al. (2013). Several obvious possibilities may explain these differences: (i) astrocytic G_i signaling triggers different cellular mechanisms depending on whether the studies are performed *in vivo* vs. *ex vivo* cultures; (ii) astrocytic G_i signaling leads to distinct responses depending on the area of the brain (e.g., substantia nigra vs. cortex); or (iii) astrocytic G_i GPCRs are involved in different functions depending on the developmental stage (mature vs. immature astrocytes). Additionally, another possibility that has not been addressed thoroughly in the field when investigating the role of astrocyte signaling in general is that different astrocytic G_i GPCRs may activate distinct signaling pathways even though they are known to couple to G_i proteins. In other words, depending on the endogenous or synthetic agonist used to active a specific G_i GPCR, this receptor may show a different bias toward other G proteins over G_i or different functional selectivity for β-arrestin recruitment over G_i activation (Allen et al., 2011; Zidar, 2011; Audet et al., 2012; Blattermann et al., 2012; Hiller et al., 2013). The depth of the complexity of GPCR signaling is now becoming familiar territory to receptor biologists, yet the application of this knowledge to the astrocyte field remains extremely limited. Therefore, findings relative to astrocytic GPCR signaling (coupling to G_q, G_i, or G_s proteins) in physiology and pathology likely reflect the complex interactions of multiple signaling pathways and provides an explanation for seemingly contradictory observations. Examples of this have already been observed relative to astrocyte Ca^{2+} signaling which is traditionally ascribed to G_q GPCR signaling pathways, yet astrocytic G_i-coupled $GABA_B$ and CB_1Rs also have been reported to evoke Ca^{2+} elevations in astrocytes (Serrano et al., 2006; Navarrete and Araque, 2008). Future studies using biased ligands in combination with astrocyte-specific cKOs of specific G proteins or β-arrestins is one approach to elucidate the role of astrocyte signaling molecules in brain function and dysfunction.

STUDYING ASTROCYTE FUNCTION THROUGH GENE EXPRESSION OR CELL-SPECIFIC RESCUE OF GLOBAL GENE KNOCKOUT

As methods for generating full or inducible conditional gene KO have been useful in deciphering some of the astrocyte gene functions *in vivo*, constitutive, inducible, and/or reversible regulation of gene expression represent other powerful approaches for understanding astrocyte function. To this end, four molecular approaches can be used: (i) expression of a genetically modified gene (transgene); (ii) reversible regulation of an endogenous gene; (iii) viral transduction of genes; or (iv) *in utero* gene electroporation (IUE).

TRANSGENE EXPRESSION

Conventional (**Figure 2A**) and inducible (**Figure 2B**) transgenesis allowing for astrocyte-specific expression of transgenes, constitutively or at specific times, have been used in the field. These approaches employ a small transcriptional unit derived from astrocyte-specific promoters. Of importance for the topic of this review, mapping of the transcriptional regulatory elements of the GFAP promoter has been critical to develop regulatory units (promoters) of small size to direct transgene expression in the majority of astrocytes *in vivo* without significant expression in other cell types in the brain (Masood et al., 1993; Brenner, 1994; Brenner et al., 1994). Other astrocyte-specific promoters have also been used successfully to drive transgene expression in astrocytes, such as Cx30, Cx43, S100b, Aldh1l1, or GLAST promoters (reviewed in Pfrieger and Slezak, 2012). The DNA construct containing a promoter fused to the transgene of interest is microinjected into the male pronucleus of fertilized eggs for random insertion in the mouse genome, followed by transfer of the fertilized eggs to the oviduct of pseudopregnant recipient females (Cho et al., 2009; **Figure 2A**). The resulting pups are identified as transgenic by polymerase chain reaction and checked for astrocyte-specific transgene expression. This constitutive gain-of-function approach however does not provide temporal control of the transgene expression, which can be problematic when studying the function of a transgene during developmental stages as mentioned above (Gotz et al., 2002; Malatesta et al., 2003; Anthony et al., 2004; Merkle et al., 2004; Casper and McCarthy, 2006). To overcome this limitation, inducible transgenic mouse models have been developed. The most commonly used method to temporally control gene expression in mouse models is based on the tet-operon/repressor and the estrogen (tetracycline) receptor ligand-binding domain (Gossen and Bujard, 1992; Baron and Bujard, 2000; Mansuy and Bujard, 2000; Saunders, 2011; **Figure 2B**). This system is bi-transgenic, which means that it involves the mating of two different transgenic mouse lines in order to produce a new tetracycline-regulated transgenic mouse line designed to activate the expression of a transgene in a specific cell type at a specific time point. The first line contains an

astrocyte-specific promoter driving the expression of the tetracycline (tet) transactivator (tTA). The second line carries the transgene of interest driven by the tet operon (TetO) DNA-binding element fused to a minimal promoter from cytomegalovirus. In this system, bigenic mice are maintained on tetracycline (doxycycline) to block transgene expression. When doxycycline is removed at a given time, tTA then binds to the TetO minimal promoter leading to targeted expression of the transgene of interest (**Figure 2B**).

Role of astrocytic G_q GPCR signaling in physiology and behavior

One important limitation in addressing the role of astrocytic GPCR signaling in neurophysiology has been the inability to pharmacologically activate astrocytic GPCRs in a cell type-specific manner. Indeed, astrocytes *in vivo* express members of most of the different families of GPCRs linked to the diverse array of intracellular signaling cascades (Porter and McCarthy, 1997) that are also known to be expressed by neurons. Therefore, the use of pharmacological approaches consisting of agonist application *ex vivo* or *in vivo* does not allow cell-specific GPCR activation. Therefore, interpretation of the findings is made difficult by direct activation of neuronal (but also other cell type) receptors by the applied agonist in addition to the intended astrocytic targets. As a consequence, it has been difficult to determine the effect of selectively stimulating astrocytic G_q GPCR-mediated Ca^{2+} signaling cascades on physiological processes such as synaptic transmission and plasticity. This complication has led investigators to consider previous reports of gliotransmission with caution.

Thus, advances in this field depend upon the development of novel tools to better address the physiological relevance of astrocytic G_q GPCR Ca^{2+} signaling. In order to overcome some of the limitations associated with traditional pharmacological approaches, two novel transgenic mouse models were developed (Fiacco et al., 2007; Agulhon et al., 2013). In the first model, a novel G_q GPCR is expressed selectively in astrocytes that is not expressed by other cell types in the brain, is not activated by endogenous ligands released in brain, and whose ligand, the peptide FMRF, does not activate endogenous brain G_q GPCRs (Fiacco et al., 2007). The novel receptor, the so-called Mas-related gene A1 (MrgA1), is a member of a family of GPCRs normally expressed in specific subsets of nociceptive sensory neurons in the spinal cord (Dong et al., 2001), but is not found in the brain. The MrgA1 receptor is targeted to astrocytes using the inducible Tet-Off system (**Figure 2B**). In one transgenic mouse line the green fluorescent protein (GFP)-tagged MrgA1 receptor is transcribed from the TetO promoter. When crossed with a second transgenic line in which tTA is targeted to astrocytes using the human GFAP promoter, a bigenic line (referred to as the MrgA1 mice) is obtained, in which MgrA1-GFP is selectively expressed in the vast majority of astrocytes in the absence of doxycycline (Fiacco et al., 2007). Several studies using this novel transgenic MrgA1 mouse model have shown that increasing astrocytic MrgA1-mediated Ca^{2+} fluxes does not lead to gliotransmission *ex vivo* and *in vivo*, suggesting that astrocytes do not release gliotransmitters in a G_q GPCR/Ca^{2+}-dependent manner (Fiacco et al., 2007; Agulhon et al., 2010; Wang et al., 2012a,b). Rather, it has been reported that astrocytic MrgA1R-mediated Ca^{2+} elevations potentiate astrocyte glutamate and K^+ uptake (Wang et al., 2012a,b, 2013; Devaraju et al., 2013), suggesting that the mechanisms by which agonist-induced astrocyte G_q GPCR activation modulates neuronal activity may be different than previously thought, in agreement with previous and most recent findings (Fiacco et al., 2007; Agulhon et al., 2010; Wang et al., 2013). It is important to bear in mind that agonist-evoked stimulation of G_q GPCRs, including MrgA1Rs, generates Ca^{2+} elevations that may not recapitulate some endogenous Ca^{2+} elevations in astrocytes which often remain confined to fine processes. One strategy in future studies could be to employ a caged version of FMRF to locally stimulate astrocyte Ca^{2+} elevations by activation of a G_q signaling pathway, which may more closely resemble the subset of microdomain astrocytic Ca^{2+} elevations which have been observed in astrocytes *in vivo*.

More recently, another transgenic mouse line has been developed in order to facilitate investigation of the role of astrocytic G_q GPCR signaling *in vivo* (Agulhon et al., 2013). One of the limitations of the MrgA1 mice is that the MrgA1 FMRF ligand does not cross the BBB, making *in vivo* studies more invasive as surgeries would be required in order to infuse FMRF into the brain of freely moving mice. Additionally, endogenous MrgA1 is expressed in sensory nerve terminals, preventing the use of MrgA1 mice to study spinal cord astrocytes (as FMRF may diffuse and activate endogenous MrgA1 in nociceptive sensory neurons). To overcome some of these obstacles, a new GFAP-hM3Dq mouse line was created (Agulhon et al., 2013). The innovation of this mouse line is based on the use of a novel genetically engineered G_q GPCR (called hM3Dq) that does not respond to endogenous ligands, but instead responds to an inert synthetic ligand (clozapine-*N*-oxide, CNO) that crosses the BBB and activates signaling cascades in a similar fashion as endogenous G_q GPCRs (Armbruster et al., 2007). Such new chemogenetic technology (called designer receptors exclusively activated by a designer drug, or DREADD) has recently led to several important discoveries in neuronal function (e.g., Alexander et al., 2009; Ferguson et al., 2011; Atasoy et al., 2012; Garner et al., 2012), and is now being applied to astrocytes for the first time (Agulhon et al., 2013). This mouse line was made through conventional transgenesis using a construct containing the human GFAP promoter driving the expression of hM3Dq (**Figure 2A**). Inducible and reversible expression of the transgene is not a constraint when using the DREADD technology, as hM3Dq can be activated postdevelopmentally through intraperitoneal CNO injection. Immunohistochemical screening in adult mice indicates that expression of hM3Dq is restricted to astrocytes within the CNS, and in non-myelinating Schwann cells of sympathetic, sensory, and sciatic nerves, as well as satellite cells in sympathethic, parasympathetic, and sensory ganglia within the PNS. Such expression is expected for a GFAP-driven transgene and allows CNO-induced global stimulation in $GFAP^+$ glial cells within the CNS and PNS. This provides a powerful approach, as it can reveal most of the physiological and behavioral phenotypes mediated by $GFAP^+$ glial cell G_q GPCR signaling. Acute CNO intraperitoneal injections of GFAP-hM3Dq mice resulted in previously unreported and long-lasting (minutes to hours) modulation of autonomic nervous system (ANS) function, including increased heart rate, blood pressure, and saliva

formation, as well as decreased body temperature. Furthermore, changes in activity-related behavior and motor coordination were observed in CNO-treated GFAP-hM3Dq mice. To address whether ANS and activity-related effects were due to Ca^{2+} or other signaling pathways downstream of G_q GPCR activation, the GFAP-hM3Dq mice were crossed with IP_3R2 KO mice in order to generate GFAP-hM3Dq mice in which IP_3R2-dependent Ca^{2+} increases were abolished. Interestingly, CNO-treated GFAP-hM3Dq/IP_3R2-deficient mice exhibited similar autonomic and motor modulation as CNO-treated GFAP-hM3Dq mice. These findings suggest that CNO-induced phenotypes are not dependent on IP_3R2-dependent Ca^{2+} increases. Thus, other (non-Ca^{2+}) signaling molecules activated by G_q GPCRs in $GFAP^+$ glial cells may be important contributors to the functional effects of CNO. Collectively, these findings open new avenues into investigation of $GFAP^+$ glial cell (astrocyte, non-myelinating Schwann cell, satellite cell) function in animal physiology. Further studies employing local CNO infusion into specific regions of the CNS or PNS, and specific blockers of G_q GPCR signaling molecules (e.g., protein kinase C or $\beta\gamma$-dependent activation of signaling cascades), will help dissect out the areas of the nervous system and the signaling pathways that are responsible for each of the CNO-induced phenotypes. Viral transduction of hM3Dq in wild-type mice is also a potential strategy to help determine what areas of the CNS vs. PNS are involved in the effects observed in CNO-treated GFAP-hM3Dq mice. Finally, when specific markers of different subpopulations of CNS astrocytes become available, it will be possible to use new promoters to create transgenic mice expressing hM3Dq in distinct subsets of astrocytes. This will allow determination of whether a specific subset of astrocytes is responsible for all of the phenotypes observed in CNO-treated GFAP-hM3Dq mice, or whether different subsets of astrocytes are responsible for distinct phenotypes. Clearly, there is much to be learned with regard to the role of astrocytes, and more generally of $GFAP^+$ glial cells, in complex physiology and behavior. Collectively, the findings of this study point to astrocytes as potential therapeutic targets for some ANS or motor dysfunctions.

CELL-SPECIFIC RESCUE OF A GENE KNOCKOUT

Another powerful molecular approach is to constitutively knock out a particular gene product and then rescue it in a specific cell population. This provides information on the contribution of a specific cell type to the phenotype produced by the full KO of the protein. This technique employs a *LoxP*-flanked selectable neo marker and transcriptional/translational stop cassette (called neostop) located in the endogenous gene of interest to generate a null mutant that can be activated by Cre-mediated recombination. This construct is placed into a targeting vector, which is inserted into ES cells and introduced into the mouse genome through homologous recombination (Müller, 1999; **Figure 1A**) to eventually generate a mouse line in which expression of the targeted endogenous gene is suppressed. When this Cre responding mouse line is crossed with a transgenic line expressing Cre recombinase under a cell type-specific promoter, recombination of *LoxP* sites excises the neostop cassette, thus re-activating expression of the endogenous gene conditionally (**Figure 1B**) and with temporal control in a specific cell population (**Figure 1C**). This approach is powerful in the sense that it enables study of important questions regarding the phenotype reversibility of certain diseases involving the loss of a single gene function, and thus has the potential to open doors for future therapeutic approaches (Dragatsis and Zeitlin, 2001). By providing gain- and loss-of-function information, this approach has led to recent important steps forward in the understanding of the role of astrocytes in autism spectrum disorders.

ROLE OF ASTROCYTE MeCP2 IN RETT'S SYNDROME

Rett's syndrome (RTT) is an X-chromosome-linked autism spectrum disorder caused by the loss of function of the epigenetic factor methyl-CpG-binding protein 2 (MeCP2; Amir et al., 1999; Chahrour and Zoghbi, 2007). MeCP2 aberrations result in a constellation of neuropsychiatric, neuroanatomical, and neurophysiological abnormalities as well as autonomic dysfunctions, such as respiratory abnormalities. Delayed neuronal maturation and synaptogenesis, sparse and short dendritic spines (Fukuda et al., 2005), impaired synaptic transmission and plasticity (Dani et al., 2005; Asaka et al., 2006; Moretti et al., 2006), and altered number of glutamatergic synapses and expression of excitatory glutamate transporter VGLUT1 (Chao et al., 2007) were detected in global MeCP2 KO mouse lines (also called RTT mouse models). Although no unifying principle on MeCP2 function has yet emerged, it has been reported that MeCP2 acts as a transcriptional repressor, activator or RNA-binding protein (Nan et al., 1998; Colantuoni et al., 2001; Chahrour et al., 2008). Most studies have been directed toward understanding the *in vivo* mechanisms of neuronal MeCP2 (Akbarian et al., 2001) using different neuronal-specific cKO mouse models, which led to the thought that the primary cause of RTT is cell autonomous, i.e., resulting from a lack of functional MeCP2 in neurons (Chen et al., 2001; Guy et al., 2001; Luikenhuis et al., 2004). However, more recent studies have shown that MeCP2 is also expressed in all glial cell types, including astrocytes, oligodendrocyte progenitor cells, oligodendrocytes, and microglia (Ballas et al., 2009; Derecki et al., 2012). In particular, *in vitro* studies have shown that astrocytic MeCP2 supports normal neuronal morphology, indicating a non-cell autonomous influence of MeCP2 on neuronal function (Ballas et al., 2009; Maezawa et al., 2009). Global re-expression of the MeCP2 gene postnatally in full MeCP2 KO mice demonstrated disease reversibility in this RTT mouse model, suggesting that the neurological defects in MeCP2 disorders are not permanent (Guy et al., 2007). Based on these studies, Lioy et al. (2011) asked whether astrocytic MeCP2 may also have a role in rescuing RTT neuropathological symptoms *in vivo* in order to determine the contribution of astrocytes to the symptoms of RTT. They used a genetically engineered mouse line in which the endogenous MeCP2 gene is globally silenced by insertion of a *Lox*P-flanked stop cassette, but can be conditionally activated by stop cassette deletion *via* Cre-mediated excision ($MeCP2^{lox-Stop}$ mice; Guy et al., 2007). When these $MeCP2^{lox-Stop}$ mice were crossed with mice expressing $CreER^{T2}$ under a human GFAP promoter (hGFAP–$CreER^{T2}$), (Hirrlinger et al., 2006), the resulting new mouse line ($MeCP2^{lox-Stop}$::hGFAP–$CreER^{T2}$) was thus specifically designed for the investigation of astrocytic function

in RTT (Lioy et al., 2011). The MeCP2$^{lox-Stop}$::hGFAP–CreER^{T2} mice are globally deficient in MeCP2 until injected with tamoxifen, which induces the excision of the Lox-stop cassette in the endogenous MeCP2 gene, restoring the expression of MeCP2 in an astrocyte-specific manner. Strikingly, it was found that re-expression of MeCP2 specifically in astrocytes significantly improved locomotion, anxiety levels, breathing patterns, and average life span, indicating that astrocytes are involved in the neuropathology of RTT and that restoring astrocyte MeCP2 can ameliorate four consistent and robust RTT-like symptoms. Additionally, restoration of MeCP2 in astrocytes in the global KO mice exerted a non-cell-autonomous positive effect on KO neurons *in vivo*, restoring normal dendritic arborization and increasing levels of VGLUT1. Altogether, these findings suggest that astrocytic MeCP2 gene replacement is well-suited as a therapeutic strategy. Therefore, these findings have major implications not only for improving the understanding of astrocyte function in pathophysiology but also have valuable clinical implications (Gadalla et al., 2013; Garg et al., 2013). Deciphering the cellular (astrocyte vs. neuron) and molecular underpinnings of RTT is likely to contribute to the understanding of the pathogenesis of a broader class of autism spectrum disorders. Finally, the use of conditional endogenous gene repair mutations has a clear application for studying astrocytic contributions to other single gene diseases.

VIRAL GENE TRANSDUCTION STRATEGIES

A new technique that is gaining momentum in the study of astrocyte function is viral-mediated delivery of transgenic constructs to express or perturb a protein or molecule of interest. This strategy offers an attractive alternative to generation of transgenic mouse lines, which can be very time consuming and expensive due to costs associated with maintenance of animal lines and genotyping. Viral mediated gene delivery is reviewed in detail in this special issue (Merienne et al., 2013) and therefore this section will focus mainly on a particular application of this approach for the study of astrocyte Ca^{2+} activity.

Briefly, in these experiments, a transgenic construct is inserted into a recombinant AAV vector which is then injected into the brain of wild-type mice. Approximately 2 weeks is sufficient for the viral vector to be incorporated into the host genome and for high expression of the transgenic construct to occur (Ortinski et al., 2010; Xie et al., 2010; Shigetomi et al., 2013a). Several AAV serotypes have been generated that show tropism preferentially for neurons or astrocytes or a combination of cells (Ortinski et al., 2010). The AAV 2/5 pseudotype shows the strongest tropism for astrocytes and therefore appears to have emerged as the AAV construct of choice for viral vector targeting of astrocytes. Substitution of the CMV minimal promoter with an astrocyte-specific promoter derived from GFAP results in astrocyte-specific expression of the transgene.

An example of an application of this technique in the study of astrocyte biochemistry and function is recording Ca^{2+} fluctuations in astrocytes. Monitoring astrocyte Ca^{2+} activity continues to be a primary technique in the study of astrocytes ever since it was discovered *in vitro* that astrocyte Ca^{2+} elevations modulate neuronal excitability (Parpura et al., 1994; Araque et al., 1998). Early approaches used commercially available membrane-permeable AM ester forms of organic Ca^{2+} indicator dyes to "bulk-load" and monitor changes in cytosolic Ca^{2+} concentration in astrocytes (Porter and McCarthy, 1996; Nett et al., 2002). This technique offered the advantage of monitoring Ca^{2+} activity in many astrocytes simultaneously, but at the cost of poor resolution of astrocyte processes that are the first responders to neuronal activity and which may operate autonomously from other astrocytic compartments. A derivative of this technique developed more recently is "bolus-loading" in which the AM Ca^{2+} indicator is pressure-ejected to load astrocytes with Ca^{2+} indicator deeper in the tissue where cells and their connections are more intact (Garaschuk et al., 2006). While this technique increases visibility of the larger astrocyte processes, it still suffers from low signal-to-noise and difficulty differentiating between domains of individual astrocytes due to high background labeling. Furthermore, a secondary label, such as sulforhodamine 101, is often required to confirm the loaded cells as astrocytes (Nimmerjahn et al., 2004), but this indicator has been shown to alter neuronal activity (Kang et al., 2010). Moreover, cell-impermeant Ca^{2+} indicators delivered via patch pipette offer excellent signal-to-noise and detection of Ca^{2+} events in small, bulk fine processes of astrocytes (Fiacco et al., 2007; Xie et al., 2012), but there is some concern about dialysis of the cellular contents by the patch pipette, especially in cases where the pipette is left in whole-cell patch clamp mode during measurements which can dampen Ca^{2+} signals. Last, while patch clamp delivery of Ca^{2+} indicator to astrocytes is relatively straightforward in brain slices, this approach is not as practical for recording astrocyte Ca^{2+} activity *in vivo*.

Viral-mediated delivery of GCaMP genetically encoded calcium indicators (GECIs) overcomes many of the limitations associated with the traditional approaches described above. It can be thought of as "high resolution bulk-loading" in that many astrocytes can be monitored at once and with the excellent signal-to-noise offered by the latest GCaMPs. Because the GECIs are delivered using an astrocyte-specific promoter, no secondary labeling is necessary to confirm the cells as astrocytes. Studies have indicated very specific transfection of astrocytes using this technique (Ortinski et al., 2010; Xie et al., 2010; Shigetomi et al., 2013a). Recently, Shigetomi et al. (2013a) used the AAV 2/5 vector to deliver a Lck membrane tethered form of GCaMP3 to astrocytes. Not only do the transfected astrocytes show significantly more frequent Ca^{2+} activity in fine processes compared to bulk-loading protocols (Shigetomi et al., 2013a), but because the indicator is membrane-tethered, it can monitor Ca^{2+} activity in the smallest "branchlets" and "leaflets" (Tong et al., 2013). Because Ca^{2+} activity monitored using the Lck-GCaMP3 indicator was diminished by an inhibitor of TRPA1 channels, the data provide evidence for astrocyte Ca^{2+} signals produced by a mechanism other than IP_3R2-mediated internal stores. It will be important to follow-up this interesting finding in future studies by determining the relative ability of the Lck-GCaMP3 to detect TRPA1 vs. IP_3R-mediated Ca^{2+} elevations or mitochondrial Ca^{2+} dynamics (Parnis et al., 2013) in astrocytes. For example, how much of the Ca^{2+} activity detected by Lck-GCaMP3 is diminished by IP_3R inhibitors? This would help rule out possible non-specific effects of HC 030031,

the drug used to block TRPA1 channels. Firmer evidence of the TRPA1-mediated Ca^{2+} elevations could be provided by transfecting IP_3R2 KO mice with the Lck-GCaMP3 Ca^{2+} indicator and determining the extent to which any remaining Ca^{2+} elevations are blocked by the TRPA1 inhibitor. In summary, the membrane-tethered version of the new astrocytic GECIs may be specialized to distinguish among different sources of astrocyte Ca^{2+}, due to a combination of its location, excellent signal-to-noise, and sensitivity to changes in Ca^{2+} concentration near the membrane.

Like any technique, viral delivery methods are not without limitations. GECIs have yet to be fully characterized. While they offer the advantage of increasing understanding of Ca^{2+} microdomains and conditions under which Ca^{2+} elevations occur, high levels of expression may result in significant calcium buffering with unintended consequences. Perhaps a more significant concern associated with the use of AAV vectors is the possibility of inducing reactive gliosis. Astrocytic Ca^{2+} signaling is disrupted in reactive astrocytes, with typically exaggerated and more widely propagating Ca^{2+} elevations and with increased propensity for gliotransmitter release (reviewed in Agulhon et al., 2012). Therefore, great care has been taken to determine the amount of reactive astrocytosis (if any) apparent after viral transfection by immunostaining for the astrocytic markers GFAP and vimentin to look for astrocytic hypertrophy (Xie et al., 2010; Shigetomi et al., 2013a). The AAV 2/5 vector was actually used purposefully to induce astrogliosis to determine the effect on astrocyte glutamine synthetase (GS) and neuronal excitability (Ortinski et al., 2010). Ortinski et al. (2010) found a significant increase in neuronal excitability caused by reduced GABA synthesis in neurons following loss of GS in reactive astrocytes. These data were confirmed by recovery of GABA transmission with exogenous application of glutamine. High titers of AAV 2/5 caused the astrogliosis and effects on neurons while low titers did not. Subsequent studies have actually used even higher titers than Ortinski et al. (2010), but reported normal astrocytic morphology suggesting that the astrocytes were not reactive (Xie et al., 2010; Shigetomi et al., 2013a). It is unclear what is behind these disparate findings but one possibility is that Ortinski et al. used the 638 bp GFAP Gfa104 promoter to target astrocytes, while Xie et al. (2010) and Shigetomi et al. (2013a,b) used the 681 bp GFAP $gfaABC_1D$ promoter. This is an important issue to continue to explore as there may be reactive changes induced by AAV in astrocytes that are underway prior to overt gliosis and hypertrophy. Because astrogliosis in itself significantly alters neuronal excitability, care needs to be taken interpreting the role of astrocyte Ca^{2+} on neuronal activity using virally transfected GECIs. Overall, the new astrocytic GECIs offer a tantalizing approach to record astrocyte Ca^{2+} activity *in vivo*. Future studies will help define limitations of viral-mediated GECI expression, leading to refinement in both methodology and interpretation of data.

IN UTERO GENE ELECTROPORATION STRATEGIES

One emerging methodology to manipulate gene expression in astrocytes *in vivo* is by IUE. IUE is a method of gene delivery into mouse embryos (Fukuchi-Shimogori and Grove, 2001; Saito and Nakatsuji, 2001; Takahashi et al., 2002), which has become a method of choice for gain and loss of function studies in embryonic CNS cell progenitors. The IUE method involves injecting a plasmid DNA vector into the ventricles of the embryonic brain, and then using electrical pulses to facilitate the transfer of the DNA into the progenitor cells of the VZ/subventricular zone (SVZ). The optimal development stage for using this method in mice is between embryonic day (E) 10.5 and E16.5. In order to obtain stable expression of a transgene of interest in highly proliferative neural precursors and their progeny, a combination of transposon-mediated gene transfer into the host genome (Cary et al., 1989) with IUE has been used. This approach enables the expression of a transgene stably and efficiently in mitotic neural precursors (radial glia) during development, and in both cortical astrocytes and oligodendrocytes, after birth (Ding et al., 2005; Cadinanos and Bradley, 2007; Wilson et al., 2007; VandenDriessche et al., 2009; Woltjen et al., 2009; Yoshida et al., 2010; Chen and LoTurco, 2012). The transposon system involves a transgene of interest from a donor plasmid and a helper plasmid that expresses a transposase under the control of a cell-specific promoter (Cadinanos and Bradley, 2007; Chen and LoTurco, 2012). For instance, by combining a plasmid containing a transposase under the control of the GLAST promoter with a donor plasmid containing the green fluorescent marker eGFP, astrocytes originating from GLAST positive progenitors were labeled with eGFP in the cortex of P27 mice (Chen and LoTurco, 2012). Using an alternate transposon system and the GFAP and S100β promoters, stable postnatal expression of eGFP was obtained in astrocytes of juvenile mice (generated from GFAP- and S100β-expressing progenitors; Yoshida et al., 2010). The relatively low number of astrocytes expressing eGFP allowed the authors to trace the long-term lineage of glial progenitors *in vivo*.

Tracing the long-term lineage of glial progenitor is of great importance in the field because of the growing evidence for astrocytic morphological, molecular, and functional heterogeneity. Whether this heterogeneity is specified during brain development is not clear. A recent study has addressed this question and analyzed cell lineages thoroughly using IUE (Garcia-Marques and Lopez-Mascaraque, 2013). Twelve plasmids containing the sequences of six fluorescent proteins, whose expression was driven by the GFAP promoter in either the cytosol or the nucleus, were used. After co-electroporation of the plasmid mixture with a plasmid encoding a transposase under the control of the ubiquitous CMV promoter into ventricles of E14 embryos, the sequences coding the fluorescent markers were randomly inserted into the genome of progenitor cells to generate many colors by a combination of the fluorescent proteins. This stochastic and combinatorial expression of six fluorescent proteins produces inheritable markers for *in vivo* long-term tracing of glial progenitor lineages (Garcia-Marques and Lopez-Mascaraque, 2013). Unanticipated and highly specific clonal distribution in specific domains was revealed in the cortex of adult mice. Moreover, the authors found that different classes of astrocytes emerge from different clones, reinforcing the view that lineage origin defines astrocyte heterogeneity. The positional identity of these clones represents an additional level of astrocytic

heterogeneity, which is likely associated with specific regional functions. Interestingly, astrocytes of the same clone often (but not always) responded equally to cortical injury, suggesting the dependence of their genetic information. While some clones exhibited strong morphological alteration following injury, other clones located similar distances from the lesion were unresponsive, suggesting that the developmentally determined features of different astrocytic clones should not be overlooked when developing brain therapies (Martin-Lopez et al., 2013).

In addition to the use of astrocyte-specific promoters to drive transgene expression in postnatal astrocytes, conditional CreER^{T2} systems have also been combined with IUE to induce cell- and time-specific expression of transgenes (Matsuda and Cepko, 2007; LoTurco et al., 2009), widening the set of possible genetic manipulations using IUE. Therefore, although the IUE technology has been used mainly for descriptive analysis so far, it is likely to become an important tool more commonly used in the field. The relative ease of implementation and inherent flexibility of a plasmid-based system should make this method valuable to many investigators interested in marking and manipulating glial progenitor lineages in the CNS of species for which Cre reporter lines are not available (e.g., rats). Additionally, one of the advantages of the IUE approach over the generation of transgenic mice is that it is both cheaper and less time consuming. Furthermore, compared to surgical viral transfer performed postnatally, it is less invasive. The introduction of transgenes in astrocytes through IUE, when the immune system is immature, does not produce overt reactive gliosis thereby enabling study of astrocytes in a more physiological context compared to viral-based approaches. However, limitations of IUE include variable electroporation efficiency, promoter leakiness, and difficulty in precisely controlling the number of labeled astrocytes.

STUDYING ASTROCYTE FUNCTION THROUGH GENETIC MANIPULATION OF HUMAN GLIAL PROGENITORS

Although the molecular approaches described above have led to important steps forward in deciphering some of the functions of astrocytes in the pathophysiology of the mammalian brain, an important question remains: can the findings obtained from studies of the rodent brain be extrapolated to human astrocytes? There is mounting evidence that rodent astrocytes have multiple key functions in the developing and adult nervous system, including roles in synapse formation and function, working memory, autonomic and locomotor functions, and cognition (Barres, 2008; Halassa and Haydon, 2010; Lioy et al., 2011; Han et al., 2012; Molofsky et al., 2012; Wang et al., 2012a; Agulhon et al., 2013; Clarke and Barres, 2013). However, human astrocytes are both morphologically and functionally distinct from those of rodents (Colombo, 1996; Oberheim et al., 2009). Therefore, do human astrocytes have unique properties compared to their rodent counterparts, which could explain the higher cognitive abilities of humans? This fascinating question in the field has been recently explored by the groups of Maiken Nedergaard and Steven Goldman (Han et al., 2013).

In this study, the authors used human glial chimeric mouse brains to ask whether human astrocytes have unique properties that can influence activity-dependent synaptic plasticity *ex vivo* and learning and memory *in vivo*. To do so, they used human glial progenitor cells (GPCs) obtained by magnetic-assisted cell sorting using antibodies against PSA-NCAM and A5B5 cell surface antigens. The A2B5$^+$/PSA-NCAM$^-$ glial cells were expanded via a cell cultured protocol that promoted differentiation into astrocytes. Prior to transplantation into brains of neonatal immune-deficient mice, GPCs were transduced using a VSVg-pseudotyped lentiviral-CMC-EGFP in order to label them with a GFP. Once transplanted, mice matured to become adult chimeras for both mouse and human astrocytes (Windrem et al., 2004, 2008), and their brains were analyzed in adult mice. Human-derived cells survived in the rodent host brains and infiltrated the cortex and hippocampus to give rise to some EGFP$^+$ astrocytes. Human astrocytes retained the large size and complex morphology that was previously reported in human brains (Oberheim et al., 2009), indicating that they matured in a cell-autonomous fashion. Additionally, human astrocytes extended processes contacting blood vessels and displayed long and tortuous processes, a phenotype typically observed in a specialized subpopulation of interlaminar astrocytes in the cortical white matter of adult human brains (Oberheim et al., 2009). Another type of engrafted human astroglial cell exhibited varicosity-studded processes (Kettenmann and Ransom, 2005; Oberheim et al., 2009). Finally, human astrocytes occupied distinct non-overlapping domains and formed gap junctions with rodent host astrocytes. At the physiological level, the input resistance of human astrocytes was twice as large as those of mouse astrocytes and Ca^{2+} signals propagated threefold faster than in mouse astrocytes. Notably, using acute hippocampal slices, Han et al. (2013) observed that the slope of fEPSPs was steeper and LTP was stronger and longer-lasting in mice grafted with human astrocytes compared to mice grafted with mouse astrocytes, indicating that excitatory synaptic transmission was enhanced in the presence of human astrocytes. Thus, enhancement of LTP was a specific characteristic of human glial cells. Interestingly, the potentiation of fEPSPs in human glial chimeric mice and the enhancement of LTP did not result from higher expression of NMDA receptors, increased synaptic release of glutamate, altered adenosine tone or increased glial release of D-serine. Rather it was found that human astrocytes facilitated synaptic insertion of the GluR1 subunit in host murine neurons through a TNFα-dependent, PKC/CaMKII-mediated pathway, consistent with the potentiation of AMPA receptor-mediated currents, thus lowering the threshold for induction of LTP in human glial chimeric mice. Strikingly, human glial chimeric mice performed better in hippocampus-mediated learning tasks compared to their untransplanted littermates. Together, these findings suggest that human astrocytes may have some unique properties to enhance cognition. This study represents an important scientific and technological step forward to study the function of human astrocytes in live adult brains, which has not been possible so far. It also illustrates the power of combining different technological advancements together in live animals. It was unclear in the study if varicose projection astrocytes were present only in cortical layers 5 and 6 as discovered previously in human temporal neocortex, or if these cells were present throughout the cortex and hippocampus in the chimeric mice. Abnormal expression of varicose

projection astrocytes in the hippocampus may in part explain some of the unique cognitive abilities in these animals. In addition, further investigation will be needed to determine whether the enhanced cognitive performance of the humanized mice is specifically due to human astrocytes, or whether the progenitor cells that did not differentiate into mature astrocytes contributed to the phenotype.

