ALL 3 TYPES OF GLIAL CELLS ARE IMPORTANT FOR MEMORY FORMATION

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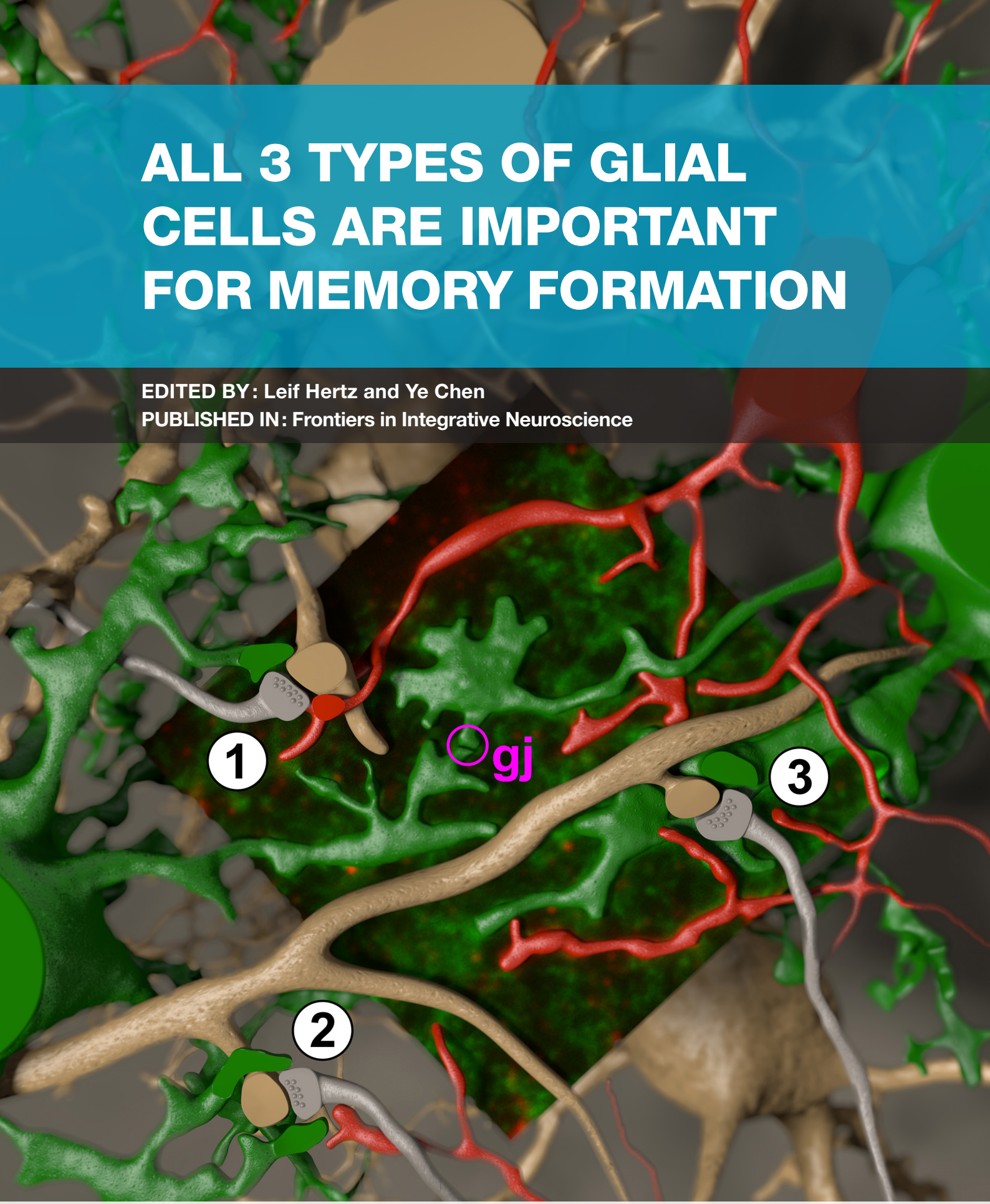

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ALL 3 TYPES OF GLIAL CELLS ARE IMPORTANT FOR MEMORY FORMATION

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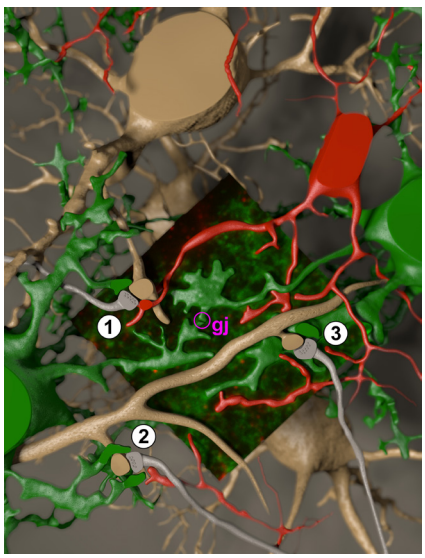

The cover image combines a real fluorescent micrograph of a brain section with a schematic drawing that illustrates mechanisms for complex astrocytic and microglial responses to cannabinoids released from postsynaptic neurons (gray). It depicts close interactions between perisynaptic astrocytic (green) and microglial (red) processes with synaptic neuronal elements, which are of importance for learning and behavior. Postsynaptic neuronal processes are shown in brown, while presynaptic processes are gray with small circles indicating pre-synaptic vesicles. Cannabinoids released from neurons act on glial cells, leading to an increase of free cytosolic glial Ca^{2+} ($[Ca^{2+}]_i$) which subsequently initiates gliotransmitter release and finally influences neuronal activity at locations far away from the initial release site. The activation of microglial CB2 receptors triggers anti-inflammatory responses and regulates synaptogenesis. Microglial effects evoked by other transmitters (e.g., ATP) are known to modify learning as described in the paper by Hristovska and Pascual. Three synapses showing specific sites of neuronal-astrocyte interactions are shown in (1), (2) and (3). The stimulation of psychoactive CB1 receptors (1) increases both astrocytic and microglial $[Ca^{2+}]_i$. The CB1 receptor-mediated rise in astrocytic $[Ca^{2+}]_i$ spreads to near-by synapses contacted by the same astrocyte (2), where it causes release of the gliotransmitter glutamate, acting on presynaptically located metabotropic receptors (mGluR1), which inhibit neurotransmitter release and spatial memory. Other types of stimulated gliotransmitter release (e.g., ATP as described in the paper by Gibbs) can promote learning. IP_3- and gap junction-mediated (gj) spread of astrocytic $[Ca^{2+}]_i$ increase to more distant perisynaptic processes (3) triggers glial release of glutamate acting on postsynaptic NMDA receptors and subsequently inducing slow inward currents with variable effects. Courtesy of Anja Scheller and Frank Kirchhoff: Endocannabinoids and heterogeneity of glial cells in brain function, with both their original and this modified image drawn by Dr. Jens Grosche, Effigos AG, Leipzig, Germany.

The vertebrate brain contains neurons and 3 classical types of glia cells, astrocytes, oligodendrocytes and microglia. Astrocytes and microglia have mainly been studied in gray matter, whereas oligodendrocytes myelinate white matter tracts. Until recently microglial effects were considered mainly during pathological conditions, but is now known that microglia plays important roles also in normal brain function. All these 3 glial cell types and their collaboration with neurons are important for learning. The concept that glia cells are important for cognitive function is not new. A glial-neuronal theory of brain function was proposed by Galambos in 1961. Hyden and Egyhazi demonstrated glial RNA changes in microdissected glia cells during learning in rats in 1963, and astrocytic and oligodendrocytic involvement of K^+-mediated effects of learning has been suggested and/or demonstrated from the 1960's and onwards as recently reviewed by Hertz and Chen (Neuroscience and Biobehavioral Reviews 71, 484-505, 2016). In 1969 van den Berg et al. showed compartmentation of glutamate in brain and thus of production of the neurotransmitters glutamate and GABA, which are essential for learning. That glutamate is synthesized in astrocytes because they in contrast to neurons express the enzyme pyruvate carboxylase was demonstrated 10-15 years later by Yu et al. in cultured astrocytes and Shank et al. in intact brain tissue. However, the present e-book focuses on more recent developments. Most information is available about astrocytic roles in learning. The importance of astrocytes in the tripartite synapse and of microglia in the tetrapartite synapse is illustrated in the front-page figure, which emphasizes the role of gliotransmitters and of Ca^{2+} transport through gap junctions, coupling astrocytes into a functional syncytium. These topics are discussed in detail in the first four papers after the editorial. The e-book subsequently describes that astrocytes are important for establishments of brain rhythms, which may differ in different cognitive tasks, and although the exact reason why knock-out of the astrocytic water channel AQP4 impairs memory remains to be established, several possibilities are discussed. The importance of the two astrocyte specific processes glutamate and glutamine formation and glycogenolysis is discussed in considerable detail. Glycogenolysis is important not only for astrocytic processes involved in learning, but also for those in neurons because glycolytically derived lactate has signaling functions in the extracellular space and may be accumulated in minute quantities into very specific and small neuronal structures. Some neurotransmitters stimulating glycogenolysis are also involved in psychiatric disease. Noradrenaline, released from locus coeruleus exerts direct effects on both astrocytes and neurons and in addition promotes secretion of corticotropin-releasing hormone and adrenocorticotrophic hormone (ACTH) in brain, and of glucocorticoids from the adrenal cortex, all of which are responsible for stress effects on learning. Lead causes memory impairment by inhibition of glutamine formation due to oxidative stress and reduced effectiveness of the glutathione system. The many adverse effects of fetal alcohol exposure on behaviour and learning are caused by a multitude of effects on all three types of glia cells. Traumatic brain injury also exerts multifactorial effects, including microglia/astrocyte-induced secretion of neuroinflammatory molecules and axonal disruption and oligodendrocytic dysfunction. In normal brain oligodendrocytes respond to the depolarization caused by neuronal activity with accelerated conduction velocity and increased compound action potentials which facilitate learning.

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EDITORIAL
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Editorial: All 3 Types of Glial Cells Are Important for Memory Formation

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Keywords: Aquaporin4 and memory, Astrocytes in memory, Brain rhythms and memory, Fetal alcohol syndrome, Glutamate, glycogen and glucocorticoids in memory, Microglia in memory, Neuro- and glio-transmitters in memory, Traumatic brain injury and memory

The Editorial on the Research Topic

All 3 Types of Glial Cells Are Important for Memory Formation

This editorial review of the research topic describes effects of the glial cells astrocytes, microglia and oligodendrocytes on memory. This includes their interactions with themselves and with neurons, and changes in their histology, physiology, and metabolism observed during learning.

ASTROCYTES

As described in Scheller and Kirchhoff astrocytes express receptors for many transmitters, including glutamate and noradrenaline, as well as for cannabinoids (CB1 and CB2 receptors). Receptor stimulation increases free cytosolic calcium $[Ca^{2+}]_i$, which is an extremely important signal involved in the activation of gliotransmitter release (see also Zorec et al.; Orellana et al.). CB1 receptors are expressed on virtually all brain cells and are partly responsible for cannabis' addictive properties. The role of this receptor in learning is not known. However, cannabis users display specific behavioral changes, including memory impairment, which is abolished in conditional mutant mice lacking CB1 in astrocytes, but conserved in mice lacking this receptor in glutamatergic or GABAergic neurons (Han et al., 2012). CB1-induced increase of $[Ca^{2+}]_i$ acts differently when it spreads in a single stimulated astrocyte to distant synapses and when it spreads through gap junctions to distant astrocytes. In the former case, it acts on presynaptic metabotropic glutamate receptors to generate persistent changes in synaptic transmission, and in the latter, the increased $[Ca^{2+}]_i$ releases glutamate which acts on postsynaptic NMDA receptors. On microglia and endothelial cells, stimulation of CB2 receptors has anti-inflammatory effects (Scheller and Kirchhoff).

Gap junctions are formed by paired connexin hemichannels. As described in Orellana et al. connexin hemichannels and pannexins transport gliotransmitters out of astrocytes. These channels also carry lactate, redox molecules and ions, and in the lateral amygdala blockade of Cx43 hemichannels inhibits consolidation of fear memory (Orellana et al.).

Like neurons, astrocytes also release transmitters from vesicles (Zorec et al.), and this release mode is energetically more effective than release via hemichannels (Guček et al., 2012). As described in Zorec et al. astrocytic gliotransmitter responses modify synaptic plasticity and function in different aspects of learning. Vesicular release from astrocytes in response to transmitter stimulation is slower than neuronal transmitter release, consistent with time-dependent memory formation.

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MICROGLIA

As described in Hristovska and Pascual microglia plays important roles in physiological brain activity although they were previously supposed to operate only under pathological conditions. Neuronal activity, sensory experience and neurotransmission modulate their high mobility, process extension and retraction and secretion of ATP among others. Their great plasticity enables them to sense activity, and to regulate synaptic inputs by the secretion of signaling molecules and pruning of synapses.

OLIGODENDROCYTES

Yamazaki et al. (2014) demonstrated that oligodendrocytes in white matter respond *in vivo* to neuronal activity with prolonged depolarization of membrane potential, which accelerates axonal conduction and increases the number of compound action potentials and thus facilitates learning.

GLIAL EFFECTS ON BRAIN RHYTHMS VIA INTERACTION WITH SYNAPSES

Tewari et al. describe in rodents that a single astrocyte can interact with 100,000 synapses and in primates and humans with up to 2 million synapses. This large number makes it impossible to experimentally study the full range of interactions. This problem, however, can be partially overcome by mathematical modeling showing that astrocytes greatly increase the amplitude and frequency of neural oscillations and moreover, that different frequencies are associated with specific aspects of learning (Tewari et al.).

Tewari and Parpura noted that neuronal spiking activity, measured as local field potentials (LFPs), represents averages of synaptic currents. These authors suggested that delta oscillations are astrocyte-dependent, but others (Lee et al., 2014) reported that astrocytes contribute to gamma oscillations and recognition memory. Probable reasons for this discrepancy and the possibility of different rhythms in different cognitive tasks or regions are discussed in Tewari and Parpura.

WATER TRANSPORT IN THE BRAIN

As discussed in Szu and Binder aquaporin4 (AQP4) is expressed in astrocytes and controls bidirectional water transport in brain. Although $AQP4^{-/-}$ mice exhibit only subtle effects on basal synaptic transmission these authors show that AQP4 influences synaptic plasticity, hippocampal long-term potentiation (LTP), long-term depression (LTD), learning and memory. The mechanisms involved are not well understood, but they suggest that in $AQP4^{-/-}$ astrocytes deficient release of brain-derived neurotrophic factor (BDNF) might impair LTP and LTD. Alternatively, glutamate transporter downregulation, excessive activation of NMDA receptors, reduced activity of the co-localized Kir4.1 channel or decreased astrocytic Ca^{2+} entry are suggested as possible inhibitors of LTP in these animals.

METABOLISM: GLYCOGEN, GLUTAMATE, LACTATE, AND GLUCOCORTICOIDS

Glycogenolysis, Glutamate, and Lactate

In astrocytes glycogenolysis is required for release of ATP as a gliotransmitter (Xu et al., 2014) and for K^+ uptake (Xu et al., 2013). Moreover, it provides metabolic energy for Ca^{2+} homeostasis (Müller et al., 2014). All of these effects, including K^+ uptake (Hertz and Chen, 2016), are important for learning. However, the most detailed account of the roles of astrocytic glycogenolysis in learning is given in the paper by Gibbs: During one-trial aversive training in day-old chickens there are 3 brief periods of transmitter-induced glycogenolysis during the first hour, all of which are prevented by the glycogenolysis inhibitor 1,4-dideoxy-1,4-imino-D-arabinitol (DAB). The first of these periods is triggered by serotonin and necessary for an increase in glutamate content, which precedes release of transmitter glutamate transferred to neurons in the glutamate-glutamine cycle. The next is activated by noradrenaline, with β_2-adrenergic stimulation activating glycogenolysis, and α_2-adrenoceptors stimulating simultaneous re-synthesis of glycogen. It probably also activates glutamate synthesis (Hertz et al., 2003), and as discussed by Robinson et al. memory formation is abolished when glutamate transfer from astrocytes to neurons is inhibited. The trigger and purpose of the third glycogenolytic period are unknown, but one function might be to release glutamate-derived lactate to the extracellular space, which is essential for learning (see papers by Steinman et al. and DiNuzzo).

As described in Steinman et al. glycogenolysis is required in rats for hippocampal memory consolidation, LTP and expression of cofilin and other memory-related genes. During glycogenolytic inhibition all of these events can be rescued by lactate administration. Lactate exits astrocytes via monocarboxylate transporters (MCT) 1 and 4 and enters neurons via MCT 2. Blockade of MCT1 or MCT4 expression inhibits memory and associated neuronal gene expressions, showing essential effects of extracellular lactate signaling and/or neuronal lactate uptake. MCT1 and 4 are upregulated by learning, but MCT2 is not (Suzuki et al., 2011; Tadi et al., 2015). Nevertheless, memory in rats is inhibited after MCT2 blockade, implying importance of neuronal lactate uptake, although memory-induced rise in extracellular lactate is modest (Steinman et al.). This suggests that only selected structures are accumulating glycogen-derived lactate, which is consistent with the lack of observed learning-induced down-regulation of MCT2. These structures might be the minute glutamatergic dendritic spines expressing AMPA glutamate receptors. They are enriched in MCT2 co-localized with GluA2/3 (Bergersen et al., 2005), and the actin depolymerizing protein cofilin is required for spine enlargement during early LTP (Rust, 2015). The spines rarely have mitochondria (Bergersen et al., 2005), but MCT2-mediated lactate accumulation and subsequent oxidation to pyruvate would rapidly produce ATP needed for remodeling of actin without interfering with glucose utilization by competition for NAD^+ (Hertz, 2004). LTP dependence on glycogenolysis has been confirmed in brain slices from young rats (Drulis-Fajdasz et al., 2015). Signaling functions of extracellular lactate are

further discussed by DiNuzzo. Stimulation of G_i-protein coupled hydroxycarboxylic acid (HCA) receptors by D- or L-lactate reduces neuronal activity. In contrast, the noradrenergic neurons of locus coeruleus (LC) are stimulated with EC_{50} ~0.5 mM by astrocyte-released L-lactate, but not D-lactate, in a glycogenolysis-dependent manner, a paradigm-shifting observation.

Glucocorticoids

Learning may be enhanced by the release of glucocorticoids from the adrenal cortex evoked by moderate acute stress, while chronic or extreme stress impairs cognitive processes as discussed in Pearson-Leary et al. This increase is caused by noradrenaline-mediated release of corticotropin-releasing hormone (CRH), promoting secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary. Both noradrenaline and glucocorticoids act on neurons, astrocytes, oligodendrocytes and microglia, where they cause release of inflammatory cytokines and immunomodulatory molecules affecting learning.

LEARNING IMPAIRMENT

Exposure to Toxic Substances

Lead is known to cause learning difficulties, which is caused by glutamine synthetase inhibition by oxidative stress and reduced effectiveness of the glutathione system as described in Robinson et al.

Fetal Alcohol Syndrome

As discussed in Wilhelm and Guizzetti alcohol is especially damaging for memory during prenatal exposure. Fetal alcohol spectrum disorders (FASD) lead to a broad spectrum of cognitive and behavioral impairments. Abnormal neuronal and glial migration reflects radial glia impairment, astrocyte proliferation or survival is reduced, and oligodendrocyte development delayed. Disrupted microglial function and survival cause deficient synaptic pruning and abnormal brain plasticity. These glial effects disrupt neuronal development and survival and brain architecture and connectivity.

Traumatic Brain Injury (TBI)

Brain trauma causes microglia-induced astrocyte reactivity and secretion of neuroinflammatory molecules that either impairs axonal regrowth and long-term cognitive outcomes or improves neuronal survival and cognitive abilities over time as discussed in Sajja et al. Axonal disruption and oligodendrocyte dysfunction impair neurotransmission by degeneration of white matter tracts. Astrocytic K^+ channel impairment contributes to cognitive impairment.

Psychiatric Disease

Chen et al. described that whereas astrocytic stimulation of the serotonergic 5-HT_{2B} receptor is essential for learning (Gibbs), down-regulation of the same receptor in these cells by fluoxetine has antidepressant effects, confirming previous findings of a relationship between psychiatric disease and learning.

CONCLUDING REMARKS

Glial effects on learning are exerted by many types of physiological and metabolic effects that influence neuronal activities. The two astrocyte-specific processes glutamate synthesis and glycogenolysis are indispensable for all forms of learning.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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OPINION
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Endocannabinoids and Heterogeneity of Glial Cells in Brain Function

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Keywords: astrocytes, microglia, endocannabinoid system, neuron-glia communication, glial heterogeneity

Attributable to their strong electrical activity, neurons have long been seen as the main determinants of brain function. Over the last decades, however, this view changed dramatically. A variety of specific roles have been assigned to different types of glial cells. Astrocytes constitute the link between the vascular system and neighboring neurons. They determine ion and transmitter homeostasis, metabolism and neuronal activity. Oligodendrocytes form the myelin sheath. They determine fast signal propagation, timing, and synchronicity. Microglial cells comprise not only the innate immune system of the brain, they also actively regulate synaptogenesis and removal of supra-numerous synapses. In general, microglial cells are quite uniformly distributed across different brain regions.

Looking at the system level of the brain, we have to take into account that the description of THE astrocyte as a uniform cell type is clearly outdated. Exploring astrocyte heterogeneity based on localization, function, age, and condition is becoming a major endeavor to fully understand brain function (Oberheim et al., 2012; Bayraktar et al., 2015; Schitine et al., 2015; Bribián et al., 2016). Astrocyte heterogeneity is not only a phenomenon between different brain regions such as cortex, hippocampus, or cerebellum, but also within a given territory. In the healthy, adult cortex the astroglial intermediate filament protein GFAP (glial fibrillary acidic protein) can be hardly detected in most of the astrocytes and only those contacting brain vasculature express significant levels (**Figure 1A**). In contrast, in the hippocampus almost all astrocytes exhibit a strong and steady expression (**Figure 1C**). Another striking example of astroglial diversity is reflected by the expression of various transporters or transmitter receptors. Perisynaptic appendages of cerebellar Bergmann glia are morphologically hard to distinguish from hippocampal astrocyte processes at the ultrastructural level. But, while the first glial cell type is characterized by high levels of AMPA-type glutamate receptor expression, the latter is completely devoid of these receptors (Matthias et al., 2003; Saab et al., 2012). Similar to the heterogeneity of astrocytes within or between given brain regions, we also have to consider a heterogeneity within a single cell given by the highly complex and polarized morphology of astrocytes bridging the gap from the brain capillaries to the neuronal synapses.

Taking into account that a cortical astrocyte contacts up to 600 dendrites, the broad and extended impact of astrocytes on neuronal plasticity becomes evident (Heller and Rusakov, 2015). It is not too tempting to speculate that this feature of astrocytes is less involved in the integration of neuronal signals rather than in modulation and synchronization of neuronal network activity of adjacent microcircuit domains of defined central nervous system (CNS) regions. While astrocytes can directly affect local synapses in the close neighborhood (<20 μm), the gap junction-coupled astroglial syncytium can bridge neighboring microcircuits (**Figure 2**; Navarrete and Araque, 2010; Navarrete et al., 2014).

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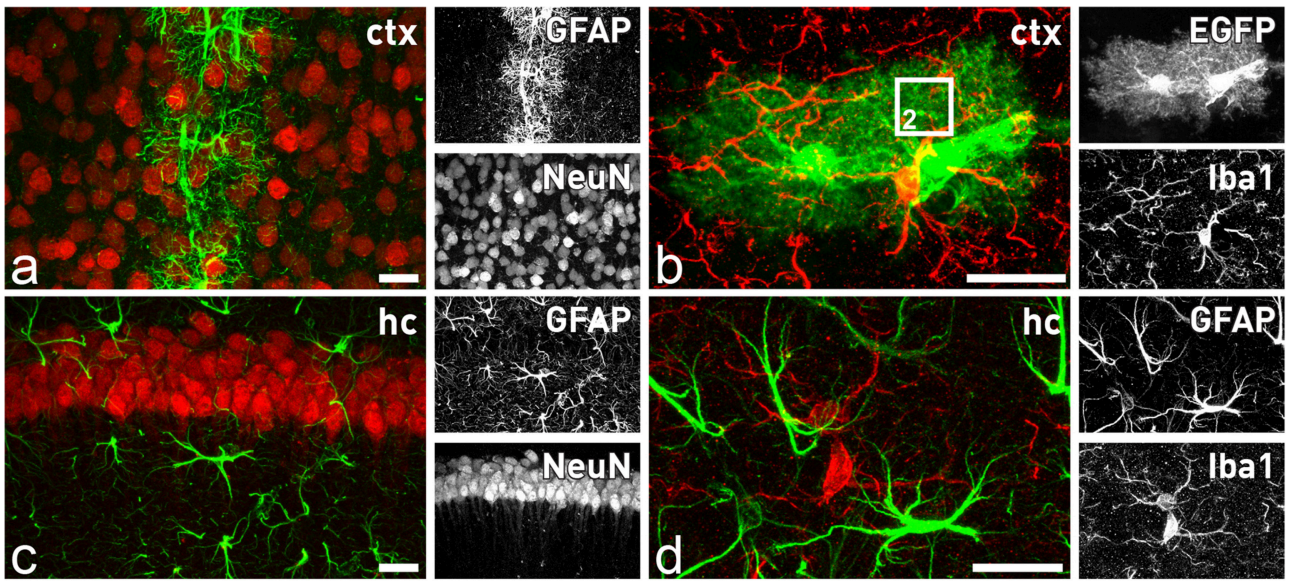

FIGURE 1 | Astrocytes and microglia in the forebrain. Distinct subtypes of astrocytes are present throughout the brain, while microglial cells seem to be more homogenously distributed. In the cortex only astrocytes in close contact to blood capillaries express significant levels of GFAP **(A)**, while all astrocytes are closely intermingled with adjacent neurons **(A)** or microglia **(B)**. In contrast, in the hippocampus all astrocytes express GFAP **(C)**. They are also in close contact to neurons **(B)** and microglial cells **(D)**. Comparison of GFAP staining **(D)** with EGFP expression in **(B)** of TgN $(GFAP\text{-}EGFP)_{GFEC}$ transgenic mice reveals only in the latter the fine arborization of perisynaptic and perivascular astrocytic processes. The square in **(B)** indicates the magnified view that is schematically depicted in **Figure 2**. Scale bars indicate 20 μm.

Common to all glial cells is the expression of a similar set of ionotropic or metabotropic receptors as their adjacent neuronal counterparts. And indeed, glutamate, GABA and ATP have been studied intensively, not only as classical transmitters but also as important molecular entities that constitute various forms of bidirectional communication among neurons and glia. Quite surprisingly, however, the most abundant metabotropic G-protein coupled receptor of the brain is sensitive to none of these important molecules. It is the cannabinoid type I (CB1) receptor which is expressed at variable levels on almost all cells of the CNS and is activated endogenously by two metabolites of arachidonic acid, N-arachidonoyl-ethanolamine (anandamide, AEA) and the more potent 2-arachidonoyl-glycerol (2-AG; Stella, 2010; Boorman et al., 2016). More commonly known is their relative which is found in *Cannabis sativa*, Δ9-tetrahydrocannabinol (THC), the main constituent of marijuana. Like THC, also AEA and 2-AG are psychoactive. While the CB1 receptor is expressed quite uniformly, the cannabinoid type II (CB2) receptor is expressed at low levels, but strongly activated in microglia or endothelial cells in CNS pathologies (Herkenham et al., 1990; Piomelli, 2003; Núñez et al., 2004; Atwood and Mackie, 2010; Zhang et al., 2014; Boorman et al., 2016; Oliveira da Cruz et al., 2016). The lipophilic nature of the endocannabinoids (ECB) together with the broad expression of the CB1 receptor results in more generalized functions in all brain regions. Dependent on the region-specific pattern of neurons and glial cells, activation of the endogenous cannabinoid signaling system can affect numerous neural circuits broadly, ranging from cognition to eating or motor behavior. Here, we would like to discuss the specific functions of CB1 and CB2 receptors on the two glial cell types, astrocytes and microglia in respect to the more recently described cellular heterogeneity.

Frequent use of marijuana by distinct human populations had provided strong insight into the function of the ECB system, the receptors as well as their ligands. Cannabis users exhibited significant distortions of their working and declarative memory. The impaired reality monitoring further resulted in a distinct susceptibility to false memories (Riba et al., 2015). In more controlled animal experiments using rodents, THC induced a combination of physiological/behavioral changes including spontaneous activity, catalepsy, hypothermia, and analgesia (Little et al., 1988; Howlett et al., 2002). Due to distinct expression of the respective receptors, CB1 and CB2, ECB signaling can determine brain functions at different levels. While expression of the CB1 receptor is held responsible for the more psychoactive behavior after activation, the CB2 receptor is more involved in anti-inflammatory processes (Buckley et al., 2000; Mackie, 2005; Buckley, 2008).

In contrast to excitatory transmitters, ECBs are generated and released from activated post-synaptic dendritic terminals and evoke a diversity of complex signaling routes involving neurons and adjacent glia (see **Figure 2**): (1) They act retrogradely at neuronal pre-synapses to control further transmitter release, resulting in *suppression* of excitation (Navarrete et al., 2014). (2) Simultaneously activated CB1 receptors on perisynaptic

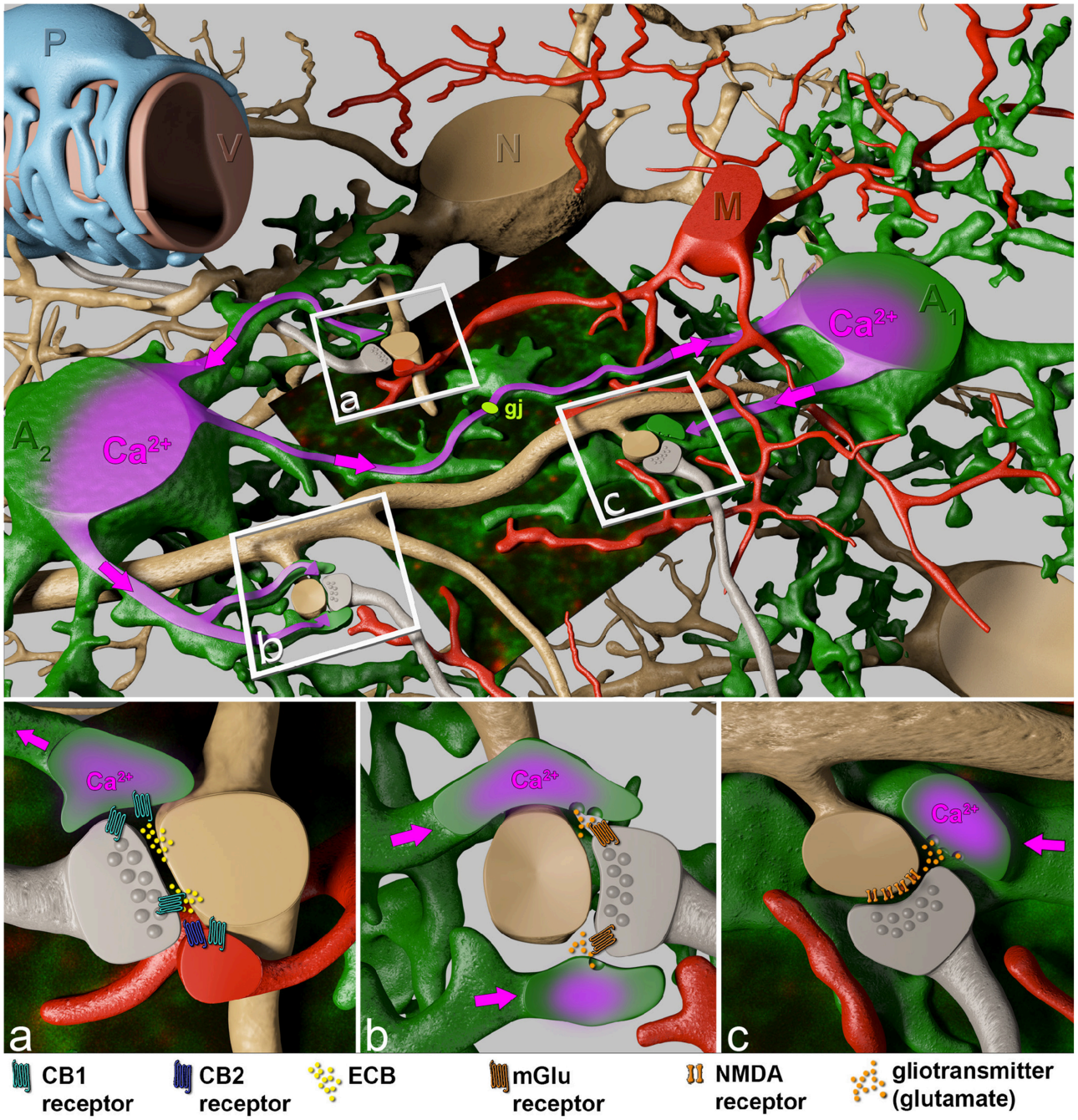

FIGURE 2 | Close interactions of perisynaptic astroglial and microglial processes with the synaptic elements of adjacent neurons. The boxed regions indicate specific sites of neuron-glial interactions. Perisynaptic astroglial processes (green) express the cannabinoid receptors CB1 (cyan), while microglial processes both, CB1 (cyan) and CB2 (blue) receptors in close contact to postsynaptic ECB (yellow) release sites **(A)**. Astrocytes respond to ECB via CB1 receptors with an increase of intracellular Ca^{2+} (purple). This intracellular Ca^{2+} rise spreads (pink arrows) through the astrocyte towards distant synapses **(B)**, where Ca^{2+}-evoked release of the gliotransmitter glutamate (orange) affects neuronal physiology via presynaptic metabotropic glutamate receptors (mGluR1, brown), and generates a persistent synaptic change **(B)**. In addition, the Ca^{2+} wave can be propagated through the gap junction-coupled astroglial syncytium (gj, light green) where on even more distant perisynaptic processes gliotransmitters (glutamate) are released as response to postsynaptic ECB liberation. Subsequently, gliotransmitters can act on postsynaptic NMDA receptors (**C**, brown) inducing slow inward currents.

astroglial processes, however, cause an intracellular Ca^{2+} release from internal stores via the $G_{q/11}$ / phospholipase C / inositol trisphosphate pathway (Navarrete and Araque, 2008, 2010) and stimulate additional release of gliotransmitters, preferentially glutamate, *triggering* presynaptically localized metabotropic glutamate receptors (**Figure 2B**) as well as

postsynaptic NMDA receptors (**Figure 2C**). The depression of excitatory neurotransmission by ECB-evoked presynaptic inhibition of neurotransmitter release impairs spatial working memory (Misner and Sullivan, 1999; Carlson et al., 2002; Takahashi and Castillo, 2006; Bajo et al., 2009; Schoeler and Bhattacharyya, 2013; Schoeler et al., 2016). This inhibiting signaling only works over short distances of less than 20 μm (Navarrete and Araque, 2010). In contrast, ECB-evoked signals in adjacent astroglial processes can affect remote synapses by using the gap junction-coupled astroglial syncytium as a bridge (Navarrete and Araque, 2010; Navarrete et al., 2014; Gómez-Gonzalo et al., 2015; **Figure 2**). Interestingly, the CB1-mediated astroglial release of glutamate can cause both, potentiation as well as depression of neuronal transmission. In the hippocampus, activation of postsynaptic NMDA receptors (**Figure 2C**) induces slow inward currents in CA1 pyramidal neurons (Navarrete and Araque, 2008; Navarrete et al., 2013, 2014), while presynaptic NMDA receptor activation causes spike timing-dependent depression (Min and Nevian, 2012). Presynaptic activation of type 1 metabotropic glutamate receptors (mGluR1) coincident with NO signaling from the postsynapse induces long-lasting synaptic potentiation. mGluR-mediated activation of presynaptic protein kinase C enhances transmitter release persistently (Gómez-Gonzalo et al., 2015). The ECB signaling on astrocytes induces highly selective, circuit-specific modulation of synaptic transmission. In the striatum the astroglial glutamate release acts only on the same subtype of medium spiny neuron (MSN) from which the ECB was released (Martin et al., 2015). The neuronal subtypes can be distinguished by their dopamine receptor expression (D1 and D2). The ECB releasing MSN and the glia-modified neuron have both to express either the D1 or the D2 receptor; no potentiation is detected if one MSN expresses the D1 and the other the D2 receptor or vice versa.

In the hippocampus, the maintenance of epileptic discharges is reduced when the neuron-to-astrocyte communication via CB1 receptor activation is pharmacologically blocked (Coiret et al., 2012). Surprisingly, despite the fact that the CB1 receptor is widely expressed on all hippocampal cells, it was only the astrocyte specific deletion of the CB1 receptor gene that completely eliminated THC-induced depression (Han et al., 2012). In detail, THC stimulates glutamate release from astrocytes by activation of its CB1. The adjacent neuron then shows long-term depression (LTD) by internalizing its AMPA-type glutamate receptors. At the behavioral level, a severe impairment of spatial working memory is observed (Han et al., 2012). But CB1 receptor expression in astrocytes is not restricted to processes at synapses. Astrocytes are also in close contact to blood vessels where the CB1 receptor has been localized to the perivascular endfeet as well (Rodriguez et al., 2001). The functional meaning for this spatial separation is not yet clear. Obviously, the function of the astroglial CB1 receptor is not restricted to neuronal transmission. By controlling local cerebral blood flow, astrocytes adjust the energy supply within a single neuronal microcircuit or even linking adjacent networks, a phenomenon that has been termed neurovascular coupling (Stella, 2010). The modulation of neurovascular coupling by targeting CB1 receptors could become important in novel strategies to combat the sequelae of ischemic insults. Similarly, it will be highly interesting to assign distinct roles of CB1 receptors which are expressed on perisynaptic processes or at the perivascular endfeet to specific behaviors. So far, only learning paradigms have been tested which would favor more the influence of CB1 receptors at the synapse, e.g., the spatial working memory in the hippocampus investigated by Han et al. (2012). It would now be very interesting, though technically challenging, to perform two-photon imaging of the neurovascular unit in experimental mice under different conditions of genetic or pharmacological CB1 receptor modulation and cognitive stress. Curiously, these experiments could be done by the same genetically modified mice (GFAP-CreERT2 × floxed CB1) that Han et al. (2012) had investigated. The GFAP-CreERT2 mouse line shows a more efficient recombination of cortical astrocytes that are part of the neurovascular unit and contact the capillaries (Jahn et al., 2015).

Another important glial cell type involved in ECB signaling are microglia. Their processes that are also in close contact with synapses and blood vessels express both, CB1 and CB2 receptors (Núñez et al., 2004; Maresz et al., 2005; Cabral et al., 2008; **Figures 1B,D**). While these innate immune cells of the CNS express only very low levels of the CB1 receptor, their major player of the ECB signaling game is the CB2 receptor. Under resting conditions the CB2 receptor is weakly expressed as well, but expression levels are highly responsive and get strongly increased upon neuroinflammatory processes associated with brain pathologies (Maresz et al., 2005; Cabral et al., 2008; Atwood and Mackie, 2010; Mecha et al., 2015; Schmole et al., 2015). Interestingly, in contrast to the CB1 receptor, selective agonists of the CB2 receptor are not psychoactive. Instead, the most potent ECB, 2-AG, exhibits strong neuroprotective effects in acute CNS injuries (Ashton and Glass, 2007; Arevalo-Martin et al., 2010). Triggering the microglial CB2 receptor reduces the release of pro-inflammatory cytokines by activated microglia. And similar to astrocytes, there is also a distinct population of microglia that surround the brain capillaries. The perivascular microglia closely interact with the capillary-forming endothelial cells that express CB2 receptors as well. And indeed, pharmacologically selective stimulation of the CB2 receptor stabilized and enhanced the efficacy of the blood-brain barrier (BBB), thereby dampening the consequences of neuroinflammatory injuries (Ramirez et al., 2012). In addition, activation of CB2 receptors signaled into the luminal side of the endothelium and reduced the homing of leukocytes to even further rescue an inflammatory response by recruiting peripheral immune cells, as it could be visualized by repeated long-term two-photon microscopy (Ramirez et al., 2012).

OUTLOOK

Obviously ECB signaling in the brain comes in different glial flavors. While CB1 receptors of perisynaptic astroglial process strongly affect different forms of neuronal plasticity, microglial

and endothelial CB2 receptors provide efficient neuroprotection by reducing neuroinflammatory processes including tightening of the BBB. However, important research questions remain for the future:

What is the function of astroglial CB1 receptors at the perivascular endfeet? In this context it is particularly intriguing that CB1 receptors are not only widely expressed throughout the brain on the cell surface, but also on mitochondrial membranes. Could it be that ECB signaling represents a major regulatory system that regulates energy demands in the brain, acting on a variety of different levels from regulating glucose uptake at the brain vasculature to fine-tuning oxidative phosphorylation in mitochondria?

Does the low level of the CB2 receptor on microglia contribute to normal brain functions? Are there synergistic interactions of the individual components of ECB signaling on different cell types? More cell-specific genetic manipulations of ECB signaling are required. In particular, specific receptor targeting as well as imaging approaches, that will help to unravel the diversity of intracellular signaling cascades, are necessary. Innovative combination of imaging and genetic approaches *in vivo* will pave the way for exciting new findings.

MATERIALS AND METHODS

This study was carried out at the University of Saarland (Center for Integrative Physiology and Molecular Medicine, CIPMM) in strict accordance with recommendations of European and German guidelines for the welfare of experimental animals. Animal experiments were approved by Saarland state's "Landesamt für Gesundheit und Verbraucherschutz" in Saarbrücken/Germany (animal license number: 71/2010). No vulnerable populations (minors, persons with disabilities or endangered animal species) were involved.

Mouse breeding and animal experiments were performed at the animal facility and the research labs of the CIPMM. For the immunohistochemical analysis heterozygous 8-week-old TgN(hGFAP-EGFP)$_{GFEC}$ mice were used (Hirrlinger et al., 2005). Mouse perfusion, tissue fixation and vibratome slice preparation (40 μm) were performed as described previously (Huang et al., 2014). For immunohistochemistry, the following antibodies were used: polyclonal rabbit anti-GFAP (1:1000, Dako Cytomation, Glostrup, Denmark) and anti-Iba1 (1:1000, Wako, Richmond, USA), monoclonal mouse anti-NeuN (1:500, Merck Millipore, Darmstadt, Germany) and anti-rabbit/mouse antibody conjugated Alexa543/633 (1:2000, Invitrogen, Grand Island NY, USA). The transgenic EGFP signal was directly recorded without additional antibody enhancement. Confocal images were taken by a laser-scanning microscope (LSM-710, Zeiss), processed with ZEN software (Zeiss) and displayed as maximum intensity projections. Figures presented in this work were modified with image processing tools of ImageJ (Fiji, www.fiji.sc).

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Integrative Neuroscience

MINI REVIEW
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Memory Formation Shaped by Astroglia

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Astrocytes, the most heterogeneous glial cells in the central nervous system (CNS), execute a multitude of homeostatic functions and contribute to memory formation. Consolidation of synaptic and systemic memory is a prolonged process and hours are required to form long-term memory. In the past, neurons or their parts have been considered to be the exclusive cellular sites of these processes, however, it has now become evident that astrocytes provide an important and essential contribution to memory formation. Astrocytes participate in the morphological remodeling associated with synaptic plasticity, an energy-demanding process that requires mobilization of glycogen, which, in the CNS, is almost exclusively stored in astrocytes. Synaptic remodeling also involves bidirectional astroglial-neuronal communication supported by astroglial receptors and release of gliosignaling molecules. Astroglia exhibit cytoplasmic excitability that engages second messengers, such as Ca^{2+}, for phasic, and cyclic adenosine monophosphate (cAMP), for tonic signal coordination with neuronal processes. The detection of signals by astrocytes and the release of gliosignaling molecules, in particular by vesicle-based mechanisms, occurs with a significant delay after stimulation, orders of magnitude longer than that present in stimulus–secretion coupling in neurons. These particular arrangements position astrocytes as integrators ideally tuned to support time-dependent memory formation.

Keywords: astroglia, memory, shape, metabolism, signaling

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MEMORY FORMATION RESULTS IN ANATOMICAL CHANGES

Memory is the process of retention and reconstruction of learned (acquired) knowledge. Studies performed in the early 1960s on patients who underwent bilateral medial temporal lobe surgery, recognized the hippocampus as a fundamental region for memory formation (Scoville and Milner, 1957). Subsequently, two distinct memory systems, declarative (explicit) memory for facts and events, for people, places, and objects ("knowing that") and non-declarative (implicit) memory, the memory for perceptual and motor skills ("knowing how"), have been defined (Dudai and Morris, 2013). Both systems rely on similar, if not identical, mechanisms associated with reinforcement of synaptic transmission, which involve morphological changes at the synapse that outlast memory stabilization (Attardo et al., 2015). This morphology-based mechanism was considered by Cajal (1894), who linked "cerebral gymnastics" (**Box 1**) with morphological alterations of dendrites and terminals of neurons.

BOX 1 | Cerebral gymnastics and memory formation

"Cerebral gymnastics are not capable of improving the organization of the brain by increasing the number of cells, because it is known that the nerve cells after the embryonic period have lost the property of proliferation; but it can be admitted as very probable that mental exercise leads to a greater development of the dendritic apparatus and of the system of axonal collaterals in the most utilized cerebral regions. In this way, associations already established among certain groups of cells would be notably reinforced by means of the multiplication of the small terminal branches of the dendritic appendages and axonal collaterals; but, in addition, completely new intercellular connections could be established thanks to the new formation of [axonal] collaterals and dendrites."

The Cronian Lecture: La fine structure des centres nerveux. *Proceedings of the Royal Society of London* 55: 444–468, 1984. Translated by DeFelipe J, Jones, E. G. (1988). *Cajal on the Cerebral Cortex*. New York, NY: Oxford University Press. p. 87.

Contemporary views assume that memory formation, although it is an outcome of a myriad of interactive processes, occurs in the form of molecular events at the level of an individual synaptic connection, which is termed synaptic plasticity. These synaptic changes integrate through multiple synaptic connections involving larger neuronal networks, and are finally expressed at the behavioral level (Kandel et al., 2014).

MEMORY FORMATION AND ASTROCYTE MORPHOLOGY

Micro-anatomical changes that are part of memory formation are not exclusively related to neurons and their parts, but involve non-neuronal cells, which in many areas of the human brain exceed the number of neurons (Azevedo et al., 2009). These non-neuronal cells include astrocytes, an abundant and arguably the most heterogeneous glial cell type in the central nervous system (CNS). It is generally acknowledged that astroglia actively participate in information processing via cytosolic calcium signals (Verkhratsky et al., 1998; Rusakov et al., 2011).

A single astrocyte is intimately associated with many neurons and with their synaptic contacts. A single rat cortical astrocyte enwraps 4–8 neuronal bodies and 300–600 dendrites (Halassa et al., 2007), and astrocytes are in contact with synapses. In the rat hippocampus, an individual astrocyte can cover (by perisynaptic processes) up to 140,000 synapses (Bushong et al., 2002). Human hippocampal astrocytes are substantially larger and a single human astrocyte may be associated with ∼2 million synapses (Oberheim et al., 2006). Abundant morphological interactions of astrocytic processes with neurons are not restricted to the hippocampus, being a widespread property of CNS tissue.

Close morphological apposition allows astrocytes to receive signals from the synaptic cleft and feedback by releasing their own signaling molecules. Release of many of these molecules occurs through a secretory pathway that uses cytoplasmic vesicles, which store chemical messengers. On stimulation, the vesicle membrane fuses with the plasmalemma, a process termed regulated exocytosis. The role of secretory vesicles in astrocytes was proposed in 1910 when Nageotte (1910) suggested, based on his microscopic observations, that glial cells (astroglia in particular) act as secretory elements in the CNS. This hypothesis has been confirmed experimentally in the last two decades by identifying vesicular release of gliosignaling molecules, which are often termed gliotransmitters (Vesce et al., 1999; Haydon, 2001; Parpura and Zorec, 2010; Zorec et al., 2012). Although there is some skepticism that this mechanism exists in astroglia (Fujita et al., 2014; Sloan and Barres, 2014), bidirectional astrocyte-neuron signaling is well accepted, and it is generally recognized that vesicle-based mechanisms participate in the heterocellular signaling that occurs at a morphofunctional unit known as the tripartite synapse (Araque et al., 1999; Perea et al., 2009). This bidirectional communication is part of the wider gliocrine system (Vardjan and Zorec, 2015), which reflects the secretory role of astrocytes, which release an extensive number of gliosignaling molecules (Verkhratsky et al., in press). These molecules are largely not involved in synaptic processes but rather regulate various brain functions through "volume" transmission (Vardjan and Zorec, 2015; Zorec et al., 2015). Astroglia-derived signaling molecules are secreted into the extracellular space and are transported throughout the tissue parenchyma to distant places in the CNS, likely taking advantage of the glymphatic convective system (Thrane et al., 2014).

During implicit memory consolidation of Pavlovian threat conditioning, astrocytic processes retract from synapses in the lateral amygdala, allowing these synapses to enlarge, suggesting that contact with astroglial processes opposes synapse growth during memory consolidation (Ostroff et al., 2014). In other words, if astrocytic processes enwrap synapses and the latter need to expand during memory formation, astrocytes may hinder this remodeling, demonstrating how astrocytic structural plasticity enables morphological remodeling of synapses associated with memory formation. Under physiological conditions, including reproduction, sensory stimulation, and learning, astrocytes display a remarkable structural plasticity. Distal astrocytic processes can undergo morphological changes in a matter of minutes, thus modifying the geometry and diffusion properties of the extracellular space and relationships with adjacent neuronal elements, especially with synapses. This type of astroglial plasticity has important functional consequences because it modifies extracellular ionic homeostasis and neurotransmission, thus ultimately modulating neuronal function at the cellular and system levels (Oliet and Piet, 2004; Theodosis et al., 2008). The mechanisms responsible for morphological changes in astrocytes are not known, but these may likely involve adrenergic receptors and generation of second messenger cAMP (Vardjan et al., 2014), which are discussed in the following section.

ASTROCYTE MORPHOLOGY, cAMP AND METABOLISM

Astrocytes are capable of a remarkable morphological plasticity. Astroglial cells *in vitro* have a flattened polygonal appearance,

however stimulation of the β-adrenergic cAMP-dependent signaling cascade results in rapid morphological remodeling with astrocytes assuming a stellate morphology with numerous processes (Shain et al., 1987; Bicknell et al., 1989; Hatton et al., 1991; Shao et al., 1994; Won and Oh, 2000; Gharami and Das, 2004; Vardjan et al., 2014). This remodeling occurs within the time frame of memory consolidation (minutes to hours) and involves cytoskeletal reorganization, including the restructuring of actin filaments, microtubules, and intermediate filaments (Goldman and Abramson, 1990; Safavi-Abbasi et al., 2001). An example of this adrenergic receptor/cAMP-mediated morphological remodeling of astrocytes is shown in **Figure 1** (Vardjan et al., 2014). Similar morphological plasticity may take place *in vivo* in long-term memory formation because noradrenaline (NA), derived from projections of neurons located in the locus ceruleus (LC), operates as a neuromodulator in Hebbian learning (Johansen et al., 2014). Under similar training conditions, changes in astrocytic shape have indeed been observed (Ostroff et al., 2014). Moreover, the existence of structural-functional changes of the astrocyte-neuron interactions during memory processes have been detected (Lavialle et al., 2011; Bernardinelli et al., 2014; Perez-Alvarez et al., 2014).

Tight association between the synaptic membranes and astrocytes is considered essential for homeostatic control of the synaptic cleft, including rapid removal of the neurotransmitter glutamate (Bergles and Jahr, 1997) and potassium from the extracellular space (Orkand et al., 1966; Verkhratsky and Nedergaard, 2014). Thus, retraction of astrocytic membrane from the synapse during memory formation (Ostroff et al., 2014) may facilitate the spillover of neurotransmitter and thus affect synaptic transmission (Rusakov and Kullmann, 1998). At the same time, memory formation is associated with morphological growth of synaptic elements together with enhanced protein synthesis and rearrangement of receptor proteins, all of which increase the energy consumption (Harris et al., 2012).

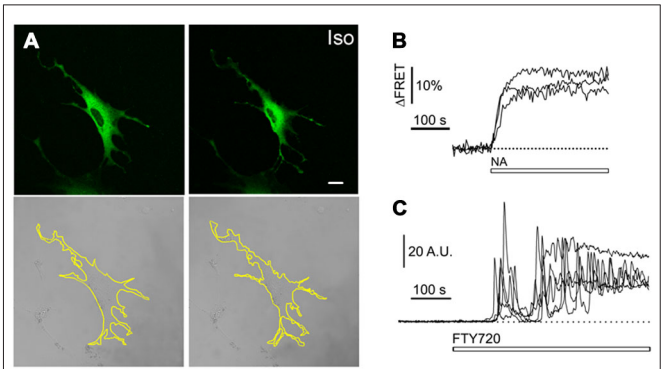

FIGURE 1 | (A) Morphological changes in astrocytes (stellation) induced by the β-adrenergic receptor (β-AR) agonist isoprenaline (Iso), which increases cAMP. Green fluorescing astrocytes transfected with the cAMP nanosensor Epac1-camps (top) and their corresponding differential interference contrast images (bottom) before (left) and after 1 μM β-AR agonist isoprenaline (Iso). Note the thinning and elongation of processes indicating astrocyte stellation. Scale bar represents 20 μm. Astrocytes were cultured from rat cortex. Modified from Vardjan et al. (2014) with permission. **(B)** Time course of cytosolic levels of cAMP. Noradrenaline (NA) persistently increases intracellular cAMP levels in astrocytes. Representative time courses of the Epac1-camps (i.e., a Förster resonance energy transfer (FRET)-based cAMP nanosensor) from three cells after the addition of 1 μM NA. Changes in FRET are expressed as percentages relative to the initial values. **(C)** Time course of cytosolic levels of Ca^{2+}. The application of fingolimod (FTY720) evokes prolonged transient increases (oscillations). Superimposed time-resolved fluorescence intensity obtained in five cells treated with FTY720 (white bar). The thin dotted line indicates the zero fluorescence level (F_0). Modified from Vardjan and Zorec (2015) with permission.

How energy substrates, needed for adenosine triphosphate (ATP) synthesis, are delivered to synapses where synaptic plasticity takes place is still an open question. A simple assumption would be that pyruvate is provided to the mitochondria by glycolysis within the neuron. However, the morphology of astrocytes, with extensive end feet plastering blood vessels, is well suited to take up glucose from blood and distribute either glucose itself, or pyruvate or lactate derived from glucose, to astrocytic processes surrounding synapses, possibly by diffusion through gap junctions integrating astroglial syncytia (Rouach et al., 2008). In support of this mechanism, diffusion of glucose within astrocytes is relatively rapid (Kreft et al., 2013) and may well support glucose delivery via interconnected astrocytes *in situ*. Although synapses are the main energy consumers in the brain, glycogen, the only CNS energy storage system, is present mainly, if not exclusively, in astrocytes. Memory consolidation in young chickens requires glycogenolysis (Gibbs et al., 2006; Hertz and Gibbs, 2009). The successful consolidation of memory from short-term to long-term memory requires neuronal NA release (Gibbs et al., 2010). Therefore, it appears that NA, released from neurons, such as those from locus coeruleus, initiates astrocytic morphological changes and activates astroglial energy metabolism. Thus, NA may be considered as an integrator of the metabolism, morphology and function of astrocytes. In the adult operational (i.e., awake) brain, NA is the main signaling molecule that triggers astroglial Ca^{2+} signaling (Ding et al., 2013), which represents the universal form of glial excitability (Verkhratsky et al., 1998).

ASTROCYTES AS HUBS FOR THE NETWORK RESET SYSTEM

The LC is the primary source of NA in the CNS. It is localized in the brainstem and projects widely, and is thus able to synchronously activate neural networks in several brain regions. This may be regarded as a functional "reset" for many brain networks (Bouret and Sara, 2005; Sara, 2015). Axons of LC neurons project to the spinal cord, the brain stem, the cerebellum, the hypothalamus, the thalamic relay nuclei, the amygdala, the basal telencephalon, and the cortex, although some cortical areas receive more abundant innervation (Chandler et al., 2014). In all these structures, synchronous activation of LC projections (Bouret and Sara, 2005) leads to coherent and synchronized electrical activity, possibly reflected by gamma waves on an electroencephalogram (Sara, 2015). LC innervation mediates arousal and the sleep–wake cycle, attention

and memory, behavioral flexibility, behavioral inhibition and stress, cognitive control, emotions, neuroplasticity, posture, and balance (Benarroch, 2009). The effects of NA are mediated through α- and β-adrenergic receptors (α/βARs) which are expressed in neurons, microglia, and astrocytes. The ARs were among the first receptors to be causally linked to astroglial Ca^{2+} signaling (Salm and McCarthy, 1989; Kirischuk et al., 1996). Increases in astroglial Ca^{2+} were observed *in vivo* after stimulation of the LC in anesthetized animals (Bekar et al., 2008). In awake animals, stimulation of LC neurons triggered (by activation of α-ARs) widespread astroglial Ca^{2+} signals, which appeared in almost all astrocytes in the field of study (Ding et al., 2013). This synchronous response may represent the means by which neural networks are coordinated. Simultaneously, through activation of β-ARs, the cAMP-dependent pathways are activated; this in turn instigates rapid degradation of glycogen, which serves as the main energy reserve in the brain (Prebil et al., 2011; Kreft et al., 2012) and initiates morphological plasticity of astrocytes (Vardjan et al., 2014).

VESICULAR RELEASE OF GLIOSIGNALING MOLECULES

By having secretory vesicles clustered close to the plasma membrane, which is a hallmark of the active zone of the presynaptic terminal, the delay between the incoming stimulus and secretion is minimized, being as short as 100 μs (Sabatini and Regehr, 1999). At the same time, vesicle-based release of chemical messengers can exhibit much longer delays in stimulus–secretion coupling. In astrocytes, the mechanism prolonging the time between the arrival of the stimulus and the release of transmitters has been naturally selected, because the maximal speed of regulated exocytosis in astroglia appears much slower than that in neurons (Guček et al., 2012; Neher, 2012; Zorec et al., 2015). Regulated exocytosis also plays a role in many forms of cell-to-cell communication besides release of transmitters, being for example critical for the delivery of transporters, ion channels and antigen presenting molecules to the cell surface (Guček et al., 2012). Vesicular trafficking and release, which have evolved ∼3 billion years ago in arhaea (Spang et al., 2015), is fundamental for signaling and communication within the relatively large eukaryotic cell volume. Communication within large cells could no longer be supported by diffusion-based processes, which provide effective and rapid transport of molecules within the submicron range. Hence the development of subcellular organelles, such as secretory vesicles, presented a solution for the "signaling problem" in the relatively large volume of eukaryotic cells, to which astrocytes belong (Guček et al., 2012).

An ideal approach to monitor the rate-limiting processes of regulated exocytosis in astrocytes at the cellular level is to measure changes in the plasma membrane area, which reflects the fusion of vesicles with the plasma membrane. This can be monitored by measuring membrane capacitance (C_m), which is linearly related to the membrane area (Neher and Marty, 1982). This technique was used in cultured astrocytes (Kreft et al., 2004) to test the hypothesis that an increase in $[Ca^{2+}]_i$, after photolysis of caged Ca^{2+} (Neher and Zucker, 1993), elicits an increase in the whole-cell C_m. A half-maximal increase in C_m of these astrocytes was attained at ∼27 μM $[Ca^{2+}]_i$, which is similar to the Ca^{2+}-dependency of regulated exocytosis in various types of neurons, recorded by a similar technique (Heidelberger et al., 1994; Bollmann et al., 2000; Kreft et al., 2003a). In contrast to neurons, however, a rather small, within 100 nM, increase in $[Ca^{2+}]_i$ from the resting level was sufficient to induce glutamate release from astrocytes, as detected by glutamatergic effects on nearby neurons, used as sniffer cells (Parpura and Haydon, 2000). A similar high-affinity Ca^{2+} sensing mechanism for vesicular release was reported in pituitary endocrine cells (Kreft et al., 2003b). At present, astrocytes appear to be the slowest secretors of all the excitable mammalian cells investigated thus far. The kinetics of C_m increase is at least two orders of magnitude slower than the kinetics of regulated exocytosis recorded by a similar technique in neurons (Kreft et al., 2004; Neher, 2012). The Ca^{2+}-dependent increases in C_m were sensitive to tetanus toxin (which cleaves synaptobrevin 2 and cellubrevin), indicating a soluble *N*-ethyl maleimide-sensitive fusion protein attachment protein receptor and Soluble NSF Attachment Protein Receptor (SNAP)-based vesicular mechanism (Kreft et al., 2004).

Why is regulated exocytosis in astrocytes so slow? One reason is the distinct slow kinetics of molecular mechanisms regulating the vesicle membrane–plasmalemma merger. The number of SNARE molecules per vesicle, which is relatively low in astrocytes (Singh et al., 2014), may also contribute to the slow kinetics of regulated exocytosis. Slow delivery of vesicles to the plasma membrane fusion sites may also play a significant role. The vesicle dynamics is an amazingly elaborate system, regulated by increases in $[Ca^{2+}]_i$ (Potokar et al., 2013; Vardjan et al., 2015). For example, the complexity of vesicle traffic regulation in astrocytes is characterized by two typical, yet opposing, properties of vesicles that contain peptides, such as atrial natriuretic peptide, and/or ATP, and those that carry amino acids, such as glutamate and D-serine, and are labeled by the glutamate transporter VGLUT1 (Potokar et al., 2005, 2013; Vardjan et al., 2012; Vardjan and Zorec, 2015). Glutamatergic vesicles speed up with an increase in $[Ca^{2+}]_i$ (Stenovec, 2007), whereas the same increase in $[Ca^{2+}]_i$ slows down peptidergic vesicles and endolysosomes (Potokar et al., 2010).

Glutamatergic and peptidergic vesicles have the capacity to recycle. The mobility of recycling peptidergic vesicles was studied in cultured astrocytes (Potokar et al., 2008) and in intact brain slices (Potokar et al., 2009). At rest, peptidergic vesicles moved faster and more directionally than after the exposure of astrocytes to ionomycin to increase $[Ca^{2+}]_i$ (Potokar et al., 2008). The effect of increased $[Ca^{2+}]_i$ was dramatic; the movement of vesicles was almost halted, with only a jitter associated with random diffusional movement remaining. At least some of the peptidergic vesicles carry ATP and a similar attenuation was observed in their mobility when astrocytes were stimulated (Pangrsic et al., 2007).