SUMMARY AND FUTURE DIRECTIONS

Important advances continue to be made in development of molecular tools to understand astrocyte function and the effect of manipulating individual astrocytic signaling molecules on neuronal activity and pathophysiology of the brain *in vivo*. Several examples have been provided in this review, including generation of conditional and inducible genetically engineered mouse lines for selective expression or removal of specific astrocyte proteins, as well as viral- or IUE-mediated gene delivery techniques for the study of astrocyte function. Study of astrocyte Ca^{2+} activity continues to be heavily emphasized, and while there is certainly more to discover in this area, there is an incredibly diverse array of other astrocytic GPCRs, ion channels, transporters, and signaling molecules to explore. Recent development of new lines using chemogenetics is one such example to further understand the importance of astrocytic signaling pathways in health and disease *in vivo*. The effect of selectively perturbing or stimulating other signaling molecules in astrocytes, such as G proteins, protein kinases, cAMP, diacylglycerol, phospholipase C, and phosphoinositol isoforms may open up new areas of research on astrocytes. Furthermore, a greater appreciation of astrocyte heterogeneity in different brain areas or even between adjacent synapses calls for the need to develop selective markers to identify astrocyte subtypes and molecular approaches to manipulate specific astrocyte subpopulations. Another recent molecular approach only just underway is understanding the effects of enhanced DNA methylation or de-methylation (epigenetic mechanisms) on astrocyte protein expression and function in development and disease. Finally, there has been a strong tendency to focus on the immediate, short-term consequences of manipulating astrocyte receptors and signaling molecules on neuronal activity and brain function. Based on the predominance of metabotropic signaling cascades in astrocytes and the role of astrocytes in homeostasis of the synaptic microenvironment, the role of astrocyte-to-neuron communication may be tuned toward long-term regulatory functions. Therefore, another area of future study is to examine the outcome of stimulating astrocytic signaling cascades on gene transcription, synthesis of new proteins, and long-term regulation of cellular processes in neurons, astrocytes, and other glial cell types.

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Efficient gene delivery and selective transduction of astrocytes in the mammalian brain using viral vectors

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Astrocytes are now considered as key players in brain information processing because of their newly discovered roles in synapse formation and plasticity, energy metabolism and blood flow regulation. However, our understanding of astrocyte function is still fragmented compared to other brain cell types. A better appreciation of the biology of astrocytes requires the development of tools to generate animal models in which astrocyte-specific proteins and pathways can be manipulated. In addition, it is becoming increasingly evident that astrocytes are also important players in many neurological disorders. Targeted modulation of protein expression in astrocytes would be critical for the development of new therapeutic strategies. Gene transfer is valuable to target a subpopulation of cells and explore their function in experimental models. In particular, viral-mediated gene transfer provides a rapid, highly flexible and cost-effective, *in vivo* paradigm to study the impact of genes of interest during central nervous system development or in adult animals. We will review the different strategies that led to the recent development of efficient viral vectors that can be successfully used to selectively transduce astrocytes in the mammalian brain.

Keywords: viral vectors, astrocytes, CNS, tropism, gene therapy

Astrocytes make up most of the cells in the brain. In addition to well-characterized roles for astrocytes in regulating brain metabolism and blood flow, there is now an increasing body of evidence that astrocytes are dynamic regulators of synaptogenesis, synaptic function and network activity. This is conceptualized in the tripartite synapse model, where pre-synaptic and post-synaptic elements of neurons are surrounded and regulated by astrocyte processes (Araque et al., 1999; Barres, 2008).

Astrogenesis occurs relatively late in development after most neurogenesis has completed (Freeman, 2010). Defects in astrocyte maturation, tripartite synapse formation and plasticity during early post-natal development may be responsible for some psychiatric and neurodegenerative diseases. There is a growing body of evidence to support the view that a loss of normal astrocyte functions or a gain of abnormal effects can contribute to disease processes, and there are now numerous examples of astrocyte contributions to pathological mechanisms in amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and brain tumors to cite few of them (for review see Sofroniew and Vinters, 2010).

Despite progress and potential significance, cellular, developmental, and systems-level studies of astrocytes still lag far behind those of neurons. New sophisticated genetic tools to label and manipulate astrocytes *in vivo* were recently developed. Additional tools that allow for temporally controlled deletion of genes, specifically in rodent astrocytes, along with improved high resolution imaging techniques, are enabling researchers to address fundamental questions in astrocyte biology for the first time. However, these tools need to be more fully expanded and exploited to better understand astrocyte biology *in vivo*. The situation is complicated by the recent findings that astrocytes do not represent a homogeneous cell population across brain regions as well as within the same brain region (Zhang and Barres, 2010). So, despite evidence showing pronounced region- and layer-specific morphological heterogeneity as well as region-specific actions of astrocytes on neuronal functions, currently available tools have had limited utility for examining functional diversity among astrocytes.

To understand the role of astrocyte signaling in brain function, it is critical to study astrocytes *in situ* where their complex morphology and intimate association with neurons remains intact. Understanding neuron–glia interactions *in vivo* requires dedicated experimental approaches to manipulate each cell type independently. These approaches include targeted transgenesis and viral

transduction to overexpress or block the expression of a specific gene in astrocytes.

The past and current approaches of targeted transgenesis were recently reviewed in a comprehensive paper (Pfrieger and Slezak, 2012) and will not be detailed here.

Yet, a very important application of transgene expression is the visualization of a large population of astrocytes *in vivo* by a fluorescent protein. The use of bacterial artificial chromosomes (BACs) for the production of transgenic mice has opened new opportunities to study gene expression and functions in the brain. The resulting gene expression central nervous system (CNS) atlas program GENSAT represents a powerful resource for the scientific community (http://www.gensat.org). However, it remains difficult and time-consuming to target specific cell subpopulations through transgenesis, and differences in recombination efficiency between transgenic lines complicate the analysis. We will therefore rather focus on an alternative approach to genetically manipulate astrocytes that relies on the use of viral vectors. Indeed, the development of highly efficient viral vectors for gene transfer in the CNS is providing new systems for localized and controlled gene expression. Even if such approach requires the stereotaxic injection of the viral vectors in each animal, it significantly reduces the costs of *in vivo* experiments, and it can be used in combination with mouse models for conditional gene targeting, providing high flexibility and versatility to replace, modify, induce, or block expression of target genes. We will therefore review the recent development in this field that led to the emergence of effective and selective viral vectors for transducing astrocytes *in vivo*.

VIRAL VECTORS: POTENT SYSTEM FOR *IN VIVO* GENE DELIVERY IN BRAIN

Viral vectors offer the possibility to control expression of a transgene in adult or developing brain areas and can exploit the unique ability of viruses to deliver genetic material into mammalian cells. Viral vectors are derived from various viruses and are engineered to preserve the transduction efficiency while preventing the original pathogenicity and, in most cases, the capacity to multiply (Davidson and Breakefield, 2003). These viral vectors are often called multiply attenuated and replication-deficient viral vectors (**Figure 1**). Among the most widely used vectors for CNS applications are the lentiviral (LVs) and adeno-associated viral vectors (AAVs) which have particularly attractive properties which include, the capacity to infect non-dividing cells, the absence of cytotoxic or immune response, long-term transgene expression and large diffusion in the brain. At least for LV, the cloning capacity is sufficient to integrate most of the genes of interest (Déglon and Hantraye, 2005). Viral vectors provide a gene transfer tool that is independent of age and species considered (Kay et al., 2001; Kirik et al., 2003; Lundberg et al., 2008). Along with somatic gene transfer in developing or adult animals, viral vectors can also be used for transgenesis in species in which classical methods are not suitable, in particular large animals (Yang et al., 2008; Wongsrikeao et al., 2011).

Natural viruses have a specific pattern of infection, which reflects the recognition and interaction between viral capsid/envelope and receptors expressed on susceptible cells. Similarly, the tropism of viral vectors is primarily determined by the interaction of the viral surface proteins with receptor molecules expressed on target cells but other mechanisms could be used for subpopulation-restricted gene transfer in the brain. In particular, cell-type-specific promoters, post-transcriptional regulatory elements, replacement of retroviral envelope proteins with heterologous viral surface proteins, a phenomenon called pseudotyping (Page et al., 1990) or the use of various serotypes (AAV and Ad harboring different capsids) have been proposed to dissect and elucidate gene functions in astrocytes.

The first viral vector was obtained by exploiting the natural tropism of brain cells from the Herpes simplex virus type 1 (HSV-1; Geller and Breakefield, 1988; Federoff et al., 1992). The HSV-1 genome is complex and large, but replication-incompetent vectors, with a partial (first generation of HSV-1 vectors) or complete (amplicons) deletion of viral genes allow the insertion of very large transgenes (around 150 kb). The HSV-1 amplicons are neither pathogenic nor toxic for the infected cells and are retrogradely transported to the CNS from the peripheral nervous system (PNS; Frampton et al., 2005). These vectors have a widespread tropism for neurons (Jerusalinsky et al., 2012) and similarly to AAV and adenoviral vectors, their genetic material does not integrate into the host genome thus reducing the risk of insertional mutagenesis (Manservigi et al., 2010). However, HSV amplicons are difficult to produce, elicit low levels of adaptive immune responses and most of the human population is seropositive which limits their clinical applications for chronic disorders (Manservigi et al., 2010).

A few years after the apparition of HSV vectors, adenoviral vectors (Ad) were derived from the Ad type 5 serotype (Le Gal La Salle et al., 1993; Horellou et al., 1994). These vectors also have a high cloning capacity (approximately 30 kb of double-stranded DNA for gutless Ad) but the tropism of these vectors is not naturally oriented to the brain (Arnberg, 2012). Interestingly, a live (replication-competent Ad) vaccine has been safely administered to humans (Rubin and Rorke, 1994). This vaccine program reflects the strong immune response induced by Ad in humans (White et al., 2011), a reason why these vectors are promising candidates for tumoral therapy, and are proposed for the treatment of glioblastoma (Candolfi et al., 2006; Kroeger et al., 2010).

In the mid 1990s, the first AAV (from serotype 2) and LVs were reported (Page et al., 1990; Kaplitt et al., 1994; Naldini et al., 1996). The AAV vectors are derived from the smallest non-enveloped viruses (approximately 20 nm) and have a cloning capacity of 5 kb of single-stranded DNA. The AAV2 naturally infects humans but is non-pathogenic. It is classified as a dependovirus because it requires a co-infection with a helper virus such as Ad or HSV to perform its infectious replication cycle. The AAV persists for years in transduced cells mostly as an extrachromosomal episome (Nakai et al., 2001; Schnepp et al., 2005). To date, more than 100 serotypes of AAV have been identified, each of them possessing a specific tropism in the CNS due to the binding of the capsid with specific receptors (Wu et al., 2006a,b). Fourteen clinical trials using AAV gene transfer were performed to assess their potential therapeutic value in various neurodegenerative diseases (Crystal et al., 2004; Tuszynski et al., 2005; Kaplitt et al., 2007). In 2012, the first AAV gene therapy product was marketed by the European Medicine Agency (EMEA) for the treatment of patients suffering from lipoprotein lipase deficiency (Yla-Herttuala, 2012).

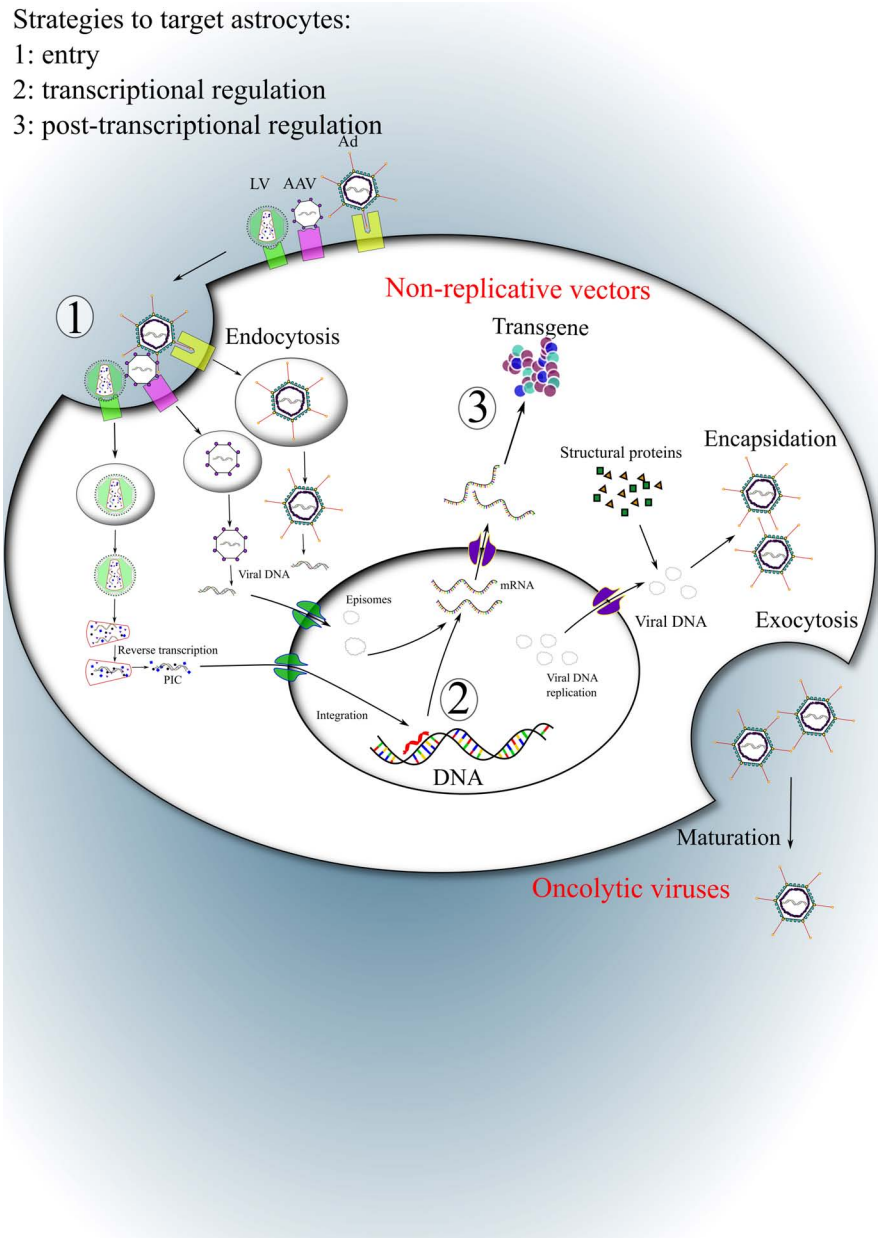

FIGURE 1 | Strategies to target astrocytes. Three steps of viral cycle are used to modify the tropism of viral vectors: (1) the entry, (2) the transcriptional and (3) post-transcriptional regulations. After binding to their respective receptors, LV, AAV, and Ad enter into host cells via receptor-mediated endocytosis. Viral DNA (AAV and Ad) or RNA (LV) are uncoated in the cytoplasm. The viral DNA remains as extrachromosomal episomes in the nucleus while viral RNA is integrated into the host genome after reverse transcription. For non-replicative vectors, in most cases only the transgene is expressed. In the case of oncolytic viruses, viral genes encoding structural proteins are necessary for the encapsidation and production of replicative particles. PIC, pre-integration complex.

Finally, the most extensively characterized LVs are derived from HIV-1, which is a subclass of retroviruses. Retroviruses are lipid-enveloped particles comprising a homodimer of linear, positive-sense, single-stranded RNA genomes of 7–11 kb. Following entry into target cells, the RNA genome is retrotranscribed into linear double-stranded DNA and integrated into the cell chromatin (Delelis et al., 2010). To decrease the risk of insertional mutagenesis, integration-deficient LVs (IDLV) were designed (Wanisch and Yanez-Munoz, 2009). These IDLVs are based on the use of integrase mutations that specifically prevent proviral integration, a process that results in the generation of increased levels of circular vector episomes in transduced cells. LVs were tested clinically for the treatment of adrenoleukodystrophy (ALD) and Parkinson's disease (PD). In the case of ALD, an *ex vivo* approach was used, with the transduction of hematopoietic CD34+ cells and re-infusion of corrected cells in the patients. An

immunological improvement occurred in the two treated children aged 9–12 months in combination with a blockage of the demyelinating lesions observed by magnetic resonance imaging (MRI), 12–16 months after gene therapy (Cartier et al., 2009, 2012). In a second study, a dopamine replacement strategy, with an LV that encodes the three enzymes responsible for the production of dopamine was tested in a phase I/II clinical trial. Increasing doses of LV were injected into the striatum of 15 patients with mid-stage PD. An improvement in motor function was observed at 6-months relative to pre-treatment assessment (Palfi, 2008; Jarraya et al., 2009 and see http://www.oxfordbiomedica.co.uk).

STRATEGIES TO TARGET ASTROCYTES

The understanding of astrocyte functions in normal and altered brain strongly relies on the availability of experimental systems to specifically target astrocytes *in vivo*. However, the first generation CNS viral vectors had a strong neurotropism *in vivo* (Naldini et al., 1996; Hermens and Verhaagen, 1997; Rabinowitz and Samulski, 1998). Indeed, the injection of AAV2 into adult rodent brains was associated with neuronal transgene expression when using ubiquitous promoters (Bartlett et al., 1998; Mandel et al., 1999; Bjorklund et al., 2000). Similarly, stereotaxic injection into rat or mouse brain of LVs pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) with CMV (cytomegalovirus) or PGK (phosphoglycerate kinase 1) promoters, leads to the specific transduction of neurons with very limited transgene expression in other cell types (Naldini et al., 1996; Kordower et al., 1999; Déglon et al., 2000). Finally, the Ad5 displays a partial neurotropism with the transduction of other cell types, especially astrocytes (Smith et al., 1996; Bohn et al., 1999; Soudais et al., 2001; Rubio and Martin-Clemente, 2002; Wang et al., 2012).

However, it is important to mention that a number of parameters could alter the tropism. These include, amongst other factors, the purity of the vector, the mode of production, the site of administration, species, the developmental stage, and normal or pathophysiological conditions. Unfortunately, data gathered in primary cultures (neurons and astrocytes) are not predictive of the *in vivo* tropism and a systematic evaluation of each vector is still required. Indeed, VSV-G/LV-GFP under the control of various promoters efficiently transduces primary rat astrocytes and to a lesser extent mouse astrocytes (Englund et al., 2000; Li et al., 2010) while transgene expression is mainly restricted to neurons *in vivo* (Naldini et al., 1996; Kordower et al., 1999; Déglon et al., 2000). This phenomenon was also observed with AAV2, which efficiently targets astrocytes *in vitro* but not *in vivo* (Gong et al., 2004). The purification method has also a major impact on the tropism of AAV8. In the mouse hippocampus, the CsCl-purified AAV8-CMV-GFP displayed an astroglial pattern in contrast to the expected neuronal expression obtained with an iodixanol purification method (Klein et al., 2008). Foust et al. (2009) found that injection of AAV9-CMV early enhancer/chicken β actin promoter (CAG)-GFP into the tail vein of adult mice mainly transduces astrocytes throughout the CNS (Foust et al., 2009), whereas the tropism is mainly neuronal after intracerebral injection or intravenous injection in neonatal mice (Klein et al., 2008). Finally, discrepancies have been observed on the transduction efficiency and tropism of various AAV serotypes between species (rodent, cat, and primates; Davidson et al., 2000; Vite et al., 2003; Burger et al., 2004; Gray et al., 2011). Additional studies are therefore still warranted to fully characterize the tropism of these vectors in the CNS. However, three strategies to direct viral vectors toward astrocytes have already been developed: shifting the tropism by favoring the entry of viruses in astrocytes, limiting transgene expression with astrocytes-specific promoters or blocking transgene expression in unwanted cells (**Figure 2**).

ALTERING THE ENTRY OF VIRAL VECTORS

The tropism of a virus is first determined by its binding with a specific receptor at the surface of the host cell (Lutschg et al., 2011; Arnberg, 2012). Knowledge of the structure and viral capsids or envelopes and their corresponding receptors provide essential information to specifically target individual cell types and/or diseased tissues. For example, the tropism of Ad5 vectors is regulated by the binding to its primary cellular receptor; the coxsackie and adenoviral vectors receptor (CAR). Tissues refractory to Ad5 infection do not express CAR. The limited expression of CAR in dopaminergic neurons of the substantia nigra of mice explains the poor transduction of these cells and transgene expression in astrocytes and other non-neuronal cells (Lewis et al., 2010). However, the expression of CAR in the nervous system and in particular in glial cells has not been extensively examined and CAR-independent forms of Ad have been developed to shift the tropism (Grellier et al., 2011).

As mentioned above, more than 100 serotypes of Ad and AAV were characterized but only a dozen of them infect cells of the CNS. Indeed, for most of them, only limited data are available concerning their receptors and their pattern of expression in the brain. The earliest and most used serotype is the AAV2, which has a natural tropism for neurons (Bartlett et al., 1998; Kugler et al., 2003). The binding of AAV2 to its primary receptor, the heparan sulfate proteoglycan (HSPG) has been well-characterized, and is centered around two amino acids on the spikes of the AAV2 capsid (Kern et al., 2003; Opie et al., 2003). However, HSPG is necessary, but not wholly sufficient, for the transduction of permissive cells. In addition, fibroblast growth factor receptor 1 (FGFR-1) was identified as a co-receptor of AAV2 (Qing et al., 1999). The tropism of AAV5 *in vivo* correlated with the pattern of expression of platelet-derived growth factor receptor (PDGFR)-alpha (Di Pasquale et al., 2003). The AAV1, 5, 7, 8, and 9 not only infect astrocytes *in vivo* but also neurons and other cells (Davidson et al., 2000; Wang et al., 2003; Shevtsova et al., 2005; Cearley and Wolfe, 2006; Gray et al., 2011). The AAV9 is unique compared to other AAV serotypes in that it is capable of crossing the blood–brain barrier and transducing neurons and/or astrocytes in the brain depending of the developmental stage (Foust et al., 2009). Recently, it has been shown that AAV9 uses galactose at the N-linked glycans as a receptor (Bell et al., 2011; Shen et al., 2011). The identification of the amino acids of the AAV9 capsid necessary for binding to galactose opens the possibility to modify the tropism (Bell et al., 2012). Finally, AAV4 and AAVrh43 preferentially target astrocytes (Liu et al., 2005; Lawlor et al., 2009) but the receptors for these serotypes are unknown. AAV4-RSV-βGal and AAVrh43-CAG-eGFP exclusively transduce astrocytes when injected into the subventricular zone (SVZ) or the striatum.

However, AAVrh43-CAG-eGFP infects approximately 3 mm^3 of the striatum and 2,000 astrocytes per mm^3 while AAV8-CAG-eGFP infects 6 mm^3 of the striatum and 150,000 neurons per mm^3 (Lawlor et al., 2009).

Lentiviral vectors are increasingly being used in neuroscience research and are unique in the sense that they are enveloped viruses that can be pseudotyped (i.e., the original envelope protein can be replaced by heterologous glycoproteins). The most used pseudotype for LV is VSV-G which confers some interesting properties to the vector (**Figure 2**). It dramatically broadens LV tropism by facilitating transduction of various cell types in different species, it stabilizes the vector particles from shear forces during centrifugation thereby allowing vector concentration and it directs LV to an endocytic pathway, which reduces the requirements of viral accessory proteins for transduction (Cockrell and Kafri, 2007). Initial studies suggest that VSV-G/LV enters into cells using phosphatidylserine (PS), but there is no correlation between the cell surface PS levels and VSV infection or binding (Coil and Miller, 2004). In addition, competition for PS using antagonists does not block the binding of VSV on target cells. Currently, the receptors responsible for VSV-G/LV entry in cells are unknown.

In the CNS, VSV-G/LVs expressing transgenes under the control of ubiquitous promoters have mainly a neuronal tropism with a limited transgene expression in astrocytes (Naldini et al., 1996; Déglon et al., 2000; Watson et al., 2002). Among the other envelopes used to pseudotype LVs, lymphocytic choriomeningitis virus (LCMV) and Mokola virus (MOK) envelopes result in a partial transduction of astrocytes. *In vivo*, LV/LCMV infects specifically astrocytes in the substantia nigra and in the striatum (Miletic et al., 2004; Cannon et al., 2011). Injection of MOK/LV into the striatum or the hippocampus leads to the infection of cells that are mainly astrocytes (Pertusa et al., 2008; Colin et al., 2009). Although no quantifications were done using LCMV/LV, 70% of cells transduced by MOK/LV are astrocytes, 20% are neurons and 10% are other cell types of the striatum. In addition, it is important to note that the titers and the transduction efficiency of these latter vectors are usually lower than VSV-G/LV.

In conclusion, specific serotypes or envelopes only partially improve the astrocytic targeting of viral vectors. However, engineering chimeric capsids or envelopes targeting astrocytes is difficult and time-consuming. In order to optimize viral vectors tropism, strategies aiming at restraining transgene expression with astrocytic promoters, or by blocking expression in unwanted cells, mainly in neurons, were developed.

TARGETING ASTROCYTES WITH TRANSCRIPTION REGULATORY ELEMENTS

Different astrocytic promoters have been used to restrict transgene expression into glial cells. However, the packaging size of each viral vector limits the type of promoters which can be inserted. Analysis of the transcriptional regulatory elements of the glial fibrillary acidic protein (GFAP) promoter reveals that 5′-flanking regions

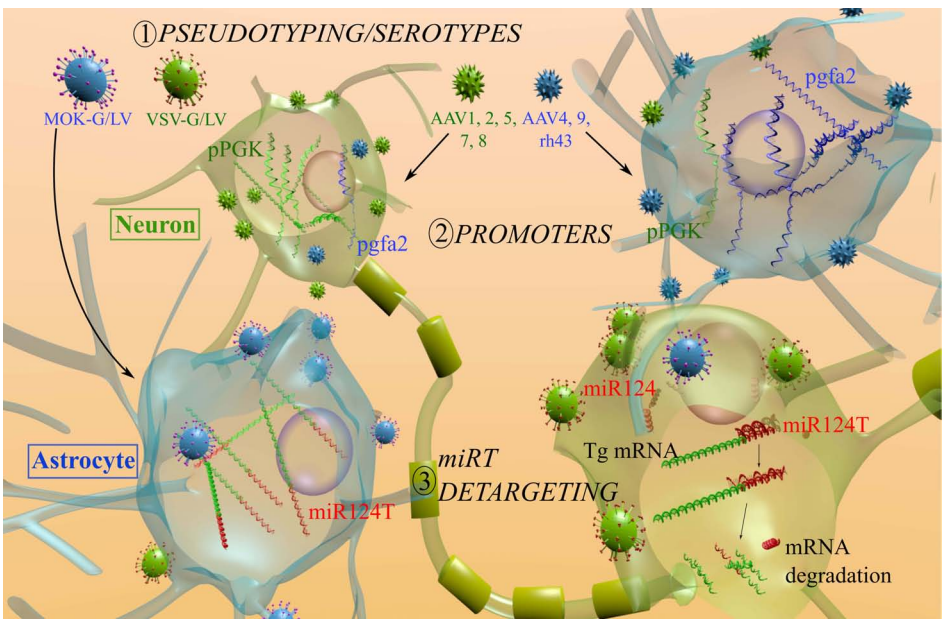

FIGURE 2 | Mechanisms used to restrain the transgene expression of AAV and LV in astrocytes. (1) To modify the entry, various AAV serotypes or LV pseudotyping with heterologous VSV-G (green) and MOK-G (blue) envelopes were used. The tropism of LV is mainly neuronal (green cells) with the VSV-G envelope and a partial shift toward astrocytes (blue cells) is observed with the MOK-G envelope. AAV1, 2, 5, 7, and 8 mainly transduce neurons (green) while AAV4, 9, rh43 display a partial astrocytic tropism. (2) To restrict transgene expression, astrocytic promoters were investigated (cells in the upper part). Transgene expression under the control of a PGK promoter (pPGK, green mRNA) leads to a preferential expression in neurons, whereas a gfa2 promoter (pgfa2, blue mRNA) results in an astrocytic expression. (3) To block the transgene expression in unwanted cells (lower part), miRNA target (miRT) sequences are integrated in the 3′-UTR of the vector (red signal on the green mRNA). The miR124 is exclusively expressed in neurons. As a consequence the miR124T is only recognized in neurons and the transgene expression is blocked (mRNA degraded). miR124, microRNA 124; miR124T, miR-124 target sequence; Tg. transgene.

of the gene are sufficient to direct transgene expression in astrocytes (Brenner et al., 1994). Two fragments compatible with AAV and LV vectors were created: gfa2 of 2.2 kb and gfaABC1D of 600 bp (Brenner et al., 1994; Lee et al., 2008). The cloning of the gfa2 fragment into Ad5 and AAVrh43 vectors restricts transgene expression in rat astrocytes (Do Thi et al., 2004; Lawlor et al., 2009; Mamber et al., 2010; Arregui et al., 2011). However, no quantification was performed to determine the number of transduced astrocytes. In the study by Lawlor et al. (2009), the gfa2 promoter was cloned into the AAV8 vector. The Gfa2-AAV8 vector infects mainly astrocytes in the striatum but a low transgene expression was still observed in neurons. The authors indicated that AAV8-gfa2-eGFP has high transduction efficiency with a wide diffusion in the striatum while AAVrh43-gfa2-eGFP transduces only a limited number of cells. It was shown recently that injection of high titer of AAV5-gfa2-eGFP into the striatum or the substantia nigra provides an astrocyte-specific expression with no residual expression into neurons or microglial cells. In addition, the expression was stable until 12 weeks post-injection. Stereological analysis of transgene expression reveals that a mean of 15,000 astrocytes per mm^3 of striatal tissue were transduced (Drinkut et al., 2012), corresponding to ∼75% of the astrocytes present in the transduced area (Savchenko et al., 2000).

Astrocytic promoters were also used in combination with LCMV and MOK pseudotyped LVs (**Figure 2**). The vector LCMV/LV-gfa2-Cre was injected into the SVZ of Rosa26 mice that express the sequence LoxP-stop-LoxP-LacZ (Stein et al., 2005). The expression of Cre in transduced cells removes the STOP cassette in Rosa26 mice and as a consequence, LacZ staining was observed in astrocytes of the SVZ after LCMV/LV injection. However, no quantification was performed although some neurons expressed the transgene. To develop an expression system activated in pathological conditions, Jakobsson et al. (2004) took advantage of GFAP up-regulation in reactive astrocytes. Using toxin-induced lesion models (6-hydroxy-dopamine and ibotenic acid lesions), they showed that the transgene expression is eight-fold higher in reactive astrocytes: a finding which correlates with the activity of the endogenous GFAP gene (Jakobsson et al., 2004). Recently, other astrocytic promoters were used in LV vectors, such as the glutamate transporter promoter, EAAT1 (Colin et al., 2009). In this study, striatal injection of MOK/LV-EAAT1-GFP leads to the expression of the transgene mainly in astrocytes (75% of the transduced cells).

In conclusion, astrocyte-specific promoters alone or in combination with an "astrocytic" capsids or envelopes, significantly shift the tropism of viral vectors toward astrocytes *in vivo*. However, the targeting is, in most cases, not complete and a residual transduction (10–40%) of non-astrocytic cells is observed. In addition, most studies rely on the use of the GFAP promoter. Large initiatives are underway to characterize the regulatory elements of the whole human genome (Gerstein et al., 2012; Whitfield et al., 2012) and new astrocyte-specific promoters were recently described. For example, the aldehyde dehydrogenase 1 family, member L1 (ALDH1L1) promoter is highly active in all mature astrocytes (Cahoy et al., 2008) while the GLAST promoter was used to express transgene in GFAP-positive but also GFAP-negative astrocytes (Liu et al., 2006; Regan et al., 2007; Buffo et al., 2008). Analysis of GLAST and GLT1-GFP mice has revealed an unexpected non-overlapping pattern between the two transporters and confirmed the differential activation of the promoters during embryogenesis and in adulthood. GLAST activity was low in the forebrain and high in the cerebellum, whereas GLT1 expression was higher in the cortex than in the cerebellum, consistent with the prominent role of GLT-1 in glutamate uptake in the forebrain. Combining data from the ENCODE project and the gene expression cartography in human and mouse brain will provide additional and essential information to identify minimal fragments necessary for cell-type-specific transgene expression in viral vectors (Hawrylycz et al., 2012). This strategy has already been developed by the Pleiade Project, which integrated information from genomic databases to construct synthetic MiniPromoters for viral vectors containing only the indispensable regulatory elements to achieve gene expression (Portales-Casamar et al., 2010).

DETARGETING STRATEGY USING MICRORNA

To further improve viral vector tropism, post-transcriptional regulatory elements have been integrated into viral vectors to block transgene expression in non-targeted cells. This strategy called "detargeting" uses microRNA (miRNA) machinery to obtain tissue-specific expression (Brown et al., 2007; **Figure 2**). miRNAs are small non-coding RNA of 19–25 nucleotides that mediates post-transcriptional gene suppression (Bartel and Chen, 2004; O'Carroll and Schaefer, 2013). Approximately 1,000 miRNAs have been identified and almost 50% of them are expressed in mammalian brains (He et al., 2012). These miRNA are differentially distributed in distinct brain regions and show cell-type specificity with even differential intraneuronal miRNA compartmentalization (Bak et al., 2008; Edbauer et al., 2010). Since miRNAs target most genes, they represent important regulators of expression and are implicated in a large range of biological activities. The negative regulation of gene expression is mediated through base-pairing with complementary regions within the 3′ untranslated region (3′-UTR) of their target protein-coding messenger RNAs (mRNAs; Bartel and Chen, 2004; Kosik, 2006; Saugstad, 2010). To restrict transgene expression in a specific cell population, a miRNA present in unwanted cells but not expressed in targeted cells is chosen. A natural target sequence (miRT) or a sequence fully complementary to the mature miRNA is cloned in the 3′-UTR of the gene of interest (Brown et al., 2007). This detargeting strategy was first demonstrated in the CNS with the neuron-specific miR124 (Colin et al., 2009). In this latter study, four copies of the natural target sequence of miR124 from the integrin-β1 gene were inserted in a LV to block transgene expression in neurons. When a miRT with a partial complementarity (bulged miRT) to its miRNA is placed in 3′-UTR of a gene of interest, repression occurs both at post-transcriptional (mRNA degradation) and translational levels. Whereas, in the case of a synthetic miRT with full complementarity with the miRNA, mRNA degradation is the main mechanism of action (Gentner and Naldini, 2012). Importantly, no saturation of miRNA machinery or adverse biological effects was reported with these miRNA-regulated LV (Colin et al., 2009; Gentner et al., 2009). The miRT threshold for saturation varies for each miRNA, perfectly complementary miRTs have a lower risk to saturate the miRNA machinery. In

addition, each miRNA has differential suppressive activity ranging from 5 up to >150-fold (Gentner et al., 2009). In this context, miR124 is a promising candidate because it is highly expressed in neurons (Lagos-Quintana et al., 2002; Smirnova et al., 2005; Deo et al., 2006). The insertion of four miR124T sequence in a VSV-G pseudotyped LV (VSV/LV-PGK-LacZ-miR124T) significantly decreases transgene expression levels and the number of β-galactosidase-positive neurons in the striatum of adult mice (Colin et al., 2009). This detargeting approach was used to shift the tropism of LV toward astrocytes. Double-immunofluorescence staining with neuronal and astrocytic markers demonstrated that combining mokola pseudotyping and miR124T (MOK/LV-PGK-LacZ-miR124T) resulted in a transgene expression that was almost exclusively restricted to astrocytes, with 89 ± 3% β-galactosidase-S100β-positive cells and 6 ± 4% NeuN-positive cells. This effect was not restricted to the striatum as similar results were obtained in the hippocampus and cerebellum.

In conclusion, the use of these three different strategies (modulation of viral vector entry, transcription and post-transcriptional regulations) has enabled the development of efficient gene transfer systems to specifically target astrocytes (**Figure 3**). Thanks to the unique features of these new viral vectors, it has already been possible to make significant advances in two areas of research related to the development of innovative therapies and the modeling of neurological disorders.

VIRAL VECTORS TARGETING ASTROCYTES: APPLICATIONS FOR BRAIN DISEASES

MODELING BRAIN DISEASES

There is evidence to support the idea that the mechanisms responsible for selective neurodegeneration in some brain disorders are non-cell autonomous and based upon pathological cell–cell interactions. The selective death of the neuronal population at risk in each disorder can be better explained by the convergence of multiple pathogenic mechanisms which provoke damage within the vulnerable neuron and neighboring cell types rather than by autonomous cell mechanisms (Ilieva et al., 2009).