What is the physiologic significance of differential mobility of vesicles carrying specific cargo, for example, classic chemical transmitter vs. neuromodulators or neuropeptides? An increase

or decrease in vesicle mobility may affect the efficiency of vesicle merger with the plasma membrane and the subsequent cargo discharge. It is possible that vesicles engaged in the dichotomous regulation of vesicle traffic exhibit different vesicle sizes, which may determine the nature of vesicle traffic and fusion with the plasmalemma, as was reported for endocrine cells (Flašker et al., 2013). Increased mobility of glutamatergic vesicles (which can quickly refill using VGLUTs) may indicate that they could be discharged at multiple loci at times of increased Ca^{2+} excitability, resulting in more diffuse signaling as opposed to spatially precise information transfer so characteristic of neuronal synaptic transmission. This speculation seems to be aligned with the ability of astrocytes to modulate synaptic transmission at a long temporal domain and via broad extrasynaptic access sites to neurons.

Impaired astrocytic vesicle traffic has been tentatively associated with intellectual deficiency (ID). Symptoms of ID appear early in life and the disease affects about 2% of the population. Family studies have demonstrated a relatively large number of X chromosome-linked forms of ID (XLID) with an incidence of about 0.9–1.4 in 1000 males (Turner, 1996). One of the first genes found to be mutated in patients with XLID is GDP dissociation inhibitor 1 (GDI 1; D'Adamo et al., 1998), which encodes for guanine nucleotide dissociation inhibitor (αGDI), a protein physiologically involved in regulating GDP-bound RAB proteins. The identification of *GDI1* association with ID suggested that vesicular traffic in neural cells is an important pathway for the development of cognitive functions (D'Adamo et al., 2002; Bianchi et al., 2009). Although the importance of αGDI in neuronal function has been demonstrated, it is unclear whether its role in glia vesicle trafficking contributes to the disease. The αGDI protein regulates the function of RAB GTPases, such as RAB 4 and RAB 5, which have been shown to regulate vesicle dynamics in astrocytes (Potokar et al., 2012), and it is likely that impaired vesicle traffic in astrocytes contributes to ID, which is linked to impaired cognitive processes involving memory formation.

CONCLUSION

Astroglial cells control homeostasis in the CNS to support many processes including memory formation. Astrocytes contribute to memory as signaling hubs and as structures that alter their morphology and recruit energy resources for memory consolidation. Excitation–secretion coupling in astrocytes is loose and this may be of particular importance to support the slowness of the overall memory-related structural dynamics in the CNS.

AUTHOR CONTRIBUTIONS

RZ, AH, NV, AV wrote the manuscript.

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Role of Astroglial Hemichannels and Pannexons in Memory and Neurodegenerative Diseases

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Under physiological conditions, astroglial hemichannels and pannexons allow the release of gliotransmitters from astrocytes. These gliotransmitters are critical in modulating synaptic transmission, plasticity and memory. However, recent evidence suggests that under pathological conditions, they may be central in the development of various neurodegenerative diseases. Here we review current literature on the role of astroglial hemichannels and pannexons in memory, stress and the development of neurodegenerative diseases, and propose that they are not only crucial for normal brain function, including memory, but also a potential target for the treatment of neurodegenerative diseases.

Keywords: connexin hemichannels, Cx hemichannels, connexin 43, pannexin, astrocytes, memory

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CONNEXIN HEMICHANNELS AND PANNEXIN CHANNELS

In the 90's, a handful of studies demonstrated that molecular and ionic interchange between the intracellular and extracellular compartments can occur via a family of plasma membrane channels called hemichannels (Goodenough and Paul, 2003). Originally known as the building blocks of gap junction channels (GJC; Revel and Karnovsky, 1967), hemichannels release relevant quantities of autocrine and paracrine signaling molecules (e.g., ATP, glutamate, NAD^+ and PGE_2) to the extracellular milieu, as well as the influx of small molecules and ions of up to ~1.5 kDa (e.g., glucose, cADPR and Ca^{2+}) (Bruzzone et al., 2001; Stout et al., 2002; Ye et al., 2003; Cherian et al., 2005; Retamal et al., 2007; Song et al., 2011; Fiori et al., 2012). Each hemichannel or connexon is comprised of six connexins (Cxs). Cxs encompass a highly conserved protein family encoded by 21 genes in humans and 20 in mice, with orthologs in other vertebrate species (Eiberger et al., 2001; Abascal and Zardoya, 2013). Recently, another gene family encoding a set of three membrane proteins, termed pannexins (Panx 1-3), was identified (Panchin et al., 2000). Pannexins form single plasma membrane channels (Bruzzone et al., 2003) termed pannexons that participate in paracrine and autocrine signaling among cells (Bao et al., 2004; Locovei et al., 2006a).

Several studies show that hemichannels and pannexons play different physiological roles in the central nervous system (CNS), including ischemic tolerance (Orellana et al., 2014), establishment of adhesive interactions (Cotrina et al., 2008), fear memory consolidation (Stehberg et al., 2012), synaptic transmission (Prochnow et al., 2012; Chever et al., 2014), spontaneous electrical activity (Moore et al., 2014), glucose sensing (Orellana et al., 2012), chemoception (Reyes et al., 2014), blood-brain barrier (BBB) permeability (De Bock et al., 2011), redox sensing (Retamal et al., 2006) and neuronal migration (Liu et al., 2012).

HEMICHANNELS AND PANNEXONS IN ASTROCYTES

Astrocytes play key roles in CNS function by providing nutrients (e.g., lactate; Pellerin, 2008) and redox molecules (Wilhelm and Hirrlinger, 2012), maintaining ionic balance (Kimelberg, 2005), K^+ clearance mediated by Na^+/K^+-ATPase (Sibille et al., 2014), glucose and lactate metabolism (Allaman et al., 2011), and neurotransmitter recycling of the two most abundant neurotransmitters in the brain: glutamate and GABA (Simard and Nedergaard, 2004). They also regulate cerebral microcirculation (Takano et al., 2006), and BBB permeability (Alvarez et al., 2013), among many other roles essential for normal brain function.

Additionally, it has been proposed that astrocytes actively participate in neuronal transmission and synaptic plasticity (Barres, 1989; Nedergaard, 1994; Parpura et al., 1994), via the release of molecules, known as gliotransmitters, into the synaptic cleft. In this context, Araque et al. (1998a,b) found that astrocytes surround synaptic buttons and release molecules into the synaptic cleft, modulating both pre- and post-synaptic activity. The term "tripartite synapse" was coined to describe synapses between neurons and astrocytes (Araque et al., 1999). Henceforth, several studies have proposed different pathways of gliotransmitter release from astrocytes, which appear to act in parallel and include P2X7 receptors (Duan et al., 2003), pannexons (Iglesias et al., 2009), Cx43 hemichannels (Cotrina et al., 1998), transporters (Szatkowski et al., 1990), and vesicles (Parpura et al., 1994). For a summary of main gliotransmitter release mechanisms see **Figure 1A**.

Astrocytes show the highest level of Cx expression among brain cells, with Cx43 being the most abundantly expressed both *in vitro* and *in vivo* (Dermietzel et al., 1991; Naus et al., 1991). Astrocytes also express Cx30 (Nagy et al., 1999), Cx26 (Rash et al., 2001), and may also show Panx1 (Iglesias et al., 2009), at least after stress (Orellana et al., 2015). Cx43 and Panx1 form functional hemichannels and pannexons in astrocytes *in vitro* and *ex vivo* (Contreras et al., 2002; Iglesias et al., 2009; Orellana et al., 2011a).

Embedded in the "tripartite synapse", astrocytes express a plethora of receptors (reviewed in Moraga-Amaro et al., 2014) and respond locally to neurotransmitters through the above mentioned mechanisms of gliotransmitter release, including the activation of hemichannels and pannexons (Malarkey and Parpura, 2008). Indeed, several gliotransmitters such as D-serine, glutamate, ATP and lactate have been reported to be released via astrocytic hemichannels (Stout et al., 2002; Ye et al., 2003; Karagiannis et al., 2015) or pannexons (Iglesias et al., 2009; Pan et al., 2015) *in vitro*. This gliotransmitter release has been proposed to be necessary for different CNS functions *in vivo* (Orellana and Stehberg, 2014; Montero and Orellana, 2015). Other *in vitro* studies have reported Cx43 hemichannels to be permeable to NAD^+ (Bruzzone et al., 2001), glucose (Retamal et al., 2007), taurine (Stridh et al., 2008), and Ca^{2+} (Schalper et al., 2010). Moreover, given that GJCs have been shown to allow for the passage of small peptides with a molecular weight of up to 1.8 kDa (Neijssen et al., 2005) and short interfering RNAs (Valiunas et al., 2005), it is possible that hemichannels may also allow the passage of such molecules, hypothesis that has not been tested so far.

Most early studies on hemichannels were performed *in vitro*, and suggested that Cx43 hemichannels have a low open probability in physiological conditions, requiring depolarized membrane potentials as high as +60 mV. Given that astrocytes are considered non-excitable cells in terms of membrane potential, their opening under physiological conditions was considered virtually impossible. However, later studies showed hemichannel opening at negative membrane potentials as measured by hemichannel-mediated dye uptake and ionic currents (Contreras et al., 2003; Retamal et al., 2007).

Recent *in vitro* studies have shown that astroglial Cx43 hemichannel activity changes in response to general anesthetics (Liu et al., 2016) antidepressants (Jeanson et al., 2015) and modafinil (Duchêne et al., 2016), suggesting that they may also be drug targets.

EVIDENCE FOR ASTROGLIAL HEMICHANNEL FUNCTION *IN VIVO*

The evidence of a role for astroglial hemichannels and pannexons *in vivo* in astroglial physiology and CNS function is much more limited, and is only now beginning to emerge. A recent study reported that astroglial Cx43 hemichannels are active in hippocampal slices during basal conditions and that astroglial Cx43 hemichannel-dependent release of ATP increases basal excitatory (glutamatergic) synaptic transmission through P2 receptors (Chever et al., 2014). Similar results were reported in neurons that project to the vagal nerve (Retamal et al., 2014).

Astroglial Cx43 hemichannel opening may also contribute to neuronal oscillations. Roux et al. (2015) reported that astroglial Cx43 hemichannel opening in olfactory bulb slices increases the amplitude of slow oscillations in mitral cells, affecting their firing rate. Hemichannel activity is also necessary for maintaining spontaneous activity in the cortex during development (Moore et al., 2014). It remains unknown whether hemichannels are still relevant for spontaneous activity in the adult cortex.

Yet another example of the role of astroglial hemichannels in CNS function measured *in vivo* can be found in the retrotrapezoid nucleus, in which the firing rate of CO_2/H^+-sensitive neurons acting as chemoreceptors (Wenker et al., 2012; Reyes et al., 2014) is modulated by ATP release from astrocytes via Cx26 hemichannels (Huckstepp et al., 2010).

In a recent study by Orellana et al. (2015), we reported from *ex-vivo* hippocampal slices that acute 2 h restraint stress in mice induces opening of astroglial Cx43 hemichannels, while chronic 10-day immobilization stress—a model used to induce depression in rodents—leads to increased opening of Cx43 hemichannels, and recruitment of astroglial Panx1 channels. This increase in hemichannel activity correlated with an increase in glutamate and ATP release, being dependent on

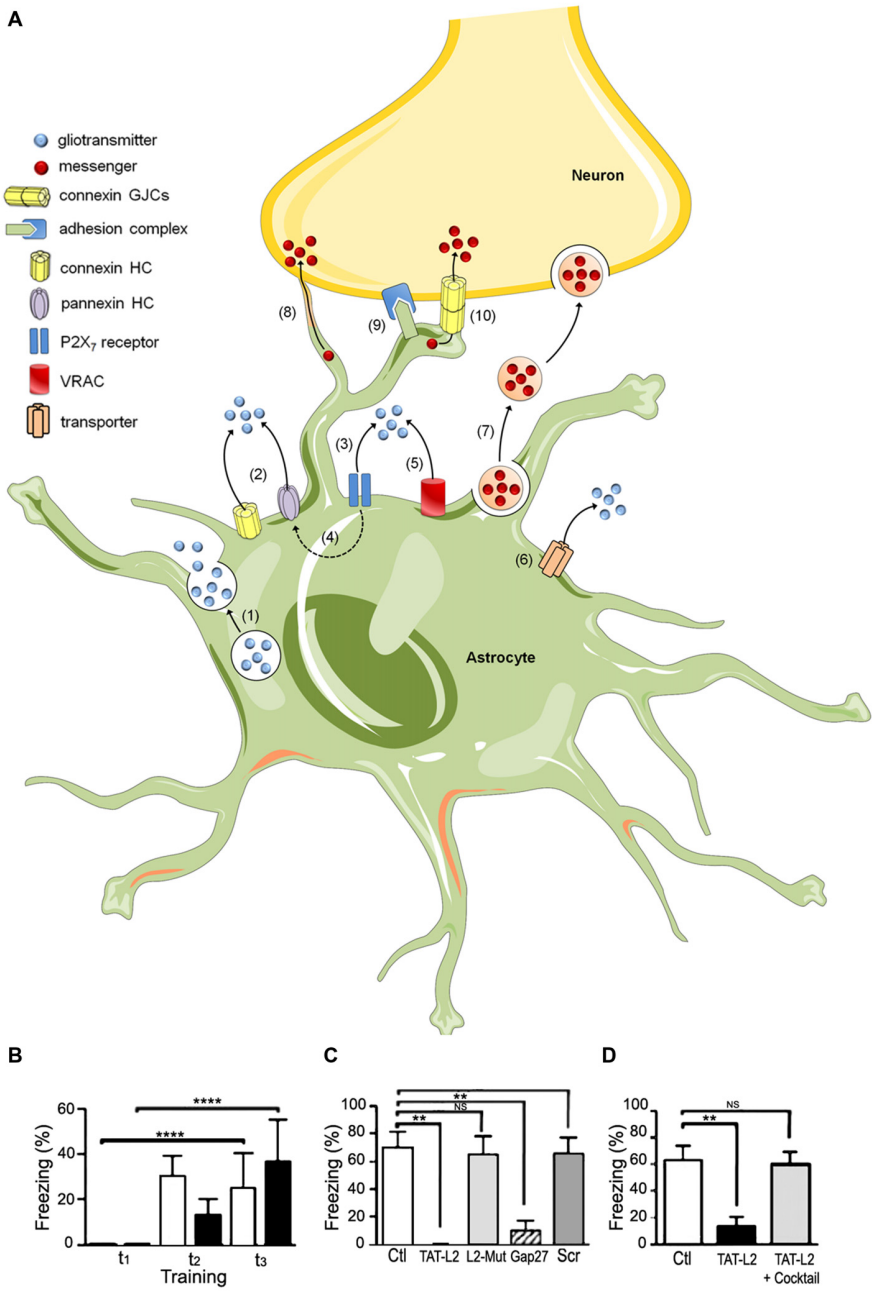

FIGURE 1 | The tripartite synapse; hemichannels, pannexons and their role in memory consolidation. (A) Astrocytes release gliotransmitters (e.g., glutamate, D-serine and ATP) through Ca^{2+}-dependent exocytosis (1) and opening of connexin (Cx) and pannexin (Panx) hemichannels (2). Long-lasting activation of P2X7 by ATP may lead to large currents and release of gliotransmitters (3), effect that may be mediated by Panx1 hemichannels (4). Gliotransmitter release may also occur through volume-regulated anion channels (VRAC) (5) and different carriers and/or co-transporters acting normally or in reverse (6) (e.g., excitatory amino-acid transporters, the cysteine-glutamate antiporter and the D-serine/chloride co-transporter). Astrocytes can also communicate with neurons via the release of vesicles (e.g., exosomes, microparticles and apoptotic bodies), containing different cellular messengers (e.g., mRNA, viruses and organelles) (7). Adjacent glial cells and neurons can communicate directly through F-actin-based transient tubular connections known as tunneling nanotubes (8), via cell-to-cell contacts between membrane-bound ligand molecules and their receptors (9) or intercellular channels known as gap junctions (10). **(B–D)** Blockade of astroglial Cx43 hemichannel opening in the basolateral amygdala by intra-BLA microinjection of TAT-L2 mimetic peptide had **(B)** no effect in short term fear conditioning memory, **(C)** but blocked fear memory consolidation as assessed 24 h after training. This amnesic effect was also found after injection of the more unspecific hemichannel blocker GAP27, but was absent when a scrambled peptide was used (scr) or a similar peptide to TAT-L2 with two aminoacids mutated (L2-mut) to interfere with its affinity for Cx43. **(D)** A minimal dose of TAT-L2 still capable of blocking memory consolidation (TAT-L2) was co-injected into the basolateral amygdala with a mixture of TAT-L2 and various putative gliotransmitters (TATL2 + cocktail), including glutamate, D-serine, glycine, ATP, etc. The microinjection of the mixture prevented the amnesic effects of intra-amygdalar TAT-L2 peptide (reprinted from Stehberg et al., 2012 with permission). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

glutamatergic N-methyl-D-aspartate (NMDA) and purinergic receptor signaling (Orellana et al., 2015). Moreover, Garré et al. (2016) showed that FGF-1 promotes inflammatory responses in acute spinal cord slices by a mechanism that involves release of ATP through Panx1 channels. Finally, in another study we shall discuss in more detail later, we showed that Cx43 hemichannels are necessary for fear memory consolidation in the basolateral amygdala (Stehberg et al., 2012).

As can be deduced from the above paragraph, *in vivo* evidence supporting a role for hemichannels and pannexons in CNS function is very recent, still limited in number but growing fast.

Astroglial hemichannels open in response to local increments in intracellular Ca^{2+}. Astrocytes express receptors and respond to most neurotransmitters known to be relevant for memory (for a review see Moraga-Amaro et al., 2014) via fast and local Ca^{2+} oscillations or inter-astrocytic Ca^{2+} waves at speeds matching neuronal activity (Winship et al., 2007). Thus, astroglial activation may trigger the opening of hemichannels or pannexons and the concomitant release of D-serine, glutamate, ATP and lactate, among various other gliotransmitters (Orellana and Stehberg, 2014). D-serine is a co-agonist of NMDA receptors critical for synaptic plasticity (Henneberger et al., 2010). Glutamate is the main excitatory neurotransmitter in the CNS and lactate is critical for brain metabolism and acts as a neurotransmitter activating NMDA receptors (Yang et al., 2014), all of which are critical for synaptic plasticity and memory. ATP mediates propagation of inter-astrocytic Ca^{2+} waves by activating astroglial purinergic receptors, whereas its conversion into adenosine may activate neuronal purinergic receptors (Zhang et al., 2003). As a consequence, it is likely that these functions are mediated by astroglial hemichannels and pannexons, but direct *in vivo* evidence is still lacking.

ASTROGLIAL HEMICHANNELS AND PANNEXONS IN MEMORY

As mentioned earlier, astroglial hemichannels and pannexons allow for the delivery of a wide variety of gliotransmitters to the extracellular milieu. However, the role of these channels in brain function under physiological conditions, and particularly in memory, has only recently begun to be elucidated. In 2012, we demonstrated that blockade of Cx43 hemichannels at the basolateral amygdala *in vivo*, using a mimetic peptide known as TAT-L2, had no effects on short term memory (**Figure 1B**), yet blocked memory consolidation, inducing amnesia for auditory fear conditioning 24 h after training (**Figure 1C**). Interestingly, the amnesic effect of the peptide was prevented by co-injecting it together with a cocktail of gliotransmitters, including glutamate, D-serine, glycine, lactate, ATP and glutamine (**Figure 1D**; Stehberg et al., 2012). This indicates that the opening of Cx43 hemichannels permits the release of gliotransmitters necessary for memory consolidation, but we were not able to identify the gliotransmitter or combination of gliotransmitters that is critical for memory. In this respect, a previous study by Henneberger et al. (2010) reported that preventing calcium oscillations in astrocytes averted long-term potentiation (LTP, a model of synaptic plasticity associated to memory) in hippocampal slices. This effect was reverted by exogenous administration of D-serine to the preparation (Henneberger et al., 2010). D-serine is a co-agonist of glutamate NMDA receptors which is secreted by astrocytes and is critical for LTP induction (Henneberger et al., 2010; Kang et al., 2013). There is currently no direct evidence that D-serine can be released via Cx43 hemichannels, but it is possible, as astroglial pannexons have been reported to release D-serine *in vitro* (Pan et al., 2015).

Genetic studies affecting Cx expression have had limited value in deciphering the role of hemichannels, as current genetic approaches affect the expression of both hemichannels and GJCs and do not allow for the distinction of the effects of either. Double knockout mice for both Cx43 and Cx30, the two main Cxs expressed in astrocytes, show enhanced synaptic transmission, attenuated LTP and increased long-term depression (LTD) in hippocampal CA1 pyramidal cells (Pannasch et al., 2011), which are critical for memory formation.

It is still debated whether Pannexons form GJCs *in vitro* (Sosinsky et al., 2011; Sahu et al., 2014). Unlike Cx43, which is expressed mainly in astrocytes (also reported in microglia, radial glia and neural progenitors; Nadarajah et al., 1997; Boucher and Bennett, 2003), Panx1 is expressed in both astrocytes and neurons (Zoidl et al., 2007; Iglesias et al., 2009; Santiago et al., 2011). Thus, Panx1 deficiency by genetic knockout or pharmacological approaches cannot distinguish neuronal from astroglial pannexons. Both pharmacological blockade and genetic deficiency of Panx1 channels induce increased excitability, enhanced LTP, reduced LTD and impairments in object recognition and spatial memory (Prochnow et al., 2012; Ardiles et al., 2014). This evidence depicts a clear role for Panx1 in synaptic plasticity and memory, regardless of whether they originate from astrocytes, neurons, or both.

HEMICHANNELS AND PANNEXONS IN PSYCHIATRIC DISORDERS ASSOCIATED WITH COGNITIVE DYSFUNCTION

Thus far no study has reported a direct role for astroglial hemichannels in psychiatric disorders that can be associated with memory. However, in Orellana et al. (2015), we showed that astroglial Cx43 hemichannel activity in the hippocampus is increased after acute restraint stress in mice, effects that were associated with a Cx43-dependent increase in extracellular levels of glutamate and ATP (Orellana et al., 2015). Interestingly, when mice underwent a protocol of chronic restraint stress commonly used to induce depressive-like symptoms in mice, an even greater increase in Cx43 hemichannel activity was induced, together with Panx1 channel opening, with the concomitant Cx43- and Panx1-dependent release of glutamate and ATP (Orellana et al., 2015). This study was followed by the work of Quesseveur et al. (2015), reporting that knockdown of Cx43 in mice induced anxiolytic- and antidepressant-like effects and an increase in

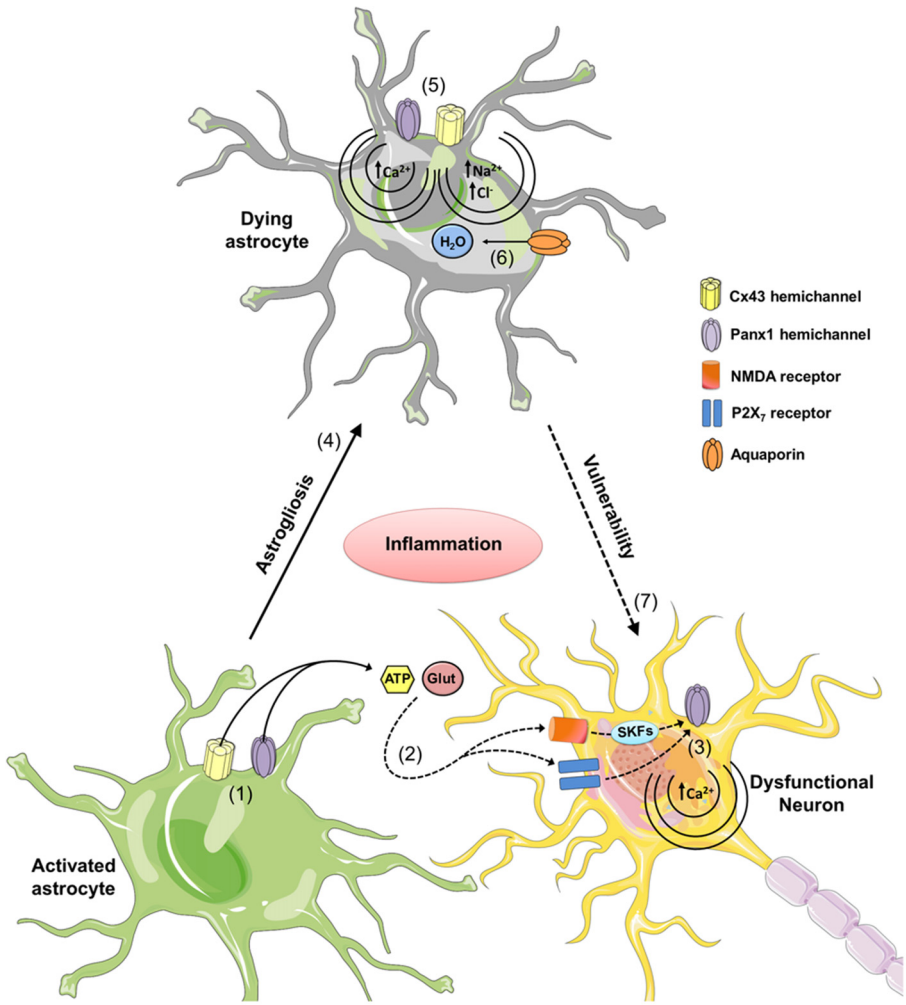

FIGURE 2 | The role of astrocytic hemichannels and pannexons during neurodegeneration. During the early stages of various neurodegenerative diseases, increased inflammation opens astrocytic Cx43 hemichannels and Panx1 channels (1). This results in the release of the gliotransmitters ATP and glutamate, and increases activation of neuronal NMDA and P2X7 receptors (2). It is hypothesized that NMDA and P2X7 receptor activation increases the opening of neuronal Panx1 channels through phosphorylation of Panx1 by Src family kinases (SFKs) and direct protein-to-protein interactions, respectively (3). These P2X7-related protein interactions could affect intracellular Ca^{2+} homeostasis resulting in cell death. Uncontrolled activation of astrocytes may result in reactive astrogliosis and further cell death by a mechanism related to the opening of connexons and pannexons (4). In particular, dysregulated opening of Cx43 and Panx1 channels could elicit cellular damage by different mechanisms. At one end of the connexon, the entry of Ca^{2+} via the Cx43 hemichannels or Panx1 channels. The added Ca^{2+} activates phospholipase A_2, thus generating arachidonic acid and activating the cyclooxygenase and lipoxygenase pathways, resulting in increased free radicals, lipid peroxidation and plasma membrane damage (5). At the other end of the connexon, Na^+ and Cl^- entry via Cx43 hemichannels or Panx1 channels may trigger cellular swelling due to an increased influx of H_2O via aquaporins (6). Finally, given that astrocytes provide support to neurons, astroglial cell damage associated with hemichannel/pannexon opening could indirectly increase neuronal susceptibility and vulnerability due to the homeostatic imbalance occurring during neurodegeneration.

freezing in the fear-conditioning paradigm. Interestingly, it was found that chronic corticosterone administration (another model used to induce depression in rodents), caused an increase in the expression of phosphorylated Cx43 in the hippocampus, effect that was reversed by successful antidepressant treatment (Quesseveur et al., 2015). This is further supported by a recent study showing that antidepressants affect astroglial Cx43 GJC and hemichannel activity (Jeanson et al., 2015). The above findings suggest that hippocampal Cx43 hemichannel activity may be important in stress responses and for the pathophysiology of depression. How they may contribute to arousal-induced memory enhancements, post-traumatic stress disorder or depression-associated cognitive impairments are exciting questions that may be answered in the near future.

HEMICHANNELS AND PANNEXONS IN NEURODEGENERATIVE DISEASES

Many neurodegenerative diseases are characterized by destruction of memory related areas, including the hippocampus,

prefrontal cortex, mediotemporal lobe, nucleus basalis, basal ganglia, etc. (for reviews on areas involved in memory see Packard and Knowlton, 2002; Jeong et al., 2015). For example, in Alzheimer's disease (AD), extensive damage to the hippocampus and cortical areas has been associated with cognitive deficits (reviewed in Pini et al., 2016). Likewise, in frontotemporal dementia, damage to frontal and anterior temporal lobes was also associated with cognitive deficits (Finger, 2016), while in Parkinson's disease (PD), damage to basal ganglia and frontal connectivity has also been correlated with cognitive deficits (Zgaljardic et al., 2003).

Various studies have linked dysregulation of hemichannel and pannexon permeability and expression, with the progression of different neurodegenerative diseases (Orellana and Stehberg, 2014; Penuela et al., 2014). However, the mechanisms by which astroglial hemichannels and pannexons contribute to neuronal damage remain elusive. It is possible that enhanced reactive astrogliosis evoked by neuroinflammation may alter different astroglial functions necessary for proper astrocyte-to-neuron crosstalk and neuronal survival, including synaptic gliotransmission, Ca^{2+} and NO signaling, as well as antioxidant and inflammatory responses. Hemichannels and pannexons are both affected by multiple inflammatory mediators released by reactive astrocytes and microglia (e.g., cytokines, NO and ROS; Retamal et al., 2007; Abudara et al., 2015). Inflammatory conditions could increase astroglial hemichannel/pannexon opening, leading to an uncontrolled influx of potentially toxic agents, as is the case of Ca^{2+}. Because hemichannels are permeable to Ca^{2+} (De Bock et al., 2012; Fiori et al., 2012), and their opening is controlled by intracellular Ca^{2+} (De Bock et al., 2012), it is possible that overactivation of hemichannels/pannexons results in intracellular Ca^{2+} overload and the subsequent impairment of vital functions for astroglial survival; including energy metabolism, Ca^{2+} handling, osmotic regulation and antioxidant defense. Consistent with this notion, hemichannel and pannexon activity has been linked to an alteration in Ca^{2+} dynamics and cell death in reactive astrocytes (Orellana et al., 2010; Abudara et al., 2015; Rovegno et al., 2015). In addition, osmotic and ionic imbalances induced by uncontrolled influx of Na^{+} and Cl^{-} through these channels could result in further cell swelling and plasma membrane breakdown. Given that astrocytes provide metabolic, synaptic and trophic support to neurons and maintain the extracellular microenvironment, astroglial cell damage or dysfunction associated with hemichannel and pannexon opening could increase neuronal susceptibility to different pathological conditions (Contreras et al., 2004; Orellana et al., 2009).

Alternatively, uncontrolled opening of hemichannels and pannexons induced by inflammatory conditions may trigger excessive release of molecules at toxic levels, such as glutamate and ATP. Consistent with this idea, astrocytes stimulated with amlyloid-β (Aβ) peptide exhibit increased Cx43 hemichannel-dependent release of glutamate and ATP, which are toxic for hippocampal and cortical neurons (Orellana et al., 2011a). Similarly, a follow-up work showed that astrocytes pre-treated with conditioned media from Aβ peptide-stimulated microglia release neurotoxic amounts of glutamate and ATP via Cx43 hemichannels when subjected to hypoxia in high glucose conditions (Orellana et al., 2011b). Interestingly, their release reduced neuronal survival via activation of neuronal NMDA/P2X7 receptors and Panx1 channels in neurons (Orellana et al., 2011a,b). Neurons express functional hemichannels formed by Cx36 and pannexons formed by Panx1 (Thompson et al., 2006; Zappalà et al., 2006), and the opening of Panx1 channels could occur via protein-protein interactions with activated P2X7 receptors (Iglesias et al., 2008), through increases in intracellular Ca^{2+} or phosphorylation triggered by activation of P2Y (Locovei et al., 2006b) or NMDA receptors (Weilinger et al., 2012). For a scheme of proposed roles for hemichannels and pannexons in neurodegeneration, see **Figure 2**.

CONCLUDING REMARKS

Exciting research on astrocytes and particularly on astroglial hemichannels and pannexons characterizes the last few years. Although hemichannels and pannexons initially appeared to be one of the many cellular mechanisms used by astrocytes to share their gliotransmitters, accumulating evidence indicates that astroglial hemichannels play a key role in brain function under physiological conditions, and in pathology. In normal conditions astroglial hemichannels and pannexons are important for memory consolidation, stress response, and possibly even for the pathophysiology of depression. Given their role in NMDA-dependent plasticity, they may also prove to be relevant in depression-associated memory impairments. Yet in pathological conditions, they appear to have a central role in the development of neurodegenerative diseases. Although many questions remain unanswered regarding their role in memory and in cognitive dysfunction, it is clear that astroglial hemichannels and pannexons play essential roles, in sickness and in health, until death do us part.

AUTHOR CONTRIBUTIONS

Review was divided into equal parts, which were combined and edited by JS. JAO made **Figure 2**; JS and JAO made **Figure 1**. All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Deciphering Resting Microglial Morphology and Process Motility from a Synaptic Prospect

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Microglia, the resident immune cells of the central nervous system (CNS), were traditionally believed to be set into action only in case of injury or disease. Accordingly, microglia were assumed to be inactive or resting in the healthy brain. However, recent studies revealed that microglia carry out active tissue sampling in the intact brain by extending and retracting their ramified processes while periodically contacting synapses. Microglial morphology and motility as well as the frequency and duration of physical contacts with synaptic elements were found to be modulated by neuronal activity, sensory experience and neurotransmission; however findings have not been straightforward. Microglial cells are the most morphologically plastic element of the CNS. This unique feature confers them the possibility to locally sense activity, and to respond adequately by establishing synaptic contacts to regulate synaptic inputs by the secretion of signaling molecules. Indeed, microglial cells can hold new roles as critical players in maintaining brain homeostasis and regulating synaptic number, maturation and plasticity. For this reason, a better characterization of microglial cells and cues mediating neuron-to-microglia communication under physiological conditions may help advance our understanding of the microglial behavior and its regulation in the healthy brain. This review highlights recent findings on the instructive role of neuronal activity on microglial motility and microglia-synapse interactions, focusing on the main transmitters involved in this communication and including newly described communication at the tripartite synapse.

Keywords: microglia, motility, neuronal activity, ATP, glutamate

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INTRODUCTION

Microglia are the resident immunocompetent cells of the central nervous system (CNS) and they comprise around 5–12% of the glial cell population (Gomez-Nicola and Perry, 2015). Microglia emerge from two sources: erythromyeloid precursors of the embryonic yolk sac, and myeloid progenitors that invade the CNS and proliferate during embryonic and postnatal development (Ginhoux et al., 2013).

Considered as the resident immune cells of the brain, microglia have been mostly studied in immune and inflammatory contexts (Prinz and Priller, 2014). However, recent *in vivo* data indicate that under physiological conditions, microglial cells exhibit a highly ramified morphology characterized by motile processes that constantly monitor their immediate surrounding by extending and retracting their processes (Davalos et al., 2005; Nimmerjahn et al., 2005).

This constant movement of microglial processes while the soma remains stationary is called microglial motility (**Figure 1**). The unexpected finding of microglial process motility led scientists to enquire and identify new roles in the non-pathological brain (Kettenmann et al., 2013). Since then, microglia were shown to be involved in the phagocytosis of synaptic elements during the entire lifespan, and the formation of learning-dependent synapses in the mature brain, as well as maturation and plasticity of excitatory synapses (Tremblay et al., 2010; Paolicelli et al., 2011; Hoshiko et al., 2012; Schafer et al., 2012; Parkhurst et al., 2013). These functions require localized fine-tuning involving specialized cellular function to deliver targeted messages at individual synapses. This targeted delivery could be compatible with the rapid process motility described earlier. A growing body of evidence also suggests that process motility and the frequency and duration of physical contacts with synaptic elements are regulated by neuronal activity, sensory experience and neurotransmission. It is thus crucial to better understand the mechanisms guiding neuron-to-microglia communication to further comprehend microglial functions in a healthy brain. In this review, we synthetize the recent discoveries on the properties of microglia in physiological conditions. Then, we report findings on the instructive role of neuronal activity on microglial motility and microglia-synapse interactions. Finally, we describe the current understanding of the molecular mechanisms underlying these interactions.

MICROGLIA, HIGHLY MOTILE CELLS THAT CONTACT SYNAPSES

Until fairly recently, due to lack of proper tools to study the microglial cells in a healthy brain, these cells were believed to be immunologically quiescent and were qualified as resting/dormant under physiological conditions. Two pioneer studies using two-photon microscopy observation of microglial motility in the intact cortical micro-environment reversed the common belief that "resting" microglia in the healthy brain were morphologically static (Davalos et al., 2005; Nimmerjahn et al., 2005). They shifted the concept of microglia from "resting" to "surveying" (Hanisch and Kettenmann, 2007). Visualization of microglia was made possible by the development of $CX3CR1^{GFP}$ and $Iba1^{GFP}$ transgenic mice (Jung et al., 2000; Hirasawa et al., 2005). Small-shaped soma of microglial cells remained still overtime, with only 5% moving by 1–2 μm/h (Nimmerjahn et al., 2005). In contrast, microglial processes were morphologically plastic with considerable motility. They presented a similar rate of extension and retraction of around 1.47 μm/min (Nimmerjahn et al., 2005). These findings position microglial cells as the most dynamic CNS cells as no other cells display such morphological plasticity *in vivo*. The dynamism of microglial processes was also confirmed in the mouse spinal cord and retinal explants (Davalos et al., 2005; Lee et al., 2008) and in the zebrafish embryo (Peri and Nüsslein-Volhard, 2008).

These groundbreaking discoveries confirmed the morphological plasticity of microglial ramifications under physiological conditions in distinct CNS structures and species led scientists to envisage a possible contribution in neuronal physiology. Specific, direct and activity-dependent microglia-to-synaptic element contacts in the adult mouse visual and somatosensory cortex *in vivo* were thus demonstrated for the first time (Wake et al., 2009). Using $CX3CR1^{GFP}/Thy1^{YFP}$ mice, Tremblay et al. (2010) were able to distinguish microglial cells from neurons in the superficial layers of the developing mouse visual cortex at 4 weeks of age. Intriguingly, microglial processes made physical contacts especially with axon terminals and dendritic spines, but also with perisynaptic astrocytic processes and synaptic clefts. They preferentially localized

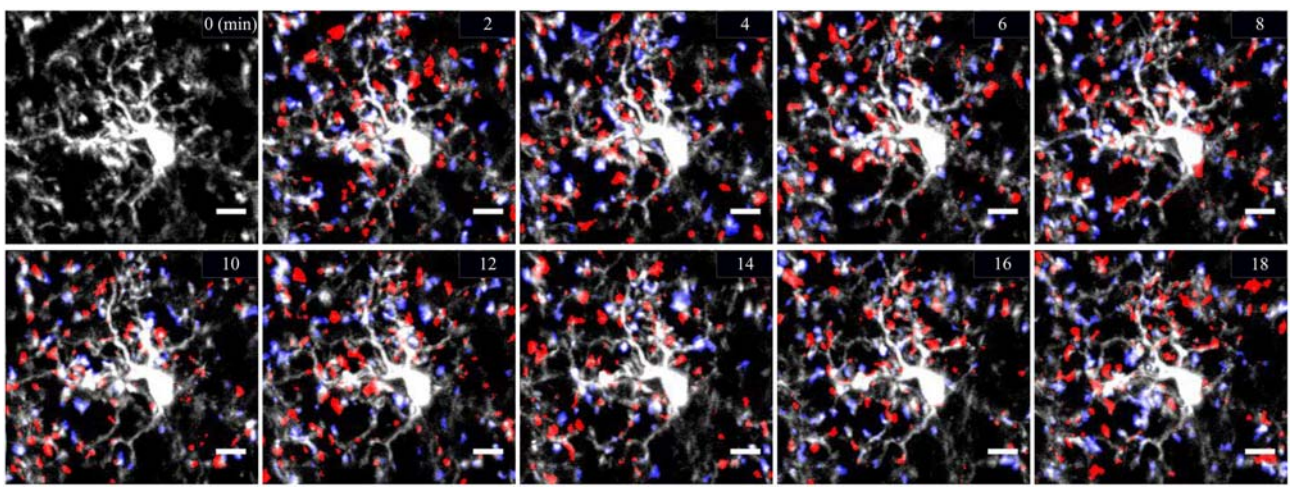

FIGURE 1 | Cortical two-photon imaging on awake mouse. Resting microglia are highly ramified and motile in the mouse cerebral cortex. Microglia are labeled by enhanced green fluorescent protein (eGFP) expressed under the control of microglial promoter CX3CR1. Microglial motility is shown by means of representative color-coded time-lapse images of a single microglial cell showing rapid process extensions (in blue) and retractions (in red) with a 2 min interval over the time course of 18 min. Scale bar = 10 μm.

at the proximity of small, more motile and more frequently eliminated dendritic spines rather than larger dendritic spines. Microglial distal processes enveloped dendritic spines by forming finger-like protrusions (Tremblay et al., 2010). Microglia-neuron contacts were brief and transient, at a frequency of one per hour (Wake et al., 2009), whereas in the developing visual cortex contacts varied in duration between 5 and 30 min (Tremblay et al., 2010). These data suggest that under physiological conditions *in vivo*, microglial processes are highly motile and make direct contacts with synaptic elements at regular frequencies during all stages of life.

MICROGLIAL MOTILITY IS MODULATED BY NEURONAL ACTIVITY

The discovery of the incessant dynamism of microglial processes and the transient contacts with synapses under physiological conditions led to new questions: are microglial processes specifically guided by neuronal activity and do they respond to the functional status of synapses? To investigate whether neuronal activity instructs microglial motility and contact with synaptic elements, global excitatory and inhibitory activity were modulated by pharmacological, physiological and genetic means. Initial results were rather negative. Silencing neuronal activity by applying tetrodotoxin (TTX) onto cortical surface *in vivo* had no impact on microglial motility (Nimmerjahn et al., 2005), as well as high frequency stimulation inducing long term potentiation (LTP) in acute hippocampal slices (Wu and Zhuo, 2008). Likewise, reduction of basal activity in the visual cortex *in vivo* by several independent approaches, including binocular eye enucleation, retinal TTX injection and reduction of body temperature, had no effect on basal velocity of microglial processes (Wake et al., 2009). However, with a simultaneous visualization of neurons and microglia, Wake et al. (2009), showed a reduced frequency of microglia-synapse contacts. Since this effect resulted from the aforementioned manipulations of neuronal activity, apart from TTX application, neuronal activity could at least modulate microglia-synapse interaction.

Using physiological approach by modulating sensory experience, Tremblay et al. (2010), studied neuronal activity-dependent microglial behavior in the developing mouse visual cortex. Mice were housed in dark conditions and thus deprived of visual experience during a critical period of development. During deprivation, average sampling and motility of microglial processes were significantly reduced, but the frequency and duration of microglia-synapse contacts remained unchanged. The synaptic target changed: microglia no longer localized next to small spines, but interacted preferentially with larger dendritic spines that subsequently shrank. Re-exposure to daylight restored microglial motility and contact with small dendritic spines.

Microglial motility and activity-dependent interactions with synaptic elements were also studied in the zebrafish optic tectum. By simultaneously monitoring GFP-labeled microglia and levels of spontaneous activity by Ca^{2+} imaging, Li et al. (2012) found that neurons with high level of spontaneous activity steer microglial processes, causing an increased contact frequency between these two elements. Conversely, reducing global activity by overexpressing human inward rectifier K+ channel (Kir2.1) in tectal neurons lowered the likelihood of physical contact (Hua et al., 2005). Visual stimuli increased the total number of bulbous endings, inferring microglia-neuron interaction, which were considerably reduced by TTX application (Li et al., 2012).

Overall, these findings suggest that under physiological conditions the motility of microglial processes and their interaction with synaptic elements can be modulated by neuronal activity.

NEUROTRANSMITTERS AND MECHANISMS AFFECTING MICROGLIAL MOTILITY

The molecular cues which maintain the movement of microglial processes and by which neuronal activity may signal to microglia are under investigation. A variety of classical neurotransmitter and neuromodulator receptors are expressed at the surface of microglia from culture assays, which in turn can cause changes in cytokine release, in membrane potential, cellular morphology and motility (Kettenmann et al., 2011). However, these *in vitro* preparations should be interpreted with caution because microglial phenotype resembles activated state (Ransohoff and Perry, 2009). Recent *in situ* studies indicate that microglia express functional purinergic receptors, whereas local application of other neurotransmitters did not elicit electrical responses, most probably reflecting lack of neurotransmitter receptors (Fontainhas et al., 2011).

ROLE OF PURINES

Adenosine triphosphate (ATP), a neurotransmitter of the CNS, has been identified as the key regulator of microglial morphology and baseline dynamics. Disruption of ATP-dependent signaling in the presence of ATP/ADP hydrolyzing enzyme apyrase decreased the basal motility of microglial processes (Davalos et al., 2005) while application of ATP increased basal motility and cell complexity (Fontainhas et al., 2011). ATP is also involved in directed microglial process outgrowth because focal applications of ATP caused a striking extension of microglial processes towards the source of ATP (Davalos et al., 2005; Dissing-Olesen et al., 2014) and process outgrowth persisted as long as ATP was applied (Dissing-Olesen et al., 2014). In addition, ATP critically mediated microglial process outgrowth towards sites of increased neuronal activity (Li et al., 2012; Eyo et al., 2014). Finally, the extension of microglial processes was found to be propelled by a cell autonomous release of ATP contained in lysosomes, serving ultimately as a motor for motility (Dou et al., 2012).

ATP acts at specific ionotropic (P2X) and metabotropic (P2Y) purinergic receptors that are largely distributed in neurons and glial cells. Initial studies *in vitro* showed that ATP signaling through P2YR induced microglial membrane ruffling (Honda et al., 2001), and that P2YR inhibition, but

not P2XR, affected the number and motility of microglial processes towards ablation site (Davalos et al., 2005). The requirement of P2R signaling for neuronal activity oriented motility and formation of bulbous contacts was also observed in the zebrafish optic tectum (Li et al., 2012). A major receptor candidate is P2Y12R, selectively expressed by microglia in the physiological brain. Following ATP release, the extension of microglial processes, but not basal motility, was critically dependent on the activation of P2Y12R, as shown by experiments performed on acute hippocampal slices from P2Y12 KO mice (Haynes et al., 2006; Eyo et al., 2014). P2Y12R accumulated at the tip of microglial processes during ATP-induced process outgrowth, along with Rho GTPase Rac, a key molecule in the cytoskeleton reorganization, whose downregulation abolished oriented microglial process movement in response to neuronal activity (Li et al., 2012; Dissing-Olesen et al., 2014).

Additional factors such as gradient formation and generation of ATP metabolites may be important in mediating motility. ATP is quickly catabolized to other purine molecules by ecto-nucleotidases in the extracellular space (Dunwiddie et al., 1997). Constant release of non-hydrolysable ATP in presence of apyrase was unable to attract microglial processes (Davalos et al., 2005). Furthermore, the contribution of ATP hydrolysis products seem to be critical because blocking ATP hydrolysis using selective ectonucleotidases inhibitor altered microglial outgrowth (Dissing-Olesen et al., 2014). When ATP and metabolites diffuse in the extracellular space and form a chemotactic gradient critical for microglial process outgrowth, their elimination reduced the extent and speed of microglial processes (Davalos et al., 2005). Adenosine seems to be a potential candidate for regulating microglial motility because high levels of adenosine receptors A1 and A3 are expressed on microglia in physiological conditions (Hammarberg et al., 2003) and an interplay of simultaneous purinergic stimulation of both A3 and P2Y12 receptors was found necessary for process outgrowth (Ohsawa et al., 2012). Adenosine was also involved in the retraction of microglial processes in the pathological brain due to signaling involving A2A receptors (Orr et al., 2009).

ATP appears to be released in an activity-dependent manner by neurons and astrocytes through hemichannels (pannexin and connexin), transporters and secretory vesicles (Burnstock, 2008). Studies reviewed below focus on ATP release from hemichannels, since this particular mode of communication has been predominantly investigated in relation to microglial motility. The precise distribution of the three pannexin subtypes (Panx1 to Panx3) between cell types and subcellular location has not been fully understood, but could partly account for discrepancies in the literature (Penuela et al., 2013). Initial studies found that probenecid, a non-selective pannexin channel antagonist, caused a general decrease in morphological parameters and basal velocity (Fontainhas et al., 2011). In the zebrafish optic tectum, glutamate uncaging-induced movement of microglial processes and the formation of bulbous endings were abolished using probenecid and more importantly, by specifically downregulating *pannexin-1* expression while keeping unchanged basal cell area and velocity (Li et al., 2012). Consequently, pannexin-1 emerged as the main hemichannel mediating ATP release and subsequently microglial outgrowth. However, a recent study found that during pannexin-1 blockage and in pannexin-1 deficient mice, microglial outgrowth following NMDAR activation in acute hippocampal slices was unaffected (Dissing-Olesen et al., 2014). This mechanism remained probenecid-dependent, raising possibilities for involvement of other pannexin channels mediating ATP efflux, such as pannexin-2 or prepackaged vesicles (Dissing-Olesen et al., 2014; Eyo et al., 2014).

Another important component affecting microglial dynamics could involve connexin hemichannels that mediate ATP released from astrocytes. They seem to be of particular importance for basal velocity of microglial processes *in vivo* because pharmacological blockade of connexins decreased it significantly (Davalos et al., 2005). However, fluoroacetate, an astrocytic function blocker, did not affect glutamate-induced microglial process extension (Eyo et al., 2014). In acute hippocampal slices, blocking connexin channels did not prevent NMDA-mediated microglial extension (Dissing-Olesen et al., 2014; Eyo et al., 2014). The discrepancy in these findings could be due to tissue specificity, microglia heterogeneity, *in vivo* and *ex vivo* preparation and the type and concentration of the pharmacological substances utilized.

These studies clearly demonstrate that purinergic signaling, in particular ATP and its derivates, are crucial for mediating microglial basal motility and neuronal activity-oriented motility. Further studies need to be performed to address the specific effects and contribution of purinergic molecules, as well as the exact downstream signaling pathways elicited in microglia.

ROLE OF GLUTAMATE AND GABA

Direct action of glutamate and γ-aminobutyric acid (GABA) neurotransmission on microglial morphology and motility was also investigated. Initial studies failed to demonstrate any effect of local application of glutamate and GABA on microglial motility. No change was provoked by enhancing neuronal activity with the application of GABA antagonist on the cortical surface (Nimmerjahn et al., 2005) or the application of glutamate and GABA on acute hippocampal slices and spinal cord dorsal horn (Wu and Zhuo, 2008; Chen et al., 2010). A more recent study found that glutamatergic and GABAergic neurotransmission modulated microglial morphology and motility in retinal explants, an *ex vivo* model with minimal CNS damage: GABA application decreased microglial motility, whereas bicuculline increased both the size and basal velocity of microglial processes (Fontainhas et al., 2011). Both agonist and antagonist of ionotropic glutamatergic receptors affected the size and motility of microglial processes mainly through fast AMPA/kainate receptors in retinal explants. This effect was mediated to a much lesser extent through NMDA receptors. Strangely, application of glutamate alone did not result in

any change of the above-mentioned parameters (Fontainhas et al., 2011). We must note that variations exist between brain areas. In the hippocampus, two recent studies used acute slices to show an important glutamate-induced microglial process outgrowth requiring NMDAR activation that was independent of AMPA/kainate receptor activation (Dissing-Olesen et al., 2014; Eyo et al., 2014). These differences between structures may attest for striking tissue-specific regulation of microglial motility through fast AMPA/kainate receptors in the retina and slow NMDA receptors in the hippocampus (**Figure 2**). Glutamate signaling also affected microglial motility in the zebrafish optic tectum. Glutamate uncaging, a noninvasive approach for upregulating neuronal activity, caused outgrowth of microglial processes towards the glutamate source as well as formation of bulbous endings (Li et al., 2012).

Studies have suggested that glutamate and GABA neurotransmission do not signal directly to microglial cells, but affect microglial motility and outgrowth by modulating extracellular levels of nucleotides, such as ATP and metabolites. Local application of glutamatergic or GABAergic agonists did not induce detectable electrophysiological responses in microglia. This is in agreement with the discovery that ionotropic glutamatergic receptors were not present on microglial processes and soma (Fontainhas et al., 2011; Eyo et al., 2014). On the contrary, ATP induced large inward currents in microglia and seems to be indeed implicated downstream glutamatergic transmission because reduction of process length and motility, obtained with glutamate receptor blockade, was inversed by ATP (Fontainhas et al., 2011).

Future studies should investigate whether the activation of all three main ionotropic glutamatergic receptors can activate a common pathway.

OTHER NEUROTRANSMITTERS

Other than glutamate and GABA, very few studies have investigated the impact of neurotransmitter application on microglial motility. Acetylcholine, norepinephrine or serotonin did not cause changes in the dynamism of microglial processes in the spinal dorsal horn (Chen et al., 2010). However, Gyoneva and Traynelis (2013) has shown that norepinephrine was responsible for the retraction of microglial processes in acute brain slices in physiological conditions through β2 adrenergic receptors present in resting microglia. Process extension caused by ATP was inhibited by co-application of norepinephrine. Propranolol, an antagonist of β2 receptor, reversed this effect. These findings raise an interesting question: can signaling mediated by norepinephrine modify the microglial reactivity to ATP release *in vivo*? We believe further studies of the influence of various neurotransmitters in more physiological conditions are needed to obtain a more complete understanding of the mechanisms underlying neuron-to-microglia communication.

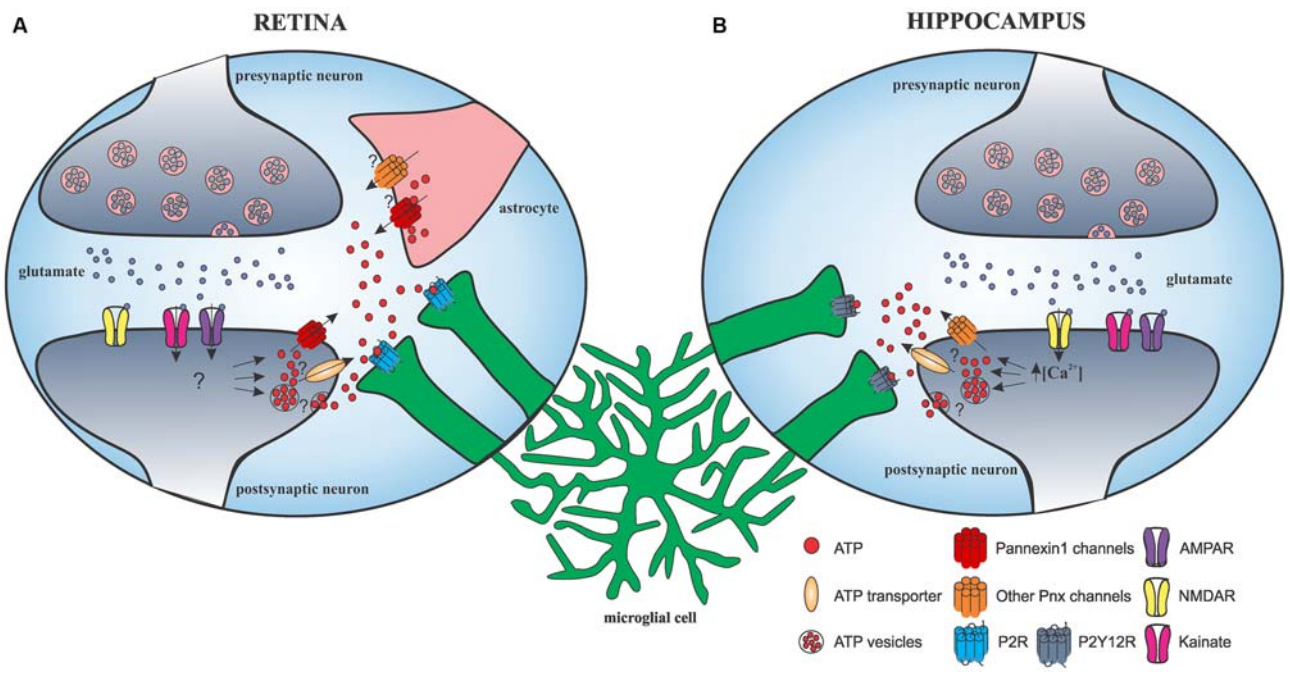

FIGURE 2 | Schematic representation of glutamatergic neurotransmission-induced microglial process outgrowth in the retina and hippocampus. **(A)** In the retina, AMPAR/kainate activation leads to release of ATP through pannexin-1 and possibly other mechanisms from neurons and astrocytes, ultimately leading to microglial response through P2 receptors. **(B)** In the hippocampus, glutamate-induced microglial process outgrowth is dependent on NMDA receptor. NMDAR activation leads to a significant Ca^{2+} influx that is required for ATP release through currently unknown mechanisms but independent of pannexin-1 and astrocyte hemichannels. ATP diffuses into the extracellular space and activates microglial purinergic receptor P2Y12, eliciting microglial process extension.

MICROGLIA-ASTROCYTE INTERACTIONS

In addition to interacting structurally with pre- and postsynaptic elements, microglia closely affix perisynaptic astrocytic processes (Tremblay et al., 2010). Accumulating evidence demonstrate that astrocytes not only play an important role in maintaining homeostasis, but also in regulating synaptic transmission (Perea et al., 2014; Oliveira et al., 2015). Thus, it appears that these glial cells may cooperate in the modulation of synaptic function taking into consideration their occasional structural proximity and emerging roles. Despite the possible influence of astrocytic ATP on microglial motility and morphology previously described, astrocyte-derived ATP was also found to induce microvesicle shedding from microglial cells, ultimately generating an increase of excitatory transmission (Bianco et al., 2005; Antonucci et al., 2012). Two recent studies provided compelling data on microglia-to-astrocyte interactions. Pascual et al. (2012) found that LPS stimulation of microglia in acute hippocampal slices resulted in a rapid ATP release. ATP bound to astrocytic P2Y receptor, which led to glutamate release by astrocytes, ultimately increasing excitatory transmission (Pascual et al., 2012). Another study found that fractalkine signaling, mediated exclusively by microglia, caused adenosine release, which in turn increased the release of D-serine most probably from both astrocytes and microglia, finally resulting in potentiation of NMDAR function (Scianni et al., 2013). Future studies must elucidate the context and molecular mechanisms governing interactions between microglia and astrocytes, as well as the functional consequences at the regulation of synaptic transmission and neural circuits.

CONCLUSION

Considerable progress has been made to decipher signaling mechanisms that regulate microglia-synapse interactions, which is only a token of its complexity. It is now crucial to better understand these mechanisms because the degree to which and how microglia interact with other cell types is most probably dependent on their morphology and motility. Several studies reveal functional importance of microglia in physiological conditions as they contribute to the fine-tuning of neuronal circuits and engage in synaptic and structural plasticity. These microglial functions are at least partly mediated by their motile processes, which can engulf synaptic terminals, thus homeostatically regulating neuronal activity and secrete a plethora of signaling molecules, including cytokines, neurotrophines, microvesicles etc. Their dynamism and functional capabilities position them perfectly to regulate individual synapses and to be undoubtedly involved in optimizing information processing, learning and memory, and cognition.

AUTHOR CONTRIBUTIONS

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Mathematical Modeling in Neuroscience: Neuronal Activity and Its Modulation by Astrocytes

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Research in neuroscience has come a long way since it was first hypothesized, in the early twentieth century, that dynamic changes in ion permeability underlie an event termed as action potential (Bernstein, 1912). Research along the same lines in the 1950s by Hodgkin and Huxley (1952) elucidated the dependence of action potential on the permeability of potassium and sodium ions—a theory achieved using quantitative analysis of potassium, sodium, and leak currents. Using mathematical modeling, they suggested that potassium and sodium conduits exist in distinct states (open, closed, or inactive) during an action potential; this was at a time when the composition of excitable membrane was largely unknown. Their mathematical model revolutionized the field of neurobiology and still forms the basis for many of the current mathematical models. Over the past several years, as more information on different channel types became available, more complex neuronal action potential models accounting for several channel types have been built (Traub et al., 1994; Bower and Beeman, 1995); a comparison between the original Hodgkin-Huxley model and a more detailed contemporary model is shown in **Figures 1A–C**.

With the hindsight, detailed models have been successful in emulating neuronal firing patterns observed *in vivo* or *in situ*. Primarily, computation of such detailed models has been possible due to significant technological advances that help solve differential equations in multiple dimensions. On one hand, detailed mathematical models are necessary to account for all the known proteins but on the other hand these detailed models possess redundant parameters which will lead to an unnecessary increase in the cost of computation. For example, consider the situation in **Figures 1B,C** where a detailed hippocampal CA3 region pyramidal neuron model is simulated under control conditions (B) and by knocking out its calcium-activated potassium channel (C). It is apparent from the figure that under both the conditions the action potential generated is unchanged, which indicates that incorporating all of the "known" proteins need not necessarily lead to a significant change in the model output of interest (in this case, an action potential). It is worth mentioning that the model described above involves only 897 ordinary differential equations, which is far less than the number of equations in a model accommodating all the neurons in the CA3-CA1 region [estimated to be $\sim 20 \times 10^6$ (West and Gundersen, 1990)]; the amount of redundancy in the latter model could be overwhelming and pruning the less significant proteins would aide in generating a region specific or whole-brain simulation. Of course, it is a "brigade" of interconnected neurons (identified experimentally by local field potential recordings), but not a single neuron *per se*, that plays a role in performing a task (Miller and Cohen, 2001; Buschman et al., 2012). This may contribute to an additional level of redundancy that needs to be assessed and optimized in order to successfully model the CA3-CA1 region.

An important player left out of the discussion above is the peri-synaptic astrocyte. In recent years, it has been demonstrated that the astrocytes can: (1) facilitate or depress synaptic plasticity (De Pittà et al., 2015), (2) synchronize CA1 neuronal firing (Fellin et al., 2004), (3) modulate extracellular field potentials (Lee et al., 2014), (4) repair damaged synapses (Wade et al., 2012),

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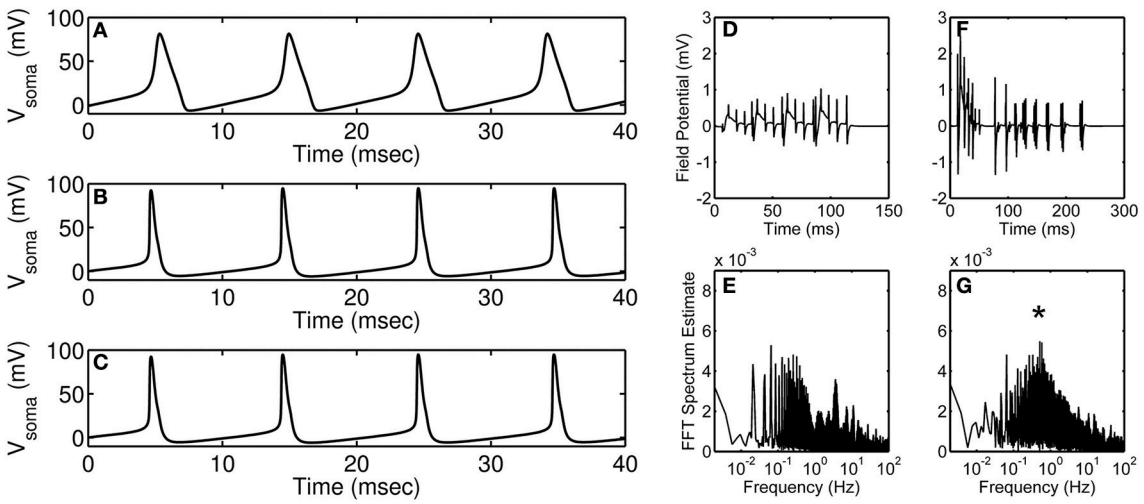

FIGURE 1 | Modeling of action potential discharges and the effect of astrocyte on accumulated neural discharges. (A) Hodgkin-Huxley squid axon model (Hodgkin and Huxley, 1952) injected with 35 μA·cm^{-2} current. **(B,C)** Branching dendrite model of a rodent CA3 pyramidal neuron (Traub et al., 1994) injected with 15 nA current into the soma compartment; **(B)** Control (wild-type) neuron modeling, and **(C)** Modeling of action potential discharges of a neuron with calcium-activated potassium channel knocked out. Simulations were obtained using published models and parameters. Model simulated field potentials of CA1 pyramidal neurons in the absence **(D)** and the presence **(F)** of peri-synaptic astrocyte. FFT spectrum analysis of the model simulated field potentials in the absence **(E)** and the presence **(G)** of astrocyte. **(D–G)** are modified and reproduced from Tewari and Parpura (2013). Asterisk denotes the astrocyte induced peak in the FFT spectrum.

and/or (5) initiate epileptic discharges (Reato et al., 2012; Tewari and Parpura, 2013). It has also been hypothesized that astrocytes can (1) store memories (Caudle, 2006), (2) promote motor-skill learning (Padmashri et al., 2015), and (3) modulate sleep (Halassa et al., 2009). Considering this overwhelming evidence of the involvement of astrocytes in brain activity, it has become important to integrate the effect of astrocyte signaling while simulating region specific neural oscillations or whole-brain rhythms. In the past decade, efforts have been made to integrate detailed models of different neurons on a large scale to mimic brain region specific neural oscillation patterns (Traub et al., 2005; Reimann et al., 2013), however, astrocytes have been left out of their calculations.