In order to dissect out the specific role of different cell populations *in vivo* (neurons, astrocytes, microglia), two different strategies were recently used. The first one relies on the use of the Cre/loxP system to silence the expression of the mutant protein in specific cell types by crossing different Cre-expressing transgenic mice with transgenic mice expressing the mutant protein flanked by loxP sites in all cell types. The opposite strategy consists of selectively expressing the mutant protein in specific cell types using either specific promoters such as GFAP or by crossing different Cre-expressing transgenic mice with transgenic mice expressing the mutant protein after a STOP cassette flanked by loxP sites.

These two strategies were useful in providing evidence that astrocytes play a key role in the pathogenesis of ALS (Ilieva et al.,

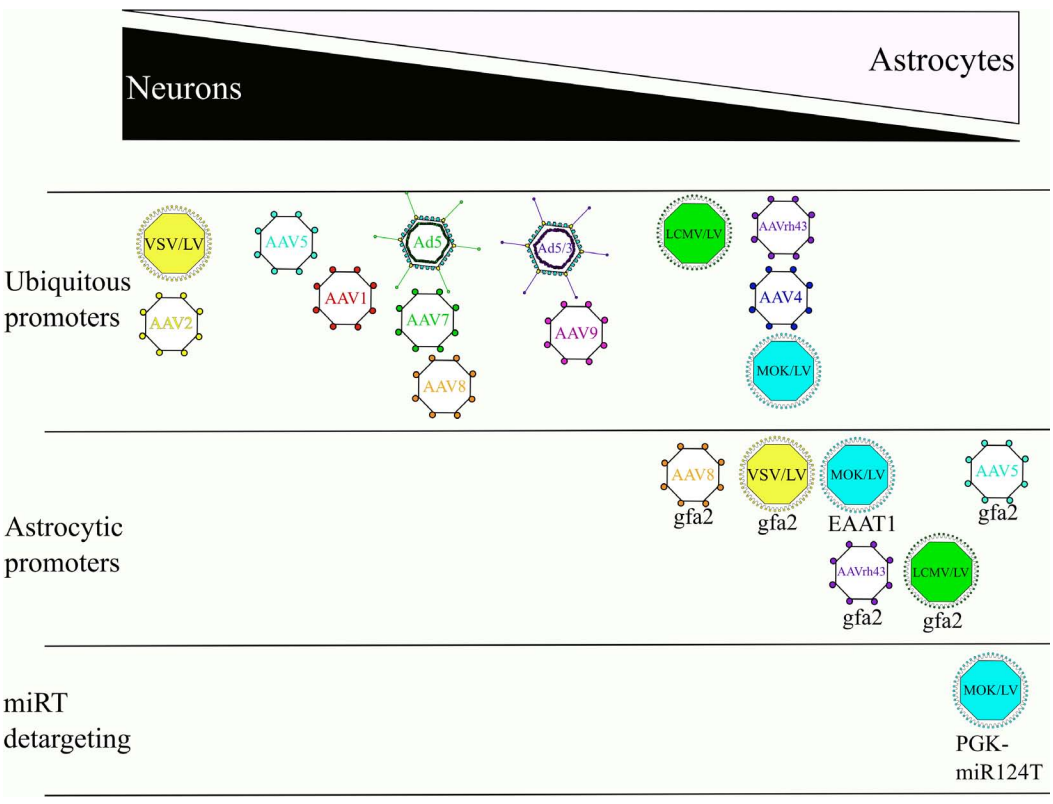

FIGURE 3 | Effects of the envelope/serotype, promoter, and miRT detargeting on the cellular tropism of LV, AAV and Ad. Overview depicting the tropisms of viral vectors in the CNS. References used for this figure are detailed and cited in the text.

2009), spinocerebellar ataxia 7 (Custer et al., 2006), HD (Gu et al., 2005; Bradford et al., 2009, 2010), and taupathies (Forman et al., 2005; Dabir et al., 2006). However, an alternative strategy based upon the use of viral vectors to selectively and locally express the mutant protein has also proven to be very useful and complementary to the development of transgenic mice in particular to test whether a local expression is sufficient to induce pathological mechanisms. Through the use of a newly developed LV (Colin et al., 2009), a short form of the mutant protein huntingtin (mHtt, responsible for HD), was expressed only in striatal astrocytes and not in neurons (Faideau et al., 2010). It has been shown that these glial cells developed a progressive phenotype of reactive astrocytes that was characterized by a marked decreased expression of both glutamate transporters, GLAST and GLT-1, and of glutamate uptake. This reactive phenotype was associated with neuronal dysfunction, as observed by a reduction in DARPP-32 and NR2B expression. Consistent with the above findings, a histological re-evaluation of potential astrocyte reactivity within postmortem brains of HD patients showed the presence of astrogliosis in the caudate nucleus of Grade 0 patients and confirmed the colocalization of mHtt in astrocytes with a grade-dependent reduction in GLT-1. Through the use of viral vectors that target astrocytes locally, we were able to show that the presence of mHtt in astrocytes is sufficient to alter the glial glutamate transport capacity early in the disease process and may contribute to pathogenesis of HD.

GLIOBLASTOMA MULTIFORM

Glioblastoma multiform (GBM) is the most common primary tumor developing in the brain from astrocytes. Due to the quick proliferation and its infiltrative nature, complete ablation by surgery is almost impossible. The prognosis is very poor, with a median survival of 14.6–19.6 months and an inevitable relapse within a few months after the resection (Grossman et al., 2010). Viral-mediated gene therapy aiming to reduce glial proliferation represents, therefore, an alternative therapy (Murphy and Rabkin, 2013). Indeed, GBM is a good candidate for gene therapy because tumor cells rarely develop metastasis outside of the brain and most cells in the CNS are post-mitotic, reducing side effects of therapeutic strategies targeting dividing cells.

However, appropriate viral vectors for the treatment of GBM are different from those developed for the treatment of neurodegenerative diseases. For GBM therapy, the aim is to mediate destruction of proliferating cells. Glial targeting is achieved either by the injection of the vector into the tumor mass, by choosing a vector which target dividing cells or having a partial tropism for glial cells, as it is the case for Ad (Asadi-Moghaddam and Chiocca, 2009).

The first studies used a replication-deficient mouse moloney leukemia virus (MLV) that infected dividing cells and expressed a suicide gene (thymidine kinase, TK; Ram et al., 1993). Thymidine kinase is a phosphotransferase enzyme that incorporates dGTP analogs in the presence of ganciclovir instead of cellular dGTP and leads to the blockade of cellular replication (Boivin et al., 1993). But the low transduction efficiency neither improved tumor progression nor the overall survival time (Ram et al., 1993, 1994; Gunzburg et al., 1995). To improve the efficacy of the treatment, vector-producing cells (VPC releasing MLV particles expressing the TK suicide gene) were injected into the brain after surgical resection of the tumor. However, no significant decrease of tumor mass occurred despite the bystander effect (Ram et al., 1997; Klatzmann et al., 1998; Shand et al., 1999; Packer et al., 2000; Rainov, 2000; Martinet et al., 2003). As an alternative therapy, Ad-TK was administered directly to GBM patients but the phase III trial showed no positive outcome (Cottin et al., 2011). Interestingly, it was shown that the preferential transduction of glioma cells is not dependent on the expression of known Ad receptors on tumor cells (Candolfi et al., 2006). Expressing the therapeutic suicide gene under the control of a strong ubiquitous promoter in combination with an immune stimulator may increase therapeutic efficacy and prevent relapse (Candolfi et al., 2006; Ghulam Muhammad et al., 2009).

As an alternative strategy to improve the therapeutic efficacy, conditionally replicative or replicative viruses were developed. The principle of oncolytic therapy is to inject directly into the tumoral cells a lytic replicative-competent cytotoxic virus, such as HSV, VSV, Ad, or retroviruses, which will induce apoptosis in proliferative cells during replication (Parker et al., 2009; Zemp et al., 2010; Castro et al., 2011; Russell et al., 2012). HSV were initially used as lytic viruses in GBM therapy (Zemp et al., 2010). However, the high worldwide HSV seropositivity limits their use in the clinic and as a consequence has led to the development of other oncolytic viruses. A deletion of E1B region on Ad genome (Ad-ONYX-15) was introduced to favor apoptosis in infected glioma cells but the efficiency of this approach was too low to reach a phase II of clinical trial (Moran, 1993; Chiocca et al., 2004). In addition, replicative adenoviral vectors expressing therapeutic genes were used to mediate tumoral cells destruction. The candidate genes are inserted in the E3 deleted region and a CAR-independent entry mechanism enhancing the transduction efficiency of tumoral cells has been proposed for these new generation oncolytic viruses. To favor replication in GFAP-positive cells, three copies of glial specific B enhancer were added on the gfa2 promoter (gfa2B3), leading to a decreased growth of glioma cells (Horst et al., 2007).

GENE THERAPY FOR NEURODEGENERATIVE DISORDERS

Degeneration of the nigro-striatal projection represents the major pathological hallmark of PD. Preclinical rodent and non-human primate models demonstrated a strong protective effect of glial cell line-derived neurotrophic factor (GDNF) on the nigro-striatal dopaminergic system (Gash et al., 1996; Kirik et al., 2000). However, intrathecal infusion of GDNF protein or viral vector-mediated expression of neurturin in the striatum of late stage PD patients showed no significant clinical benefit (Lang et al., 2006; Marks et al., 2010). Current gene therapeutic trials in the brain predominantly use AAV2 due to its proven safety record. In the animal and human CNS, AAV2 predominately transduces neurons. However, the expression of neurotrophic factors in neurons may impose a serious safety issue since the factors can be secreted from the soma, unmyelinated projections, or synaptic sites of transduced neurons, thereby delivering a complex signaling-inducing molecule to potential off-target sites. One alternative strategy would be to restrict their impact to the immediate vicinity of the site of the lesion. Through the use of an AAV5 expressing

GDNF under the expression of GFAP, Drinkut et al. (2012) demonstrated the same efficacy as neuron-derived GDNF. In terms of safety, unilateral striatal GDNF expression in astrocytes did not result in delivery of bio-active GDNF to the contralateral hemispheres (potential off-target sites) as was the case when GDNF was expressed in neurons. This suggests that astrocytic neurotrophic factor expression achieved by a viral vector can be considered an efficient alternative to current gene therapeutic strategies.

Astrocyte activation, characterized by hypertrophic somata and processes, is an early hallmark in most neurodegenerative conditions. The functional impact of this activation on the progression of these diseases is still elusive and their therapeutic potential is yet unexploited. A recent study has taken advantage of the strong astrocytic tropism of AAV2/5 expressing the astrocyte-specific promoter Gfa2 to test the potential of astrocyte-targeted therapeutics in an intact animal model of Alzheimer's disease (AD; Furman et al., 2012). It was shown that the bilateral administration of AAV2/5 Gfa2–VIVIT (a synthetic peptide that blocks the calcineurin (CN)/nuclear factor of activated T cells (NFAT) pathway which regulates several components of the activated astrocyte phenotype) into the hippocampus of 7- to 8-month-old APP/PS1 mice, was associated with reduced glial activation, lower amyloid levels, improved synaptic plasticity, and an improved cognitive function at 16–17 months of age. This result represents a proof-of-principle that astrocytes can be considered as significant therapeutic targets not only in AD but also for other neurodegenerative diseases. Because of its specificity, lack of toxicity and capacity for widespread and long-lasting transgene expression, AAV appears to be an ideal vehicle for directing therapeutics to astrocytes.

CONCLUSION AND PERSPECTIVES

The growing importance of astrocytes in crucial brain functions and also dysfunctions has led to the development of new genetic tools to label and manipulate these glial cells *in vivo*. Thanks to these tools that include targeted transgenesis and viral transduction, considerable advances were made in the understanding of astroglial biology. This first generation of astrocytic viral vectors was instrumental to start depicting their role in specific brain regions of different species. However, a better determination of the numerous functions played by astrocytes during development, in adulthood and disease will require new viral vectors that can further resolve the intimate relationship between neurons and glia in the maturing brain (Molofsky et al., 2012). One important issue relates to the recent but well-accepted notion that astrocytes do not represent a homogenous population of cells. This is, of course, thoroughly demonstrated for neurons (Miller and Gauthier, 2007) but is just starting to be studied for astrocytes in particular because of the lack of reliable markers to follow these different cell populations. The launching of recent initiatives such as the Human Brain Project and ENCODE will increase our knowledge on the functions of astrocytes and may help to refine strategies previously developed to drive transgene expression into specialized astrocytes at different stages of development either in normal or diseased states. A comprehensive mapping of the cell-type-specific expression of miRNAs, the development and *in vivo* assessment of efficient miRT sequences will also permit one to ameliorate the detargeting strategy. Similarly, the identification of the receptors required for the binding of the viral particles to astrocyte subpopulations will represent a major step toward the production of more efficient astrocytic viral vectors. In addition to these strategies which are already used to drive the tropism of viral vectors toward astrocytes, new viral vectors could be developed. Among these emerging viral vectors, baculoviral vectors take advantage of their natural tropism for astrocytes (Boulaire et al., 2009). Their large genome size (140 kb) is suitable for the incorporation of large genes of interest and complex regulatory elements (Wang and Wang, 2006). Clinical observations in patients suffering from neurological pathologies following viral infections suggest that other viruses could have a cerebrotropism (e.g., alphaviruses or arboviruses; Das et al., 2010; Walker et al., 2012). This illustrates the need for multidisciplinary programs that would share the expertise of neurobiologists, virologists, geneticists, and clinicians in order to overcome the limitations of current vectors and discover innovative gene transfer systems. Considering how much more might be discovered about the functions of normal or diseased astrocytes, it is tempting to suggest that we are just at the beginning of the development of astrocentric viral vectors.

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A cellular star atlas: using astrocytes from human pluripotent stem cells for disease studies

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What roles do astrocytes play in human disease? This question remains unanswered for nearly every human neurological disorder. Yet, because of their abundance and complexity astrocytes can impact neurological function in many ways. The differentiation of human pluripotent stem cells (hPSCs) into neuronal and glial subtypes, including astrocytes, is becoming routine, thus their use as tools for modeling neurodevelopment and disease will provide one important approach to answer this question. When designing experiments, careful consideration must be given to choosing paradigms for differentiation, maturation, and functional analysis of these temporally asynchronous cellular populations in culture. In the case of astrocytes, they display heterogeneous characteristics depending upon species of origin, brain region, developmental stage, environmental factors, and disease states, all of which may render experimental results highly variable. In this review, challenges and future directions are discussed for using hPSC-derived astroglial progenitors and mature astrocytes for neurodevelopmental studies with a focus on exploring human astrocyte effects upon neuronal function. As new technologies emerge to measure the functions of astrocytes *in vitro* and *in vivo*, there is also a need for a standardized source of human astrocytes that are most relevant to the diseases of interest.

Keywords: human stem cells, astrocytes, RASopathies, disease models, synaptogenic proteins, neurological disorders, regenerative medicine, developmental disorders of the brain

INTRODUCTION

With the recent technological advances for astrocyte manipulation, generation, purification, and functional analyses in normal and diseased states, there is a need for standardization and optimization of the experimental system. In regards to this review, the system of interest is the differentiation of astrocytes from human pluripotent stem cells (hPSCs), either embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). During neurodevelopmental studies, it would be ideal to compare cell differentiation of control and experimental groups beginning with limited differences except for the variable of interest. Inevitably, epigenetic variations will exist within the hPSC lines prepared from different cellular origins (Kim et al., 2010) and those cultured with different methods (Nazor et al., 2012; Tomoda et al., 2012), all of which may lead to altered differentiation potentials. Also, genetic variability will always occur among human cellular sources, which has led to the design of methods for generating isogenic control hPSC lines through genetic correction technology (Zwaka and Thomson, 2003; Hockemeyer et al., 2009, 2011). These technologies (e.g., homologous recombination, zinc finger nucleases, and TALENS) are designed to target and replace a mutation of interest with wild-type sequence while keeping the rest of the genome unmodified. In the subsequent stage of directed neural induction and differentiation of hPSCs into neuroepithelia/neural stem cells (NSCs), multiple protocols have been designed with varying environmental factors and techniques which can alter the developmental timing and identity of the final cell type of interest [for example, the generation of CNS neural cells (Zhang et al., 2001) vs. PNS (Lee et al., 2007)]. Although variability has the advantage of masking non-specific phenotypes in disease models, and is also a better representation of variation in nature, it may lead to false positive experimental results when using a low number of replicates. While keeping in mind this heterogenous source material, we will focus this review on examining the variability and heterogeneity that occurs between the stages from hPSC-derived NSCs to astrocytes, and discuss the advantages and disadvantages of using an *in vitro* system to enumerate major experimental variables that should be taken into account when designing disease-related studies.

For which neurological diseases is an examination of astrocyte function relevant? There are numerous methods to mimic disease states in cultured astrocytes including scratch assays (Yang et al., 2012), mechanical stretch (Wanner et al., 2008), and treatments with inflammatory factors (Falsig et al., 2004), but the main benefit of using patient-specific iPSCs is to study specific disease-causing genetic mutations. The most obvious disorder to target is Alexander Disease, which is referred to as a "primary astrocyte disease" because it is caused by mutations of the semi-specific astrocyte protein GFAP, but also eventually leads to damage in oligodendrocytes and neurons through yet unknown mechanisms (Messing et al., 2012). On the other hand, it is becoming clear that many, if not all, neurodevelopmental and neurodegenerative diseases may be directly or indirectly affected by glial function (Molofsky et al., 2012; Verkhratsky et al., 2012). Whether the

observed astrocytic phenotypes are disease-specific or generic consequences of a stressed "reactive" astrocyte (referred to here as astrogliosis) that contributes downstream to neighboring cells is a major question that should be examined in each case. For example, it has been observed in some amyotrophic lateral sclerosis models that astrocytes either secrete toxic factors [i.e., lipocalin 2 (Bi et al., 2013)] or have a deficiency in providing support to motoneurons, leading to neuronal degeneration. Whether these factors are the main cause of motoneuron loss and how they specifically affect these neurons is still not clear (Sica, 2012; Phatnani et al., 2013). In the case of neurodevelopmental disorders, an altered timing of astrocyte differentiation likely leads to changes in the number of adult astrocytes and/or in their impact upon neurons, as described in more detail below. For the purposes of this review, we will provide examples for experimentation using one of the most common classes of neurodevelopmental disorders that are likely affected by both developmental and functional changes in neural cells. These syndromes are commonly referred to as "RASopathies" because they all involve alterations in the Ras/MAPK signaling pathway and lead to mental impairments among other phenotypes (Tidyman and Rauen, 2009). Mouse models have shown that astrocyte progenitors have an accelerated development and/or proliferation in a number of these syndromes including Noonan syndrome (Gauthier et al., 2007), Neurofibromatosis-1 (Hegedus et al., 2007), Costello syndrome (Paquin et al., 2009), and cardiofaciocutaneous syndrome (Li et al., 2012; Tien et al., 2012), though the astrocyte-specific functional consequence on neurons in these contexts, especially in a human cellular system, is unknown.

What phenotypes should be examined? There are at least three major levels of cellular examination that can be addressed when comparing diseased and control astrocytes; (1) intrinsic changes within an individual cell such as gene expression and cell signaling, (2) population networks that include heterogeneous cell types and long range coupling, and (3) extrinsic factors released from astrocytes that affect other cell types including neurons, oligodendrocytes, microglia, or those that make up the vasculature. These levels are also temporally dynamic during differentiation and the functional consequences may depend on development, brain region, and environmental conditions (Zhang and Barres, 2010; Oberheim et al., 2012; Theis and Giaume, 2012). In some cases the appropriate choice of analysis is obvious when the specific cause of disease is known, i.e., astrocytes from an ALS model (mutant TDP-43) iPSC lines have an increased expression and mislocalization of TDP-43 protein (Serio et al., 2013), and astrocytes from Alzheimer's disease models (sporadic cases and mutant APP) have Aβ oligomer accumulation (Kondo et al., 2013). Though for the majority of other cases, finding a disease-related phenotype may take various large scale profiling methods. Below, using examples with RASopathy-specific cells, we will discuss the advantages and shortcomings of utilizing hPSC-derived astrocytes to study specific functional aspects both intrinsically and upon other cell types. Ultimately, the phenotypes should be confirmed in an *in vivo* environment with methods such as transplantation of the human astrocytes into rodent or primate brain.

CONSEQUENCES OF FITTING A STELLAR UNIVERSE IN A DISH

One major advantage of studying *in vitro* astrocyte progenitor differentiation from NSCs in a culture system is that the intrinsic developmental order (neurons, then glia) and timing (several months for human) correlates with *in vivo* development, due to a combination of transcriptional and epigenetic regulations (Sauvageot and Stiles, 2002; Okano and Temple, 2009). For developmental studies, this extended temporal program allows for human cellular profiling during differentiation at the gene expression (qPCR, microarray, RNA-seq), protein (immunocytochemistry, Western, proteomics), and epigenetic (methylation, histone modifications) levels at discrete stages (Krencik and Zhang, 2006). During RASopathy studies, this profiling could be used to determine whether abnormal Ras/MAPK signaling cause temporal shifts in astrogliosis, as observed in mouse models, and these data sets can provide baselines for comparisons after genetic or drug screening with the aim of modifying the abnormal developmental timing back to control levels. For example, cells may be acutely or chronically treated with compounds safe for clinical trials including inhibitors of farnesyl transferase, MEK, and ERK (Rauen et al., 2011), or expression levels may be hampered with siRNA technologies. One should bear in mind that one major drawback is that the population of neural progenitors derived from hPSCs are non-synchronous, e.g., there will always be a mixture of cells at slightly different stages of development (**Figure 1**). On the other hand, this phenomenon may have some silver linings. For example, three-dimensional differentiation of neuronal cultures in a cluster generates cells intrinsically organized in a spatial and temporal polarization that mimics radial glial and neuronal layering in the cortex (Eiraku et al., 2008), potentially producing a more accurate model of *in vivo* development. This structural recapitulation may also occur during astroglial differentiation although it has not been extensively investigated. With an *in vitro* system it is possible to recapitulate normal development by examining the temporal expression pattern of progenitor (NFIA, S100B) and more mature (GFAP) markers over time (Krencik et al., 2011). The scarcity of astrocyte markers does not yet allow for more precise examination of subtypes that occur throughout the CNS (one likely exemption are regionally-specific developmental transcription factors), but it may be possible to recapitulate the distinct morphological characteristics displayed by human astrocytes *in vivo*, which depends on their cortical location (Oberheim et al., 2009).

Perhaps the most important pressing issues in this field are how to properly identify a mature astrocyte and how to standardize this definition between research laboratories. GFAP has long been the gold standard as an astrocyte marker (Eng et al., 2000), even though its levels change during development, aging, and stress. At what point should a glial progenitor cell be termed an astrocyte using this marker? For immunocytochemical analysis, the appearance of GFAP protein is commonly used since there are many commercially available antibodies that consistently work well and the cellular protein content is highly abundant. Unfortunately, during early stages of the hPSC differentiation process, GFAP can be observed diffusely throughout the cells with high antibody concentrations and high exposure times, then

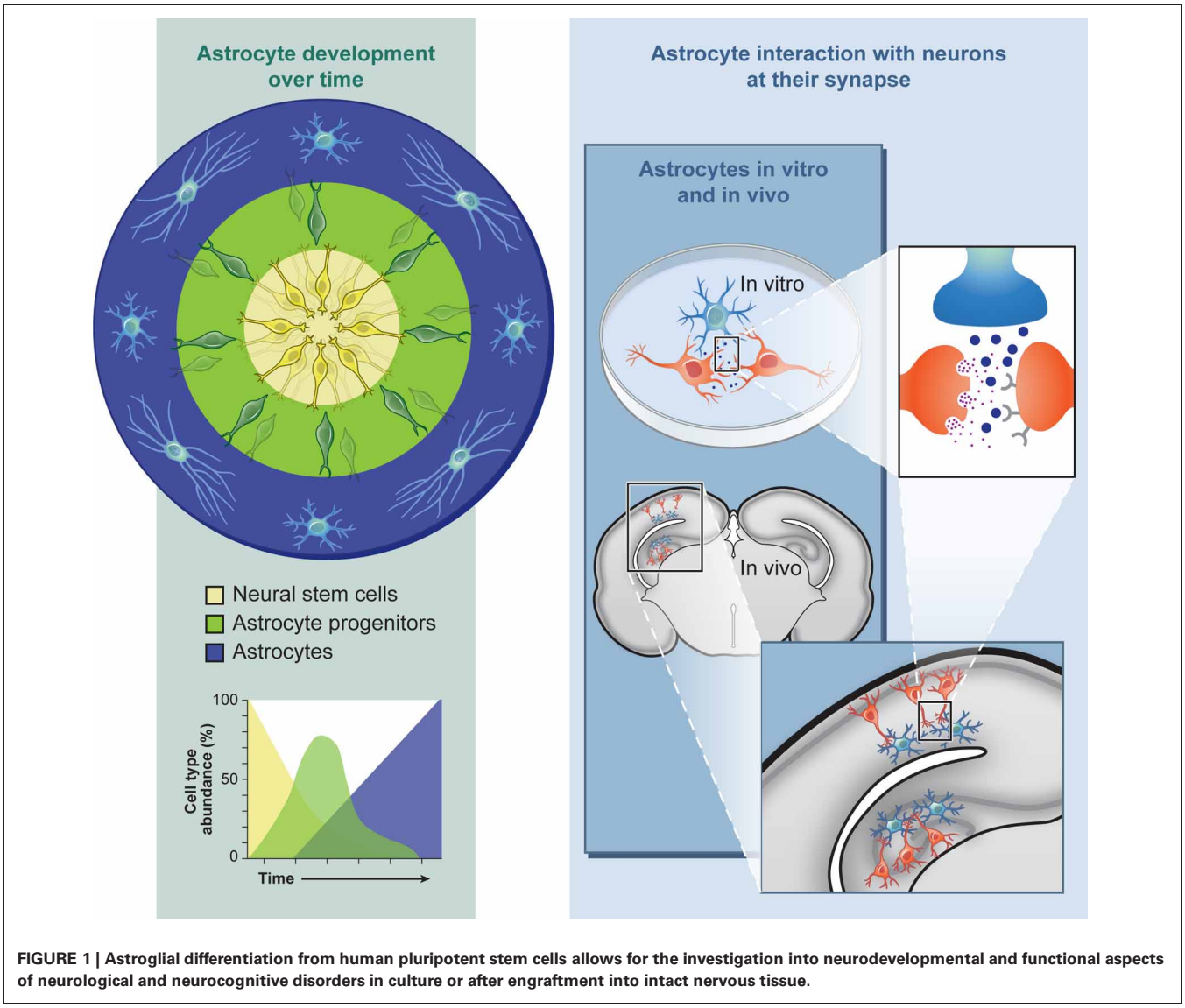

FIGURE 1 | Astroglial differentiation from human pluripotent stem cells allows for the investigation into neurodevelopmental and functional aspects of neurological and neurocognitive disorders in culture or after engraftment into intact nervous tissue.

gradually localizes in a filamentous pattern while becoming more intense (Krencik, pers. observation), thus the identification of an astrocyte using GFAP as a marker is not absolute. Other proteins used as markers also display shifting localization as the cells mature, for example CD44 localizes in a punctuate/ruffled manner after the receptor inserts into the cell surface membrane. Thus, standards for identifying onset of these markers may be unknowingly disparate among research groups that have thus far generated hPSC-derived astroglial progenitors for studies (Krencik et al., 2011; Gupta et al., 2012b; Juopperi et al., 2012; Serio et al., 2013; Shaltouki et al., 2013; Wang et al., 2013), leading to examination of cells at different developmental stages, levels of astrogliosis, or may even falsely identifying non-neural contaminating cells which can appear in this culture system (Krencik and Zhang, 2011). One suggestion for standardizing identification is to first measure primary astrocyte cultures that usually contain GFAP+ and GFAP- cells to determine maximum and minimum cutoffs during imaging, and then assemble information on the relative intensity of filamentous GFAP for each individual cell in an imaging field. Unfortunately, other astrocyte-specific markers such as Glt-1 and Aldh1L1 are more difficult to use for immunochemistry (Krencik, pers. observation) and less is known about their expression in human cells, yet tools based on these markers are effective reporters in mouse transgenic studies (Yang et al., 2011). Likewise, S100B is a useful progenitor marker, but it is also expressed in oligodendrocyte progenitors (Deloulme et al., 2004) and NG2 cells (Hachem et al., 2005). The pressing need to identify more markers of both rodent and human astrocytes to further investigate these cells under various conditions is evident. Ultimately, hPSC-derived astrocytes should be identified by astrocyte-specific functional outputs, but traditional readouts such as glutamate uptake and promotion of synaptogenesis are also functions of progenitor cells and other glial types to differing extents.

Unfortunately for researchers interested in generating purified clonal mature astrocytes as quickly as possible for functional

studies, the prolonged developmental timeline of this heterogenous culture system mentioned above (approximately 4–6 months depending on the protocol used) is an inconvenience. The astrocyte differentiation process can be accelerated by prolonged treatment with gliogenic factors including CNTF, BMP, and LIF, or possibly by imposing epigenetic changes to occur (Gupta et al., 2012a). However, these treatments could have some effect upon disease phenotypes or even mask them. To date, no systematic comparison has been made of astroglial cells derived from the same starting material with differing methods. For a more temporally pure population it may be possible to clonally culture and expand a single cell, although proliferation of human astrocytes appears to be density dependent (Krencik, pers. observation). Other possible options are to cell-sort progenitors based on distinct cell type markers as has been done with CD44 (Yuan et al., 2011) or to transform fibroblasts directly into astrocytes using transcriptional codes similar to what has been done for the transdifferentiation of human fibroblasts to neurons (Pang et al., 2011). Regardless of the techniques used, methods should be considered that most accurately produce the cell type of interest for disease studies and detailed method descriptions should be provided for repeatability and data comparison between research groups.

It is understood in quantum mechanics that any observation of a system will have some change on the system itself, thus affecting the final measurement. In cellular neuroscience, we obviously disturb the cellular order by designing simplified models outside of the natural state. The major shortcoming of using hPSC-derived neural cells (and primary cultures from rodent or human origin) for disease modeling is the changes that occur in culture, which is usually a more stressful environment compared to the normal *in vivo* nervous system. Yet, the use of cultured cells confers many advantages including cell type purity, control of environmental factors, and easy access for experimentation. Astrocytes were first cultured from early postnatal rodent brain by taking advantage of their adhesiveness, survivability, and enhanced growth rates compared to other neural types while cultured in serum conditions (McCarthy and de Vellis, 1980) or after subsequently switching to serum-free defined conditions (Morrison and de Vellis, 1981). Serum varies between batches and leads to changes in morphological and proliferative properties, and therefore it is not suggested for use with primary astrocyte cultures; yet many studies are still conducted in serum containing conditions for its ease of use. More recently, an immunopanning technique has been designed for a rapid serum-free purification, revealing major gene expression changes in the presence of serum (Foo et al., 2011). High serum also affects hPSC-derived astrocytes in their morphology, adhesiveness, proliferation, and usually results in the expansion of non-neural contaminants at early differentiation stages (Krencik, pers. observation). Another stressor is that cells are typically cultured at higher oxygen levels than physiological conditions, which may impact progenitor proliferation and/or differentiation (Studer et al., 2000). The choice of culture media and additives will likely affect the differentiation process as well. For example, long term expansion of human NSCs requires the presence of growth factors such as EGF and FGF2 (Caldwell et al., 2001), but variability in the starting concentrations and metabolism of these factors, and the time between media replacement, will likely be inconsistent between different cultures and researchers. One advantage of using these growth factors is that for RASopathy studies the presence of these factors directly activates the Ras/MAPK pathway through receptor tyrosine kinase receptors, thus this pathway is chronically stimulated and may expose disease-related phenotypes. Taken together, although these culture factors may produce a system that is dissimilar to the natural environment, the stressful conditions may accelerate or expose disease phenotypes that normally do not appear until adulthood in humans and evolve over years such as neurodegenerative diseases. Even though all neurodegeneration-related phenotypes will be unlikely to be present in this relatively short-term culture system, cellular phenotypes have been observed with Parkinson's disease (Devine et al., 2011; Nguyen et al., 2011) and ALS cellular models (Bilican et al., 2012). It is important to note that even after astrocytes are generated, semi-purified, and prepared for experimentation using the method of choice, the issue still remains as to whether the final product is the best functional model for the disease of interest.

HOW TO TRAVERSE THIS DIVERSE MULTIVERSE

For modeling region-specific diseases *in vitro*, the most relevant cell subtype to generate is one that displays similar dysfunctional properties as cells in the natural disease system. Precise directed differentiation of specific neuronal subtypes from hPSCs is increasingly attainable due to the ease of subtype identification using expression of neurotransmitter-related factors as markers; for example, observing the presence of choline acetyltransferase in motoneurons (Li et al., 2005), tyrosine hydroxylase in dopaminergic neurons (Zeng et al., 2004) or GABA in striatal interneurons (Aubry et al., 2008). Though astrocytes are known to secrete various gliotransmitters including ATP and D-serine, these are unlikely ideal markers because questions still remain about the physiological role of gliotransmission due to possible experimental artifacts including astrogliosis (Agulhon et al., 2012), and whether these gliotransmitters are variably expressed between subtypes. However, it is known that astrocytes display heterogenous enzymatic activities (Hansson, 1984) and responses to neurotransmitters in regionally distinct subtypes, correlating with their adjacent neuronal subtypes (Matyash and Kettenmann, 2010; Oberheim et al., 2012). Since hPSC-derived NSCs can be regionally specified at the neuroepithelia stage by the application of morphogens and then further matured into neurons or glia that maintain this identity (Liu and Zhang, 2011), regional distinctions that can be measured *in vivo* may also occur *in vitro* if these functions are endowed through intrinsic mechanisms. For example, the promoter activity for the astrocyte specific glutamate transporter Glt-1 is lower in spinal cord astrocytes compared to those in the brain (Regan et al., 2007) and the inward rectifying potassium channel Kir4.1 protein is more abundant in ventral spinal cord compared to dorsal regions (Olsen et al., 2007). Morphology and proliferation rates are also region-specific (Emsley and Macklis, 2006). *In vitro*, it has been shown that astrocytes prepared from different regions exhibit differential effects upon neurons including neuronal outgrowth (Qian et al., 1992), dendritic arborization (Le Roux and Reh, 1995),

and differentiation (Castelo-Branco et al., 2006). Whether these functional distinctions can be recapitulated by hPSC-derived astrocytes specified to distinct subtypes is still unknown. Together, some of these specific markers and functions may be useful for identification after directed differentiation of hPSCs toward the distinct type of interest.

Does astrocyte regional heterogeneity have relevance for disease studies? *In vivo*, the degree of astrocytic responses in disease states may be due to both intrinsic diversity and responses to the local environment. For example, midbrain astrocytes may respond differentially to changes in dopamine levels in early stages of Parkinson's disease due to variable MAO-B levels (Mallajosyula et al., 2008; Vaarmann et al., 2010) and spinal cord astrocytes may not be able to properly reduce glutamate levels during ALS-induced excitotoxicity due to a low level of Glt-1 (Regan et al., 2007). This heterogeneity also has relevance for regenerative medicine. Midbrain astrocytes can secrete neurotrophic factors that protect dopaminergic neurons from degeneration including GDNF and CDNF (Lin et al., 1993; Lindholm et al., 2007), although these factors are also expressed in other regions. Developmental studies have revealed that astrocyte functional diversity may at least partially depend on their domain of origin. For example, dorsally and ventrally located astrocyte progenitors differentially express the guidance cues Slit1 and Reelin (Hochstim et al., 2008) and the extracellular matrix protein tenascin C (Karus et al., 2011). It is unknown whether these differences also depend on environmental cues, though it is interesting that astrocytes continue to occupy their distinct subregional domains determined in development and do not migrate to other domains after injury or depletion of adjacent cells (Tsai et al., 2012). With regards to RASopathy modeling, astrocytomas in NF1 predominantly occur around or near the optic nerve, thus, it may be most relevant for cancer studies to direct hPSCs to the optic stalk neuroepithelium, the probable source of optic nerve astrocytes (Horsburgh and Sefton, 1986). Though it is unknown why gliomas preferentially occur in the optic nerve, there is evidence of heterogenous astrocyte expression of NF-1 (Yeh et al., 2009), region specific effects of NF1 on astrocyte differentiation (Lee da et al., 2010), subtype astrogliosis (Rizvi et al., 1999) and differential responses from the local environment (Simmons et al., 2011) which may all play some role. As another example, abnormal vision is very common in Noonan syndrome including refractive errors (Sharland et al., 1992), therefore astrocytes differentiated from hPSC-derived retinal and optic nerve progenitor cells (Lamba et al., 2006; Meyer et al., 2009; Nakano et al., 2012) may be a promising candidate for investigation.