Recently, there have been attempts to include astrocytes in modeling of synaptic transmission. For example, **Figures 1D–G** show simulations from Tewari and Parpura (2013) performed in the absence (**Figures 1D,E**) and the presence (**Figures 1F,G**) of astrocyte signaling. Briefly, these model simulations show an effect of extra-synaptic signaling on the amplitude and the frequency of neural oscillations. The amplitude of neural oscillations is represented by field potentials and the frequency of neural oscillations is estimated using fast Fourier transform (FFT). It is apparent from these simulations that the presence of astrocyte increases the amplitude of neural oscillations (compare **Figure 1D** and **Figure 1F**) and also orchestrates neural firing to occur at a certain frequency (note "asterisk" in **Figure 1G**). Note that these simulations were performed under minimal recurrent synapses within a neural population which suggests that both neural oscillation amplitude and frequency would change depending upon astrocyte input.

Two types of modeling issues have been introduced above, (1.) which arises due to redundant model parameters and (2.) which arises due to the structural limitations of the model (e.g., ignoring glio-transmission in a model of CA3-CA1 region). To avoid these issues, as mentioned earlier, two things need to be considered before even initiating mathematical analysis of an experimental dataset:

(a) The extent to which a given mathematical model can mimic a biological response.
(b) Level of details that can be ignored in an experimental data-set without significant loss in the biological question being addressed.

Action potentials generated using the famous Hodgkin-Huxley model (shown in **Figure 1A**) could be considered today as the case (2) as some of the details we know today are not included in the model. However, it is noteworthy that the time taken to simulate the Hodgkin-Huxley model is more than 500 times less as compared to the more elaborate Traub model (shown in **Figure 1B**), which can be considered as the case (1). Although the action potentials generated using these two models are noticeably different, there are methods available that can help build on (improve) the Hodgkin-Huxley model in a parsimonious way or reduce the Traub model to be computationally less expensive. The first step toward building a new model (or modifying an old model) should be to gather as much data as possible (from literature and/or experiments). The models developed should be checked for parsimony using Akaike information criterion (Bozdogan, 1987) or Bayesian information criterion (Neath and Cavanaugh, 2012). The best practice for estimating the parameters is to use a global optimization (Vinnakota and Bugenhagen, 2013) technique along with a local optimization technique (Byrd et al., 1999). Moreover, sensitivity analysis methods are very useful in identifying sensitive or insensitive

parameters to facilitate model reduction (Saltelli et al., 2008), and should be applied before parameter estimation.

Future models of synaptic plasticity need to integrate the effect of astrocytes on the frequency of neural synchrony in different brain regions. It is understandable that prediction of region specific firing patterns would involve the integration of all possible neuron-astrocyte-neuron interactions in the region of interest which can be computationally challenging. For example, simulation of the network model presented in **Figures 1D–G** takes about 48 h on a laptop computer. Therefore, it is really important to employ modeling strategies which lead to simplified, computationally tractable and biologically relevant mathematical models.

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All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Astrocytes Modulate Local Field Potential Rhythm

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Ever since memory deficits were characterized in patient H.M. (Scoville and Milner, 1957), it has become clear that the hippocampal region is an important player in memory acquisition and retrieval. Not to mention that memory is of paramount importance for maintaining relationships, achieving personal and professional goals, and carrying out even simple day-to-day activities. Scientists have been interested in understanding the basic mechanism by which our brain perceives and processes information, stores, and then later on (when required) retrieves it. Sometimes it is not possible to retrieve relevant information unless we recall events (context) associated with the memory trace. This begs a few questions: Why is the memory retrieval depressed or enhanced under some conditions? Is this a function of long-term synaptic facilitation or depression? If so, what are the processes that underlie synaptic facilitation or depression?

With the technological advancements made in the past 3–4 decades, it has been possible to start addressing these questions by recording spiking activity from a single neuron or a population of neurons (i.e., Local Field Potentials, LFPs) during memory acquisition and retrieval (Fried et al., 1997). It is widely believed that LFPs are the spatially weighted averages of synaptic currents (Kraskov et al., 2007). These synaptic currents are in some cases facilitated even though the amplitude and frequency of stimulating protocol is unchanged—a phenomenon widely known as long-term potentiation (Bliss and Collingridge, 1993). Synchronized synaptic activity within specialized brain regions, recorded as LFPs, have been reported during events of memory acquisition and retrieval (Eldridge et al., 2000; Kraskov et al., 2007) but the fundamental processes involved in this coordinated activity of neurons are still mainly unknown (Uhlhaas and Singer, 2006).

Astrocytes have emerged as an important player in synaptic plasticity in the past two decades. These glial cells were considered merely a "glue" for the most part of the last century till it was demonstrated that astrocytes respond to glutamate, a neurotransmitter released by excitatory neurons, by increasing their cytosolic calcium concentration (Cornell-Bell et al., 1990) and providing a feedback to neuron-neuron signaling by, for example: (1) secreting glutamate and activating metabotropic glutamate receptors (mGluRs) on pre-synaptic neurons (Perea and Araque, 2007) and/or (2) secreting D-serine and modulating N-methyl-D-Aspartate receptors (NMDARs) on post-synaptic neurons (Henneberger et al., 2010). Moreover, individual astrocytes are known to have fine processes that can enwrap asymmetrical (mainly excitatory) synapses (Fellin et al., 2006). Thus, strategically, astrocytes are ideally positioned to receive signal and relay it onto a small population of neurons during specific cognitive tasks.

The majority of experiments conducted so far have suggested that astrocytes contribute to short-term (Perea and Araque, 2007; Sibille et al., 2014, 2015) and long-term potentiation of individual synapses (Perea and Araque, 2007; Henneberger et al., 2010; Shigetomi et al., 2013). Several mathematical models have been developed (Nadkarni and Jung, 2007; Volman et al., 2007; De Pittà et al., 2009; Wade et al., 2011; Tewari and Majumdar, 2012b) to explore the tripartite synapse, a conventional neuron-neuron synapse with an add-on of a peri-synaptic astrocyte. However, relatively few experiments on hippocampal slices (Fellin et al., 2004; Sasaki et al., 2014)

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are available which report on the effect of astrocyte signaling on neuronal synchrony. These experiments hint at a role of astrocytes in tuning LFPs during events of memory acquisition and/or retrieval (see **Table 1** for a list of LFP rhythms and associated cognitive function).

As stated earlier, there are several mathematical models which quantify the effect of astrocytes at a single synapse. However, there are not many mathematical models which attempt at integrating the effect of astrocytes on a network of synapses and possible LFP rhythm generation and its modulation. One such mathematical model was developed by Tewari and Parpura (2013) that simulated the effect of astrocyte signaling on neuronal synchrony and LFP generation using published biophysics-based models of neurons and astrocytes. Briefly, four CA1 pyramidal neurons (with recurrent synapses) were stimulated by a single CA3 pyramidal neuron with one astrocyte present in the *stratum oriens* wrapping the CA3-CA1 synapses. The mathematical model of pyramidal neurons was the branching dendrite model (Traub et al., 1991, 1994) and astrocyte wrappings were modeled using the tripartite synapse model (Tewari and Majumdar, 2012a,b). LFP computation of their small CA1 network suggested astrocyte-dependent delta oscillations (Tewari and Parpura, 2013). Otherwise, the modeled neuronal network exhibited a theta rhythm. It should be noted that CA3 pyramidal neuron firing is essential for a recall of memory related patterns (Makara and Magee, 2013). Therefore, recurrent CA3 pyramidal neuron activity may affect the rhythm of CA1 LFP during recall of memory related patterns. Of note, the sole existing model (Tewari and Parpura, 2013) did not account for these recurrent connections within CA3 pyramidal neurons.

It is interesting that a recent experimental study has also demonstrated that the ablation of astrocytic vesicular release leads to the impairment of cognitive abilities in awake mice (Lee et al., 2014). More specifically, Lee et al. (2014) observed astrocyte-dependent tuning of cortical neurons to gamma rhythm, a result which appears to be in contrast with the model predictions of Tewari and Parpura (2013). However, the differences can be reconciled by: (1) the presence of neocortical interneurons known to oscillate at gamma frequency (Bartos et al., 2007) and/or (2) the size of network used to compute field potentials. It is quite possible that different brain regions may have different firing patterns in response to a given task. An alternate explanation for this difference in LFP rhythm observed in the model (Tewari and Parpura, 2013) and data (Lee et al., 2014) can be merely due to the choice of parameters used in the modeling approach; e.g., number of concurrent CA1 synapses, density of extra-synaptic NMDAR current etc.

TABLE 1 | List of different LFP types and their proposed behavioral function.

Rhythm type	Frequency	Proposed role
Delta oscillations	1–4 Hz	Attention, salience detection, and subliminal perception (Knyazev, 2012).
Theta oscillations	4–12 Hz	Active behavior, spatial navigation, memory, and sleep (Tort et al., 2008).
Gamma oscillations	30–100 Hz	Sensory binding, selective attention, neuronal assembly, information transmission and storage (Tort et al., 2008).
Sharp wave ripples	120–200 Hz	Consummatory behavior, immobility, slow wave sleep (Buzsáki et al., 2003).

To summarize, it has now become quite clear, from experiments and modeling simulations, that astrocytes do play an important role in modulating mammalian cognitive and behavioral abilities. But whether their contribution is specific to certain cognitive tasks than others remains to be seen. Availability of such information could be beneficial in treating conditions that pertain to memory deficit disorders.

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REVIEW
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The Role of Astrocytic Aquaporin-4 in Synaptic Plasticity and Learning and Memory

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Aquaporin-4 (AQP4) is the predominant water channel expressed by astrocytes in the central nervous system (CNS). AQP4 is widely expressed throughout the brain, especially at the blood-brain barrier where AQP4 is highly polarized to astrocytic foot processes in contact with blood vessels. The bidirectional water transport function of AQP4 suggests its role in cerebral water balance in the CNS. The regulation of AQP4 has been extensively investigated in various neuropathological conditions such as cerebral edema, epilepsy, and ischemia, however, the functional role of AQP4 in synaptic plasticity, learning, and memory is only beginning to be elucidated. In this review, we explore the current literature on AQP4 and its influence on long term potentiation (LTP) and long term depression (LTD) in the hippocampus as well as the potential relationship between AQP4 and in learning and memory. We begin by discussing recent *in vitro* and *in vivo* studies using AQP4-null and wild-type mice, in particular, the impairment of LTP and LTD observed in the hippocampus. Early evidence using AQP4-null mice have suggested that impaired LTP and LTD is brain-derived neurotrophic factor dependent. Others have indicated a possible link between defective LTP and the downregulation of glutamate transporter-1 which is rescued by chronic treatment of β-lactam antibiotic ceftriaxone. Furthermore, behavioral studies may shed some light into the functional role of AQP4 in learning and memory. AQP4-null mice performances utilizing Morris water maze, object placement tests, and contextual fear conditioning proposed a specific role of AQP4 in memory consolidation. All together, these studies highlight the potential influence AQP4 may have on long term synaptic plasticity and memory.

Keywords: aquaporin-4, synaptic plasticity, long term potentiation, long term depression, learning, memory

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INTRODUCTION

Astrocytes were previously thought to act only as support cells, however, over the years, increasing amount of evidence have implicated more dynamic functions of these glial cells (Volterra and Meldolesi, 2005). It is now well known that astrocytes play various vital roles in the central nervous system (CNS) such as providing metabolic and structural support (Barker and Ullian, 2010; Scharfman and Binder, 2013) and regulating the blood-brain barrier (BBB; Abbott et al., 2006). In addition to these roles, multiple studies have also shown that astrocytes can impact synaptic plasticity. Modulation of synapses by astrocytes can be observed by their structural organization. Astrocytic processes contact multiple neuronal membranes and form the tripartite

synapse where the astrocytic foot process envelopes the pre and postsynaptic terminal (Halassa et al., 2007, 2009). The intimate contact between astrocytes and neurons allow astrocytes to directly influence the strength of the synapse (Barker and Ullian, 2010). For example, astrocytes can secrete factors that can directly influence the formation of synapses (Barker and Ullian, 2010; Scharfman and Binder, 2013). Glia-derived secreted factors that promote synaptogenesis include cholesterol coupled to apolipoprotein-E (Mauch et al., 2001) and thrombospondins (Christopherson et al., 2005) whereas inhibitory synapses are influenced by astrocyte conditioned media by increasing vesicular GABA transporter and $GABA_A$ receptor through TrkB signaling (Elmariah et al., 2005). One of the hallmarks of synaptic plasticity involves the trafficking of AMPA receptors (AMPAR) to the synaptic sites (Malinow and Malenka, 2002; Malinow, 2015). Astrocyte-released cytokine tumor necrosis factor-α (TNFα) is involved in increasing surface expression of AMPAR (Beattie et al., 2002). Furthermore, TNFα has been shown to be involved in homeostatic synaptic scaling where neurons adjust the strength of their synapses to maintain its electrical output (Stellwagen and Malenka, 2006; Achour and Pascual, 2010). Other factors, such as glutamate, ATP, and D-serine, are also released by astrocytes. Termed 'gliotransmitters,' these molecules can regulate neuronal and synaptic activity (Perea et al., 2009; Achour and Pascual, 2010; Barker and Ullian, 2010; Parpura and Zorec, 2010).

Taken together, these roles suggest that astrocytes may be a key player in synaptic plasticity by modulating neuronal function. Moreover, studies have proposed that long lasting changes in synaptic plasticity underlies the basis of learning and memory. For example, long term potentiation (LTP) has been linked to acquisition and learned behaviors (Lu et al., 2008). Therefore, while the role of astrocytes in cellular communication and synapse regulation may be well established, the role of astrocyte-specific proteins, specifically aquaporin-4 (AQP4), in synaptic plasticity and learning and memory remains unclear. In this review we explore current literature on AQP4 and its impact on LTP and long term depression (LTD) as well as the potential relationship between AQP4 and in cognitive functions.

AQUAPORIN-4

The aquaporins (AQP) are a family of small, hydrophobic, membrane-spanning proteins involved in fluid transport (∼30 kDA/monomer; Verkman and Mitra, 2000; Verkman, 2005, 2008, 2011) Aquaporin-4 (AQP4) is one of 13 members of the AQP family and serves as a bidirectional water-selective transporter (Verkman et al., 2006; Verkman, 2011) and is heterogeneously expressed throughout the CNS with highest expression levels in the cerebellum and significantly lower protein levels in the hippocampus, diencephalons, and cortex (Hubbard et al., 2015). Additionally, the water channel protein is primarily associated with brain-fluid interfaces such as the BBB and ependymal-cerebrospinal fluid (CSF) barriers (Verkman, 2011) and is largely expressed by astrocytes predominantly at the perivascular end-feet in direct contact with blood vessels (Verkman et al., 2006; Nagelhus and Ottersen, 2013; Yao et al., 2015). Studies utilizing transgenic mice lacking AQP4 have provided a deeper understanding of this protein in various functions and diseases.

Using both *in vitro* and *in vivo* studies, researchers have identified an important role of AQP4 in astrocyte migration. A modified Boyden chamber was utilized for the transwell migration assay where astroglia migrated through a porous filter in response to a chemoattractant. Results from this assay revealed a slower migration of AQP4-null astrocyte as compared to wild-type (WT) astrocyte. Using a wound healing assay, confluent astroglial monolayers were injured by removing ∼1 mm of cells across the well. Glial scar formation by reactive astrocytes is crucial in wound healing. Results showed impaired wound healing and migration in AQP4-null astroglia. Additionally, the *in vivo* stab injury model also demonstrated a decrease in cell migration in AQP4-null mice (Saadoun et al., 2005).

What functional role does AQP4 play in cell migration? Early literature has shown localized swelling of the lamellipodia as a cause of cell movement (Oster and Perelson, 1987). Because AQP4 mediates the bidirectional transport of water, it is possible that migration of astrocytes is caused by increased water permeability leading to increased transmembrane water flux which ultimately moves the cell. Additionally, small extracellular osmotic gradients can also affect the speed of astrocyte movement in which astrocyte migration is accelerated toward hypo-osmolality (Saadoun et al., 2005).

Water homeostasis maintenance is critical in the CNS. Increases in brain water content can result in deleterious effects and thus understanding water regulation via AQP4 is of great significance. The role of AQP4 in brain edema has been heavily investigated in various studies using AQP4-null mice. There are two main types of cerebral edema: cytotoxic (cellular) edema and vasogenic edema. Cytotoxic edema results in an intracellular accumulation of water across an intact BBB whereas vasogenic edema results from fluid leaking across a compromised BBB (Verkman, 2005, 2008; Papadopoulos and Verkman, 2007). Studies of cytotoxic edema showed that AQP4-null mice were protected from cellular swelling and had improved neurological outcome (Manley et al., 2000, 2004; Papadopoulos and Verkman, 2005; Yao et al., 2015) while vasogenic edema worsens in mice lacking the water channel protein (Papadopoulos et al., 2004a,b). Hydrocephalus, a specialized form of vasogenic edema, results from obstruction of CSF drainage. Studies utilizing mice deficient in AQP4 showed accelerated progression of hydrocephalus, enlargement of ventricles, and increased intracranial pressure (Verkman et al., 2006).

While AQP4 can certainly play a significant role in edema formation, it is possible that AQP4 can also take part in edema elimination due to its function of bidirectional water transport. The traditional view of excess brain water elimination is believed to be through the bulk flow of fluid through the extracellular space (ECS) and the glial limitans, into the ventricles, and eventually into the blood through AQP4 located at the astrocytic endfeet (Verkman et al., 2006; Smith et al., 2015). This theory is

supported by studies using models of brain edema and ischemia where electron microscopy findings demonstrate endfeet swelling of astrocytes suggesting AQP4-dependent mediated osmotic water uptake (Manley et al., 2000; Ito et al., 2011) and early induction of AQP4 reduced the development of edema formation (Hirt et al., 2009). The glymphatic hypothesis suggests that brain water elimination is by way of hydrostatic pressure and osmotic forces that drives water through AQP4 (Thrane et al., 2014, 2015). In addition to fluid elimination, the glymphatic hypothesis also provides a possible pathway for interstitial solute clearance from the brain that is also AQP4 dependent. For example, Iliff et al. (2012) demonstrated significant reduction of clearance of ^{125}I-amyloid β_{1-40} in AQP4-null mice as compared to WT and AQP4-null amyloid-beta (Aβ) precursor protein/presenilin 1 transgenic mice had increased amyloid plaque deposition in the hippocampus and cortex as compared to WT animals (Xu et al., 2015). This suggests that interstitial soluble Aβ is removed via the gliovascular pathway in which clearance of interstitial fluid and solutes are driven by convective bulk interstitial fluid flow that is aided by AQP4-dependent water flux (Iliff et al., 2012). This hypothesis is further supported by mathematical modeling which considers the intracellular and extracellular water pathways between the arterial and venous paravascular space in astrocyte networks (Asgari et al., 2015).

AQP4 has also been associated with other neurological disorders such as epilepsy (Binder et al., 2012). Seizure phenotype of Aqp4-null mice was observed using the convulsant ($GABA_A$ antagonist) pentylenetetrazole (PTZ). Interestingly, AQP4-null mice showed a longer latency to generalized seizure when given PTZ as compared to WT mice (Binder et al., 2004a). Furthermore, AQP4 has been shown to be colocalized with the inwardly rectifying potassium channel Kir4.1. The colocalization of these two proteins suggest that AQP4 contributes to the coupled influx of water and K^+ after neuronal activity. Prolonged increases in $[K^+]_o$ in response to electrical stimulation were observed as well as an increased electrographic seizure threshold and electrographic seizure duration after stimulation. These findings suggests a deficit in extracellular K^+ clearance in AQP4-null animals (Binder et al., 2006). Indeed, the brain is sensitive to changes in extracellular osmolarity as this would alter the cell volume and subsequently the ECS (Traynelis and Dingledine, 1989; Schwartzkroin et al., 1998). Changes in ECS volume significantly impacts the extracellular concentration of solutes. A multitude of solutes are released upon stimulation, including potassium, that are known to be cotransported with water (Haj-Yasein et al., 2012). Thus altered ECS has been correlated to brain tissue excitability (Binder et al., 2004b; Haj-Yasein et al., 2012). In a study using cortical fluorescence recovery after photobleaching method, ECS diffusion was reduced after glutamate and seizure-induced neuronal activity. Mice lacking AQP4 also exhibited faster ECS diffusion than WT suggesting an ECS expansion (Binder et al., 2004b).

Compelling evidence from these studies show that AQP4 not only control water and ion homeostasis but also plays a role in neural signal conduction that may potentially contribute to synaptic transmission that is regulated by water transport.

AQP4 AND SYNAPTIC PLASTICITY

While there are mounting evidence that suggest a role of astrocytes in synaptic plasticity there are only a few studies that implicate a direct relationship of AQP4 in LTP and LTD. Here, we explore recent studies that present compelling data that support the hypothesis that AQP4 plays an important role in regulating synaptic plasticity in the hippocampus and amygdala. **Table 1** outlines the comparison of synaptic plasticity between WT and AQP4-null mice.

Basal Transmission in AQP4 WT and KO Mice

Using age-matched hippocampal slices Skucas et al. (2011) showed no differences in Schaffer collateral (SC) transmission between AQP4 WT and AQP4-null mice in extracellular recordings. There were no significant differences in field excitatory postsynaptic potentials (fEPSP) slope, amplitude, area, total duration, or half-duration. Additionally, there were no differences detected in the mean amplitude or latency to peak of the fiber volley, or the change in fiber volley amplitude with increasing fEPSP slope or amplitude. Furthermore, there were no significant differences in the paired-pulse facilitation (PPF), amplitude, area under the curve, or duration of both WT and AQP4-null slices. Whole-cell recordings from CA1 pyramidal cells also revealed no significant differences in frequency, amplitude, and cumulative probability of spontaneous postsynaptic currents and miniature postsynaptic currents (Skucas et al., 2011).

Fan et al. (2013) saw similar findings using hippocampal slices prepared from WT and AQP4-null mice. In their study, there were no differences in fEPSP slope between the two genotypes. Normal PPF was also observed in AQP4-null mice indicating that lack of the water channel protein did not cause a change in basal synaptic transmission (Fan et al., 2013).

Results from Yang et al. (2013) were parallel to findings from Fan et al. (2013). Basal synaptic transmission were not statistically different in AQP4 WT and AQP4-null hippocampal slices from the perforant path-dentate gyrus pathway (PP-DG) as there were no differences in fEPSP slope in both genotypes (Yang et al., 2013).

In a different study, Li et al. (2012) explored basal synaptic transmission in the thalamo-lateral amygdala (LA) pathway in slices taken from age and weight-matched littermates of WT and AQP4-null mice. As with findings reported above, fEPSP amplitude and PPF were not significantly different between WT and AQP4-null animals (Li et al., 2012).

These studies show that AQP4 deficiency does not cause a general defect in basal synaptic plasticity in various pathways in the brain due to undistinguishable differences in fEPSP and PPF between AQP4 WT and AQP4-null mice. Lack of AQP4, therefore, does not alter the probability of neurotransmitter release in the presynaptic neuron (Commins and O'Mara, 2000; Li et al., 2012; Scharfman and Binder, 2013). Thus, impairment in synaptic plasticity in AQP4-null mice might possibly be due to changes in the postsynaptic responses (Li et al., 2012). While these findings provide strong support that an absence of AQP4

TABLE 1 | AQP4 in synaptic plasticity.

Study	Genotype	Stimulation paradigm	Pathway	Results
Skucas et al., 2011	WT	TBS	SC-CA1 (*in vitro*)	LTP
		HFS	SC-CA1 (*in vitro*)	LTP
		LFS	SC-CA1 (*in vitro*)	LTD
	KO	TBS	SC-CA1 (*in vitro*)	Reduced LTP with
		HFS	SC-CA1 (*in vitro*)	delayed LTD
		LFS	SC-CA1 (*in vitro*)	LTP
		LFS + TrkB-Fc	SC-CA1 (*in vitro*)	LTD with delayed LTP
		LFS + K252a	SC-CA1 (*in vitro*)	LTD
				LTD
Fan et al., 2013	WT	TBS	SC-CA1 (*in vivo*)	LTP
		TBS	PP-DG (*in vivo*)	LTP
	KO	TBS	SC-CA1 (*in vitro*)	Reduced LTP
		TBS	PP-DG (*in vivo*)	Reduced LTP
Yang et al., 2013	WT	TBS	PP-DG (*in vivo*)	LTP
		TBS + Cef	PP-DG (*in vivo*)	LTP
		TBS + DHK	PP-DG (*in vivo*)	Reduced LTP
	KO	TBS	PP-DG (*in vivo*)	Reduced LTP
		TBS + Cef	PP-DG (*in vivo*)	LTP
Li et al., 2012	WT	HFS	Thalamo-LA	LTP
	KO	HFS	Thalamo-LA	Reduced LTP
		HFS + Cef	Thalamo-LA	LTP

Comparisons of synaptic potentiation in wild-type (WT) and KO mice.

does not affect basal synaptic transmission *in vitro*, data from *in vivo* studies could offer a more valuable assessment.

AQP4 in Long Term Potentiation and Long Term Depression

To determine the role of AQP4 in synaptic plasticity Skucas et al. (2011) evaluated LTP and LTD from hippocampal slices of age-matched WT and AQP4-null mice using theta-burst stimulation (TBS) and high-frequency stimulation (HFS) in the SC synapse in CA1. Using the TBS-LTP paradigm, the authors noted no significant differences in post-tetanic potentiation (PTP) during the first 3 min, however, a reduction in LTP was observed in slices from AQP4-null mice at 60 min. Additionally, the incidence of LTP was reduced in AQP4-null mice. The authors also observed an unexpected delayed LTD in AQP4-null mice after TBS. To further evaluate this surprising observation, additional experiments were conducted in AQP4-null slices to assess fEPSP decay. The authors saw no significant decay in fEPSP slope and thus established the conclusion that TBS evoked LTD in KO mice (Skucas et al., 2011).

The authors further evaluated LTD using low-frequency stimulation (LFS) due to the unanticipated finding of LTD induction after TBS. Skucas et al. (2011) saw no significant differences in short-term depression but LTD was reduced in AQP4-null mice. Moreover, the incidence of LTD was also lower in these animals. Interestingly, the authors also saw a delayed LTP in AQP4-null mice using LFS. Finally, using HFS, Skucas et al. (2011) saw no significant differences in PTP, incidence of LTP, and LTP amplitude 60 min after HFS in both genotypes (Skucas et al., 2011).

Increasing evidence from studies of the neurotrophin brain-derived neurotrophic factor (BDNF) have implicated a role of BDNF-TrkB signaling in synaptic plasticity (Purcell and Carew, 2003; Minichiello, 2009; Park and Poo, 2013), however, the influence of AQP4 on activity-dependent BDNF-TrkB synaptic plasticity is only beginning to be elucidated. The first line of evidence, to our knowledge, that illustrates a direct impact of AQP4 on BDNF-dependent synaptic plasticity is demonstrated by Skucas et al. (2011). In their study, Skucas et al. (2011) asked if either scavenging BDNF or antagonizing TrkB would rescue LTD as it was shown that LFS resulted in a delayed LTP in AQP4-null mice. Using the BDNF scavenger TrkB-Fc the authors saw a rescue of LTD in AQP4-null mice. Furthermore, application of K252a, a Trk antagonist, also rescued LTD. Western blots for TrkB and immunoprecipitation/Western blots for P75NTR, a receptor for neurotrophins (Binder and Scharfman, 2004), were analyzed to determine if LTP and LTD in AQP4-null mice were influenced by levels of TrkB receptors. Western blots revealed no differences in full-length or truncated TrkB between WT and AQP4-null mice. However, immunoprecipitation/Western blots data showed reduced levels of p75NTR in AQP4-null animals (Skucas et al., 2011).

Fan et al. (2013) also observed impaired LTP in AQP4-null mice. LTP was induced using two different models; (1) in the SC-CA1 pathway *in vitro* and (2) in the PP-DG pathway *in vivo*. TBS in SC-CA1 resulted in a reduction of LTP in AQP4-null mice. There were also no differences in I/O curves between the two genotypes at different stimulation intensities. TBS-induced LTP in the PP-DG *in vivo* also resulted in impaired LTP in AQP4-null mice. An initial increase in population spike (PS) amplitude was observed immediately after TBS, however, the PS amplitude was significantly lower in AQP4-null mice as compared to WT. Furthermore, the potentiation of the PS amplitude remained significant in both WT and AQP4-null animals but there was significantly less LTP of PS amplitude in AQP4-null mice. These

results suggest that AQP4 is involved in LTP induced by TBS in the DG *in vivo* (Fan et al., 2013).

Impaired LTP was also observed in TBS-induced LTP in the PP-DG *in vivo* by Yang et al. (2013). An initial increase of PS amplitude immediately after TBS also remained significant after 60 min in both genotypes. Similar to findings reported previously, LTP was greatly reduced in AQP4-null mice although both genotypes exhibited LTP after TBS. (Yang et al., 2013). It is well documented that glutamate plays a significant role in synaptic potentiation (Bear and Malenka, 1994; Perea et al., 2009; Achour and Pascual, 2010) and the high affinity glutamate transporter-1 (GLT-1) has been shown to be colocalized with AQP4 (Zeng et al., 2007). Furthermore, decreased expression levels of GLT-1 has been reported in AQP4-null mice which results in reduced glutamate uptake by astrocytes (Zeng et al., 2007; Papadopoulos and Verkman, 2013). Studies reported in this review thus far have shown impaired LTP in AQP4-null mice. Therefore, Yang et al. (2013) asked whether ceftriaxone (Cef), a β-lactam antibiotic that has been shown to up-regulate GLT-1 expression in astrocytes (Rothstein et al., 2005), can rescue AQP4 deficiency induced synaptic plasticity impairment. WT and AQP4-null mice underwent TBS in PP-DG *in vivo* after receiving daily injections of Cef for 7 days. PS amplitude was strongly increased in AQP4-null animals immediately and 60 min after TBS. Cef treatment did not further increase PS amplitude in WT animals. Furthermore, daily injections of dihydrokainate (DHK), a GLT-1 inhibitor, decreased PS amplitude in WT mice immediately and 60 min after TBS (Yang et al., 2013). These astounding results provide evidence that LTP impairment in AQP4-null mice can be rescued by Cef.

Li et al. (2012) also observed an impaired LTP in AQP4-null mice in the thalamo-LA pathway. After HFS, LTP was markedly reduced in AQP4-null slices as compared to WT slices that was not due to basal synaptic transmission as previously discussed. Similar to studies conducted by Yang et al. (2013), chronic Cef treatment reversed the impairment of LTP in AQP4-null mice (Li et al., 2012).

An outline describing LTP and LTD in both genotypes are reported in **Table 1**.

POTENTIAL MECHANISMS UNDERLYING IMPAIRED LTP IN AQP4 KO MICE

NMDAR-Dependent Synaptic Plasticity

It is well recognized that LTP and LTD induction is dependent on postsynaptic NMDA receptor (NMDAR) activation and the subsequent rise in intracellular calcium (Bear and Malenka, 1994; Lamprecht and LeDoux, 2004; Taniike et al., 2008; Paoletti et al., 2013). High increases in calcium activates various protein kinases which results in new AMPAR insertion into the postsynaptic membrane and ultimately leads to LTP while low increases of calcium activates protein phosphatases which dephosphorylates AMPAR and induces LTD (Bear and Malenka, 1994; Lamprecht and LeDoux, 2004; Taniike et al., 2008). It is possible that the lack of AQP4 could lead to NMDA dysregulation and consequently impaired LTP after TBS through reduced calcium entry. The attenuated LTP in response to TBS in hippocampal slices from AQP4-null mice could also account for NMDAR being less activated. This may result from impaired bicarbonate transport (Scharfman and Binder, 2013). During neuronal activity, increases in extracellular pH promotes NMDAR activation (Sinning and Hübner, 2013). Bicarbonate acts as a pH buffering system (Sinning and Hübner, 2013) and is regulated by the electrogenic Na^+/HCO_3^- cotransporter which drives water into astrocytes through AQP4 after taking up sodium and bicarbonate (Nagelhus et al., 2004). The absence of AQP4 could then affect pH homeostasis at the synapse during TBS.

Although postsynaptic activation of NMDAR is critical for LTP induction, excessive activation of these receptors may possibly contribute to the impairment of LTP seen in the AQP4-null mice. NMDARs have a higher affinity for glutamate compared to AMPARs (Patneau and Mayer, 1990). GLT-1 expression levels are reduced in APQ4-null mice (Zeng et al., 2007) and the downregulation of GLT-1 plays a significant role in synaptic plasticity as GLT-1 is responsible for the largest proportion of glutamate transport (Danbolt, 2001). Thus, large accumulation of glutamate, as a result of the reduced levels of GLT-1 in AQP4 deficient mice, results in intense activation of NMDAR (Danbolt, 2001). Strong NMDAR activation by excessive accumulation of glutamate was also observed in mice lacking GLT-1 (Katagiri et al., 2001). Additionally, increases in NMDAR-mediated currents was observed by Li et al. (2012) and Yang et al. (2013) in AQP4 deficient mice. Both studies measured the ratio of NMDAR to AMPAR mediated EPSC amplitudes to evaluate synaptic strengths. The authors found that NMDAR/AMPAR ratio was significantly larger in AQP4-null mice as compared to WT mice and that lack of AQP4 resulted in a selective increase in NDMAR-dependent EPSCs (Li et al., 2012; Yang et al., 2013). Moreover, low concentration of NMDAR antagonist reversed the impairment of LTP in AQP4-null mice (Li et al., 2012). These findings further support the hypothesis that excess glutamate in the synaptic cleft is due to the reduction of GLT-1 expression in AQP4-null mice which results in increased NMDAR-mediated currents in the hippocampus and the amygdala which may contribute to the impaired LTP in AQP4-null mice (Li et al., 2012; Yang et al., 2013). While the importance of synaptic NMDAR in synaptic plasticity is evident, the role of extrasynaptic NMDAR and glutamate is also critical in understanding synaptic potentiation. Elevated extracellular concentration of glutamate has been shown to strongly activate extrasynaptic NR2B-mediated NMDAR (Li et al., 2012). A correlation between the NR2B subunit and LTP has also been observed. Previous studies have shown that selective reduction in NR2B expression levels in the hippocampus disrupts LTP which consequently resulted in a decline in spatial learning behavior (Clayton et al., 2002). Thus, the impaired LTP seen in AQP4-null animals may also involve the NR2B subunit of the NMDAR (Li et al., 2012).

Effect of Potassium Dysregulation in Synaptic Plasticity

Another essential role of astrocytes is maintaining potassium homeostasis. This is achieved by either net uptake of potassium or potassium spatial buffering (Macaulay and Zeuthen, 2012; Bedner and Steinhäuser, 2014; Cheung et al., 2015). The net uptake of potassium involves a variety of cotransporters that includes the Na^+/K^+ pumps as well as the $Na^+/K^+/Cl^-$ cotransporters (Macaulay and Zeuthen, 2012; Bedner and Steinhäuser, 2014). In the spatial potassium buffering model (Orkand et al., 1966) extracellular potassium is taken up by Kir4.1 and redistributed to adjacent astrocytes via gap junctions (Bedner and Steinhäuser, 2014; Cheung et al., 2015). Therefore, potassium dysregulation could also possibly explain the impairment in synaptic potentiation seen in AQP4-null animals. Induction of LTP is related to neuronal excitability which is also sensitive to changes in extracellular potassium (Li et al., 2012). Previous studies have reported slowed K^+ reuptake in AQP4-null mice (Binder et al., 2006) that may also be contributed by the Na^+/K^+ pumps (Strohschein et al., 2011). Reduced potassium reuptake would lead to an increase in extracellular potassium that would depolarize neurons and glial cells. High levels of $[K^+]_o$ would result in tonic depolarization of neurons which may improve LTP due to greater postsynaptic depolarization. However, tonic depolarization, by high levels of extracellular potassium, may also impair LTP by reducing the driving force of the fEPSP or inactivating sodium channels, which would decrease the postsynaptic firing during LTP induction (Scharfman and Binder, 2013). Certainly, LTP has been shown to be expunged by high $[K^+]$ (Harrison and Alger, 1993). Although AQP4 has been shown to colocalize with the inwardly rectifying potassium channel Kir4.1 (Nagelhus et al., 2004; Zhang and Verkman, 2008) the function of Kir4.1 does not seem to play a role of impaired potassium kinetics seen in AQP4-null mice (Zhang and Verkman, 2008). Furthermore, conditional Kir4.1-null slices exhibited defects during short-term plasticity indicating that Kir4.1 plays an essential role in potassium buffering during the early stages of LTP (Djukic et al., 2007).

Contribution of GLT-1 and Ceftriaxone in Synaptic Potentiation

As previously stated, GLT-1 is responsible for reducing the spillover of glutamate at the excitatory synapses preventing excitotoxicity and ultimately controlling synaptic currents (Omrani et al., 2009). Previous studies have shown that AQP4 deletion resulted in a reduced expression of GLT-1 that led to an accumulation of extracellular glutamate (Zeng et al., 2007). Therefore, impaired LTP in AQP4-null mice may be explained by the reduction of glutamate uptake by glial cells. Other studies have also noted late LTP in GLT-1 deficient mice (Katagiri et al., 2001). Additionally, Rothstein et al. (2005) showed an impressive upregulation of GLT-1 by chronic treatment of Cef (Rothstein et al., 2005) which implicate a notable role of GLT-1 in synaptic plasticity. The effect of Cef treatment on LTP was seen in the studies conducted by Li et al. (2012) and Yang et al. (2013) where the authors noted that Cef treatment attenuated the deficits of LTP in the DG (Yang et al., 2013) and the LA (Li et al., 2012). Conversely, upregulation of GLT-1 by chronic treatment of Cef impaired LTD after LFS in the mossy fiber (MF)-CA3 pathway. Moreover, LTP was reduced after HFS in MF-CA3 but not in SC-CA1. Mechanisms underlying the induction of LTP in MF-CA3 is presynaptic, metabotropic glutamate receptors-dependent, and involves the release of glutamate, whereas LTP induction in SC-CA1 is primarily postsynaptic (Omrani et al., 2009). Additionally, LTP induction differs in different regions of the hippocampus. For example, LTP in SC-CA1 is NMDAR and glutamate dependent while LTP in MF-CA3 is NMDAR independent (Ota et al., 2013). Therefore, the reduced expression of GLT-1 in AQP4-null mice may affect LTP after TBS but have little affect after HFS (Omrani et al., 2009).

BDNF-Dependent Synaptic Plasticity

Neurotrophins are a family of four structurally related proteins that include BDNF, nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5; McAllister et al., 1999; Binder and Scharfman, 2004; Park and Poo, 2013). The neurotrophins bind to the lower-affinity p75NTR receptor and each bind to one or more of the high-affinity Trk family of receptor tyrosine kinases (McAllister et al., 1999; Binder and Scharfman, 2004; Minichiello, 2009; Park and Poo, 2013). NGF binds to TrkA, BDNF and NT-4 binds to TrkB, and NT-3 binds to TrkC (Binder and Scharfman, 2004; Park and Poo, 2013) and with low-affinity binding to TrkB (Binder and Scharfman, 2004). Additionally, there are two different classes of TrkB receptors; (1) full-length TrkB receptors that contains all the canonical motifs of tyrosine kinase receptors and (2) two alternatively spliced truncated receptors that lack the entire kinase catalytic region (Binder and Scharfman, 2004; Minichiello, 2009). Moreover, activation of the Trk receptors promote pro-survival signals while activation of p75NTR imparts anti-survival signals (Berk et al., 2015).

Multiple studies have recognized BDNF as a central player in regulating synaptic plasticity in the CNS. For example, mice targeted with a disruption of the BDNF gene displayed defects in the basal synaptic transmission and LTP at the SC-CA1 synapse which can be rescued by exogenous application of recombinant BDNF (Patterson et al., 1996). BDNF-TrkB activation is also regulated through PLCγ, PI3K, and MAPK pathways (Yoshii and Constantine-Paton, 2010). For instance, studies conducted by Ying et al. (2002) not only confirmed that BDNF is implicated in LTP but that activation of the MAPK pathway is required for the induction of LTP (Ying et al., 2002).

Again, it is interesting to note that impaired potentiation in AQP4-null mice was only observed in specific types of plasticity (TBS-LTP induced a delayed LTD and LFS induced a delayed LTP in AQP4-null mice; Skucas et al., 2011) and the roles of BDNF and TrkB could offer explanations to these differences. TBS-LTP attenuated LTP when TrkB functions are blocked, however, synaptic potentiation using HFS-LTP is relatively insensitive to perturbed TrkB functions (Kang et al., 1997). Thus, TBS-LTP appears to be dependent on BDNF (Scharfman and Binder, 2013). Furthermore, BDNF and TrkB also plays a significant role in early and late phase LTP. Blockade of TrkB functions

results in impaired LTP by TBS as early as 15 min after LTP induction (Kang et al., 1997) and sustained release of BDNF, either presynaptically or postsynaptically, appears to induce and maintain late-LTP (Kang et al., 1997; Barco et al., 2005). Furthermore, while both BDNF and NT-4/5 bind to TrkB, there are stark functional differences between the two *in vivo* (Fan et al., 2000) and *in vitro* (Xie et al., 2000). Therefore, one cannot exclude the possibility that AQP4 may affect synaptic potentiation in NT-4/5 and NT-3 with TrkB signaling.

While there were no differences in TrkB receptor levels, the reduced levels of p75NTR could also explain the delayed LTD observed after TBS-LTP seen in KO mice. Studies have shown that binding of proBDNF, the precursor to mature BDNF, to p75NTR results in LTD (Pang et al., 2004; Woo et al., 2005; Yang et al., 2014). But because scavenging BDNF and antagonizing Trk rescued LTD after LFS, reduced levels of p75NTR is thus not necessary to cause the LTD defect seen in AQP4-null mice (Skucas et al., 2011). Furthermore, increased mRNA and protein levels of p75NTR was observed in hypoosmolar conditions (Ramos et al., 2007) which is comparable to an expanded ECS seen in AQP4-null mice (Binder et al., 2004b). Hypoosmolarity could ultimately dilute extracellular molecules (Scharfman and Binder, 2013) which presents as a plausible explanation for the changes in potentiation seen after TBS and LFS.

It is no surprise that cells undergo various changes when placed in hypoosmotic environments but how these conditions affect BDNF and synaptic plasticity remains unclear. Recent studies in retinal Müller cells may provide clues to explain for the deficits in synaptic plasticity seen in AQP4-null animals. For example, Berk et al. (2015) showed that BDNF inhibited osmotic swelling of retinal Müller cells. The somata of these cells did not alter much when they were challenged in a hypoosmotic condition. However, after blocking Kir channels with barium, Müller cell somata swelled significantly. Additionally, exogenous BDNF prevented barium-induced hypoosmotic swelling of the cells that was selective to TrkB (Berk et al., 2015). Furthermore, AQP4 has been shown to synergistically interact with the transient receptor potential isoform 4 (TPRV4) in Müller cells during glial swelling. Under hypotonic stress, water influx via AQP4 activates TPRV4 (which calcium enters through) and further promotes swelling (Jo et al., 2015). Moreover, calcium influx has also been show to modulate BDNF expression that is mediated by the activation of CREB (Shieh et al., 1998; Tao et al., 1998).

Although evidence of osmotic swelling inhibition by BDNF are still currently unknown, striking results from Berk et al. (2015) and Jo et al. (2015) could possibly link the relationship of BDNF and AQP4 in synaptic plasticity. Indeed, ionic flux coupled with water can alter the ECS and ultimately modulate neuronal activity (Andrew and MacVicar, 1994; Schwartzkroin et al., 1998; Dmitriev et al., 1999). The defect in LTD seen in AQP4-null animals (Skucas et al., 2011) could be a consequence of excess BDNF binding to TrkB during LFS to induce LTP but which was masked by the resulting LTD effect (Scharfman and Binder, 2013). The excess BDNF can also be explained through increases in calcium influx that promotes transcription of BDNF by CREB. Additionally, it is important to note that potassium mediated currents by Kir channels were blocked in Müller cells in the study conducted by Berk et al. (2015). Therefore, although the impaired potassium reuptake and altered water regulation was observed in AQP4-null mice could be of some agreement with the study, the enhanced ECS in AQP4-null mice seems to challenge the notion of osmotic swelling inhibition by BDNF. This could also be due to the difference in potassium kinetics in retinal Müller cells as compared to brain astrocytes. The mechanisms underlying excessive BDNF and swelling and its relationship to AQP4 remain to be resolved.

It is also well established that mature BDNF modulates LTP at the SC-CA1 synapse (Kang and Schuman, 1995; Jiang et al., 2003). Studies have demonstrated that release of mature BDNF leads to LTP (Kang and Schuman, 1995) and inhibits LTD (Jiang et al., 2003) and LFS suppresses the release of mature BDNF (Aicardi et al., 2004; Yang et al., 2013). Therefore, LTD induced by LFS was expected in WT mice, however, the delayed LTP observed in AQP4-null mice was unanticipated (Scharfman and Binder, 2013). Mature BDNF is known to be released by glial cells (Parpura and Zorec, 2010; Perea and Araque, 2010), hence, robust release of mature BDNF after LFS in AQP4-null mice might expound the observed LTP after LFS in AQP4-null slices (Scharfman and Binder, 2013). The data presented here suggests a tight regulation of pro and mature BDNF release for the competition of either LTP or LTD and that there may be a functional role of AQP4 in BDNF release in modulating synaptic potentiation.

Figure 1 depicts possible mechanisms of impaired LTP due to the absence of AQP4.

AQP4 IN SPATIAL LEARNING AND MEMORY

Astrocyte dysfunction plays a major role in various neurological disorders that may affect synaptic plasticity. Since AQP4 has been shown to affect LTP and LTD, spatial learning and memory alterations in AQP4-null mice seems likely. To address this hypothesis, several studies have been conducted using different behavioral tasks to investigate the influence of AQP4 on cognitive functions. The Morris water maze (MWM) is a widely used method used to validate certain neurological conditions. The hippocampus has been suggested as the primary brain region for spatial memory acquisition and retrieval as well as memory storage and consolidation (D'Hooge and Deyn, 2001) and the hidden platform task of the WMW produces long lasting spatial memories (Kee et al., 2007). Fear conditioning is also a valuable technique in identifying neural circuits underlying synaptic plasticity in learning and memory particularly in the amygdala and hippocampus (Phillips and LeDoux, 1992; Maren, 2001; Pham et al., 2009). Finally, the hippocampal-dependent object placement (OP) task is useful in assessing cognition in spatial memory and discrimination and is also suitable for identifying memory alterations (Antunes and Biala, 2012). Behavior assessments from these tasks can help further elucidate the potential influence of AQP4 in synaptic plasticity and learning and memory.

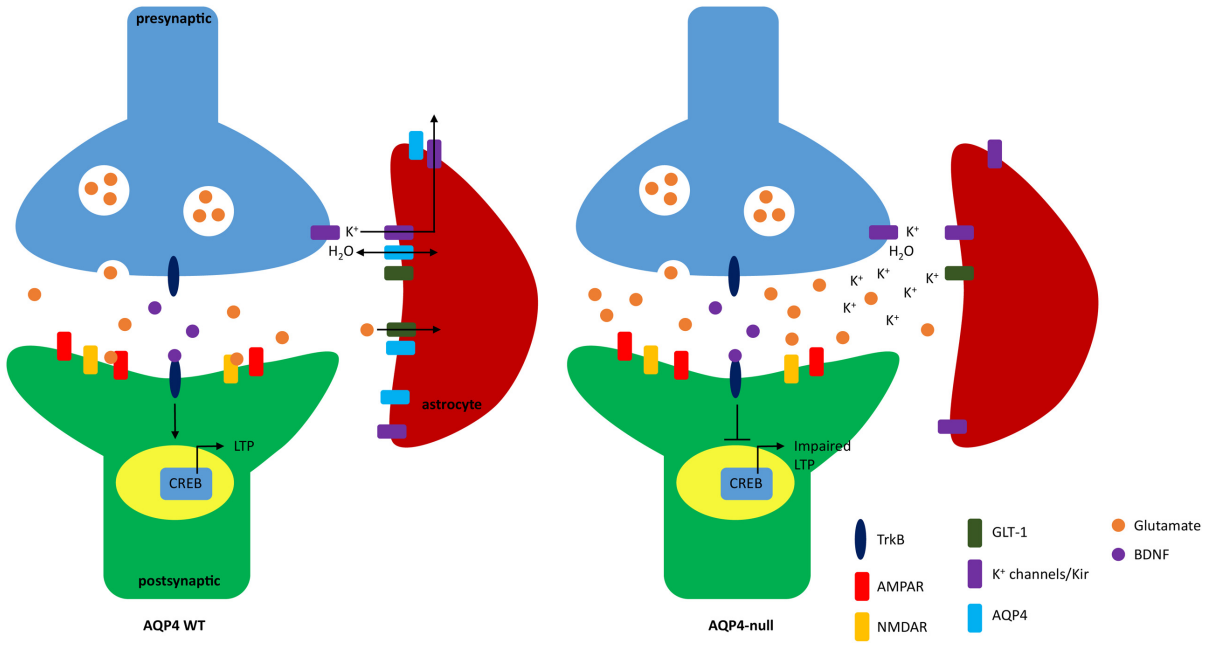

FIGURE 1 | Schematic of impaired long term potentiation (LTP) due to lack of AQP4. In WT mice, AQP4 facilitates the bidirectional transport of water and is seen colocalized with GLT-1 and Kir4.1. Extracellular glutamate and potassium is taken up by GLT-1 and Kir4.1, respectively. Binding of glutamate to NMDA receptor (NMDAR) and AMPA receptors (AMPAR) increases calcium influx and promotes surface expression of AMPAR (not shown). The influx of calcium also modulates brain-derived neurotrophic factor (BDNF). Binding of BDNF to TrkB is regulated through three pathways: PLCγ, PI3K, and MAPK which activates the transcription factor CREB that further promotes gene expression to induce LTP. In AQP4-null animals potassium reuptake in astrocytes is impaired. NMDAR is also dysregulated which may result in loss of calcium influx and ultimately lack of BDNF-TrkB binding which inhibits CREB activation and downstream effects important to synaptic plasticity.

Morris Water Maze

Skucas et al. (2011) used the MWM to assess hippocampal-dependent behaviors. Mice were trained twice a day for 9 days with the platform in the same location. Animals were then subjected to a probe test 24 h after the acquisition phase to measure their retention. The authors found that both WT and AQP4-null mice had similar performances and that both groups reached a plateau at approximately day seven that were not statistically significant. Swimming speed, conducted during the probe test, was also not significantly different between genotypes (Skucas et al., 2011).

In a different study, Liu et al. (2012) used a model for Alzheimer' disease (AD) to determine the effects of AQP4 in spatial learning and memory. Five month old female WT AQP4-null mice were first subjected to a bilateral ovariectomy (OVX) followed by daily injections of D-galactose (D-gal) 1 week after OVX for 8 weeks which was then proceeded by spatial memory testing using the MWM. High levels of D-gal, a reducing sugar in the body, results in the generation of a superoxide anion and oxygen-derived free radicals which ultimately produces brain oxidative stress-induced memory deficits. Control groups of WT and AQP4-null mice received sham operation and were treated with saline (Liu et al., 2012).

The training paradigm for the MWM, conducted by Liu et al. (2012), consisted of six consecutive days of training with four trails per day. The first 2 days of training were performed with a visible platform while the remaining 4 days utilized a hidden platform for testing. Escape latency, swim distance, swim speed, and swim patterns were analyzed. Genotype and treatment effects on motor ability and visual-spatial function was determined using the visible platform test. Swimming distance and escape latency only had an effect on training days and not on genotype or treatment. Additionally, there were no difference in swim speed in all groups during the first 2 days of training. This suggest that motor and/or visual deficits in adult mice were not due to OVX with D-gal treatment and AQP4 deficiency (Liu et al., 2012).

Spatial learning ability was then assessed in the hidden platform test. The authors found that escape latency and swim distance gradually decreased over the 4 days of training in all groups. WT and AQP4-null OVX plus D-gal treated groups required more time and distance to locate the hidden platform as compared to the vehicle treated control groups. Additionally, longer escape latency and swim distance was observed in AQP4 null-treated animals as compared to WT-treated animals, however, there was no difference between WT and AQP4-null control groups. Finally, swim speed was not affected by treatment or genotype demonstrating that swimming ability was unaffected and not related to spatial learning caused by OVX and D-gal treatment (Liu et al., 2012).

Swim patterns in the hidden platform tests was assessed to determine if delays in finding the hidden platform was associated

with abnormal search pattern and deficits in OVX and D-gal-treated mice. WT and AQP4-null control groups swam within the inner portion of the pool with weaving or looping search patterns indicating that the control animals had learned the location of the hidden platform after training. For the OVX and D-gal-treated mice, particularly the AQP4-null group, the mice swam in random patterns suggesting the animals found the platform by chance. The random swimming pattern was observed throughout the last trial on day 6. Swim patterns reveals further information regarding memory deficits in each group of animals. Control mice tend to swim directly to the target quadrant to search for the hidden platform, whereas the OVX plus D-gal-treated mice swam in repetitive looping patterns to reach the target quadrant. These results indicate greater spatial learning and memory defects in OVX plus D-gal treated mice which were greater in AQP4-null mice. Finally, a probe test was conducted 1 h following the training trials on day 6 to assess whether a mouse had learned the location of the platform. WT and AQP4-null OVX plus D-gal treated mice spent a smaller percentage of time in the target quadrant and higher percentage of time in the adjacent quadrants as compare to the vehicle control mice. Additionally, WT-treated mice spent greater time in the target quadrant as compared to the AQP4 null-treated mice (Liu et al., 2012).

Fan et al. (2013) also employed MWM to investigate the correlation between AQP4 and memory consolidation between WT and AQP4-null mice. In this experiment mice were trained for four trails per day for five consecutive days. A probe trial was tested 24 h after the acquisition phase where the platform was removed and the mouse was allowed to swim for 60 s. One day after the first probe test, mice were trained for the reversal phase where the platform was removed and placed in the opposite quadrant used during the acquisition phase. Again, 24 h after the reversal phase, a second probe test was conducted. In another subset of mice a visible platform task was tested 24 h after the probe tests for two consecutive days where the platform was made visible using a black cubic landmark (Fan et al., 2013).

The authors noted a disruption in memory consolidation in the AQP4-null mice through analysis and measurement of the total time latency of the swim path during the acquisition and reversal phases. During the training period in the acquisition phase, WT mice showed improvement in their performance being able to reach the hidden platform faster over time while AQP4-null mice displayed a significantly shorter time of swim path compared to the WT only during the early stages of the acquisition phase. Similar to the acquisition phase results, AQP4-null mice had a shorter time of swim path in the early stages of the reversal phase as compared to WT. However, after comparing each trial, the authors noted that the AQP4-null mice seemed to forget the escape position more easily than the WT mice even though both genotypes were able to find the hidden platform. The probe tests also revealed impaired spatial retention in mice deficient in AQP4. The latency to cross the hidden escape platform was longer in AQP4-null mice as compared to the WT but the numbers of crosses were less in the AQP4-null mice. Furthermore, AQP4-null mice did not show preference toward the trained quadrants as the time spent in the quadrants were less than the WT. Finally, there was no difference in performance during the visible platform task in both genotypes which suggests that the AQP4-null mice presented learning deficits as compared to the WT (Fan et al., 2013).

Zhang et al. (2013) continued investigations from Fan et al. (2013) testing the hypothesis that AQP4 modulates the aversive motivation in MWM. The authors used two MWM testing paradigms; (1) hidden platform training (acquisition training with reward) and (2) non-platform training (swimming only without reward). In both MWM training protocols, WT and AQP4-null mice were trained for four trials a day for 9 days. A probe test was conducted on day 10. On days 11 and 12, a cued platform training was performed where the platform was made visible and moved to the opposite quadrant from the training quadrant. A different subset of WT and AQP4-null mice were used for the non-platform training. The latency to the hidden platform, distance moved, and the mean swimming velocity was measured and analyzed. The authors considered that reaching the hidden platform to be rewarding and thus the motivation to reach the reward would be measured by the mean swimming velocity (Zhang et al., 2013).

Similar to findings from Fan et al. (2013) and Zhang et al. (2013) also reported a shorter latency to the hidden platform in AQP4-null mice during the first day of training and a longer escape latency during the last training days. Although both genotypes showed progressive improvement in escape latency, there were no significant differences between WT and AQP4-null mice. Additionally, both genotypes displayed an increase in shorter pathways during the training phase; AQP4-null mice, surprisingly, traveled a significantly shorter distance than the WT mice. No differences in distance traveled to the platform was observed during the later training days in both genotypes. Swimming velocity was significantly different in both genotypes during the latter 7 days of the training period. WT mice had increased swimming velocity over the course of the acquisition phase while AQP4-null mice displayed a decrease in swimming velocity. The increased escape latency in AQP4 could be attributed to the decreased swimming velocity. These findings suggests that impairment of spatial learning and reduction of motivation could be associated with a lack of AQP4 in mice (Zhang et al., 2013).

In the non-platform MWM task, AQP4-null mice exhibited a significant decrease in their mean swimming distance and velocity. There were no differences in swimming distance or velocity between the WT and the AQP4-null during the first day of training. While both genotypes demonstrated the same length in swimming during the four-trial sessions, the AQP4-null mice showed a gradual reduction in travel length during the later training periods (Zhang et al., 2013).

To further analyze spatial learning, Zhang et al. (2013) used the probe test to measure memory retention in WT and AQP4-null from both MWM training. After the acquisition phase in the hidden platform task (reward), the probe test revealed that WT mice preferred the target quadrant. WT mice displayed more time and more distance traveled in the target quadrant. Additionally, WT mice showed more numbers of platform crossed as compared to AQP4-null mice. In the non-platform task (no reward), both

WT and AQP4-null mice showed no preference to any of the four quadrants (Zhang et al., 2013).

Motivation of WT and AQP4-null animals during the probe test was then explored through analysis of swimming distance and velocity. There were no significant differences in swimming distance and velocity in WT mice. AQP4-null mice in the non-platform (swimming only without reward) training displayed a significantly shorter swimming distance and velocity as compared to AQP4-null mice that were trained with the hidden platform (reward). Furthermore, while AQP4 KO mice in the hidden platform task showed a reduction in swimming velocity, there was no significant differences in swimming velocity as compared to WT mice in the same hidden platform training. A possible explanation could be due to the fact that WT mice were decreasing their swimming speed while AQP4-null mice were increasing their swimming speed on their last training day prior to the probe test (Zhang et al., 2013).

Finally, the authors subjected the animals to two trials of the cued platform task where a visible platform was moved to the opposite quadrant from the training quadrant. During the first trial, escape latency, swimming distance, and swimming velocity did not differ between WT and AQP4-null mice that had undergone acquisition training (hidden platform with reward). In contrast, mice that were subjected to swimming only training (no reward) had a greater escape latency compared to animals that had acquisition training. Furthermore, in the group trained without reward, WT mice displayed a greater distance traveled compared to AQP4-null mice, however, by the second trial, WT mice had shorter distance travel path to the platform. WT mice trained without reward also showed an increase in swimming velocity compared to AQP4-null mice trained without reward. Escape latency and swimming velocity did not differ between genotypes in both training paradigms on the second trial. These findings suggest that acquisition training can improve the animals' ability to reach the cued platform (Zhang et al., 2013). A summary of the MWM findings are listed in **Table 2**.

Contextual Fear Conditioning

Skucas et al. (2011) also performed contextual fear conditioning (CFC) on the same group of WT and AQP4-null mice 2–3 weeks after MWM testing (see above; Skucas et al., 2011). In this CFC protocol, mice were placed into the conditioning chamber and received three 2 s, 0.75 mA scrambled footshocks 2.5, 3.5, and 4.5 min after placement into the chamber. During the retention test (24 h after training), the animals received one 5 min exposure to the chamber without footshocks (Pham et al., 2009). CFC testing revealed that AQP4-null mice were more immobile than the WT during the conditioning phase, however, the levels of immobility between the two genotypes were not statistically significant (Skucas et al., 2011) suggesting that AQP4-null mice had normal long-term memory for contextual fear (Scharfman and Binder, 2013).

Using a different CFC method, Yang et al. (2013) observed contrasting results as compared to Skucas et al. (2011). In their study, mice were trained and tested in the conditioning chambers for two consecutive days. During the training period, the animals were exposed to the conditioning chamber for 3 min followed by a 2 s, 1.0 mA constant current foot shock. Memory test was performed 24 h after the training period by re-exposing the mice to the conditioning chamber for 3 min with the absence of the foot shock. Here, AQP4-null mice showed pronounced decreased freezing behavior as compared to WT mice. The results from this study suggests that AQP4 deletion impairs associate fear memory formation. Additionally, immobility in AQP4-null mice treated with Cef daily for a week had increased significantly. WT mice treated with Cef increased freezing behavior whereas WT mice treated with DHK decreased immobility. (Yang et al., 2013). These findings indicates that Cef may have therapeutic benefits in rescuing hippocampal-dependent memory deficits through increasing GLT-1 expression.

Using a light fear conditioning protocol, Li et al. (2012) determined if amygdala-dependent learning behavior is altered in AQP4-null mice. During the conditioning period, mice were allowed to explore the conditioning chambers for 3 min followed by a light conditioned stimulus (CS) which was produced by an 8 W white light bulb that was presented for 30 s and co-terminated with a single electric foot shock (0.7 mA, 1 s). The light-cued fear memory was tested 2 and 24 h after conditioning and freezing behavior was monitored for 3 min with the presentation of the light. Prior to CS, both WT and AQP4-null mice did not exhibit any significant differences in baseline behavior. During training, both genotypes displayed increased freezing behavior, however, differences in immobility were not statistically significant between WT and AQP4-null mice suggesting that normal acquisition of cued fear memory was not altered in AQP4-null mice. Freezing behavior was also unaltered in WT and AQP4-null mice 2 h after training, however, AQP4-null mice displayed reduced immobility 24 h after conditioning indicating that consolidation of associate fear memory is impaired in AQP4-null mice (Li et al., 2012).

To corroborate the findings that the freezing behavior was specific to fear-associated learning, the authors also performed an open field test, elevated plus maze, and a nociception test. A 10 min analysis in locomotive behavior during the open field test demonstrated no differences in locomotive activity between WT and AQP4-null mice. The results suggests that hyperlocomotive activity was not a factor in the decreased freezing behavior seen in AQP4-null mice. Elevated plus maze also resulted in no significant differences between WT and AQP4-null mice indicating unaltered level of innate fear and anxiety in AQP4-null mice. Finally, pain thresholds as assessed through vocalization and jump responses to increased intensity of electric shocks were not statistically significant between the two genotypes indicating that pain sensitivity does not influence fear memory impairment in AQP4-null mice (Li et al., 2012).

Object Placement

The OP test was also performed in WT and AQP4-null mice by Skucas et al. (2011) using standard methods with a 1 or 24 h interval between the two trials (Scharfman et al., 2007). During the first trial, WT and AQP4-null mice spent the same percent of time exploring the objects when first presented. During the second trial (1 h interval), WT mice spent more time exploring the moved object while AQP4-null mice did not show

TABLE 2 | Summary of findings from Morris water maze.

Study	Findings
Skucas et al., 2011	1. No significant differences between genotypes
Liu et al., 2012	1. No significant differences between genotypes during the visible platform test. 2. No significant differences between genotypes and treatment during the hidden platform test. 3. KO-treated animals had longer escape latency and swim distance. 4. KO-treated mice had higher random swim pattern. 5. KO-treated mice spent less time in target quadrant.
Fan et al., 2013	1. KO mice had shorter swim path in earlier stages of training. 2. KO mice had longer escape latency. 3. KO mice spent less time in target quadrant. 4. No significant differences in performance between genotypes during the visible platform task.
Zhang et al., 2013	1. No significant differences in escape latency between genotypes (hidden platform). 2. KO mice traveled shorter distances (hidden platform). 3. KO mice had significantly decreased swimming distance and velocity (hidden platform). 4. KO mice displayed gradual reduction in travel length (non-platform). 5. No significant differences between genotypes for quadrant preference (non-platform). 6. No significant differences between genotypes in escape latency, swimming, distance, and swimming velocity during cued platform task (hidden platform). 7. Wild-type mice had greater distance traveled compared to KO mice on first day of cued platform task (non-platform). 8. WT mice had increased swimming velocity compared to KO mice on first day of cued platform task (non-platform). 9. No significant differences in escape latency and swimming velocity between genotypes on second day of cued platform task (hidden platform and non-platform).

Comparison of findings from the Morris water maze in WT and KO mice.

preference toward the moved object. During the 24 h interval, WT and AQP4-null mice spent similar time exploring the objects. However, during the second trial, WT mice again spent more time exploring the moved object (Skucas et al., 2011). These results indicate that WT mice can differentiate between objects in a familiar and new location and that there is a defect in object placement memory in the AQP4-null mice (Scharfman and Binder, 2013).

AQP4 REGULATION IN COGNITIVE FUNCTIONS

Behavioral tasks findings between WT and AQP4-null mice varied between each study. Groups that utilized the MWM all saw impairment in spatial memory except for Skucas et al. (2011). Additionally, Skucas et al. (2011) also observed no significant differences between WT and AQP4-null mice during CFC while Li et al. (2012) and Yang et al. (2013) reported evident differences in immobility between WT and AQP4-null mice. Finally, OP test revealed defects in object placement memory as reported by Skucas et al. (2011).

Impaired cognitive function observed by Liu et al. (2012) in both WT and AQP4-null OVX plus D-gal treated animals can be correlated to decreased expression of the presynaptic vesicle protein synaptophysin (SYP) and the postsynaptic density protein-95 (PSD-95; Liu et al., 2012). These proteins are known to be altered in the hippocampus and cause memory deficits during the progression of AD (Sze et al., 1997; Xu et al., 2015). Another study of AD proposed a pathway including PSD-95, BDNF, and NMDAR. In this model, NMDAR stimulation recruits TrkB to the synapse and initiates BDNF signaling through the PI3K pathway to transport new PSD-95 to the synapse where it acts as a scaffold for BDNF receptors (Yoshii and Constantine-Paton, 2010). Decreased expression of SYP and PSD-95 were reported in both WT and AQP4-null OVX-treated mice, however, the reduced expression of these two proteins were more pronounced in AQP4 deficient animals which is consistent with the decline in spatial learning and memory (Liu et al., 2012). The deficits in cognitive function of these animals can be attributed to the lack of new PSD-95 to further strengthen the responsiveness of the synapse to BDNF to promote LTP.

Additionally, the cholinergic system has been linked to cognitive deficits in AD (McKinney, 2005; Schliebs and Arendt, 2006) and is also vulnerable to oxidative damage (McKinney, 2005). Interestingly, cholinergic neurons have also been associated with endogenous levels of estrogen (Gibbs, 1998) which has been correlated to BDNF and memory functions (Bekinschtein et al., 2007; Francis et al., 2012; Luine and Frankfurt, 2013). The OVX treatment performed by Liu et al. (2012) could reduce levels of BDNF which is mediated by activation of CREB (Shieh et al., 1998; Tao et al., 1998) and sublethal accumulation of Aβ has been shown to suppress activation of CREB (Tong et al., 2004). Additionally, the authors have observed a significant reduction of cholinergic neurons as well as increased brain oxidative stress and reduced antioxidative capabilities in AQP4-null-treated mice as compared to WT-treated mice and control animals. Therefore, an increase in Aβ in the hippocampus (a hallmark of AD) and brain oxidative stress with a decrease in SYP, PSD-95, and cholinergic neurons can be attributed to a lack of AQP4 in regulating astrocytic functions (Liu et al., 2012).

Studies performed by Fan et al. (2013) demonstrated significant memory consolidation impairment in AQP4-null mice as compared to WT mice. In particular, AQP4 deficient mice had an obvious dissociation between memory acquisition and spatial retention as assessed by the MWM. The authors

stated that these findings can be attributed to the impaired hippocampal TBS-LTP *in vivo* (PP-DG) and *in vitro* (SC-CA1) and suggest that AQP4 may act downstream of glutamate receptors to regulate LTP memory formation and consolidation (Fan et al., 2013). The DG plays a fundamental role in memory storage and acquisition and is the site of neurogenesis (Kee et al., 2007; Jessberger et al., 2009; Fan et al., 2013). Studies have shown that as new neurons mature they are incorporated into the spatial memory circuits (Kee et al., 2007) and inhibiting neurogenesis in the DG results in impaired spatial and object recognition (Jessberger et al., 2009). Furthermore, AQP4 is expressed in adult neural stem cells (ANSCs) and has been shown to be involved in neurogenesis (Zheng et al., 2010) and participating in various vital roles such as neuronal migration (Saadoun et al., 2005; Zheng et al., 2010). Even though the authors did not observe alterations in the number of neural stem cells after MWM in either genotype, they deduced that the absence of AQP4 could possibly block the recruitment of new neurons to the spatial memory circuits that ultimately contributes to memory processing in the DG. The results indicate that ANSCs were not recruited into the DG memory circuit and that neuronal proliferation was inhibited (Fan et al., 2013). These findings can be validated by the previously mentioned roles of AQP4. The impaired memory consolidation observed in the AQP4-null mice may be a consequence of the lack of AQP4 in promoting cell migration and proliferation which would eventually inhibit the recruitment of ANSCs into the spatial memory network in the DG to stabilize memory trace (Fan et al., 2013).

Subsequent studies by Zhang et al. (2013) confirmed the dissociation between acquisition and spatial retention in APQ4-null mice that was observed by Fan et al. (2013). The authors noted significant reduction in swimming velocity in the AQP4-null mice as compared to the WT mice which they attributed to be a deficit in aversive motivation. During the first day of training in the hidden platform test the AQP4-null mice were more capable in finding the platform, however, their performance levels on escape latency was not comparable to the WT animals which resulted in reduced swimming velocity. Additionally, AQP4-null mice also showed slower swimming velocity during the swimming only task. The authors suggested that the AQP4-null mice "gave up" during a difficult task implying a lack of motivation (Zhang et al., 2013). Dopamine has been highly regarded in learning and motivation (Dayan and Balleine, 2002; Wise, 2004) and the regulation of dopamine has been correlated to AQP4 (Fan et al., 2005, 2008). Previous studies using AQP4-null mice have reported increased basal extracellular levels of dopamine (Fan et al., 2005; Ding et al., 2007), however, the correlation between increased levels of dopamine and motivation remains elusive (Cagniard et al., 2006; Treadway et al., 2012).

The hippocampus and the LA have both been implicated in the neural circuit of fear memory (Phillips and LeDoux, 1992). Although Skucas et al. (2011) observed an increase in immobility in both WT and AQP4-null mice during the testing trial of the CFC, the differences between the two genotypes were not statistically significant. On the other hand, Yang et al. (2013) observed contrasting findings. In their study, AQP4-null mice had a significant reduction in freezing behavior as compared to WT mice, however, this was rescued by chronic treatment of the GLT-1 activator Cef (Yang et al., 2013). The inconsistency in findings between the two studies could be due to different testing protocols. In another study, Li et al. (2012) also observed an increased in immobility in WT and AQP4-null mice, however, the AQP4-null mice had a significant reduction in freezing behavior 24 h after training as compared to WT mice. Similarly, Cef treatment rescued the impairment in mice lacking AQP4 (Li et al., 2012). Findings from Li et al. (2012) further solidified the hypothesis that GLT-1 is a key player in the mechanisms underlying AQP4 regulated synaptic plasticity and memory formation. Finally, the differences in findings between these studies are not entirely surprising as the discrepancies lies in the specific fear conditioning protocol. The hippocampus is known to be dependent on contextual fear memories while the LA is dependent on cued fear memories. Therefore, the mechanisms for the retrieval of the fear memory for either the hippocampus or the LA are not identical (Phillips and LeDoux, 1992; Hall et al., 2001; Li et al., 2012).

Memory impairment was only observed in the AQP4-null mice during the OP task and not the MWM or CFC in the study conducted by (Skucas et al., 2011). Associating the deficit to the short time interval during training and testing seems unlikely since the CFC was also tested during a 24 h interval. This may also be correlated to the early phase of LTP after TBS [60 min after induction (Scharfman and Binder, 2013)]. Nonetheless, the impairment observed during the OP task may be related to the different neural circuitry involved in this specific task as compared to MWM and CFC. Interestingly, the mechanisms underlying memory performance in OP task has been linked to BDNF (Bekinschtein et al., 2007; Francis et al., 2012; Luine and Frankfurt, 2013).

The studies presented here thus far have provided great insight to the role of AQP4 in learning and memory in hippocampal and amygdaloid-dependent tasks. While there are discrepancies between studies one must recognize that behavior performances from animals can be influenced by various factors such as differences in animal (strain, sex, age) and experiment protocols (differences in training). For example, consideration of mouse strain when interpreting data in specific behavioral tasks is imperative as different strains tend to exhibit marked differences in performances (Adams et al., 2002; Patil et al., 2008, 2009). Furthermore, defects in LTP, LTD, and behavioral tasks are not always entirely correlated to each other (Jun et al., 1998; von Engelhardt et al., 2008; Leiva et al., 2009). Despite the varied findings all groups have concluded that the lack of AQP4 results in cognitive deficits and these data have shed some light into the possible role of AQP4 in regulating learning and memory.

CONCLUSION

AQP4 is the major water channel in the CNS and it is now established that AQP4 possesses greater functions beyond regulating water homeostasis in the brain. It is clear that the

absence of AQP4 plays a unique role in synaptic plasticity and learning and memory although the exact mechanisms remain unclear. These emerging studies provides a glimpse into the potential role of AQP4 in LTP, LTD, and cognitive functions that was once elusive.

In reviewing the findings from these studies, it is evident that synaptic plasticity and learning and memory seems to be, in part, regulated by AQP4. For example, the fundamental basis of LTP induction requires NMDAR activation, however, mechanisms underlying the impairment of LTP in AQP4-null mice seems to be an indirect consequence from the lack of AQP4. In particular, the down regulation of GLT-1 and the subsequent elevation of glutamate in the ECS results in the strong activation of NMDAR which seems to inhibit LTP in KO mice. Furthermore, defects in potassium homeostasis plays a role in different stages of LTP. Finally, the neurotrophin BDNF is also observed as a key player in modulating LTP which may be associated with cellular swelling. Moreover, impairment of LTP observed in AQP4-null mice was followed by memory decline as assessed by MWM, fear conditioning, and object placement tasks, reiterating the potential role of AQP4 in cognition. While convincing studies reveal a critical role of AQP4 in synaptic plasticity and learning and memory, the mechanisms underlying the cellular and behavioral changes in mice lacking this astrocyte water channel is still unknown.

The interest of AQP4 in synaptic plasticity and cognition is also critical from a public health standpoint. AQP4 has been implicated in various neurological disorders such as epilepsy, cerebral edema, and Alzheimer's disease. The effects of AQP4 in learning and memory are only beginning to be elucidated, therefore, ongoing research efforts is of great importance to the clinical field as there may be potential therapeutic benefits that may modulate this protein. And while there are certainly compelling evidence from these studies future investigations are required to further understand the precise role of AQP4 in synaptic plasticity and learning and memory.

AUTHOR CONTRIBUTIONS

JS performed a complete literature review and drafted the manuscript. DB conceived of the manuscript and provided feedback on its content.

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frontiers in Integrative Neuroscience

REVIEW
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Role of Glycogenolysis in Memory and Learning: Regulation by Noradrenaline, Serotonin and ATP

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This paper reviews the role played by glycogen breakdown (glycogenolysis) and glycogen re-synthesis in memory processing in two different chick brain regions, (1) the hippocampus and (2) the avian equivalent of the mammalian cortex, the intermediate medial mesopallium (IMM). Memory processing is regulated by the neuromodulators noradrenaline and serotonin soon after training glycogen breakdown and re-synthesis. In day-old domestic chicks, memory formation is dependent on the breakdown of glycogen (glycogenolysis) at three specific times during the first 60 min after learning (around 2.5, 30, and 55 min). The chicks learn to discriminate in a single trial between beads of two colors and tastes. Inhibition of glycogen breakdown by the inhibitor of glycogen phosphorylase 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) given at specific times prior to the formation of long-term memory prevents memory forming. Noradrenergic stimulation of cultured chicken astrocytes by a selective β_2-adrenergic (AR) agonist reduces glycogen levels and we believe that *in vivo* this triggers memory consolidation at the second stage of glycogenolysis. Serotonin acting at 5-HT_{2B} receptors acts on the first stage, but not on the second. We have shown that noradrenaline, acting via post-synaptic α_2-ARs, is also responsible for the synthesis of glycogen and our experiments suggest that there is a readily accessible labile pool of glycogen in astrocytes which is depleted within 10 min if glycogen synthesis is inhibited. Endogenous ATP promotion of memory consolidation at 2.5 and 30 min is also dependent on glycogen breakdown. ATP acts at P_2Y_1 receptors and the action of thrombin suggests that it causes the release of internal calcium ($[Ca^{2+}]_i$) in astrocytes. Glutamate and GABA, the primary neurotransmitters in the brain, cannot be synthesized in neurons *de novo* and neurons rely on astrocytic glutamate synthesis, requiring glycogenolysis.

Keywords: astrocytes, glycogen re-synthesis, noradrenaline, ATP, serotonin, memory processing, consolidation, day-old chickens

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INTRODUCTION

The major fuel for all cells in the brain is glucose and both astrocytes and neurons metabolize glucose via glycolysis and oxidative phosphorylation. Neuronal transmission of information is undoubtedly important for memory but astrocytes are also critically involved in memory processing. Memory is dependent on metabolic functions including Na/K-ATPase activity and

glucose uptake, which occur in both neurons and astrocytes. It is also reliant on glycogen breakdown and re-synthesis. It is recognized that glycogen has an important role in the brain (e.g., Brown, 2004; Brown and Ransom, 2007; Hertz et al., 2007; Oz et al., 2007; Morgenthaler et al., 2009). Normally only astrocytes can store glucose as glycogen. Glycogen in the brain is stable and has slow turnover under resting conditions (Watanabe and Passonneau, 1973; Choi et al., 2003; Oz et al., 2007). Glycogen is unlikely to be acting solely as a slowly available energy source. The breakdown of glycogen increases substantially during sensory activation in the brain (Swanson, 1992; Swanson et al., 1992; Cruz and Dienel, 2002) and it appears to have an active role in astrocytic function. It is necessary for memory formation in both chickens and rodents (O'Dowd et al., 1994; Gibbs et al., 2006; Suzuki et al., 2011; Newman et al., 2011; Duran et al., 2013). Both Suzuki et al. and Newman et al. assumed that glycogen-derived lactate is used as a metabolic fuel by neurons after release from astrocytes via the astrocytic monocarboxylate transporters MCT 1 and 4 and uptake in neurons via the neuronal MCT 2 (i.e., operation of the astrocyte-to-neuron lactate shuttle (ANLS)) suggested by Pellerin et al. (1998) and Pellerin and Magistretti (2012). However, a recent study by Tadi et al. (2015) showed that learning in mice increased expression of MCT 1 and 4 without affecting MCT 2. Thus astrocytic lactate release is important for learning but it is doubtful whether any neuronal uptake occurs. Moreover convincing evidence has recently been obtained for activity-dependent glucose phosphorylation in neurons (Patel et al., 2014), which focuses attention on recently discovered signaling functions of extracellular lactate (Tang et al., 2014; Bergersen, 2015). In this context it is interesting that the lactate signaling demonstrated by Tang et al. (2014) like memory in the chicken is abolished not only by the glycogenolysis inhibitor DAB (see below) but also by D-lactate (Gibbs and Hertz, 2008). The action of these and other drugs used in the studies to be reviewed are summarized in **Table 1**.

MEMORY FORMATION IN THE CHICKEN FOLLOWING ONE-TRIAL DISCRIMINATED AVOIDANCE LEARNING

The experiments described in this review use avoidance training in the day-old chick. Chicks are precocious and can discriminate between colors soon after hatch, and they do so after a single 10 s learning experience. At training a red bead is tainted with a compound of bitter taste (methyl anthranilate) and presented to the chick for 10 s. They peck at this bead once or twice (**Figure 1A**) before registering the bitter taste and turning away in disgust (**Figure 1B**). On test after a predetermined interval they are then presented with *clean* red (**Figure 1C**) and blue (**Figure 1D**) beads, each for 10 s. The chickens will avoid the second (untainted) red bead when presented, but they will continue to peck when presented with a neutral blue bead. Memory is measured as the ratio of pecks at red and blue beads on test. A high discrimination ratio reflects a good memory and avoidance of pecking at the red bead, whereas a discrimination ratio close to 0.5 reflects an increased rate of pecking at the red bead- such that red and blue beads are pecked at equally on the 10 s test (for details see Gibbs and Summers, 2002a; Gibbs et al., 2008d). It is not uncommon for chicks to give up to 10 pecks at the blue bead, and when they have forgotten they can give up to 10 pecks on the clean red bead.

The chicks are kept in pairs and used in groups of 20, or more recently, in groups of 16 chicks. The data from chicks not pecking the blue bead on test or not pecking the red bead on training are excluded from the data analysis at the completion of the experiment. Each data point on the behavioral graphs represents a single group of chicks with generally no more than 2–3 chicks excluded on the basis on not training or avoiding the blue bead, i.e., $N \geq 13$–14 when the original number in the group was 16. The very short training period enables exact timing of biochemical events correlated with learning.

The bead is either strongly aversive, i.e., the bitter taste is caused by application of 100% anthranilate, or weakly aversive when anthranilate is diluted to 20% with alcohol. When separate groups of chicks are tested at defined periods post-training after strongly reinforced training three stages of memory are revealed, and the memory remains for days, indicating that this corresponds to normal learning. However, even in normal learning there are brief periods of low discrimination ratios measured on testing at 15 or at 55 min. With weak reinforcement, with the exception of the test at 15 min after training, the memory is good on tests up to 30 min after which memory disappears (**Figure 1E**).

Based on the demonstration of different biochemical events (Gibbs and Ng, 1977) we have defined three stages of memory consolidation during normal learning : short-term (STM)-lasting for 10 min intermediate term memory (ITM), which has two different stages, ITMA between 20 and 30 min and ITMB between 30 and 50 min. The transition is normally triggered by neuromodulatory transmitters, including noradrenaline and serotonin, as will be reviewed here. Weakly reinforced learning (**Figure 1E**) includes ITMA but not ITMB, another indication that these two stages depend upon different metabolic events. The advantage of studying weakly reinforced learning is that it can be converted to normal learning by different procedures, providing information on possible underlying mechanisms. Normal learning can on the other hand be disrupted by other interventions, again providing clues about the underlying events. Accordingly, both types of learning will be discussed in this review.

Drugs are injected into the hippocampus in 1 μl volumes (Gibbs et al., 2008a; **Figure 1F**) or in 5 or 10 μl volumes into the intermediate medial mesopallium (IMM), a cortical integration area in the avian brain serving the same functions as the cortex in mammals (Puelles et al., 2000; Reiner et al., 2004; Jarvis et al., 2005). This area of the brain has been traditionally used for studies on memory (see Gibbs, 2008; Gibbs et al., 2008d). By injecting different groups of chicks at different times, we have been able to determine when memory is vulnerable to either inhibition in the case of strongly reinforced training or

TABLE 1 | Individual effects of drugs on glycogenolysis and memory in imm and hippocampus (Hp).

Receptor agonists		
ARC239	α_{2C}-AR agonistActivates glycogen synthesis	IMM and HP
RO363	β_2-AR agonist consolidates ITMA	HP not IMM
Zinterol	β_2-AR agonist consolidates ITMB Activates glycogenolysis	IMM and HP
CL316243	β_3-AR agonist consolidates ITMA	IMM and HP
5-HT	Serotonin, an agonist on many subtypes, activates glycogenolysis, but probably not glycogen synthesis	IMM, HP unknown
Fluoxetine	5-HT_{2B} agonist	IMM, HP unknown
Paroxetine	5-HT_{2B} agonist	IMM, HP unknown
ATP	ATP receptor agonist, promotes consolidation 2.5 and 30 and 35 min after training	IMM and HP
ADP| 3S	P2Y1 receptor agonist, promotes consolidation 2.5 and 35 min after training	HP
ATPγS	P2Y2 receptor agonist, promotes consolidation 2.5 and 30 min after training	HP
Thrombin	Stimulates release of internal calcium ($[Ca^{2+}]_i$) in astrocytes, promotes consolidation 2.5 and 35 min after training	IMM and HP
Phaclofen	$GABA_B$ receptor antagonist inhibits memory during STM and ITMA injected up to 25 min after training	IMM and HP
Receptor antagonists		
CGP20712A	β_1-ARs, antagonist inhibits STM	HP not IMM
ICI118551	β_2-AR antagonist inhibits ITMB	IMM and HP
SR59230A	β_3-AR antagonist inhibits ITMA	IMM and HP
SB221284	5-$HT_{2B/C}$ R antagonist inhibits STM and ITMB	IMM HP unknown
MRS2179	P2Y1 receptor antagonist, inhibits consolidation 2.5 and 35 min after training	STM and HP
D-AVP	NMDA receptor antagonist, inhibits 2.5 and 30 min after training	IMM and HP
Metabolic inhibitors		
DAB	Inhibits glycogenolysis	IMM and HP
Fluoroacetate	Inhibits astrocytic oxidative metabolism and memory during STM and ITMA injected up to 20 min after training	IMM and HP

consolidation in the case of weakly reinforced training. When injections of inhibitory agents are made after strongly reinforced training, memory is poor on test either at 120 min or at specified times after training; consolidation of weakly reinforced training maintains memory after ITMA. **Figure 1F** shows details of the injection procedure for hippocampal injections and it can be seen that tissue damage due to the injection is minimal (Gibbs et al., 2008a). All procedures reviewed here have been approved by the Monash University Animal Ethics Committee and comply with the 1997 Australian Code of Practice for the care and use of animals for scientific purposes. All efforts were made to minimize both the suffering and the number of animals used. Chicks were killed at the completion of each experiment by CO_2 inhalation.

Key points of our findings are (1) the involvement of glycogen in memory processing and in synthesis of glutamate, a neurotransmitter essential for learning (Riedel et al., 2003) and long-term potentiation, LTP (Bashir et al., 1993), (2) the role of neuromodulators in glycogenolysis, glycogen synthesis and learning, and (3) mechanisms by which the neuromodulators influence glycogen storage and breakdown and thus the processes during learning which are glycogenolysis-dependent. A crucial role of glycogenolysis in glutamate synthesis (**Figure 2**) was first shown in chick brain during learning (Gibbs et al., 2007) and its role in support of glutamatergic transmission was confirmed in co-cultures of neurons and astrocytes (Sickmann et al., 2009) and in the mouse brain (Sickmann et al., 2012). That synthesis of transmitter glutamate as well as return of previously released transmitter glutamate depends on astrocytic metabolism has been known for a long time (reviewed by Gibbs et al., 2008b; Hertz, 2013; Hertz and Rothman, 2015), however that the astrocytic-neuronal flux carrying glutamate from astrocytes to neurons equals the rate of total neuronal glucose consumption (or 75% of total gray matter oxygen uptake) has only been established more recently (Sibson et al., 1998; Rothman et al., 2011). This shows the intensity of the astrocytic-neuronal interactions required during learning.

MEMORY FORMATION REQUIRES THE BREAKDOWN OF GLYCOGEN

Measurement of the change in glycogen levels, expressed as % of pretraining levels revealed two periods where total forebrain levels decreased and later rose to former pretraining levels (O'Dowd et al., 1994; Hertz et al., 2003). There is a large, rapid decrease 2.5 min after training where glycogen levels remain low for 10 min and then return to previous levels within 5 min (**Figure 3A**). There is a second transient decrease at 55 min. This fits wth the turnover of brain glycogen increasing during activation of brain tissue (Swanson et al., 1992; Dienel et al., 2003, 2007). A third tendency toward a decrease at 30 min is not significant.

The importance of the breakdown of glycogen for learning is seen when an inhibitor of glycogenolysis or glycogen breakdown is injected into the forebrain at different times after training. The glycogen phosphorylase inhibitor DAB (Andersen et al., 1999; Fosgerau et al., 2000) blocks glycogenolysis but is known not to affect glycolysis.

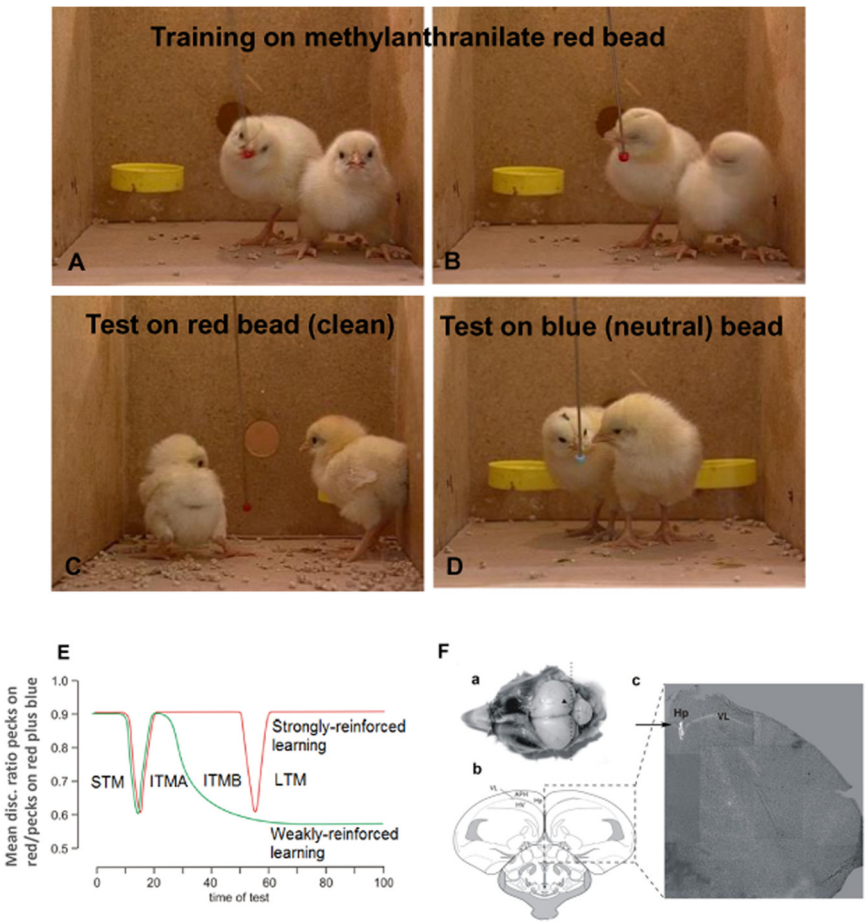

FIGURE 1 | Memory model established from single trial learning in day-old chicks. Prior to training chicks are presented once with clean red and blue beads to ensure they will peck at beads of both colors. For training they are presented for 10 sec with a red bead lightly coated in methyl anthranilate **(A)**. They peck at this once or twice before registering the bitter taste and turning away in disgust **(B)**. On test they are then presented with *clean* red **(C)** and blue **(D)** beads, each for 10 s and the number of pecks on each counted using an electronic counter and converted by computer to discrimination ratios (DRs). Perfect learning equals a DR of 1, and complete forgetting or inhibition of learning a DR of 0.5. Normally the DR after unimpaired learning is ∼0.9. The chicks are kept in pairs and 8–10 pairs are included in the group used in each experiment, which allows reliable determination of significance. Each data point on subsequent graphs represents one group. **(E)** Memory stages following strongly reinforced (red line) after exposure to undiluted aversant and weakly reinforced training (green line) after exposure to diluted aversant. The loss of labile, weakly reinforced memory coincides with the transition between two phases of intermediate memory (ITM A and B) 30 min post-training. Drugs are used to inhibit strongly reinforced learning or rescue weakly reinforced learning, as indicated by memory retained 120 min after training. **(F)** Illustration of injection for hippocampal administration of drugs. (a) Image of head with scull removed and injection site indicated by arrowhead. Dotted line indicates coronal section presented in panels (b) and (c). see Gibbs et al., 2008a for details. (1F from Gibbs et al., 2008a).

In chicks where DAB was injected into IMM 5 min before training or 55 min after training, there was very poor memory 2 h later, but injection between 25 and 45 min after training also decreased memory (**Figure 3B**). When does this memory deficit occur? The early blockade of glycogenolysis 5 min prior to training leads to an inability to retrieve memory from short-term storage until after 10 min after training, with no amnestic effect being seen between 20 and 30 min (**Figure 3C**); we concluded therefore that memory retrieval throughout the first part of intermediate memory (ITMA) does not depends on glycogenolysis, but does so on tests from 40 min on. Effects occur early and some of the consequences appear during STM, not ITMA but later during ITMB. However, DAB has effects on memory beyond this time. When memory is monitored after injections made 25 min after training, memory loss is seen at the earliest test made 35 min after training, i.e., 10 min after injection and with injections 55 min after training, memory is lost 15 min later – the earliest time tested (Gibbs et al., 2006).

DAB injected into the hippocampus also leads to memory loss (Gibbs et al., 2008a). However, the pattern of susceptibility in the hippocampus is not not the same as in IMM (see **Figure 3D**). There are three important times where there is coincidence – 5, 25, and 55 min post- training, but there are differences between the two brain areas. DAB inhibits in the hippocampus when injected during ITMA in contrast to IMM, whereas during ITMB (30–50 min after training) DAB is inhibitory in the cortex but not in the hippocampus. When DAB is injected into the hippocampus 5 min before training, memory is present tested at 20 min

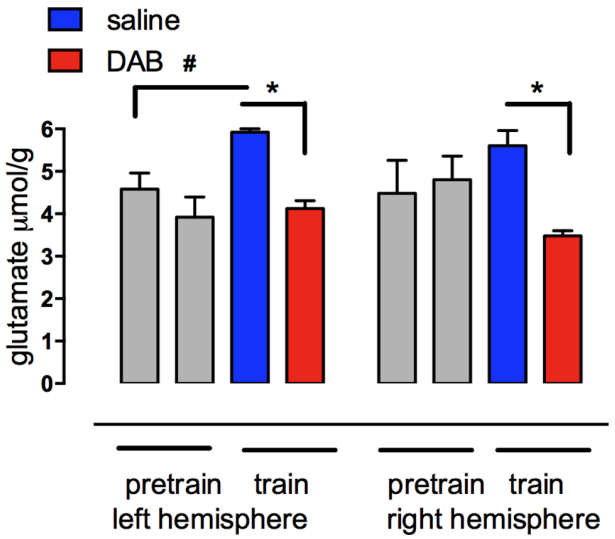

FIGURE 2 | Glutamate content measured with HPLC in left and right forebrain hemispheres of chicks before and after training in the presence of DAB or saline. Chicks were injected with either saline or DAB 5 min before strongly reinforced training and brains rapidly removed 5 min after training. The increase in glutamate in the left hemisphere is prevented by DAB. No concomitant decrease occurred in aspartate or glutamine. $^{\#,*}P < 0.05$. (From Gibbs et al., 2007).

but as with IMM injection memory is absent when chicks are tested 5 and 120 min after training (Gibbs et al., 2008a). DAB does not appear to produce a long acting inhibition of glycogen phosphorylase, since when it is injected 10 min before training it has no effect on memory. These data and results mentioned below suggest that there is a time lag of 5–10 min before glycogenolysis is significantly inhibited.

The breakdown of glycogen is clearly important for learning. Two of the susceptible periods when long-term memory is prevented occur when DAB is administered during STM (0–10 min post-training) or at the end of intermediate memory (ITMB) 55 min after training, and both periods correlate with the changes in glycogen levels in the forebrain. However, there is a second period when memory is sensitive to DAB (around 20–25 min post-training) but is not associated with a significant decrease in glycogen content, suggesting that glycogen synthesis may be going on at this time, offsetting the inhibitory effect on glycogenolysis. This is supported by the observation that an inhibitor of glycogen synthesis inhibits memory 10–20 and 40–60 min after training (see below).

What is the trigger for glycogenolysis? Evidence is presented here for the involvement of noradrenaline, serotonin and ATP in the breakdown of glycogen for memory processing.

NORADRENERGIC TRIGGER OF GLYCOGENOLYSIS

There is evidence that glycogenolysis is stimulated by noradrenaline (Magistretti, 1988; Quach et al., 1988; Subbarao and Hertz, 1990), and in the chicken, via the β_2 -adrenergic receptor (Gibbs et al., 2006) at the time of transition between the first and second phases of intermediate memory, i.e., around 25–30 min after training. Noradrenaline acts on two α and three β receptor subtypes, and in extensive studies we have shown that it can do so via all five subtypes (Gibbs and Summers, 2002a, 2005) with effects differentiated by time and brain location.

Injected into the cortex, noradrenaline promotes formation of memory after weakly- reinforced training by activation of β_2- and β_3-adrenergic receptors as well as by α_1-ARs. Consolidation of weakly reinforced training is achieved with injection of either β_2-(zinterol) or β_3-AR (CL316243) receptor agonists (**Figure 4A**). Both agonists consolidate when injected at any time up to 30 min after training with the exception of a lesser effect with injection given at 15 min post-training. In the avian brain there is a predominance of β_2-ARs (see Gibbs et al., 2008a), whereas in the mammalian brain mainly the β_1- subtype of the receptor is expressed (Quach et al., 1978). However, the β_1-AR agonist RO363 was unable to promote consolidation at any of the times injected into IMM (Gibbs and Summers, 2002a) but there are β_1- AR effects on memory in other brain areas such as the Medial Striatum and the hippocampus (see below). The specific antagonist for the β_2-AR (ICI 118551) and for the β_3-AR (SR59230) both inhibited strongly reinforced learning when injected 5 min after training, with the β_2-AR antagonist inhibiting up to 25 min after training, i.e., over the duration of ITMA, but memory was spared when the antagonists were given 5 min before training (**Figure 4B**).

In the hippocampus injection of the β-AR agonists promoted consolidation of weakly reinforced training; zinterol and CL316243 both promoted consolidation although with slightly different timing compared to that seen in the IMM. In addition, the β_1-AR agonist RO363 also promoted consolidation during STM and the antagonist CGP20712A inhibited strongly reinforced training over the same time course (**Figures 4C,D**). Varying the time of injection clearly shows that both the agonists and antagonists had different memory response patterns in the hippocampus and the IMM.

Although the β_2-AR agonist in IMM can consolidate memory injected up to 25 min post-training (**Figure 4A**), the time at which the antagonist is effective has a close correlation with the time when DAB is ineffective (**Figure 3B**). In the hippocampus glycogenolysis is critical for underwriting the STM and ITMA memory periods as well as at 55 min post-training (**Figure 3D**). The β_2-AR agonist was only effective for a short time when injected during ITMA, whereas the β_3-ARs were active during STM and ITMA. The β_1-AR agonists promoted consolidation with injection during STM only (**Figure 4C**).

Behavioral experiments to confirm that glycogenolysis is associated with β_2-adrenoceptors involved challenging the selective agonists with a weak dose of DAB or saline. A dose level of DAB that was not sufficient to block memory itself was injected at a time when it has been shown not to affect memory. In the cortical area (IMM) β_2- and β_3-AR agonists were injected 25 min after weakly reinforced training when they normally promote consolidation. DAB reduced the ability of zinterol to consolidate memory (**Figure 5A**), but

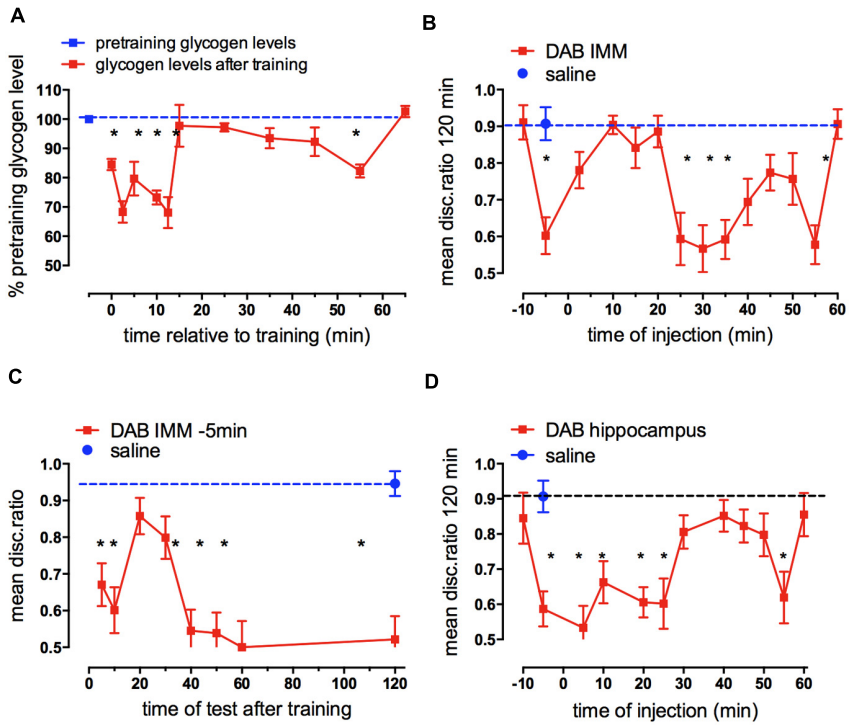

FIGURE 3 | Glycogen levels after training in chick forebrain and effect on memory consolidation of inhibition of glycogen breakdown. (A) Glycogen levels in combined left and right hemispheres after training compared with the glycogen content at pre-training. Glycogen was measured as described by Lo et al. (1970) with minor modifications. Brains were excised and transferred to pre-weighted tubes and their weight determined by the increase in weight. After digestion of the tissue, glycogen was precipitated by the addition of 95% ethanol, hydrolyzed to glucose in a phenol-sulphuric acid solution and the absorbance measured at 490 nm and calibrated by aid of a standard glucose curve (From O'Dowd et al., 1994; Hertz et al., 2003). **(B)** Times of injection of DAB into IMM after strongly reinforced training showing those times at which memory processing requires glycogen breakdown; **(C)** Times of test after strongly reinforced training following DAB injection 5 min before training revealing when memory is affected by DAB; **(D)** Times of injection of DAB into the hippocampus following strongly reinforced training. (From Gibbs et al., 2007, 2008b). In this figure and all subsequent graphs the dashed line represents saline control value for strongly reinforced training (high DR) or weakly reinforced training (DR approaching 0.5) $^{*}P < 0.05$.

had no effect on the ability of the CL316243 to consolidate memory (**Figure 5B**). In the hippocampus, a low dose of DAB did not affect the ability of CL316243 or the β_1-AR agonist RO363 to consolidate memory, but as in IMM, DAB reduced the ability of zinterol to promote consolidation (Gibbs et al., 2008a). Since DAB prevents activation-induced glycogen breakdown and decreases memory, we conclude that glycogen breakdown achieved by stimulation of β_2-ARs is necessary for memory.

GLYCOGEN CONTENT AND SYNTHESIS IN CULTURED CHICKEN ASTROCYTES

The effects on glycogen levels measured in chick astrocyte primary cultures, in the presence of DAB or in the presence of zinterol (with DAB or saline added 20 min prior to zinterol exposure) are shown in **Figure 6A**. As can be seen DAB did not alter the basal glycogen levels but it did prevent the zinterol-induced decrease in glycogen level (Gibbs et al., 2006) but had no significant effect in the presence of the β_3-adrenergic antagonist CL316243.

If stimulation of noradrenergic β_2-ARs stimulates memory consolidation via the breakdown of glycogen with no dramatic decrease in glycogen levels (at 30 min post-training), it is likely that there is some cellular mechanism to stimulate the synthesis of glycogen. This increased synthesis is achieved by noradrenaline released from neurons stimulating astrocytic α_2-ARs (post-junctional receptors; Hertz et al., 2007; Hutchinson et al., 2011; Gibbs and Hutchinson, 2012). At the same time β_3-adrenergic stimulation increased uptake of glucose, securing the glucose presence needed for the synthesis of glycogen (Gibbs and Summers, 2002a,b; Gibbs et al., 2008c).

Noradrenaline increased glycogen formation (total ^{14}C incorporation) in astrocytic culture (Hutchinson et al., 2008, 2011). This was not inhibited by the β_1-/β_2-AR antagonist propranolol nor by the β_3-AR antagonist SR59230A, suggesting that the effect of noradrenaline to increase glycogen turnover is not mediated by β-ARs (Hutchinson et al., 2011). Insulin was used as the positive control (**Figure 6B**). When the effects on glycogen levels were compared, insulin and clonidine significantly increased glycogen levels but the other agonists had no effect (**Figure 6C**). A timecourse experiment with noradrenaline, isoprenaline and zinterol demonstrated

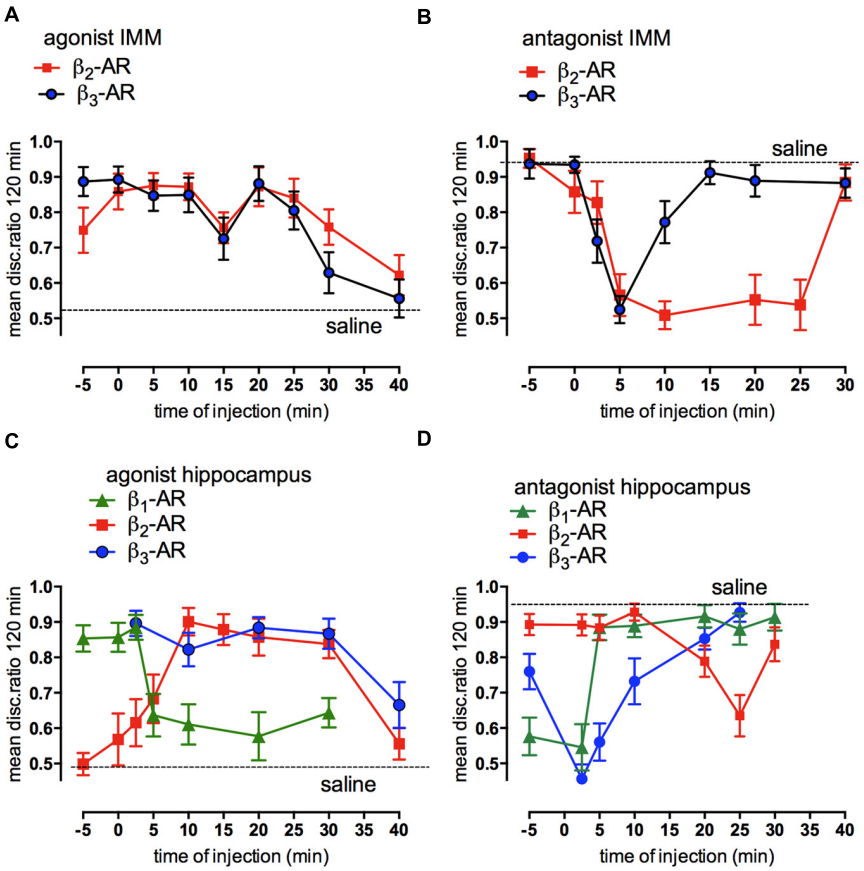

FIGURE 4 | Involvement of β-adrenoceptors in weakly- and strongly reinforced memory in both avian cortex (IMM) and hippocampus. Injections of selective β-AR agonists at different times after weakly reinforced training in IMM **(A)** or hippocampus **(C)** and of selective antagonists for β-ARs in IMM **(B)** or hippocampus **(D)** after strongly reinforced learning. Memory tested 120 min after training. (From Gibbs, 2008).

significantly decreased glycogen levels following 5 and 10 min of stimulation (Hutchinson et al., 2008). In experiments where noradrenaline and subtype selective agonists were incubated with astrocytes, only noradrenaline and the α_2-AR agonist clonidine increased glycogen synthesis measured as total ^{14}C incorporation (% of basal) into glycogen, whereas the β-AR agonists had no significant effect. Noradrenaline stimulates glycogenolysis and formation of cAMP in similar cultures of astrocytes (O'Dowd et al., 1995), consistent with the glycogenolytic mechanism shown in **Figure 7A**.

The α_2-AR agonist clonidine activates both α_{2A}- and α_{2C}-ARs and both are present in areas of the brain important for memory processing (Scheinin et al., 1994; Hutchinson et al., 2011). AR α_{2A}-and α_{2C}-AR subtypes are found both pre and post-synaptically on neurons as well as post-junctionally on astrocytes (Enkvist et al., 1989; Aoki, 1992; Lee et al., 1998a,b; Hertz et al., 2010). In chick astrocytes clonidine stimulation of glycogen synthesis was blocked by the α_{2C}-AR subtype selective agonist ARC239 (Gibbs and Hutchinson, 2012). α_2-Adrenergic stimulation activates glycogen synthesis by stimulation of the AKT pathway and subsequent down-regulation of GSK (**Figure 7B**). This is consistent with the observations that the potent α_2-AR agonist, dexmedetomidine induces ERK phosphorylation both in cultured astrocytes and in brain slices, but not in cultured neurons (Li et al., 2008; Du et al.,

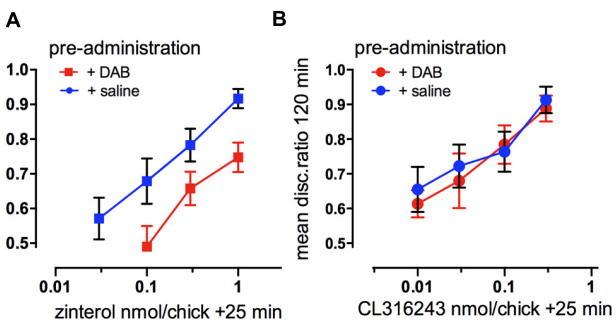

FIGURE 5 | Functional selectivity of drug interaction. (A) Consolidation of memory for weakly reinforced training with the β_2-AR agonist zinterol or **(B)** the β_3-AR agonist CL316243, challenged by preadministration at 20 min of a suboptimal dose of DAB. Zinterol in the presence of DAB, inhibiting the breakdown of glycogen, was less effective in promoting memory consolidation. (From Gibbs et al., 2008c).

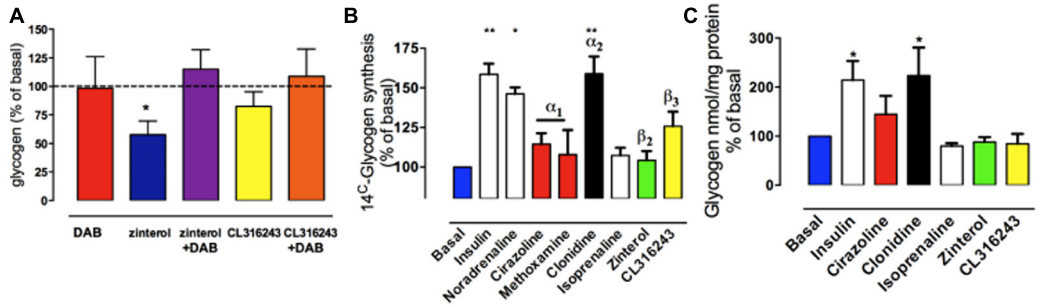

FIGURE 6 | The effect of adrenoceptor agonists on glycogen levels turnover and new synthesis in cultured astrocytes from chick forebrain. **(A)** Astrocytes were incubated for 2 h under basal conditions with no drug added, or for 2 h in the presence of zinterol or CL316243. DAB was added 20 min prior to the 2 h incubation (adapted from from Gibbs et al., 2006). **(B)** Glycogen synthesis assessed by [^{14}C]-glucose incorporation into glycogen in response to 3 hr stimulation with AR agonists, noradrenaline or insulin. **(C)** Glycogen levels expressed as % of basal after incubation with AR agonists or insulin. $^{*}P < 0.05$; $^{**}P < 0.001$. (From Hutchinson et al., 2011).

2009), and that the ERK and AKT pathways interact in astrocytes (Dai et al., 2013).

EFFECT OF ARC239 INHIBITION OF α_{2C} ARS ON MEMORY PROCESSING AND COMPARISON WITH DAB TIMECOURSE

Inhibition of the synthesis of new glycogen, caused by injection of the selective α_{2C}-AR antagonist ARC239 into IMM revealed a clear time-dependence. Memory at 120 min was inhibited by ARC239 injected at two time periods after training, (i) when injected 10–20 and (ii) when injected 40–50 min after strongly reinforced training (**Figure 8A**). On the other hand, preventing glycogen breakdown with DAB, inhibited memory when injected at three different times: 5 min before, 25–35 min after and at 55 min after training in IMM. The effects of ARC239 and DAB mirror each other *but* with a lag time of about 10 min (Hertz and Gibbs, 2009). ARC239 prevented memory at quite discrete time points, which suggests that its inhibitory action is not sustained for long periods in the brain. In the presence of ARC239 there is insufficient accessible glycogen, so β_2-ARs although activated cannot induce glycogenolysis and promote consolidation. Since noradrenaline will activate α_2-AR as well as β_2-ARs at the same time, any measurement of glycogen levels will reflect the net balance between synthesis and degradation.

There is a slow turnover of glycogen during resting conditions in the brain (Brown, 2004; Oz et al., 2007), but it can obviously be accelerated to underwrite memory processing in the hippocampus and cortex, as illustrated in **Figure 3**. DAB is only active in the brain for a short time period as when injected 10 min before training DAB had no effect on memory. In agreement with this idea, DAB injected 5 min prior to training memory is only shown to be effective when first tested 5 min after training, suggesting that it does not remain active in the brain for long and therefore only acts on memory processes very close to the time of injection, even though the consequences appear later.

Our data clearly show that glycogen has an active role in astrocytic function. This may in turn also influence neuronal metabolism because glycogenolysis may inhibit astrocytic glucose consumption and thereby make more glucose available for neurons (DiNuzzo et al., 2012, 2015). Since glycogen turnover is slow under resting conditions, the question arises as to how fast it can be recruited in cells and are there readily accessible stores with fast turnover in brain regions important for memory. It is possible that high turnover could occur in specific brain regions during neural activation. Glycogen phosphorylase can be activated within seconds and is stimulated during various behavioral activities (Walling et al., 2006). In chick astrocyte cultures, we have shown an initial decrease in glycogen levels after 5–10 min of incubation with noradrenaline, this decrease is then followed by an increase in glycogen levels (Hutchinson et al., 2011). The fall in glycogen levels, but not the later increase, also occurs with incubation with zinterol. These biochemical results suggest that the onset of breakdown of glycogen can occur quickly.

We designed experiments where the glycogen synthesis inhibitor ARC239 was challenged with zinterol (increasing glycogenolysis and promoting weakly reinforced learning) in two different paradigms (Gibbs and Hutchinson, 2012; **Figures 8B,C**) to ask two questions: firstly, can prevention of glycogen re-synthesis reduce glycogen levels sufficiently so that zinterol is unable to promote weakly reinforced learning, and at when does this occur after training? When we prevented glycogen synthesis by injecting ARC239, given 5 min before training, zinterol was able to promote consolidation given either immediately or 5 min after training, but it was unable to promote memory when given later than this, at 10, 15, or 20 min after training (**Figure 8B**), although zinterol will normally do so at these times. These results suggest that the ARC239-mediated inhibition following its injection 5 min before training took 10 min to reduce the readily available glycogen stores.

Secondly, we asked by what mechanisms and at what times can inhibition of glycogen synthesis affect the ability of zinterol to promote consolidation after weakly reinforced training? Zinterol was injected 20 min after training to promote consolidation

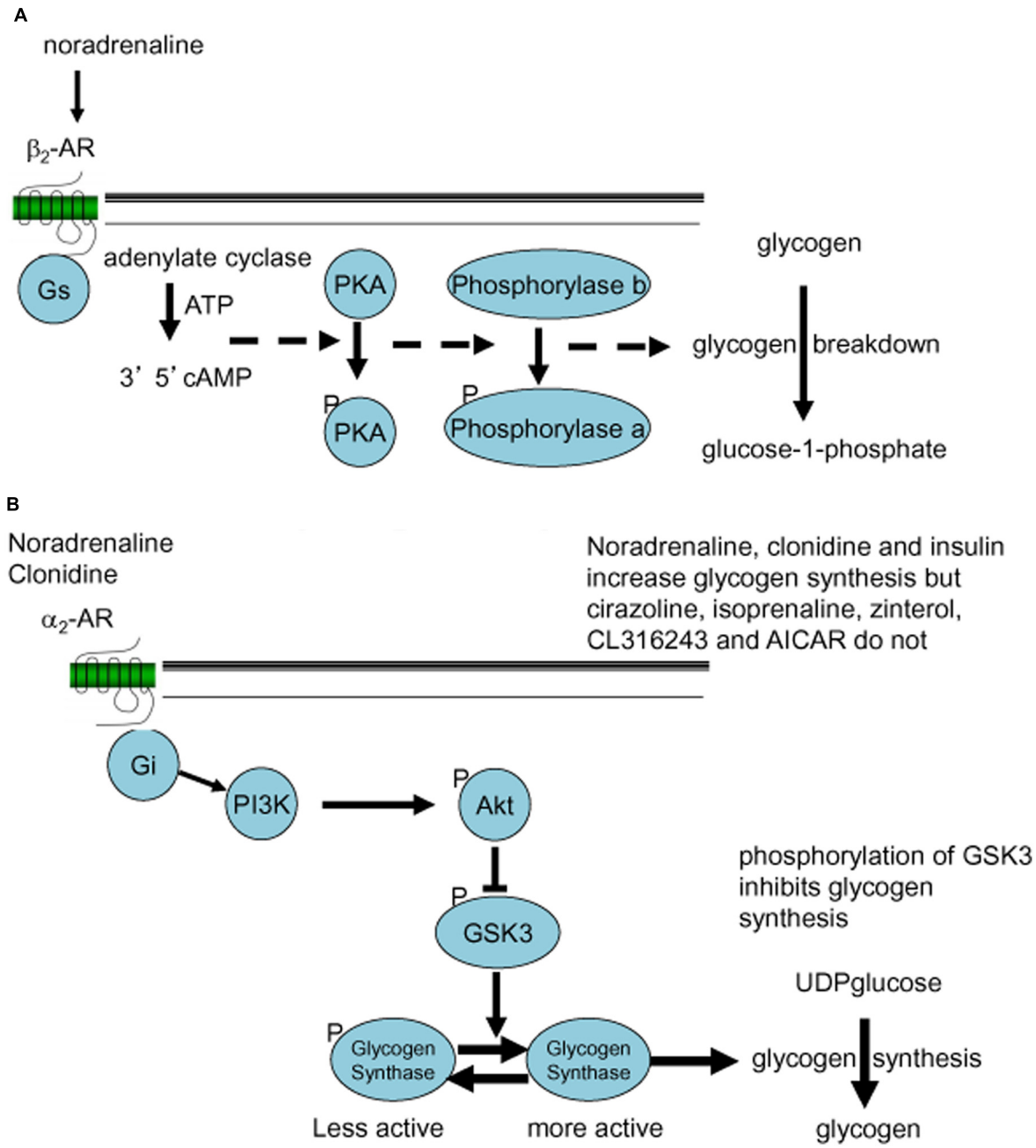

FIGURE 7 | Noradrenergic signaling stimulating glycogenolysis (A) or glycogen synthesis (B). (A) β_2-Adrenergic G_s-mediated formation of cAMP and phosphorylation of proteinkinase A (PKA) leads to conversion (phosphorylation) of phosphorylase b to the active phosphorylase a, which stimulates glycogen breakdown (conversion of glycogen to glucose-1-phosphate). **(B)** α_2-Adrenergic G_i-mediated stimulation of the PI3K-AKT pathway leads to phosphorylation of glycogen synthase kinase (GSK) and dephosphorylation of glycogen synthase, which stimulates glycogen synthesis (incorporation of UDPglucose into glycogen).

and ARC239 at times between 5 min before and 15 min after training (i.e., 20–5 min before zinterol) prevented the β_2-AR agonist promoting consolidation at all times except 5 min before training (**Figure 8C**). ARC239 given up to 10 min before zinterol prevented it from promoting consolidation. These results again suggest that depletion of accessible glycogen stores takes place quickly, i.e., within 10 min of injection.

Serotonin and Glycogenolysis

Serotonin is another neuromodulatory transmitter that promotes memory consolidation (Gibbs and Hertz, 2014). Like noradrenaline, serotonin activates glycogenolysis in brain tissue and in astrocytes (Quach et al., 1982; Cambray-Deakin et al., 1988; Magistretti, 1988; Chen et al., 1995; Kong et al., 2002; Darvesh and Gudelsky, 2003). Although the β_2-AR activation

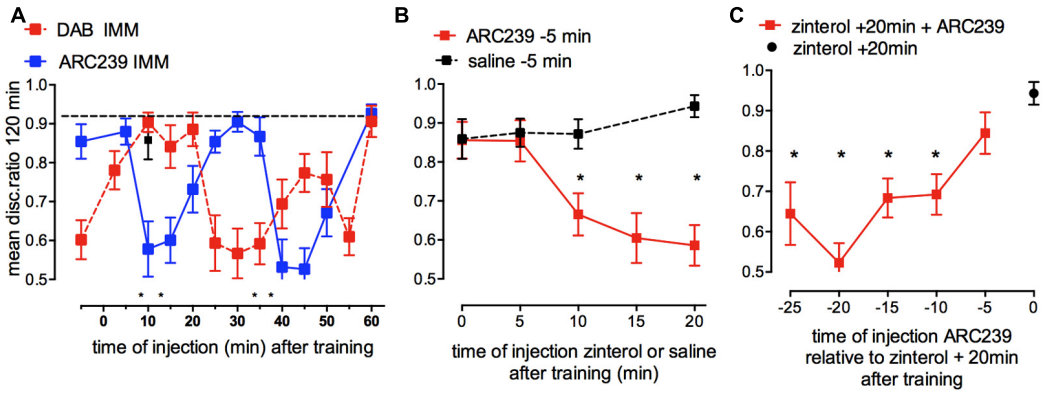

FIGURE 8 | Effect of inhibition of glycogen re-synthesis by the $\alpha_{2B/C}$-AR antagonist ARC239 on strongly reinforced memory and challenges to inhibition of re-synthesis by β_2-AR stimulation (glycogen breakdown) and vice versa. (A) ARC239 was injected into IMM at different times after training. The timing of the effect of ARC239 is compared with that of DAB (From Gibbs et al., 2008b; Hertz and Gibbs, 2009). **(B)** ARC239 or saline were injected 5 min before weakly reinforced training and zinterol injected into different groups at various times after training. Inhibiting re-synthesis of glycogen prevented β_2-AR stimulation from promoting memory consolidation up to 10 min prior to zinterol injection. Normal learning is indicated by black squares. **(C)** Zinterol was injected into all groups 20 min after weakly reinforced training and ARC239 had been injected at times from 25 to 5 min before zinterol (i.e., from 5 min before to 15 min after training). $^*P < 0.05$. (From Gibbs and Hutchinson, 2012).

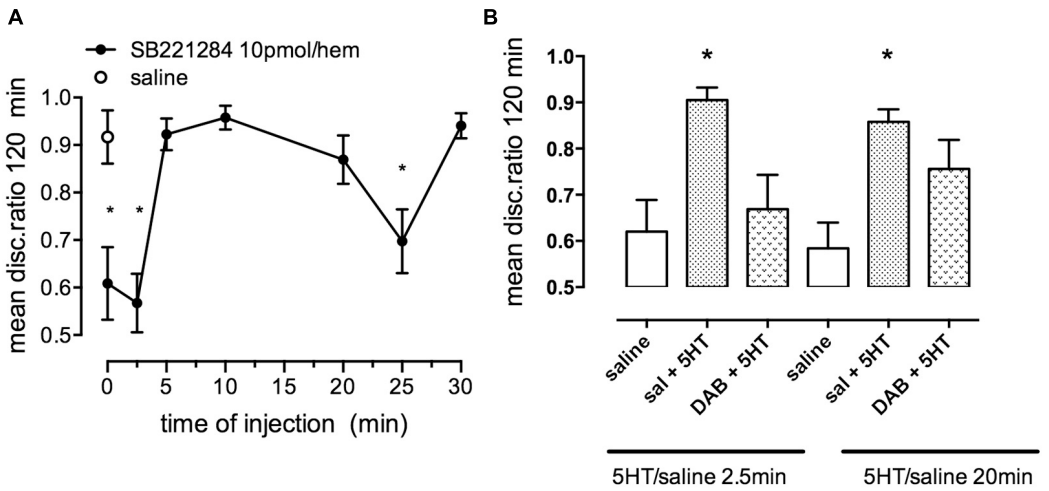

FIGURE 9 | Effect of the selective 5-$HT_{2B/C}$ receptor antagonist SB221284 on strongly reinforced memory. (A) Time of injection of SB221284 following strongly reinforced training. **(B)** Ability of the inhibitor DAB to prevent consolidation of weakly reinforced memory. A suboptimal dose of DAB was given either before or 15 min after training, serotonin was given 2.5 or 20 min after training. DAB only interfered with serotonin induced consolidation at the early period. $^*P < 0.05$. (From Gibbs and Hertz, 2014).

appears to be the signal leading to the second glycogenolytic period in IMM, noradrenaline is not implicated in the first period close to training. Serotonin has effects on memory in the chick dependent on the dose. Low doses of serotonin promote memory consolidation when injected up to 25 min after weakly reinforced training whereas high doses inhibit memory (Gibbs and Hertz, 2014). Serotonin thus appears to act on at least two different 5HT receptors during learning stimulating a high affinity serotonin receptor, identified below as the $5HT_{2B}$ receptor and inhibiting a lower-affinity serotonin receptor, possibly a 5-HT_1 receptor.