Another important variable to consider includes the maturation state of the astrocyte. hPSC-derived astrocytes are likely a mix of NSCs and astroglial progenitors during the differentiation process as described above (**Figure 1**), making measurements of mature functions variable in this mixed culture system. At the genomic level, astrocytes isolated at different stages of development differ in expression of numerous genes (Cahoy et al., 2008). Even after further maturation of hPSC-derived astrocytes in prolonged culture, these cells are likely immature compared to human adult astrocytes since they have been differentiating for only a few months and have not received signals from neurons that are known to affect astrocyte-specific proteins (Stipursky et al., 2012). For example, the Glt-1 protein remodels its localization near neighboring neuronal synapses during development (Benediktsson et al., 2012). Conversely, immature astrocytes may be better suited for studying synaptogenesis because immature astrocytes, but not mature, secrete the synaptogenic factor thrombospondin (Christopherson et al., 2005). It has also been revealed that developing and adult astrocytes functionally differ based on differential expression of glutamate receptors (Sun et al., 2013). Importantly, the key factor in modeling disease is that after generating the most relevant cell of interest, it would be critical to determine whether these cultured hPSC-derived astrocytes are exhibiting distinct responses to specific disease paradigms or whether they are only displaying generic responses independent of the stress paradigm (i.e., increases in oxidative stress, ER stress, or cell death), though there is now evidence of specific transcriptional changes that occur depending on the type of astrocyte stimulus (Lavisse et al., 2012; Zamanian et al., 2012).

EXTRACELLESTIAL CONTACT

Even though cultured astrocytes imperfectly recapitulate cells in the brain environment as discussed above, the use of cultured primary astrocytes has been instrumental in elucidating their influence on other cell types in both normal and diseased conditions, as thoroughly reviewed elsewhere (Lange et al., 2012). Importantly, the use of human-specific astrocytes from hPSCs may uncover unique phenotypes, including those that are known to exist *in vivo* (Oberheim et al., 2009), which can be masked in non-human backgrounds or otherwise difficult to measure due to limited resources of human tissue. In the case of RASopathies, immunochemical analysis with human NF1 brains has revealed an increase of astrogliosis, though how this contributes to neuronal abnormalities is unknown (Nordlund et al., 1995). Structural MRI studies of Costello syndrome brains usually uncover macrocephaly, ventriculomegaly, and Chiari 1 malformation which suggests an increase of astrocyte progenitor number (Gripp et al., 2010) similar to what has been observed in mouse models. Another experimental option for human cellular studies is use of human fetal NSCs for analysis. Recent use of this system has revealed that the Ras/MAPK pathway may be dysregulated in both Fragile X and Down syndrome genetic backgrounds (McMillan et al., 2012); suggesting this pathway plays a major disease role outside of RASopathies. In light of these issues, the use of hPSC-derived astrocytes would be a convenient human specific system to study intrinsic changes in RASopathy genetic backgrounds including astrogliosis and/or proliferation. Besides intrinsic cellular changes, disease modeling can shed light on what factors diseased astrocytes bestow upon other cells types. Because it is difficult to separate the effects of specific cell types *in vivo*, the system can be used for identifying these factors in either coculture, with use of astrocyte conditioned media (ACM), or transplantation studies (**Figure 1**).

Astrocytes receive and send signals to numerous cell types throughout the nervous system; thus simplified coculture systems may be a means to reveal important signaling components while optimizing additional extrinsic factors. One major route of communication apt for examination is between the brain

vasculature system and astrocytic end feet (termed the gliovascular unit) which plays diverse roles in normal and diseased states. These functions include the coupling of neuronal activity to blood flow regulation and maintenance of the blood-brain-barrier (BBB) (Iadecola and Nedergaard, 2007; Kovacs et al., 2012). In order to study the effect of astrocytes on BBB formation and maintenance, numerous direct and indirect co-culture systems have been designed for studies with brain endothelial cells, as extensively reviewed elsewhere (Naik and Cucullo, 2012; Lippmann et al., 2013), although the specific molecules they provide is unclear. In the other direction, cultured endothelial cells induce astrocyte differentiation possibly through LIF secretion (Mi et al., 2001). Astrocytes also play major roles in synaptogenesis and synaptic plasticity (Allen and Barres, 2005; Eroglu, 2009; Barker and Ullian, 2010). Astrocyte-neuronal cocultures and/or ACM have been used to uncover numerous synaptogenic factors released by astrocytes which include cholesterol (Mauch et al., 2001), TNFα (Beattie et al., 2002), thrombospondins, (Christopherson et al., 2005), Hevin, Sparc (Jones et al., 2011; Kucukdereli et al., 2011), and glypicans (Allen et al., 2012). How these and other factors that alter synaptic plasticity [including extracellular matrix molecules and cytokines (Wiese et al., 2012)] change during development and disease in human cellular backgrounds are unclear. One method to investigate these changes may be quantitative secretomics via mass spectrometry as has been conducted with mouse astrocytes (Dowell et al., 2009; Jha et al., 2012). Simplified coculture studies between differing cell types may lead to discovery of drug targets to inhibit or activate these specific signaling pathways, as has been conducted in the case of a thrombospondin receptor (Eroglu et al., 2009).

Ultimately, cell–cell interactions should be studied in a more natural environment for proper cell–cell communication to limit astrogliosis and provide the human astrocytes with extracellular matrix factors (**Figure 1**). One potential experimental model system includes hippocampal slice cultures, which have been previously used from human epilepsy patient tissue to measure functional changes in astrocytes (Hinterkeuser et al., 2000). Since live human brain tissue is rarely available for other neurological

Table 1 | Atlas for using hPSC-astrocytes in normal and disease states.

Stages	Challenges	Recommendations
hPSCs	Heterogeneity between lines	Use similar source material (cell type, age of donor, passage number, etc.) Use non-integrative reprogramming Generate control lines with pharmacological or genetic correction technology
Neuroepithelia/NSCs	Regional heterogeneity	Generate/select for CNS-specific cells instead of PNS neural crest Specify to distinct dorsal-ventral and anterior-posterior axis if needed
Astrocyte progenitors	Stressful culture environment	Use serum free conditions plus additives such as antioxidants Lower oxygen conditions, avoid acidity Limit stress during passaging
	Non-synchronous mixed culture	Cell sort if purification is desired Mature long term with factors such as CNTF to induce GFAP Remove adherent non-neural cells via astrosphere culture method (Krencik and Zhang, 2011)
Mature astrocytes	Identification of mature vs. immature	
	Identification of mature vs. reactive	Not yet well-defined. Conduct quantitative measurement of GFAP and other markers over time
	Functional characterization	Not yet well-defined Recommended assays include proliferation, synaptogenic studies with neuron cocultures, receptor/transporter stimulation followed by electrophysiological measurements or calcium imaging (Krencik et al., 2011), BBB formation/maintenance of endothelia
Engrafted astrocytes	Test for functional integration	Measure: Glutamate uptake after synaptic stimulation Calcium wave propagation between endogenous and engrafted human astrocytes Endfeet formation on blood vessels, constriction assay after stimulation Rescue a mouse disease model
Diseased states	Determine disease-specific phenotype	Assay for known disease-related phenotype in monoculture, coculture, or post-engraftment Profile at various levels for reactive signature
	Rescue the phenotype	Screen pharmacological or genetic (siRNA, etc.) methods

diseases, it would be more feasible to inject or overlay hPSC-derived astrocytes into rodent slice cultures to allow functional integration followed by characterization with electrophysiological recordings, as has been conducted with mouse stem cell-derived glial progenitors (Scheffler et al., 2003; Husseini et al., 2008). For example, this coculture system has been utilized with hPSC-derived neurons in order to measure their resultant neuronal orientation and differentiation over time (Shi et al., 2012). Better yet, cells can be directly transplanted into a live animal for long term functional integration as has been previously conducted (Weick et al., 2011). What functional measurements would be informative after engraftment of human astrocytes into the rodent nervous system? Potential measurements could include the response of diseased astrocytes to synaptic activity via glutamate uptake measurements (Bergles and Jahr, 1998) or while calcium imaging during different experimental paradigms (Duffy and MacVicar, 1995; Torres et al., 2012), interactions with brain vasculature (Mulligan and MacVicar, 2004; Krencik et al., 2011), or the response of adjacent neurons after astrocyte stimulation with optogenetic tools (Figueiredo et al., 2011; Sasaki et al., 2012). Besides investigating questions about diseases, this system could also be used for regenerative medicine. For example, human astrocytes may be used as neuroprotective tools by transplanting them into a diseased system (Lepore et al., 2008) or as vehicles to deliver neurotrophic compounds (Drinkut et al., 2012).

CONCLUSIONS

Astrocyte differentiation includes most of the same advantages and drawbacks that exist when generating heterogenous neural and non-neural cell types from hPSCs, although as described above they have additional technical challenges that include an extensive developmental timeline, limited cell specific tools, and sensitivity to stressful stimulations that leads to reactive astrogliosis. By summarizing these challenges and the best techniques with which to meet them (**Table 1**), this review can be used as an atlas to accordingly plan studies to produce the best model system possible, while keeping efforts of the researchers at a minimum. With careful preparation, functional analysis of astrocytes during disease studies can be conducted with high standards to account for cellular and temporal heterogeneity, although understandably most studies cannot address all the issues listed above. Typically, neurodevelopmental diseases such as RASopathies and neurodegenerative diseases have been modeled using neuronal cell types, but the roles of astrocytes in other less studied pathologies may also be targeted such as neuropathic pain (Watkins and Maier, 2003), sleep, and memory (Ben Achour and Pascual, 2012). Future technologies including high throughput screens, robotic apparatuses, computer models, and improved data analysis programs will undoubtedly increase the scale and accuracy of data collection/interpretation. Together with improved techniques to image and monitor healthy and diseased human astrocytes in culture or engrafted into a donor nervous system, the potential for glial studies in regenerative medicine and future disease discoveries can reach to the stars.

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Multifunctional role of astrocytes as gatekeepers of neuronal energy supply

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Dynamic adjustments to neuronal energy supply in response to synaptic activity are critical for neuronal function. Glial cells known as astrocytes have processes that ensheath most central synapses and express G-protein-coupled neurotransmitter receptors and transporters that respond to neuronal activity. Astrocytes also release substrates for neuronal oxidative phosphorylation and have processes that terminate on the surface of brain arterioles and can influence vascular smooth muscle tone and local blood flow. Membrane receptor or transporter-mediated effects of glutamate represent a convergence point of astrocyte influence on neuronal bioenergetics. Astrocytic glutamate uptake drives glycolysis and subsequent shuttling of lactate from astrocytes to neurons for oxidative metabolism. Astrocytes also convert synaptically reclaimed glutamate to glutamine, which is returned to neurons for glutamate salvage or oxidation. Finally, astrocytes store brain energy currency in the form of glycogen, which can be mobilized to produce lactate for neuronal oxidative phosphorylation in response to glutamatergic neurotransmission. These mechanisms couple synaptically driven astrocytic responses to glutamate with release of energy substrates back to neurons to match demand with supply. In addition, astrocytes directly influence the tone of penetrating brain arterioles in response to glutamatergic neurotransmission, coordinating dynamic regulation of local blood flow. We will describe the role of astrocytes in neurometabolic and neurovascular coupling in detail and discuss, in turn, how astrocyte dysfunction may contribute to neuronal bioenergetic deficit and neurodegeneration. Understanding the role of astrocytes as a hub for neurometabolic and neurovascular coupling mechanisms is a critical underpinning for therapeutic development in a broad range of neurodegenerative disorders characterized by chronic generalized brain ischemia and brain microvascular dysfunction.

Keywords: astrocytes, brain oxidative metabolism, glutamate-glutamine shuttle, neurovascular coupling, Alzheimer's disease, ischemia, epilepsy

INTRODUCTION

The brain receives 10% of cardiac output but consumes 20% of total blood glucose and oxygen during cerebral activity to restore ion gradients after action potential conduction and neurotransmission (Magistretti et al., 1999; Magistretti, 2006). Large metabolic demand requires that brain blood flow remain constant despite variations in blood pressure (autoregulation) and that areas of high neuronal activity have correspondingly high metabolic rate and local blood supply (Magistretti, 2006). Astrocytes are multi-functional regulators of neurometabolic coupling that control uptake and release of neurotransmitters (Anderson and Swanson, 2000), influence local blood supply (Zonta et al., 2003; Mulligan and Macvicar, 2004; Takano et al., 2006; Gordon et al., 2008), and directly supply neurons with substrates for oxidative phosphorylation (Pellerin et al., 1998a).

Several characteristics of astrocytes confer suitability for sensing and satisfying neuronal metabolic needs. Protoplasmic astrocytes are highly organized into nearly unique three-dimensional domains (Oberheim et al., 2006) with limited overlap (Ogata and Kosaka, 2002). This feature places astrocytes non-randomly in virtually all central nervous system (CNS) 3D space, which is an ideal anatomical scenario for cells engaging in regional brain activity monitoring and/or corresponding nutritive distribution. Astrocyte process extensions from the soma define domain extremities and extensively ensheath central synapses (Ventura and Harris, 1999) producing a synaptic structure referred to as the "tripartite synapse" (Araque et al., 1999; Oberheim et al., 2006), in which astrocyte processes are located in close enough proximity to communicating nerve terminals that they receive neurotransmitter input. Astrocyte processes also envelop parenchymal brain arterioles and capillaries in unique spatial domains, extending terminal structures known as endfeet that are directly in contact with the vascular basal lamina (Simard et al., 2003; Oberheim et al., 2006). Endfeet express surface proteins, such as glucose transporters, for uptake of energy substrates from the endothelium (Kacem et al., 1998), and are capable of releasing transmitters that influence local blood flow (Simard et al., 2003; Zonta et al., 2003; Mulligan and Macvicar, 2004; Metea and Newman, 2006;

Gordon et al., 2008). Astrocytes are therefore also uniquely positioned for bidirectional communication across the blood-brain barrier, as well as being participants in synaptic transmission. In addition, single hippocampal or cortical astrocytes are in contact with up to 600 dendrites (Halassa et al., 2007) and over 100,000 synapses (Bushong et al., 2002), and extend multiple processes to blood vessels (McCaslin et al., 2011). This provides a single-cell linkage between the locus of neuronal activity and sites that can leverage additional energy supply in an arrangement known as the neurovascular unit. This anatomy provides an astrocyte-mediated communication link for energy substrate transfer between blood supply and synaptic terminals (Tsacopoulos and Magistretti, 1996; Simard et al., 2003; Rouach et al., 2008).

Protoplasmic astrocytes also form a functional syncytium, where distal processes are connected by connexin gap junctions permitting diffusion of ions and metabolites between neighboring astrocytes (Giaume and McCarthy, 1996; Scemes et al., 1998). This creates a conduit for intercellular communication and flow of metabolites, but also allows intracellular communication through autocellular junctions between processes of the same cell (Wolff et al., 1998; Rouach et al., 2002). Connexin proteins also form hemichannels, which do not connect to adjacent cells, but allow release of small molecules from the cytoplasm into the extracellular space (Contreras et al., 2002; Rouach et al., 2002; Ye et al., 2003). This network of gap junctions is central to astrocyte function and control of brain metabolism, facilitating communication and movement of molecules within and around astrocyte domains.

Peri-synaptic or vascular astrocyte distributions would not be functionally relevant without mechanisms for receiving input. Astrocytes achieve this by expressing numerous types of neurotransmitter receptors that initiate electrically silent activation of astrocytes by enhancing intracellular Ca^{2+} levels. These broad receptor categories are coupled to G-proteins and activate a wide array of intracellular second messenger pathways, including inositol trisphosphate production and release of Ca^{2+} into the cytoplasm from endoplasmic reticulum stores (Sheppard et al., 1997; Idestrup and Salter, 1998). This permits astrocytes to respond to synaptic transmission through elevated cytosolic Ca^{2+}. Astrocyte GPCR receptors involved in Ca^{2+} signaling cover a diverse range of neurotransmitters such as $GABA_B$ receptors (Kang et al., 1998; Bettler et al., 2004; Meier et al., 2008), acetylcholine muscarinic receptors (Takata et al., 2011; Navarrete et al., 2012), α-adrenergic receptors (Duffy and Macvicar, 1995; Bekar et al., 2008), H1 histamine receptors (Shelton and McCarthy, 2000), endocannabinoid receptors (Navarrete and Araque, 2008, 2010), purinergic P2Y receptors binding adenine nucleotides (Guthrie et al., 1999), and metabotropic glutamate receptors (mGluRs) (Porter and McCarthy, 1996; Perea and Araque, 2007). Many papers implicate mGluR5 as a major activator of astrocyte Ca^{2+} (Bezzi et al., 1998; Zonta et al., 2003; Takano et al., 2006; Gordon et al., 2008; Liu et al., 2011), however, there is recent work suggesting mGluR5 expression decreases with age and does not stimulate Ca^{2+} signals in adult cortical and hippocampal astrocytes (Sun et al., 2013). More work on this is required before a consensus can be reached.

Ca^{2+} elevations may represent the fulcrum of a multi-faceted repertoire of potential astrocyte responses to sensory input. There is broad consensus that increased astrocytic intracellular Ca^{2+} triggers release of gliotransmitters such as glutamate, ATP, and D-serine (Bezzi et al., 2004; Mothet et al., 2005; Jourdain et al., 2007). Gliotransmitters, in turn, can affect synaptic activity (Parpura et al., 1994; Araque et al., 1999; Panatier et al., 2006; Henneberger et al., 2010; Sasaki et al., 2011; Fossat et al., 2012), produce constriction or dilation of local blood supply vessels (Zonta et al., 2003; Mulligan and Macvicar, 2004; Takano et al., 2006; Gordon et al., 2008) or have an autocrine effect to amplify Ca^{2+} signals (Suadicani et al., 2006). Additionally, elevation of Ca^{2+} in a single astrocyte is capable of initiating a similar response in surrounding astrocytes in a regenerative wave-like fashion. This process is primarily dependent on connexin 43 (Scemes et al., 1998; Blomstrand et al., 1999; Haas et al., 2006; Gosejacob et al., 2011) and release of extracellular gliotransmitters, including ATP (Hassinger et al., 1996; Guthrie et al., 1999) and may mediate fast, long-distance intercellular communication between astrocytes (Scemes and Giaume, 2006). It is important to note that recent data challenge the view that astrocyte Ca^{2+} modulates neuronal activity (Petravicz et al., 2008; Agulhon et al., 2010; Nedergaard and Verkhratsky, 2012) or even that adult astrocytes express Ca^{2+}-mobilizing metabotropic glutamate receptors shown previously to be critical for synaptic effects of astrocytes (Sun et al., 2013). These findings are fueling debate about the functional roles of astrocytic Ca^{2+} responses in adult animals *in vivo*. Finer spatial resolution of astrocytic Ca^{2+} levels may reveal that local responses are limited to process microdomains and not necessarily the cell soma (Shigetomi et al., 2010, 2012; Di Castro et al., 2011), which could partially explain apparent discrepancies. Regional differences in astrocytic physiology and developmental changes in astrocytic expression of neurotransmitter receptors may also be factors. Systematic attention to animal age, brain regions imaged and spatial resolution of astrocyte Ca^{2+} imaging *in vivo* will greatly help resolve these issues.

Architectural organization, neurotransmitter receptor expression, and gliotransmitter release are features enabling astrocytes to be prime regulators of synaptic environment and transmission (Araque et al., 1999; Anderson and Swanson, 2000; Henneberger and Rusakov, 2010), neurovascular coupling (Zonta et al., 2003; Mulligan and Macvicar, 2004; Takano et al., 2006; Gordon et al., 2008), blood-brain barrier function (Ballabh et al., 2004) and carbon source shuttling to neurons in high demand periods (Pellerin et al., 1998a; Rouach et al., 2008). We will discuss the influence of astrocytes on the synaptic environment and cerebral bioenergetics, including how astrocytes handle glutamate, supply neurons with oxidative energy substrates and store glycogen. Mechanisms by which astrocytes couple glutamatergic neurotransmission with neuronal energy metabolism and blood flow regulation will also be discussed. Finally, we will survey astrocyte dysfunction in brain diseases and injuries, including ischemic stroke, epilepsy, and Alzheimer's Disease.

ASTROCYTES CONTROL CEREBRAL GLUTAMATE LEVELS

Glutamate is quantitatively the dominant excitatory CNS neurotransmitter (Fonnum, 1984). Unregulated synaptic glutamate levels, however, can cause neuronal excitatory cell death in multiple diseases (Dong et al., 2009). Therefore, regulation of

synaptic glutamate is crucial. Under normal conditions, glutamate balance in the neuropil is tightly controlled by astrocytes. Astrocytic processes enveloping glutamatergic synapses express active amino acid transport proteins that are the main route of extracellular glutamate removal (Rothstein et al., 1994; Danbolt, 2001). The primary glutamate transporters are Na^+/glutamate co-transporters of the SLC gene family, termed excitatory amino acid transporter 1 and 2 (EAAT1 and 2) in human tissue (Shashidharan et al., 1994) or glutamate transporter-1 (GLT-1) and L-glutamate/L-aspartate transporter (GLAST) in rodents (Pines et al., 1992; Storck et al., 1992). These proteins rely on the Na^+ electrochemical gradient, maintained by Na^+/K^+ ATPase activity, to co-transport 1 glutamate molecule and 3 Na^+ ions. Glutamate uptake is energetically expensive, as ATP is consumed by Na^+/K^+ ATPases, but with sufficient energy supply, perisynaptic astrocyte processes prevent excitotoxic accumulation of glutamate in the neuropil. A Na^+-independent, glutamate/cystine antiporter is also expressed by astrocytes but this is considered a secondary mechanism of glutamate uptake as these transporters primarily conduct cystine (Cho and Bannai, 1990).

Neurons may also take up glutamate through EAAT3 (EAAC1 in rodents) (He et al., 2000; Chen and Swanson, 2003), EAAT4 (Furuta et al., 1997; Nagao et al., 1997; Jackson et al., 2001), or EAAT5 (Arriza et al., 1997); however, the expression and localization of these transporters vary throughout the brain. For example, EAAT5 is mainly located in the retina (Arriza et al., 1997) and EAAC1 and EAAT4 are found on extrasynaptic neuronal membranes, particularly in the cerebellum, and are believed to modulate glutamate release and post-synaptic excitation (Tong and Jahr, 1994; Overstreet et al., 1999). EAAT3 also readily takes up cysteine (Chen and Swanson, 2003), which is used for glutathione production, suggesting EAAT3 has a central role in neuronal antioxidant defense (Aoyama et al., 2006).

Once synaptic glutamate enters astrocytes, one-third is used as a substrate for oxidative metabolism (Schousboe et al., 1993; Hertz and Zielke, 2004; Hertz et al., 2007). Glutamate can be converted to α-ketoglutarate by glutamate dehydrogenase or aspartate aminotransferase to replenish components of the tricarboxylic acid (TCA) cycle (Faff-Michalak and Albrecht, 1993; McKenna et al., 2006a). An additional portion of salvaged glutamate is recycled for neurotransmission through a process known as the glutamate-glutamine shuttle (**Figure 1**). Glutamate is converted to glutamine by astrocytic glutamine synthase (Martinez-Hernandez et al., 1977). Glutamine is then transported from the astrocytic cytoplasm by system N transporters and removed from the extracellular space by neuronal system A neutral amino acid transporters (Chaudhry et al., 2002). Neuronal glutamine is converted back to glutamate by phosphate-activated glutaminase (Kvamme et al., 2000) and repackaged into vesicles (Fremeau et al., 2004) for synaptic release (McKenna, 2007). This shuttle process is vital for proper synaptic glutamate release because neurons do not express enzymes for *de novo* synthesis of glutamate, so neuronal glutamate is entirely derived from astrocyte glutamine or α-ketoglutarate (Yu et al., 1983; Shank et al., 1985). Astrocytes produce *de novo* glutamate or glutamine from glucose via pyruvate conversion to oxaloacetate by pyruvate carboxylase (Yu et al., 1983; Hertz, 2011).

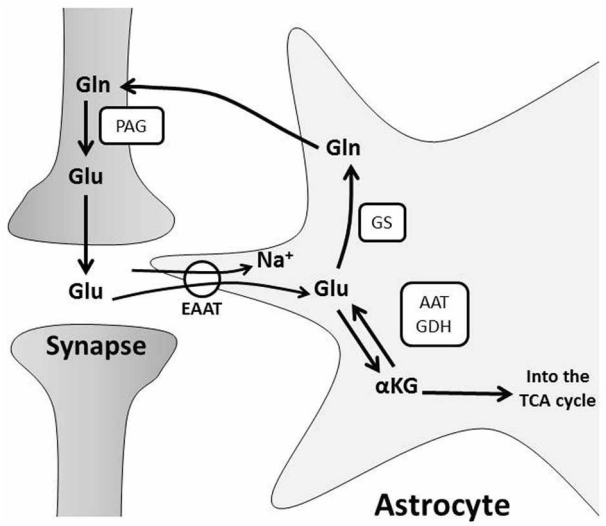

FIGURE 1 | The glutamate-glutamine cycle. Glutamate (Glu) from pre-synaptic neurons stimulates post-synaptic neurons, and the signal is terminated by uptake of Glu from the synaptic cleft into astrocytes. Glu is primarily transported into astrocytes through Na^+-dependent excitatory amino acid transporters, EAATs. This disrupts the astrocyte Na^+ gradient and energy is consumed by the Na^+/K^+ ATPase to restore ionic concentrations. Glu is converted to: (a) glutamine (Gln) via glutamine synthase (GS) or (b) alpha-ketoglutarate (α-KG) by glutamate dehydrogenase (GDH) or aspartate aminotransferase (AAT) for subsequent oxidative metabolism in the TCA cycle. Gln is shuttled to neurons for glutamate production by phosphate-activated glutaminase (PAG) and the resulting Glu is repackaged in vesicles for further synaptic release.

The glutamate-glutamine cycle not only drives neurotransmitter recycling, but also influences brain metabolism. Astrocytes metabolize glutamate to TCA cycle intermediates (Schousboe et al., 1993; Hertz and Zielke, 2004; Hertz et al., 2007), which diminishes the glutamate pool, and may drive astrocytic glucose consumption, ATP production and *de novo* glutamate synthesis (Hertz, 2011). Neurons also utilize glutamine and/or glutamate as energy substrates during glucose deprivation *in vitro* (Peng et al., 2007) or ischemia *in vivo* (Pascual et al., 1998). They similarly use glutamine or glutamate to replenish intermediates of the TCA cycle during metabolism of other substrates *in vitro* (Shokati et al., 2005). These observations suggest that the glutamate-glutamine shuttle impacts neuronal metabolism. Glutamate uptake by cultured astrocytes also correlates with increased glycolysis and lactate production (Pellerin and Magistretti, 1994). This is a separate mechanism of glutamate-driven astrocyte-neuron metabolic coupling that will be discussed below.

ASTROCYTE LACTATE FUELS NEURONAL METABOLISM

Synaptic glutamate is a direct signal of neuronal activity and, therefore, of metabolic demand. Astrocytes surveying synaptic activity respond with elevated glucose utilization, glycolysis (Pellerin and Magistretti, 1994; Cholet et al., 2001), and lactate production (Pellerin and Magistretti, 1994; Schurr et al., 1999; Voutsinos-Porche et al., 2003; Caesar et al., 2008). Enhanced astrocytic metabolism is thought to result from intracellular

Na^+ accumulation associated with Na^+/glutamate co-transport (Voutsinos-Porche et al., 2003; Langer and Rose, 2009). This elevates ATP consumption by Na^+/K^+ ATPase activity resulting in increased glucose uptake, enhanced glycolytic rate, and lactate generation (Pellerin and Magistretti, 1994; Chatton et al., 2000; Loaiza et al., 2003; Porras et al., 2008). Intercellular Na^+ waves are also generated throughout the astrocyte syncytium, elevating glucose uptake, and metabolism in neighboring astrocytes as well (Bernardinelli et al., 2004; Scemes and Giaume, 2006). Furthermore, K^+ released during neurotransmission is taken up by astrocytes, which stimulates glycolysis and lactate export (Bittner et al., 2011; Ruminot et al., 2011).

Glutamatergic neurotransmission increases both neuronal and astrocytic energy consumption, but the primary neuronal energetic substrate during normal and pathological conditions has been debated. One hypothesis is that neurons and astrocytes utilize systemically delivered glucose and oxygen from the extracellular space for metabolism by oxidative phosphorylation (Chih and Roberts, 2003). The second hypothesis proposes astrocytes convert glucose to lactate in an activity-dependent, glutamate-mediated manner for delivery to neurons (Pellerin and Magistretti, 1994; Pellerin et al., 1998a; Magistretti and Pellerin, 1999). This is known as the astrocyte-neuron lactate shuttle hypothesis (ANLSH) and suggests lactate is more than a potentially damaging final metabolite of anaerobic glycolysis (**Figure 2**; Kasischke, 2008).

In light of the ANLSH, a large body of literature pertaining to production and neuronal use of lactate has accumulated over the last 20 years. Several points have been made. First, there is a correlation between synaptic activity and extracellular lactate concentrations. At rest, the extracellular space around neurons and astrocytes has a homogenous concentration of lactate and glucose (Simpson et al., 2007; Barros and Deitmer, 2010). Extracellular lactate decreases slightly during short periods of brain activation *in vivo* (Hu and Wilson, 1997; Mangia et al., 2003), possibly because neurons are utilizing lactate for oxidative metabolism (Kasischke et al., 2004). However, extracellular lactate rapidly rises as neuronal stimulation continues for longer periods (Prichard et al., 1991; Mangia et al., 2007). Oxygen levels remain unchanged, suggesting brain activation stimulates aerobic glycolysis (Hu and Wilson, 1997). Second, *in vitro* studies demonstrate that glutamate induces glucose transporter (GLUT1) activity and uptake rates in astrocytes (Loaiza et al., 2003), while inhibiting neuronal glucose transporter activity (Porras et al., 2004). This suggests glutamatergic transmission may increase astrocyte glucose availability and reduce neuronal glucose metabolism. Third, lactate can support neuronal survival. In rodent brain slices, inhibition of lactate transport and glycolysis during exposure to glutamate caused a permanent loss of neuronal function (Schurr et al., 1999), while addition of lactate maintained synaptic activity in the absence of glucose (Schurr et al., 1988; Fowler, 1993; Izumi et al., 1997), preventing neurotoxicity (Schurr et al., 1997; Maus et al., 1999; Cater et al., 2001). Fourth, neurons express protein machinery necessary for lactate metabolism. Lactate metabolism is mediated by lactate dehydrogenase (LDH), which reversibly converts pyruvate to lactate with oxidation of NADH to NAD^+ (Tsacopoulos and Magistretti, 1996). Several different LDH isoforms are located in the brain; LDH1 is the main isoform in neurons, while LDH1 and LDH5 are found in astrocytes (Bittar et al., 1996; Tsacopoulos and Magistretti, 1996). Lactate *consumption* is favored by neuronal LDH1, which promotes conversion of lactate to pyruvate (Bittar et al., 1996). In contrast, there is evidence that astrocytes favor *production* of lactate (Walz and Mukerji, 1988; Peng et al., 1994), likely due to the properties of LDH5, which has a higher affinity for pyruvate than lactate (Bittar et al., 1996). Lactate is transported between the intracellular and extracellular spaces by monocarboxylate transporters (MCT). MCT are symporters that co-transport lactate anions with H^+, suggesting lactate transport is driven by pH (Schneider et al., 1993; Barros and Deitmer, 2010). The distribution of MCTs in the brain is heterogeneous: MCT1, MCT2, and MCT4 are expressed by astrocytes, while neurons express predominately MCT2 (Broer et al., 1997; Gerhart et al., 1998; Pellerin et al., 1998b; Bergersen et al., 2001; Pierre et al., 2002). MCT2 co-localizes with post-synaptic density proteins in dendritic spines and has the highest affinity for lactate of all MCTs (Bergersen et al., 2001). Together, MCT2 and LDH1 provide neurons with lactate protein machinery ideally suited to remove and metabolize lactate from active synapses. Lastly, a recent study found that an important activator of glycolysis, 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase isoform 3 (Pfkfb3), is continually degraded in neurons (Herrero-Mendez et al., 2009). This suggests glucose metabolism is shifted toward the pentose phosphate pathway and antioxidant production, and that neurons have a low glycolytic rate, necessitating utilization of lactate for aerobic respiration.

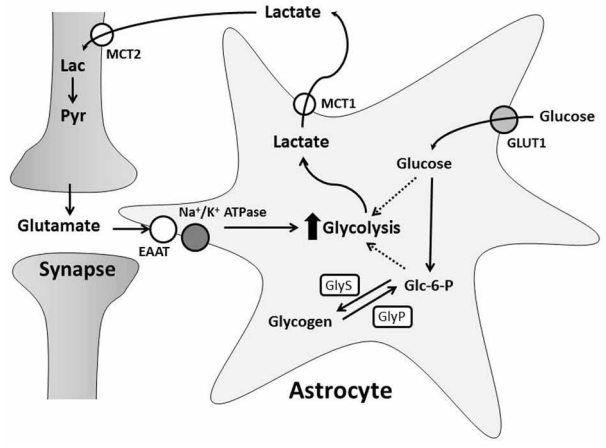

FIGURE 2 | The Astrocyte to Neuron Lactate Shuttle Hypothesis. Free glucose is taken up by astrocytes through GLUT1 transporters and converted to glucose-6-phosphate (Glc-6-P). Glc-6-P is stored as glycogen synthesized by glycogen synthase (GlyS). During greater energy demand, glycogenolysis, mediated by glycogen phosphorylase (GlyP), creates Glc-6-P for glycolysis. Synaptic transmission induces astrocyte glycolysis and lactate production through glutamate uptake. This increases glucose consumption and/or glycogen breakdown in astrocytes. Astrocyte lactate is transported into the extracellular space by MCT1 and taken up through MCT2 by neurons. Neurons can convert lactate (Lac) to pyruvate (Pyr) for oxidative phosphorylation.

There is also a correlation between changes in neuronal and astrocytic redox states and lactate transport and metabolism that may support the ANLSH (Hirrlinger and Dringen, 2010). During glycolysis, cytosolic NADH is produced and must be oxidized back to NAD^+ in order for glycolysis to continue. NAD^+ is replenished by lactate production or redox shuttle systems (glycerol-3-phosphate and malate-aspartate shuttle, MAS) which transfer reducing equivalents to the mitochondrial electron transport complexes. While the level of involvement of the glycerol-3-phoshpate shuttle in neuronal NAD^+ homeostasis is not clear (Cammer and Zimmerman, 1982; Waagepetersen et al., 2001; Nguyen et al., 2003), the MAS is important for regenerating NAD^+ for glutamate neurotransmitter renewal and energy metabolism (Palaiologos et al., 1988; McKenna et al., 2006b). Inhibition of the MAS-elevated cytosolic NADH, disrupting the redox balance and limiting lactate consumption (McKenna et al., 2006b) without affecting glucose metabolism in synaptic terminals (McKenna et al., 1993). Moreover, disruption of the MAS in malate-aspartate carrier (aralar) deletion mice resulted in impaired neuronal development (Gomez-Galan et al., 2012), reduced dopamine levels (Llorente-Folch et al., 2013), and hypomyelination (Ramos et al., 2011), indicating this pathway affects neuronal function in a profound way. There is no clear evidence that this is related to lactate metabolism, however.