Strongly reinforced memory is inhibited by the selective $5HT_{2B,C}$ antagonist SB221284 injected immediately, 2.5 or 25 min after (**Figure 9A**). Conversely, weakly reinforced learning can be rescued by administration of serotonin (**Figure 9B**). In contrast to the β_2-AR involvement, it is only during the first period where SB221284 inhibits (2.5 min after training) that the rescue by serotonin is challenged by a sub-optimal dose of DAB. In contrast, during the second glycogenolytic period where serotonin plays only a minor role, memory is not significantly affected when DAB is administered together with serotonin (**Figure 9B**). On the other hand the 5-HT_{2B} agonists fluoxetine and paroxetine (Li et al., 2008; Diaz et al., 2012) can consolidate weakly reinforced learning. It is interesting that fluoxetine and paroxetine which are better known as serotonin-specific

antidepressants (SSRIs) also consolidate weakly reinforced learning in the chicken (Gibbs and Hertz, 2014). Fluoxetine also increases glycogenolysis in cultured astrocytes (Chen et al., 1995) by a 5-HT_{2B} receptor-mediated effect (Kong et al., 2002).

ATP AND GLYCOGENOLYSIS

As with the neurotransmitters, noradrenaline and serotonin, memory consolidation is also modulated by endogenous adenosine triphosphate (ATP) acting at purinergic receptors in the hippocampus (Gibbs et al., 2011) and in the cortex (IMM; Cronin et al., 2011). ATP is important for communication between neuronal and glial circuits and is released from both neurons and astrocytes (Fields and Burnstock, 2006; North and Verkhratsky, 2006). ATP released from astrocytes acts as a widespread gliotransmitter, triggering, and maintaining calcium signaling and calcium oscillations (Bowser and Khakh, 2004; Burnstock, 2007; Zorec et al., 2012). Release of transmitter ATP from cultured astrocytes is inhibited by DAB (Hertz et al., 2014b; Xu et al., 2014). On the other hand ATP is also known to trigger glycogenolysis (Hertz et al., 2015c). ATP injected into either the hippocampus (**Figure 10A**) or IMM promoted consolidation of weakly reinforced training at two time periods: 0–2.5 and 25–30 min post-training. The two non-hydrolyzable agonist analogs, ATPγS and ADPβS (**Figure 10B**) produced similar but not identical effects on memory time courses, in particular with ADPβS promoting consolidation at 35 but not at 30 min, whereas ATPγS promoted consolidation when injected at 30 but not at 35 min. Challenge to the action of ADPβS by the selective ADPβS antagonist MRS2179 showed that it acts via purinergic P_2Y_1 receptors (Gibbs et al., 2011).

Astrocytes are a major source of the released ATP in hippocampal slices in rodents (Bowser and Khakh, 2007), and evidence suggesting astrocytes are the source of the ATP promoting memory comes from the effect of thrombin on memory processing. Thrombin selectively activates calcium release from intracellular stores in astrocytes (Bowser and Khakh, 2004) and thrombin injected into IMM (Gibbs and Bowser, 2010) or the hippocampus (**Figure 10C**; Gibbs et al., 2011) like ADPβS, promoted memory consolidation when injected at 2.5 and 35 min after training. These results strongly suggest that astrocytes are the source of the ATP facilitating memory. Thrombin consolidation could also be successfully challenged by a low dose of the P2Y1 antagonist MRS2179. The inhibitory effect of fluoroacetate, which inhibits astrocytic oxidative metabolism, also confirms astrocytic involvement in promotion of memory by ATP (Gibbs and Bowser, 2009). Neither ADPβS nor thrombin were able to promote consolidation with prior administration of fluoroactate (Gibbs et al., 2011).

Further support for astrocytic involvement in purinergic memory formation comes from the successful challenge by DAB of both ADPβS and thrombin rescue of weakly reinforced learning (**Figures 10D,E**). DAB did not challenge ATPγS suggesting that the source of the ATP mimicked by ATPγS could therefore be neurons, which co-release ATP with glutamate or noradrenaline (Burnstock, 2007). Incubation of astrocytes with ATPγS or ADPβS resulted in an increase in intracellular calcium $[Ca^{2+}]_i$, with the effect of ADPβS being blocked by fluoroacetate and also by the P2Y1 antagonist, again implicating a specific role for astrocytic P2Y1 receptors in the calcium response. We suggested that the source of the ATP itself acting on neural P2Y1 receptors is most likely astrocytes, since thrombin selectively increases $[Ca^{2+}]_i$ in astrocytes but not in neurons (Bowser and Khakh, 2004). Astrocytic $[Ca^{2+}]_i$ must accordingly play an important role in the consolidation of short-term to long-term memory through activation of astrocytic P2Y1 receptors (Gibbs et al., 2011). An increase in $[Ca^{2+}]_i$ is also a prerequisite for stimulation of glycogenolysis (Hertz et al., 2015c). An important pathway for entry of Ca^{2+} is store-operated Ca^{2+} channels, and store-operated Ca^{2+} entry (SOCE) triggers astrocytic glycogenolysis in cortical astrocyte cultures (Müller et al., 2014). Accordingly administration of DAB reduces the amount of Ca^{2+} loaded into the sarco/endoplasmic reticulum.

GLYCOGEN FACILITATION OF *DE NOVO* SYNTHESIS OF GLUTAMATE USED FOR MEMORY PROCESSING AND INVOLVEMENT WITH ASTROCYTIC Na,K-ATPase ACTIVITY

It was shown earlier (**Figure 2**) that glycogenolysis is essential for the production of glutamate and thus also of its metabolite GABA, two important neurotransmitters. Glutamate is supplied by astrocytes to neurons, which are unable to synthesize glutamate *de novo* as will be discussed below. If the breakdown of glycogen in astrocytes is compromised, then so is neural transmission (Hertz et al., 2003; Hertz and Zielke, 2004; Sickmann et al., 2009). This process would require relatively rapid turnover of glycogen *in vivo*.

Astrocytic glycogenolysis supports increased glutamate and glutamine synthesis at both 5 min (**Figure 2**; Hertz et al., 2003; Gibbs et al., 2007) and probably also after 30 min after training. Both these times are consistent with the times at which glutamate is released in trained chickens (Daisley et al., 1998; Daisley and Rose, 2002). The reduction in glycogen levels in the chick forebrain coincides with a transient increase in glutamate in the left forebrain over the first 5 min after training, and since there is no concomitant decrease in aspartate or the glutamate precursor glutamine, it indicates *de novo* synthesis of glutamate (Hertz et al., 2003; Gibbs et al., 2007). The importance of glycogenolysis for glutamate synthesis has been confirmed in the rat (Sickmann et al., 2012).

Since glycogen is responsible for the increased glutamate content in neurons occurring at specific times during memory consolidation, it would be expected that glutamine as a glutamate precursor should be able to rescue memory impairment caused by DAB. Our data show that glutamine can indeed rescue DAB-impaired memory when administered up to 2.5 min after training as well as during the second window for rescue 30–55 min after training. At times when DAB does not inhibit,

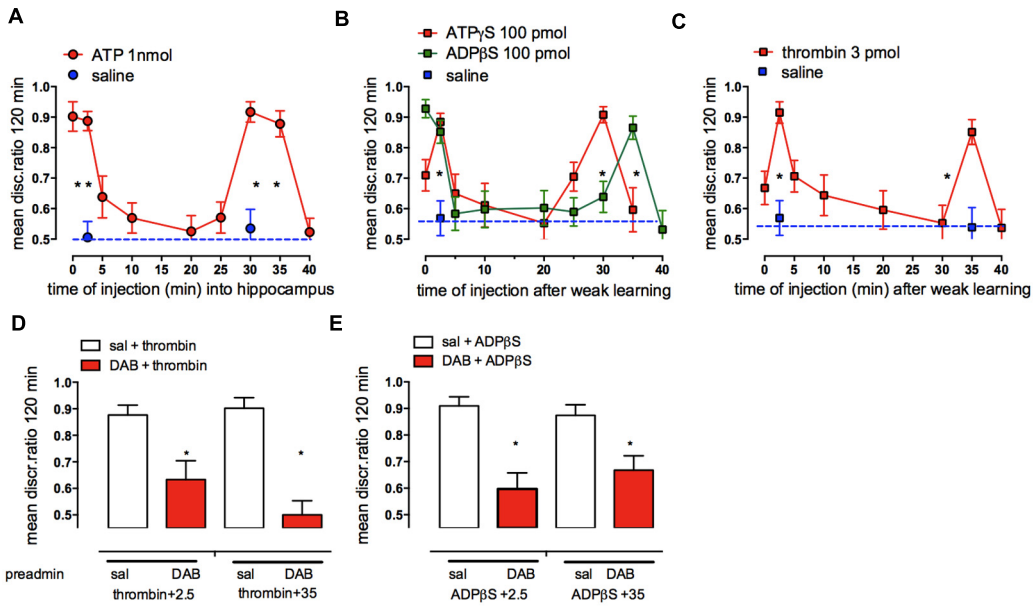

FIGURE 10 | Effect of hippocampal injection of ATP, ATPγS, ADPβS, or thrombin (A–C) on consolidation of weakly reinforced training and the effect of DAB on the ability of thrombin, ADPβS to promote consolidation (D,E). Injections over two time periods after weakly reinforced training resulted in consolidation of memory 120 min after training. **(A)** ATP, **(B)** ADPβS and ATPγS and **(C)** thrombin. **(D,E)** A sub-optimal dose of DAB (red columns) was injected subcutaneously 5 min before weakly reinforced training or 5 min before injection of thrombin or ADPβS into the hippocampus 2.5 or 35 min after weakly reinforced training, i.e., at times when they normally promote consolidation (open columns). $^{*}P < 0.05$. (From Gibbs et al., 2011).

i.e., when glycogen breakdown is not critical for memory consolidation (5–25 min) post-training glutamine does not rescue (**Figure 11A**) even though glutamine enhances weakly reinforced learning over this time period (5–30 min; Gibbs et al., 2006, 2007, 2008b). Additional experiments have shown that DAB is not successfully challenged by either aspartate or the astrocyte-specific metabolic substrate acetate alone. However, DAB-induced memory impairment can be rescued by acetate together with aspartate, with acetate needed as a precursor for the pyruvate carboxylase product oxaloacetate, because acetate alone cannot support pyruvate carboxylation (Gibbs et al., 2008b). These results suggest that a major function for glycogen during memory consolidation is to enable synthesis of glutamate and GABA via glutamate production in the astrocytes. This does not preclude that glycogen might also have a major role as a substrate for energy production. Moreover, both glutamate synthesis and its degradation are oxidative processes resulting in ATP production (Hertz et al., 2007). However, the relatively low rates of glycogenolysis in brain (Oz et al., 2012) should be kept in mind. Even the fast decline in glycogen occurring in the chick brain soon after training (Hertz et al., 2003) only amounts to ∼1.0 μmol/min per g wet wt, which is similar to the stimulated rate of glucose utilization in rat brain (Hertz and Dienel, 2002). A major role for glycogen might also be to support the energetic needs of astrocytes, including those related to their signaling (DiNuzzo et al., 2012; Xu et al., 2013). If the role of extracellular lactate is, indeed, a signaling one (as suggested above), then glycogenolysis might also promote signaling to neurons.

Lactate also rescues DAB induced impairment of memory when injected either immediately after training or 10–20 min later (**Figure 11B**). So, in contrast to the action of glutamine, lactate rescues memory from loss caused by DAB only at times when memory is NOT susceptible to DAB. These are the same time periods over which lactate normally enhances weakly reinforced learning (5–20 min; Gibbs et al., 2008b). Because of the difference in timing of the effect of lactate and glutamine, it is unlikely that they are producing the same end result of increasing glutamate. Lactate is readily taken up by neurons and astrocytes, but lactate uptake into astrocytes inhibits glycolysis (Hertz et al., 2014a). In that paper we discussed the fluxes of lactate into astrocytes, neurons, and between astrocytes and the multifunctional roles for lactate. Transport of lactate between brain cells is mainly between astrocytes via gap junctions and release into extracellular space leads to significant exit of lactate from the brain via the blood and via the perivascular-lymphatic drainage system (Ball et al., 2010).

Many years ago we found evidence that inhibition of the Na,K-ATPase abolished memory (Mark and Watts, 1971; Gibbs and Ng, 1977). Recent experiments have suggested that β_2-adrenergic activity may partly alleviate the impaired memory in day-old chickens resulting from Na,K-ATPase inhibition (Hertz et al., 2015b). This would suggest involvement of the astrocytic Na,K-ATPase, which may require glycogenolysis for its function (DiNuzzo et al., 2010; Xu et al., 2013; Hertz et al., 2015b). Clearance of extracellular K^+ increases signal to noise ratio and thus is likely to play an important role in synaptic plasticity (and hence in triggering memory formation).

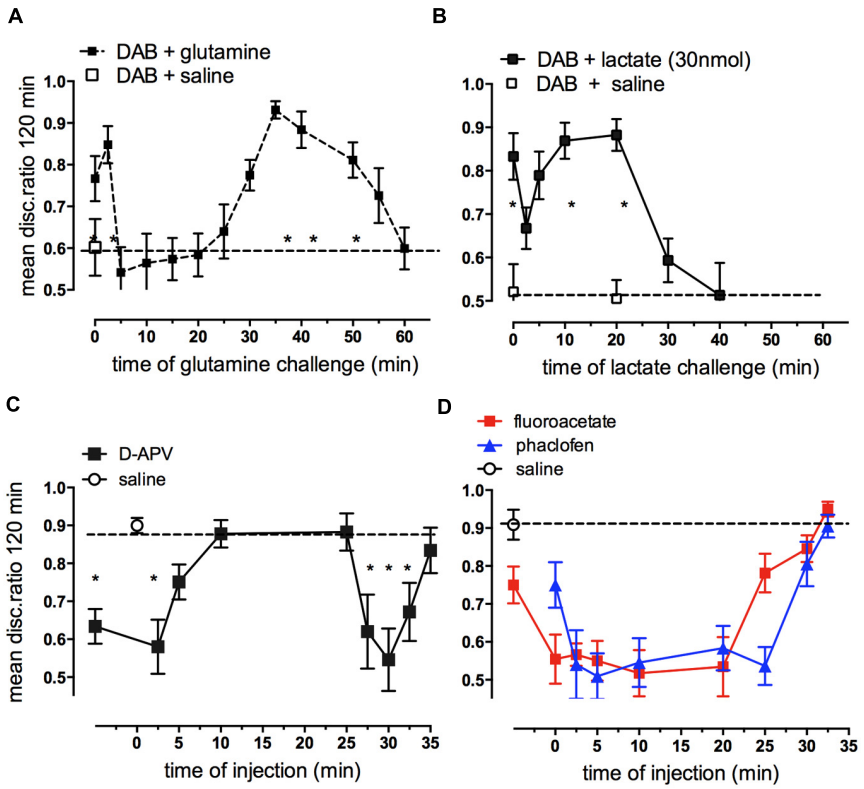

FIGURE 11 | Ability of glutamine or lactate to rescue memory following DAB inhibition of glycogenolysis (A,B) and effect of hippocampal injection of antagonists of NMDA receptors, $GABA_B$ receptors or fluoroacetate on strongly reinforced training (C,D). DAB was injected into IMM 5 min before strongly reinforced training and challenged at various times by glutamine **(A)** or L-lactate **(B)** (From Hertz and Gibbs, 2009). **(C)** The NMDA angtagonist D-APV injected into the hippocampus inhibited memory given up to 5 min post-training and again when given 27.5–32.5 min post-training. **(D)** Both the $GABA_B$ receptor antagonist, phaclofen and fluoroacetate inhibited memory when given between 2.5 and 25 min post-training. $^*P < 0.05$. (From Gibbs et al., 2008a; Gibbs and Bowser, 2009).

GLYCOGEN AS THE SUBSTRATE FOR *DE NOVO* SYNTHESIS OF GLUTAMATE AND GABA

Both glutamate and GABA are involved in neurotransmission in the central nervous system and roles for glutamatergic NMDA (**Figure 11C**) and AMPA receptors (Gibbs et al., 2008a), mGluR and $GABA_B$ (Gibbs and Bowser, 2009) receptors have been described for memory processing in the chick hippocampus. The importance of glutamate in memory formation up to 30 min post-training is highlighted by its effects at the two time periods 0–2.5 and 25–30min, i.e., during STM and at the ITM transition from ITMA to ITMB. ATP and thrombin are similarly involved at these two times (Gibbs et al., 2011). A recent report has shown that DAB inhibition of glycogenolysis prevents LTP beyond 30 min in the rat hippocampus (Suzuki et al., 2011). We have shown above that one important reason why consolidation of memory is dependent on glycogenolysis is because of the brain's requirement for glutamate formation. However, both fluoroacetate (**Figure 11D**) and DAB (**Figure 3D**) injected into the hippocampus impair memory when given in between these times, implicating astrocytic activity and glycogenolysis over this period. The involvement of glycogen breakdown in memory is more extensive in the hippocampus than in the cortical IMM, and this may reflect on the requirement for astrocytes to supply GABA for interneurons. It is of interest that in IMM, fluoroacetate loses its inhibitory effect earlier than in the hippocampus and by 10 min after training it is ineffective in disrupting memory (Gibbs and Ng, 1977). GABA produced from glutamate is also dependent on glycogenolysis and inhibition of $GABA_B$ receptors by the selective antagonist phaclofen resulted in memory inhibition injected at all times between 2.5 and 25 min after training (**Figure 11D**; Gibbs and Bowser, 2009).

WHY ASTROCYTES ARE REQUIRED FOR GLUTAMATE SYNTHESIS

The reason neurons are unable to synthetize glutamate (and thus also GABA) and therefore are dependent upon glycogenolysis-mediated glutamate synthesis is that they lack an enzyme, pyruvate carboxylase, which is present in astrocytes (Yu et al., 1983; Shank et al., 1985) and most cell types outside the brain. Pyruvate carboxylase activity is required for direct conversion of

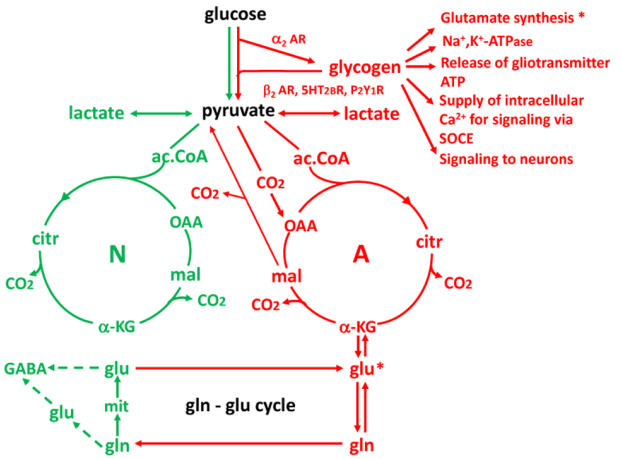

FIGURE 12 | Cartoon showing key features of glucose metabolism in neurons (N and green) and astrocytes (A and red) and interactions between the two cell types. Three reactions of importance in the present review are astrocyte-specific: (1) glycogenolysis (and thus its stimulation by noradrenergic, serotonergic and purinergic subtype-specific agonists); (2) formation of the tricarboxylic acid (TCA) cycle intermediate oxaloacetate (OAA) by condensation of pyruvate with CO_2 (pyruvate carboxylation); and (3) formation of glutamine from glutamate by glutamine synthetase. Reaction (1) is important for the transmitter actions on memory and for glutamate synthesis documented in this review, and for partial alleviation by β_2-adrenergic activity of DAB-impaired memory resulting from Na,K-ATPase inhibition and inhibition of release of gliotransmitter ATP and of SOCE by DAB referred to. It is also important for release of lactate acting on neurons. Reaction (2) is essential for creation of a new molecule of a TCA cycle constituent OAA, allowing another molecule of a TCA cycle constituent, α-KG to leave the cycle and form glutamate. *Indicates the importance of glycogenolysis for glutamate synthesis and glutamate synthesis from α-KG. And reaction (3) is a crucial step in supplying neurons with both newly synthesized and previously released glutamate. A fourth astrocyte-specific reaction is conversion of malate to pyruvate after its exit from the TCA cycle. This reaction allows metabolic degradation of accumulated glutamate but is not discussed in the review. The Figure should not give the impression that one half of brain energy metabolism is neuronal and one half astrocytic. Astrocytes account for a smaller fraction of the volume and therefore only for about one quarter of total glucose metabolism in gray matter (reviewed by Hertz, 2011), and other cell types like microglia also contribute to total energy metabolism.

pyruvate to oxaloacetate (OAA), which in turn is needed for the astrocytic production of glutamate (**Figure 12**). Metabolism of pyruvate by the pathway via acetyl coenzyme A (ac.CoA) which is used for energy production does not allow production of a *new* tricarboxylic acid (TCA) constituent because the two carbon atoms which are introduced into the TCA cycle with ac.CoA are released (providing energy) during its subsequent turn to OAA. Such a new molecule of a TCA cycle constituent is necessary for glutamate formation because glutamate is formed from another TCA cycle constituent, α-ketoglutarate, α-KG, which accordingly is removed from the cycle.

After its formation in astrocytes glutamate is converted by the astrocyte-specific glutamine synthetase (Norenberg and Martinez-Hernandez, 1979; Anlauf and Derouiche, 2013) to glutamine which is released from astrocytes and accumulated into neurons by specific, regulated transporters (Nissen-Meyer and Chaudhry, 2013) in the glutamine-glutamate cycle (gln-glu cycle) and re-converted to glutamate and GABA (with slightly different pathways used in glutamatergic and GABA-ergic neurons). It should be emphasized that not all transmitter release depends on the *de novo* synthesis, since the transmitters are also re-utilized. However, especially glutamate is accumulated into astrocytes, not the neurons from which it was released, and again transferred to neurons after conversion to glutamine in astrocytes. This process accounts for ∼75% of the flux in the gln-glu cycle whereas the remaining ∼25% represents *de novo* synthesis (reviewed by Gibbs et al., 2008b; Hertz, 2013; Hertz and Rothman, 2015).

The similarly astrocyte-specific glycogenolysis (Pfeiffer-Guglielmi et al., 2003) is also shown in **Figure 12**, and its correlation with glutamine synthesis indicated by asterisks, although it is probably not glutamate formation from α-KG but rather pyruvate carboxylation that requires glycogenolysis. This review has documented the importance of glycogenolysis for both glutamate formation and Na,K-ATPase activity and references have been made to its importance for release of gliotransmitter ATP and for supply of intracellular Ca^{2+} by store-operated Ca^{2+} entry (SOCE). Similarly, the possibility that glycogen-derived lactate may be an important signal to neurons has been discussed. **Figure 12** summarizes these mechanisms (red text) as well as stimulation of glycogenolysis by β_2-AR, 5-HT_{2B} receptors and P2Y1 receptors and of glycogen synthesis by α_2AR, all documented in the review.

CONCLUDING REMARKS

The excitatory and inhibitory neurotransmitters, glutamate, and GABA, are mainly responsible for information transfer and communication between nerve cells, but it is the modulation of synaptic activity by neurotransmitters including noradrenaline, serotonin and ATP that determines whether information in short-term or intermediate memory is consolidated into permanent storage or allowed to fade. These neuromodulatory transmitters act via astrocytes, stimulating the breakdown of glycogen that enables the synthesis of glutamate and glutamine that is essential for maintaining normal neuronal levels of glutamate and GABA and also for the regulation of potassium homeostasis (Hertz et al., 2015a).

Serotonin is responsible for the glycogen breakdown required for the transition from STM to ITM 2.5 min after training and noradrenaline for the transition from intermediate to long-term memory 30 min after training. Even if glycogen's major role should be to provide energy, it clearly has another role in the brain where it is responsible for the synthesis of glutamine, the precursor for glutamate in neurons that are unable to synthesize glutamate *de novo* and most likely also for astrocytic K^+ uptake (Xu et al., 2013; Hertz et al., 2015a), which constitutes an essential component of K^+ homeostasis in the brain.

All noradrenergic input into the forebrain comes from cell bodies located in the locus coeruleus situated in the medulla. These fibres radiate out to brain areas including the hippocampus and the cortex where noradrenaline released acts on the

noradrenergic receptors located on neurons, astrocytes, and microvessels and therefore can influence memory (Gibbs et al., 2010). The effect of noradrenaline on different cell types depends on the location of the different adrenergic receptors. The effects of noradrenergic stimulation on astrocytes, neurons and microglia, has been reviewed recently by O'Donnell et al. (2012).

In the avian cortex and hippocampus acts released noradrenaline acts on both β_2 and α_2 adrenergic receptors to cause the breakdown and the re-synthesis of glycogen. It promotes the transition of intermediate to long-term memory, whereas serotonin-induced glycogenolysis is responsible for the earlier consolidation of STM to intermediate memory, probably without any concomitant effect of serotonin on glycogen re-synthesis. This might explain why the level of glycogen is reduced 5 min after training (when serotonin mediates glycogenolysis) but not 30 min after training, when noradrenaline mediates glycogenolysis as well as glycogen synthesis (**Figure 3A**).

AUTHOR CONTRIBUTIONS

MG is the sole author of this review and which constitutes published work with a number of others.

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frontiers in Integrative Neuroscience

REVIEW
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The Role of Lactate-Mediated Metabolic Coupling between Astrocytes and Neurons in Long-Term Memory Formation

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Long-term memory formation, the ability to retain information over time about an experience, is a complex function that affects multiple behaviors, and is an integral part of an individual's identity. In the last 50 years many scientists have focused their work on understanding the biological mechanisms underlying memory formation and processing. Molecular studies over the last three decades have mostly investigated, or given attention to, neuronal mechanisms. However, the brain is composed of different cell types that, by concerted actions, cooperate to mediate brain functions. Here, we consider some new insights that emerged from recent studies implicating astrocytic glycogen and glucose metabolisms, and particularly their coupling to neuronal functions via lactate, as an essential mechanism for long-term memory formation.

Keywords: astrocyte, neuron, learning, memory, lactate, glucose, glucose metabolism

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INTRODUCTION

A newly formed memory is temporarily labile, and can become a long-term, stable memory if it undergoes a process known as *consolidation*. Memory consolidation is a fundamental, evolutionarily conserved process that occurs in different types of long-term memories and depends upon an initial phase of gene expression, which, if interrupted, results in amnesia (Davis and Squire, 1984; McGaugh, 2000; Alberini, 2009). At the cellular level, long-term memory has been studied using long-term potentiation (LTP) that produces activity-dependent increases in synaptic strength, generally evoked by high frequency stimulation (Malenka, 1994). Although not always fully corresponding, both long-term memory and LTP share the initial *de novo* gene expression requirement, involve the activation of similar molecular pathways such as MAPK, CaMKIIα, PKA and PKC (Martin et al., 1997), and are accompanied by synapse morphological modifications (Toni et al., 1999). Thus, memory consolidation has historically been considered a product of neuronal changes, and has been studied with a near total emphasis on neuronal responses.

However, particularly during the last 10 years, it has become increasingly better established that the glial cells known as astrocytes actively participate in complex brain functions, including cognitive ones, once attributed solely to neurons (Haydon and Nedergaard, 2015). Notably, the greater focus on astrocytes has brought new insights into non-neuronal contributions to learning and memory and synaptic plasticity. Both long-term memory and plasticity, in fact, require astrocytic transmission and processing of signals (Stehberg et al., 2012; Moraga-Amaro et al., 2014). Astrocytes also critically contribute to the morphological remodeling associated with synaptic plasticity, hence possibly functioning as spatial and temporal integrators of

neural activity and plasticity (Bourne and Harris, 2008; De Pittà et al., 2015). In addition, synaptic plasticity and memory rely on astrocytic regulations of nutrient availability; one that is particularly well-studied is glucose entry into the brain and its subsequent metabolism (Nedergaard et al., 2003; Halassa and Haydon, 2010). In this review, we will discuss evidence and questions concerning how glucose and glycogen metabolism in the brain, via production of lactate, are implicated in long-term synaptic plasticity and memory formation.

BRAIN GLUCOSE AND GLYCOGEN METABOLISM, AND THE HYPOTHESIS OF AN ASTROCYTE-NEURON LACTATE SHUTTLE (ANLS)

The glucose that astrocytes import from systemic blood has traditionally been believed to serve as the foremost substrate of energy directly supplied to and used by neurons. This, however, is now highly debated (e.g., Chih et al., 2001; Pellerin and Magistretti, 2012). Several studies in the last decades, including ours focusing on memory formation (Suzuki et al., 2011), have supported the hypothesis that, at least in physiological conditions of high-energy demands, that may not be the case. These studies indicate that L-lactate might in fact be preferentially utilized in these conditions. Many excellent reviews report the debated issues and different views (Pellerin et al., 1998; Chih et al., 2001; Dienel and Hertz, 2001; Chih and Roberts, 2003; Dienel and Cruz, 2004; Kimelberg, 2004; Bonvento et al., 2005; Fillenz, 2005; Hertz and Dienel, 2005; Pellerin et al., 2007; Barros and Deitmer, 2010; Belanger et al., 2011; Pellerin and Magistretti, 2012; Barros, 2013; Schurr, 2014; Dienel, 2015; Magistretti and Allaman, 2015; Weber and Barros, 2015; Kasparov, 2016) and we refer to these for a comprehensive overview of the debates and questions that still remain to be addressed. Our data, collectively, are in agreement with many studies summarized and discussed by Schurr (2014) and the conclusion that L-lactate plays a critical role in the physiology of brain functions and that "*glycolysis, in cerebral and other tissues, is a singular pathway, in the presence or absence of oxygen, which begins with glucose as its substrate and terminates with the production of lactate as its main end product*".

Pellerin and Magistretti (1994) proposed that electrophysiological activity links activated glutamatergic neurons and astrocytes through lactate, which is produced glycolytically by astrocytes and shuttled to neurons to be used as a significant source of energy (the astrocyte-neuron lactate shuttle or the ANLS; Pellerin and Magistretti, 2012). The ANLS hypothesis originally derived from findings that glutamate triggered uptake of labeled glucose and release of L-lactate by cultured astrocytes (Pellerin and Magistretti, 1994). In line with this hypothesis, earlier work demonstrated that L-lactate was sufficient to maintain synaptic activity in rat hippocampal slices (Schurr et al., 1988), and that during focal physiologic neural activity the consumption of glucose is non-oxidative (Fox et al., 1988). Fox and Raichle (1986) had previously described a focal physiological uncoupling between cerebral blood flow and oxidative metabolism upon somatosensory stimulation in humans; thus, it was subsequently proposed that the temporal mismatch between glucose uptake and oxygen consumption in the brain observed in positron emissions tomography (PET) scans reflects the fact that glucose is taken up by astrocytes and metabolized through aerobic glycolysis prior to use by neurons (Magistretti and Pellerin, 1999). According to this idea, glucose uptake does indeed occur as brain regions activate, though it may not occur primarily in neurons, but, rather, in astrocytes. This has been confirmed in rat barrel cortex after whisker stimulation using a real-time method for measuring the uptake of the fluorescent glucose analog 6-[N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-6-Deoxyglucose (6-NBDG) in astrocytes and neurons *in vivo*. During resting conditions the 6-NBDG uptake in astrocytes and neurons is similar, but during intense neuronal activity, triggered by whisker stimulation, astrocytes increase their uptake, whereas neurons do not, suggesting that stimulation elicits glucose uptake primarily in astrocytes (Chuquet et al., 2010). In contrast with these findings, a recent study demonstrated that whisker stimulation in awake mice causes neurons to take up near-infrared 2-deoxyglucose analog at a higher rate than astrocytes (Lundgaard et al., 2015), and identified the neuron as the principal locus of glucose uptake. One important caveat of using glucose analogs is that a change in size and structure may lead to their uptake via endocytosis rather than transport (Tadi et al., 2015). As endosomal trafficking is highly specialized in neurons (Yap and Winckler, 2012) further studies are needed to sort out if and in which functional conditions astrocytes or neurons have differential glucose uptake. In agreement with preferential uptake of glucose in astrocytes, downregulation of the expression of glial glutamate transporters impairs the 2-deoxyglucose autoradiographic signal in barrel field following stimulation of the corresponding whisker, indicating that glutamate uptake into astrocytes is the trigger for glucose uptake by the brain parenchyma (Voutsinos-Porche et al., 2003).

In contrast, studies have reported a preferential uptake of glucose by neurons during periods of activity. For example Bak et al. (2009) showed that administration of NMDA elicits increased neuronal uptake of glucose but not L-lactate; however it has been suggested that the dose of NMDA used in these experiments is excitotoxic (Bouzier-Sore and Pellerin, 2013). Another study showed that following seizure, nerve terminals extracted from rats had increased hexokinase-mediated phosphorylation of glucose analog 2-fluoro-2-deoxy-D-glucose indicating neuronal glucose oxidation (Patel et al., 2014). It is important to point out, however, that seizure activity may also be excitotoxic (Meldrum, 1991), and that the metabolic demands and regulations of excitotoxicity or seizure may not be comparable to physiological activity underlying learning and memory.

Astrocytes are also poised to be a source of lactate for neurons because in the hippocampus and cortex they have been shown to express the type 5 isoform of lactate dehydrogenase (LDH-5; Bittar et al., 1996), which favors the conversion of pyruvate into L-lactate (Cahn et al., 1962). Neurons, on the other hand, do not

express this isoform (Bittar et al., 1996), although both neurons and astrocytes express LDH-1, which catalyzes formation of pyruvate from L-lactate (Cahn et al., 1962). Thus, the differential expression of these enzymes also supports the idea that lactate is produced by astrocytes and shuttled to neurons, where it is then metabolized. The selective expression of other enzymes such as 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (Pfkfb3) and the M2 form of Pyruvate kinase (PKM2) provides astrocytes, in contrast to neurons, with a high capacity for aerobic glycolysis leading to L-lactate production (for review, see Magistretti and Allaman, 2015). However, a recent study demonstrated that neurons in resting conditions express higher hexokinase mRNA and protein levels than astrocytes, suggesting that neurons could at least initiate glycolysis (Lundgaard et al., 2015).

L-lactate shuttles among cells through monocarboxylate transporters (MCTs), which bidirectionally transport molecules with one carboxylate group, in particular lactate, pyruvate and ketone bodies. The transport occurs via diffusional, saturable co-transport of H^+ until equilibrium is reached. In the brain, MCT4 is mainly expressed by astrocytes, whereas MCT2 is mainly expressed by neurons (Pierre and Pellerin, 2005; Rinholm et al., 2011), and MCT1 is expressed in astrocytes, oligodendrocytes, and endothelial cells of blood vessels (Gerhart et al., 1997; Rinholm et al., 2011). These MCTs are found not only in the brain but also in several other tissues of many species throughout evolution. While the expression distribution of MCT1 suggests that this transporter has a special role in lactic acid oxidation, the localization of MCT4 suggests that this transporter is found where lactic acid efflux predominates. Finally, MCT2 has a tenfold higher affinity for substrates than MCT1 and MCT4 and is found in cells where rapid uptake at low substrate concentrations may be required, including the proximal kidney tubules, neurons and sperm tails (Halestrap and Price, 1999). In **Figure 1** a graphical model of L-lactate shuttling between astrocytes and neurons via MCTs is depicted.

Overall the question of how glucose and glycogen metabolism is regulated in neurons and astrocytes in resting conditions as well as under different conditions of stimulation remains debated (Chih and Roberts, 2003; Schurr, 2014). As we mentioned before, in this review we will not discuss the debates referring to different conditions of stimulation, as these arguments have been extensively described. In this review, we intend to discuss the literature and recent findings, including ours, related to the metabolism of glycogen, glucose and lactate in synaptic plasticity and memory formation in physiological conditions. Concerning the debates, we suggest that different paradigms and conditions of stimulation likely reflect different states of metabolism regulation. For example the metabolic regulations associated with oxidative stress conditions (e.g., stroke or traumas leading to neurodegeneration) or excessive activation of glutamate receptors (e.g., seizure; Patel et al., 2014), which causes cell damage, are very likely not comparable to those of physiological learning events. Thus, disagreements in the field of glucose/glycogen metabolism regulation in the brain may reflect, and hence be reconciled, by taking into account the differential regulations of glucose/glycogen metabolism in different stimulation conditions.

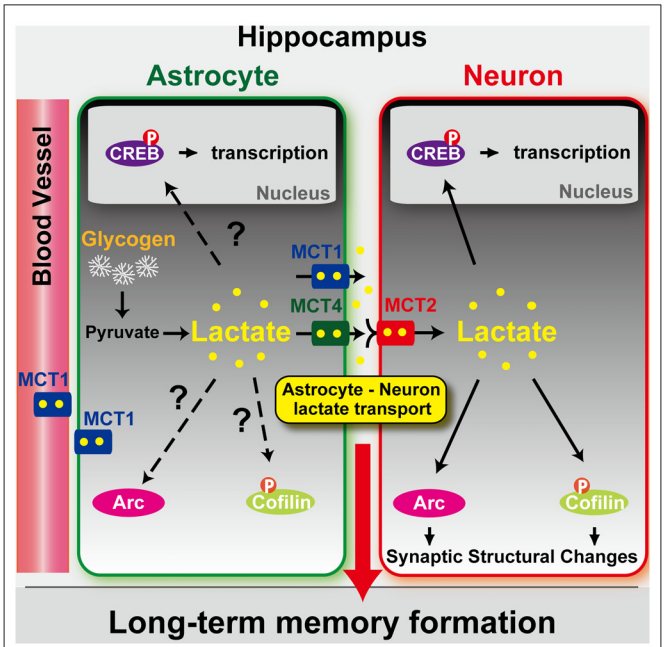

FIGURE 1 | Model depicting the role of astrocyte-derived lactate in long-term memory formation. Astrocytic glycogen breakdown and lactate release are essential for long-term but not short-term memory formation, and for the maintenance of long-term potentiation (LTP) of synaptic strength elicited *in vivo*. Disrupting the expression of the astrocytic lactate transporters monocarboxylate transporter 4 (MCT4) or MCT1 causes amnesia, which, like LTP impairment, is rescued by L-lactate but not equicaloric concentration of glucose. Disrupting the expression of the neuronal lactate transporter MCT2 also leads to amnesia that is unaffected by either L-lactate or glucose, suggesting that lactate import into neurons is necessary for long-term memory. Glycogenolysis and astrocytic lactate transporters are also critical for the regulation of molecular changes required for long-term memory formation, including the induction of phospho-CREB, Arc, and phospho-cofilin. Adapted from Suzuki et al. (2011).

GLYCOGEN, LACTATE AND ASTROCYTIC-NEURONAL MONOCARBOXYLATE TRANSPORT IN PLASTICITY AND MEMORY

The use of lactate to metabolically couple astrocytes and neurons during high energy-stimulus-dependent demands, like the formation of persistent modifications in the brain after learning, which is at the basis of long-term memory consolidation and storage, is very intriguing because it could offer an explanation for concerted actions that occur at brain circuitry and whole system levels. This mechanism may represent a key regulation of memory processes for at least two reasons. First, memory consolidation and storage have high-energy demands, especially when they take place in conditions of arousal or stress, a critical regulator of long-term memories. Second, like other tissues that have high-energy functional requirements, the brain stores glucose in the form of glycogen, and does so mostly in astrocytes

but not in neurons (Brown, 2004; Vilchez et al., 2007; Magistretti, 2008). Notably, it was reported in cell culture that breakdown of glycogen stores via glycogenolysis is associated with release of L-lactate but not glucose, suggesting that glycogen is functionally a lactate reservoir (Dringen et al., 1993).

As many studies that identified and characterized the role of lactate in astrocyte-neuronal coupling have been done in cell culture, conclusions for *in vivo* functioning in learning and memory needed *in vivo* validation. The first evidence for an important role of *in vivo* glycogenolysis in memory formation was the finding that glycogen stores diminish 30 min after taste aversion training in day-old chicks (O'Dowd et al., 1994; Hertz et al., 2003). 1,4-dideoxy-1,4-imino-D-arabinitol (DAB), an inhibitor of glycogen phosphorylation and breakdown, impaired taste aversion memory in day-old chicks when infused into the multimodal forebrain association region intermediate medial mesopallium (IMM), known to be required for memory consolidation (Gibbs et al., 2006). In this case glutamine was sufficient to rescue memory, presumably by contributing to a rescue of the glutamate/glutamine shuttle, which may also be affected by DAB. A subsequent study demonstrated that L-lactate was sufficient to rescue memory in the chick taste aversion model, both in the presence of DAB or 2-deoxyglucose, which impairs glucose phosphorylation and glycolysis (Gibbs et al., 2007). Notably, administration of D-lactate, the non-biologically active form, impaired chick taste aversion memory with a time delay that suggested it was inhibiting L-lactate metabolism and not uptake (Gibbs and Hertz, 2008).

Inspired by these studies, a few years ago we became interested in determining whether astrocytic mechanisms contribute to long-term memory formation *in vivo*, and specifically to hippocampal consolidation of a long-term episodic, aversive memory, using inhibitory avoidance (IA) in rats, a paradigm we had been studying for several years. IA is a long-lasting memory evoked by a single training trial, in which the animal associates the experience of a footshock with a given context upon entering it, and thus avoids it in future presentations. This memory requires intact hippocampus and amygdala, and has been extensively investigated in studies of arousal and stress-dependent modulation (Izquierdo et al., 1997; Roozendaal and McGaugh, 2011). IA models a behavioral response of an episodic aversive or stressful memory: its hippocampus-dependence makes it a useful model of emotional complex aversive memories, and its amygdala-dependence makes it an interesting model of emotional regulation of long-term hippocampal memory. Furthermore, this memory undergoes hippocampal-cortical system consolidation, a process that is typical of episodic and explicit memories, (those that in humans include declarative and autobiographical). In fact, in addition to the gene expression-dependent phase necessary for cellular consolidation, complex hippocampus-dependent memories undergo another consolidation process, which initially requires the role of the hippocampus, but with time becomes hippocampal-independent, while it maintains the engagement of cortical brain areas for memory storage and processing (Squire, 2004; Frankland and Bontempi, 2005).

Focusing on the dorsal hippocampus, because this region is key for contextual associative learning, we found that training leads to an increase in extracellular L-lactate, as measured by *in vivo* microdialysis. This increase in lactate lasted at least 50 min after training, and was completely blocked by inhibition of glycogenolysis with DAB (Suzuki et al., 2011; **Figure 2A**). DAB also blocked long-term—but not short-term—IA memory indicating that glycogenolysis in the dorsal hippocampus is required for long-term memory consolidation (**Figure 2B**).

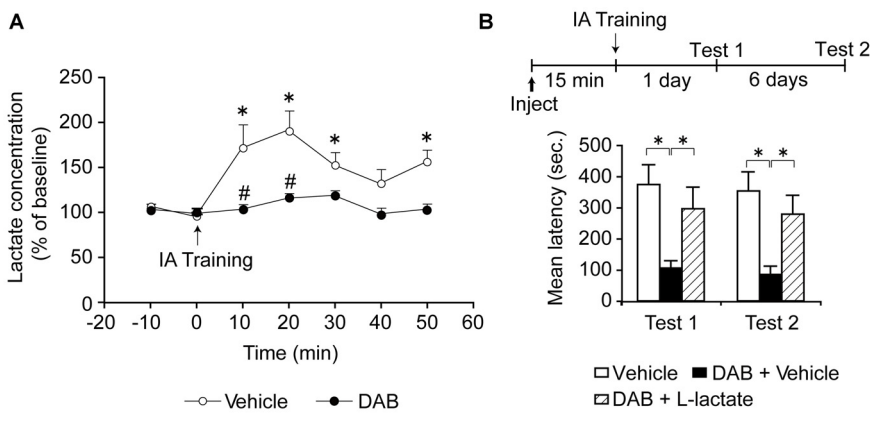

FIGURE 2 | (A) Extracellular lactate levels expressed as % of baseline ± SEM (mean of the first two samples set 100%) within the dorsal hippocampus of freely moving rats measured by microdialysis. Rats were injected bilaterally into the dorsal hippocampi with either vehicle or 1,4-dideoxy-1,4-imino-D-arabinitol (DAB, 300 pmol). Dialysate fractions were collected 10 min prior to training (0 min, ↑) to determine baseline levels and collection then continued every 10 min for 50 min. Training significantly increased lactate levels over baseline (*$p < 0.05$) and this effect was abolished by DAB (#$p < 0.05$; $n = 4$/group). **(B)** Long-term memory retention expressed as mean latency values ± SEM in seconds (s) tested 24 h (Test 1) as well as 7 days (Test 2) after training. Vehicle, DAB (300 pmol), or DAB (300 pmol) + L-lactate (100 nmol) was administered bilaterally into the dorsal hippocampus 15 min prior to inhibitory avoidance (IA) training (*$p < 0.05$; $n = 12$/group). Adapted from Suzuki et al. (2011).

Similar results also done in rats were reported by Newman et al. (2011) on a different learning paradigm, spontaneous alternation, a spatial working memory task that requires the ventral hippocampus, and recently by Zhang et al. (2015a) and Boury-Jamot et al. (2015) in both cocaine-induced conditioned place preference (CPP) and self-administration consolidation or reconsolidation. Reconsolidation is a process of stabilization that occurs after memory retrieval and shares many similarities with consolidation (Alberini, 2005).

Studies in rodents had revealed that extracellular glucose levels in the hippocampus are depleted when rats learn and memorize tasks such as on a four-arm spontaneous alternation maze (McNay and Gold, 2002; Gold, 2014). Following up on these results, Newman et al. (2011) showed that extracellular L-lactate rapidly increases in the hippocampus with spontaneous alternation, while glucose decreases. The levels were determined using L-lactate and glucose-sensitive biosensors that measure current from a probe coated with lactate or glucose oxidase, respectively, and interestingly, allowed for temporal resolution of lactate and glucose concentration on the order of seconds. This temporal resolution revealed that the lactate increase slightly precedes the decrease in glucose, suggesting that the astrocytic responses may anticipate energy needs rather than responding to them (Newman et al., 2011). DAB also impaired memory for this task, and the impairment was rescued by ventral hippocampal injection of L-lactate, but, contrary to what we found with IA in the dorsal hippocampus, the memory impairment was also rescued by an equicaloric concentration of glucose, suggesting that, in this case, glucose was equivalent to L-lactate.

A subsequent experiment done both in our laboratory with IA and by Newman et al. (2011), asked whether L-lactate transport into neurons is necessary for memory formation, a critical prediction for the ANLS model. Using different approaches and tasks we both first tested whether MCT2 was necessary for memory formation. The conclusion from both studies was affirmative. MCT2 blocked either pharmacologically with α-cyano-4-hydroxycinnamate (4-CIN; Newman et al., 2011) or genetic targeting with antisense oligodeoxynucleotides (Suzuki et al., 2011) caused a significant and profound impairment in memory retention. Second, we, as well as Newman et al. (2011), asked whether L-lactate or glucose could rescue the amnesia. These experiments were addressing two important alternative explanations: the first was whether there was a critical flow of lactate in the opposite direction, from neurons to other cells such as astrocytes, as proposed by the neuronal-astrocytic lactate shuttle (Simpson et al., 2007; Mangia et al., 2009). If L-lactate exported via neuronal MCT2 played a critical role in these other cells contributing to memory consolidation, then the amnesia would be rescued by lactate and/or glucose. The second question was whether glucose entering into neurons was a preferential substrate for long-term memory formation. In this case, as glucose import into neurons is not stopped by the MCT2 blockade, the administration of glucose should have resulted in memory recovery. We both found that the amnesia caused by blocking MCT2 persisted in the presence of either lactate or glucose. Thus, these findings provided strong support that L-lactate transported into neurons via MCT2 is necessary for memory formation (**Figure 3A**).

In addition, when we blocked translation of MCT1 or MCT4 in the hippocampus using antisense oligodeoxynucleotides, which led to selective knock down of the respective protein, we found a blockade of long-term IA memory, as well as of the molecular cascades underlying long-term memory formation, indicating, again in agreement with the ANLS hypothesis, that the function of these transporters is necessary for memory formation. The memory deficit was in this case rescued by exogenous administration of L-lactate, but not of equicaloric glucose (**Figures 3B,C**). Moreover, with MCT1 knockdown, equicaloric concentration of glucose had no effect on memory impairment; however high concentrations of glucose (three times higher than equicaloric) rescued the memory loss, but the effect was transient (**Figure 3D**). While dorsal hippocampal administration of glucose led to a transient memory enhancement, administration of L-lactate did not change long-term memory retention compared to vehicle-injected controls (**Figure 3C**). From all these results, we concluded that the lactate produced by glycogenolysis, and therefore exported from astrocytes, must be imported into neurons in order to produce long-term memory formation, while an equicaloric concentration of glucose administration is not able to replace the effect of lactate.

We also found that astrocytic glycogenolysis is required for the maintenance of LTP at the Shaffer Collateral-CA1 synapse *in vivo* (Suzuki et al., 2011). In the presence of DAB, which did not affect baseline neurotransmission, tetanic stimulation produced a strong initial potentiation, which, however, rapidly decayed. Co-injection of L-lactate with DAB before LTP induction reversed this decay.

Together, all these data strongly supported the conclusion that lactate-mediated mechanisms and lactate transport from astrocytes into neurons are essential for long-term plasticity and long-term memory consolidation.

Some alternate explanations of our results have been offered in a recent review by Dienel (2015). One question raised is that the results should be considered in the context of noradrenaline (NA) release from locus coeruleus projections to the hippocampus and the multiple regulations that this would target. Indeed, as also detailed below, the role of NA and its receptors in memory formation and modulation as well as stimulation of astrocytic glycogenolysis is well documented (Sorg and Magistretti, 1991; Gibbs and Summers, 2002; McGaugh and Roozendaal, 2002). IA has actually been extensively investigated as a model of memory modulation by arousal and stress (Gold and Van Buskirk, 1975; McGaugh and Roozendaal, 2002). We in fact agree that NA may act through astrocytic metabolic mechanisms. Ongoing studies in our laboratory investigating how NA as well as β adrenergic receptors (βARs) expressed by hippocampal astrocytes play a critical role in memory formation suggest that they function by engaging the ANLS. We will return to this point below.

A second issue raised is that the dose of L-lactate used for memory rescue in our study (Suzuki et al., 2011) is excessive and possibly pathophysiological and may suppress neuronal

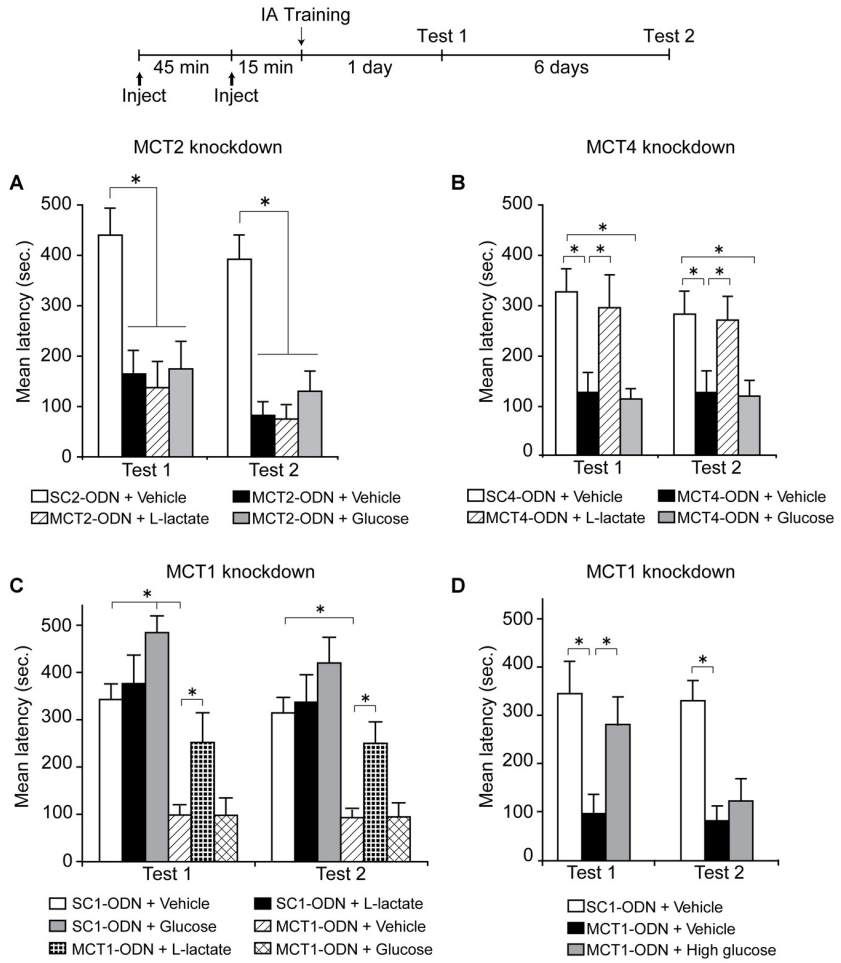

FIGURE 3 | Long-term memory retention expressed as mean latency values ± SEM in seconds (s) tested 24 h (Test 1) as well as 7 days (Test 2) after training. (A) Dorsal hippocampi were injected bilaterally with scrambled MCT2 ODN (SC2-ODN; 2 nmol) 1 h before training + Vehicle 15 min before training, or antisense MCT2 ODN (MCT2-ODN; 2 nmol) 1 h before training + Vehicle, L-Lactate (100 nmol) or Glucose (50 nmol) 15 min before training (n = 6–8 per group). **(B)** Dorsal hippocampi were injected bilaterally with scrambled MCT4 ODN (SC4-ODN; 2 nmol) 1 h before training + Vehicle 15 min before training, or antisense MCT4 ODN (MCT4-ODN; 2 nmol) 1 h before training + Vehicle, L-Lactate (100 nmol) or Glucose (50 nmol) 15 min before training (n = 10–12/group). **(C)** Dorsal hippocampi were injected bilaterally with scrambled MCT1 ODN (SC1-ODN; 2 nmol) 1 h before training + Vehicle, L-Lactate (100 nmol) or Glucose (50 nmol) 15 min before training, or antisense MCT1 ODN (MCT1-ODN; 2 nmol) 1 h before training + Vehicle, L-Lactate (100 nmol) or Glucose (50 nmol) 15 min before training (n = 7–13/group). **(D)** Dorsal hippocampi were injected bilaterally with scrambled MCT1 ODN (SC1-ODN; 2 nmol) 1 h before training + Vehicle 15 min before training, or antisense MCT1 ODN (MCT1-ODN; 2 nmol) 1 h before training + Vehicle or High glucose (150 nmol) 15 min before training (n = 8–11/group). *$p < 0.05$. Adapted from Suzuki et al. (2011).

firing. However, the concentrations of L-lactate we used were within range of physiological levels (approximately 5 mM), and did not elicit any tissue damage or altered response such as seizure. Similar concentrations of L-lactate used by Newman et al. (2011) also enhanced physiological responses such as spontaneous alternation memory, again excluding a toxic effect. Finally, a similar concentration of L-lactate with DAB in *in vivo* electrophysiology experiments did not preclude LTP induction, which also argues against a suppression of neuronal firing.

Lastly, it was indicated that: "*lactate transport was not measured* and *shuttling was assumed to occur*." Unfortunately techniques that directly detect the flux of lactate are still missing. However, our results based on transport blockers and directional rescuing experiments, in agreement with evidence from cell culture studies and slice preparations, are consistent with the conclusion that lactate is transported from astrocytes to neurons. Astrocytes can generate and release lactate (reviewed in Pellerin et al., 2007), which neurons are capable of utilizing as an energy source (Schurr et al., 1988; Izumi et al., 1997) while at the same time they appear to have a lower enzymatic capacity to generate lactate (Bittar et al., 1996). In agreement, a recent study *in vivo* in the anesthetized mouse reported evidence for a lactate gradient from astrocytes to neurons (Mächler et al., 2016). Hence, collectively our results, in line with others, indicate that lactate released from astrocytes and entered into neurons is critical for memory consolidation. Nevertheless, as other substrates such as

pyruvate and ketone bodies can be transported via MCTs (Poole and Halestrap, 1993), it cannot be excluded that they may also critically contribute to memory consolidation.

Recently, Zhang et al. (2015a) and Boury-Jamot et al. (2015) repeated the experimental design we carried out in Suzuki et al. (2011) to ask whether glycogenolysis, lactate release and MCT1, 4 and 2 in the amygdala play a critical role in the consolidation and/or reconsolidation of cocaine-induced CPP or self-administration, and found that to be the case (Zhang et al., 2015a). Furthermore, Tadi et al. (2015) using IA in mice found that mRNA levels of MCT1, MCT4, the α2 subunit of the Na/K-ATPase and glucose transporter type 1 increase at 24 h following IA training. They also showed that mice heterozygous for an MCT1 targeted disruption have impaired IA memory, again supporting the conclusion that genes involved in glia metabolism play a critical role in memory formation.

An important additional mechanism was recently described (Sotelo-Hitschfeld et al., 2015), by which L-lactate can be released in the interstitial space in response to cell depolarization through permeable ion channels, which suggests a neuromodulatory and gliotransmitter role of lactate. The identification of the channel/s will allow for testing whether this release also plays a role in learning and memory.

Indeed, L-lactate may not only be an energy substrate but may provide additional important regulations: a number of recent studies suggest that lactate may be critical because in addition to providing energy it can: (1) act as a transmitter of metabolic information by modulating prostaglandin action and cerebral vasodilatation causing cerebral blood flow to increase; (2) regulate the NADH/NAD+ redox ratio by conversion to pyruvate; and finally (3) activate the G-protein-coupled receptor GPR81 (also known as hydroxycarboxylic acid receptor 1) in astrocytes, capillaries and neurons which inhibits cAMP production (Gordon et al., 2008; Bergersen and Gjedde, 2012; Lauritzen et al., 2014). Thus, lactate physiologically produced by astrocytes may play multiple functions including providing energy, function as a signaling and/or modulatory molecule (Barros, 2013), and act as a preferential substrate, because it might spare glucose for other more specific uses such as glutamate synthesis (Bouzier-Sore et al., 2003).

All these functions may be critically involved in parallel, or regulated in different conditions and/or stages of synaptic plasticity and memory formation, and the field is now in a position to test all these hypotheses.

THE FUNCTIONS OF LACTATE TRANSPORTED INTO ACTIVATED NEURONS FOR MEMORY FORMATION: TRANSLATION AND MOLECULAR CHANGES INDUCED BY LEARNING

What is the function of lactate entering activated neurons? What does lactate provide, once inside the neurons that is critical for long-term memory formation? Is this mechanism general for different tasks, brain areas and memory modulations?

First, as lactate is a glycolytic metabolic substrate, an obvious question is whether it supplies energy. If that is the case, the next question is: what is this energy necessary for in long-term memory consolidation? Astrocytes are spatially well placed to couple neuronal activity with neuronal metabolic needs because they have processes that enwrap synapses as well as endfeet that enwrap blood vessels (Belanger et al., 2011). The shape and function of neurons suggests that when they are under high energy consumption, such as with excitatory postsynaptic potentials, the oxidation of glucose present in the neuron may not be sufficient, and an additional source of energy is needed; astrocytes have the structure and ability and have been shown to function precisely as an energy storage and supplier for neurons when they are in high energy demands. We refer to several excellent reviews that provide extensive descriptions of this matter (Magistretti, 2006; Belanger et al., 2011; Barros, 2013).

To begin addressing the question of the critical role of lactate in long-term memory formation, we reasoned that, first, the energy generated by lactate may be necessary for high-energy demanding functions required to form long-term memories; for example *de novo* transcription and translation, as well as the resulting structural modification of synapses. Translation occurs at activated synapses, where the process of gene expression regulation critical for memory formation seems in fact to rapidly start with activity (Martin et al., 2000). Thus, we have asked whether fundamental molecular changes required for long-term synaptic plasticity and memory formation are dependent upon lactate released by training. It is well established, in many species and different types of memories, that consolidation requires the activation of gene cascades, including that mediated by the transcription factor cAMP response element binding protein (CREB), which plays an evolutionarily conserved role in memory formation (Alberini, 2009). Additional critical mechanisms of long-term memory formation include the activation of the actin severing protein p21-activated kinase-cofilin (Chen et al., 2007), as well as the induction of the immediate early gene activity-regulated cytoskeletal protein (Arc/Arg3.1), both of which are key regulators of structural changes at synapses (Fischer et al., 2004; Chen et al., 2007; Bramham et al., 2008; Mantzur et al., 2009). Intra-dorsal hippocampal administration of DAB did not affect baseline levels of these markers in untrained rats, but completely blocked the activation of CREB and cofilin as well as the induction of Arc in rats trained in IA. These changes were reversed by co-administration of L-lactate (Suzuki et al., 2011). Notably, Arc is a neuronal protein (Vazdarjanova et al., 2006); thus our data indicated that neuronal molecular mechanisms occurring following learning depend upon glycogenolysis and lactate release.

In agreement with the conclusion that lactate controls molecular modifications in neurons, Yang et al. (2014) reported that administration of L-lactate to primary neuronal cortical cultures, or into mouse sensory-motor cortex, induces the expression of Arc along with the other immediate early genes c-Fos and Zif268. Notably, these changes are mediated by N-methyl-D-aspartate receptor (NMDAR) activation, and the

engagement of the extracellular-signal-regulated kinase 1/2 (ERK1/2) signaling, indicating that they are activity-dependent.

Lactate may also have concerted actions as a signaling molecule. In the mitochondria, lactate is oxidized to pyruvate while regenerating NADH, thus affecting the cellular and organelle redox state. In cultures, application of lactate is associated with increased intracellular levels of NADH, and application of NADH alone induces similar effects as lactate on NMDAR signaling (Yang et al., 2014). Therefore, lactate transport may serve as a signaling mechanism between cells and intracellularly via changes in redox balance (Brooks, 2009). For example, lactate oxidation can directly activate reactive oxygen species (ROS) production, and although excessive ROS production leads to oxidative damage, physiological levels of ROS are required for synaptic plasticity (Massaad and Klann, 2011). Thus, further studies should investigate whether lactate functions also as a regulator of redox balance and of signaling in models of memory formation. Lactate in fact is also known to act at GPR81, which is expressed on post-synaptic membranes of excitatory synapses, and the activation of this receptor is known to reduce cAMP levels (Gundersen et al., 2015; Morland et al., 2015). However, a role for GPR81 in synaptic plasticity and memory has not been investigated, and further studies are necessary to understand this role.