The role of the malate-aspartate shuttle in astrocytes is currently debated. Several groups show that astrocytes express low levels of aralar (Ramos et al., 2003; Berkich et al., 2007), which limits MAS activity and requires elevated lactate production to replenish cytosolic NAD^+ (Schurr, 2006; Lemire et al., 2008). In support, astrocyte lactate-to-pyruvate ratios were unchanged in aralar knockout mice, compared to wild type controls (Pardo et al., 2011). In contrast, a recent paper suggests adult cultured astrocytes express aralar and the MAS could be functional (Li et al., 2012). This makes the importance of MAS in astrocytes difficult to determine at this point. Nevertheless, there is a clear correlation between cytosolic redox states and lactate production in astrocytes. In cultured astrocytes, inhibition of oxidative phosphorylation (which elevates cytosolic NADH) increases lactate production and regenerates NAD^+ (Dringen et al., 1993). High levels of NADH also influence transcription factors, including Clock and NPAS2, which activate LDH1 expression in astrocytes (Rutter et al., 2001), further potentiating lactate production. Again, extracellular lactate increases during longer periods of neuronal stimulation ($\sim$10 s), and a corresponding elevation of astrocytic cytosolic NADH concentrations is also observed (Kasischke et al., 2004). This means astrocytes may replenish extracellular lactate pools for shuttling to neurons during prolonged activation (Pellerin et al., 1998a; Magistretti and Pellerin, 1999; Magistretti et al., 1999; Bouzier-Sore et al., 2002).

Mathematical modeling has been used to approximate the flux of energy metabolites between neurons and astrocytes based on known mass balances and enzyme/transporter kinetics, with the goal of linking *in vitro, in vivo,* and functional imaging results. Several models have recently been presented, but with varying results. One model describes energy substrates (lactate, glucose, pyruvate), oxygen, and NADH concentrations within the neuronal and astrocyte energy compartments, while also considering the subcellular compartments (cytosol and mitochondria) (Aubert et al., 2007), glutamate transport, and astrocyte glycogen (Cloutier et al., 2009). Results from this model support the ANLSH (Aubert et al., 2007; Cloutier et al., 2009) and the flow of lactate from astrocytes to neurons. The second model focuses on glucose and lactate transport between the blood-brain barrier, neurons, and astrocytes and suggests that neurons primarily metabolize glucose and export lactate (Simpson et al., 2007; Mangia et al., 2009). This supports a neuron to astrocyte lactate shuttle hypothesis (NALSH) (Simpson et al., 2007; Mangia et al., 2009). A third model attempts to combine metabolism rates and concentrations from the first model with transporter kinetics and metabolite diffusion equations from the second model and the results also support a neuron to astrocyte lactate shuttle (Dinuzzo et al., 2010). While the outcomes and design of these mathematical models continue to be debated, each model succeeds in raising questions to be addressed by future experiments. Most notably, there is evidence that neurons can utilize lactate as an energy source during periods of activation, but the question remains: do astrocytes produce lactate for neuronal consumption? Clearly, neurons and astrocytes produce and utilize lactate differently based on the expression profiles and properties of LDH and MCT isoforms, but due to experimental limitations of lactate detection, it is not possible to distinguish lactate producers from the cell type that utilizes lactate, or if these roles change depending on region or activity (Barros and Deitmer, 2010). Measurement of radiotracer kinetics *in vivo* suggest neurons consume lactate during activation (Wyss et al., 2011), and further *in vivo* studies may elucidate the complex flux of brain metabolites. In particular, experiments involving awake animals may more accurately reflect brain metabolic states, as anesthetics are known to decrease metabolic rates (Alkire et al., 1995, 1997, 1999). It would also be beneficial to directly visualize *in vivo* glucose and lactate levels (possibly via fluorescent sensors for glucose or lactate) to determine metabolite concentrations in different cell populations in various brain regions during activation (Barros et al., 2013; San Martin et al., 2013).

Astrocyte lactate is not only a potential energy substrate, but also acts as a signaling molecule in other brain bioenergetic processes, including blood flow regulation (discussed in detail later) (Gordon et al., 2008), blood glucose sensing (Lam et al., 2005, 2007), and sodium sensing in the subfornical organ (SFO; Shimizu et al., 2007). Brain lactate is involved in a brain-liver signaling axis. Hypothalamic arcuate nuclei projections to the brainstem signal to vagal hepatic efferents (Schwartz et al., 2000; Grill et al., 2002) to regulate blood glucose levels (Lam et al., 2005) and insulin signaling (Pocai et al., 2005). Elevated blood glucose leads to increased glial glucose uptake (Chari et al., 2011) and lactate production in the rodent hypothalamus (Lam et al., 2005). Lactate is transported into hypothalamic neurons for conversion to pyruvate. This process is required to activate neuronal ATP-sensitive K^+ channels (K_{ATP}) (Lam et al., 2005), and K^+ flux that induces hyperpolarization and reduces firing (Pocai et al., 2005). Resulting hepatic vagal stimulation (Pocai et al., 2005) reduces gluconeogenesis and glycogenolysis rates (Lam et al., 2005; Pocai et al., 2005), leading to secretion of very-low density lipoprotein (Lam et al., 2007) and reduced expression

of hepatic enzymes for endogenous glucose production, including glucose-6-phosphatase (Lam et al., 2005; Pocai et al., 2005; Kishore et al., 2011). This provides a lactate-mediated brain-liver negative feedback axis (Lam et al., 2007), which has implications in obesity and hepatic insulin resistance. In particular, hypothalamic glial GLUT1 expression and glucose uptake are decreased during hyperglycemia in rodents *in vivo*, and this could form the basis of blood glucose dysregulation in diabetes (Chari et al., 2011). Also, intracerebroventricular injection of lactate decreased blood glucose levels in animal models of uncontrolled diabetes and diet-induced insulin resistance, independent of insulin signaling (Chari et al., 2008), which suggests that hypothalamic lactate could be a future therapeutic target.

In the SFO of the brain periventricular region, lactate influences salt intake behavior and blood Na^+ sensing (Shimizu et al., 2007). Glial cells of the SFO express atypical sodium (Na_x) channels (Hiyama et al., 2004), which have a concentration-sensitive, extracellular sodium threshold of 150 mM (Hiyama et al., 2002). SFO glial Na_x channels interact with Na^+/K^+ ATPase and progressive Na^+ influx upon elevated extracellular Na^+ triggers anaerobic glucose metabolism and lactate production (Shimizu et al., 2007). Lactate and Na_x channels mediate salt-intake behavior, since Na_x-knockout mice continue to ingest salt when dehydrated (Hiyama et al., 2004) and they have reduced SFO lactate concentrations compared to wild type animals (Shimizu et al., 2007). Salt-intake behavior is reduced when glial lactate stimulates inhibitory neurons in the SFO by a MCT-dependent mechanism (Shimizu et al., 2007). This mechanism may also involve inhibition of K_{ATP} channels by lactate-induced ATP production (Shimizu et al., 2007); however, further experiments are required to determine the involvement of these channels in the pathway. These studies of the role of lactate in glucose and sodium sensing and food intake behaviors indicate an exciting new role for lactate as a signaling molecule to neurons and suggest the importance of lactate in the brain may be underestimated.

ASTROCYTE GLYCOGEN PRODUCTION FUELS NEURONAL METABOLISM

Glycogen is the main cellular storage depot of glucose in mammals (Brown and Ransom, 2007). When glucose is in excess of immediate energy requirements, it can be stored as glycogen; glycogen is mobilized to glucose when glucose levels cannot meet energy demands (Brown and Ransom, 2007). Astrocytes are the main glycogen repository in the adult brain (Phelps, 1972; Koizumi, 1974). Astrocytes express both glycogen synthase (GlyS, for glycogen formation) and glycogen phosphorylase (GlyP, for glycogen degradation) (Pellegri et al., 1996) and glycogen stores are primarily located in regions of high synaptic density, such as gray matter (Phelps, 1972; Sagar et al., 1987). Astrocyte glycogen is critical for maintaining neuronal survival and synaptic activity during hypoglycemia *in vitro* (Swanson and Choi, 1993) and *in vivo* in cortex, hippocampus (Suh et al., 2007) and optic nerve (Wender et al., 2000). Similarly, during periods of increased brain activity and local glucose depletion, astrocyte glycogen stores can be rapidly degraded to provide a temporary energy supply (Shulman et al., 2001; Brown et al., 2003, 2005).

Glycogen cycling occurs when astrocytes acquire glucose through the glucose transporter, GLUT1, and rapidly phosphorylate it to glucose 6-phosphate in the first steps of glycolysis, preventing it from leaving the cell (Vannucci et al., 1997). Glucose-6-phosphate can be converted to glycogen through a process catalyzed by GlyS (**Figure 2**). GlyS exists in both an inactive phosphorylated form and an active dephosphorylated form. Astrocyte glycogen formation is therefore regulated by enzymes that dephosphorylate and activate GlyS, most notably protein phosphatase 1 which acts via the regulatory subunit Protein Targeting to Glycogen (PTG) (Allaman et al., 2000). Expression of PTG is stimulated by numerous molecules such as vasoactive intestinal peptide, norepinephrine, and adenosine, which increase glycogen production (Sorg and Magistretti, 1992; Allaman et al., 2000). Similarly, GlyP can be regulated by phosphorylase kinase, which converts GlyP from its inactive form to its active, phosphorylated form (Brown and Ransom, 2007). GlyP is only expressed in astrocytes, solidifying the specialization of these cells in glycogen utilization. Glycogenolysis results in glucose-6-phosphate, which can be metabolized within astrocytes to lactate (Dringen and Hamprecht, 1993; Tekkok et al., 2005) or free glucose (Ghosh et al., 2005). This suggests astrocyte glycogen-derived substrates can be supplied to other brain cells for oxidative metabolism.

The astrocytic glycogen reservoir is dynamic under normal brain activity and euglycemic conditions (Brown et al., 2005), and is influenced by glutamatergic neurotransmission and uptake. Glutamate triggers glycogenolysis to meet the energy demand of the glutamate-glutamine cycle and Na^+ gradient restoration, in addition to the mechanisms proposed in the ANLSH (Shulman et al., 2001). Glycogenolysis fuels glutamate uptake by enhancing active transport-mediated recovery from the extracellular space, since inhibition of glycogenolysis-elevated extracellular glutamate concentrations (Sickmann et al., 2009; Schousboe et al., 2010). Glycogenolysis also facilitates *de novo* synthesis of glutamate and glutamine (Sickmann et al., 2005; Gibbs et al., 2006, 2007). Therefore, astrocyte glycogen is important for supporting the energetic needs of glutamatergic neurotransmission.

Recent studies have found glycogen-derived lactate is central to higher cognitive function and memory formation (Gibbs et al., 2006; Newman et al., 2011; Suzuki et al., 2011). In day old chicks, a bead discrimination learning task for memory consolidation was impaired after inhibition of glycogenolysis (Gibbs et al., 2006, 2007) or injection of poorly metabolized D-lactate (which competes with L-lactate for transport) (Gibbs and Hertz, 2008). An *in vivo* study of rats during an inhibitory avoidance test found learning-induced glycogenolysis and lactate release that was important for long-term memory formation (Suzuki et al., 2011). This was determined by administering inhibitors of glycogen phosphorylation or knocking down expression of MCT1/4 or MCT2, which induced amnesia. Inhibition of glycogen phosphorylation also reduced long-term potentiation (LTP), which was rescued by lactate injection (Suzuki et al., 2011). In another rat study during a spontaneous alternation task to assess spatial working short-term memory, lactate concentrations increased during the task and inhibition of glycogenolysis and lactate transport decreased task success (Newman et al., 2011). These results suggest astrocyte glycogenolysis and lactate transport to neurons

is required for working memory processing and long-term memory consolidation.

While debate over the primary neuronal energy source will likely continue, it is clear that there is situational activity-dependent regulation of neuronal metabolism by astrocytes involving glycogen cycling, lactate production, and the glutamate-glutamine shuttle. This metabolic coupling of astrocytes and neurons appears to be important for higher cognitive function.

ASTROCYTES MEDIATE VASOMOTOR RESPONSES BASED ON TISSUE ENERGY DEMAND

Neuronal activity is tightly coupled to increased local blood flow by neurovascular coupling in a response termed functional hyperemia. Neurovascular coupling is a complex, multi-modal response involving numerous identified signaling pathways and resulting in vasodilation of penetrating arterioles upstream of regions with enhanced of activity, and vasoconstriction in regions with abundant substrate supply and lower activity (Devor et al., 2007). The net effect of this response is to enhance glucose and oxygen delivery from blood to meet neuronal and glial energy demands.

Astrocytic spatial architecture permits relay of signals from synapses to penetrating arterioles and capillaries. As part of the multi-faceted response of astrocytes to increased neuronal activity, synaptic neurotransmission triggers elevated intracellular astrocyte Ca^{2+} through diverse receptor types including $GABA_B$ receptors (Kang et al., 1998; Bettler et al., 2004; Meier et al., 2008), acetylcholine muscarinic receptors (Takata et al., 2011; Navarrete et al., 2012), α-adrenergic receptors (Duffy and Macvicar, 1995; Bekar et al., 2008), H1 histamine receptors (Shelton and McCarthy, 2000), endocannabinoid receptors (Navarrete and Araque, 2008, 2010), $mGluR_5$ (Zonta et al., 2003), and P2Y receptors (Simard et al., 2003). Astrocyte cytosolic Ca^{2+} elevations (Simard et al., 2003; Zonta et al., 2003; Filosa et al., 2004; Schummers et al., 2008), and inositol-3-phosphate signaling (Straub et al., 2006) are central to neurovascular coupling, stimulating release of vasoactive compounds that dilate or constrict neighboring arterioles (Zonta et al., 2003; Mulligan and Macvicar, 2004; Metea and Newman, 2006; Takano et al., 2006; Gordon et al., 2008). The polarity (i.e., constriction vs. dilation) of these vascular responses involves multiple pathways, discussed in later sections.

ARACHIDONIC ACID METABOLITES

Elevated astrocyte cytosolic Ca^{2+} stimulates activity of phospholipase A_2 (PLA_2), which hydrolyzes phospholipids to produce arachidonic acid (AA) (Mulligan and Macvicar, 2004; Sun et al., 2005). AA metabolism by several enzymes produces different molecules with variable vascular effects (**Figure 3**). In brain slices and *in vivo*, a non-selective cyclooxygenase (COX) or COX-1 inhibitor blocked arteriolar vasodilation after astrocyte Ca^{2+} stimulations, suggesting AA is metabolized by astrocyte COX-1 to prostaglandin E_2 (PGE_2) (Zonta et al., 2003; Takano et al., 2006; Gordon et al., 2008). In cortical astrocytes and retinal glia, AA is also metabolized by cytochrome P450 epoxygenase to vasodilator, epoxyeicosatrienoic acids (EETs) (Peng et al., 2002; Metea and Newman, 2006; Liu et al., 2011). Both PGE_2 and EETs open smooth muscle large conductance Ca^{2+}-sensitive K^+ (BK_{Ca}) channels, triggering hyperpolarization and decreased voltage-gated calcium channel (VGCC) activity (Gebremedhin et al., 1992; Miura and Gutterman, 1998; Higashimori et al., 2010). EETs also indirectly stimulate BK_{Ca} channels by increasing Ca^{2+} sparks (Earley et al., 2005). AA metabolism can also cause vasoconstriction. AA can diffuse to smooth muscle cells and be rapidly metabolized by ω-hydroxylase (another cytochrome P450 enzyme) to produce 20-hydroxyeicosatetraenoic acid (20-HETE) (Mulligan and Macvicar, 2004; Metea and Newman, 2006). 20-HETE causes smooth muscle contraction by inhibiting vascular BK_{Ca} K^+ channels, leading to depolarization and increased Ca^{2+} entry through VGCC.

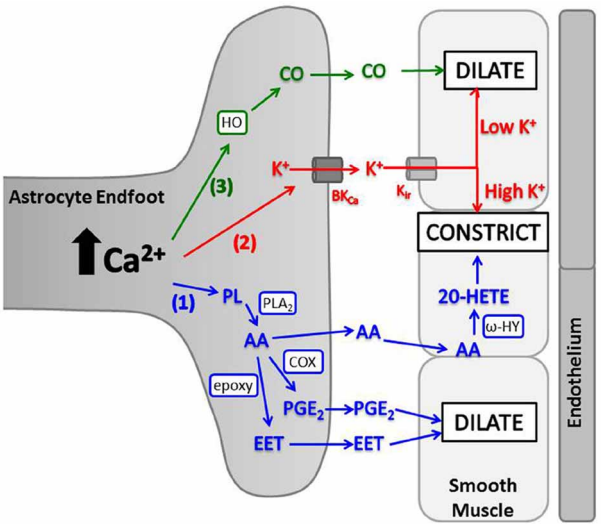

FIGURE 3 | Astrocyte intracellular Ca^{2+} elevations trigger release of vasoactive molecules. (1) PLA_2 is activated by Ca^{2+} and converts phospholipids (PL) to AA. AA is metabolized in astrocyte endfeet to PGE_2 (by COX) or EET [by cytochrome P450 epoxygenase (epoxy)] which dilate arterioles, or AA can diffuse to smooth muscle where ω-hydroxylase (ω-HY) converts it to 20-HETE and causes constriction. **(2)** K^+ is released from astrocyte endfeet through BK_{Ca}, and the amount of K^+ released is directly proportional to astrocyte Ca^{2+} level. K^+ is taken up into smooth muscle through K_{ir} and causes dilation at low concentrations and constriction at high concentrations. **(3)** HO is activated by Ca^{2+} and produces CO, which diffuses to smooth muscle and triggers dilation.

At first, these opposing effects of astrocyte AA metabolism on vascular lumen diameter represented a confusing dichotomy in the field. However, the last 5 years have brought some mechanistic clarity showing that the directional control of AA metabolism is finely controlled by metabolic need and nitric oxide (NO). In brain slices and retinal preparations equilibrated with 95–100% oxygen, elevated astrocyte Ca^{2+} led to vasoconstriction mediated by 20-HETE production (Mulligan and Macvicar, 2004; Gordon et al., 2008; Mishra et al., 2011). However, in brain slices and retinal preparations treated with 20% oxygen, astrocyte Ca^{2+} elevations caused vasodilation induced by PGE_2 produced from COX-1 activity (Gordon et al., 2008; Mishra et al., 2011). Vasodilation induced by direct astrocyte Ca^{2+} stimulation *in vivo* was also mediated by COX-1 (Takano et al., 2006).

Interestingly, part of the mechanism for dictating response directionality appears to be related to lactate production by astrocytes, revealing another critical role for lactate alongside the ANLSH. At 20% oxygen, astrocytes oxidize glucose and produce lactate (Gordon et al., 2008). Astrocyte endfeet express a prostaglandin-lactate transporter that exchanges intracellular lactate for extracellular PGE_2 (Chan et al., 2002). Thus, at 20% oxygen, increased extracellular lactate from astrocyte glycolysis inhibits the prostaglandin-lactate transporter, resulting in elevated extracellular PGE_2 and vasodilation (Gordon et al., 2008). Current consensus suggests astrocytes maintain vascular tone equilibrium (between vasodilation and vasoconstriction) under physiological conditions. When synaptic activity is minimal and oxygen consumption is low, vasoconstriction by 20-HETE is favored because PGE_2 is taken up rapidly through prostaglandin-lactate transporters. During periods of elevated activity, oxygen is depleted and lactate is released from astrocytes, leading to inhibition of the prostaglandin-lactate transporter, more extracellular PGE_2, and vasodilation. This mechanism couples cerebral blood flow regulation and the ANLSH, since astrocyte lactate production may act as a neuronal energy source and signaling molecule to increase blood flow.

While astrocytes may "sense" the oxygen content in their local environment by producing variable lactate levels *in vitro* (Gordon et al., 2008), the relevance of this mechanism *in vivo* is not clear. *In vivo*, oxygen levels may not influence neurovascular coupling like they do *in vitro* preparations (Lindauer et al., 2010; Mishra et al., 2011). In *ex vivo* retinal preparations, for example, while incubation with 100% oxygen increases tissue partial pressure of oxygen (pO_2) 16-fold, administering 100% oxygen to anesthetized rats only modestly elevates retinal pO_2 (Mishra et al., 2011). Consequently, retinal neurovascular coupling favors vasodilation under normoxic and hyperoxic conditions *in vivo*, in contrast to vasoconstriction *in vitro* under high pO_2. In addition, physiologic cerebral oxygen levels are between 12 and 38 mmHg (Jamieson and Vandenbrenk, 1963; Metzger et al., 1971; O'Hara et al., 2005), suggesting 20-HETE synthesis, which is dependent on binding of molecular oxygen as a cofactor and has a K_mO_2 (Michaelis constant for oxygen) of 60–70 mmHg (Harder et al., 1996), is low in normoxia. Conversely, production of dilatory prostaglandins and EETs, both with $K_mO_2 \leq 10$ mmHg (Harder et al., 1996; Juranek et al., 1999), would be favored at physiologic oxygen. This suggests that the effect of oxygen on the kinetics of AA metabolism may be sufficient to dictate vascular response polarity as observed *in vitro*; however, the influence of oxygen on responses *in vivo* may favor dilation and requires further investigation.

The role of NO in functional hyperemia further complicates neurovascular signaling, as NO also modulates AA metabolism (Metea and Newman, 2006). Traditionally, NO has been considered a direct vasodilator, stimulating vascular smooth muscle guanylyl cyclase leading to activation of K^+ channels and hyperpolarization (Ignarro et al., 1999). However, NO can also inhibit cytochrome P450 enzymes, such as ω-hydroxylase, thereby reducing 20-HETE production (Alonso-Galicia et al., 1998, 1999), or cytochrome P450 epoxygenase, mitigating EET production (Udosen et al., 2003). Additionally, NO weakly activates COX-1, while suppressing COX-2 (Fujimoto et al., 2004), which may affect prostaglandin levels. Overall, cerebral vasodilation by NO likely involves both smooth muscle effects and inhibition of 20-HETE production, thereby favoring lumen expansion by prostaglandins and EETs (Sun et al., 2005; Attwell et al., 2010). The opposite effect of NO on AA metabolism, that is inhibition of dilatory metabolism in deference to 20-HETE, may also occur at elevated tissue oxygen *in vitro*. In retinal preparations maintained in 95% O_2, NO-enhanced constriction produced by glial activation in a manner thought to result from inhibition of EET formation (Metea and Newman, 2006). There is evidence that all NOS isoforms (i.e., nNOS, eNOS, or iNOS) could be involved in 20-HETE modulation. Reduction of vasodilation *in vivo* by an nNOS inhibitor was reversed by 20-HETE inhibition, suggesting neuronal NO inhibits 20-HETE production in live animals (Liu et al., 2008). Recent evidence from brain slices also indicates that eNOS permits dilation through suppression of 20-HETE synthesis (Stobart et al., 2013). iNOS is more likely to be involved in neurovascular coupling during pathological conditions. Inhibition of iNOS, which is elevated in retinal glia of diabetic animal models, rescued functional hyperemia, possibly by attenuating EET production (Mishra and Newman, 2010).

POTASSIUM

Extracellular K^+ is generated by working neurons and is an effective vasodilator, giving it suitable properties as a neurovascular coupling mediator. Astrocytes have long been known to regulate neuronal membrane potential by removing synaptic K^+ (Amedee et al., 1997; Kofuji and Newman, 2004), and astrocytes express inwardly rectifying $K_{ir}4.1$ K^+ channels (Sontheimer and Waxman, 1993; Sontheimer, 1994) and large conductance Ca^{2+}-sensitive K^+ channels (BK_{Ca}) on vascular endfeet (Price et al., 2002) as a potential egress route for vasodilatory K^+. In retinal preparations, it was suggested vasodilation can be triggered via K^+ efflux through glial endfoot $K_{ir}4.1$ channels in response to neurotransmission (Newman et al., 1984; Paulson and Newman, 1987), but studies of $K_{ir}4.1$ knockout mice failed to support this idea (Metea et al., 2007). A second mechanism was proposed involving Ca^{2+}-dependent astrocyte BK_{Ca} channels (Filosa et al., 2006). The idea is that astrocytic Ca^{2+} increases lead to BK_{Ca} channel activation, K^+ release, and smooth muscle relaxation. Moderate astrocytic Ca^{2+} increases indeed triggered BK_{Ca} channel-induced dilation of neighboring arterioles, but larger astrocyte Ca^{2+} signals produced greater BK_{Ca} channel opening, higher astrocyte K^+ release and vasoconstriction (**Figure 3**) (Girouard et al., 2010). This polarity was dictated by a threshold extracellular K^+ ($[K^+]_o$) concentration of 20 mM. Lower than this threshold, conductance of smooth muscle inward rectifying $K_{ir}2.1$ K^+ channels (Bradley et al., 1999) was enhanced, causing hyperpolarization, reduced VGCC activity, and vascular smooth muscle relaxation (Girouard et al., 2010). In contrast, $[K^+]_o$ larger than 20 mM caused smooth muscle depolarization, increasing VGCC conductance, and vasoconstriction (Knot et al., 1996; Knot and Nelson, 1998). This represents another potential mechanism of activity-dependent vasodilation mediated by astrocytes. Moreover, it is another mechanism by which astrocytes

could theoretically select for dilation or constriction based on magnitude of K^+ release (Dunn and Nelson, 2010).

AA metabolite and K^+ signaling occur in parallel to regulate cerebral blood flow (Filosa et al., 2006) and may interact since AA metabolites also affect smooth muscle ion conductance. In renal arteries, PGE_2 can induce smooth muscle BK_{Ca} channel- mediated dilation through EP2 or EP4 prostanoid receptors (Zhang et al., 2005), but this mechanism has not been tested in cerebral arteries. Also, astrocyte BK_{Ca} channel activity is increased by EETs (Higashimori et al., 2010), suggesting AA metabolites can modulate K^+ release into the perivascular space, but the vascular implications of this interaction have not been studied.

CARBON MONOXIDE

Carbon monoxide (CO) is produced by heme oxygenase (HO) and can have vasoactive effects. On a cellular level, CO can relax vascular smooth muscle by increasing coupling between smooth muscle BK_{Ca} channels and local Ca^{2+} transients, similar to EET activity (**Figure 3**; Jaggar et al., 2002; Wu et al., 2002; Xi et al., 2010). CO-mediated vasorelaxation has been observed in peripheral tissues such as liver (Suematsu et al., 1994, 1995) and carotid arteries (Brian et al., 1994), but cerebrovascular results are varied (Brian et al., 1994; Leffler et al., 1999; Ishikawa et al., 2005; Leffler et al., 2006a; Li et al., 2008; Xi et al., 2010, 2011; Morikawa et al., 2012). Brain arteries from rabbits and dogs demonstrated no response to CO (Brian et al., 1994), while arteries from rats and piglets dilated in response to CO (Leffler et al., 1999; Jaggar et al., 2002; Holt et al., 2007; Li et al., 2008; Xi et al., 2010, 2011) or constricted based on CO-induced inhibition of NO dilation pathways (Ishikawa et al., 2005). In piglet studies, glutamate-induced vasodilation was mediated by CO, as HO inhibitors blocked lumen diameter increases in isolated arteries (Fiumana et al., 2003) and pial arteries *in vivo* (Leffler et al., 1999; Robinson et al., 2002). Glutamate stimulates endothelium-dependent dilation through CO production from endothelial and smooth muscle cells (Fiumana et al., 2003; Leffler et al., 2003), but also induces CO production in astrocyte endfeet (Leffler et al., 2006b; Parfenova et al., 2012) by Ca^{2+} and calmodulin-dependent activation of HO (Xi et al., 2011). This astrocyte-specific response can reportedly mediate vasodilation *in vivo* (Li et al., 2008) indicating CO is another diffusible, vasoactive molecule, released upon astrocytic activation by neurotransmission. Astrocyte CO production and dilation of piglet pial arteries *in vivo* can be enhanced by adenosine diphosphate (Kanu and Leffler, 2009), NO (Barkoudah et al., 2004; Leffler et al., 2005a,b), AA and PGE_2 (Kanu et al., 2006; Kanu and Leffler, 2011), suggesting an interaction between other dilatory mechanisms and HO activity. A study of adult rat pial arteries *in vivo* indicated CO-induced cerebral vasoconstriction by inhibiting NO production (Ishikawa et al., 2005), and similar results were observed in piglets, but after prolonged exposure to CO (Knecht et al., 2010; Leffler et al., 2011). Therefore, there may be a polarity to CO-mediated cerebrovascular effects, akin to similar effects seen with AA metabolism and K^+ effects.

In summary, astrocytes are not only important for regulating synaptic environments and the supply of energy metabolites to neurons, but they are also central to the regulation of neurovascular coupling by releasing several molecules, including AA metabolites, K^+, and CO, in response to synaptic transmission. We are only just beginning to understand how these pathways work in concert to fine-tune regulation of cerebral blood flow.

ASTROCYTE CONTROL OF CEREBRAL BIOENERGETICS CAN CONTRIBUTE TO DISEASE

Multiple brain diseases and injuries are associated with aberrant energy metabolism, dysfunctional glutamate cycling by astrocytes, and altered neurovascular coupling. Here, we discuss the major bioenergetic changes and astrocyte dysfunction in Alzheimer's disease (AD), cerebral ischemia, and epilepsy.

ALZHEIMER'S DISEASE

AD is the most common form of dementia, characterized by declining cognitive performance and memory (McKhann et al., 1984). AD pathology is characterized by two types of lesions—amyloid-β (Aβ) plaques, consisting of insoluble, extracellular deposits of Aβ peptide fibrils, and neurofibrillary tangles, composed of intracellular neuronal deposits of hyperphosphorylated and crosslinked tau protein (Merz et al., 1983; Braak and Braak, 1988). Aβ peptides are linked to synaptic dysfunction, activation of microglia and astrocytes, and oxidative stress, but the precise contribution of plaque formation to disease pathogenesis remains controversial (Fuller et al., 2009).

During AD, astrocytes undergo morphological changes, related to proximity of Aβ deposits. In dementia patients and transgenic mice, extensive reactive gliosis appears near Aβ plaques (Rodriguez et al., 2009; Simpson et al., 2010), while astrocytes farther away display dystrophic changes such as decreased complexity, surface area, and volume of cell processes (Senitz et al., 1995; Rodriguez et al., 2009). In many cases, abnormal glial morphology occurs early in disease on-set before amyloid deposition is apparent (Scheff et al., 2007; Rodriguez et al., 2009). Astrocyte dystrophy and reactive astrogliosis may greatly impair astrocytic modulation of synaptic environments and neuronal metabolism, exacerbating AD progression (Fuller et al., 2009; Steele and Robinson, 2012). For example, brain glucose metabolism is diminished in pre-clinical patients (Mosconi et al., 2008) and cerebral glucose uptake in transgenic AD mice (Merlini et al., 2011) and AD patients (Alexander et al., 2002) is significantly reduced, often before Aβ plaques or neurofibrillary tangles are detected (Small et al., 2000). Glycogen-derived lactate is important for memory formation in healthy brain (Gibbs et al., 2006; Newman et al., 2011; Suzuki et al., 2011), and dysfunction of this pathway could contribute to AD pathogenesis. Transgenic AD mice demonstrate decreased brain lactate release during neuronal stimulation (Merlini et al., 2011). In day-old chicks treated with Aβ1–42 peptide, memory consolidation was rescued upon injection of energy substrates, such as acetate, a substrate oxidized specifically by astrocytes (Gibbs et al., 2009). This suggests Aβ may damage astrocyte glycolysis and lactate production, reducing brain metabolism, and impairing memory.

The astrocyte glutamate-glutamine shuttle is also altered during AD. Expression of astrocyte glutamate transporter, EAAT2, is reduced in both transgenic mice and dementia patients,

suggesting astrocytes take up less synaptic glutamate (Li et al., 1997; Masliah et al., 2000; Simpson et al., 2010). Also, both glutamine synthetase activity (Smith et al., 1991) and the concentration of glutamine in cerebrospinal fluid is reduced in AD patients (Csernansky et al., 1996; Jimenez-Jimenez et al., 1998). The confluence of these events results in a dysregulation of glutamate homeostasis and reduced transfer of glutamine to neurons from astrocytes. Neurons in AD brains aberrantly express astrocyte proteins, including the amino acid transporter, EAAT1 (Scott et al., 2002), and glutamine synthetase (Robinson, 2000), possibly in an attempt to normalize glutamate handling and limit excitotoxicity. Neuronal expression of EAAT1 is correlated with neurofibrillary tangle formation (Scott et al., 2002), while glutamine synthetase expression corresponds with plaque formation (Robinson, 2000). Since these enzymes and transporters are critical for glutamate uptake and the glutamate-glutamine shuttle, such dramatic changes in cellular distribution suggest profound astrocyte dysfunction and impaired glutamate handling during AD. In combination with reduced energy metabolism, this may greatly affect neuronal viability and synaptic transmission (Rodriguez et al., 2009).

Impaired vascular reactivity, reduced neurovascular coupling, and diminished resting blood flow are all associated with AD (Mentis et al., 1996; Warkentin and Passant, 1997; Niwa et al., 2000, 2001; Iadecola, 2004), and could be attributed to astrocytes and hemodynamic dysfunction. Cultured astrocytes treated with Aβ peptides (1–42 and 25–35) (Abramov et al., 2003; Chow et al., 2010) and *in vivo* astrocytes from transgenic AD mice exhibit increased frequency of spontaneous, focal intracellular Ca^{2+} responses not coupled with neuronal activity (Takano et al., 2007; Kuchibhotla et al., 2009). Intercellular Ca^{2+} waves between astrocytes were also increased in frequency and amplitude in both cultured cells and *in vivo* (Haughey and Mattson, 2003; Kuchibhotla et al., 2009). Furthermore, Aβ40-peptide accumulates in blood vessel walls (Selkoe and Schenk, 2003; Agyare et al., 2012) causing endothelial cell deformity, smooth muscle deterioration (Farkas and Luiten, 2001; Merlini et al., 2011), and pericyte toxicity (Wilhelmus et al., 2007). This is linked to reduced free NO and vasoconstriction (Thomas et al., 1996; Niwa et al., 2001), and suggests that Aβ accumulation may alter the functional neurovascular unit. The concentration of reactive oxygen species (ROS) also increases in AD transgenic mice (Park et al., 2004), which are known to reduce production of vaso-active molecules, as observed *in vitro* (Fleming, 2004; Sun et al., 2008). Thus, dysfunctional neurovascular coupling during AD could be caused by altered astrocyte Ca^{2+} signaling, increased ROS, and gross vascular abnormalities, which change normal intrinsic vascular tone. Astrocyte dysfunction appears to be central to AD initiation and progression, and these cells have now become future therapeutic targets (Fuller et al., 2009).

CEREBRAL ISCHEMIA

During cerebral ischemia, blood flow is restricted by cortical or subcortical occlusion, chronically impaired vascular reactivity or cardiac arrest. Bioenergetic failure results (Hertz, 2008) in a cytotoxic cascade characterized by lactate and proton acidification (Silver et al., 1997) and ROS generation, (Abramov et al., 2007), inhibition of Na^+/K^+ ATPases, membrane depolarization (Silver et al., 1997) and elevation of extracellular glutamate due to depolarization-induced vesicular release and non-vesicular egress mechanisms. This initiates further membrane depolarization, mitochondrial damage, excitotoxicity, and neuronal death (Schild et al., 2003; Brookes et al., 2004; Nicholls, 2004; Nicholls et al., 2007). Neurons are very sensitive to this chain reaction, while astrocytes are more resistant because they can increase their glycolytic rate (Walz and Mukerji, 1990) or utilize alternate energy substrates for ATP production (Edmond et al., 1987; Hertz, 2003; Hertz and Hertz, 2003). Astrocytes also exploit glutathione stores to limit ROS damage (Juurlink, 1997). In early ischemic stages, astrocytes may help ailing neurons, but prolonged ischemic stress damages astrocytes, which may contribute to neuronal demise (Rossi et al., 2007). As described below, astrocytes affect neuronal survival and metabolism during ischemia through glutamate handling, lactate shuttling, and glycogen breakdown, and the transport of metabolites through gap junctions.