Collectively, these results suggest that the metabolic functions of lactate may be needed to supply the necessary energy required for cellular mechanisms (including neuronal mechanisms) evoked by training and critical for processing memory consolidation. This distinctive role of lactate may actually be an important differential mechanism that gates the changes necessary to make and maintain long-lasting structural modification, as opposed to feeding the preservation of survival or homeostatic mechanisms of cells. We suggest that when activity-dependent changes involving high-energy demands occur, such as those required for episodic long-term memory consolidation in the hippocampus, glycogenolysis and glycolysis leading to lactate production by astrocytes is a necessary step that allows the molecular and morphological modifications underlying memory storage to occur. We also suggest that, consistent with the results found in cell cultures, additional regulatory mechanisms such as redox regulation and signaling are likely to be activated by lactate, and that lactate may not be solely a resource for energy in memory formation. We speculate that the coordinated action of all lactate-mediated changes may be necessary for the multiple cellular and system effects that lead to the long-lasting biological changes necessary for memory storage.

THE FUNCTIONS OF LACTATE IN MEMORY FORMATION: GLUTAMINE/GLUTAMATE SHUTTLE

In addition to providing an energy source for molecular regulations, lactate coupling between neurons and astrocytes may function to regulate and monitor glutamate-glutamine turnover. Glutamatergic neural activity underlies formation of LTP (Milner et al., 1998), and is therefore essential for memory consolidation. Given that glutamate activity is believed to drive the ANLS (Pellerin et al., 2007) it appears the neurotransmitter contributes to both providing a signal for the production of lactate and as a mechanistic target of lactate. Astrocytes participate in the glutamate-glutamine shuttle wherein they take up glutamate from the active synapse and convert it into glutamine, which is then transported back to neurons to be metabolized back into glutamate (Gibbs et al., 2008b). This cycle uses the same principles of metabolic compartmentalization as the ANLS, given that astrocytes, but not neurons, express glutamine synthetase with which to metabolize glutamate (Norenberg and Martinez-Hernandez, 1979). A model has been proposed which postulates that cotransport of glutamate and Na^+ into astrocytes results in enhanced activity of the Na^+-K^+ ATPase and associated generation of lactate via aerobic glycolysis (Magistretti, 2009). During periods of elevated ammonium levels in the brain, which can lead to memory impairment (Aguilar et al., 2000; Muñoz et al., 2000), both lactate levels and glutamine synthetase activity are increased, illustrating a codisruption of the astrocyte neuronal coupling cycles under metabolic insult. Lactate generation essentially tracks glutamatergic activity and could potentially act as a signal for the rate at which astrocytes must import synaptic glutamate for purposes of recycling it and preventing excitotoxicity. Additionally, rather than be converted into glutamine, glutamate can also be directed toward the citric acid cycle (Gibbs et al., 2008b). Interestingly, cultured cortical astrocytes supplied with 0.2–0.5 mM glutamate converted a portion of the glutamate into lactate, which was released into the culture media (McKenna et al., 1996). We have not tested the effect of glutamine with DAB in our IA paradigm, but our data do not exclude that this may in fact function in parallel with the lactate-dependent changes we have reported (Suzuki et al., 2011). Taken together the glutamate-glutamine shuttle and the ANLS may be two subcomponents of a broad metabolic coupling between neurons and astrocytes, which have significant importance in memory consolidation.

THE FUNCTIONS OF LACTATE IN MEMORY FORMATION: STRESS, NEUROMODULATION, NORADRENALINE AND GLUCOCORTICOIDS

Another domain with important ramifications for memory formation and modulation, where the ANLS may play a significant role, is stress. Stress affects all aspects of physiology, including brain functions, and hence, memory formation, retrieval and storage. In humans, as in animal models, a large body of literature has shown that, for complex memories such as those processed by the hippocampal-dependent system, the degree of stress and the degree of memory retention are related by an inverted-U function (de Kloet et al., 2005). Accordingly, the stress hormones NA and glucocorticoids modulate memory retention: low concentrations of these hormones enhance memory while high concentrations impair memory retention (de

Kloet et al., 2005; Andreano and Cahill, 2006; Roozendaal and McGaugh, 2011; Herman, 2013).

Blocking ARs in the amygdala blocks IA memory enhancement by stress (Liang et al., 1986) or by glucocorticoid receptors (GRs) agonism (Quirarte et al., 1997). While the memory enhancing effects of NA in the basolateral amygdala have been more extensively investigated, less is known about the effects of NA in the hippocampus, despite projections from the locus coeruleus releasing NA in the hippocampus where it directly modulates hippocampal-dependent memories (Sara, 2009). As mentioned before, the hippocampus is critical for episodic, explicit and declarative memory formation, and engages in crosstalk with the amygdala to encode and store emotionally relevant information (McGaugh, 2013). This crosstalk has been suggested by evidence of the synchronized theta activity found in both the amygdala and hippocampus after fear conditioning (Paré et al., 2002; Seidenbecher et al., 2003; Narayanan et al., 2007), as well as by links in codependent molecular responses. For example, Arc increases in the dorsal hippocampus following injections of β_2 adrenergic agonists into the basolateral amygdala (McIntyre et al., 2005). Moreover, studies directly targeting the hippocampus showed that stress mechanisms in this region change emotional memories: infusion of NA into the dorsal hippocampus increases contextual fear learning (Yang and Liang, 2014), and in fact regulates hippocampal molecular mechanisms critical for long-term plasticity, such as CREB activation (Kabitzke et al., 2011). The NA effect on the hippocampus is mediated by βARs, as the antagonist propranolol given either systemically or into the dorsal hippocampus blocks contextual fear memories including IA, as well as spatial memories (Stuchlik et al., 2009; Kabitzke et al., 2011; Chen et al., 2012). This suggests that not only the amygdala but also the hippocampus is a main target of stress regulation. This is in line with the large body of literature showing that chronic stress suppresses dentate gyrus neurogenesis and causes dendrites of hippocampal (and medial prefrontal cortical) neurons to shrink, whereas it causes basolateral amygdala neurons to increase in dendritic complexity and sprout new synapses (Miller and McEwen, 2006). Thus, the NA-mediated role in memory consolidation or enhancement, through the action of amygdala and/or hippocampus, may be differentially regulated by distinct cell types, and therefore by distinct NA receptor subtypes (Gibbs et al., 2008c; Hutchinson et al., 2008; McReynolds et al., 2010). Further studies are needed to address this important issue.

As detailed below, there is evidence suggesting that stress regulates lactate formation and in turn, lactate seems to be critically involved in regulating responses to stress. We suggest that the hippocampal ANLS may participate in both adaptive and maladaptive stress-mediated allostasis. When arousal or stress occurs, the release of adrenaline from adrenal glands produces changes that are necessary for the organism to adapt to the new condition: one of these changes is the breakdown of glycogen stores in the liver, which leads to an increase in blood glucose levels. This glucose is a source of energy for all the necessary responses. The peripheral increase in adrenaline is paralleled by a release of NA and glucocorticoids in the brain, the stress hormones that are required for memory consolidation, as well as its modulation (McGaugh and Roozendaal, 2009; Chen et al., 2012). Notably, both NA and glucocorticoids regulate the metabolism of energy substrates necessary to brain cells for processing the experience to be remembered. Thus, a rise in blood glucose subsequent to adrenaline may mediate, at least in part, the effects of the hormone on memory (Gold, 1995).

It has been suggested that the increased glucose metabolism after stress may take place in the brain, like in the liver, through the recruitment of glycogenolysis (Allaman et al., 2004). In the brain glycogenolysis occurs in astrocytes, which would therefore provide lactate as an energy substrate to neurons. In other words, it is possible that stress increases lactate release in the brain through astrocytic glycogenolysis and glycolysis. In agreement with this idea, restraint stress (De Bruin et al., 1990; Elekes et al., 1996) and electric shock (Krugers et al., 1992) stimulate intrahippocampal lactate formation, indicating that NA release and lactate formation during stress are indeed potentially linked. Furthermore, L-lactate seems to regulate NA availability because lactate released by activated astrocytes in the locus coeruleus leads to neuronal excitation and release of NA (Tang et al., 2014).

How do stress and lactate come together in processing memory consolidation? In pioneering studies performed in chicks, Gibbs and colleagues revealed that noradrenergic activation via both α and βARs is critical for memory processing through distinct effects on metabolism (Hourani et al., 1990; Gibbs et al., 2008b). As with mammals, a memory elicited by a weak training in chick can be enhanced by NA (Crowe et al., 1990; Gibbs, 1991). Infusion of the β_2AR agonist zinterol or the β_3AR agonist CL316243 into the intermediate hyperstriatum ventral/medial neostriatum (IMVH) of chicks enhances taste aversion memory consolidation, indicating that NA promotes memory consolidation via these receptors (Herman, 2013). Furthermore, different subtypes of βAR were found implicated in the effects of lower or higher doses of NA: subcutaneous administration of the $\beta_1 + \beta_2$AR antagonist propranolol interfered with NA-mediated memory consolidation at medium doses of NA infused into the IMHV, whereas the β_3AR antagonist SR59230 impaired consolidation at the low doses (Herman, 2013). It was subsequently demonstrated that the memory enhancing effects of NA are achieved through metabolic mechanisms. β_2AR appears well positioned to regulate lactate availability during memory formation. Subcutaneous administration of zinterol augmented memory consolidation in the chick taste aversion paradigm (Gibbs et al., 2008c), but this effect was mitigated by DAB injection into the IMM, suggesting that glycogenolysis underlies the memory enhancement. In agreement, DAB also prevented a zinterol-induced reduction in glycogen in cultured astrocytes (Gibbs et al., 2008a,c; Gibbs, 2008). In contrast, the memory enhancing effects of β_3AR agonism were blocked by the glucose transport inhibiting compounds cytochalasin B, phloretin, or phlorizin

(Gibbs et al., 2008c). Cytochalasin B inhibits GLUT 1, a glucose transporter found on astrocytes and endothelial cells, whereas phloretin is a general GLUT inhibitor and phlorezin inhibits Na^+/energy-dependent endothelial glucose transport (Gibbs et al., 2008c). Whether or not β_3AR mediated uptake of glucose by astrocytes serves to provide these glial cells with a substrate for lactate production or for glycogen synthesis is an intriguing question. The same pattern of β_2AR playing a role in glycogenolysis and β_3AR supporting glucose uptake was found in the avian hippocampus (Gibbs et al., 2008a).

Prior to the work in cultured chick astrocytes, NA had been found to stimulate glycogenolysis in cultured mammalian cortical cells (Magistretti and Morrison, 1988). This effect was blocked by βAR as well as α_2AR antagonism (Subbarao and Hertz, 1990). Antagonism of α_1AR had no effect, which is consistent with behavioral impairment of taste aversion memory via α1AR (Subbarao and Hertz, 1990). Hence, it is possible that excess levels of NA under very stressful circumstances could impair memory consolidation through α_1AR-mediated inhibition of glycogenolysis. We are suggesting that the degree of a stressor could potentially affect the strength of memory consolidation by regulating the levels of lactate available within the brain to support metabolism and plasticity. At higher levels of stress memory could be impaired due to dysregulation of lactate formation, which may then result in a dysregulation of its metabolism.

Glucocorticoids appear to synergize with NA in the mobilization of energy during a stress response (Joëls and Baram, 2009). Astrocytes express GRs (Bohn et al., 1994; Simard et al., 1999) and corticotrophin releasing hormone receptors (Kapcala and Dicke, 1992), and respond to glucocorticoids with calcium waves (Simard et al., 1999) and peptide release (Chatterjee and Sikdar, 2013). The breakdown of glycogen following NA stimulation is followed by a seemingly compensatory increase in rates of glycogenesis (Sorg and Magistretti, 1992), which is inhibited by dexamethasone (Allaman et al., 2004), a synthetic form of glucocorticoid (Sapolsky et al., 2000). It is tempting here to speculate that glucocorticoids as well as NA affect memory formation and modulation by acting through astrocytic functions and a critical one is the glycolytic pathway that provides lactate to neuronal functions.

Consequently, we can expect that in maladaptive conditions, for example prolonged or chronic stress, the lactate metabolic role may be significantly altered. In fact, it has been proposed that glycogen depletion following prolonged stress and glucocorticoid exposure could lead to pathological states of the brain (Allaman et al., 2004). In accordance with this hypothesis, mice chronically exposed to glucocorticoids exhibited reduced levels of hippocampal glycogen, which appeared to derive from reduced glycogenic and increased glycolytic activity (Zhang et al., 2015b). These changes corresponded with increased immobility in forced swim test and tail suspension test wherein high levels of immobility are taken to suggest depression-like behavior (Zhang et al., 2015b). Additionally, prolonged social defeat stress in tree shrews (*Tupaia belangeri*, Czeh et al., 2006) and rats (Araya-Callís et al., 2012) is associated with significantly reduced counts of astrocytes that stain for glial fibrillary acidic protein (GFAP), a glial cytoskeletal element. In tree shrews these astrocytes also exhibit a 20% reduction in somal volume (Czeh et al., 2006), implying that alteration of astrocytic function critically contributes to the effect of stress and of stress-induced psychopathologies.

Together, these results offer support for some intriguing hypotheses for conditions of high or prolonged stress, which as mentioned before, critically contribute to psychopathologies: the failure to adapt in the long term could result in glycogen depletion, hence a shortage of available fuels required for coping over the longer term. The hippocampus is especially sensitive to the deleterious effects of glucocorticoids, and is a principle target in the brain for glucocorticoid dysregulation (Sapolsky, 1990).

In sum, several lines of evidence report an important contribution of lactate in the brain in the regulation of stress hormone-mediated responses, including memory formation in adaptive conditions but also, possibly through distinct mechanisms, in acute high or prolonged stress.

CONCLUSION

Work exploring the role of lactate in long-term memory formation supports the conclusion that memories that are formed after salient or stressful experiences critically engage glycogenolysis and glycolysis to support several mechanisms that lead to long-term morphological synapse modification. As glycogenolysis leading to lactate formation is a function of astrocytes, we suggest that the ANLS hypothesis provides important insights into the basic metabolic physiology that underlies the formation of memories. We speculate that the energy required for making long-term structural changes in synaptic networks is a metabolic signature of memory consolidation, and this is provided by lactate generation from astrocytes and its transfer into activated neurons. There remains much to be elucidated regarding how the ANLS may contribute to memory formation and processing under normal physiological conditions and then by extension, how it performs in pathological states. This knowledge will likely provide explanations and suggestions for new therapies targeting metabolic, stress and brain disorders.

AUTHOR CONTRIBUTIONS

CMA conceived and wrote the review; MQS and VG wrote the review.

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OPINION
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Astrocyte-Neuron Interactions during Learning May Occur by Lactate Signaling Rather than Metabolism

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Biology is full of instances of exaptation, the functional shift or co-optation of a trait during evolution (Gould and Vrba, 1982). Exaptation played a critical role in human brain evolution. For example, hominin brain expansion is thought to have happened opportunistically upon food resources rich in brain-selective nutrients (Tattersall, 2010). Prehensile hands and bipedalism were other enabling factors in this process, as both features preceded the expansion of the brain, and notably, the development and utilization of tools (Wood, 2010). Similarly, central and peripheral vocal structures, initially used for a variety of non-linguistic reasons (chewing, larynx protection, size exaggeration), were pre-existing conditions to, and provided the anatomical basis for, the evolution of language (e.g., MacNeilage, 2010). The very emergence of abstract cognitive abilities in humans are hypothesized to have evolved from faculties originally developed for other purposes (Pinker, 2010).

The same mechanisms were likely involved in the evolutive selection (or exploitation) of glutamate as the principal excitatory neurotransmitter of mammalian brain (reviewed by Mangia et al., 2012). Notably, glutamate is a central compound in amino acid metabolism in virtually all organisms, even those that lack a nervous system and even in unicellular organisms. In multicellular organisms, signaling through glutamate receptors existed well before the divergence between animal and plant phyla (Chiu et al., 1999). Of course, not all molecules that became neurotransmitters had distinct and pre-existing roles in cell metabolism. For example, there is no trace of noradrenaline (NE) receptors until multicellular organisms and cell-to-cell communication (Venter et al., 1988). Similarly, not all molecules with a specific role in cell metabolism eventually entered signaling pathways. Lactate was thought to be one of such molecules, and for many years it was regarded as a waste end-product of anaerobic glycolysis (reviewed by Schurr, 2006).

In the brain *in vivo*, lactate is constantly produced in spite of adequate oxygenation, and local increases in neural activity rapidly (i.e., within seconds) and transiently elevate lactate levels around the activated cells (Li and Freeman, 2015). *In vitro*, cultured neurons and astrocytes both release lactate. Although astrocytic release is higher under basal conditions, during metabolic uncoupling with dinitrophenol, the neuronal lactate release becomes as high as the astrocytic one (Walz and Mukerji, 1988). In 1994, Pellerin and Magistretti reported that lactate release and concomitant glucose uptake in astrocytic cultures were stimulated by sodium-coupled uptake of glutamate (Pellerin and Magistretti, 1994). Different laboratories attempting to replicate these findings either confirmed or refuted them, possibly because of the employment of distinct culture preparations (reviewed by Dienel, 2012). That glutamate can pay for its own uptake in cultured astrocytes (McKenna, 2013) is evident from comparison between uptake (by the same carrier) of the metabolizable L-glutamate and non-metabolizable D-aspartate showing that glutamate caused no increase in glycolytic rate, whereas D-aspartate did (Peng et al., 2001). However, the stimulation of glycolysis during glutamate uptake shown by Pellerin and Magistretti (1994) triggered the hypothesis of an astrocyte to neuron lactate shuttle, setting the stage for subsequent research

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and debate in the field. During the last two decades a large number of studies by many different investigators have been carried out to prove or disprove this hypothesis. Whatever the study and the specific outcome, the intercellular trafficking of lactate was always interpreted as movement of fuel, i.e., energy carbons useful for yielding most of the ATP that is achievable from oxidative metabolism of glucose.

Although astrocyte-neuron lactate transfer in the brain is relevant under some circumstances (e.g., during development; see Medina and Tabernero, 2005) and involves also oligodendrocytes (Sánchez-Abarca et al., 2001), recent experimental evidence indicates that cerebral lactate has signaling functions that are independent of its role as energy source (Bergersen and Gjedde, 2012). In particular, the brain expresses G_i-protein coupled hydroxycarboxylic acid (HCA) receptors, the activation of which inhibits adenylate cyclase (Lauritzen et al., 2013). Thus, the increase in brain lactate levels that follows focal neural activation might have been co-opted during evolution to serve signaling purposes. The brain has high respiratory capacity and the increase in lactate occurs through aerobic glycolysis, i.e., it is not due to oxygen insufficiency (Dienel, 2012). This argument suggests a specific role for glycolysis and lactate production in the brain, which was maintained even when it eventually became dispensable. Similar receptors evolved in adipose tissue to mediate the insulin-induced inhibition of lipolysis (Ahmed et al., 2010). A role for lactate as neuro/glio-transmitter in brain is a paradigm-shifting concept that will require re-evaluation of data obtained in the past decades that were interpreted only as a result of the metabolic nature of lactate (i.e., its caloric content). Elevated lactate was previously found to suppress neuronal firing in hippocampus (Gilbert et al., 2006), and a direct HCA1/GPR81 isoform-mediated inhibitory effect of lactate (either L-lactate or its stereoisomer D-lactate) on neuronal firing rate, with the relatively high $IC_{50} \sim 4.2$ mmol/L, has recently been demonstrated in cultured glutamatergic and GABAergic neurons from cerebral cortex (Bozzo et al., 2013). In contrast, the noradrenergic neurons of the pontine locus coeruleus (LC) were found to be stimulated by astrocyte-released lactate, with $EC_{50} \sim 0.5$ mmol/L, seemingly through a still unknown G_s-protein coupled receptor (Tang et al., 2014). As cerebral cortex and hippocampus are extensively innervated by LC axons it is conceivable that a minor, physiological increase in cortical lactate concentration exerts an excitatory effect on noradrenergic innervations, whereas higher concentrations have an inhibitory effect on pyramidal cells and interneurons, which would be useful as a negative feedback for homeostatic control of excitation and associated energy consumption. Astrocytes are primary targets for NE signaling in the cerebral gray matter, and in these cells NE potently stimulates breakdown of glycogen (reviewed by DiNuzzo et al., 2015). Astrocytic glycogen has been proposed to play a role in the rapid buffering of cellular ATP as well as in the production of lactate and/or sparing of glucose (Swanson et al., 1992; Shulman et al., 2001), although the role of brain glycogen is not yet established in detail (Dienel and Cruz, 2015).

Glycogenolysis in astrocytes plus glycolysis in neurons are proposed to contribute to the stimulation-induced rise in extracellular lactate observed during learning (Bergersen, 2015), and both glycogen and lactate are necessary for memory consolidation. In particular, inhibition of glycogen phosphorylase by 1,4-dideoxy-1,4-imino-d-arabinitol (DAB) or isofagomine resulted in short-term and long-term memory impairment during different learning protocols and animal models (Gibbs et al., 2006; Newman et al., 2011; Suzuki et al., 2011). These studies showed that memory could be rescued by injection of lactate, although the effect is dependent upon spatiotemporal variables and is partly recapitulated by other compounds including glucose, acetate and glutamine (aspects that are not discussed here). The main point is that the capacity of lactate to reverse memory impairment was regularly attributed to its relevance as an energy fuel. To this end, several studies examined the consequences of interfering with intercellular lactate trafficking through inhibition of monocarboxylate transporter (MCT) proteins.

In the brain, neurons predominantly express the MCT2 isoform, whereas astrocytes express both MCT1 and MCT4 isoforms. Intrahippocampal injection of the non-selective MCT inhibitor α-cyano-4-hydroxycinnamate (4-CIN, ∼60 μmol/L) caused memory impairment that could be partly rescued, though not significantly, by lactate (Newman et al., 2011). This finding was interpreted as supporting the requirement for neuronal lactate uptake, because the affinity of MCT2 for 4-CIN is much higher ($K_i = 24$ μmol/L) than that of MCT1 and MCT4 ($K_i =$ 425 μmol/L and 350–990 μmol/L, respectively) (Bröer et al., 1999; Dimmer et al., 2000; Manning Fox et al., 2000). However, in addition to plasmalemmal MCTs 4-CIN potently inhibits pyruvate uptake by mitochondrial MCTs ($K_i = 6$ μmol/L) and oxidative metabolism (Halestrap, 1975). Notably, both MCT1 and MCT2 colocalize with the mitochondrial inner membrane marker cytochrome oxidase in brain (Hashimoto et al., 2008). Yet, neurons and astrocytes are likely affected differently by inhibition of mitochondrial pyruvate uptake, as for example astrocytes but not neurons are capable of malate production from pyruvate due to much higher expression of cytosolic malic enzyme (Vogel et al., 1998), which can be followed by malate entry into mitochondria via dicarboxylate carrier and reversal of mitochondrial malic enzyme for regeneration of pyruvate.

Similar reasoning can be applied to the finding that reduction in the expression of MCT2 by 25% was sufficient to impair long-term memory formation (Suzuki et al., 2011). Under these conditions lactate was unable to rescue memory, whereas it reversed memory impairment after reduction in the expression of either MCT1 or MCT4. It is difficult to understand how a reduction of neuronal MCT2 as small as 25% could totally abolish memory, especially if this outcome is interpreted as precondition for lactate uptake in neurons. The higher affinity of MCT2 for lactate ($K_m = 0.74$ mmol/L) compared to MCT1 and MCT4 ($K_m = 3.5$–5.6 mmol/L and 28–34 mmol/L) implies that lactate flow through neuronal MCT2 is already saturated (i.e., cannot increase with increasing lactate) at resting brain lactate level (about 1 mmol/L; Bröer et al., 1997, 1999; Dimmer et al., 2000; Manning Fox et al., 2000). Therefore, such an

exceptional sensitivity to MCT2 levels is difficult to reconcile with the observation that memory consolidation is accompanied by increases in expression of MCT1 and MCT4 but not MCT2 (Tadi et al., 2015).

The importance of oxidative metabolism during learning is supported by the fact that memory is impaired, in addition to the non-transportable 4-CIN, also by D-lactate, which is transported but only slowly metabolized by D-lactate dehydrogenase, and whose inhibition can be counteracted by addition of different metabolic substrates (Gibbs and Hertz, 2008). Much like 4-CIN, D-lactate also competitively inhibits brain mitochondrial MCTs (Ling et al., 2012). Moreover, astrocytes have a high capacity for lactate uptake from extracellular fluid as well as for lactate dispersal via the astrocytic syncytium (Gandhi et al., 2009), and trafficking of glucose and its metabolites through astroglial networks via gap-junction (GJ) subunit proteins connexin 43 and 30 sustains synaptic transmission in hippocampus (Rouach et al., 2008). These observations are consistent with the inhibition of memory formation by the GJs uncoupler 18-α-glycyrrhetinic acid (Hertz and Gibbs, 2009), which was found to damage mitochondrial function in both astrocytes and neurons (Blanc et al., 1998). A need for lactate transport through astrocytes, not only out of astrocytes, might also be the reason for the expression of the type 5 isoform of the lactate dehydrogenase, which is the isoform of the enzyme that has the highest efficiency to catalyze pyruvate transformation to lactate (e.g., Koukourakis et al., 2003). Trans-astrocytic transport also entails that the effect of drugs, such as 4-CIN or D-lactate, is blunted in GJ-coupled astrocytes due to rapid dilution within these cells, something that cannot happen in neurons. It is noted that GJ proteins also mediate astrocytic release of lactate and other compounds that are relevant to learning. For example, inhibition of connexin 43 hemichannels was found to abolish long-term, but not short-term, memory formation and this effect was prevented by a mixture of several gliotransmitters, including glutamate, glutamine, lactate, D-serine, glycine, and ATP (Stehberg et al., 2012).

Activation of gene expression and associated protein synthesis is a fundamental process underlying the acquisition of new memories, which includes induction of phosphorylated cAMP response element-binding protein (pCREB), activity-regulated cytoskeleton-associated protein (Arc) and brain-derived neurotrophic factor (BDNF), among others. The induction of these plasticity-related genes depends on the activity of the LC-noradrenergic system (Cirelli and Tononi, 2000). Support for the view that noradrenergic signaling stimulates intracortical glycogenolysis and increase in lactate comes from the important observations that brain NE and lactate rise during wakefulness, rapid eye movement (REM) sleep or sleep deprivation, and decline during slow-wave non-rapid eye movement (NREM) sleep (Cirelli et al., 2005; Naylor et al., 2012; Wisor et al., 2013), while glycogen has the opposite dynamics (Kong et al., 2002). Retention of new information is possible only during wakefulness while it is largely impaired during NREM sleep (Emmons and Simon, 1956; Simon and Emmons, 1956; Portnoff et al., 1966; Koukkou and Lehmann, 1968). Similarly, hippocampal long-term potentiation (LTP) occurs during wakefulness but not during NREM sleep (Leonard et al., 1987; Bramham and Srebro, 1989). Notably, LTP and memory acquisition are impaired in glycogen synthase-deficient mice (Duran et al., 2013).

In conclusion, the rise of extracellular lactate level in cerebral cortex and hippocampus appears to be necessary for memory formation. While the importance of the noradrenergic system in learning has long been undisputed, evidence that glycogen is an important link in the causal chain between NE and lactate has only recently been established. The literature about brain lactate described in this short communication is necessarily incomplete, but it demands that intellectual efforts be aimed at further investigating its receptor-mediated signaling not only its cellular uptake.

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OPINION
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Sequential Astrocytic 5-HT$_{2B}$ Receptor Stimulation, [Ca^{2+}]$_i$ Regulation, Glycogenolysis, Glutamate Synthesis, and K$^+$ Homeostasis are Similar but Not Identical in Learning and Mood Regulation

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INTERRELATION BETWEEN ANXIETY AND MEMORY

A close interrelation between anxiety and memory was first suggested by Kalueff and Nutt (1996), reviewing effects of γ-aminobutyrate (GABA) on both conditions. Wall and Messier (2000) showed subsequently that pretreatment with an opioid kappa receptor antagonist was anxiogenic and disrupted working memory. Additional papers supporting interactions between anxiety and memory were cited by Kalueff (2007). They included demonstration of memory improvement by serotonin, whereas a decreased ability to increase serotonin is a model of anxiety.

SEROTONIN AND THE 5-HT$_{2B}$ RECEPTOR

Serotonin acts on many different receptors. The present paper specifically deals with the 5-HT$_{2B}$ receptor, which is expressed in human brain (Schmuck et al., 1994; Bonhaus et al., 1995). Its mRNA expression is two times higher in freshly isolated (Lovatt et al., 2007) mouse astrocytes than in neurons (Li et al., 2012). It is necessary for consolidation of one-trial aversive learning in day-old chickens (Gibbs and Hertz, 2014) at an early stage of memory consolidation. It is also required for the therapeutic effect of serotonin-specific reuptake inhibitors (SSRIs) in major depression (Diaz et al., 2012; Li et al., 2012; Hertz et al., 2015b), a disease often accompanied by anxiety. The 5-HT$_{2B}$ receptor in cultured astrocytes is stimulated by fluoxetine (Li et al., 2008: Qiao et al., 2015) and all other SSRIs (Zhang et al., 2010). Chronic treatment of mice with fluoxetine for 14 days up-regulates the astrocytic, but not the neuronal 5-HT$_{2B}$ receptor, although this receptor is expressed in both cell types (Li et al., 2012; Hertz et al., 2015b). Decrease of its astrocytic gene expression parallels development of a depressive phenotype in a mouse model of Parkinson's disease (Zhang et al., 2015), and Pitychoutis et al. (2015) reported schizophrenia-like symptoms in mice lacking the 5-HT$_{2B}$ receptor gene or treated with a receptor inhibitor. Schizophrenia is often associated with depressed mood (Fortunati et al., 2015).

5-HT_{2B} RECEPTOR, $[Ca^{2+}]_i$, GLYCOGENOLYSIS, GLUTAMATE, K^+, AND LEARNING

Inhibition of learning by a 5-$HT_{2B/C}$ receptor antagonist (SB221284) and equipotent rescue of impaired learning by the 5-HT_{2B} receptor agonists fluoxetine and paroxetine (Gibbs and Hertz, 2014) injected intracerebrally at specific times shows the importance of this receptor for establishment of memory soon after training. The similar potency of the two drugs is important, because they have widely different affinities for SERT (Wong and Bymaster, 1995) whereas all SSRIs have similar affinity for the 5-HT_{2B} receptor (Zhang et al., 2010). Another SSRI, citalopram, counteracts spatial memory deficits (Ren et al., 2015). Mice lacking the 5-HT_{2B} receptor gene show learning disabilities (Pitychoutis et al., 2015).

Fluoxetine increases free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) and stimulates glycogenolysis (Chen et al., 1995) with similar potency by stimulation of 5-HT_{2B} receptors (Kong et al., 2002; **Figure 1A**). $[Ca^{2+}]_i$ regulates many astrocytic functions, including gliotransmission and glycogenolysis (Gucek et al., 2012; Hertz et al., 2015a). Inhibition of glycogenolysis with DAB (1,4-dideoxy-1,4-imino-D-arabinitol) prevents 5-HT_{2B}-receptor-mediated memory enhancement by serotonin or fluoxetine during the early part of memory formation after one-trial aversive learning in the day-old chicken, a precocious animal (Gibbs and Hertz, 2014). In brain both glycogen and its degrading enzyme glycogen phosphorylase are virtually confined to astrocytes (Ibrahim, 1975; Pfeiffer-Guglielmi et al., 2003). Induction of glycogenolysis by fluoxetine occurs both in our cultured astrocytes, differentiated by dibutyryl cyclic AMP and in astrocytes grown in the absence of this agent (Allaman et al., 2011). The association with increased $[Ca^{2+}]_i$ (Chen et al., 1995) is important because increased $[Ca^{2+}]_i$ is a requirement for stimulation of glycogenolysis in astrocytes (Xu et al., 2014a; Hertz et al., 2015a) as in muscle (Ozawa, 2011). In rat brain 5-HT_2 receptor stimulation similarly induces glycogenolysis (Darvesh and Gudelsky, 2003). The enhanced glycogenolysis is accompanied by an increased lactate release (Allaman et al., 2011). This might affect neurons either by use of lactate as an additional metabolic fuel, as suggested by Suzuki et al. (2011) and Newman et al. (2011), or by lactate signaling (Tang et al., 2014; Bergersen, 2015). The signaling mechanism established by Tang et al. (2014) is, like memory (Gibbs et al., 2006; Newman et al., 2011; Suzuki et al., 2011; Gibbs and Hutchinson, 2012; Duran et al., 2013), glycogenolysis-dependent, and its signaling is specifically directed to neurons releasing noradrenaline. Noradrenaline has effects on both neurons and astrocytes (O'Donnell et al., 2012).

Glycogenolysis is also required for formation of glutamate, and its metabolite GABA (**Figure 1B**) in the brain *in vivo* (Gibbs et al., 2007, 2008) at a time when glutamate production must be evoked by 5-HT_{2B} stimulation (Gibbs and Hertz, 2014). It also increases uptake of glutamate into cultured astrocytes and neurons as well as release of lactate from astrocytes (Sickmann et al., 2009). Glutamate is synthesized intracerebrally from glucose. This can only occur in astrocytes, because neurons lack an enzyme, pyruvate carboxylase, needed for its synthesis (reviewed by Gibbs et al., 2008; Hertz, 2013). Glutamate is subsequently converted to glutamine and carried to neurons (green arrow in **Figure 1B**) in an extremely active glutamine-glutamate/GABA cycle, which also returns released transmitter glutamate to neurons after its initial astrocytic accumulation (reviewed by Hertz, 2013; Hertz and Rothman, in press). The importance of glutamate receptor activity for memory is beyond doubt (Riedel et al., 2003), and interruption of the glutamine-glutamate/GABA cycle by inhibition of either glutamine synthetase (Kant et al., 2014) or astrocytic glutamate uptake (Gibbs et al., 2004) abolishes learning. GABA is also important for learning (Kalueff and Nutt, 1996; Gibbs and Bowser, 2009), and besides its neuronal effects stimulates glycogenolysis in cultured astrocytes and brain slices (Xu et al., 2014a).

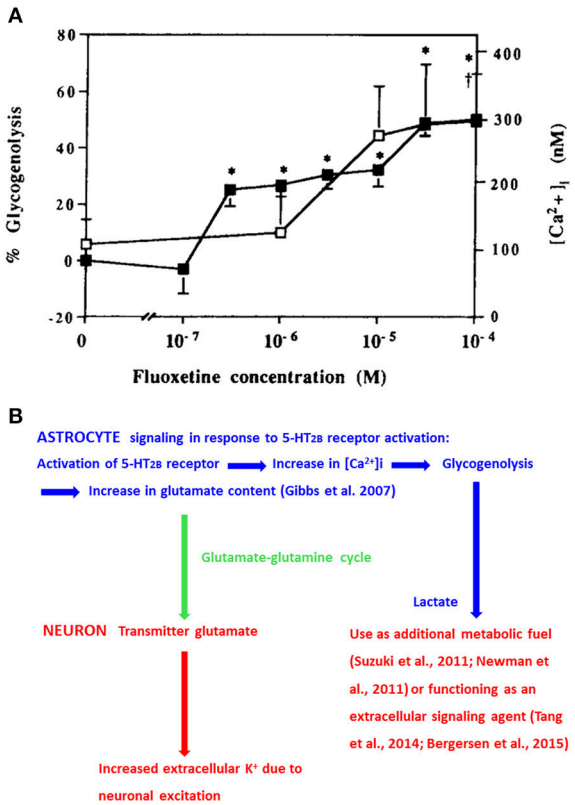

FIGURE 1 | (A) Effects of fluoxetine concentrations between 100 nM and 100 μM on glycogenolysis (filled squares and left ordinate) and $[Ca^{2+}]_i$ (open squares and right ordinate) in well differentiated cultures of mouse astrocytes. Values significantly different from baseline are indicated by * for glycogenolysis and by † for $[Ca^{2+}]_i$ (From Chen et al., 1995). **(B)** Chart showing 5-HT_{2B} receptor-mediated effects on $[Ca^{2+}]_i$, glycogenolysis, and glutamate content in astrocytes (blue) and effects of glutamate transfer to neurons (green arrow) and of glycogenolysis-evoked release of lactate on neurons (red). These effects are acutely important for learning, and drug-induced chronic effects of 5-HT_{2B} receptor stimulation have therapeutic effect, also on impaired memory, in major depression (fluoxetine) and in schizophrenia (clozapine). However, as discussed in "Concluding remarks" in these situations it appears that it is a decreased effect on the receptor or on $[Ca^{2+}]_i$that is therapeutically effective.

A major role of glutamate (**Figure 1B**) is stimulation of postsynaptic glutamate receptors, leading to increases in brain metabolism (Howarth et al., 2012) and in extracellular K^+ concentration (Hertz et al., 2015c and references therein). Cellular re-accumulation of K^+ includes an initial uptake mediated by the astrocytic Na^+,K^+-ATPase (MacAulay and Zeuthen, 2012; Hertz et al., 2015c), release of astrocytically accumulated K^+ by Kir4.1 channels (Bay and Butt, 2012) and neuronal reuptake. The astrocytic Na^+,K^+-ATPase is important for learning (Moseley et al., 2007; Schaefer et al., 2011; Hertz et al., 2013; Tadi et al., 2015). Extracellular K^+ concentrations high enough to stimulate the Na^+, K^+, 2 Cl^- co-transporter NKCC1 (>10–12 mM) also causes release of gliotransmitters (Song et al., 2014; Xu et al., 2014b; Liu et al., 2015). Both astrocytic release of glutamate (Lee et al., 2014) and ATP (Gibbs et al., 2011; Stehberg et al., 2012) are crucial for learning. Facilitation of learning by K^+-mediated depolarization of oligodendrocytes and increased myelination at high extracellular K^+ concentration (Roitbak, 1984) and attributed to increased ability of the myelinated axon to carry out rapid impulse conduction has recently been confirmed and characterized by Yamazaki et al. (2014).

5-HT_{2B} RECEPTOR, $[Ca^{2+}]_i$, GLYCOGENOLYSIS, GLUTAMATE, K^+, AND MOOD DISORDERS

Fluoxetine is better known for its antidepressant effect, which in contrast to the acute stimulation of the 5-HT_{2B} receptor during learning takes several weeks to materialize. During this time many changes occur in gene expression and editing, as shown in mice chronically treated with fluoxetine. Studies in neuronal and astrocytic cell fractions freshly obtained from these mice (Lovatt et al., 2007) showed that most of these alterations occurred in astrocytes, although some neuronal changes also took place (Li et al., 2012; Peng et al., 2014; Hertz et al., 2015b). This finding suggests that astrocytes play a major role in the antidepressant effects of SSRIs (Li et al., 2012; Hertz et al., 2015b), a conclusion in agreement with results by many other authors (e.g., Ongür et al., 1998; Kugaya and Sanacora, 2005; Ongür et al., 2007; Valentine and Sanacora, 2009; Rajkowska and Stockmeier, 2013; Rajkowska et al., 2013; Bernstein et al., 2015; Hertz et al., 2015b and references therein). It is especially interesting that Bechtholt-Gompf et al. (2010) found that blockade of astrocytic glutamate uptake in rats induces signs of anhedonia (a component of depression that is easily measurable in animals) *and* impaired spatial memory.

Some of the editing changes reduced normally occurring effects of transmitters. Li et al. (2011) showed that in astrocyte cultures treated for sufficient length of time with fluoxetine, the effects on $[Ca^{2+}]_i$ by acute administration of several transmitters or ryanodine receptor agonists are reduced or abolished. On the other hand, the effect of an increased extracellular concentration of K^+ was increased. Thus, chronic treatment with an SSRI diminishes or alters some of the normal responses of the 5-HT_{2B} receptor to stimulation. This might partly be explained by inhibition of capacitative Ca^{2+} entry, mediated by glycogenolysis-dependent (Müller et al., 2014) TRPC1 channels, which causes depletion of Ca^{2+} stores. Due to this inhibition refilling of depleted Ca^{2+} stores by addition of 2 mM $CaCl_2$ to the medium was greatly reduced (Li et al., 2011). All effects of chronic fluoxetine administration could be replicated by TRPC1 channel antibody. However, the expression of $Ca_v1.2$, a gene of an L-channel for Ca^{2+} which is stimulated by elevations in extracellular K^+ concentrations of at least 10 mM is increased (Du et al., 2014), probably explaining the enhanced K^+ effect on $[Ca^{2+}]_i$ described above. The 5-HT_{2B} receptor itself is also edited by chronic fluoxetine treatment, rapidly reducing the effects of its stimulation of the IP_3 receptor (Peng et al., 2014). Since chronic SSRI treatment improves memory in depressed patients (Table in Krysta et al., 2015), inhibition of glutamate-induced increase in astrocytic $[Ca^{2+}]_i$ and thus in release of gliotransmitter glutamate (Peng et al., 2012) has no deleterious effect on learning, at least not when combined with $[Ca^{2+}]_i$ increase by elevation of the extracellular K^+ concentration. In this connection it seems of considerable interest that Medina et al. (2015) described down-regulation of mRNA expression of glutamate transporters, K^+ channels and gap junction proteins in hippocampus of patients having suffered from major depression. Most of these genes are selectively expressed in astrocytes. Abnormalities of Na^+,K^+-ATPase function in depressed patients have been described by De Lores Arnaiz and Ordieres (2014)

RELATED ASTROCYTIC MECHANISMS IN SCHIZOPHRENIA

Schizophrenia is treatable both by the dopamine antagonist haloperidol and atypical antipsychotics like clozapine, which is an antagonist at the 5-HT_{2B} receptor in the fundus of the stomach (Villazón et al., 2003). Again, acute stimulation of the 5-HT_{2B} receptor is likely to increase $[Ca^{2+}]_i$, glycogenolysis and glutamate formation (**Figure 1B**). An increase in $[Ca^{2+}]_i$ by stimulation of astrocytic dopamine receptors is reduced by exposure to clozapine (Reuss and Unsicker, 2001), and this seems also to be the case after clozapine activation of 5-HT_{2B} receptors. A resulting reduced production of glutamate (**Figure 1B**) in mice lacking 5-HT_{2B} receptors may explain a decreased content of glutamate in some brain areas (Pitychoutis et al., 2015), which may contribute to the impairment of learning.

CONCLUDING REMARKS

Activation of the astrocytic 5-HT_{2B} receptor stimulates an *increase* in $[Ca^{2+}]_i$, glycogenolysis, glutamate formation, and the effect of glutamate on extracellular K^+, all of which are involved in learning (**Figure 1B**). However, Sibille et al. (2015) found that acute *inhibition* of Ca^{2+} signaling in astrocytes by $[Ca^{2+}]_i$ chelation potentiates excitatory synaptic transmission. This apparent contradiction may be explained by the complexity of astrocytic $[Ca^{2+}]_i$ regulation (Volterra et al., 2014). An important difference between Gibbs and Hertz (2014) and Sibille et al. (2015) is that the latter authors elicited astrocytic increase in $[Ca^{2+}]_i$ in response to adjacent neuronal activity

during $GABA_A$ receptor inhibition, whereas the former described transmitter-induced, glycogenolytic (and thus Ca^{2+}-dependent) effects on learning without $GABA_A$ receptor inhibition.

Drugs used for treatment of symptoms of major depression (fluoxetine) and of schizophrenia (clozapine), which included memory impairment, interfered with 5-HT_{2B} receptor-activated functions, but in different manners: the SSRI fluoxetine edited and thereby reduced some normal effects of this receptor, whereas clozapine caused a decrease in $[Ca^{2+}]_i$. This effect is consistent with the enhancement of excitatory synaptic transmission described by Sibille et al. (2015). Disposition to both major depression and schizophrenia is probably inborn, and perhaps these patients display quantitative and/or qualitative abnormalities in 5-HT_{2B}-mediated signaling, which might also affect learning processes. In agreement with this notion 5-HT_{2B} receptors play a major role during brain development (Lauder et al., 2000).

AUTHOR CONTRIBUTIONS

All authors planned or carried out reviewed experiments. YC and LH wrote the paper and MEG edited it.

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Inhibition of Astrocytic Glutamine Synthetase by Lead is Associated with a Slowed Clearance of Hydrogen Peroxide by the Glutathione System

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Lead intoxication in humans is characterized by cognitive impairments, particularly in the domain of memory, where evidence indicates that glutamatergic neurotransmission may be impacted. Animal and cell culture studies have shown that lead decreases the expression and activity of glutamine synthetase (GS) in astrocytes, yet the basis of this effect is uncertain. To investigate the mechanism responsible, the present study exposed primary astrocyte cultures to a range of concentrations of lead acetate (0–330 μM) for up to 24 h. GS activity was significantly reduced in cells following 24 h incubation with 100 or 330 μM lead acetate. However, no reduction in GS activity was detected when astrocytic lysates were co-incubated with lead acetate, suggesting that the mechanism is not due to a direct interaction and involves intact cells. Since GS is highly sensitive to oxidative stress, the capacity of lead to inhibit the clearance of hydrogen peroxide (H_2O_2) was investigated. It was found that exposure to lead significantly diminished the capacity of astrocytes to degrade H_2O_2, and that this was due to a reduction in the effectiveness of the glutathione system, rather than to catalase. These results suggest that the inhibition of GS activity in lead poisoning is a consequence of slowed H_2O_2 clearance, and supports the glutathione pathway as a primary therapeutic target.

Keywords: astrocytes, glutamine synthetase, glutathione, glutamate, oxidative stress, toxicity

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INTRODUCTION

A primary function of astrocytes is to recycle synaptically-released glutamate (Pow and Robinson, 1994). Unlike neurones, astrocytes express glutamine synthetase (GS; Norenberg and Martinez-Hernandez, 1979; Ong et al., 1993; Robinson, 2000), which catalyzes the ATP-dependent condensation of glutamate and ammonia to form glutamine (Rose et al., 2013), which is subsequently released for uptake by neurones and deamidation to glutamate, in the glutamate-glutamine cycle (Westergaard et al., 1995; Hertz et al., 1999). GS is particularly sensitive to inactivation by iron-mediated oxidative stress (Fernandes et al., 2011), and consequently depleted GS activity levels in tissue have often been used as an indicator of oxidative stress (Schor, 1988; Robinson, 2000).

Studies have linked the toxicity of lead to elevated oxidative stress, with lead exposure correlating with increased production of free radicals and a lowering of antioxidant reserves

(Patrick, 2006; Rubino, 2015). In the brain, astrocytes contribute to the defense against toxic metals, xenobiotics and oxidative stress (Dringen et al., 2014). Hydrogen peroxide (H_2O_2), a common source of oxidative stress, is generated during erobic metabolism by the action of superoxide dismutases and several oxidases. H_2O_2 is broken down in iron-catalyzed reactions to form hydroxyl radicals, which react readily with proteins, lipids and DNA, and are thus toxic to cells. To limit free radical formation from H_2O_2, astrocytes utilize two main antioxidant systems. The first of these, catalase, rapidly degrades H_2O_2, even when the peroxide is applied acutely at high concentrations (Dringen and Hamprecht, 1997). The rate of H_2O_2 breakdown is significantly slowed by pre-incubating astrocytes with the catalase inhibitor 3-amino-1,2,4-triazole (3AT), and this is correlated with an increase in cell death (Liddell et al., 2006).

The second antioxidant system is the glutathione pathway, whereby H_2O_2 is reduced to water in a reaction catalyzed by glutathione peroxidase (GPx). Glutathione (GSH) serves as the electron donor, and is itself oxidized to glutathione disulfide (GSSG). GSSG is reduced to GSH through the action of glutathione reductase (GR; Dringen and Hamprecht, 1997; Dringen et al., 2014). Astrocytes pre-incubated with buthionine sulfoximine (BSO), a GSH synthesis inhibitor, show a marked reduction in their rate of H_2O_2 clearance and an increase in cell death (Liddell et al., 2006). Application of 3AT and BSO to inhibit both antioxidant systems, futher retards H_2O_2 clearance rates and exacerbates cell death (Liddell et al., 2006). The high cellular GSH content of astrocytes, combined with their efficiency in breaking down peroxides, protects astrocytes and neighboring cells from oxidants and toxins (Dringen et al., 2014).

While several animal and cell culture studies have shown that application of lead diminishes the expression and activity of GS (Engle and Volpe, 1990; Sierra and Tiffany-Castiglioni, 1991), the basis of this effect is uncertain. The decrease in GS activity may be due to lead binding to cysteine residues on GS, thereby interfering with its catalytic activity (Tang et al., 1996; Qian et al., 1999). Alternatively, GS may be inactivated by reactive oxygen species that result from a diminished effectiveness of the GSH system. Lead binds to sulfhydryl groups, giving it an affinity for GSH which then cannot act an antioxidant (Patrick, 2006). Lead can also bind to the catalytic site of GR, irreversibly inhibiting the enzyme and preventing it from reducing GSSG to GSH (Rubino, 2015). Impairment of the glutathione system will decrease cellular antioxidative capacity and reduce protection from oxidative stress (Scortegagna et al., 1998; Aykin-Burns et al., 2003).

It is possible that lead binds to the active site of GS inhibiting it directly, or the inhibition of lead may be a downstream event, secondary to inactivation of the glutathione system. The present study conducted experiments to discriminate between these alternatives. Primary astrocyte cultures were exposed to a range of concentrations of lead for up to 24 h. We confirmed that GS activity is significantly lowered in cultures following incubation with lead acetate. However we found that this effect is not replicated when astrocyte lysates are exposed to lead, suggesting that, when in the presence of other cellular components, lead does not directly interfere with the catalytic activity of GS. We also demonstrated that lead limits the capacity of astrocytes to degrade H_2O_2, and that this appears to be due to an impairment of the GSH system.

MATERIALS AND METHODS

Materials

This study was carried out in accordance with the guidelines of the National Health and Medical Research Council (NHMRC) of Australia. The protocol was approved by Monash University's Psychology Animal Ethics Committee. Primary astrocyte cell cultures were derived from newborn Wistar rat pups obtained from Monash Animal Services. Constituents of growth media were obtained from Gibco (Carlsbad, CA, USA): Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), streptomycin sulfate and penicillin G. Triton X-100 was obtained from Ajax Finechem (Seven Hills, Australia). Lead (II) acetate trihydrate, BSO, 3AT, H_2O_2 and all other chemicals were obtained from Sigma (Australia). Twenty four-well cell culture dishes and 96-well microtiter plates were obtained from Greiner Bio-One (Frickenhausen, Germany).

Cell Cultures

Primary astrocyte cultures were obtained from the brains of newborn Wistar rat pups (<24 h old) as previously described (Hamprecht and Löffler, 1985). Astrocytes were seeded at approximately 3×10^5 cells/well in 24-well culture plates. Cells were grown in humidified incubators (Heraeus Instruments) at 10% CO_2. Growth medium was replaced every 6th or 7th day until cultures were confluent, at which time they were used for experimentation (14–21 days).

Cell Viability and Protein Content

The activity of lactate dehydrogenase (LDH) released by cells into media by treated cells was measured to determine the extent of cell death, as previously described (Dringen et al., 1998). Hundred percent cell death values were derived from cells lysed with Triton X-100. Cellular protein content per well was determined via the Lowry method (Lowry et al., 1951).

GS Activity

GS activity was measured with a colorimetric assay (Fernandes et al., 2011). To determine the effect of lead on GS activity, cultures were incubated with 0, 33, 100 or 330 μM lead acetate in DMEM for 2 or 24 h. Cells were washed with ice-cold PBS and frozen at −20°C for 30 min before being warmed to 37°C and lysed with 200 μl of 50 mM imidazole/ HCl buffer (IHB), pH 7.2. After 5 min, 200 μl of a reaction mix was added to the lysates (50 mM L-glutamine, 2 mM manganese chloride, 25 mM sodium arsenate, 0.16 mM ADP and 25 mM NH_2OH*HCl). Following 30 min incubation with the reaction mix, 800 μl of a solution of 0.37 M ferric chloride ($FeCl_3$), 0.67 M HCl and 0.2 M trichloroacetic acid was added to halt the reaction.

Samples were transferred to microfuge tubes and centrifuged for 5 min at 15000 g. Three hundred microliters of the supernatant was transferred to a microtiter plate. Absorbances were measured spectrophotometrically (Multiskan Ascent plate reader, Thermo Labsystems) at 500 nm and the samples were compared to standard solutions of the reaction product γ-glutamylhydroxymate (0 and 1000 nmol), which had been processed identically to the samples.

To examine the direct effect of lead on GS activity, a variation of the method of (Santoro et al., 2001) was performed on lysed astrocytes. Since 10 μM lead is reported to produce direct and complete inhibition of GS (Sierra and Tiffany-Castiglioni, 1991), low concentrations of lead acetate were prepared to test the concentration-dependency of lead inhibition: 0, 2.5, 5, 7.5 and 10 μM. To allow comparison with the incubations on live cultures, lead concentrations of 33, 100 and 330 μM were also analyzed. Untreated cell culture plates were washed, frozen and brought to 37°C as described above, then lysed with 100 μl of 100 mM IHB for 5 min. One Hundred microliters of lead acetate in IHB was added to achieve the final concentrations listed above and the lysate was incubated for a further 5 min. Two hundred microliters of GS reaction mix was then applied for 30 min, with the remaining steps as described for lead-incubated cell cultures.

For cell culture and lysed cell experiments, final GS activity values were standardized against protein samples from cells or lysates incubated with equivalent lead acetate concentrations, thereby correcting for the loss of GS due to cell death. GS activity was then expressed as a percentage of the control values (the 0 μM lead acetate treatments), to provide values for specific GS activity.

H_2O_2 Clearance

In control cultures, the H_2O_2 clearance system was examined independently of lead, including conditions that partially inhibited H_2O_2 clearance for subsequent comparison to the effects of lead. 3AT completely inhibits catalase activity within 2 h, whereas BSO requires 24 h to deplete GSH to 14% of control levels (Dringen and Hamprecht, 1997). Therefore, the BSO-containing treatments were pre-incubated for 18 h with BSO in DMEM followed by a further 6 h with the addition of 3AT and/or lead acetate, as appropriate. For comparability, conditions not containing BSO received a 18 h pre-incubation in DMEM only. Similarly, to study the effect of lead on H_2O_2 clearance, lead-treated cultures were pre-incubated for 18 h with DMEM only, then incubated with 0, 10, 33, 100 or 330 μM lead acetate for 6 h, with or without the addition of 10 mM 3AT, prior to the addition of H_2O_2.

For each replication of this experiment, the incubations were carried out on three sets of cells. One set was used for the H_2O_2 clearance assay, the second set for protein estimates to standardize H_2O_2 clearance values, while the third set was used to provide a 100% cell death control condition for the LDH assay (Dringen et al., 1998).

The clearance of H_2O_2 was determined as previously described (Dringen et al., 1998). After the incubations described above, culture media was collected for the subsequent measurement of released LDH. Cells were washed with incubation buffer (IB; 20 mM HEPES, 145 mM NaCl, 0.8 mM Na_2HPO_4, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 5 mM glucose, pH 7.4) before adding a bolus of 500 μl IB containing 100 μM H_2O_2. Ten microliters samples were taken from each well after 2, 4, 6, 8, 10, 20 and 60 min and transferred to microtiter wells containing 170 μl 25 mM sulfuric acid to halt further degradation of H_2O_2. One hundred and eighty microliters of a freshly made reaction mixture (100 mM sorbitol, 25 mM H_2SO_4, 0.25 mM $(NH_4)_2Fe(SO_4)_2$ and 100 μM xylenol orange) was added to each well for 45 min. The resulting color change was proportional to the concentration of H_2O_2 remaining in the sample. Absorbances were measured at 550 nm and compared to standards of known H_2O_2 concentration.

To compare rates of H_2O_2 degradation across treatments and cultures, results were standardized against protein values, and half-times were calculated as previously described (Dringen et al., 1998). Specific detoxification rate constants (*D*-values) were calculated by taking the inverse of the product of protein content and the half-time of H_2O_2 clearance. The *D*-values represent the concentration of H_2O_2 that is cleared per minute, per mg of protein.

Statistical Analysis

Each treatment condition and time point was performed in triplicate and replicated on astrocyte cultures or lysates derived from three independently prepared cultures. Data provided in the results section represent mean ± SD. Values for GS activity in cell cultures and cell lysates were analyzed using one-way independent samples ANOVAs, with *post hoc* corrections, to determine the effect of each lead concentration on GS activity at each time point. Such analyses were also applied to data obtained from the same cultures and processed through the LDH cell death assay.

D-values calculated after H_2O_2 detoxification experiments were analyzed with one-way independent samples ANOVAs with treatment type (BSO, DMEM, BSO/3AT, 3AT, 10 μM lead/3AT, 33 μM lead/3AT, 100 μM lead/3AT, 330 μM lead/3AT, 10 μM lead, 33 μM lead, 100 μM lead or 330 μM lead) being the independent factor.

RESULTS

Effect of Lead Acetate on Specific GS Activity and Cell Viability

In cultures treated with 0–330 μM lead acetate for 2 h, no significant differences in specific GS activity were observed between the control treatment and any of the lead concentrations ($F_{(3,32)} = 0.396$, $p > 0.05$). However, after 24 h, cells treated with 100 or 330 μM lead acetate displayed a marked reduction in specific GS activity (40–50%) when compared to control cells ($F_{(3,32)} = 11.052$, $p > 0.05$; **Figure 1A**).

Cell viability was examined after incubation with lead. After 2 h, 330 μM lead acetate caused a modest yet significant increase in LDH release when compared to untreated cells and the other lead acetate concentrations ($F_{(16,19)} = 6.415$, $p < 0.05$; **Figure 1B**).

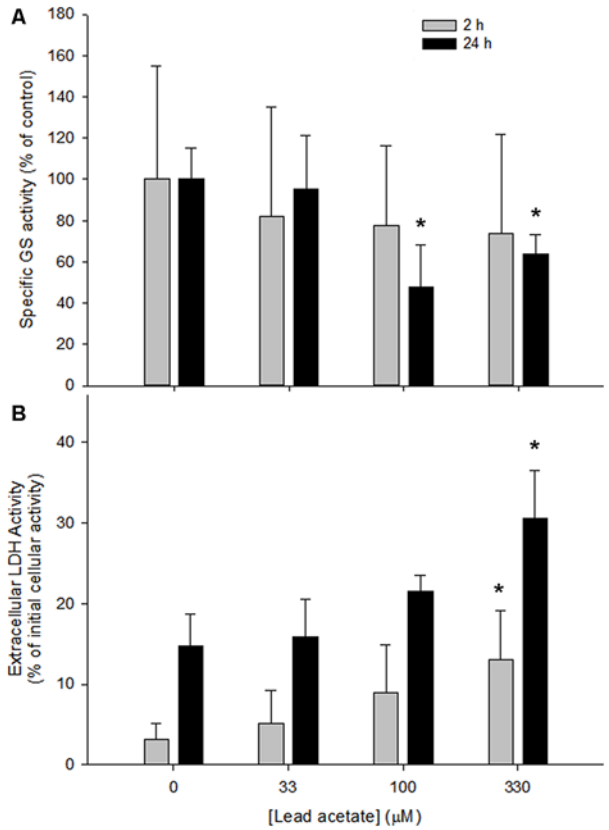

FIGURE 1 | Specific glutamine synthetase (GS) activity and cell viability in rat astrocyte cultures after 2 or 24 h incubation with four concentrations of lead acetate (0, 33, 100 and 330 μM). (A) Specific GS activity expressed as a percentage of that in untreated cells. Each bar represents mean ± SD of $n = 9$ samples. **(B)** Cell death as determined by activity of Lactate dehydrogenase (LDH) released into the media. Each bar represents means ± SD of $n = 6$ samples. *Significant differences between lead-treated and untreated cells at their respective timepoint ($p < 0.05$).

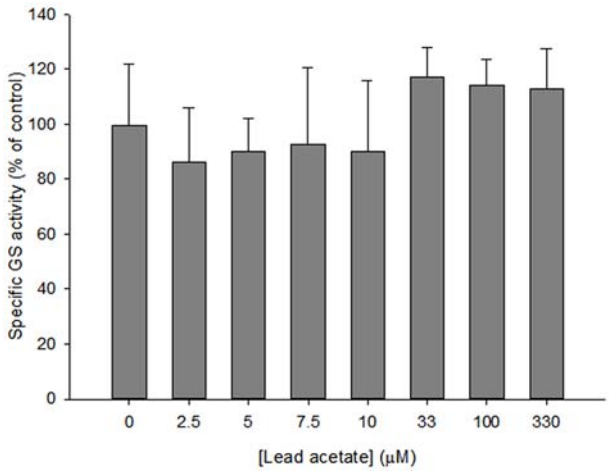

FIGURE 2 | Specific GS activity in astrocyte lysates incubated with 0–330 μM lead acetate for 35 min. Bars show means ± SD of $n = 6$ samples. No significant difference was found between control and treated lysates.

By 24 h, 330 μM lead had caused a doubling of LDH release ($F_{(18,21)} = 15.762$, $p < 0.05$; **Figure 1B**), whereas values for 33 and 100 μM lead exposure did not differ significantly from controls. The detectable activity of extracellular LDH showed a remarkable linear correspondence as a function of lead concentration at both time points examined. Thus at 2 h the correlation coefficient was $r = 0.957$ and at 24 h the correlation coefficient was $r = 0.990$.

Specific GS activity in astrocyte lysates was examined after treatment with lead acetate. Compared to controls (0 μM lead), no significant reduction of GS activity was found in lysates for any lead acetate concentration ($F_{(9,61)} = 1.714$, $p > 0.05$; **Figure 2**).

Effect of Lead Acetate on H_2O_2 Clearance by Astrocytes

The influence of lead on the capacity of astrocytes to degrade H_2O_2 was examined. The peroxide clearance curves (**Figure 3**) revealed that in all conditions investigated, except for BSO + 3AT (**Figure 3A**), all of the H_2O_2 applied was cleared within 60 min. However, the rates of peroxide clearance differed between conditions. While cultures treated with lead acetate demonstrated a slightly slower rate of peroxide clearance in the first 20 min compared with control cells (**Figure 3B**), the rates of clearance were slowed substantially when the cells had been exposed to both lead and the catalase inhibitor 3AT (**Figure 3C**), indicating an additive effect.

Analysis of specific detoxification rate constants (*D*-values; **Figure 4**), derived from the half-times of extracellular H_2O_2 degradation and the specific protein values, revealed a significant effect of the treatments ($F_{(11,87)} = 148.180$, $p < 0.05$). Exposure of astrocytes to 10 or 100 μM lead acetate for 6 h significantly slowed the rates of H_2O_2 clearance when compared to astrocytes treated without lead (**Figure 4**). Furthermore, all of the lead + 3AT treatments yielded significantly slower rates of H_2O_2 detoxification than treatment with 3AT alone, but faster rates than those observed after treatment with BSO + 3AT (**Figure 4**). The *D*-values of astrocytes treated with different lead concentrations did not differ significantly from each other (**Figure 4**, columns 2–5). Therefore, increasing lead concentrations do not cause greater impairment of peroxide clearance. Under the conditions investigated, the lowest lead concentration applied (10 μM) caused maximal effects on peroxide clearance in both the presence and the absence of 3AT.