During ischemia, neuronal ionic gradients are disrupted by Na^+/K^+ ATPase inhibition, elevating extracellular glutamate concentrations (Bosley et al., 1983; Goldberg et al., 1988; Hillered et al., 1989). In early stages, astrocytes take up and accumulate intracellular glutamate (Hertz et al., 1998; Volobouева et al., 2007) in an attempt to balance the extracellular environment, but they continue to shuttle glutamine to neurons, facilitating additional glutamate release (Haberg et al., 2001). Prolonged ischemia disrupts the glutamate-glutamine cycle (Gorovits et al., 1997) due to depleted ATP levels, accumulation of intracellular Na^+ and reversal of GLT1 and GLAST to cause facilitated extrusion of glutamate (Anderson and Swanson, 2000; Phillis et al., 2000; Bonde et al., 2003). Furthermore, astrocytes swell and release yet more glutamate through volume-regulated anion channels (Kimelberg et al., 1990). These observations have generated interest in astrocyte glutamate handling as a potential ischemic therapeutic target, since upregulated expression or activity of glutamate transporters or inhibition of volume-regulated anion channels may decrease glutamate excitotoxicity (Rossi et al., 2007).

Progression of neuronal death during ischemia is dependent on availability of energy substrates. Experimental inhibition of lactate transporters (MCTs) during ischemia exacerbates neuronal death and astrocytes display increased conversion of glycogen to lactate (via glucose-6-phosphate) during this time (Brown et al., 2005; Tekkok et al., 2005; Suh et al., 2007), suggesting lactate and glycogen are important for maintaining ATP levels and neuronal survival. Lactate can also diffuse through astrocyte gap junctions (Rouach et al., 2008), which remain open during ischemia (Cotrina et al., 1998), facilitating the beneficial flux of lactate within the astrocytic network. As oxygen is depleted, astrocytes appear to be able to sustain neuronal function via anaerobic glycolysis (Rossi et al., 2007). However, there is a fine balance between benefit and injury and eventually lactate builds to concentrations which induce acidosis and cellular damage (Li and Siesjo, 1997). Further experimental testing is required to determine the role of astrocyte glycogen during ischemia. Brain regions with higher than normal glycogen concentrations are more resistant to ischemic damage (Swanson et al., 1989), and increasing glycogen stores in cultured astrocytes reduces neuronal death

during glucose deprivation (Swanson and Choi, 1993). Protective effects *in vivo* may also be enhanced by increasing glycogen stores, either through inhibition of GlyP (Suh et al., 2007) or by elevating glycogen synthase activity (Rossi et al., 2007). In cell culture models of ischemia, propagation of signals and metabolites through the glial network is increased through hemichannels (Contreras et al., 2002). This may exacerbate tissue damage as increased hemichannel activity allows Na^+ and Ca^{2+} to diffuse into astrocytes, while glutamate flows out, furthering excitotoxicity (Ye et al., 2003). Also, in astrocyte cultures, glutathione (an important astrocytic antioxidant) is lost over time through hemichannels, limiting ROS protection (Rana and Dringen, 2007). While both hemichannels and gap junctions respond to ischemic signals, they are difficult *in vivo* therapeutic targets as both are inhibited by the same antagonists, obscuring potential benefits (Rossi et al., 2007).

Reperfusion after ischemia is characterized by reduced blood flow (Leffler et al., 1989) due to disruption of the neurovascular unit via neuronal and vascular ischemic damage (Del Zoppo, 2010). Reduced neurovascular coupling exacerbates ischemic injury, which may increase infarct size. Blood flow is partly reduced because fibrin, activated platelets and/or leukocytes occlude capillaries and venules (Del Zoppo and Mabuchi, 2003). Evidence also suggests that AA metabolite (EETs and 20-HETE) signaling is altered during ischemia, which contributes to decreased blood flow and neurovascular coupling. Recent therapeutic studies have elevated EET levels using inhibitors of soluble epoxide hydrolase (sEH), an enzyme that degrades EETs (Imig and Hammock, 2009). sEH inhibitors are beneficial regardless of administration time, since infarct size is decreased in rodents when the drug is given chronically, shortly after the ischemic insult or during reperfusion (Dorrance et al., 2005; Zhang et al., 2007, 2008; Simpkins et al., 2009). EETs mediate this protection, as inhibition of CYP epoxygenase (the EET synthesis enzyme) prevents sEH benefits (Zhang et al., 2007, 2008). This protective mechanism increases astrocyte survival (Liu and Alkayed, 2005), elevates antiapoptotic factors (Simpkins et al., 2009) and increases neurovascular coupling (Zhang et al., 2007, 2008). Conversely, 20-HETE is elevated during ischemia (Tanaka et al., 2007), and inhibition of 20-HETE production is also neuroprotective in rodent models (Miyata et al., 2005; Poloyac et al., 2006; Tanaka et al., 2007; Dunn et al., 2008; Renic et al., 2009). Reduction of 20-HETE inhibits ROS production (Dunn et al., 2008), limits vasoconstriction and increases blood flow during reperfusion (Miyata et al., 2005; Dunn et al., 2008). Taken together, evidence suggests AA metabolite signaling is dysfunctional during and after cerebral ischemia, whereby EETs are decreased and 20-HETE is elevated. By inhibiting EET degradation and 20-HETE production, functional hyperemia can be restored, and these pathways make promising therapeutic targets.

Focal cerebral ischemia causes altered glutamate handling and lack of energy substrates, which triggers neuronal excitotoxicity, ATP depletion, and ROS production (Hertz, 2008). In early stages of ischemia, astrocytes are less susceptible to damage and may help protect neurons through glutamate uptake, glycogen hydrolysis to lactate for energy, and conduction of protective molecules through gap junctions. However, prolonged ischemia damages the neurovascular unit reducing blood flow and functional hyperemia during reperfusion. Current therapeutic targets are meant to promote astrocyte protection of neurons and help restore proper circulation after stroke.

EPILEPSY

Epilepsy is characterized by sudden, temporary synchronization of electrical charges in groups of neurons, which may manifest as seizures. The origins of this disorder are not completely understood (McCormick and Contreras, 2001; Scharfman, 2007), but neuronal hyperexcitability is believed to be caused by disequilibrium between glutamatergic and GABAergic neurotransmission, either by decreased inhibitory (GABA) circuits or excessive glutamatergic release (Dudek et al., 1999; Uhlhaas and Singer, 2006). Dysfunctional astrocyte glutamate-glutamine cycling is also involved (Tian et al., 2005), as astrocyte expression of EAAT2 is diminished in epilepsy patients (Proper et al., 2002; Fotheringham et al., 2007), and knock-down of glutamate transporters [EAAC1 (Sepkuty et al., 2002), GLT-1 (Tanaka et al., 1997), and GLAST (Watase et al., 1998)] in animal models exacerbates neuronal excitability. Also, glutamine synthetase expression is reduced by 40% in astrocytes of epilepsy patients, suggesting that glutamate degradation is greatly diminished (Eid et al., 2004). Therefore, dysfunctional glutamate metabolism in astrocytes could contribute to neuronal synchronization and hyperexcitability.

Ion homeostasis by astrocytes is altered during epilepsy. Particularly, both K_{ir} currents and aquaporin 4 expression are reduced, (D'Ambrosio, 2004; Eid et al., 2005) and this results in elevated extracellular K^+, decreased water homeostasis, and reduced seizure thresholds (Binder and Steinhauser, 2006). Astrocytes also display elevated intracellular Ca^{2+} signals before and during seizure activity in rodents (Tian et al., 2005; Gomez-Gonzalo et al., 2010, 2011), which are mediated by mGluR and purinergic receptors, and may further exacerbate neuronal activation by triggering gliotransmission (Gomez-Gonzalo et al., 2010). Interestingly, common antiepileptic drugs, such as valproate and phenytoin, reduce astrocytic Ca^{2+} increases (Tian et al., 2005).

Cerebral bioenergetics are aberrantly regulated in epilepsy, but the precise changes remain unknown. Epilepsy patients display high levels of glucose uptake and hypermetabolism during seizures (Engel et al., 1983), and low levels of glucose uptake and hypometabolism between seizures (Engel et al., 1982). In animal models of epilepsy, astrocyte glycogen accumulates before the onset of seizures for possible conversion to neuronal energy substrates (Bernard-Helary et al., 2000). Glycolytic inhibitors, such as 2-deoxy-D-glucose, have antiepileptic properties (Garriga-Canut et al., 2006), suggesting glycolysis is necessary for neuronal hyperexcitability and synchronization. Also, glucose flux from blood vessels to neurons through astrocytic gap junctions can partially sustain epileptiform activity in brain slices (Rouach et al., 2008). However, connexin knockout mice experience spontaneous interictal bursts and neuronal hyperexcitability, which has been attributed to decreased buffering of extracellular K^+ and glutamate (Wallraff et al., 2006; Cloix and Hevor, 2009; Pannasch et al., 2011; Bedner and Steinhauser, 2013). Gap junction trafficking is reportedly altered in epilepsy, possibly permitting elevated

extracellular K^+ and glutamate, but how this effects the flow of energy substrates remains unclear (Bedner and Steinhauser, 2013).

Epileptiform activity triggers increased blood flow and deoxygenates hemoglobin (Suh et al., 2006) to meet energy and oxygen demand of active neurons (Kuhl et al., 1980). However, hyperemia may not fully support neurons, since some studies suggest chronic epilepsy may cause ischemic-like tissue damage (Suh et al., 2006). A lag time was identified between astrocyte endfeet Ca^{2+} elevations and vasodilation of pre-constricted arterioles during synchronous bursts in rat brain slices treated with 95% oxygen, indicating astrocyte-independent neurovascular coupling mechanisms may be more prevalent in epilepsy (Gomez-Gonzalo et al., 2011). However, the cellular pathways influencing the hemodynamic response during epilepsy have not been investigated (Kovacs et al., 2012).

Astrocytes may play an important role in epilepsy, but it is unclear if they promote neuronal excitability, or merely sustain seizures and epileptogenesis. Several astrocyte functions are altered during epilepsy including glutamate-glutamine shuttle, ion homeostasis, and movement of metabolites, but the role of astrocytes in functional hyperemia during seizure activity is unknown. In the future, astrocyte glutamate uptake, blood flow control, or metabolism could be targeted to limit neuron excitability.

CONCLUSION

Astrocytes were once considered the "glue" of the brain with little importance to brain function; however, they have emerged as modulators of brain bioenergetics, blood flow, and neuronal survival. Based on spatial orientation, gap junction connections, and complexity, astrocytes are well-situated to influence synaptic environments and function as "gatekeepers" of neuronal metabolism and blood flow. This involves complex, multi-modal mechanism where astrocytes "listen" to synaptic activity and respond through (a) glutamate uptake and recycling via the glutamate-glutamine cycle, (b) increased glycolysis and shuttling of metabolites to neurons for oxidative phosphorylation, and (c) elevated Ca^{2+} signaling and release of vasoactive molecules for blood flow control. These responses ensure astrocytes tightly couple neuronal metabolic need with enhanced supply. Furthermore, astrocyte dysfunction may contribute to aberrant neuronal metabolism and neurovascular coupling in disease and injury and these pathways are promising therapeutic targets.

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Unraveling the complex metabolic nature of astrocytes

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Since the initial description of astrocytes by neuroanatomists of the nineteenth century, a critical metabolic role for these cells has been suggested in the central nervous system. Nonetheless, it took several technological and conceptual advances over many years before we could start to understand how they fulfill such a role. One of the important and early recognized metabolic function of astrocytes concerns the reuptake and recycling of the neurotransmitter glutamate. But the description of this initial property will be followed by several others including an implication in the supply of energetic substrates to neurons. Indeed, despite the fact that like most eukaryotic non-proliferative cells, astrocytes rely on oxidative metabolism for energy production, they exhibit a prominent aerobic glycolysis capacity. Moreover, this unusual metabolic feature was found to be modulated by glutamatergic activity constituting the initial step of the neurometabolic coupling mechanism. Several approaches, including biochemical measurements in cultured cells, genetic screening, dynamic cell imaging, nuclear magnetic resonance spectroscopy and mathematical modeling, have provided further insights into the intrinsic characteristics giving rise to these key features of astrocytes. This review will provide an account of the different results obtained over several decades that contributed to unravel the complex metabolic nature of astrocytes that make this cell type unique.

Keywords: astrocytes, energy metabolism, aerobic glycolysis, lactate, glycogen, glucose

As often in physiology, the role(s) of specific cell types is(are) suggested initially by their morphology, localization and interactions with other elements in the tissue where they are found. This was made possible by the development of microscopy and various histological techniques. Astrocytes are no exception and it is quite instructive to recollect the historical descriptions (by those who made them) that led to the hypothesis of an important metabolic role of astrocytes in the central nervous system. For a more extensive historical perspective about the emergence of the concept of Neuroglia, the reader is referred to Somjen (1988) or Kettenmann and Verkhratsky (2008).

A STAR IS BORN

The first description of a distinct tissue from neurons was attributed to the german anatomist Rudolf Virchow as he named it "nervenkitt" or "neuroglia" (Virchow, 1856) to reflect the suggested function of scaffold material. But the identification of glial cells as a distinct cell population will be made possible by the development of specific histological stainings such as the silver impregnation method by Golgi (1873). Taking advantage of it, Andriezen (1893a) will distinguish in fact two populations of glial cells that will become known as the protoplasmic and fibrous astrocytes. But the term astrocytes will be coined by von Lenhossek (1893) based on their starlike morphology. Interestingly, Held (1904) proposed that neuroglia rather constitute a syncytium (instead of separate cells), a notion that will be revived later with the discovery of gap junctions between them and will turn out to be important for their metabolic role (Giaume et al., 2010).

Golgi (1886) also made two other important observations. First, he described that each neuroglial cell is in direct contact with a blood vessel through one fine process. With the advent of immunocytochemistry as well as fluorescence and electronic microscopy, not only the confirmation of the presence of astrocytic end-feet on blood vessels was made, but also it was realized that the surface of all capillaries is covered at 99% with these glial elements (Kacem et al., 1998). Moreover, because neuroglial cells are characterized by many fine, dendrite-like, processes and no axons, in contrast to neurons, and that dendrites were assumed to fulfill a nutritive function, by analogy Golgi (1886) hypothesized that neuroglial cells would be dedicated to this role. In accordance with the views of Golgi (1886); Andriezen (1893b) formally proposed that neuroglia (yet not identified as astrocytes) would assume a nutritive function, allowing the transfer of metabolites from the circulation to neurons. He wrote: "*The development of a felted sheath of neuroglia fibers in the ground-substance immediately surrounding the blood vessels of the Brain seems therefore . . . to allow the free passage of lymph and metabolic products which enter into the fluid and general metabolism of the nerve cells.*" Lugaro (1907) added another aspect by suggesting that neuroglial cells play an essential role in the homeostasis of extracellular milieu, by degrading or taking up substances released by nerve cells for their communication, ensuring a buffer role. Despite these early insights based on histological observations, the metabolic roles of astrocytes were quite controversial at the time and some important scientific figures, like Ramon y Cajal, preferred to consider them rather as sole electrical insulator for nerve cells. More progress on the putative metabolic roles of astrocytes will need to wait for

the development of a new field of investigation associated with biochemistry and its methodologies: neurochemistry.

THE CHEMICAL FACTORY

One major obstacle to determine the functions of astrocytes was the difficulty to study them independently of other cell types within the nervous tissue. In contrast to neurons that are excitable and exhibit complex electrophysiological responses that can be studied individually with fine electrodes *in situ*, astrocytes have more limited electrophysiological features. Astrocytes appeared more interesting from a metabolic point of view but to probe their metabolic characteristics was requiring a distinct approach to be able to study them in isolation. An elegant methodological solution will be proposed by the Swedish scientist Hyden et al. (2000). In the late 1950s, he was able to acutely isolate from the vestibular nucleus of adult animals both neurons and glial cells using thin wires under a stereomicroscope (Hyden, 1959). Using this approach, he was able to determine the metabolic characteristics of each cell type before and after stimulation, using enzymatic measurements. He observed that stimulation led to enhancement of glycolytic capacity in glial cells, and of oxidative capacity in neurons (Hamberger and Hyden, 1963). Based on these results, he postulated the existence of a metabolic cooperation between neurons and glial cells, although the precise nature of the interactions would remain unknown for almost three decades. Indeed, Tsacopoulos et al. (1988) will take advantage of the well-structured organization of the honeybee drone retina to unravel the metabolic role of glial cells. In this preparation, photoreceptor cells are surrounded by a necklace-like set of glial cells easily distinguishable by light microscopy (Tsacopoulos et al., 1988). Using 2-deoxy-D-[5,6-^{3}H]glucose (an unmetabolizable analog of glucose) and autoradiography, he could show that all this radioactive tracer was trapped within glial cells (Tsacopoulos et al., 1988). He went on to show that glial cells, that only exhibit glycolytic metabolism, transfer alanine to photoreceptor cells that depend entirely on oxidation of this substrate as source of energy (Tsacopoulos et al., 1994). These data clearly provided the proof of principle that glial cells in general, but eventually astrocytes, do fulfill a metabolic role toward neurons.

With the advent of primary cultures of various brain cell types, it became possible to further explore their individual metabolic properties, including those of astrocytes (McCarthy and de Vellis, 1980). Thus, it was possible to show that astrocytes exhibit a high glycolytic rate with an important production of lactate, as compared to neurons (Walz and Mukerji, 1988). Moreover, they were shown to contain significant levels of glycogen (in contrast to neurons) and this energy reserve could be mobilized by various neuroactive signals including noradrenaline, vasoactive intestinal peptide, adenosine or elevated potassium levels (Magistretti et al., 1981; Sorg and Magistretti, 1991; Hof et al., 1988). Interestingly, the consequence of glycogenolysis in astrocytes was neither an oxidation of the mobilized glycosyl residues nor their release in the extracellular medium. Rather, it was observed that lactate was the end product of glycogenolysis in astrocytes and it was exported outside the cell (Dringen and Hamprecht, 1993; Magistretti et al., 1993). Thus, such a compartmentalization of glycogen, the main energy reserve in the brain, in astrocytes together with the strong production of lactate upon glycogenolysis suggested that these cells may play an important role as energy substrate suppliers for neurons, the main energy consumers of the central nervous system.

Apart from energy supply, astrocytes were also shown to play other important metabolic roles. One of them is glutamate recycling. Indeed, as glutamate is the major excitatory neurotransmitter in the central nervous system, its extracellular concentration needs to be tightly controlled. This is done through a very efficient reuptake system located in astrocytes. High-affinity, sodium-dependent glutamate transporters known as GLT-1 and GLAST were shown to be expressed by astrocytes (Danbolt, 2001). Moreover, the enzyme glutamine synthetase that allows the conversion of glutamate to glutamine was found to be exclusively present in astrocytes (Martinez-Hernandez et al., 1977). Glutamine is then released by astrocytes via a particular aminoacid transporter system, the system N transport (SN1) to be taken up in neurons by a different transport system (system A) before being converted back to glutamate by the enzyme glutaminase (Bröer and Brookes, 2001). It was determined that the great majority (∼80%) of glutamate taken up by astrocytes is converted to glutamine (McKenna et al., 1996). The rest however is oxidized very efficiently and the proportion of oxidized glutamate increases with its concentration. In order to compensate for this cataplerotic use of glutamate, an anaplerotic pathway must exist to replenish the glutamate pool. Astrocytes are able to synthesize glutamate (and glutamine) from glucose via the TCA cycle and aspartate aminotransferase (Pardo et al., 2011). This capacity to maintain glutamate levels for neurotransmission through both the recycling and synthesis of glutamine has been known as the glutamate-glutamine cycle and astrocytes are key elements to support this important neurochemical function.

GENETIC AND BIOCHEMICAL PROFILING – ESTABLISHING A METABOLIC IDENTITY

It became quite evident that astrocytes appear to be very versatile cells in terms of metabolism. Although they have an important oxidative metabolism especially toward glutamate as described above, they also exhibit a clear aerobic glycolysis capacity. In order to further understand which characteristics are responsible for giving rise to these metabolic responses, both transcriptomic and biochemical investigations have provided some exquisite informations about how astrocytes can combine what appears to be a Pasteur effect with a Warburg effect. Indeed, raising oxygen levels promote oxidative metabolism in astrocytes at the expense of anaerobic glycolysis and lactate production (Pasteur effect). But even in presence of supraphysiological levels of oxygen (e.g., 21% O_2 in culture conditions), aerobic glycolysis with lactate production was shown to take place in astrocytes (Warburg effect), which can be further enhanced under certain circumstances (e.g., glutamate exposure). The capacity to exhibit both processes may depend on the expression of particular subsets of proteins that need to be specifically identified.

The possibility to explore the level of expression of thousands of genes at once in a selected population of cells using microarrays technology has been applied to acutely isolated, adult astrocytes, thus bypassing the caveats of primary astrocytes in cultures that

are essentially obtained from newborn preparations. These studies revealed several interesting points. First of all, they showed that astrocytes express high levels of mitochondrial tricarboxylic acid cycle enzymes, thus confirming the high oxidative capacity of these cells (Lovatt et al., 2007; Cahoy et al., 2008). But at the same time, they showed that astrocytes also strongly express enzymes involved in glycolysis and glycogen metabolism. At the biochemical level, several observations were made that refined the transcriptomic findings. In contrast to neurons, astrocytes maintain high levels of the PFKFB3 protein, a key regulator of glycolysis (Herrero-Mendez et al., 2009). Moreover, the activity of pyruvate dehydrogenase, the key enzyme for the entry into the TCA cycle, is maintained low in astrocytes through its high level of phosphorylation (Itoh et al., 2003; Halim et al., 2010). Finally, it was shown that an important component of the malate-aspartate shuttle in mitochondria, the aspartate glutamate complex Aralar, exhibits a very low expression in astrocytes compared to neurons (Ramos et al., 2003), contributing to a low level of malate-aspartate shuttle activity (Berkich et al., 2007). As a consequence, in order to maintain their high glycolytic rate, astrocytes will prominently convert pyruvate into lactate, thus regenerating the NAD cofactor. Thus, it appears that most of the glucose utilized by astrocytes will not be oxidized within the astrocyte to produce energy. Rather, glucose- or glycogen-derived pyruvate will be converted to lactate and exported, as a consequence of the aforementioned state of key biochemical steps that favor such a metabolic fate. This is further supported by the selective expression of the lactate dehydrogenase B isoform (Bittar et al., 1996; Laughton et al., 2007; O'Brien et al., 2007) and the monocarboxylate transporter MCT4 (Bergersen et al., 2002; Pellerin et al., 2005) by astrocytes which concur with the high glycolytic rate and lactate production capacity of these cells. Although the kinetic characteristics *per se* of these isoforms DO NOT determine metabolite flux direction, their presence is nevertheless indicative of a prevalent metabolic profile, as their properties would be better exploited within such a specific metabolic environment.

ATTRACTIVE ASTROCYTES – PROBING THE METABOLIC NATURE OF ASTROCYTES WITH MAGNETS

A large part of evidence that astrocytes do fulfill a metabolic role towards neurons was achieved by nuclear magnetic resonance (NMR) spectroscopy. ^{13}C-NMR spectroscopy in particular is a unique tool to study the metabolism of glucose and metabolic interactions between neurons and astrocytes in the brain. However, sensitivity of the carbon-13 nucleus is low. To overcome these disadvantages, 99%-^{13}C enriched substrates, such as [1-^{13}C]glucose or [2-^{13}C]acetate for example, are used. Added to the cell culture medium, or intravenously injected, this magnetic active isotope will permit analyzing cellular metabolism over time using ^{13}C-NMR spectroscopy. Indeed, all ^{13}C -labeled metabolites derived from the ^{13}C-labeled precursor will be detected on a single ^{13}C-NMR spectrum; each carbon will respectively give a signal (peak) at a different place on the NMR scale depending on their position within every metabolite. (**Figure 1A**).

Moreover, it is also possible to detect on the same spectrum if one carbon 13 is linked to an unlabeled carbon 12 or to another carbon 13. In this latter case, homonuclear spin coupling patterns will appear (**Figure 1B**). For example, a ^{13}C with one ^{13}C neighbor will lead to a doublet (instead of a singlet if linked to a carbon 12); with two ^{13}C neighbors, the peak will become a triplet and so on, the rule being n+1 peaks where n equals the number of ^{13}C neighbors. ^{13}C-NMR spectroscopy is therefore a powerful technique which can be applied *in vitro*, *ex vivo* and *in vivo* to follow up labeled carbons in metabolites and examine their fate through different metabolic pathways.

IN VITRO STUDIES

As indicated in the first part of this review, astrocytes exhibit a clear aerobic glycolysis. NMR spectroscopy is particularly suitable to estimate the rate of glycolysis in astrocytes, by measuring the rate of lactate formation. ^{1}H-NMR spectroscopy allows detecting on the same spectrum, the ^{13}C-labeled lactate synthetized from glycolysis of the administered ^{13}C-labeled glucose, and also, the unlabeled lactate coming from unlabeled precursors. Indeed, as shown in **Figure 2**, on carbon 3 of the unlabeled lactate, the protons of the methyl group will give a doublet at 1.32 ppm, rising from their homonuclear coupling (^{1}H/^{1}H) with the neighbor ^{1}H linked to carbon 2 (**Figure 2**, in red). On the other hand, the [3-^{13}C] lactate will lead to two doublets, at 1.21 and 1.43 ppm due to the heteronuclear coupling (^{1}H/^{13}C, different coupling value $J = 128$ Hz).

The first experiments using NMR spectroscopy on brain cell cultures were conducted *in vitro* in the early 90's. The metabolism of [1-^{13}C]glucose by astrocytes, neurons and mixed astroglial/neuronal cultures derived from the striatum of fetal rats was studied by Leo et al. (1993). Interestingly, they found that neuronal cultures consumed glucose much slower than the astrocytic or the mixed cultures. In the study of Martin et al. (1993) they investigated the metabolism of [1-^{13}C]glucose in rat cerebellum astrocytes and granule neurons. Results showed that the ^{13}C-specific enrichment of lactate C3 (% of ^{13}C incorporated into the carbon position 3 of lactate from the precursor [1-^{13}C]glucose enriched at 99%) was higher in astrocytes compared to neurons, demonstrating that astrocytes were more glycolytic than neurons. Moreover, although acetylCoA C2 and lactate C3 had very similar enrichments in granule cells, acetylCoA C2 enrichment in astrocytes was 60% lower than that of lactate C3. These data indicate that the labeling at the pyruvate node was mainly directed toward the TCA cycle in neurons, which was not the case for astrocytes. This glycolytic feature of astrocytes was also demonstrated on mouse primary cultures (Sonnewald et al., 1993). When astrocytes were incubated with [1-^{13}C]glucose, lactate C3 was found to be enriched at 30%. Since the maximum theoretical labeling value is 50% (as one [1-^{13}C]glucose molecule gives rise to two pyruvate, and thus to two lactate molecules, one labeled and one unlabeled), we can calculate that 60% of glucose was converted into lactate. Based on the rates of glucose consumption and lactate production, this percentage was found even higher in another study (ranging between 67 and 84%), also performed on primary cultures of mouse astrocytes (Teixeira et al., 2008). A more recent study, combining NMR and metabolic flux analysis, confirmed that astrocytes showed a high glycolytic flux, converting most of the glucose to lactate (Amaral et al., 2011). This particular astrocytic metabolic characteristic was also observed even if a high

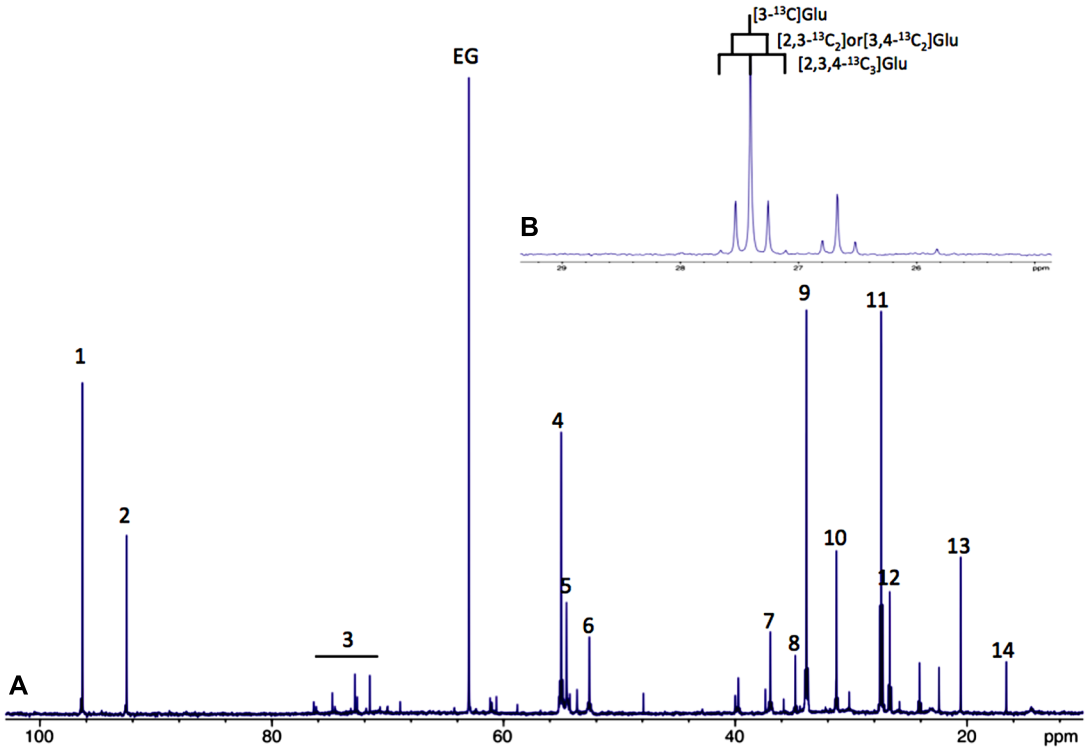

FIGURE 1 | (A) Typical ^{13}C-NMR spectrum of rat brain perchloric extract, after perfusion with [1-^{13}C]glucose. (1) Glucose C1α, (2) glucose C1β, 3: glucose C2, C3, C4, C5 and C6, 4: Glu C2, 5:Gln C2, 6: Asp C2, 7: Asp C3, 8: GABA C2, 9: Glu C4, 10: Gln C4, 11: Glu C3, 12: Gln C3, 13: lactate C3 and 14: Ala C3. **(B)** ^{13}C-^{13}C coupling figures allow to distinguish between different isotopomers (example on glutamate C3).

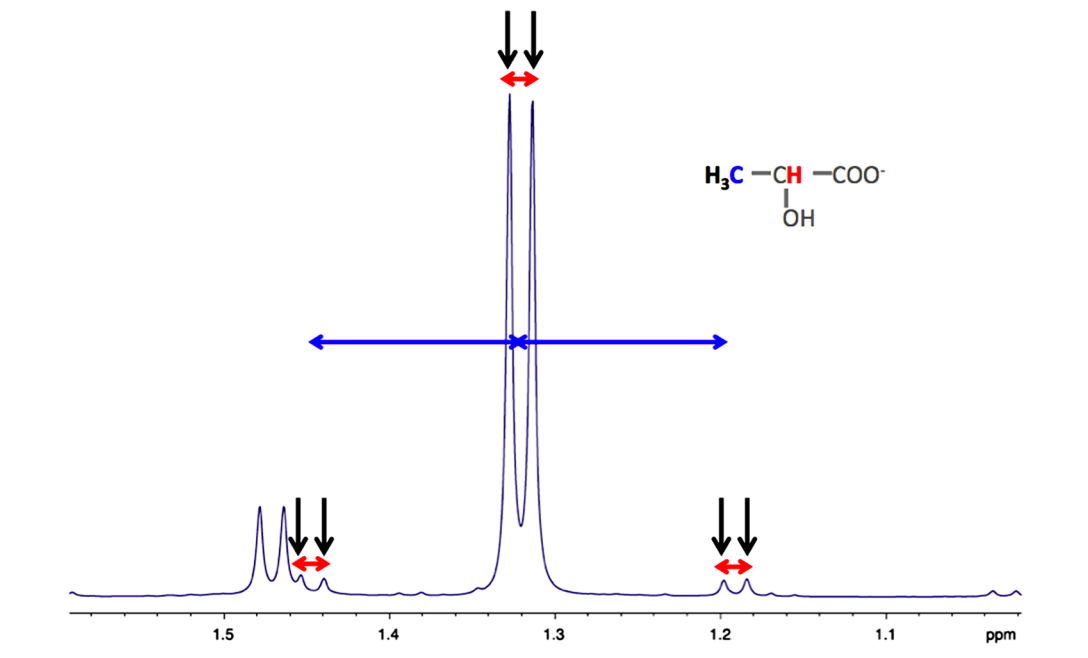

FIGURE 2 | Typical high resolution at the magic angle spinning (HRMAS) ^{1}H-NMR spectrum of rat brain biopsy after [3-^{13}C]lactate perfusion. Protons of the methyl group of lactate are detected (black arrows), centered at 1.32 ppm. The doublet is coming from the homonuclear spin coupling (J_{H-H} = 7 Hz, red arrows). When a ^{13}C is located on lactate carbon 3, then a doublet of doublet is appearing (^{13}C satellites of H_3 lactate), due to the heteronuclear spin coupling (J_{H-C} = 128 Hz, horizontal blue arrows).

concentration of lactate was present (Alves et al., 1995). In this latter study, astrocytes were incubated in a medium containing 6 mM of [1-^{13}C]glucose and 10 mM of lactate. After 6 h, [3-^{13}C]lactate was detected in the medium and its specific enrichment was 6.5%. Combining this value with the total amount of lactate present at $t = 6$ h (162 mmol/mg prot) and the rate of glucose consumption (136 mmol/mg prot in 6h), we can estimate that 10.5 mmol/mg prot of [3-^{13}C]lactate were produced in 6h and that around 15% ($10.5 \times 2/136$) of glucose was converted into lactate and exported out of the cell. This experiment reinforces the glycolytic nature of astrocytes even if high concentrations of lactate are present in the medium.

Beside [1-^{13}C]glucose, other ^{13}C-labeled substrates were tested. The fate of [3-^{13}C]alanine was followed and compared between primary cultures of astrocytes, neurons and co-cultures (Zwingmann et al., 2000). In astrocytes, 90% of the [3-^{13}C]alanine was converted into [3-^{13}C]lactate, whereas only a 12.5%-conversion was measured in neurons. The increased glycolytic activity in astrocytes induced by the uptake of alanine was suggested to contribute to the synthesis of releasable lactate. This alanine-lactate shuttle might constitute a way to transfer nitrogen from neuron to astrocytes (Zwingmann et al., 2001; Bak et al., 2005), which may promote, in return, the glutamate-glutamine cycle between these two cell types.

The fate and metabolism of ^{13}C-labeled lactate was also explored. Primary cultures of mouse cortical astrocytes were incubated during 4 h in a medium without glucose and containing 1 mM [U-$^{13}C_3$]lactate (Waagepetersen et al., 1998a). The incorporation of ^{13}C into glutamate was only 50% of the corresponding one observed in cultured neocortical neurons cultured under the same conditions (Waagepetersen et al., 1998b). In parallel to the high glycolytic activity and lactate production in astrocytes, this result suggests that lactate is predominantly employed as an oxidative substrate in neurons. From these two studies, we can also compare the ^{13}C-NMR spectra of neurons incubated with either 1 mM [U-$^{13}C_3$]lactate (Waagepetersen et al., 1998b) or 1 mM [U-$^{13}C_6$]glucose (Waagepetersen et al., 1998a); we can clearly observe that more carbon-13 was incorporated into glutamate in the lactate-labeled condition. To determine which is the preferential neuronal substrate, a competition between glucose and lactate was performed (Bouzier-Sore et al., 2003). Both substrates were added to the culture medium, but alternatively labeled ([1-^{13}C]glucose + lactate or glucose + [3-^{13}C]lactate). When glucose and lactate concentrations were equal (5.5 mM), results clearly indicated that neurons in the presence of both substrates preferentially use lactate as their main oxidative substrate. The same result was found under physiological concentrations of glucose and lactate (1.1 mM; Bouzier-Sore et al., 2006). Using a mathematical model, the relative contribution of exogenous glucose and lactate to neuronal oxidative metabolism was measured to be 25% for glucose and 75% for lactate.

Metabolism on brain slices can also be explored by NMR spectroscopy (Badar-Goffer et al., 1992). Guinea-pig cerebral-cortical slices were incubated with either [1-^{13}C]glucose or [2-^{13}C]acetate, a more specific glial substrate (Waniewski and Martin, 1998), under resting or depolarization conditions. When [1-^{13}C]glucose was the labeled substrate, an intense and much higher lactate resonance was observed on the ^{13}C-NMR spectrum during activation compared to resting conditions. Lactate C3 specific enrichment was 45% (close to the theoretical 50% value). Moreover, under depolarization, it was evidenced that glucose metabolism in glia was selectively stimulated: a significant increase in ^{13}C-incorporation was occurring into metabolites of the glial pool.

Altogether, these *in vitro* results obtained on separate astrocytic and neuronal cultures support the idea that astrocytes exhibit a clear aerobic glycolysis and produce lactate. It can then be used as a supplementary fuel by neurons since lactate appears to be a more efficient oxidative substrate for them compared to astrocytes.

EX VIVO AND *IN VIVO* STUDIES

Glial-neuronal metabolic interactions can be studied using ^{13}C-labeled substrates and high resolution or *in vivo* ^{13}C-NMR techniques. Compared to *in vitro* studies, *ex vivo* or *in vivo* experiments are more complicated to interpret since all metabolites from the different cell types are present on the same spectrum. However, it is possible to distinguish astrocytic from neuronal metabolism since a metabolic and enzymatic compartmentalization exists between neurons and astrocytes. Indeed, the existence of two distinct cerebral pools of glutamate was first determined; a small one (around 10%) attributed to the astrocyte compartment and a large neuronal one (Berl et al., 1962; Van den Berg et al., 1969). Thereafter, it was shown that glutamine synthetase and glutaminase were exclusively glial (Martinez-Hernandez et al., 1977) and mainly neuronal (Patel et al., 1982), respectively. The key outcome of this enzymatic compartmentalization is the glutamate-glutamine cycle between neurons and astrocytes. Moreover, since glutamate is in rapid equilibrium with the TCA cycle intermediate α-ketoglutarate, the neuronal TCA cycle flux can be estimated from the kinetics of ^{13}C enrichment of total cerebral glutamate (Fitzpatrick et al., 1990; Mason et al., 1992, 1995; Sibson et al., 1998, 2001). Since glutamine synthetase is exclusively located in astrocytes, glutamine will reflect the astrocytic compartment. Moreover, pyruvate carboxylase (PC) was found to be also only in astrocytes (Yu et al., 1983; Shank et al., 1985). The presence of this enzyme will lead to a different fate of the ^{13}C compared to neurons and to a higher incorporation of the ^{13}C into the carbon position 2 compared to carbon position 3 in glutamine. Such imbalance cannot be evidenced for glutamate, which reflects the neuronal compartment, where PC activity is not present. Astrocytic metabolism can also be distinguished from the neuronal one using ^{13}C-labeled acetate since this substrate is only transported to glial cells (Waniewski and Martin, 1998). This substrate enters the TCA cycle directly at the citrate level, bypassing thus the PC and PDH steps. Using all these tools, neuronal and astrocytic TCA cycle rates can be estimated either from rat brain extracts (Kunnecke et al., 1993; Preece and Cerdan, 1996) or directly *in vivo* after infusion of ^{13}C-labeled substrates (Mason et al., 1992, 1995; Sibson et al., 1997; Shen et al., 1999; Lebon et al., 2002). Glial TCA cycle rate was found to be 0.4 and 0.14 mmol/min/g, in rat brain extracts and human brain, respectively, whereas neuronal TCA cycle rate was 1 mmol/min/g in rat brain extracts and ranging from 0.6 to 1.6 mmol/min/g in *in vivo* experiments. This demonstrated

a higher neuronal oxidative metabolism, compared to glia's one (Rodrigues et al., 2012).

The exclusive presence of the PC enzyme in astrocytes was used to analyze the fate of [3-^{13}C]lactate in rat brain extracts (Bouzier et al., 2000; Hassel and Brathe, 2000) and *in vivo* in humans (Boumezbeur et al., 2010). When rats received an intravenous infusion of [3-^{13}C]lactate, the analysis of the ^{13}C-NMR spectrum of the brain extracts indicated that no imbalance between glutamine carbon 2 and carbon 3 could be evidenced. Such data indicate that there was no entry of ^{13}C into the astrocytic TCA cycle via the PC pathway, and therefore that [3-^{13}C]lactate was metabolized in a PC-deprived compartment, i.e., neurons. This lactate consumption has also been confirmed to be more neuronal specific *in vivo* in humans (Boumezbeur et al., 2010). Interestingly, a correlation was found between the use of endogenously synthetized lactate and level of activity (Serres et al., 2004). Finally, it was recently shown that during rat brain activation (whisker stimulation) there was an average 2.4-fold increase in lactate content in the activated area. Furthermore, this increase was arising from newly synthetized lactate during brain activation from blood ^{13}C-labeled glucose (Sampol et al., 2013).

SEEING IS BELIEVING – THE CONTRIBUTION OF FLUORESCENCE IMAGING

Despite the power of NMR to obtain metabolic information, this technique is unable to give an answer at the cellular level. The need to visualize metabolic responses from individual cells especially *in situ* became essential. Different optical approaches have been exploited to attain this goal. Another advantage of optical methods is that they allow the characterization of fast metabolic events, which can be applied on preparations with mixed populations of cells. This could be particularly important since the metabolic maturation of astrocytes might depend on signals from other cell types (Brix et al., 2012). A first method is based on the intrinsic fluorescence produced by the metabolic co-factor NADH. Major changes in intracellular NADH fluorescence have been attributed to alterations in mitochondrial activity (Mayevsky and Rogatsky, 2007). However, a cytosolic NADH fluorescence signal can be evidenced in astrocytes and associated with an enhancement of glycolysis (Kasischke et al., 2004; Requardt et al., 2010). These characteristics will be exploited in combination with two-photon microscopy to study the metabolic responses of brain cells and specifically in astrocytes upon stimulation both *ex vivo* (in slices) and *in vivo*.

It was demonstrated in hippocampal brain slices that electrical stimulation produced a biphasic signal of intrinsic NADH fluorescence (Kasischke et al., 2004; Brennan et al., 2006). An early dip in NADH fluorescence was observed followed by a delayed increase of the signal. The early decrease in NADH signal was associated with enhanced oxidative metabolism in neurons (Kasischke et al., 2004; Brennan et al., 2006), most likely due to enhanced lactate utilization (Galeffi et al., 2007). Although the enhancement in NADH signal subsequently taking place could be largely due also to oxidative metabolism in neurons (Brennan et al., 2006), a delayed increase of the fluorescence signal originating from the cytosol of astrocytes also occurred in parallel (Kasischke et al., 2004). Such a response in astrocytes seems to be caused by an enhancement of glycolysis in these cells, as revealed both in cultured astrocytes and in cortical brain slices stimulated with dopamine (Requardt et al., 2010). A stunning confirmation of this sequence of events was provided *in vivo* in the cerebellum using flavoprotein autofluorescence imaging (Reinert et al., 2011). The first part of the response observed, called the on-beam light phase, could be attributed essentially to activation of oxidative metabolism in neurons. Of note, lactate oxidation in neurons seems to participate to the on-beam light phase signal. The second part identified as the on-beam dark phase appears to be dependent, at least in part, on activation of glutamate transporters in glia and could be caused by the reduction of flavoproteins via an increase in glycolysis, although the origin of the dark phase signal cannot be attributed specifically either to glia or neurons. It was also suggested (but not demonstrated) that elevated extracellular potassium could be another factor contributing to the on-beam dark phase via its stimulation of glial glycolysis.

An important question to be addressed was the degree of glucose utilization by both neurons and astrocytes. Indeed, based on the estimated energy expenditures of each cell type, it is predicted that the majority (>70%) of glucose consumption should occur in neurons while the remaining (<30%) should take place in glial cells, assuming that glucose is entirely oxidized (Attwell and Laughlin, 2001; Pellerin and Magistretti, 2003). Two approaches have been developed in order to evaluate glucose uptake and utilization by each cell type. First of all, FRET nanosensors can be used to measure the intracellular concentration of glucose and estimate glycolytic rates in specific cells, including astrocytes and neurons (Bittner et al., 2010). The use of fluorescent glucose analogs such as 2- and 6-NBDG can also be used to evaluate the relative glucose uptake and utilization by neurons vs. glia. Thus, it was shown first in cerebellar slices that most glucose uptake and utilization takes place in Bergmann glia and not in Purkinje neurons (Barros et al., 2009). More recently, a follow-up study was performed in both cerebellar and hippocampal slices in which glucose transport and metabolism was found to be faster in Bergmann glia and astrocytes than in neurons (Jakoby et al., 2013). The results led to the conclusion that preferential glucose transport and metabolism takes place in glia. Interestingly, it was demonstrated that 6-NBDG, the glucose analog used to estimate glucose transport, largely underestimates glucose transport in astrocytes compared to neurons. Thus, it is clear that the rate of glucose transport and utilization is largely superior in astrocytes vs. neurons. Such a conclusion has important consequences. As stated above, if glucose is the sole energy substrate used by brain cells, it is expected that glucose transport and utilization should be proportional to the cell energy needs. Clearly, this is not the case. The most likely explanation to resolve this paradox is to admit that astrocytes convert a substantial amount of the glucose they use into lactate. Then, the lactate released by astrocytes can be used by neurons as an additional oxidative substrate to satisfy their large energy needs (Pellerin and Magistretti, 2003).

Data above provided indications about the glycolytic capacity of astrocytes *in vitro* and *ex vivo* (in slices) under resting condition. It was necessary to obtain further insight *in vivo* under both resting and activated conditions. Two-photon microscopy imaging was performed over the rat somatosensory cortex upon

infusion of 6-NBDG (Chuquet et al., 2010). At rest, the amount of 6-NBDG accumulating in astrocytes and neurons was equivalent. But based on the higher affinity of 6-NBDG for the glucose transporter expressed by neurons (GLUT3) compared to the one found on astrocytes (GLUT1), it seems that already at rest, the largest proportion of glucose is taken up by astrocytes. Upon whisker stimulation, most of the increased 6-NBDG accumulation took place in astrocytes. These results provide a strong evidence that astrocytes are the major site of glucose uptake and utilization in the brain. They also respond to neuronal activation by enhancing their glucose uptake and utilization. The mechanisms explaining such a specific metabolic response of astrocytes have been clarified over the years by the use of cell culture preparations.

First of all, using isotopic methods, glutamate had been clearly shown to cause an enhancement of glucose utilization in cultured astrocytes by a mechanism involving its uptake and an activation of the Na^+/K^+ ATPase (Pellerin and Magistretti, 1994; Takahashi et al., 1995). Such an effect of glutamate on astrocytes was confirmed *in vivo* (Voutsinos-Porche et al., 2003). In contrast, potassium was found to have either a small (Brookes and Yarowsky, 1985) or no effect (Takahashi et al., 1995) on glucose utilization in cultured astrocytes. With the advent of optical methods allowing measurements with high temporal resolution, a better characterization of the role of each substance could be performed. Indeed, taking advantage of a FRET glucose nanosensor, the group of Felipe Barros was able to show that while potassium caused a rapid but transient enhancement in the glycolytic rate (explaining why it was overlooked in isotopic studies), glutamate had a delayed but long-lasting effect (Bittner et al., 2011). Moreover, using the same approach, the same group was able to demonstrate that the glycolytic action of potassium in astrocytes requires the implication of the Na^+/HCO_3^- cotransporter NBCe1, while the Na^+/K^+ ATPase only plays a permissive role in this case (Ruminot et al., 2011). Interestingly, while they could also observe using the fluorescent glucose analogs 2- and 6-NBDG the enhancing effect of glutamate on glucose transport in cultured astrocytes (Loaiza et al., 2003), they found just the opposite in cultured neurons (Porras et al., 2004). These data are consistent with the concept that while neuronal activity triggers an enhancement of glucose uptake and glycolysis in astrocytes, it rather prevents glucose utilization in neurons under physiological conditions. As mentioned earlier, in contrast to astrocytes, neurons normally expressed low levels of the key regulator of glycolysis PFKFB3 (Herrero-Mendez et al., 2009). It is only under excitotoxic conditions leading to overstimulation of NMDA receptors that neuronal glycolysis can be activated (Rodriguez-Rodriguez et al., 2012, 2013) and neuronal glucose utilization be increased (Bak et al.; 2009), but this condition leads to neuronal cell death (Rodriguez-Rodriguez et al., 2012, 2013).

Most results converge toward the idea that astrocytes are the main brain cell type not only consuming glucose but also exhibiting glycolytic responses upon neuronal activation. In contrast, neurons appear to be highly oxidative cells that would prefer to oxidize lactate rather than produce it from glucose. A key question that arises is what are the key metabolic features that determine the apparently different (but complementary) metabolic phenotypes of astrocytes and neurons. Possible hints are emerging from modeling studies.

CALCULATE ME AN ASTROCYTE – MATHEMATICAL MODELING

Different modeling efforts have attempted to capture the role that astrocytes might play as suppliers of energy substrates for neurons along with their other metabolic functions. A first approach was proposed by developing a model of compartmentalized brain energy metabolism whereby astrocytes and neurons have been dissociated and assumed to exhibit slightly different metabolic features, based on the experimental data available (Aubert and Costalat, 2005). In such case, it was found that despite assumptions highly unfavorable to a popular concept of energy substrate supply between brain cells known as ANLS (for astrocyte-neuron lactate shuttle; see Pellerin and Magistretti, 2012), neuronal activation led to a robust lactate flux from astrocytes to neurons that can be either continuous or phasic, depending of the degree of neuron vs. astrocyte activation. This model was pushed one step further to address the question of brain lactate kinetics. Taking this time into account the distribution and kinetics of monocarboxylate transporters involved in lactate transport as well as the variations in extracellular lactate levels, it could be concluded that neurons represent the most likely compartment where lactate is consumed while astrocytes would be a plausible source (Aubert et al., 2005). Such a cellular compartmentalization of brain energy metabolism was supported by another modeling approach based rather on brain glucose and oxygen utilization (Jolivet et al., 2009). The authors concluded that glycolysis must take place in large part in astrocytes (while oxidative metabolism would predominate in neurons) and that glucose-derived metabolites must be transferred from glial cells to neurons. Independently, other authors have shown with their modeling approach that lactate shuttling from astrocytes to neurons could be advantageous for neurons, both under normoxia and hypoxia, further extending the validity of the concept to pathological situations (Genc et al., 2011). They also emphasized the fact that astrocytes and neurons might switch between a more classical, glucose alone-based mode of metabolism to a metabolic interaction mode, depending of the situation.

However, another contrasting view has been proposed following a different series of modeling analyses. It has been argued, based on a distinct set of data, that there is probably very little shuttling of lactate from astrocytes to neurons (DiNuzzo et al., 2010a). If anything, it was even proposed that it is rather the neurons that export lactate while astrocytes would oxidize it (Mangia et al., 2009). Similarly, it was proposed that glycogenolysis, a process known to occur only in astrocytes, would serve the purpose of funneling glucose to neurons instead of shuttling lactate to them (DiNuzzo et al., 2010b), in sharp contrast to the well established data showing that lactate rather than glucose is released by astrocytes following glycogenolysis as indicated above (Dringen and Hamprecht, 1993; Magistretti et al., 1993). This controversy gave rise to a heated debate, each side providing arguments to dismiss the conclusions of the other (Jolivet et al., 2010; Mangia et al., 2011; Pellerin and Magistretti, 2012). Some authors have attempted to

reconcile the two points of view by applying a more probabilistic approach of modeling (Somersalo et al., 2012). After a rigorous and thorough analysis of each model, their conclusion is that there is such variability in the system that each one might capture only one part of the reality, advocating for stochastic models rather than deterministic ones.

Nevertheless, there are still some interesting points that have been highlighted, confirming for example experimental data. Thus, it was confirmed that astrocytes have a TCA cycle rate several orders of magnitude lower than neurons (Occhipinti et al., 2007), as was determined previously both *in vitro* (Bouzier-Sore et al., 2006) and *in vivo* (Tyson et al., 2003). This is not to say that they are devoid of oxidative activity as it was previously demonstrated (Wyss et al., 2009) but at least it does not compare to the degree observed in neurons. Moreover, a recent modeling study has tackled the critical question of which biochemical steps determine whether a cell is rather oxidative (thus oxidizing both glucose and lactate) or exhibit some glycolytic features (by exporting rather than consuming lactate; Neves et al., 2012). This work has revealed that the flux through the pyruvate dehydrogenase-catalyzed reaction as well as the mitochondrial NADH shuttling rate are essential in determining the preference for oxidation rather than for export of lactate (**Figure 3**). Varying the importance of these two reactions by a modest value allowed to observe a switch in metabolic phenotype. Interestingly, it was observed that astrocytes in general exhibit characteristics for these two steps (i.e., low pyruvate dehydrogenase and mitochondrial NADH shuttling activities; see above Itoh et al., 2003; Ramos et al., 2003; Halim et al., 2010) that are consistent with the prominence of aerobic glycolysis in this cell type, in contrast to neurons that are in most cases essentially oxidative in nature. In addition to these features that determine the overall metabolic profile in resting state, there is also other mechanisms that come into play in a transient manner during activated states and further reinforce these characteristics. This is the case in astrocytes for which it was shown that in parallel with glutamate uptake that follows glutamatergic activity, an intracellular acidification takes place that spreads over mitochondria (Azarias et al., 2011). As a consequence, the cytosol-to-mitochondrial matrix pH gradient is abrogated, reducing oxidative metabolism in this cell type. Such a mechanism would favor glycolysis in astrocytes and spare oxygen for its use by neuronal oxidative metabolism. Modeling of these metabolic transients might provide us with further insights about the dynamic aspect of these adaptive metabolic characteristics of astrocytes.

THE INDISPENSABLE ASTROCYTE – IMPLICATIONS IN VARIOUS BRAIN FUNCTIONS

The putative roles related to the metabolic characteristics of astrocytes are just beginning to be explored but there is already a number of conditions for which their importance as started to be highlighted. Apart from the well-characterized role as lactate supplier for active neurons (Pellerin and Magistretti, 2012), the

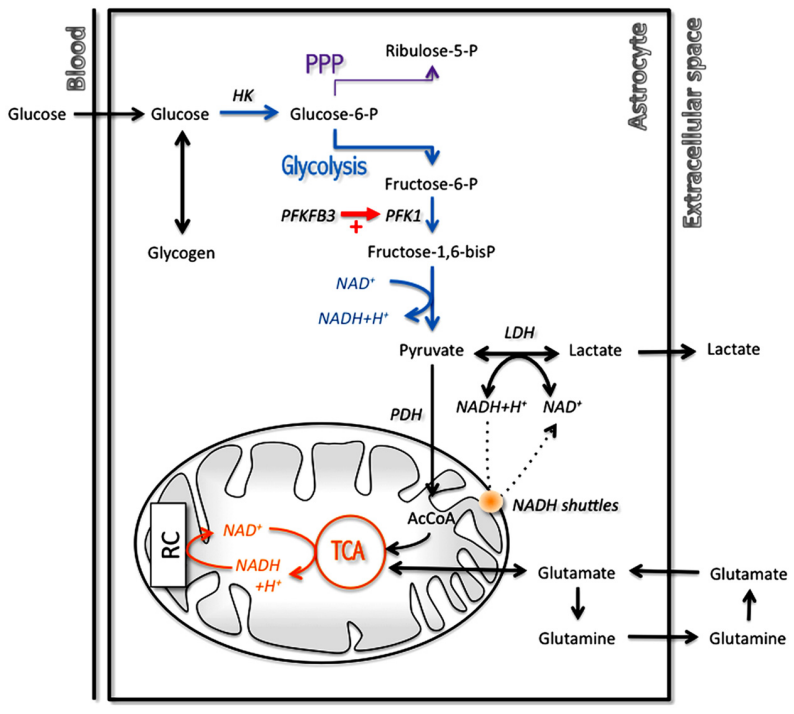

FIGURE 3 | Main metabolic pathways implicated in energy production in astrocytes with a prominent role for glycolysis. In order to regenerate cytosolic NAD^+ levels and maintain glycolytic rate, astrocytes have two options: transfer cytosolic NADH in mitochondria through specific mitochondrial NADH shuttles or convert pyruvate into lactate in the cytosol. AcCoA, acetylCoenzyme A; HK, hexokinase; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; PFK1, phosphofructokinase 1; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; PPP, pentose phosphate pathway; RC, respiratory chain; TCA, tricarboxylic acid cycle.

possibility that lactate produced by astrocytes could be a coupling factor to link neuronal activity to increased blood flow has been revealed (Gordon et al., 2008). Thus, it appears that astrocytes, through their metabolic response, represent key elements in both the neurovascular and the neurometabolic coupling, two mechanisms at the basis of functional brain imaging (Bonvento et al., 2002).

It was previously suggested that enhanced glycolysis and glycogenolysis in astrocytes were essential for the formation of memory in neonate chicks (O'Dowd et al., 1994a,b). More recently, it was shown that lactate, produced by astrocytes by those two metabolic pathways and transferred to neurons via monocarboxylate transporters, is essential for memory formation in rodents (Newman et al., 2011; Suzuki et al., 2011). Other central functions have also been shown to be dependent on proper astrocyte-neuron metabolic interactions. Thus, it was shown that orexin neurons, that play a key role in arousal, are sensitive to astrocyte-derived lactate and modify their firing activity (Parsons and Hirasawa, 2010). Sleep is another centrally controlled condition that might be regulated by the energetic responses of astrocytes (Scharf et al., 2008). Indeed, the metabolism of glycogen, which is essentially present in astrocytes, has long been associated with sleep/wake cycle regulation (Benington and Heller, 1995).

Different peripheral functions controlled by the central nervous system seem also to be regulated via metabolic responses of astrocytes. This is the case of glucose sensing that is regulated at the level of the hypothalamus. It was shown that regulation of blood glucose depends on the conversion of glucose into lactate, presumably in astrocytes, and lactate metabolism in neurons (Lam et al., 2005). Similarly, respiration control was shown to depend on proper metabolic interactions between astrocytes and neurons (Erlichman et al., 2008). Thus, it was demonstrated that in the retrotrapezoid nucleus, astrocytes participate to the medullary central chemosensory stimulus by providing lactate to neurons. Sodium homeostasis is another essential function for the organism and it is regulated centrally at the level of the subfornical organ. It was shown that elevation of sodium level in body fluids is detected by astrocytes and ependymal cells in the subfornical organ and transmitted to neurons via a lactate signal, allowing to regulate their activity, and set in motion the appropriate adaptative responses (Shimizu et al., 2007).

It is likely that if so many important brain functions depend on appropriate astrocyte-neuron metabolic interactions, some pathological situations might be caused by a dysfunction or failure in this process at one level or another. It could be the case for Alzheimer's disease as it was recently demonstrated (Allaman et al., 2010). Indeed, it was found that β-amyloid aggregates alter the metabolic phenotype of astrocytes and in return, affect neuronal viability. As this case illustrates, astrocytes and their metabolic properties might represent an interesting therapeutic target in various neurological diseases. Some studies have already shown the putative neuroprotective impact of modifying the intrinsic metabolic characteristics of astrocytes, either by exposing them to specific trophic factors (Escartin et al., 2007) or by overexpression of intrinsic metabolic components (Bliss et al., 2004).

Based on what we have seen so far about their metabolic capacities, it is clear that astrocytes have not finished to surprise and fascinate us. And this is precisely what we expect from the stars of the brain.

AUTHOR CONTRIBUTIONS

Anne-Karine Bouzier-Sore and Luc Pellerin conceived the manuscript, wrote and corrected the text.

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D-serine as a gliotransmitter and its roles in brain development and disease

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The development of new techniques to study glial cells has revealed that they are active participants in the development of functional neuronal circuits. Calcium imaging studies demonstrate that glial cells actively sense and respond to neuronal activity. Glial cells can produce and release neurotransmitter-like molecules, referred to as gliotransmitters, that can in turn influence the activity of neurons and other glia. One putative gliotransmitter, D-serine is believed to be an endogenous co-agonist for synaptic *N*-methyl-D-aspartate receptors (NMDARs), modulating synaptic transmission and plasticity mediated by this receptor. The observation that D-serine levels in the mammalian brain increase during early development, suggests a possible role for this gliotransmitter in normal brain development and circuit refinement. In this review we will examine the data that D-serine and its associated enzyme serine racemase are developmentally regulated. We will consider the evidence that D-serine is actively released by glial cells and examine the studies that have implicated D-serine as a critical player involved in regulating NMDAR-mediated synaptic transmission and neuronal migration during development. Furthermore, we will consider how dysregulation of D-serine may play an important role in the etiology of neurological and psychiatric diseases.

Keywords: gliotransmission, D-serine, NMDA receptors, schizophrenia, depression and anxiety disorders, neural development, glycine site, astrocyte–neuron interactions

INTRODUCTION

Two decades ago a revolutionary discovery identified the presence of the D-amino acid D-serine in mammalian brain. The discovery that D-serine is an abundant amino acid in the brains of rodents and humans led to extensive experimental inquiry into how this amino acid, which was previously thought to have no specific biological function, might be involved in normal brain development and function. In this review we will consider the development of analytical techniques that have been used to identify and localize D-serine within mammalian brains and review what is now known about its important biological function during brain development.

D-SERINE LOCALIZATION WITHIN GLIA IN SPECIFIC BRAIN AREAS IN PROXIMITY TO NMDARs

With the development of sensitive analytical techniques, such as gas chromatography and mass spectrometry, D-serine was discovered in mammalian brains about 20 years ago. In particular, Hashimoto et al. (1992) showed, using these two techniques, that D-serine was present in rodent and human brains at significantly higher concentrations than other D-amino acids, such as D-aspartate and D-alanine (Hashimoto et al., 1992, 1993a). Moreover, they showed that the distribution of D-serine paralleled the distribution of *N*-methyl-D-aspartate (NMDA) type glutamate receptors (Hashimoto et al., 1993a; **Figures 1A** and **B**). While previous work had determined that D-amino acids were capable of binding the glycine modulatory site of the NMDA receptor (Kleckner and Dingledine, 1988), the findings of Hashimoto et al. (1992, 1993a,b) pointed to D-serine as a potential endogenous co-ligand for the NMDA receptor. This role for D-serine as an endogenous NMDA receptor co-ligand helped explain the observation that glycine is not typically enriched in brain regions that have high levels of NMDA receptor expression (Schell et al., 1997; **Figure 1C**). Furthermore, D-amino acid oxidase (DAAO), the enzyme that degrades D-serine, had been discovered in mammals (Weimar and Neims, 1977; Horiike et al., 1987) before this demonstration of endogenous D-serine. Thus, with the discovery of D-serine, the ability of endogenous NMDA receptors to function in the absence of glycine and the presence of DAAO in the central nervous system (CNS) were both demystified.

Since the initial detection of D-serine in the CNS, subsequent immunostaining studies have more precisely localized D-serine and DAAO within specific brain areas and specific cell types. In the adult brain, areas with particularly high levels of NMDARs, including the cerebral cortex, hippocampus, thalamus, hypothalamus, amygdala, and retina are enriched in D-serine whereas other brain regions, such as the hindbrain, pons, and medulla have virtually undetectable levels of D-serine (Schell et al., 1995). Intriguingly, the localization of DAAO is opposite that of D-serine; areas including the cortex, which have high levels of D-serine have very low levels of DAAO.

After looking more closely at the distribution of D-serine amongst different cell types Schell et al. (1995) made the striking and important discovery that D-serine is localized principally within glial cells. Specifically, they found that type-2 astrocytes, cultured from cerebral cortex, expressed particularly high levels of

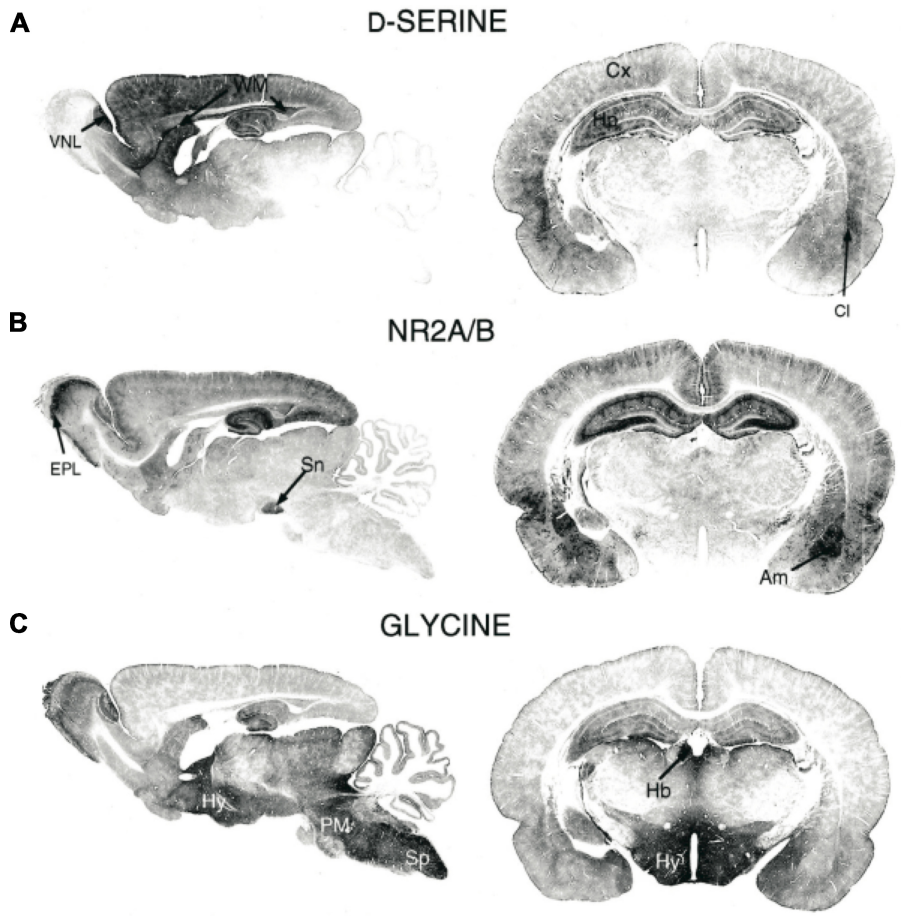

FIGURE 1 | Immunohistochemical staining of (A) D-serine, (B) NMDAR subunits GluN2A/B, and (C) glycine in rat brains (P21). Am, amygdala; Cl, claustrum; Cx, cortex; EPL, external plexiform layer; Hb, habenula; Hy, hippocampus; PM, pons/medulla; Sn, substantia nigra; Sp, spinal cord; WM, white matter; VNL, vomeronasal nerve layer. Figure modified from Schell et al. (1997).

D-serine. Additional studies in other brain areas have also localized D-serine within astrocytes. In the retina, Stevens et al. (2003) found D-serine in astrocytes and Müller glia cells. Furthermore, studies in the hippocampus, hypothalamus, vestibular nuclei (VN), and cerebellum have all placed the main expression of D-serine within glia cells (Mothet et al., 2000; Kim et al., 2005; Panatier et al., 2006; Puyal et al., 2006). Accumulating evidence, however, suggests that the synthesis, storage, and release of D-serine may not be limited exclusively to astrocytes, but rather may involve distinct functions for different cells.

D-SERINE SYNTHESIS AND DEGRADATION

The key enzyme thought to be responsible for the synthesis of D-serine is serine racemase (SR). SR, which converts L-serine to D-serine, was initially found in astrocytes and microglia in the mammalian brain (Wolosker et al., 1999; Stevens et al., 2003; Wu et al., 2004; Panatier et al., 2006). More recently, however, SR was also identified in neurons (Kartvelishvily et al., 2006; Yoshikawa et al., 2007; Dun et al., 2008; Miya et al., 2008; Wolosker et al., 2008; Rosenberg et al., 2010) challenging the traditional view that D-serine is generated solely by astrocytes. In fact, SR was found to be expressed at significantly higher levels in neurons than in astrocytes. A study that compared SR and D-serine levels in cell type-specific SR knockout mice found that SR levels were lower in mice with neuronal knockout of SR than in mice with astrocyte-specific knockout of SR (Benneyworth et al., 2012). Surprisingly, despite a significant reduction of SR protein in neuronal SR knockout mouse brains, D-serine levels were only minimally reduced indicating that neurons are not the sole source of D-serine.

REGULATION OF SR

A number of proteins that interact directly with SR have been identified, including the Golgin subfamily A member 3 protein (Dumin et al., 2006), the glutamate receptor interacting protein (GRIP; Kim et al., 2005), and the protein interacting with C-kinase 1 (PICK1; Fujii et al., 2006; Hikida et al., 2008). Furthermore, a more recent study showed that protein kinase C (PKC), which interacts directly with PICK1, is able to phosphorylate SR, leading to a corresponding decrease in D-serine levels *in vitro* and *in vivo* (Vargas-Lopes et al., 2011). There is also evidence suggesting that activation of erythropoietin-producing hepatocellular carcinoma (Eph) receptors decreases interaction between Eph–PICK1 and

instead favors the SR–PICK1 interaction, resulting in a subsequent increase in D-serine synthesis in cultured hippocampal astrocytes (Zhuang et al., 2010).

THE D-SERINE SHUTTLE HYPOTHESIS

In addition to catalyzing the conversion of L-serine to D-serine, SR can also cause the degradation of serine through the biochemical elimination of water, resulting instead in the production of pyruvate and ammonia (De Miranda et al., 2002). This degradation function of SR is likely to be important in regions of the brain, including the forebrain, that have with low levels of DAAO expression (Hashimoto et al., 1993b; Nagata et al., 1994). Astrocytes, having lower levels of SR compared to neurons, would be ideally suited for the safe storage of D-serine, effectively sequestering it from degradation by neuronal SR.

Interestingly, L-serine and its precursors are not abundant in neurons but found primarily in glial cells suggesting that although neurons have high levels of SR they require an external source of L-serine. For example, 3-phosphoglycerate dehydrogenase (Phgdh) an enzyme that catalyzes the formation of L-serine from glucose is localized almost exclusively in astrocytes (Furuya et al., 2000; Yamasaki et al., 2001) and a recent study has shown that a conditional deletion of Phgdh results in a significant decrease in both L- and D-serine levels in adult cerebral cortex and hippocampus (Yang et al., 2010). It has been suggested that the biosynthetic pathway for L-serine may be located in astrocytes but not neurons, requiring the transport of astrocytic L-serine to neurons where it can then be converted to D-serine for subsequent storage back in astrocytes.

Taken together there is accumulating evidence supporting a "D-serine shuttle hypothesis" which proposes that D-serine synthesized in neurons is shuttled to astrocytes where it is stored and released (Wolosker, 2011; **Figure 2**). Amino acid transporters have been identified in astrocytes and neurons (Yamamoto et al., 2004) and are thought to play an important role the transfer of amino acids between neurons and glia. Specifically, Na^+-dependent ASCT1 and ASCT2 and Na^+-independent alanine–serine–cystein transporter-1 (Asc-1) are two types of transporters that regulate D-serine levels. Of these, Asc-1, which is found exclusively in neurons, has a higher affinity for D-serine than ASCT1 and ASCT2 (Fukasawa et al., 2000; Helboe et al., 2003) and activation of Asc-1 by D-isoleucine has recently been shown to increase D-serine levels and to play a role in modulating synaptic plasticity (Rosenberg et al., 2013).

While it is generally agreed that astrocytic D-serine is necessary for normal glutamatergic transmission, the relative contribution of neuron- versus astrocyte-derived D-serine remains controversial and is likely to change over development and to differ by brain region.