Toxicity of Treatments with H_2O_2

Prior to treatment with a bolus of H_2O_2, the amount of LDH released by cultures exposed to lead and/or to 3AT was within the normative range for all treatments (less than 10% LDH release), indicating that the treatments did not cause significant cell death (**Figure 5A**). However, after 60 min incubation with 100 μM H_2O_2, LDH levels were

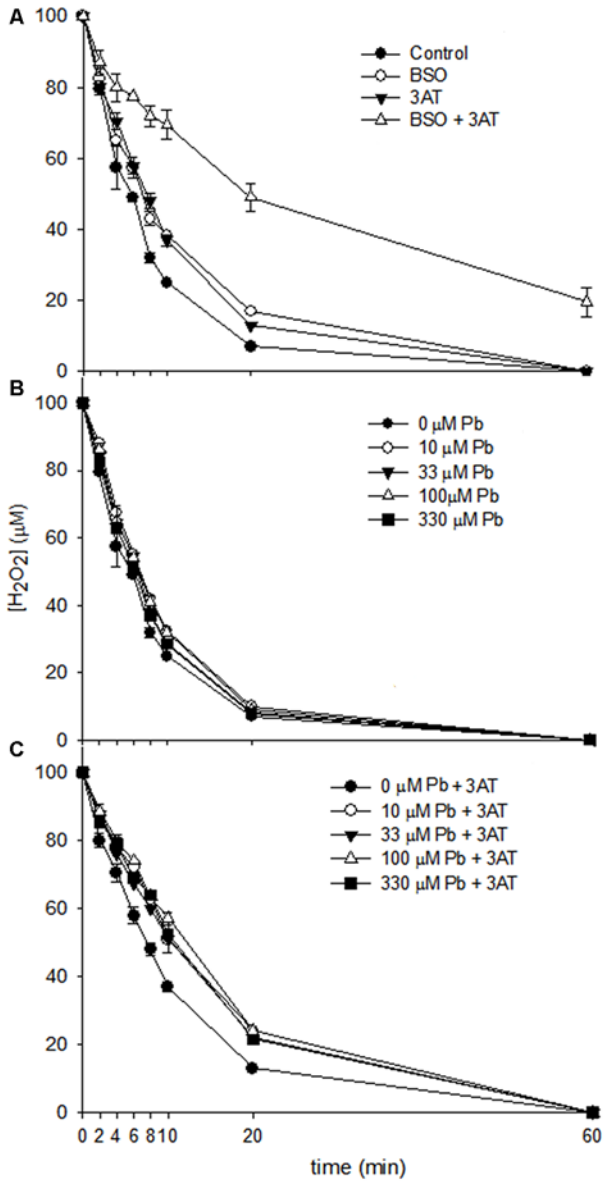

FIGURE 3 | Clearance of H_2O_2 by rat astrocyte cultures. Cells were incubated for 60 min with 500 µl of 100 µM H_2O_2, and media were collected at the specified time points for measurement of H_2O_2 concentration. **(A)** Dulbecco's modified eagle medium (DMEM) only control, 1 mM Buthionine sulfoximine (BSO), 10 mM 3AT or 1 mM BSO + 10 mM 3AT. **(B)** 10–330 µM lead acetate. **(C)** 10–330 µM lead acetate + 10 mM 3AT. Each data point represents mean ± SD of $n = 9$ samples.

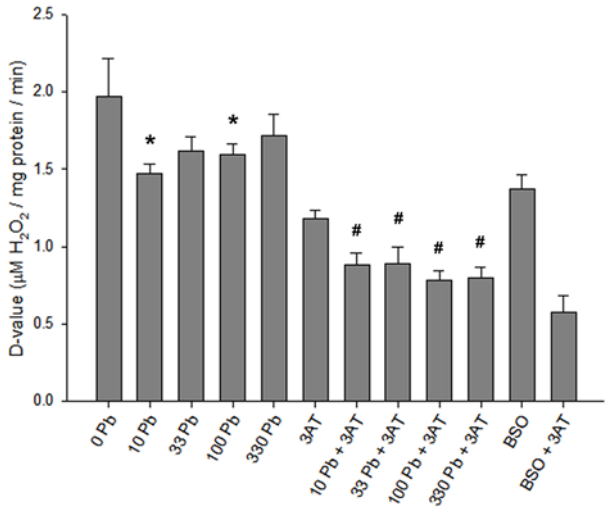

FIGURE 4 | *D*-values for H_2O_2 clearance in rat astrocyte cultures. Standardized H_2O_2 clearance values were derived from H_2O_2 half-times. The higher the *D*-values, the faster the rate of detoxification. Incubation conditions were the same as in **Figure 3**. Treatment concentrations: 1 mM BSO, 10 mM 3AT; concentrations for lead (Pb) are in µM. Statistical analyses were performed on lead-treated conditions. Significant differences ($p < 0.05$) between lead-treated and untreated control are represented by *. Significant differences ($p < 0.05$) between lead + 3AT-treated and 3AT-treated controls are represented by #. Each bar represents a mean ± SD of $n = 9$ samples, except for those treated with 10 µM lead where $n = 6$.

elevated in all conditions (**Figure 5B**). When these levels were analyzed, a one-way ANOVA indicated a significant effect of treatment ($F_{(11,90)} = 12.052$, $p < 0.05$). Dunnett's T3 *post hoc* analyses demonstrated that none of the lead treatments significantly increased the extent of cell death compared to the respective control condition ($p > 0.05$). However, after 60 min incubation with H_2O_2, the BSO-treated group demonstrated a significant increase in LDH release, both in the presence of 3AT ($T3 = 7.483 + 1.073$, $p < 0.05$) and in the absence of 3AT ($T3 = 6.207 + 0.930$, $p < 0.05$; **Figure 5B**).

DISCUSSION

While lead intoxication is known to impair GS activity, uncertainty exists regarding the basis of this impairment. It is possible that lead binds to the active site of GS inhibiting it directly, or the inhibition of lead may be a downstream event, secondary to inactivation of the glutathione system. The present study conducted experiments to discriminate between these alternatives. It has been shown that low concentrations of lead are capable of reducing GS activity in astrocytes that have been cultured with lead for a week or more (Engle and Volpe, 1990; Sierra and Tiffany-Castiglioni, 1991). The present study investigated whether astrocytes incubated with lead for shorter periods (2 or 24 h) also show an altered GS activity. Our results revealed that the effect of lead on specific GS activity in cultured astrocytes is dose- and time-dependent, with 100 and 330 µM lead acetate significantly reducing GS activity following 24 h incubation, but not after 2 h. The slowness of this effect is inconsistent with a direct inactivation of GS by lead. For instance, direct inhibitors of GS such as methionine sulfoximine can inhibit GS activity in intact tissue within minutes (Barnett et al., 2000).

Previous studies have speculated that lead might bind to cysteine residues on GS, thereby directly impairing the function

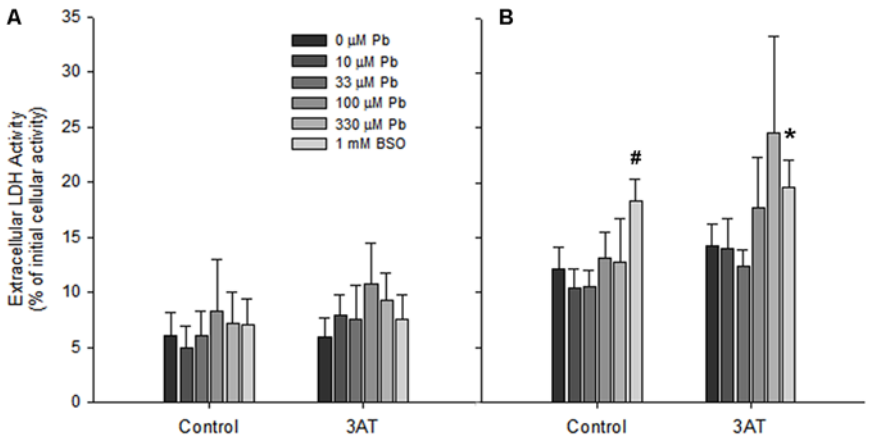

FIGURE 5 | Cell viability in rat astrocyte cultures before and after 100 µM H_2O_2 treatment. Incubation conditions were the same as in **Figure 3**. **(A)** Extracellular LDH immediately before application of 100 µM H_2O_2. **(B)** Extracellular LDH 60 min after application of 100 µM H_2O_2. Significant differences ($p < 0.05$) between treatments and the untreated controls are represented by # for the no-3AT control group, and by * for the 3AT group. Each bar represents a mean ± SD of $n = 9$ samples, except for those treated with 10 µM lead where $n = 6$.

of this enzyme (Sierra and Tiffany-Castiglioni, 1991; Tang et al., 1996). This possibility was investigated in the present study by lysing astrocyte cultures to release their GS and subsequently incubating the lysates with lead for 35 min. It was expected that the inhibitory effect of lead would be rapid and would require lower concentrations than those effective on living astrocytes, which require time to accumulate lead. The present results, however, demonstrated that lead does not inhibit GS activity in cell lysates. Regardless of the concentration of lead used, GS activity did not differ from control lysates. These results are inconsistent with an earlier report (Sierra and Tiffany-Castiglioni, 1991) that direct application of 10 µM lead is sufficient to maximally inhibit the activity of GS in astrocyte lysates within 30 min. Those researchers used a radio ligand assay for GS activity, whereas we used a colorimetric assay, testing for an enzymatic side reaction of GS which appears to be unaffected by lead. Regardless of the reason for this discrepancy, the present results imply that the inhibition of GS is not caused by lead binding to cysteine residues at the active site of the enzyme, and instead may be an indirect consequence of a secondary mechanism.

While studies have noted that lead toxicity is accompanied by oxidative stress, there is uncertainty regarding how lead promotes this, since lead itself is not redox-active. Much of the evidence points to the fact that lead has an affinity for sulfhydryl groups and hence may bind readily to GSH and enzymes in the GSH system (Patrick, 2006; Rubino, 2015), thereby reducing the cellular capacity to clear H_2O_2 (Tang et al., 1996). For example, the blood and tissues (including brain) of mice exposed to toxic concentrations of lead demonstrate significantly lower levels of endogenous GSH (Penugonda and Ercal, 2011). The results of the present study indicate that when compared to lead-free controls, lead acetate decreased H_2O_2 clearance rates, and that the extent of slowing was about half that caused by the GSH synthesis inhibitor BSO, regardless of the presence or absence of 3AT in the system. These observations are consistent with the hypothesis that lead targets the GSH system by affecting the synthesis and regeneration of GSH. By slowing the rate of detoxification, metabolically-produced H_2O_2 will remain in cells for a longer period, giving it an increased potential to cause oxidative stress and to oxidize GS. It should be noted however, that GS activity in astrocytes is unaffected by the presence of H_2O_2 alone; inactivation of GS requires the presence of iron (Fernandes et al., 2011).

Compounds that block GS activity cause profound memory impairments and cognitive deficits (e.g., Gibbs et al., 1996; reviewed by Robinson, 2000). The glutamate-glutamine cycle can be interrupted by inhibiting astrocytic GS with methionine sulfoximine (Pow and Robinson, 1994). The subsequent depletion of glutamate stores and interruption of glutamatergic neurotransmission occurs with surprising rapidity. For instance, 40 mM doses of methionine sulfoximine delivered to the retina of rats results in blindness within 2 min, which can be reversed by the administration of glutamine (Barnett et al., 2000). Similarly, delivery of methionine sulfoximine into the hyperstriatum of chicks prevents memory consolidation, an effect that is reversed by the administration of glutamine, demonstrating the essential contribution of astrocytic GS to learning and the consolidation of memories (Gibbs et al., 1996; Hertz et al., 1996; Gibbs and Hertz, 2005). In rats the inhibition of GS impairs the temporal component of memories (Kant et al., 2014).

Verbal and nonverbal memory is impaired in individuals who have been chronically exposed to lead, with the greatest deficits being evident in children, particularly with respect to their global intelligence and capacity to learn (Mason et al., 2014). The effects of lead intoxication on memory have been amply demonstrated in animal models (Kuhlmann et al., 1997;

Barkur et al., 2011). While neurones are sensitive to the toxicity of lead, they preferentially accumulate it in lysosomes, whereas astrocytes accumulate lead at a higher rate than neurones and concentrate it in the nucleus and throughout the cytoplasm (Holtzman et al., 1987). The results of the present study support the conclusion that the impairment of learning and memory in lead intoxication is due to the inactivation of astrocytic GS. Since the inactivation of GS by lead appears to be a secondary consequence of inhibition of the GSH system, it might be possible to reduce the effects of lead on cognition by providing supplements that boost the activity of the GSH system. Indeed, (Penugonda and Ercal, 2011) have shown that when lead-intoxicated rats are provided with N-acetylcysteine, a precursor of GSH, the depletion of GSH in the brain and other tissues is reversed. However, it remains to be shown whether such supplements can lessen the deficits in learning and memory caused by lead intoxication in rats and humans.

AUTHOR CONTRIBUTIONS

SRR designed and supervised the research project, reviewed the data analysis and was the lead author of the manuscript. AL undertook the experiments, collected and analyzed the data and drafted an early version of the manuscript. GMB supervised the research, assisted with data analysis and drafting of the manuscript. HC trained AL in the analytical techniques used, assisted with the preparation of figures and drafting the manuscript. RD provided technical advice and assisted with drafting the manuscript.

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REVIEW
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Role of Glia in Stress-Induced Enhancement and Impairment of Memory

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Both acute and chronic stress profoundly affect hippocampally-dependent learning and memory: moderate stress generally enhances, while chronic or extreme stress can impair, neural and cognitive processes. Within the brain, stress elevates both norepinephrine and glucocorticoids, and both affect several genomic and signaling cascades responsible for modulating memory strength. Memories formed at times of stress can be extremely strong, yet stress can also impair memory to the point of amnesia. Often overlooked in consideration of the impact of stress on cognitive processes, and specifically memory, is the important contribution of glia as a target for stress-induced changes. Astrocytes, microglia, and oligodendrocytes all have unique contributions to learning and memory. Furthermore, these three types of glia express receptors for both norepinephrine and glucocorticoids and are hence immediate targets of stress hormone actions. It is becoming increasingly clear that inflammatory cytokines and immunomodulatory molecules released by glia during stress may promote many of the behavioral effects of acute and chronic stress. In this review, the role of traditional genomic and rapid hormonal mechanisms working in concert with glia to affect stress-induced learning and memory will be emphasized.

Keywords: glia, memory, stress, norepinephrine, hippocampus, glucocorticoids

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INTRODUCTION

Over the past several years, consensus on the role of glia in cognitive function has shifted from viewing them as primarily supportive of neuronal actions to recognizing them as critical players in neural function and behaviors such as learning and memory (Fields et al., 2014) (**Figure 1**). Astrocytes exert a variety of influences on neural processes critical for memory, including regulation of extracellular K^+ concentration, provision of metabolic support (i.e., glucose and/or lactate) to neurons, and recycling of glutamate and GABA (Moraga-Amaro et al., 2014). Ramified (previously known as "resting") microglia can control synaptic plasticity through release of several different cytokines that can modulate memory (Morris et al., 2013). An expanding field of research demonstrates that other types of glia, such as oligodendrocytes, can also influence processes underlying learning and memory (Fields et al., 2014). Conversely, stress can have a profound impact on glial structure and function (particularly astrocytes and microglia), which may affect the glial contribution to learning and memory. The impact of stress and stress-associated molecules, such as glucocorticoids (GCs) and norepinephrine (NE), on glial function has been the target of substantial

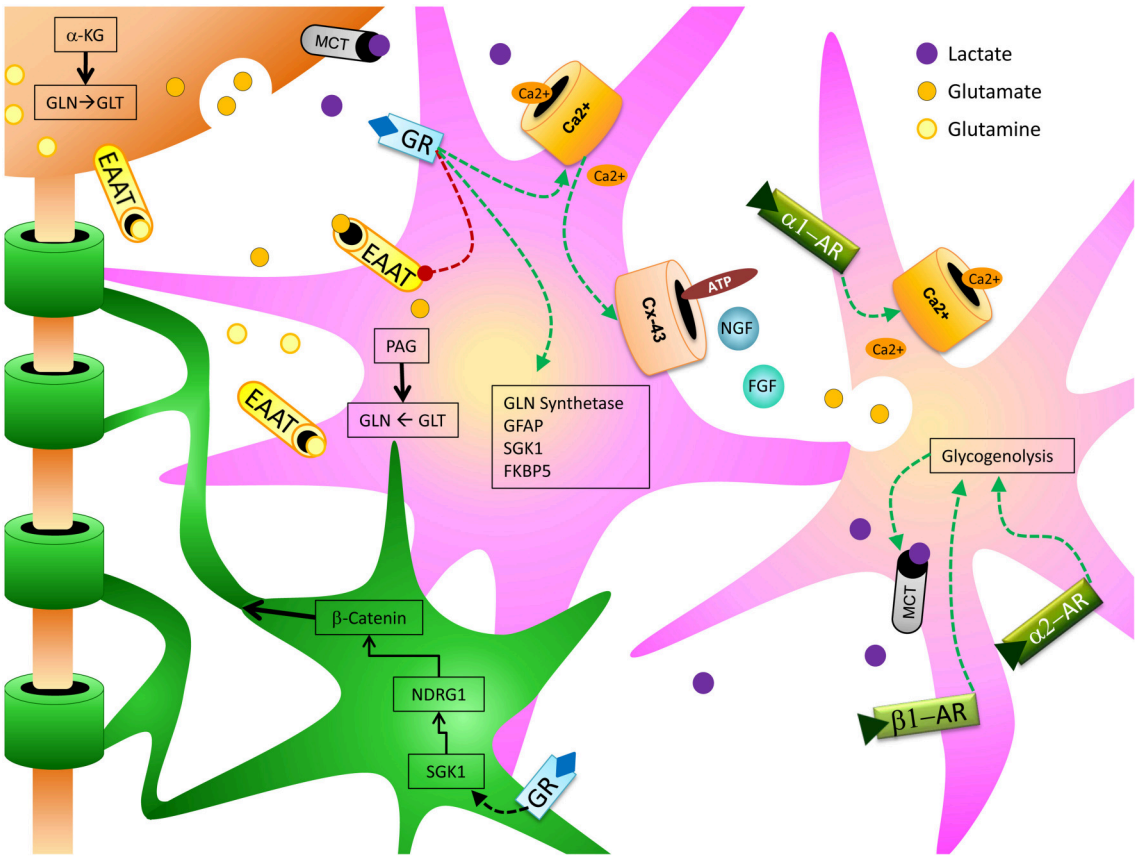

FIGURE 1 | Depicts the relationship between stress hormone effects on astrocytes (pink) and oligodendrocytes (green) and how they can support and enhance neuronal (orange) function to produce cognitive enhancing effects.

investigation (Jauregui-Huerta et al., 2010). Many of the effects that glia exert on memory following stress may be mediated through neuroinflammatory processes (Frank et al., 2012), and the interaction between glia and stress hormones is likely to be bidirectional: many neuroimmune molecules released by glia, such as interleukin-1 (IL-1) and IL-6, can activate the hypothalamic-pituitary-adrenal axis (HPA) (Dinan and Cryan, 2012).

This review focuses on the specifics of interactions between stress hormones and glia, and the impact that these interactions have on learning and memory primarily via actions in the hippocampus.

STRESS AXIS OVERVIEW

The HPA axis is the canonical regulatory system for stress hormones. Immediately following exposure to a stressor, epinephrine is secreted from the adrenal medulla and triggers a central stress response via stimulation of NE release from the locus coeruleus (LC) to multiple areas of the brain, including the hippocampus (Womble et al., 1980; Wong et al., 2012). NE in the paraventricular nucleus (PVN) then prompts the release of corticotropin releasing hormone (CRH). CRH and vasopressin promote secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary via pro-opiomelanocortin (Petrov et al., 1993). ACTH initiates the synthesis of GCs for release by the adrenal cortex. Whereas NE acts rapidly in the brain following release from the LC, GCs do not typically reach the brain for up to 10 min (Barbaccia et al., 2001), at which point they exert both signal transduction- and genomic-based effects in the brain (Salehi et al., 2010); these include feedback signaling in the hippocampus and other brain regions to negatively regulate the HPA axis and hence limit the neural impact of stress (Liberzon et al., 1999).

IMPORTANCE OF STRESS TO LEARNING AND MEMORY

Acute stress can facilitate formation of highly salient and long-lasting memories, which in extreme cases may form the basis of post-traumatic stress disorder (Oitzl and de Kloet, 1992; Sandi and Rose, 1994a,b, 1997; Roozendaal et al., 1996; Sandi et al., 1997; Oitzl et al., 2001; Lupien et al., 2002a,b; Cahill and Alkire, 2003; Cahill et al., 2003). Increases in NE and GCs, either independently or to a greater extent together, act to increase integration of input from several regions [e.g., basal lateral

amygdala (BLA), LC, cortex, PVN] whose outputs directly or indirectly converge on the hippocampus (de Kloet et al., 2005). Released early in the stress response, NE actions in the BLA are particularly important for enhanced memory consolidation following exposure to acute stress (Ferry and McGaugh, 1999; Hatfield and McGaugh, 1999; LaLumiere et al., 2003; Lalumiere and McGaugh, 2005). BLA lesions attenuate the procognitive effects of NE (Liang and McGaugh, 1983; Liang et al., 1990; Roozendaal and McGaugh, 1996), and administration of adrenergic antagonists, particularly β-adrenergic receptor (AR) antagonists to the amygdala (i) produces similar attenuation of stress enhanced memory and (ii) prevents GC-mediated increases in hippocampal-dependent learning (Liang et al., 1986; Quirarte et al., 1997; Roozendaal et al., 1999). Compared to the actions of NE in the BLA, direct effects of NE in the hippocampus are not as clear, although infusions of NE to the hippocampus can improve contextual fear learning (Yang and Liang, 2014). Again these effects appear to be dependent on β-AR activation, as propranolol administration prior to or following training diminished contextual fear performance (Stuchlik et al., 2009; Kabitzke et al., 2011).

The effects of elevated GCs on learning and memory are more complicated and time/dose sensitive than those of NE. Both mineralocorticoid (MRs) and glucocorticoid receptors (GRs) are present throughout the brain, and the hippocampus has the highest level of receptor co-localization (Sarrieau et al., 1984; Reul and de Kloet, 1985, 1986; Van Eekelen et al., 1988; Herman et al., 1989; Decavel and Van den Pol, 1990; Funder, 1994; Cullinan, 2000; Reul et al., 2000a,b; Barbaccia et al., 2001); consistent with this, GCs are potent modulators of hippocampal memory processes. Stress-mediated rises in GC levels following learning can improve memory formation, but proximate to recall GCs may impair memory retrieval (Oitzl and de Kloet, 1992; Kirschbaum et al., 1996; Sandi and Rose, 1997; Oitzl et al., 2001; Roozendaal, 2002; Joëls, 2006). GCs can also mask the mnemonic effects of NE when administered prior to NE (Borrell et al., 1984; Joels and de Kloet, 1989; Roozendaal, 2003; Richter-Levin, 2004). The effects of GCs on memory processing vary not only with the temporal relationship of increases in GCs to the event being remembered, but also according to the level of GC increase. The impact of GCs on the hippocampus (particularly CA1) follows an inverted-U-shaped dose-response curve (Joëls, 2006; Polman et al., 2013). Removal of GCs by adrenalectomy results in impaired consolidation, an effect that can be rescued by administering moderate doses of GCs (Joëls, 2006; Spanswick et al., 2011), so that low levels of GCs appear to mediate not only procognitive effects of moderate stress but also the formation of memories under baseline, non-stressed conditions. Moderate, physiological increases in GCs can improve cognitive processes, but very high elevations in GCs acutely impair hippocampal function (Salehi et al., 2010). Similarly, very high and/or prolonged GC exposure markedly impairs subsequent hippocampal function with results including cognitive deficits, hippocampal atrophy, metabolic dysfunction, and central insulin resistance (Sapolsky et al., 1985; Sapolsky, 1996; Willi et al., 2002; Joëls et al., 2004; Stranahan et al., 2008; Karatsoreos et al., 2010; Yun et al., 2010; Ye et al., 2011; Reagan, 2012).

Astrocytes are the most widely studied glial cell-type with regard to both memory processes and the effects of stress. It has been known for decades that chronic stress is associated with a decrease in hippocampal and prefrontal cortex volume (Fuchs and Flügge, 2003). More recent studies have shown that much of the brain volume reduction caused by chronic stress is accounted for by a large decrease in astrocytes, rather than neurons (Rajkowska and Miguel-Hidalgo, 2007). Similar outcomes of stress, and of elevated GCs in particular, have been observed in animal models: short-term stress increasing astrocyte volume (as measured by GFAP immunoreactivity), while chronic stress decreases astrocyte volume (Lambert et al., 2000; Jauregui-Huerta et al., 2010).

GLUCOCORTICOID-SPECIFIC ASTROCYTIC ACTIONS: STRESS AND MEMORY

Astrocytes can influence memory in a variety of ways including (i) control of glutamate reuptake, synthesis and metabolism, (ii) regulation of calcium dynamics, (iii) large-scale coordination of neural activity via release of gliotransmitters, (iv) regulation of blood flow and hence glucose supply, and (v) provision of lactate as a metabolic substrate for neurons (Sahlender et al., 2014). Both GRs and MRs are expressed by astrocytes, and GCs have potent effects on astrocytic function (Jauregui-Huerta et al., 2010). Hence, it follows that GC-mediated modulation of astrocyte activity would influence cognitive processes. Here, we will focus on the known impact of GCs on memory processes mediated by astrocytes.

Astrocytes play a key role in regulating glutamate metabolism and activity. Following neural release of glutamate into synapses, astrocytes can remove glutamate through glial-specific glutamate transporters and convert glutamate into glutamine. Glutamine can then be used as an energy substrate in astrocytes or exported to neurons, where it can be re-converted into glutamate (Schousboe et al., 1993). Many of the long-term effects of chronically elevated GCs have been linked to excitotoxicity; GC-mediated dysfunction in astrocytes may prevent optimal glutamate clearance and therefore promote excitotoxicity (Popoli et al., 2012). Moreover, GC-induced dysfunction of astrocytes may also affect calcium metabolism and regulation, which will also impair glutamate regulation and thus increase the risk of excitotoxicity. Indeed, an important candidate mechanism for transduction of mnemonic regulation by astrocytes is calcium signaling. Regulation of calcium release and sequestration is also affected by GCs and stress. In both neurons and astrocytes, GCs control calcium homeostasis and signaling (Simard et al., 1999; Chameau et al., 2007; Suwanjang et al., 2013). Activated GRs can increase mitochondrial buffering capacity, leading to a reduction in cytosolic calcium (Psarra and Sekeris, 2009), and GCs can increase astrocytic calcium waves (Simard et al., 1999). These effects feed back into regulation of glutamate, discussed above: astrocytic calcium signaling controls release of glutamate (Volterra and Meldolesi, 2005). Calcium influx in astrocytes promotes release of gliotransmitters, which can

include amino and nucleic acids, ATP, growth factors, glutamate, and/or peptides (Parpura et al., 2010, 2011). Gliotransmitters have been associated with regulation of the multipartite synapse, where they regulate neural excitability and can hence modulate memory processing (Hassanpoor et al., 2014). Inhibition of gliotransmitter release by blocking Cx-43 hemichannels (which release gliotransmitters) in astrocytes prevents the formation of long-term fear memories (Stehberg et al., 2012).

Stress can affect a variety of growth factors involved in learning and memory. For example, nerve growth factor (NGF) and fibroblast growth factor (FGF) are both increased following exposure to GCs or stress (Mocchetti et al., 1996; Molteni et al., 2001; Gubba et al., 2004; Chang et al., 2005; Kirby et al., 2013; Hashikawa et al., 2015). FGF in particular is critical for homeostatic regulation of astrocytes, and changes in FGF are largely responsible for the transition of astrocytes from a nonreactive to a reactive state (Kang et al., 2014a,b). These findings are intriguing because NE signaling can increase astrocyte reactivity (Griffith and Sutin, 1996); thus suppression of astrocyte reactivity by GC-induced release of FGF may moderate increased overall astrocyte reactivity following prolonged sympathetic activation. NGF has many roles, not limited to effects on glia; these include increasing both survival and differentiation of newly-maturing neurons and supporting hippocampal-dependent memory processes and cholinergic signaling (Chao, 2003; Mufson et al., 2003; Capsoni and Cattaneo, 2006; Schindowski et al., 2008; Aboulkassim et al., 2011).

GRs in the nucleus act as a transcription factor. Because different cell types have unique transcriptomes, it is worth discussing how GRs specifically influence gene transcription in astrocytes. In mature astrocytes GCs have been found to regulate a specific subset of genes that differs from that regulated in neurons. Early work demonstrated that transcription of glutamine synthetase and glial fibrillary acidic protein, both astrocyte-specific, is under GC-mediated control (Nichols et al., 1990; Laping et al., 1994), suggesting that both metabolic function and morphology of astrocytes can be influenced by GCs at the level of mRNA. Of the numerous genes affected by GCs in hippocampal astrocytes, some stand out as playing a prominent role in cognition and are under differential control by GCs based on dose/duration of exposure (Carter et al., 2013). Acute exposure to GCs, at doses sufficient to activate GRs, results in significantly increased astrocytic mRNA levels of adenosine 2b receptor, FK506 binding protein (FKBP5), pyruvate dehydrogenase kinase 4, and serum/glucocorticoid-inducible kinase-1 (Sgk1), while significantly decreasing early growth response protein 2 (Egr2) and wingless-related MMTV integration site 7a (Wnt7a). In contrast, chronic exposure to GCs produced effects that in some cases are opposite of the acute effects. Chronic GCs decrease hippocampal RNA expression of growth associated protein 43 (Gap43), histone deacetylase 7 (Hdac7), and synapsin II. Additionally, chronically elevated GCs decrease adenosine receptor 2b and Sgk1, while the effects of acute vs. chronic are the same for FKBP5 and Wnt7a. Similar effects were observed in the cortex, demonstrating that acute and chronic exposure to GCs have different effects on astrocytic gene expression (Carter et al., 2012, 2013) including that of several genes whose products are important in memory processing: in general, these changes are consistent with enhancement of memory processing after acute elevation in GCs but impairment after chronic GC elevation. Moreover, prolonged elevations in GCs can lead to a reduced number of astrocytes (Unemura et al., 2012). Given the data discussed in this review showing the importance of astrocytes in mediating neural processes critical for memory, it is likely that changes in gene expression and a reduction in astrocyte quantity and function following prolonged stress and GC exposure may underlie some of the cognitive deficits observed following long-term stress.

As noted, many of the astrocytic genes changed by GCs have products that are involved in memory processing. FKBP5 is particularly interesting as it can control stress reactivity (Schmidt et al., 2015). FKBP5 is a chaperone protein required to shuttle GRs to the nucleus (Binder, 2009). It has also been heavily implicated in early life stress programming, epigenetic regulation of stress responding (Klengel et al., 2013), and susceptibility to chronic stress later in life (Hartmann et al., 2012; Guidotti et al., 2013; Radley et al., 2013). Patients with PTSD have decreased FKBP5 expression, and successful cognitive-behavioral therapy in PTSD patients increases FKBP5 expression and hippocampal volume (Levy-Gigi et al., 2013). It is unclear, however, whether many of these effects of FKBP5 are mediated through glia or neurons. Sgk1 has an ever-growing body of evidence to indicate that it is integral to GC effects to enhance cognition, which includes actions to activate CREB and increase AMPAR and NMDAR receptors at the plasma membrane (Strutz-Seebohm et al., 2005; Yang et al., 2006; Lee et al., 2007; Tai et al., 2009; Lang et al., 2010). Currently, it is unclear as to whether the pro-cognitive activities of Sgk1 are mediated by neurons or astrocytes. Future research should examine the specific role of Sgk1 in astrocyte function given the robust increase in astrocytic Sgk1 following both acute and chronic GCs (Carter et al., 2013).

A further mechanism by which astrocytes may contribute to learning and memory is via metabolism of glucose leading to export of lactate, which will both regulate cerebral blood flow and potentially provide metabolic support to active neurons (Iadecola and Nedergaard, 2007). Some data suggest that acquisition of long-term fear memories may require astrocytically-derived lactate (Suzuki et al., 2011). Intriguingly, it is unknown as to what extent stress-induced molecules such as GCs can affect efficacy of lactate export from astrocytes. Astrocytes form a functional unit with neurons and blood vessels, which has been referred to as the neurovascular unit (Iadecola and Nedergaard, 2007). Many neuroinflammatory molecules that are released during stress are known to affect the neurovascular unit, and can increase "leakiness" of the blood-brain barrier (Kröll et al., 2009). In the amygdala, GCs produced by chronic stress can impair efficacy of the neurovascular unit by preventing vasodilation in response to neural activity (Longden et al., 2014). It is currently unknown whether neurovascular units in other brain regions respond similarly/differently to that of the amygdala, but such effects are likely to markedly impact cognitive processing. As a side note, such effects are also important to consider when interpreting

fMRI or PET studies in which patients may be stressed, which is likely to be the case in the majority of such studies.

Overall, GCs exert a variety of effects on astrocytic function, from acute effects via calcium dynamics and release of gliotransmitters to transcription-mediated events, neurovascular control, and regulation of glutamate metabolism and glucose flux.

ASTROCYTE-SPECIFIC NORADRENERGIC ACTIVITIES

Using rodents and chicks, respectively, Dr. Leif Hertz's and Dr. Marie Gibbs' groups have elucidated many roles for noradrenergic signaling in regulating astrocyte function and metabolism (Huang and Hertz, 1995; Hertz et al., 2007, 2010; Gibbs and Bowser, 2010). While most of this work has been aimed at unraveling cognitive aspects of astrocytic function under baseline conditions, these data can likely be extended to permit speculation on the impact of LC activation and subsequent NE release on cognitive function during stress.

Noradrenergic cell bodies are primarily located within the LC and project throughout the cerebral cortex, limbic system, and cerebellum (Swanson and Hartman, 1975), with prefrontal cortical and hippocampal regions receiving large amounts of noradrenergic innervation (Gibbs et al., 2010). A simplified primary function of LC-activation during stress may be to "boost brain power" and direct cognitive processes toward enhanced attention, improved vigilance, and a shift in memory processing toward retrieval of information relevant to the stressor (O'Donnell et al., 2012). NE may promote some of these cognitive effects via astrocytic release of glutamate, lactate production and transport to active neurons, and an increase in both glycogen metabolism and gliotransmitter release (O'Donnell et al., 2012; Moraga-Amaro et al., 2014): overall, one major effect is to increase glucose metabolism to meet the demands of cognitive processing. Hippocampal memory processes are well-established to be limited by glucose availability, and the procognitive effects of exogenous glucose administration have been suggested to be via glia rather than neurons (McNay and Gold, 2002).

NE acts via G-protein coupled receptors; α- and β- type 1 and 2 adrenergic receptors (ARs). Astrocytes primarily express β1, α1, and α2 (Hertz et al., 1984, 2010; Deecher et al., 1993). Like GCs, NE regulate astrocytic calcium signaling (Gibbs and Bowser, 2010): administration of the α1 agonist phenylephrine increases intracellular Ca^{2+}, and similarly stimulation of the LC increases intracellular astrocytic Ca^{2+} via an α1-dependent mechanism (O'Donnell et al., 2012). Because NE activation during stress precedes GC activation, it is possible that the reduction in intracellular calcium and increased mitochondrial calcium buffering induced by GCs acts to control increases in intracellular Ca^{2+} caused by NE signaling to prevent overexcitation and/or apoptotic events.

NE also has effects on astrocytic metabolism, and effects of both α-receptor and β-receptor signaling on astrocytic metabolism have been documented (Subbarao and Hertz, 1990, 1991; Hutchinson et al., 2007, 2008; Gibbs et al., 2008). Activation of α2 receptors on astrocytes can both promote glycogen storage and increase astrocytic glycogen breakdown (Hertz et al., 2007). Astrocytic β-receptor-mediated glycogenolysis is more effective than α-receptor mediated glycogenolysis (Subbarao and Hertz, 1990). Astrocytic glycogenolysis is critical for glutamate cycling, as well as providing lactate that may be especially important as a rapidly-utilizable energy source during cognitive demand. Both α1- and α2 adrenergic signaling in astrocytes can increase oxidative metabolism including lactate production (Subbarao and Hertz, 1991). NE can also increase glutamine uptake in astrocytes, which is another important energy metabolite in astrocytes (Huang and Hertz, 1995). Because energy provision is a rate-limiting step in neural activity, the several actions of NE to increase metabolic support for both astrocytes and neurons are likely key to producing increased strength of memories formed at times of moderate stress (Osborne et al., 2015). The specificity of NE receptor subtypes found on astrocytes offers a potential target for therapies aimed at specifically modulating glial responses to stress, including treatments for stress-related disorders.

MICROGLIAL IMPACTS ON COGNITION

No longer regarded as solely an immune cell of the brain, several studies show that microglia are also involved in regulating neural activity (Thomas, 1992; Ilschner et al., 1996; Kettenmann, 2007), primarily through release of neuroimmune molecules such as cytokines that modulate surrounding neurons and astrocytes (**Figure 2**). Chronic unpredictable stress (CUS) promotes an increase in microglial proliferation and activation in a variety of brain regions including the hippocampus; however, following 5 weeks of CUS exposure microglial activity declines below baseline (Kreisel et al., 2014). Treatment with a variety of microglia-activating molecules such as endotoxin, macrophage colony-stimulating factor, or granulocyte-macrophage colony stimulating factor can prevent CUS-induced depressive behaviors (Kreisel et al., 2014), suggesting that microglia may be key regulators of adaptation to chronic stress.

Interleukin-1β (IL-1β) is perhaps the best-characterized cytokine released in response to stress. Following chronic mild stress, mice have impaired memory on both object location and object recognition memory. Cognitive impairments are accompanied by increased plasma IL-1β, plasma tumor necrosis factor-alpha, and IL-6 (Li et al., 2008). After chronic mild resident-defeat stress, a subset of rats that develop pro-depressive behaviors shows increased brain IL-β expression, and inhibition of brain IL-1β signaling prevents the pro-depressive symptoms (Wood et al., 2015). Independent of stress, studies have reported that IL-1β can impair memory, have no effect on memory, or enhance memory (Ross et al., 2003; Goshen et al., 2007; Huang and Sheng, 2010; Ben Menachem-Zidon et al., 2011; Bitzer-Quintero and González-Burgos, 2012; Pascual et al., 2012; Arisi, 2014; Jones et al., 2015), indicating that further work is needed.

Other microglial-released cytokines and immune molecules, such as IL-6 and TNF-α, can affect memory (Tonelli and Postolache, 2005; Nelson et al., 2013; Williamson and Bilbo, 2013; Arisi, 2014; Grinan-Ferre et al., 2015; Smith et al., 2015). In general, effects of cytokines are highly dependent on timing,

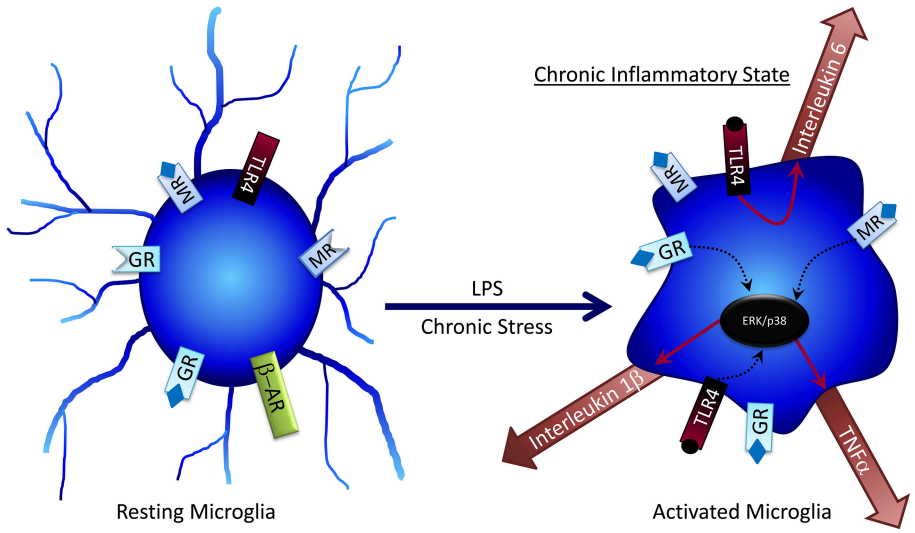

FIGURE 2 | Microglia can become activated by factors such as LPS or chronic stress, where they become desensitized to the anti-inflammatory effects of glucocorticoids. Activated microglia can release pro-inflammatory cytokines, which at high concentrations can have negative effects on cognitive processes.

dose, and duration; but the potentially confounding effects of inflammation can make interpretation difficult and are a major contributing factor to every major neurological disease (Bibi et al., 2014; Daulatzai, 2014; Legido and Katsetos, 2014; Nisticò et al., 2014; Stuart and Baune, 2014; Patterson, 2015; Walker and Lue, 2015). Chronic stress, poor diets, and acute traumatic stress can all induce elevated cytokine release, negatively affecting learning outcomes (Boitard et al., 2014; Hsu et al., 2015; Jones et al., 2015; Yazir et al., 2015).

Important to discuss, as a component of microglial effects following stress, is the kynurenine pathway (KP). Following chronic stress, tryptophan can be directed toward the KP (Miura et al., 2008), primarily driven by cytokine induction of indoleamine 2,3,-dioxygenase (IDO). In astrocytes, the KP increases production of the NMDA receptor agonist kynurenic acid; in microglia, the KP increases production of the NMDA receptor agonist quinolinic acid (Jo et al., 2015). Given the key role of NMDA receptors in hippocampal memory processing, it is not surprising that recent data suggests that activation of the KP can affect memory processes, and thus may be a novel pathway with importance for stress-related memory dysfunction (Heisler and O'Connor, 2015; Varga et al., 2015).

MICROGLIA AND GLUCOCORTICOIDS

GCs have well-established anti-inflammatory effects that decrease microglial activation. GCs provide master control over several inflammatory and anti-inflammatory factors (**Figure 3**). Microglia express both MRs and GRs (Sierra et al., 2008), and GCs can suppress central inflammation through microglia (Goujon et al., 1996; Kawai and Akira, 2010). GC administration to microglial cultures suppresses nitric oxide release by blocking the expression of inducible nitric oxide synthase, which likely leads to a reduction in microglial-mediated cell death (Drew and Chavis, 2000). In mice lacking functional GRs on microglia, lipopolysaccharide treatment (LPS; a treatment that produces "active" microglia) can increase neuroinflammation and neural toxicity relative to wildtype mice treated with LPS (Carrillo-de Sauvage et al., 2013). In other studies, GR antagonism attenuated the effects of LPS-induced microglial activation including CA1 pyramidal cell loss, JNK and p38 activation and decreased Akt and CREB phosphorylation (Espinosa-Oliva et al., 2011); taken together, these findings strongly support a role for GCs in modulating microglia function, but suggest that the impact of GCs may—as with e.g., astrocytes—be critically dependent on dosage and timing.

Chronic stressors can attenuate or prevent the anti-inflammatory effects of GR activation on microglia. Microglia can readily become sensitized to GC over-secretion to the point where GCs no longer prevent pro-inflammatory microglial activity, but rather promote it, as is the case in neurodegenerative disease and obesity (Munhoz et al., 2006; Frank et al., 2012; Dey et al., 2014). Understanding of the links between diet-induced obesity, cognitive dysfunction, and neurodegenerative disease continues to expand and now encompasses a greater role for microglia in these events. Sustained GC release and impaired HPA negative feedback are hallmarks of obesity and Type 2 Diabetes (Bruehl et al., 2009; de Guia et al., 2014; Paredes and Ribeiro, 2014; Martocchia et al., 2015), cognitive dysfunction (McEwen and Sapolsky, 1995; Dumas et al., 2010) and Alzheimer's disease (Notarianni, 2013). These pathological conditions are also characterized by increased cytokine release, inflammation, and microglial activation (Xiang et al., 2006; Rodriguez et al., 2010; Buckman et al., 2014; Erion et al., 2014; Hwang et al., 2014; Heneka et al., 2015; Kälin et al., 2015; Lee et al., 2015; Ramos-Rodriguez et al., 2015).

MICROGLIA AND NOREPINEPHRINE

NE has several effects on microglia that may contribute to cognitive deficits following stress (**Figure 4**). Generally, NE is capable of subduing inflammatory response genes in microglia and astrocytes (Hetier et al., 1991; Feinstein et al., 2002; Tynan et al., 2012), such as the major histocompatibility complex (Frohman et al., 1988), IL-1β (Ballestas and Benveniste, 1997), and TNF-α (Ballestas and Benveniste, 1997; Tynan et al., 2012) via β-AR activation. While in a "resting" phase, microglia predominately express β2 and β1 ARs (Tanaka et al., 2002), but this can change to α2 expression following inflammatory activation (i.e., during stress). Bath application of NE to brain slices results in microglial process retraction and potential reversal of stress-induced inflammation (Mori et al., 2002). NE can also attenuate microglial process extension mediated by the gliotransmitter ATP (Gyoneva and Traynelis, 2013); conversely, loss of NE decreases microglial migration to sites of inflammation (Heneka et al., 2010), confirming a key role for NE in microglial regulation. NE administration to cultured rat microglial cells decreases mRNA expression of several pro-inflammatory cytokines including IL-6 and TNF-α (Mori et al., 2002). Although under normal, non-pathological conditions these pro-inflammatory factors can have important procognitive roles, in disease or chronic stress states their effects can become pathogenic and lead to impaired cognitive function and cell

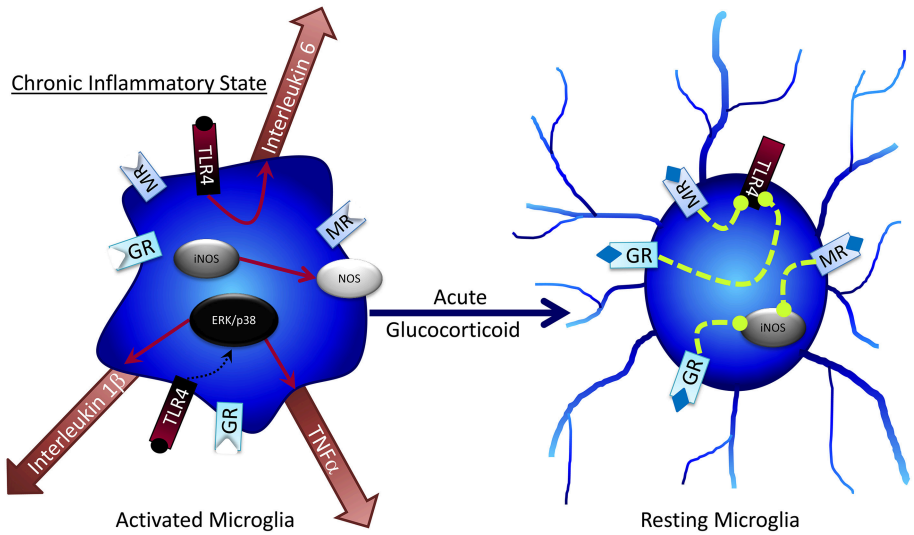

FIGURE 3 | Acute glucocorticoid exposure can return activated microglia to resting states.

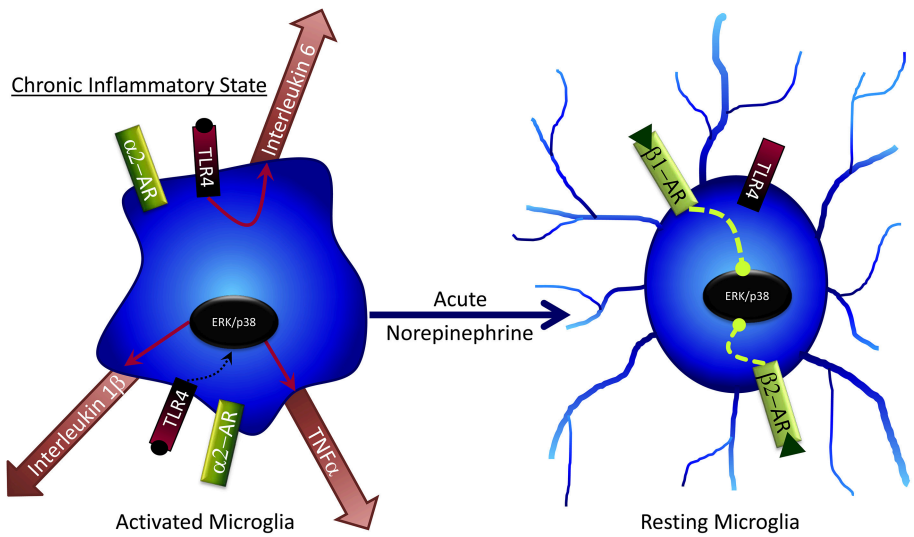

FIGURE 4 | Acute norepinephrine exposure can decrease release of cytokines by microglia via inhibition of ERK/p38 signaling.

death. Therefore, NE may decrease pathogenic microglial action following stress (Heneka et al., 2010). Taken together with findings of GCs on inhibiting microglial activation, it is intriguing that both NE and GCs can suppress microglial activation amidst a milieu of pro-inflammatory stimuli (e.g., pro-inflammatory cytokines, increased BBB permeability, and immune cell influx into the brain) that occur following stress. These findings may suggest that NE and GCs, via glia, act in part as anti-inflammatory stimuli that prevent pathogenic inflammatory activity in the brain following stress; the impaired response to these hormones seen after chronic and/or very high stress may include diminished ability to protect against brain inflammation, with deleterious consequences.

OLIGODENDROCYTES AND STRESS

While most attention in this field has been given to the impact of astrocytes and microglia on cognitive function, there is evidence that oligodendrocytes are also affected by stress and could modulate learning and memory. Oligodendrocytes' list of known functions extends beyond axon myelination, and now includes direct modulation of neuronal function (de Hoz and Simons, 2015).

Although stress and/or GC administration almost ubiquitously decreases neurogenesis (Anacker et al., 2013; Lehmann et al., 2013; Schoenfeld and Gould, 2013; Anacker, 2014; Chetty et al., 2014), stress has the opposite effect on oligodendrogenesis (Chetty et al., 2014). As with neurons, corticosterone increases activation of SGK1 in oligodendrocytes and subsequently induces abnormal morphological changes in arborization via NDRG1 and catenin signaling (Miyata et al., 2011). This increased arborization has been linked to increased depression-like behavior in stressed mice (Miyata et al., 2015). Other research suggests that GCs provide important survival signals that can aid oligodendrocyte and oligodendrocyte precursor survival against cytokine toxicity (Melcangi et al., 2000; Mann et al., 2008). Further investigation into the role oligodendrocytes play in cognition is required, both at baseline and after stress, but early evidence supports a vital function for this cell type in cognitive responses to stress.

CONCLUSION

Glia are increasingly recognized as critical regulators of cognitive processes; understanding of the multiple cell types involved in behavioral regulation and the molecular processes involved continues to expand. Glia are quickly becoming pharmacologically-relevant cellular targets for treatments of a variety of psychiatric disorders (Koyama, 2015) and offer a potential opportunity to regulate neural and cognitive responses to stress including treatment of stress-induced behavioral disorders. Such approaches are likely to take advantage of the potentially increased specificity offered by modulation mechanisms unique to glia rather than also affecting neurons.

AUTHOR CONTRIBUTIONS

JP-L, DO, and EM all planned, drafted, and edited the manuscript.

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REVIEW
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Fetal Alcohol Spectrum Disorders: An Overview from the Glia Perspective

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Alcohol consumption during pregnancy can produce a variety of central nervous system (CNS) abnormalities in the offspring resulting in a broad spectrum of cognitive and behavioral impairments that constitute the most severe and long-lasting effects observed in fetal alcohol spectrum disorders (FASD). Alcohol-induced abnormalities in glial cells have been suspected of contributing to the adverse effects of alcohol on the developing brain for several years, although much research still needs to be done to causally link the effects of alcohol on specific brain structures and behavior to alterations in glial cell development and function. Damage to radial glia due to prenatal alcohol exposure may underlie observations of abnormal neuronal and glial migration in humans with Fetal Alcohol Syndrome (FAS), as well as primate and rodent models of FAS. A reduction in cell number and altered development has been reported for several glial cell types in animal models of FAS. *In utero* alcohol exposure can cause microencephaly when alcohol exposure occurs during the brain growth spurt a period characterized by rapid astrocyte proliferation and maturation; since astrocytes are the most abundant cells in the brain, microenchephaly may be caused by reduced astrocyte proliferation or survival, as observed in *in vitro* and *in vivo* studies. Delayed oligodendrocyte development and increased oligodendrocyte precursor apoptosis has also been reported in experimental models of FASD, which may be linked to altered myelination/white matter integrity found in FASD children. Children with FAS exhibit hypoplasia of the corpus callosum and anterior commissure, two areas requiring guidance from glial cells and proper maturation of oligodendrocytes. Finally, developmental alcohol exposure disrupts microglial function and induces microglial apoptosis; given the role of microglia in synaptic pruning during brain development, the effects of alcohol on microglia may be involved in the abnormal brain plasticity reported in FASD. The consequences of prenatal alcohol exposure on glial cells, including radial glia and other transient glial structures present in the developing brain, astrocytes, oligodendrocytes and their precursors, and microglia contributes to abnormal neuronal development, reduced neuron survival and disrupted brain architecture and connectivity. This review highlights the CNS structural abnormalities caused by *in utero* alcohol exposure and outlines which abnormalities are likely mediated by alcohol effects on glial cell development and function.

Keywords: astrocyte, microglia, oligodendrocyte, fetal alcohol syndrome, fetal alcohol spectrum disorders, neurodevelopment, animal models

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INTRODUCTION

Prenatal alcohol exposure causes neurodevelopmental and physical alterations in the resultant offspring. The neurodevelopmental effects of *in utero* alcohol exposure are long-lasting affecting individuals throughout life and represent the major concerns associated with alcohol exposure during gestation (Guerri et al., 2009). Fetal Alcohol Syndrome (FAS) was the first characterized consequence of *in utero* alcohol exposure (Jones and Smith, 1973, 1975; Jones et al., 1973). Diagnosis of FAS requires the presence of three characteristics: growth retardation, distinct facial malformations (dysmorphia) and evidence of central nervous system (CNS) dysfunction. Both the Centers for Disease Control and Prevention (CDC) and the Institute of Medicine (IOM) have published guidelines for FAS diagnosis (Hoyme et al., 2005).

Alcohol-related neurodevelopmental disorder (ARND) is diagnosed when a maternal history of alcohol is established (Stratton et al., 1996). These individuals suffer from behavioral and cognitive impairments similar to those observed in FAS, yet lack the facial dysmorphia and/or growth retardation characteristic of FAS. For the diagnosis of ARND, structural, neurological or functional impairment in at least three of the followings domains must be present: achievement, adaptive behavior, attention, cognition, executive functioning, language, memory, motor skills, multisensory integration, or social communication (Warren et al., 2011).

More recently the term Fetal Alcohol Spectrum Disorders (FASD) has been introduced to describe the array of physical, behavioral, and learning impairments deriving from *in utero* alcohol exposure. Due to disruption of normal brain development, individuals with FASD may have a range of neurobehavioral deficits including impairments in attention, reaction time, visuospatial abilities, executive functions, motor skills, memory, language, and social and adaptive functions, and reduced IQ (Riley and McGee, 2005). As seen with other aspects of FASD, individuals afflicted with neurobehavioral deficits do not necessarily possess the characteristic facial features. However, those with prototypical FAS features generally exhibit more severe neurobehavioral deficits (Mattson and Riley, 1998). Approximately 25% of individuals with FAS fit the criteria for intellectual disability (an IQ lower than 70; Streissguth et al., 1997), making FAS the most common preventable cause of intellectual disability in the general population (Abel and Sokol, 1987). Given the range of cognitive impairments described above, it is not surprising that prenatal alcohol exposure is coincident with reduced academic performance and an increased frequency of learning disabilities (Howell et al., 2006). Indeed, attention deficit hyperactivity disorder (ADHD) is often diagnosed in FASD individuals with concordance rates ranging from 65–95% (Coles et al., 2002; Fryer et al., 2007; Rasmussen et al., 2010). Executive function is also impaired in individuals with FASD with deficits in response inhibition, concept formation, set shifting and planning (Guerri et al., 2009; Mattson et al., 2011). Individuals with FASD also exhibit weak grasp, poor hand/eye coordination, tremors, as well as gait and balance difficulties that can persist into adulthood and are indicative of motor control problems (Guerri et al., 2009; Mattson et al., 2011). Better therapeutics and biomarkers are needed to improve recognition and treatment for those suffering from FASD.

While FASD is not a diagnostic term, it should be noted that the American Psychiatric Association introduced for the first time into the appendix of the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5) the new category called Neurobehavioral Disorder associated with Prenatal Alcohol Exposure (ND-PAE). The diagnosis requires evidence of prenatal alcohol exposure and CNS impairment specifically in three areas: cognition, self-regulation, and adaptive functioning.

With increased recognition, the estimated combined prevalence of FAS and partial FAS (pFAS) individuals with confirmed prenatal alcohol exposure, substantial CNS anomalies and most, though not all of the growth impairments and facial abnormalities characteristic of FAS may reach as high as 5% in the USA and Western Europe (May et al., 2009). Some South African communities exhibit FAS/pFAS prevalences between 6.8 and 8.9% (May et al., 2007).

STRUCTURAL BRAIN ABNORMALITIES IN FETAL ALCOHOL SPECTRUM DISORDERS

Brain Irregularities Associated with FASD

Autopsies of infants born with FASD paint a grim picture of the effects of alcohol on the brain. In these, the most severe cases of FASD, damage is ubiquitous throughout the brain (Riley and McGee, 2005). A general CNS disorganization is observed, with errors in neuronal migration, neuroglial heterotopias, microcephaly, and abnormalities of the brainstem, cerebellum, basal ganglia, hippocampus and corpus callosum, pituitary gland and optic nerve (Jones et al., 1973). The degree to which these characteristics are present in individuals that do not die during development or early childhood is unclear, but these findings are reflective of the widespread disruption of normal brain development that results from alcohol exposure.

Attempts to identify the degree of damage in living subjects with FASD have been carried out with magnetic resonance imaging (MRI). These reports indicate that individuals with FASD exhibit a reduction in the cranial vault as well as a corresponding decrease in the overall size of the brain (Mattson et al., 1992; Archibald et al., 2001). More recent reports indicate reduced gyrification of the cortex (Infante et al., 2015) and a reduction in the surface area of the anterior cingulate cortex (Migliorini et al., 2015) among adolescents with heavy prenatal alcohol exposure. Reduced activation of some cerebellar areas during rhythmic vs. non-rhythmic finger tapping (du Plessis et al., 2014) and global reductions in gray matter (Soh et al., 2015) are observed in subjects with FAS and FASD. Interestingly it appears that shrinkage is not uniform throughout the brain, but that certain areas such as the cerebellum and regions of the cortex are disproportionately affected (Archibald et al., 2001). The volume of the parietal lobe in the cerebral cortex is consistently reduced in FASD individuals (Archibald et al., 2001). Further,

the composition of cortical brain regions is also impacted, with FASD brains exhibiting increases in gray matter, but reductions in white matter in the perisylvian cortices of the parietal and temporal lobes (Sowell et al., 2001). Such disruptions of normal cortical development are thought to underlie deficits in executive functions, verbal learning and recall, visuospatial processing and language present in individuals with FASD (Riley and McGee, 2005; Norman et al., 2009; Lebel et al., 2011).

The cerebellum is a primary target of alcohol effects; cerebellar volumes are significantly reduced and graded as with other deficits, with more severe shrinkage (>15%) present in subjects with FAS, and less loss among subjects with FASD that lack facial dysmorphology (Mattson et al., 1992; Archibald et al., 2001; Riley and McGee, 2005). Such disrupted cerebellar development is associated with deficiencies in balance, coordination, learning (eye-blink), verbal learning and memory and attention (Riley and McGee, 2005; Norman et al., 2009; Lebel et al., 2011).

The corpus callosum is another structure severely impacted by prenatal alcohol exposure. Agenesis (lack of formation) of the corpus callosum or anterior commissure, hypoplasia, volume reduction, heightened variability and displacement has been reported (Riley and McGee, 2005; Norman et al., 2009; Lebel et al., 2011). Due to consistent effects of *in utero* alcohol exposure on the corpus callosum, some have suggested that impaired formation of the corpus callosum may be a sensitive diagnostic indicator of prenatal exposure (Bookstein et al., 2002). White matter in general appears to be targeted by prenatal alcohol exposure, as diffusion tensor imaging and fractional anisotropy studies revealed white matter microstructural abnormalities and differences in white matter morphology in children and adolescents with FASD (Sowell et al., 2008).

Deficiencies in the basal ganglia have also been noted in subjects with FASD (Riley and McGee, 2005). Significant volume reductions are observed in the caudate of subjects with FASD (Archibald et al., 2001). Recent studies also suggest asymmetric formation of the caudate among subjects with either one trimester or all three trimesters of alcohol exposure *in utero* (Willford et al., 2010).

Unilateral decreases in hippocampal volume have been observed (Riley and McGee, 2005; Norman et al., 2009; Lebel et al., 2011). Scattered reports also suggest reduced hippocampal volume, though sometimes only when values are uncorrected for the reduced overall brain volume, or irregular brain shape associated with FASD (Joseph et al., 2014).

Brain Irregularities in FASD Animal Models

To gain a greater understanding of alcohol effects on brain development and behavioral effects of prenatal alcohol exposure, several animal models have been developed. Models are present for nonhuman primates and sheep, which are advantageous due to gestation periods comparable to humans; however this is offset by substantially higher costs. Paralleling the human gestation period, the brain growth spurt in nonhuman primates and sheep occurs prenatally (Cudd, 2005). Other animal models have also been developed in *Drosphila*, zebrafish, guinea pigs and avian embryos (Fabregues et al., 1985; Cudd, 2005; Smith, 2008; McClure et al., 2011; Cole et al., 2012). The majority of FASD studies however, make use of mice and rats. Animal models of FASD target and expose animals to blood alcohol concentrations observed in humans during specific developmental hallmarks and then examine subsequent behavioral and structural changes induced by alcohol exposure (**Table 1**). These studies recapitulate several of the behavioral deficits observed in subjects with FASD in these animal models, with impairments observed in attention, inhibition, motor tasks, learning and social interactions. A full review of the behavioral consequences of alcohol in animal models of FASD was recently published (Patten et al., 2014).