RELEASE OF D-SERINE

A number of studies have now clearly shown that the release of D-serine from astrocytes can be stimulated with the application of non-NMDA glutamate receptor agonists (Schell et al., 1995; Ribeiro et al., 2002; Mothet et al., 2005; Sullivan and Miller, 2010).

Using a sensitive chemoluminescence assay, Mothet et al. (2005) were able to demonstrate that D-serine release from cortical cultured astrocytes is evoked by glutatmate, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) or kainic acid application, and is inhibited in the presence of AMPA blockers. The AMPA-evoked release of D-serine has been further supported by studies in other brain areas. Using capillary electrophoresis in the intact retina Sullivan and Miller (2010) have shown AMPA stimulates D-serine release and that release is abolished in the presence of a glial toxin. Furthermore, in primary glial cultures from cerebellum, activation of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPARs) has also been shown to trigger activation of SR by binding to GRIP to drive subsequent efflux of D-serine (Kim et al., 2005).

One important but somewhat contentious observation is the finding that release of D-serine from astrocytes may involve vesicular trafficking. Using the chemiluminescence assay for measuring D-serine, Mothet et al. (2005) found evidence that D-serine release is a SNARE (soluble NSF attachment protein receptor) protein-dependent process that requires calcium influx. D-serine release from astrocytes is impaired by applying tetanus toxin light chain, an endopeptidase that cleaves SNARE proteins necessary for vesicular fusion (Martineau et al., 2008). Furthermore, more recent studies in hippocampal slices have provided additional evidence that at least some of astrocytic D-serine release relies on vesicle-associated membrane protein (VAMP)-dependent exocytosis (Henneberger et al., 2010). In contrast, Rosenberg et al. (2010) showed that activation of volume-regulated anion channels (VRACs) resulted in a significant amount of D-serine released from astrocytic cultures, but that blocking vesicular filling using bafilomycin and concanamycin, which are inhibitors of vacuolar ATPase, did not affect D-serine release. Notably, Rosenberg et al. (2010) highlighted the fact that while both neurons and astrocytes can release D-serine, the mechanisms mediating D-serine release from neurons are likely to be different from those in astrocytes. They found that depolarization of neurons in cortical slices using veratridine, which induced D-serine release by neurons but not by astrocytes, resulted in 10-fold greater levels of D-serine release than AMPA receptor activation, which is thought to evoke release from astrocytes.

A recent series of studies has provided new lines of evidence that astrocytes and neurons can release D-serine through different mechanisms. In particular, a detailed analysis of the contents of rat cortical astrocytes has revealed that these cells contain storage vesicles that share features similar to synaptic vesicles. Moreover, they found that cortical astrocytic vesicles contain high levels of D-serine, in addition to other neuromodulators including glutamate and glycine (Martineau et al., 2013). In contrast, Rosenberg et al. (2013) has used both cultured cells and acute hippocampal slices to demonstrate that Asc-1 mediates release of D-serine from cytosolic pools in neurons.

Additional studies are still needed to determine the relative importance and function of astrocytic versus neuronal D-serine. To date, much of what is known about D-serine release comes from *in vitro* studies but it will become increasingly important to accurately measure D-serine concentrations *in vivo*. For example, *in vivo* microdialysis has been used to measure extracellular

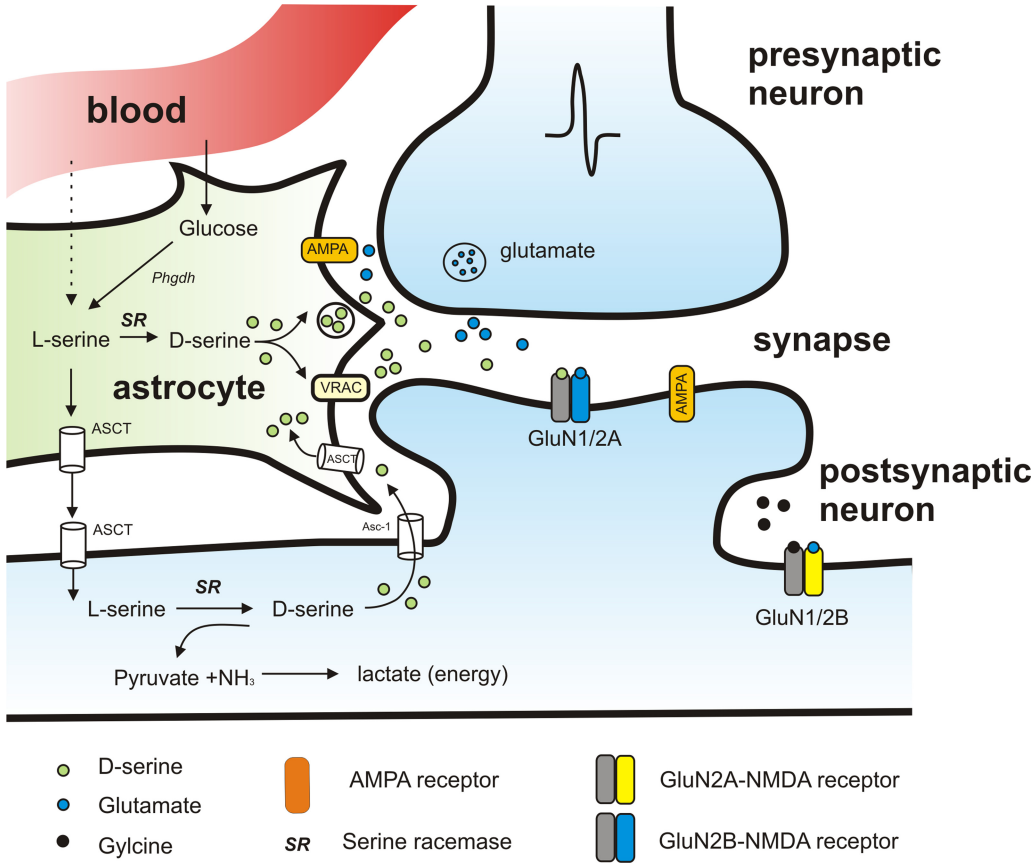

FIGURE 2 | Schematic model of the proposed pathways mediating D-serine synthesis and release. Activation of presynaptic neuron results in release of glutamate that binds to AMPA receptors on neighboring astrocytes and causes release of D-serine. D-serine released from astrocytes binds to synaptic NMDAR-containing GluN2A subunits. Extrasynaptic receptors containing GluN2B preferentially bind glycine instead of D-serine. SR localized in neurons synthesizes D-serine from L-serine. L-serine is shuttled to neurons from astrocytes through amino acid transporters (ASCT). SR is also responsible for the degradation of serine resulting in production of pyruvate and ammonia.

concentrations of D-serine in mouse barrel cortex (Takata et al., 2011). It was found that stimulation of the nucleus basalis of Meynert resulted in an increase in D-serine concentration that could not be induced in inositol-1,4,5-trisphosphate receptor type 2 knockout mice, in which astrocytic calcium fluctuations are reduced.

Recently, implantable amperometric biosensors have been developed which will be extremely valuable for monitoring *in vivo* release of D-serine (Pernot et al., 2008, 2012; Zain et al., 2010). These biosensors consist of a thin platinum wire coated with a semi-permeable layer of poly-*m*-phenylene-diamine (PPD) and a layer of DAAO. Degradation of available D-serine is catalyzed by DAAO, generating hydrogen peroxide that is oxidized at the surface of the platinum wire. The resulting hydrogen peroxide oxidation current corresponds to the D-serine concentration in the surrounding environment. To date, biosensors implanted in the rat brain have been used to detect D-serine *in vivo* (Zain et al., 2010; Pernot et al., 2012). Alternatively, the temporal resolution of *in vivo* microdialysis has been recently improved to the point that it is now possible to detect D-serine levels with a resolution of several seconds (Rosenberg et al., 2010). Additional studies determining what factors influence and modulate D-serine levels *in vivo* are sure to follow shortly.

D-SERINE IS A CRITICAL PLAYER IN NMDAR-MEDIATED SYNAPTIC PLASTICITY

NMDARs are unique in that they require the binding of two agonists to be fully functional. In particular, activation of NMDARs requires the binding of glutamate to the GluN2 subunit as well as the binding of a separate co-agonist to the glycine site of the GluN1 subunit (Johnson and Ascher, 1987). While D-serine was originally identified as an effective co-agonist of the NMDAR (Kleckner and Dingledine, 1988), glycine was thought to be a more likely endogenous candidate because, at the time, more was known about its presence in mammalian brain. In light of the considerable accumulation of evidence that D-serine is indeed present in many mammalian brain areas, D-serine has come to be accepted as an endogenous co-agonist for synaptic NMDARs, involved in modulating synaptic transmission and plasticity. Further support for this idea came from a pioneering study that monitored NMDAR-mediated synaptic transmission in hippocampal cell cultures after the selective degradation of

endogenous D-serine by application of DAAO (Mothet et al., 2000). It was found that when cells were exposed to DAAO, the NMDAR currents were significantly reduced and this reduction could be reversed with application of exogenous D-serine. More recent evidence from Papouin et al. (2012) implicates D-serine as the endogenous co-agonist specifically at synaptic NMDARs in the hippocampus.

As NMDARs are known to be a key player in mediating excitatory transmission and synaptic plasticity, such as long-term potentiation (LTP; Constantine-Paton et al., 1990), astrocyte-derived D-serine is also likely to play an important modulatory role. Yang et al. (2003) designed an elegant experiment to address this question *in vitro*. Specifically, the ability to evoke LTP in cultured neurons was compared between cells grown in direct contact with astrocytes and those grown without direct contact. Strikingly, they found that LTP could not be induced in the neurons that were not in direct contact with astrocytes unless the cells were supplemented with an exogenous source of D-serine (Yang et al., 2003). The important contribution of D-serine to activity-induced synaptic plasticity has since been further confirmed in other brain areas including the hypothalamus, retina, and prefrontal cortex (Panatier et al., 2006; Henneberger et al., 2010; Stevens et al., 2010; Fossat et al., 2012).

In the hypothalamus, Panatier et al. (2006) took advantage of the fact that the physical association of astrocytes with neurons in the supraoptic nucleus (SON) changes during pregnancy. In particular, during lactation the release of the hormone oxytocin causes a retraction of astrocytic processes from synaptic sites in the SON, which results in a decrease in available D-serine at the synaptic cleft. Panatier et al. (2006) found a corresponding decrease in NMDAR-mediated synaptic activity as well as an increase in the threshold required to induce LTP in the SON.

More recently, Henneberger et al. (2010) have extended these findings by providing evidence that the release of D-serine from astrocytes in the hippocampus is likely to be a calcium-dependent process and that an individual astrocyte can contribute to NMDAR-dependent plasticity in many surrounding neuronal synapses. Specifically, using a technique they developed called "calcium-clamping" where intracellular astrocytic calcium was chemically clamped at a fixed concentration, and therefore unable to fluctuate rapidly, they were able to show that LTP could no longer be induced in neighboring neurons unless exogenous D-serine was supplemented. A similar result was obtained by loading astrocytes with tetanus toxin to prevent VAMP-dependent vesicular release. Notably, they also showed that one astrocyte in the hippocampus appeared to be responsible for a specific territory of neighboring neurons and that neurons found outside of this specific area (i.e., $>200\ \mu m$) were unaffected by these manipulations. These specific findings were somewhat surprising since the anatomical territory of an astrocyte in the hippocampus is generally considered to be only about 50–100 μm large, raising the possibility that communication between adjacent astrocytes may participate in this phenomenon.

An interesting feature of NMDARs is that they are found both synaptically and extrasynaptically. The NMDAR has been shown to have different physiological functions depending on its location. While NMDARs found at synapses have a well-established role in mediating synaptic LTP, extrasynaptic NMDARs have been implicated in other forms of plasticity, as well as in the pathogenesis of certain neurodegenerative diseases including Huntington's and Alzheimer's disease (Kaufman et al., 2012).

Recent results have shed some light as to why synaptic versus extrasynaptic NMDARs may have distinct physiological functions. Papouin et al. (2012) have shown that synaptic and extrasynaptic NMDARs in the hippocampus preferentially bind different co-agonists. In particular, by applying enzymes to selectively degrade either D-serine or glycine they were able to demonstrate that D-serine preferentially affects synaptic NMDARs whereas glycine preferentially affects extrasynaptic receptors. When D-serine was degraded using DAAO, synaptically mediated NMDAR functions, including LTP, were abolished. Conversely, the application of glycine oxidase, an enzyme that specifically degrades glycine, had no effect on synaptic excitatory potentials.

N-methyl-D-aspartate receptor affinity for binding D-serine versus glycine depends on its GluN2 subunit composition. While NMDARs composed of GluN2A subunits exhibit a high affinity for D-serine, NMDARs composed of GluN2B subunits preferentially bind glycine (Madry et al., 2007). Papouin et al. (2012) took advantage of the existence of antagonists with preferential affinities for GluN2A and GluN2B to further determine the respective contributions of D-serine and glycine in regulating synaptic versus extrasynaptic NMDAR activation. They found that NMDAR-mediated post-synaptic potentials were reduced in the presence of free Zn, an antagonist of GluN2A-containing receptors, but were unaffected in the presence of Ro25-6981, a selective GluN2B antagonist. To study the endogenous co-agonist of extrasynaptic NMDARs, synaptic receptors were silenced using MK801. Under synaptic NMDAR blockade, the spared extrasynaptic NMDA-evoked responses were decreased when glycine was specifically degraded. These results indicate that that while D-serine is an important co-agonist of synaptic NMDARs, glycine may function as the principal partner for extrasynaptic receptors.

DEVELOPMENTAL EXPRESSION OF D-SERINE AND THE DEVELOPMENT OF GLUTAMATERGIC SYNAPSES

During early development neurons undergo extensive synaptic refinement and maturation as they establish functional neuronal circuits. It has been well-established that NMDARs play a critical role in the development of neuronal circuits. Blocking their activity has been shown in many cases to disrupt normal activity-dependent map formation (Constantine-Paton et al., 1990; Ruthazer and Cline, 2004; Espinosa et al., 2009; Erzurumlu and Gaspar, 2012).

Immunohistochemical localization studies of D-serine in rat brain have illustrated that D-serine levels change over different stages of development. For example, the VN, an area specialized in controlling balance and spatial orientation, have high levels of D-serine during the first 3 weeks of post-natal development, which gradually decrease with age (Puyal et al., 2006). Furthermore, in the cerebellum, a brain region important for motor coordination and learning, D-serine levels are high during the first weeks after birth but then rapidly decline to almost undetectable levels by P26. As in other brain areas, D-serine has been localized

within specific glia cells bodies of the cerebellum. In particular, high levels of D-serine are found in the radial processes and end feet of Bergmann glia surrounding Purkinje cell dendrites (Schell et al., 1997; Wolosker et al., 1999). Notably, the drop in D-serine levels parallels the increase in expression of DAAO in cerebellum (Weimar and Neims, 1977; Horiike et al., 1987).

During early post-natal development the cerebellum undergoes a series of important developmental changes that are essential for forming a functional cerebellar circuit. It is a time when parallel and climbing fibers form stereotypic synaptic connections with Purkinje cells. This establishment of functional synapses has been shown to be dependent on NMDARs since blocking the receptors prevents the formation of proper synapses (Rabacchi et al., 1992). Given the tight physical interaction between Bergmann glia and Purkinje cells it has been proposed that D-serine release from Bergmann glia may be an important player in the development of functional synapses in the cerebellum (Schell et al., 1997), however, the specific involvement of D-serine in establishing connections in the cerebellum remains to be examined. Consistent with the idea that D-serine may play a role in promoting the formation of functional synapses is the recent evidence that synaptogenesis induced by transforming growth factor β-1 (TGF-β1) is dependent on D-serine (Packard et al., 2003; Diniz et al., 2012). In cultured neurons Diniz et al. (2012) have shown that TGF-β1 secreted from astrocytes promotes the NMDAR-dependent formation of synapses. Interestingly, in addition to inducing synaptogenesis, TGF-β1 also results in an increase in extracellular levels of D-serine. Furthermore, application of D-serine alone was sufficient to induce the formation of synapses, similar to TGF-β1 treatment. The synaptogenic property of TGF-ß1 was eliminated when D-serine was reduced by DAAO treatment, suggesting that D-serine release is responsible for TGF-β1-mediated synaptogenesis.

The development and refinement of functional neuronal circuits is mediated, at least in part, by neurotrophins. In particular, brain-derived neurotrophic factor (BDNF) has been found to be an activity-regulated neurotrophin that plays a key role in promoting synapse formation and maturation and in regulating the functional development of neuronal circuits (Huang et al., 1999; Kaneko et al., 2008; Cohen-Cory et al., 2010; Schwartz et al., 2011; Park and Poo, 2012). Consistent with evidence that NMDAR activation can regulate BDNF production, a recent study found that SR knockout mice ($SR^{-/-}$) have a reduction in total BDNF protein levels (Balu et al., 2012). This result further underscores the importance of D-serine as a potential mediator in the establishment of precise neuronal connections during development.

The subunit composition of NMDARs changes over development and by brain region, suggesting that NMDAR subtypes may play different developmental roles. In the hippocampus GluN2B expression peaks early in development while GluN2A expression occurs later. This shift in GluN2A/2B subunit expression has been correlated with the maturation of neuronal circuits and the control of a number of important developmental events (van Zundert et al., 2004; Yashiro and Philpot, 2008). For example, in the rodent visual cortex the developmental switch from GluN2B to GluN2A is delayed by extended dark-rearing (Quinlan et al., 1999).

Recent evidence has shown that the availability of D-serine and glycine preferentially influences the diffusion of NR2B versus NR2A subunits (Burnet et al., 2011; Papouin et al., 2012), suggesting that D-serine may play a role in the development of neuronal circuits potentially by influencing the subunit make-up of glutamatergic synapses. Experiments by Papouin et al. (2012) revealed that, increasing the amounts of D-serine or glycine selectively inhibited surface trafficking of GluN2B and GluN2A, respectively. The mechanisms by which D-serine and glycine inhibit the diffusion of GluN2A or 2B are unknown, however, conformational changes in the receptor have been proposed as a means by which diffusion could be prevented. Furthermore, a DAAO knockdown in mouse cerebellum, resulting in elevated D-serine levels has been shown to lead to a decrease in GluN2A mRNA levels (Burnet et al., 2011). These experiments reveal that D-serine and glycine exert a surprising degree of control over functional NMDAR subunit composition at multiple levels, ranging from synthesis to trafficking to activation.

D-SERINE AND THE DEVELOPMENT OF DENDRITIC MORPHOLOGY

NMDARs are required for the proper development of dendritic arbors. NMDAR blockade in the developing brain results in a drastic decrease in dendritic growth (Rajan and Cline, 1998; Sin et al., 2002). Recently, the importance of D-serine in mediating normal dendritic development has been examined in $SR^{-/-}$ mice (DeVito et al., 2011; Balu and Coyle, 2012). The dendrites of pyramidal neurons in prefrontal cortex, as well as primary somatosensory cortex, of $SR^{-/-}$ mice are less complex and have a shorter total length (Balu et al., 2012). $SR^{-/-}$ animals have a small but significantly reduced cortical volume. As expected, the dendritic arbor changes were not as severe when neuronal SR was deleted in older mice after weaning, indicating that there is an early post-natal critical period in which SR is most effective in affecting dendritic development. The relative contributions of neuronal and astrocytic SR to normal dendritic development and maintenance remain to be fully explored.

NEURONAL MIGRATION

In addition to its important role in the formation and maturation of synaptic contacts, D-serine has been demonstrated to contribute to earlier stages neuronal circuit construction, as a regulator of neuroblast migration in the developing brain. High levels of D-serine have been localized in the radial processes and end feet of Bergmann glia surrounding Purkinje cell dendrites (Schell et al., 1997; Wolosker et al., 1999). During the period of early post-natal development the cerebellum undergoes a series of important developmental changes that are essential for forming a functional cerebellar circuit. Granule cells, the primary excitatory neurons in the cerebellar circuit, migrate from the external to internal granule cell layer and this migration processes has been shown to occur along radial glial cells which give rise to Bergmann glia.

The completion of granule cell migration has been found to correspond to decreasing levels of D-serine suggesting that D-serine may be an important player in the granule cell migration process. Indeed, Kim et al. (2005) found that when D-serine was degraded by DAAO this resulted in a reduction in neuronal

migration by ∼60%, which could be reversed with application of exogenous D-serine. Furthermore, inhibition of SR also was found to result in a reduction in migration. Overall, the evidence suggests that D-serine acts as chemokinetic stimulus to granule cells (Kim et al., 2005).

The cerebellum is a clear and well-studied example of a brain area where neurons migrate along glia to reach their mature position in an organized neuronal circuit. However, radial cell migration is not limited to the cerebellum and thus it will be interesting to further determine how D-serine may be involved in neuronal migration in other brain areas that depend on directed migration, such as neocortex.

D-SERINE AND DISORDERS OF GLUTAMINERGIC FUNCTION

D-serine has emerged as an influential player in the context of psychiatric disease. Motivated in large part by the hypothesis that schizophrenia and depression may represent dysregulation of glutamatergic systems, with a particular emphasis on NMDAR transmission (Carlsson and Carlsson, 1990; Bennett, 2009; Gunduz-Bruce, 2009), there has been an increasing number of studies exploring D-serine, both endogenously as a potential cause and exogenously as a potential therapy for these disease conditions.

SCHIZOPHRENIA

Schizophrenia is characterized by disintegration of thought processes and emotional responsiveness (Lewis and Lieberman, 2000; van Os and Kapur, 2009). The etiology of schizophrenia is believed to involve a combination of genetic and environmental factors. Schizophrenia symptoms can be categorized as positive and negative: positive symptoms include delusions and hallucinations whereas negative symptoms include anhedonia, asociality, alogia, avolition, and blunted emotional response. In addition, schizophrenia patients suffer from varying degrees of cognitive deficit. A model of glutaminergic hypofunction with accompanying disinhibition of the dopaminergic system has gained significant support (Carlsson and Carlsson, 1990; Lisman et al., 2008; Bennett, 2009; Gunduz-Bruce, 2009). This hypothesis, which proposes that NMDAR hypofunction may be responsible in part for schizophrenia, is premised upon the observation that NMDAR antagonists like phencyclidine (PCP) and ketamine are able to induce a wide spectrum of schizophrenia-like symptoms in normal human subjects (Javitt and Zukin, 1991; Krystal et al., 1994; Coyle, 1996; Olney et al., 1999). This hypothesis initially led to the use of D-serine as a potential therapeutic for alleviating schizophrenia (Contreras, 1990). Subsequently, a deficiency in endogenous D-serine availability was proposed as a potential underlying cause of NMDAR hypofunction.

Consistent with this hypothesis, several studies have revealed a reduction in D-serine levels in the plasma and cerebrospinal fluid of schizophrenic patients (Hashimoto et al., 2003; Bendikov et al., 2007; Calcia et al., 2012). This may be explained in part by excessive degradation of D-serine, as supported by observations that DAAO levels and activity have been found to be elevated in post-mortem hippocampus and cortex samples from schizophrenia patients (Madeira et al., 2008; Habl et al., 2009).

Association studies have identified several mutations in human D-serine metabolic enzymes as risk factors for schizophrenia. These include single-nucleotide polymorphism (SNP) variants of SR (Morita et al., 2007), DAAO (Boks et al., 2007; Caldinelli et al., 2013), and the DAAO interacting protein G72 (Muller et al., 2011). Notably, the schizophrenia and depression susceptibility gene Disrupted in schizophrenia-1 (DISC1) appears to directly bind SR, protecting it from ubiquitin-mediated degradation (Ma et al., 2012).

D-serine, as an NMDAR co-agonist, has been tested as a potential therapeutic agent for schizophrenia. D-serine, in combination with antipsychotics, was reported to be more efficient in relieving numerous symptoms of schizophrenia than antipsychotics on their own (Tsai et al., 1998; Heresco-Levy et al., 2005; Kantrowitz et al., 2010). In mice, D-serine successfully reversed schizophrenia-like symptoms, such as the reduction in prepulse inhibition and certain cognitive deficits, caused by administration of NMDAR antagonists (Lipina et al., 2005; Hashimoto et al., 2008; Kanahara et al., 2008). However, chronic administration of D-serine can result in desensitization as well as kidney damage in patients. Thus, a number of alternative NMDAR glycine site agonists have been tested. The antibiotic D-cycloserine and a synthetic amidated tetrapeptide GLYX-13 are both partial agonists of the NMDAR glycine site and therefore do not function identically to the full agonist D-serine (Kanahara et al., 2008; Zhang et al., 2008). The assessment of D-cycloserine effects in schizophrenia patients has not been straightforward (Duncan et al., 2004). It has been proposed that D-cycloserine may only be efficient when used in treatment together with a training paradigm (Gottlieb et al., 2011). Glycine, also a full agonist at the NMDAR glycine site, has been reported to relieve negative and positive schizophrenia symptoms in patients and to do so more efficiently than D-cycloserine (Heresco-Levy and Javitt, 2004). A meta-analysis reviewing a multitude of trials with schizophrenia patients receiving D-serine, glycine and D-cycloserine came to the conclusion that the full agonists were more effective at relieving negative symptoms of schizophrenia than D-cycloserine, but that no glutaminergic therapy successfully attenuated the positive symptoms (Tuominen et al., 2005).

DEPRESSION AND D-SERINE

The etiology of mood disorders [major depressive disorder (MDD), bipolar disorder and dysthymic disorder] is also not well-understood (Hidaka, 2012). MDD constitutes a disturbance of mood accompanied by a selection of psychophysiological changes, such as slowing of speech and action, insomnia or hypersomnia, fatigue, psychomotor agitation or retardation, and loss or increase of appetite (Cassano and Fava, 2002; Belmaker and Agam, 2008). Common psychological symptoms include loss of concentration, perfectionism/obsessiveness, increased sensitivity to criticism, feelings of worthlessness, anhedonia, and suicidal thoughts (Cassano and Fava, 2002). MDD is currently one of the leading causes of disability worldwide (Hidaka, 2012). MDD is generally considered to reflect a malfunction of the monoaminergic systems, but growing evidence also points toward the involvement of excitatory amino acids (Kugaya and Sanacora, 2005). Altered, usually elevated, levels of glutamate

metabolites have been detected in post-mortem human prefrontal cortex (Hashimoto et al., 2007) and blood serum (Mauri et al., 1998). A proton magnetic spectroscopy study, which enabled quantification of glutamate and GABA levels in live depression patients, also revealed higher than normal glutamate concentrations in the prefrontal cortices of MDD patients, together with an abnormal inhibitory/excitatory neurotransmitter ratio (Sanacora et al., 2004).

Chronically stressed animals, considered to be a model for depression, exhibit profound changes in neuroplasticity, specifically impairment in hippocampal and prefrontal cortical LTP induction, and a facilitation of spike-timing dependent long-term depression (Shors et al., 1990; Shors, 2004; Pittenger and Duman, 2008; Marsden, 2013). Chronic restraint stress was shown to result in NMDAR-dependent dendritic atrophy in rat medial prefrontal cortex, that could be prevented by CPP administration (Martin and Wellman, 2011). The evidence for NMDAR involvement in animal depression models inspired the testing of NMDAR binding compounds. MK-801, AP-7, and 1-aminocyclopropanecarboxylic acid (ACPC) were all found to be as effective as common antidepressants in increasing the performance of mice in stress paradigms like the forced swim and the tail suspension test (Trullas and Skolnick, 1990). Ketamine has been repeatedly shown to relieve depression and anxiety in about 70% of human patients (Sappington et al., 1979; Zarate et al., 2006; Diazgranados et al., 2010).

Interestingly, whereas ketamine, MK-801 and AP-7 are all NMDAR antagonists, ACPC is a partial agonist for the NMDAR glycine site. Subsequently D-serine was tested on the so-called "Flinders Sensitive rat" strain that displays symptoms similar to human depression patients, for example reduced interest in reward and increased fear response (Overstreet et al., 2005). This rodent model for depression exhibits increased hippocampal glutamatergic activity. Its hippocampi appear to contain lower than normal levels of D-serine, as measured by enantioselective chromatography, perhaps due to a homeostatic compensation for the elevated glutamate, and are impaired for CA1 LTP induction. Flinders Sensitive rats were found to have worse object-recognition memory, which could be rescued by an acute injection of D-serine (Gomez-Galan et al., 2012). Similar benefits of acute D-serine injection were demonstrated in the Wistar Kyoto rat, another stress-sensitive animal model for depression studies (Malkesman et al., 2012). Importantly in this latter study, NMDARs in the forebrain were confirmed as the site of action of the D-serine treatment by demonstrating that rescue was not effective in forebrain targeted NR1 knockout mice. While it is difficult to distinguish between a specific requirement for NMDAR transmission and general excitatory transmission, such results provide support for the idea that disrupted NMDAR function may be an underlying factor in depression.

COMMON SENSITIVITY TO D-SERINE IN SCHIZOPHRENIA AND DEPRESSION

It is intriguing to note that while depression is thought to constitute a hyperglutaminergic condition, relieved by NMDAR antagonists, schizophrenia is seen as a hypoglutaminergic disease worsened by NMDAR antagonists. Nonetheless, D-serine appears to mitigate both of those disorders. Similarly, whereas low plasma and cerebrospinal fluid (CSF) levels of D-serine are reported in schizophrenic patients, the Flinders Sensitive rat model for depression also exhibited lower than normal levels of D-serine in hippocampus. In the latter case, this was speculated to be the consequence of a protective downregulation of D-serine to spare neurons from excitotoxic effects in this hyperglutamatergic strain (Gomez-Galan et al., 2012). Binding of the glycine site on the NMDAR has been demonstrated to reduce NMDAR desensitization (Mayer et al., 1989). Thus, one possible way to reconcile the paradox of D-serine's dual efficacy in schizophrenia and depression would be if the effects of hyperglutaminergia were in part mediated by NMDAR desensitization, with D-serine both attenuating desensitization and enhancing neurotransmission.

It is also likely that despite similar outcomes of helping restore more normal functional states, the major therapeutic targets of D-serine in schizophrenia and depression are different or at distinct locations. For example, the main site of dendritic atrophy in post-mortem samples from schizophrenia patients is reportedly the prefrontal cortex (Garey et al., 1998; Glantz and Lewis, 2000; Broadbelt et al., 2002; Garey, 2010), whereas in chronically stressed animals, spine and dendrite loss is mostly observed in hippocampus (Magarinos and McEwen, 1995; Magarinos et al., 1996; Luo and Tan, 2001; Chen et al., 2008; Bedrosian et al., 2011) and in some cases in prefrontal cortex (Cook and Wellman, 2004; Martin and Wellman, 2011). Hippocampal neuropil damage is supported by magnetic resonance imaging (MRI) studies in humans suffering from depression (Sheline et al., 1999; von Gunten et al., 2000; Stockmeier et al., 2004; MacQueen et al., 2008). Based on the important contributions of D-serine to normal dendrite formation during development (DeVito et al., 2011; Balu and Coyle, 2012; Balu et al., 2012), it offers great promise as a potential means for helping restore normal dendritic structure and function in schizophrenia and depression, albeit in heterogenous brain areas.

APPLICATIONS OF D-SERINE THERAPY IN OTHER DISEASES

Due to the success of D-serine treatment in relieving symptoms of schizophrenia, its use has been extended to treat a variety of diseases. NMDAR antagonists have been effective in ameliorating Parkinsonian symptoms in animal models (Johnson et al., 2009). A pilot study involving 10 Parkinson disease patients treated with 30 mg/kg D-serine for 6 weeks in addition to their usual medications demonstrated improvements in motor and behavioral deficits (Gelfin et al., 2012). Using an identical treatment regimen, the same group reported anxiolytic properties for D-serine in a group of 22 post-traumatic stress disorder patients (Heresco-Levy et al., 2009). D-cycloserine has been found to have anxiolytic properties in patient cohorts with different phobias or obsessive-compulsive disorder. Interestingly, D-cycloserine seems to be effective only when combined with exposure therapy involving the object of fear or obsessiveness (Kushner et al., 2007; Guastella et al., 2008). Finally, autism has also been viewed by some as a hypoglutaminergic disorder (Carlsson, 1998). In concordance with that idea, high doses of both D-serine and D-cycloserine were found to improve the sociability of an inbred Balb/C mouse strain that exhibits

autism-like behaviors (Jacome et al., 2011; Deutsch et al., 2012). The pre-clinical glycine site partial agonist GLYX-13 likewise has been reported to reverse autism-like symptoms in selectively bred non-social rats (Moskal et al., 2011).

NEURODEGENERATIVE DISORDERS: THE DARK SIDE OF D-SERINE

In addition to alleviating several diseases and conditions, D-serine may have its dark side in neurodegenerative disease. Amyloid beta (Aβ) and secreted amyloid precursor protein appear to induce the release of D-serine and glutamate from cultured microglia by increasing transcription and stabilization of the microglial SR (Wu et al., 2004). This could contribute to excitotoxic neuronal death in Alzheimer's disease. D-serine may play a similar role in amyotrophic lateral sclerosis (ALS) where a principal cause for motoneuron death is considered to be excitotoxicity (Bruijn et al., 2004). The cerebrospinal fluid of ALS patients has been reported to contain elevated glutamate levels (Rothstein et al., 1990). Interestingly, it has been observed that spinal cord in the G93A-SOD1 transgenic mouse model for ALS has several-fold higher concentrations of D-serine than control animals (Sasabe et al., 2007). In these mice, D-serine was found to be produced by activated microglia. The presence of endogenous D-serine has been shown to be necessary for most, if not all, NMDAR-mediated excitotoxicity observed in rat brain slices and *in vivo* (Katsuki et al., 2004; Hama et al., 2006; Inoue et al., 2008). Pretreatment of rat hippocampal organotypic slices with D-serine deaminase was shown to practically abolish NMDA-mediated excitotoxicity, suggesting that D-serine, rather than glycine, participates in these excitotoxic events in the hippocampus (Shleper et al., 2005). Furthermore, SR knockout mice exhibited a 50–60% reduction in the volume of brain damaged in a transient cerebral artery occlusion-induced stroke model. These SR knockout mice were protected against focal cerebral ischemia despite a fourfold increase in NR1 subunits and elevated NMDAR sensitivity (Mustafa et al., 2010). D-serine has also been hypothesized to participate in epileptogenesis. In animals with pilocarpine-induced epilepsy increased D-serine content in astroglia or neurons, but interestingly not microglia, has been reported (Liu et al., 2009).

SUMMARY

Once thought to have no neurological significance, D-serine is now recognized as an important player in normal brain development and function. D-serine is localized in areas of the brain that have high NMDAR expression and is considered to be an important endogenous co-agonist of NMDARs in many brain regions, including the forebrain and hippocampus. D-serine regulates NMDAR-mediated synaptic transmission and plasticity. It has also been shown to be a key mediator in neuronal migration in the cerebellum. Just as our understanding of the various roles for D-serine in brain development has grown more sophisticated, our comprehension of the mechanisms underlying D-serine synthesis and release has also evolved. It has been proposed that neurons, which contain high SR, may play an important role in synthesizing D-serine while glial cells appear to play a more important role in its release (**Figure 2**). Still, our understanding of the specific roles of neuronal versus glial D-serine remain incomplete. Progress has been made using *in vitro* models, but the key issue of how signaling events influence and modulate D-serine levels *in vivo* is still an open question. Overall, there is a great deal of accumulated evidence implicating D-serine as an important player in neuronal development, however, its specific roles in the refinement of functional neuronal circuits remains to be fully explored.

In light of its critical role in the normal development of neuronal circuits, it is hardly surprising that D-serine also participates in adult psychiatric health. D-serine regulation has been investigated extensively as a causative factor and in some cases as a potential therapeutic in schizophrenia, as well as a broad spectrum of other neurological disorders. Curiously, it appears to be beneficial for both NMDAR hypofunction (schizophrenia) and hyperfunction (depression) disease models. The explanation for this might lie in its differential effects on neuronal and glial subpopulations or in the particular brain regions impacted. It is tempting to speculate based on the important roles played by D-serine in the developing brain that its ability to remediate disease may in part depend on enhancing functional connectivity by supporting NMDAR-dependent synaptic plasticity, dendritic arborization, and synaptic transmission in the mature brain.

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