The effects of alcohol are detrimental throughout the developing nervous system and therefore heavy alcohol exposure can be harmful to the embryo and fetus at any stage of gestation. At the structural level, rodent models of FASD exhibit similar brain alterations to those of human FASD subjects as with craniofacial abnormalities similar to FAS (Sulik, 2005), hypoplasia or aplasia of the corpus callosum (Qiang et al., 2002; Deng and Elberger, 2003; Sulik, 2005; Livy and Elberger, 2008), microencephaly (Bonthius and West, 1990), alterations of the neocortex (Miller, 1986, 2007; Miller and Potempa, 1990; Miller and Robertson, 1993; Mooney and Miller, 2007), hippocampus (Berman and Hannigan, 2000), cerebellum

TABLE 1 | Effects of alcohol exposure during critical neurodevelopmental periods.

CNS developmental events	Consequences of alcohol exposure in animal models
Neural tube and crest formation	Craniofacial abnormalities Hypoplasia or aplasia of the corpus callosum Decrease in somatosensory neurons Malformation of the anterior cingulate cortex and hippocampus Anterior commissure, corpus callosum, and hippocampal commissure alterations and reduced myelination Sulik (2005), Miller (2007), and Cao et al. (2014)
CNS differentiation	Decreased neurogenesis
Neuronal migration	Radial glia alterations
Neurogenesis surge	Reduced migration and survival of neocortical, hippocampal and primary sensory neurons Miller (1986), Miller and Potempa (1990), Miller and Robertson (1993), and Sulik (2005)
Significant brain growth	Neuronal apoptotic cell death
Myelination	Decreased complexity of dendritic arborization
Dendritic arborization	Behavioral impairment
Synaptogenesis	Microencephaly
Gliogenesis (astrocytes and oligodendrocytes)	Apoptotic cell death of differentiating oligodendrocytes Delayed expression of myelin proteins Davies and Smith (1981), West et al. (1986), Bonthius and West (1990), Smith and Davies (1990), Ikonomidou et al. (2000), Granato et al. (2003), Cui et al. (2010), Hamilton et al. (2010), and Creeley et al. (2013)

(Guerri, 1998; Luo, 2012) and basal ganglia (Creeley and Olney, 2013). Imaging studies of mouse brains also revealed reduced myelin in major midline white matter tracts after prenatal alcohol exposure (Cao et al., 2014).

Significant differentiation of the CNS occurs during GD 11–21 in rats and is highlighted by a burst of neurogenesis and population of the cerebral cortex and hippocampus by migrating neurons (Guerri, 1998). Neurogenesis during this time is wide-spread in the developing rat brain with the exception of the hippocampal dentate gyrus and granule cells of the cerebellum. These newborn cells then differentiate into neurons and glia and begin to mature, forming axonal and dendritic processes. With the direction of radial glia fibers, neurons of the cerebral cortex migrate from the germinal zone (Nadarajah and Parnavelas, 2002). Alcohol exposure during this period results in decreased neurogenesis and disrupted radial glia, as well as reduced migration and survival of neurons of the neocortex, hippocampus and principal sensory nucleus of the trigeminal nerve (Miller, 1986; Miller and Potempa, 1990; Miller and Robertson, 1993; Sulik, 2005).

Key maturation that occurs during the third trimester of gestation in humans instead takes place postnatally in rats. Exposure to alcohol during key developmental periods results in characteristic abnormalities consistent with the ongoing ontogenic events. A substantial increase in brain size, dendritic arborization and synaptogenesis, which corresponds to proliferation of astrocytes and oligodendrocyte precursors and initiation of myelination occurs from late gestation up to postnatal days (PNDs) 9 in rats (Bayer et al., 1993; Rice and Barone, 2000; Kelly et al., 2009). Alcohol exposure during the early postnatal period results in decreased neuronal number throughout the hippocampus (West et al., 1986), apoptotic cell death in several brain regions (Ikonomidou et al., 2000), microencephaly (Bonthius and West, 1990) and cerebellar anomalies (Bonthius and West, 1990). Prenatal and/or early postnatal alcohol exposure in rodents affects the structural plasticity of the brain as previously reviewed (Medina, 2011). Reduced dendritic arborization and dendritic spine density and complexity have been reported in hippocampal and neocortical pyramidal neurons after prenatal and/or postnatal alcohol exposure (Davies and Smith, 1981; Granato et al., 2003; Whitcher and Klintsova, 2008; Cui et al., 2010; Hamilton et al., 2010). This short overview does not cover the whole literature available on preclinical FASD studies but focuses on alcohol-induced anomalies that can be attributed to alterations in glial cells.

GLIAL CONTRIBUTIONS TO BRAIN DEVELOPMENT

Radial Glia and Other Transient Glial Cells in the Developing Brain

Radial glia cells are widespread in the developing CNS where they differentiate directly from the neuroepithelial cells lining the ventricles. Radial glia exhibit processes that "radiate" in a directed, polarized fashion, thereby giving the cells their name. Radial glia are progenitors of neurons and several glial cell types, including astrocytes, oligodendrocytes and ependymal cells (Rowitch and Kriegstein, 2010). Radial glia are also essential for neuronal migration and the formation of the cerebral cortex. Indeed, radial glia have their cell body near the ventricular zone and extend their long process to the pial surface; newly differentiated post-mitotic neurons in the ventricular zone wrap around and migrate along the radial glia process to reach their location in the cortex (Rakic, 1972; Norris and Kalil, 1991). Radial glia persist in few specific locations in the adult brain (adult stem cells of the subventricular zone, Müller cells of the retina, and Bergman glia of the cerebellum; Sild and Ruthazer, 2011).

Midline glial cell populations that guide callosal axons are essential to the successful formation of the corpus callosum. Callosal axons extend toward and cross the midline in response to various molecular cues produced by midline glia cells. The glial wedge, one of the glial midline structures encountered by callosal axons, repels ipsilateral callosal axons towards the midline and guide them towards the contralateral cortex (Suárez et al., 2014). The glial wedge is composed of specialized astrocytes that develop from cortical radial glia between E13 and E17 (Shu et al., 2003). Defects in the glial wedge lead to the agenesis of corpus callosum (Chinn et al., 2015).

A transient midline raphe glial structure has been described (Van Hartesveldt et al., 1986), which is hypothesized to contribute to the development of the serotoninergic neurons of the raphe nuclei by providing trophic support for developing serotoninergic neurons through the release of glial-specific S100β, a growth factor for serotoninergic neurons (Azmitia et al., 1990), and by guiding neuronal migration (Van Hartesveldt et al., 1986).

Astrocytes in Brain Development

The ratio of macroglia (astrocytes and oligodendrocytes) to neurons increases as the evolutionary complexity of the species increases (Sherwood et al., 2006), suggesting that astrocytes play an important role in higher order cognition and brain development. Indeed, recent studies indicate that astrocytes play a critical role in coordinating neuronal growth and migration with regulated secretion of extracellular matrix (ECM) proteins and trophic factors (Higgins et al., 1997; Pfrieger and Barres, 1997; Booth et al., 2000; Ullian et al., 2001; Martinez and Gomes, 2002; Yang et al., 2003; Christopherson et al., 2005; Pascual et al., 2005; Stellwagen and Malenka, 2006; Guizzetti et al., 2008). In addition, astrocytes are tuned to sense neuronal cues as evidenced by the diverse array of neurotransmitter receptors they express (Steinhauser and Kettenmann, 2009). As such, astrocytes are critical to proper neuronal circuit development.

The manner by which astrocytes and neurons communicate is still under heavy investigation. We recently identified a novel astrocyte-neuron interaction by which cholinergic activation leads to induction of multiple signaling cascades that enhance neurite outgrowth of hippocampal neurons (Guizzetti et al., 2008; Giordano et al., 2011). The majority of the factors secreted by astrocytes are proteases, protease inhibitors and ECM components (Moore et al., 2009).

Following cholinergic stimulation of cultured astrocytes, we observed increases in ECM protein expression (fibronectin and laminin) both intracellularly and in the media and upregulation of factors that prevent degradation of the ECM (Guizzetti et al., 2008). Astrocyte-released proteins also play a major role in the regulation of synaptogenesis (reviewed in Guizzetti et al., 2014), the maintenance of brain homeostasis, neuroimmune function and blood-brain barrier development (Obermeier et al., 2013; Engelhardt and Liebner, 2014).

The brain is very rich in cholesterol (the brain contains 15–20% of the total body cholesterol, but represents only about 5% of the total body weight); cholesterol is necessary for proper brain health and development (Martín et al., 2014). Cholesterol circulates through the brain in association with astrocyte-produced lipoproteins (Martín et al., 2014). Both microglia and astrocytes express apolipoprotein E (apoE), which is a major component of brain lipoproteins (Diedrich et al., 1991; Nakai et al., 1996). The process by which cholesterol is removed from the brain is important for overall brain health and homeostasis (Dietschy, 2009).

Brain lipoproteins generally exert positive effects on CNS functions and are protective against neurodegenerative diseases. Astrocyte-produced lipoproteins, however, also induce a net efflux of cholesterol from astrocytes and neurons (Guizzetti et al., 2007; Kim et al., 2007; Chen et al., 2013). During development, cholesterol is essential for several brain functions including cell proliferation, neuronal survival, and activation of the sonic hedgehog pathway. Genetic defects in cholesterol synthesizing enzymes, such as the one causing Smith-Lemli-Opitz-Syndrome (SLOS), result in low levels of cholesterol in all the tissues, and are associated with altered brain development, intellectual disability and behavioral disorders (Nowaczyk et al., 1999). Therefore, increased brain lipoproteins may be deleterious to the developing brain because it may reduce brain cholesterol levels (Guizzetti and Costa, 2007).

Oligodendrocytes and Myelination in Brain Development

Myelin is formed by the plasma membrane of oligodendrocytes (in the CNS) or Schwann cells (in the peripheral nervous system) wrapping around axon segments many times (Szuchet et al., 2015) and forming a compacted insulating sheath, that serves to improve the speed and efficiency of electrical signal conduction along the myelinated axon. Myelination is a dynamic process that can be modulated by the axonal release of neurotransmitter and by environmental factors. Oligodendrocytes and their myelin sheaths also provide supportive factors to maintain axonal health (Rosenbluth, 1999; Simons and Trotter, 2007; Fields, 2010; Nave, 2010; Mitew et al., 2013; Nualart-Marti et al., 2013).

Developmentally, myelination occurs as one of the final stages of brain development. In humans, most myelination takes place during the first 20 years of postnatal life (Lebel et al., 2008). While the bulk of myelination occurs in relatively early postnatal life, it is now clear that myelination continues throughout life (Bartzokis et al., 2012; Young et al., 2013). Coinciding with their later development, oligodendrocytes are the final cells of the CNS to mature. Nevertheless, oligodendrocyte precursor cells (OPCs) are present at embryonic day 12.5 in mice. Production of OPCs is localized to distinct areas, thus, migration of this cell type throughout the brain is an important phase of development. OPCs are generated throughout life and may contribute to myelination in adulthood (El Waly et al., 2014). Genetic influences appear to strongly regulate myelination, however, mounting evidence suggests that experiential factors also influence myelination. Control of oligodendrocyte differentiation and myelination occurs through a diverse array of transcription factors, extracellular signals and intracellular pathways (Mitew et al., 2013).

Microglia in Normal Brain Development

Microglia are of hematopoietic origin and are the resident "professional" immune cell, composing roughly 10% of all cells in the brain (Benarroch, 2013). Under normal conditions, microglia exhibit a ramified morphology, allowing them to sample and survey from the environment for signs of infection or insult. Upon activation, microglia become rod-like or amoeboid, with a multinucleated or epithelioid appearance (Benarroch, 2013). Activated microglia also exhibit changes in gene expression, function and produce an array of pro-inflammatory cytokines, chemokines and reactive oxygen species (ROS). Microglia polarize, much like T-cells, becoming relatively pro- or anti-inflammatory depending on the surrounding environmental cues present (Nakagawa and Chiba, 2015). Microglia are also capable of clearing pathogens or cellular debris via phagocytosis. Under normal conditions, microglia are important contributors to maintaining brain homeostasis, but under pathological conditions, microglia can become chronically activated, inducing inflammation and neurodegeneration when functionally impaired (Saijo and Glass, 2011).

During embryonic development in rodents, microglia appear in the activated state with an amoeboid shape and transition to a resting ramified morphology shortly after birth. Microglia also play a major role during brain development. Indeed, microglia phagocytize neural precursor cells, particularly during late stages of cortical neurogenesis therefore regulating the size of the neural precursor cell pool in the developing cortex (Cunningham et al., 2013). Microglia also instruct neuronal apoptosis, engulf and phagocytize apoptotic neurons (Bessis et al., 2007; Marín-Teva et al., 2011) and induce synaptic pruning (Paolicelli et al., 2011).

GLIAL DYSFUNCTION IN FASD

Effects of Alcohol on Radial Glia and Other Glial Structures in the Developing Brain

Heavy prenatal alcohol exposure alters neuronal migration and induces glial heterotopia (Jones et al., 1973). Radial glia are targets of alcohol damage and are critical to proper neuronal migration. Radial glia cells derived from 13-day rat fetuses exhibited reductions in GFAP, but not vimentin expression both *in vivo* and in culture (Valles et al., 1996). Prenatal

alcohol exposure *in vivo* also causes a decrease in GFAP fibers in cerebellar Bergman glia on PND 15, reflecting a delayed maturation of these cells that may contribute to the delayed migration of granule cells (Shetty and Phillips, 1992). Developmental exposure to alcohol lead to decreased density and fasciculation of radial glial processes that run from the ventricular surface to the pial surface (Miller and Robertson, 1993) during the first week following birth. This was accompanied by a decrease in vimentin staining on PND 5 compared to non-exposed control animals. Chronic exposure to alcohol via liquid diet led to decreased cell division in radial glia cultured from E12 telencephalon, and also reduced the number of multipotent progenitor cells derived from neurospheres, with concurrent decreases in the progenitor-maintaining proteins Notch1 and fibroblast growth factor 2 (Rubert et al., 2006). This study also observed a greater percentage of cells derived from alcohol-exposed embryos remaining as radial glia in culture as opposed to differentiating into neurons or astrocytes following 2 days of culture compared to control cultures (Rubert et al., 2006). Alcohol exposure damages neural progenitor cells, limiting their survival and impeding their differentiation into astrocytes (Taléns-Visconti et al., 2011; Nash et al., 2012). Culture models using neurospheres also demonstrate that selective exposure of neural precursor cells to alcohol impairs cell division and astrocyte formation (Vemuri and Chetty, 2005). Thus, alcohol exposure disrupts the development and maturation of radial glia cells that act both as precursor cells for neurons, astrocytes and oligodendrocytes, as well as aid in the migration of other progenitor cells throughout the developing CNS (**Table 2**).

The formation of the corpus callosum is strongly affected by prenatal alcohol exposure (Riley and McGee, 2005; Norman et al., 2009; Lebel et al., 2011). While no studies have investigated the effect of alcohol on midline glial cell populations directing the formation of the corpus callosum, such as the glial wedge, it is possible to speculate that alcohol may affect the release or expression of guiding molecules by these cells.

Interestingly, the number of glial cells in transient midline raphe glial structures expressing S100β, a trophic factor for serotoninergic neurons (Azmitia et al., 1990), is decreased. Expression of S100β is reduced in the midline raphe of prenatal alcohol exposed rats and mice (Eriksen et al., 2000; Zhou et al., 2001; Tajuddin et al., 2003), which may underlie the reduced number of serotoniergic neurons as well their migration and development observed in FASD animal models (Tajuddin and Druse, 1999, 2001; Zhou et al., 2001).

TABLE 2 | Cell specific consequences of alcohol exposure.

Cell type	Consequences of developmental alcohol exposure
Radial Glia	Reduced GFAP expression Decreased density and fasciculation of processes Reduced cell division Decreased density of multipotent progenitor cells and associated factors Impaired differentiation into astrocytes Miller and Robertson (1993), Valles et al. (1996), Vemuri and Chetty (2005), Rubert et al. (2006), Taléns-Visconti et al. (2011), and Nash et al. (2012)
Oligodendrocytes	Reduced and disorganized white matter Delayed myelination Decreased myelin thickness Ultrastructural anomalies in myelin Altered myelin biochemical profile Decreased oligodendrocyte differentiation Delayed and reduced expression of key myelin proteins Cell death Druse and Hofteig (1977), Hofteig and Druse (1978), Gnaedinger et al. (1984), Samorajski et al. (1986), Phillips and Krueger (1992), Lancaster (1994), Phillips (1994), Zoeller et al. (1994), Parson et al. (1995), Riley et al. (1995), Pinazo-Duran et al. (1997), Archibald et al. (2001), Guerri et al. (2001), Dalitz et al. (2008), Sowell et al. (2008), Bichenkov and Ellingson (2009), and Creeley et al. (2013)
Astrocytes	Reduced proliferation and survival of progenitor cells Decreased production and release of neurotrophic factors Increased production and release of inhibitors of neurite outgrowth Activation of inflammatory signaling pathways (TLR-4, nitric oxide (NOS), cyclooxygenase 2) Decreased production of the primary brain antioxidant glutathione Disruption of cholesterol homeostasis Reduced astrocyte differentiation Altered expression of GFAP Altered production of extracellular matrix proteins Davies and Smith (1981), Renau-Piqueras et al. (1989), Miller and Potempa (1990), Saez et al. (1991), Fletcher and Shain (1993), Goodlett et al. (1993, 1997), Lokhorst and Druse (1993), Resnicoff et al. (1994), Montoliu et al. (1995), Guizzetti and Costa (1996), Vallés et al. (1997), Kötter and Klein (1999), Granato et al. (2003), Blanco et al. (2004, 2005), Tomás et al. (2005), Watts et al. (2005), Rathinam et al. (2006), Guizzetti et al. (2007, 2011), Pascual and Guerri (2007), Whitcher and Klintsova (2008), Cui et al. (2010), Hamilton et al. (2010), Zhou et al. (2014), and Topper et al. (2015)
Microglia	Increased phagocytosis Reduced survival Increased production of inflammatory mediators and oxidative stress Increase in microglia-mediated hypothalamic neuron death Decreased production of neurotrophic factors Boyadjieva and Sarkar (2010), Boyadjieva and Sarkar (2013a,b), and Kane et al. (2011)

Role of Astrocytes in FASD

Glial Fibrillary Acidic Protein (GFAP) Expression

Glial fibrillary acidic protein (GFAP), an intermediate filament often used as a marker for astrocytes whose upregulation is considered a marker for reactive astrogliosis, and has been investigated in several studies in relation to FASD. Astrocytes prepared from fetuses exposed *in vivo* to alcohol and maintained in culture in the presence of alcohol present decreased GFAP expression and failed to develop processes (Renau-Piqueras et al., 1989). When astrocyte cultures are exposed to alcohol, GFAP levels increase initially after which GFAP expression decreases after 21 days in culture (Saez et al., 1991). A decrease

in GFAP expression was confirmed *in vivo* after prenatal alcohol exposure in the brain of postnatal animals and was attributed to increased DNA methylation in the GFAP promoter region (Vallés et al., 1997).

Neonatal alcohol exposure lead to an increase in GFAP expression in the cortex and hippocampus when exposure was carried out via artificial rearing (Fletcher and Shain, 1993; Goodlett et al., 1993) and an increase in GFAP in the parietal cortex after alcohol exposure via intragastric intubation (Goodlett et al., 1997), but no GFAP upregulation was observed when alcohol was administered via vapor inhalation (Ryabinin et al., 1995). More recently, neonatal alcohol exposure via vapor inhalation has been shown to increase GFAP expression in the cerebellum and hippocampus during the alcohol-withdrawal period (Topper et al., 2015). From these reports it can be concluded that the reduction of GFAP levels observed *in vitro* is recapitulated by *in vivo* experiments when alcohol is administered prenatally. However, the effect of neonatal exposure to alcohol on GFAP levels is not conclusive. Studies suggest that the effects of alcohol on astrocyte GFAP levels vary depending on the developmental stage of astrocytes at the time of exposure, on alcohol levels, route of administration, length of the treatment, and alcohol-withdrawl status.

The fact that, when observed, the increase in GFAP expression is not generalized to the whole brain, but rather confined to specific areas, suggests that this may not be a direct effect of alcohol on astrocytes, but rather a secondary effect caused, for instance, by increased neuronal damage, release of proinflammatory cytokines by microglia, or damage to blood brain vessels. However, the question remains regarding the significance of changes in alcohol-induced GFAP expression in astrocytes and whether these changes play a positive (protective) or negative role in alcohol-induced developmental brain injury. Several studies during the last 20 years have shown that astrogliosis is not a simple "all or nothing" response; astrocytes are capable of a spectrum of changes (ranging from reversible alterations in gene expression and cellular hypertrophy to cell proliferation, scar formation and permanent tissue rearrangement) tailored to the type of insult they are responding to Sofroniew (2014).

Astrocyte Proliferation

Exposure of primary astrocyte cultures to alcohol also impairs astrocyte proliferation induced by serum, IGF-1, the cholinergic agonist carbachol, and PDGF (Resnicoff et al., 1994; Guizzetti and Costa, 1996; Kötter and Klein, 1999). Alcohol appears to affect astrocyte proliferation by inhibiting specific signal transduction pathways. For instance, we have reported that alcohol selectively inhibits carbachol-stimulated phospholipase D signaling (inducing the formation of the second messenger phosphatidic acid, which activates Akt, p70S6K and PKCζ) in astrocytes, leaving unaffected other pathways activated by carbachol (Guizzetti and Costa, 2000, 2002; Guizzetti et al., 2003, 2004, 2014; Tsuji et al., 2003). Prenatal alcohol exposure has been reported to cause a decrease in astrocyte number (Miller and Potempa, 1990), an observation that supports the *in vitro* studies.

Astrocyte-Induced Neuronal Plasticity

In vivo pre- and post-natal alcohol exposure affects neuronal structural plasticity (Medina, 2011). Alcohol strongly affects the release of neuritogenic factors by astrocytes (Tomás et al., 2005). Reduced dendritic arborization and dendritic spine density and complexity is reported in hippocampal and neocortical pyramidal neurons (Davies and Smith, 1981; Granato et al., 2003; Whitcher and Klintsova, 2008; Cui et al., 2010; Hamilton et al., 2010) and may be due to alcohol-induced alterations in the secretion of factors modulating neuronal development and plasticity by astrocytes. A reduction in neuritogenesis and neuronal survival was reported in naïve neurons co-cultured with astrocytes prepared from alcohol-exposed rats (Pascual and Guerri, 2007). In an earlier study, neurons cultured in conditioned media from astrocytes exposed in culture to 100 mM alcohol for 4 days displayed reduced DNA content, neurite length, number of serotonin neurons and serotonin uptake (Lokhorst and Druse, 1993). Astrocytes also modulate the effect of alcohol on dendrite development in culture (Yanni et al., 2002). Astrocyte-specific expression of serum response factor in a ferret model of early alcohol exposure reverses alcohol-induced reductions in ocular dominance plasticity (Paul and Medina, 2012).

We have reported that alcohol exposure inhibits the ability of carbachol-treated astrocytes to foster neurite outgrowth in neurons co-cultured with astrocytes after treatment by decreasing the release of neuritogenic proteins laminin and fibronectin (Guizzetti et al., 2010), an effect reproduced in hippocampal slices (Giordano et al., 2011). This effect is in part mediated by the upregulation of the tissue-type plasminogen activator (tPA) which converts plasminogen to plasmin, an extracellular proteolytic enzyme that degrades the ECM (Zhang et al., 2014). Chondroitin sulfate proteoglycans (CSPGs) are inhibitors of neurite outgrowth. We found that alcohol upregulates the levels of CSPGs through the inhibition of the enzyme arulsulfatase B (ARSB). ARSB degrades the chondroitin sulfate moiety of CSPGs and leads to the extracellular proteolysis of the core-protein in astrocyte cultures *in vitro* and after neonatal alcohol exposure *in vivo* (Zhang et al., 2014).

Oxidative Stress

Exposure of astrocyte cultures to alcohol leads to reductions in glutathione (GSH), and induction of cyclooxygenase 2 (Cox-2) as a result of NF-κB signaling, and the formation of ROS in astrocytes (Montoliu et al., 1995; Blanco et al., 2004). The induction of reactive species and inflammatory signaling appears to be mediated via toll-like receptor 4 (TLR4) and interleukin one receptor (IL-1R1) signaling in astrocytes, as inhibition of these receptors prevents alcohol-induced AP-1 and NF-κB activation as well as induction of nitric oxide (NOS) and Cox-2 (Blanco et al., 2005). Cortical neurons undergo apoptotic cell death in response to depletion of GSH and ROS production following alcohol exposure. Co-culturing neurons with astrocytes decreased GSH depletion and attenuated

neuronal death following alcohol treatment (Watts et al., 2005; Rathinam et al., 2006).

Brain Lipid Homeostasis

Cholesterol homeostasis is maintained by astrocyte-released lipoproteins that can extract cholesterol from astrocytes and neurons. Our studies indicate that alcohol disrupts this process by increasing release of lipoprotein from astrocytes which leads to increased efflux of cholesterol from astrocytes and neurons (Guizzetti et al., 2011) and may lead to cholesterol-loaded lipoproteins exiting the brain and subsequent reductions in cholesterol (Zhou et al., 2014). The transporter ABCA1 (ATP binding cassette-A1) is essential for the generation of nascent lipoproteins in astrocytes. Cholesterol efflux is mediated by ABCG1 and ABCG4 transporters and leads to the lipidation and remodeling of nascent, lipid poor lipoproteins (Koldamova et al., 2003; Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004). Our studies indicate that alcohol upregulates expression of ABCA1 and ABCG1 in astrocyte cultures, thereby increasing cholesterol efflux and reducing brain cholesterol levels (Guizzetti et al., 2007). Astrocyte-released lipoproteins also increase cholesterol efflux from neurons through a mechanism likely mediated by ABCG4 (Zhou et al., 2014). *In vivo*, neonatal alcohol exposure increases cortical levels of ABCA1 (Guizzetti et al., 2007); while, prenatal alcohol exposure up-regulates ABCA1 and ABCG1 and reduces the levels of cholesterol in the neocortex of GD 21 females (Zhou et al., 2014).

Role of Oligodendrocytes in FASD

Examination of the impact of alcohol on oligodendrocytes and by extension, myelination has been intermittent at best, with the bulk of studies occurring in the late 1970s or early 1990s. White matter (myelin/oligodendrocytes) is another target of the developmental effects of alcohol as evidenced by lower volumes and sometimes complete lack of formation of major white matter tracts in the brain of individuals with FASD (**Table 2**; Riley et al., 1995; Sowell et al., 2008). With the availability and affordability of advanced imaging techniques, interest is once again growing and high resolution examination of myelin in FASD subjects is now possible.

As previously described, at first glance, prenatal exposure to alcohol may not be expected to exert a substantial effect on myelination in adults, given that much of the myelination that occurs in humans happens long after birth. Nevertheless, developmental exposure to alcohol permanently impacts the programming of OPCs, as imaging studies indicate widespread anomalies in children and adults with FASD (Archibald et al., 2001; Sowell et al., 2008). These findings are recapitulated in rat models of FASD, where alcohol slows myelination and disrupts the myelin ultrastructure (Lancaster, 1994; Phillips, 1994; Pinazo-Duran et al., 1997).

Developmental alcohol exposure in rats causes clear changes in myelin, with a distinct shift in the expression patterns of oligodendrocytes (Druse and Hofteig, 1977; Hofteig and Druse, 1978; Gnaedinger et al., 1984). The greatest harm to myelin and oligodendrocytes is associated with exposure during the first 10 PNDs in animal models, and is characterized by myelin malformation and morphological deficits in oligodendrocytes (reviewed in Guizzetti et al., 2014). Studies in sheep suggest that third-trimester alcohol exposure leads to myelin malformation and morphological deficits in oligodendrocytes (Dalitz et al., 2008). Myelin basic protein (MBP), which as its name implies is a critical element of the myelin sheath, is delayed and reduced in its expression following postnatal alcohol exposure in the cerebellum of PND 15 rats (Zoeller et al., 1994). Exposure of cultured oligodendrocytes to alcohol also leads to a decrease in MBP levels (Bichenkov and Ellingson, 2009). Monkeys exposed to alcohol during late stage development exhibit extensive apoptosis of oligodendrocytes in white matter regions (Creeley et al., 2013), further highlighting oligodendrocytes as targets of alcohol in the developing brain. Studies in primary mouse oligodendrocyte cultures suggest that acetaldehyde, the toxic, metabolic byproduct of alcohol is highly lethal to oligodendrocytes, while very high concentrations of alcohol are necessary to cause damage (Coutts and Harrison, 2015).

A common characteristic of individuals with FASD is impaired visual function (Strömland, 2004). Myelination of the optic nerve is disrupted in animal models of FASD, with reports of decreased thickness, as well as aberrant and fewer myelin sheaths (Samorajski et al., 1986; Phillips and Krueger, 1992; Parson et al., 1995; Pinazo-Duran et al., 1997). Developmental alcohol exposure interferes with oligodendrocyte maturation and delays expression of MBP resulting in ultrastructural harm to the myelin sheaths and a decreased number of myelinated optic nerve axons (Guerri et al., 2001). Such alterations in myelin may be responsible for the hypoplasia of the optic nerve present in FAS.

This data suggests that myelin and more specifically, oligodendrocyte development is a target of alcohol effects in the developing brain and that early exposure can lead to persistent, life-long deficiencies. Disruptions in survival and maturation of oligodendrocytes are likely to impair the formation of neurocircuitry and the efficient conduction of neuronal signals. Future studies are needed, however, to elucidate the effects of alcohol on oligodendrocytes and OPCs to understand the pathways that lead to alcohol-induced dysfunction.

Role of Microglia in FASD

Microglia possess an assortment of receptors designed to detect aberrant "danger" signals so that an appropriate response can be mounted. Recent work indicates that alcohol can induce microglial activation (Fernandez-Lizarbe et al., 2009) and even death (**Table 2**; Kane et al., 2011). Initial studies suggest this activation may occur via the Bcl-2 associated X Protein (BAX), as mice lacking BAX exhibit blunted microglial activation and pro-inflammatory signaling following alcohol exposure on PND 7 or PND 8 (Ahlers et al., 2015). In cultures, alcohol increases the release of inflammatory cytokines from microglia and reduces intracellular cAMP and brain-derived neurotrophic factor in co-cultures of hypothalamic neurons and microglia (Boyadjieva and Sarkar, 2010, 2013a). Oxidative

stress is also increased in alcohol exposed microglia (Boyadjieva and Sarkar, 2013b). Alcohol exposure also affects the viability of microglia both *in vitro* and *in vivo* following neonatal alcohol exposure (Kane et al., 2011). The response of microglia to inflammatory factors is variable, with repeated exposures leading to a sensitized response (Perry and Teeling, 2013). Some have hypothesized that prenatal alcohol exposure may lead to long-term sensitization of microglia that results in persistent inflammatory signaling in the brain following insult (Chastain and Sarkar, 2014). This priming hypothesis is consistent with a study observing increased inflammatory signaling in prenatally-exposed rats in a model of rheumatoid arthritis in adult animals (Zhang et al., 2012). Recent studies suggest that alcohol-induced microglial activation can be reduced by treatment with peroxisome proliferator-activated receptor γ agonists such as pioglitazone (Drew et al., 2015), thus identifying a potential treatment.

Alterations in microglial function also impact neuronal health. Microglia-conditioned media contributes to alcohol-induced apoptosis in immature hypothalamic neurons (Boyadjieva and Sarkar, 2010, 2013a,b). Neonatal alcohol exposure in rodents induces neurotoxicity in hypothalamic neurons *in vivo*, which appears to be mediated, at least in part, by microglia (Sarkar et al., 2007). Several excellent reviews offer greater depth and discussion of alcohol effects on microglia and the subsequent developmental impacts (Drew and Kane, 2013, 2014; Chastain and Sarkar, 2014; Crews and Vetreno, 2014).

Glial Cells in Abnormal Brain Development

FASD is not the only neurodevelopmental disorder for which glial dysfunction is a major contributing factor. Rett syndrome is a neurodevelopmental disorder characterized by seizures, motor impairment, neurogenic apneas and delayed or absent speech (Chahrour and Zoghbi, 2007). Mutations in the methyl-CpG-binding protein 2 (*MeCP2*) gene appear to be a critical factor in the disease. Interestingly, *MeCP2* is abundantly expressed in neurons, but also present in astrocytes, microglia and oligodendrocytes. Original theories regarding the disease focused almost exclusively on neurons, but recent evidence now indicates that glial cells (astrocytes, microglia and oligodendrocytes) are important contributors to disease pathology (reviewed in Guizzetti et al., 2014). Disruptions in astrocyte development and function may contribute to autism, fragile X, Down syndrome, Costello syndrome, neurofibromatosis-1, Noonan syndrome and Cardiofaciocutaneous syndrome (Garcia et al., 2010; Jacobs and Doering, 2010; Sloan and Barres, 2014). Astrocyte dysfunction is also hypothesized to partially underlie the neurodevelopmental origins of major depressive disorder (MDD) and schizophrenia as evidenced by glial anomalies in patients with these disorders (Sloan and Barres, 2014). Neurotoxicants such as diazinon, an organophosphate insecticide, and its active metabolite diazoxon, as well as manganese impair the development of hippocampal pyramidal neurons when they are co-cultured with astrocytes previously treated with these compounds (Giordano et al., 2009; Pizzurro et al., 2014). There are also genetic mutations that interfere with normal astrocyte function, such as the gain of function mutation in the GFAP gene thought to be responsible for Alexander's disease. Alexander's disease is characterized by atypical myelination, developmental delay, and macroencephaly, with astrocytes exhibiting Rosenthal fiber accumulation, and abnormally high expression of GFAP (Quinlan et al., 2007; Messing et al., 2012). The disruption of myelination highlights cross-talk that occurs between astrocytes and oligodendrocytes during neurodevelopment. Prenatal infections lead to activation of microglia and astrocytes and are associated with decreased oligodendrocyte density, abnormal myelination, and schizophrenia (Anderson and Maes, 2013; Meyer, 2013). Microglial activation has been observed in multiple brain regions of autism spectrum disorder patients (Morgan et al., 2010; Rodriguez and Kern, 2011; Suzuki et al., 2013). Prenatal stressors such as vitamin B12 deficiency, restraint stress, hypoxia, opioids and methamphetamine result in compromised myelination in the postnatal brains of exposed mothers (Lövblad et al., 1997; Baud et al., 2004; Melo et al., 2008; Sanchez et al., 2008). In conclusion, glial cells are important contributors to pathological events that may occur in the brain during development and therefore have great potential as therapeutic targets for the treatment of neurodevelopmental diseases.

CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

Several of the structural abnormalities observed in FASD and in FASD animal models are consistent with altered glial cell function. Defects in neuronal migration observed in FASD are likely due to reported effects of alcohol on radial glia. Prenatal alcohol can also induce agenesis or hypoplasia of the corpus callosum, which may be due to an effect of alcohol on midline glial populations causing them to alter the signals these cells send to the axons that need to cross the midline into the opposite hemisphere. Studies on the effect of alcohol on these structures have not been carried out, but may represent a new, interesting avenue of investigation.

Recent imaging studies of FASD children show defects in white matter fiber tracts that may arise from the reported effects of alcohol on oligodendrocytes or their precursors. Similarly, alcohol exposure during the brain growth spurt and the resultant microencephaly that occurs also highlights the glial effects of alcohol. There is evidence that reduced dendritic arborization and structural plasticity observed in FASD models may be due, at least in part, to changes in factors released by astrocytes, which are known to have a major role in neuronal development and brain maturation. Finally, the neuronal loss reported in several FASD models may be caused by reduced release of trophic factors and antioxidants by astrocytes or increased release of neuroinflammatory molecules by microglia and astrocytes.

A major challenge of glia and FASD research is to causally link the effects observed in glial cells to neuronal abnormalities in the brain. *In vitro* studies have been instrumental in demonstrating the concept that, by affecting glial cell functions, alcohol alters neuronal survival and development. However, these studies need

to be validated *in vivo* and to be linked to behavioral outcomes. Molecular approaches for manipulating glial cell signaling and functions *in vivo* (for instance, Xie et al., 2015) and methods for *ex-vivo* isolation of specific brain cell populations are becoming available and should be used in FASD research.

The role of sex in susceptibility of glial cells to the consequences of fetal alcohol exposure is almost entirely unexplored, although differences have been observed in the function of astrocytes derived from male and female animals in culture (Liu et al., 2008; Santos-Galindo et al., 2011; Wilhelm et al., 2015). Individuals with FASD exhibit different deficits in eye movement depending on whether they are male or female (Paolozza et al., 2015). Sex differences in FASD preclinical models have been reported in a number of instances; for instance, developmental alcohol exposure leads to social avoidance in females, but increased play fighting in males in a model of FASD (Varlinskaya and Mooney, 2014); females exposed to prenatal alcohol and chronic mild stress exhibit increases in learned helplessness, and disrupted social interactions (Hellemans et al., 2010), sexually dimorphic effects of prenatal alcohol on the development of the hypothalamic-pituitary-adrenal axis have been extensively documented (Weinberg et al., 2008); sex differences in spatial learning deficits after neonatal alcohol exposure have also been reported (Kelly et al., 1988; Goodlett and Peterson, 1995). The hypothesis that glial cells in males vs. females are differentially affected by alcohol during brain development is worth further examination.

We have summarized published evidence that strongly support the notion that alcohol detrimentally affects the glial cells of the developing brain and that disruption of the function of these cells leads to neuronal deficits. It is through interactions between neurons and glial cells that the CNS is successfully developed. Thus, elucidating not just the cell-specific effects of alcohol, but also the cell-cell interactions that occur is necessary to fully understand and effectively treat abnormal brain development. Unraveling the complex biological disruptions induced by fetal alcohol exposure and identifying causal links between glial dysfunction and brain structural and functional abnormalities as well as behavioral impairments is an important next step to identifying optimal targets for therapeutic development.

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MINI REVIEW
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Role of Glia in Memory Deficits Following Traumatic Brain Injury: Biomarkers of Glia Dysfunction

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Historically, glial cells have been recognized as a structural component of the brain. However, it has become clear that glial cells are intimately involved in the complexities of neural networks and memory formations. Astrocytes, microglia, and oligodendrocytes have dynamic responsibilities which substantially impact neuronal function and activities. Moreover, the importance of glia following brain injury has come to the forefront in discussions to improve axonal regeneration and functional recovery. The numerous activities of glia following injury can either promote recovery or underlie the pathobiology of memory deficits. This review outlines the pathological states of glial cells which evolve from their positive supporting roles to those which disrupt synaptic function and neuroplasticity following injury. Evidence suggests that glial cells interact extensively with neurons both chemically and physically, reinforcing their role as pivotal for higher brain functions such as learning and memory. Collectively, this mini review surveys investigations of how glial dysfunction following brain injury can alter mechanisms of synaptic plasticity and how this may be related to an increased risk for persistent memory deficits. We also include recent findings, that demonstrate new molecular avenues for clinical biomarker discovery.

Keywords: astrocytes, microglia, oligodendrocytes, traumtic brain injury (TBI), biomarkers, MRS spectroscopy, memory impairment, gliosis

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INTRODUCTION

It is generally accepted, that neurons make up less than 25% of the cells in the brain, yet are responsible for information processing and control of bodily functions. Astrocytes, which make up 30–65% of glia and are the most abundant cell type in the brain, are multifunctional cells whose roles include maintaining osmotic balance and optimal ionic conditions for neurons, information processing via neurotransmitter recycling, and metabolite homeostasis (Kimelberg, 2005; Buffo et al., 2010; Kimelberg and Nedergaard, 2010). Collectively, these functions, as well as others, make the astrocytes indirectly involved in all brain function including memory formation (Moraga-Amaro et al., 2014). Microglia compose approximately 10% of total glia in the brain and are mainly identified by their function as immune cells of the central nervous system (CNS), arriving first at the injury site to initiate the inflammatory cascade. However, evidence indicates that "resting" microglia play a critical role in regulating synaptic and structural plasticity during learning and memory (Kettenmann et al., 2011, 2013; Scheff et al., 2013). Lastly, oligodendrocytes

provide support to axons with the production of the myelin sheath, which is vital for fast impulse conduction through the white matter (WM) tracts. These rapid interactions between brain regions are required for higher order brain functions like memory formation. Because of their high metabolic rates, oligodendrocytes are susceptible to the molecular consequences of tissue damage (McTigue and Tripathi, 2008). Oligodendrocyte death causes demyelination, impairment of axonal conduction, and ultimately axon death which contribute to memory impairment. Collectively, dysfunction of glia causes morphological and functional changes which effect the neural-glial and glial-glial interactions. Synaptic disconnections, imbalances of neurotransmitter homeostasis, and potential axonal degeneration and neuronal death can ultimately lead to memory impairment. Understanding the glia response, following injury at the molecular level may provide clues to decreasing chronic memory deficits.

SECONDARY INJURY AND METABOLIC DYSFUNCTION

Traumatic brain injury (TBI) is a complex, progressive condition that consists of primary and secondary injury mechanisms. Primary injury is due to direct mechanical insult and is the initiator of secondary molecular cascades. Secondary injury is characterized largely by metabolic imbalance and neuroinflammation (**Figure 1**). Following primary insult, brain cells experience energy depletion and a loss of calcium homeostasis, both of which are principal in mitochondrial function. Mitochondrial disruption is well documented in acute stages of TBI (Colicos and Dash, 1996; Xiong et al., 1997; Sullivan et al., 1998, 2005; Singh et al., 2006; Gilmer et al., 2009; Cheng et al., 2012). While these alterations are not glia-specific, they are intensified by activated glia. Because of the surge in extracellular ATP that results from damaged cells, glia are activated leading to downstream calcium release from endoplasmic reticulum (Locovei et al., 2006). Alterations in expression of various metabotropic receptors can occur as a result (Wang et al., 2012), contributing to surges of intracellular calcium. Increased cytosolic calcium is balanced by mitochondria at the expense of mitochondrial membrane potential. Eventually, mitochondria are driven to calcium overload and injury is exacerbated through generation of reactive oxygen species (ROS). Neurons are limited in their antioxidant capacity and thus rely on astrocytes to buffer ROS (Hamby and Sofroniew, 2010). Otherwise, they become susceptible to irreversible damage. Importantly, a pro-oxidative environment contributes to lipid, protein, and nucleic acid damage manifested largely in membrane disruption (Lewén and Hillered, 1998; Miller et al., 2015) and induction of neuroinflammation (Hsieh and Yang, 2013). Studies have concentrated on elucidating the roles of cellular sensors and enzymes that modulate intracellular calcium and ROS in metabolic dysfunction associated with death (Lu et al., 2014; Angeloni et al., 2015; Rao et al., 2015). Moreover, calcium signals in glial transmission are necessary for information processing and neuronal-glial coordination. Thus, impairment of glial-neuronal transmission contributes to memory loss (Walker and Tesco, 2013; Croft et al., 2015; Gundersen et al., 2015). In addition to calcium homeostasis, it is necessary to consider the consequence of potassium imbalances in secondary injury. Astrocytes normally uptake extracellular potassium via channels and Na+/K+/ATPase which in turn contributes to volume changes characteristic of TBI (Macaulay and Zeuthen, 2012; Larsen et al., 2014). Disruptions

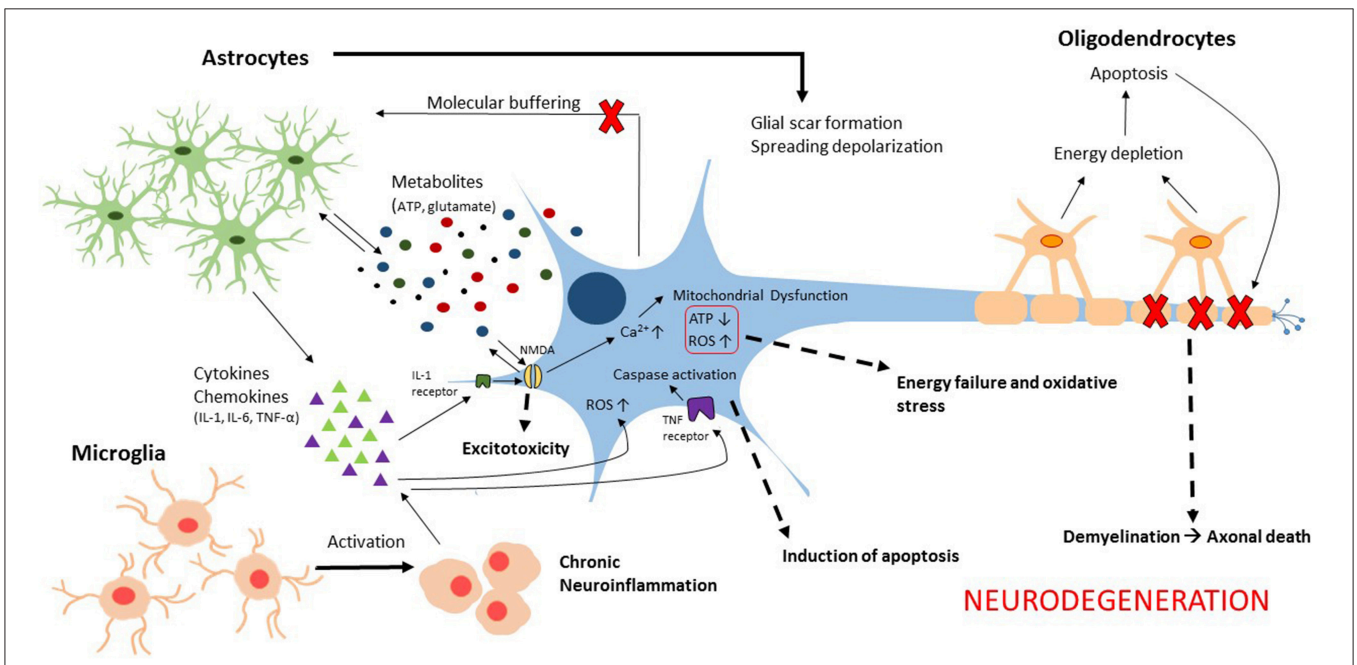

FIGURE 1 | Illustration of the glial contributions to secondary injury mechanisms associated with neurodegeneration following traumatic brain injury.

in potassium homeostasis, alongside neurotransmitter receptor activation, enhance neuronal impairment (D'Ambrosio et al., 1999; Pietrobon and Moskowitz, 2014).

TRAUMA-ASSOCIATED EDEMA

Cerebral edema is induced by water imbalance in response to trauma. Cytotoxic edema occurs in acute stages of TBI as a result of dysregulated metabolism. Often, there is a biphasic edema response in which early cytotoxic edema is followed by vasogenic edema associated with compromised blood-brain-barrier (BBB). Glia play an integral role in regulation of water and other molecules that transverse the BBB. Astrocytic endfeet directly contact brain vessels and are localized with aquaporin (AQP) proteins, which are pore proteins for water passage (Nielsen et al., 1997). Moreover, astrocytes are highly susceptible to swelling due to expression of AQPs (Suzuki et al., 2006; Satoh et al., 2007; Rao et al., 2011). Expression of AQP following trauma suggests that sustained AQP expression is critical in alleviating edema however, it is dependent on location relative to the injury, time, and variations in TBI models (Kiening et al., 2002; Sun et al., 2003; Zhao et al., 2005). A recent study reported only a small reduction in brain volume in AQP4 knockout mice with no evidence of difference in BBB disruption between AQP4 knockout and wildtype (Yao et al., 2015). Varied results may be related to a differential role of AQPs in the biphasic edema response. New theories hypothesize that AQP4 facilitates bulk flow through the glymphatic system, which poses contradiction to edema formation localized in astrocytic endfeet (Thrane et al., 2014). Evidence also suggests, that crosstalk exists between microglia and astrocytes in the regulation of AQP4 via microglial pattern recognition receptor-mediated pathways (Laird et al., 2014). Other studies are aimed to understand the effect of modulation of AQPs to ameliorate neuronal injury and cognitive deficits associated with TBI-induced edema (Tran et al., 2010; Shenaq et al., 2012).

EXTENSION OF CELLULAR DEATH

Much of the intercellular molecular buffering required for homeostasis in the brain is mediated by gap junctions (GJs), which consist of connexin (Cx) hemichannels that transverse the plasma membrane directly connect adjacent cells. Cx30 and Cx43 are expressed by astrocytes while Cx32 is expressed only by oligodendrocytes (Rash et al., 2001). GJs are necessary for the formation of astrocytic networks that interconnect neurons synapses and vessels (Giaume, 2010; Giaume et al., 2010). The role of Cx43 in CNS injury has been debated as both protective and detrimental for GJ communications (Chew et al., 2010). GJs allow for the passage of ions, metabolites, and other small molecules. Thus, an injured cell can distribute its damaging components to adjacent healthy cells. While this is potentially protective for injured cells, it also exacerbates the spread of injury. Studies have investigated the role of Cx43 expression in the expansion of cellular death (Frantseva et al., 2002; Lin et al., 2008; Sun et al., 2014; Rovegno et al., 2015). Inflammatory cytokines secreted by microglia activate Cx43 in astrocytes and can enhance N-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity in surrounding neurons (Froger et al., 2010). Additionally, Cx hemichannels are a route for the release of ATP to extracellular space, which exacerbates metabolic dysfunction and inflammation (Cotrina et al., 1998; Frantseva et al., 2002; Davalos et al., 2005; Figiel et al., 2007). It is also known, that release of transmitters, including ATP and glutamate, can perturb intercellular calcium signaling within astrocytic networks, which in turn may contribute to neuroinflammation and cell death (Choo et al., 2013; De Bock et al., 2014). There is evidence that Cx expression influences functional and cognitive outcomes from injury (Huang et al., 2012; Sun et al., 2015) as well as progressive neurodegeneration (Orellana et al., 2009).

REACTIVE GLIOSIS

Subsequent to insult, glia are transformed into a reactive state. Reactive gliosis is characterized by specific molecular and morphologic changes in microglia and astrocytes. Upon activation, microglia in combination with macrophages and astrocytes secrete cytokines (interferon-γ, tissue necrosis factor-α, interleukins-1 and 6 as well as transforming growth factor-β (TGF-β)) (Morganti-Kossmann et al., 2001; Li et al., 2009; Kumar and Loane, 2012; Aungst et al., 2014; Sajja et al., 2014b). While activation is initiated immediately upon injury, it is often sustained chronically which is linked to damaging neuronal homeostasis and memory deficits (Hanisch and Kettenmann, 2007; Ramlackhansingh et al., 2011; Mannix and Whalen, 2012; Smith et al., 2012; Johnson et al., 2013). Neuroinflammation is associated with ROS and the exacerbation of astrocyte activation. Evidence of prolonged neurotrophic effects from activated microglia has been reported (Nagamoto-Combs et al., 2007). This chronic inflammation has detrimental effects and contributes to neurodegeneration and memory impairment (Faden and Loane, 2015). Approaches to molecular and genetic influence on decreased microglial activation have resulted in decreased neuropathology (Yi et al., 2008; Dohi et al., 2010) and improved cognitive and functional outcomes (Erlich et al., 2007; Li et al., 2009; Kabadi et al., 2012; Cho et al., 2013).

Astrocyte reactivity or astrogliosis, is characterized by three hallmarks: hypertrophy, increased expression of intermediate filaments (glial-fibrillary acidic protein (GFAP), nestin and vimentin), and increased proliferation (Baldwin and Scheff, 1996; Sahin Kaya et al., 1999; Vandevord et al., 2008). Astrogliosis is dependent on interplay with activated microglia (Di Giovanni et al., 2005; Myer et al., 2006). Reactive astrocytes secrete molecules for regulation of the existing neuroinflammatory response (Panenka et al., 2001; Gorina et al., 2011), are integral in creating physical barriers associated with the BBB, as well as contribute to scar formation around injured tissue. The astrocytic scar inhibits axonal regrowth as cells will secrete growth inhibitors, such as TGF-β, thus affecting long-term cognitive outcomes. Although, most research focuses on modulation of astrogliosis, both the protective and inhibitory effects have been evaluated in the context of improved neuronal survival and cognitive abilities over time (Smith et al., 1997; Hoane et al., 2003; Wu et al., 2010; Madathil et al., 2013).

GLIAL CONTRIBUTION TO MEMORY DEFICITS

Oligodendrocyte dysfunction due to inflammation or cellular death impairs neurotransmission via degeneration of WM tracts (Smith et al., 1997; Gorina et al., 2011). Pre-clinical and clinical studies have shown axonal disruption associated with functional impairment (Lu et al., 2012; MacDonald et al., 2013; Calabrese et al., 2014). A non-human primate study reported a loss of WM integrity and astrocytic hypertrophy with increased AQP-4 contributed to cell death associated with cognitive impairment (Lu et al., 2012). Specifically, learning and memory has been shown to be associated with abnormal levels of myo-inositol, which is an astrogliosis marker (Sajja et al., 2014b). Resultants of gliosis directed toward dementia, such as tau and DNA methylation markers are found to be upregulated following TBI (Bailey et al., 2015; Sajja et al., 2015; Shultz et al., 2015). Another indicator linked to memory deficits is the disrupted homeostasis of extra and intra-cellular K+ channels in glia (D'Ambrosio et al., 1999). Furthermore, it has shown, that by blocking glial activation, cognition was improved (Homsi et al., 2010; Bedi et al., 2013). New research has shown the role of ependymal cells in contributing to memory deficits. Ependymal cells are specialized glia, that line the ventricles of the CNS. Ependymal cell lose was found to decrease ventricular flow following TBI which could negatively affect the waste and nutrient exchange within the brain (Xiong et al., 2014). Additional research that helps decipher the molecular pathways between glia and memory deficits will be vital for development of better clinical tools for gauging memory loss.

GLIA-BASED BIOMARKERS

The response of glia to TBI is multifaceted, supporting the importance of these cells to recovery. However, the intricate chemical and physical reactions of glia are very difficult to detect in the clinical setting. It is technically challenging to diagnosis and study the involvement of the glia in the recovery stages following injury and their contribution to memory deficits. Most minor TBI cases have normal findings in conventional neuroimaging [computed tomography (CT) and magnetic resonance imaging (MRI)]. While both basic and clinical research have made significant improvements, advancements are vital to fill the translational gap. Innovative technologies have emerged, such as serum biomarkers and *in vivo* magnetic resonance spectroscopy (^{1}H-MRS) which may provide the link needed to branch the basic and clinical arenas (**Figure 2**).

Serum Biomarkers

Minimally invasive techniques, such as serum biomarkers, can be used to detect brain-specific pathologies. With technological advancements in proteomics and lipidomics, finding accurate biomarkers that reflect glial health status would be tremendously valuable. GFAP is a common astrocytic marker, that has been detected in serum following TBI in both pre-clinical and clinical studies (Fraser et al., 2011; Vajtr et al., 2012; Papa et al., 2014;

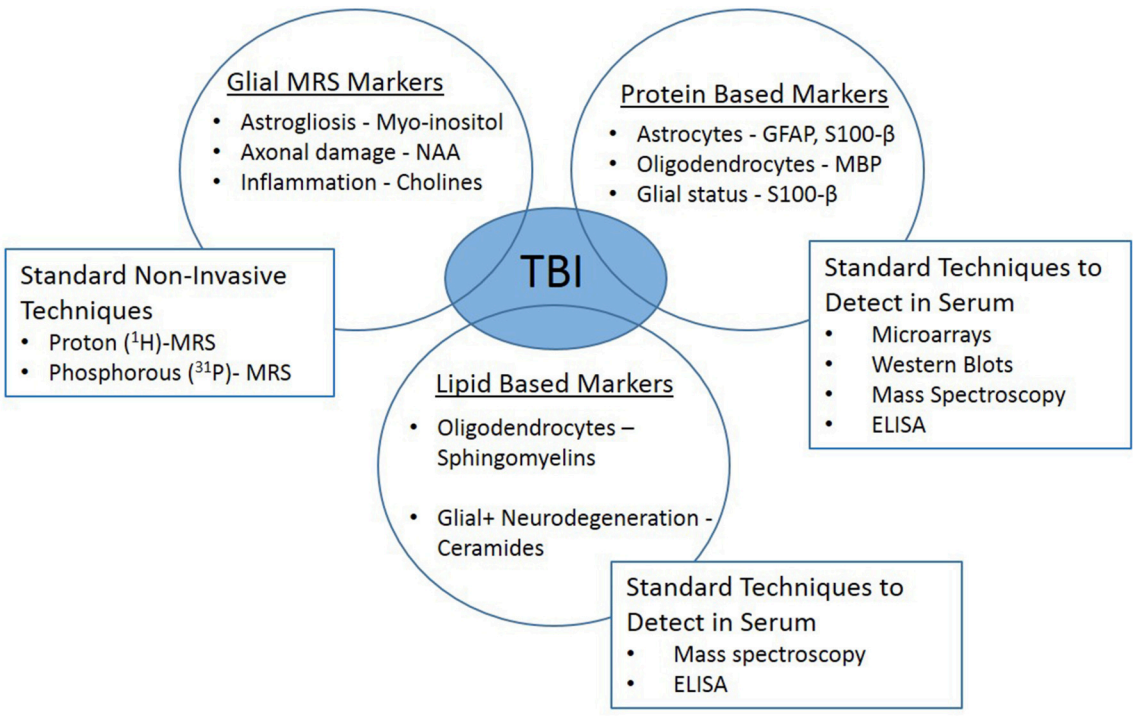

FIGURE 2 | Clinically translatable biomarkers for traumatic brain injury. *In vivo* magnetic resonance spectroscopy (^{1}H-MRS) and glial-specific serum biomarkers may provide the link needed to branch the basic and clinical research arenas.

Huang et al., 2015). Significant accumulation of GFAP persisted in blood up to 7 days post-injury (Svetlov et al., 2009; Boutté et al., 2015). Some have suggested, that the use of GFAP as a TBI biomarker yields a net benefit above clinical screening alone and may help avoid costly imaging scans without sacrificing diagnostic sensitivity (McMahon et al., 2015). S100 calcium-binding protein B (S100-β) is another serum biomarker that is clinically used to help in diagnosis of neurological disorders (Bouvier et al., 2012; DeFazio et al., 2014; Thelin et al., 2014). S100-β is expressed primarily by mature astrocytes and is present in the extracellular space surrounding glia, assisting with regulation of the cell calcium influx/efflux, but also linked to apoptotic environments (Gyorgy et al., 2011; Vajtr et al., 2012). Studies have identified S100-β as biomarker that could potentially be used in TBI diagnosis, however, others suggest, that GFAP is a better evaluator of TBI without skull fractures (Papa et al., 2014). Myelin-basic protein (MBP) is a specific marker of oligodendrocytes and was detected in blood, indicating potential disruption in myelin, thus leading to axonal injury (Gyorgy et al., 2011; Yan et al., 2014; Papa et al., 2015).

Lipid-based biomarkers such as sphingolipids, specifically sphingomyelins and ceramides, have recently become an active area of biomarker research. Sphingomyelin is abundant in the myelin membrane and abnormal levels in blood can constitute changes in myelin health and associations with oligodendrocyte injury (Haughey, 2010; Abdullah et al., 2014; Novgorodov et al., 2014; Henriquez-Henriquez et al., 2015; Koal et al., 2015). In addition, ceramide is metabolized from sphingomyelins and vice versa by sphingomyelinase. Ceramide is known to serve as a secondary messenger for intracellular activation of caspase-3 levels in cellular apoptosis (Haughey et al., 2010). Therefore, combination of changes in ceramide and sphingomyelin levels can predict the overall lipid status of myelin in the brain. Lipids are highly sensitive to changes in brain health, so they offer new diagnostic possibilities due to the development of robust and sensitive analytical methods (Touboul and Gaudin, 2014).

^{1}H-MRS

MRI is a non-invasive and widely accepted diagnostic modality to study brain abnormalities. While T1, T2, and T2* MRI can provide information related to gross anatomical changes, edema and cerebral hemorrhaging, ^{1}H-MRS provides more detailed chemical insight into the functional status and pathological prognosis of the brain (Sajja et al., 2012; Kantarci, 2013). Pre-clinical ^{1}H-MRS can resolve ∼25 and clinical ^{1}H-MRS about ∼10 metabolites depending on peak-suppression parameters (Moore and Galloway, 2002; Moffett et al., 2007; Sofroniew and Vinters, 2010).

N-acetyl aspartate (NAA) is a neurometabolite synthesized from aspartate and acetyl co-enzyme A. NAA or NAA/creatine (Cre) is trans-regulated between oligodendrocytes and neurons and can provide insight to structural integrity of WM (Charlton et al., 2006; Ariyannur et al., 2008; Kantarci, 2013). Studies have shown NAA levels in brain correlate with altered WM integrity following TBI (Pendlebury et al., 2000; Brooks et al., 2001; Shiino et al., 2004). Disruption in neuron-oligodendrocyte homeostasis can affect axon potentials and eventually neurotransmission, leading to an altered cognitive status. Since, alterations in the levels of NAA in WM-rich regions could indicate health status of oligodendrocytes and it can be measured by both pre-clinical and clinical ^{1}H-MRS, it has the potential to be an innovative translational avenue.

Reactive astrocytes rapidly accumulate in the injury region and alter their morphology, typically inducing swelling. This is related to osmolarity changes that result from edema or ischemia following TBI (Sofroniew and Vinters, 2010). Myo-inositol (Ins) is a primary metabolite that maintains brain osmolarity. Clinical studies have reported that an up-regulation of Ins correlates with astrogliosis in pathophysiological conditions such as TBI, dementia, and glioblastoma (Hattingen et al., 2008; Kantarci, 2013; Kierans et al., 2014). Pre-clinical studies have demonstrated that ^{1}H-MRS-resolved Ins was associated with astrogliosis and impaired cognition following TBI (Kierans et al., 2014; Sajja et al., 2014a).

In conjunction with astrocytes, microglia actively participate in clearing debris resulting from neuroinflammation. Changes in metabolites such as phosphoryethanolamine (PEA), glycerophosphocholine (GPC), and cholines (Cho) have been linked to microglia. PEA and GPC are involved in cell membrane turnover indicating neuroinflammation and GPC/PEA levels change depending of cell activation status (Sajja et al., 2012). Thus, they indicate compromised cellular activities.

Resolving ^{1}H-MRS peaks with lower signal-to-noise ratios depends on the field strength of the scanner, time of acquisition and number of repetitions of acquisition. Although, many metabolites can be resolved using pre-clinical MR scanners, only a small portion can be resolved with a clinical scanner which limits clinical translation. However, NAA, Ins, and Cho can be resolved with clinically available MR scanners. Thus, we highlighted the potential utility of clinical ^{1}H-MRS in combination with other modalities for differential diagnosis.

CONCLUSION

We have reviewed several glial-based molecules, that give clues to glia health status following TBI. There is a general consensus that a panel of markers will provide the most clinically relevant diagnostic tool. Thus, understanding how glial dysfunction following injury can alter mechanisms of synaptic plasticity and its relationship to an increased risk for persistent memory deficits is necessary for advancement. Researchers are actively pursuing new targets for a minimally invasive tools which can accurately and objectively detect brain injury. Combining sophisticated tools, such as serum biomarkers and MRS, will provide for an accurate differential diagnosis following TBI. Moreover, a temporal pattern of these markers could offer prognostic clues as to neuronal plasticity leading to memory formations.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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