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Review

Astroglial Glutamate Transporters in the Brain: Regulating Neurotransmitter Homeostasis and Synaptic Transmission

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Astrocytes, the major glial cell type in the central nervous system (CNS), are critical for brain function and have been implicated in various disorders of the central nervous system. These cells are involved in a wide range of cerebral processes including brain metabolism, control of central blood flow, ionic homeostasis, fine-tuning synaptic transmission, and neurotransmitter clearance. Such varied roles can be efficiently carried out due to the intimate interactions astrocytes maintain with neurons, the vasculature, as well as with other glial cells. Arguably, one of the most important functions of astrocytes in the brain is their control of neurotransmitter clearance. This is particularly true for glutamate whose timecourse in the synaptic cleft needs to be controlled tightly under physiological conditions to maintain point-to-point excitatory transmission, thereby limiting spillover and activation of more receptors. Most importantly, accumulation of glutamate in the extracellular space can trigger excessive activation of glutamatergic receptors and lead to excitotoxicity, a trademark of many neurodegenerative diseases. It is thus of utmost importance for both physiological and pathophysiological reasons to understand the processes that control glutamate time course within the synaptic cleft and regulate its concentrations in the extracellular space.

Key words: GLT-1; Astrocytes; surface trafficking; glutamate uptake

INTRODUCTION

Since the initial discovery of its excitatory action on neurons (Hayashi, 1954; Curtis and Watkins, 1960), glutamate has been found to be highly concentrated in the central nervous system of mammals, with estimates in the range of 5-15 mmol/kg (Danbolt, 2001). Yet this neurotransmitter is not uniformly distributed. Indeed, vast differences in glutamate concentrations of have been reported in extracellular, intracellular and subcellular compartments. As an example, the extracellular glutamate concentration is maintained at low values lying most likely below 100 nM (Hamberger and Nyström, 1984; Cavelier and Attwell, 2005; Herman and Jahr, 2007), while it is much more concentrated inside cells. As there are no extracellular enzymes degrading glutamate, this low extracellular concentration can be fully accounted for by specific transporters, which are widely expressed throughout the brain (Furuta et al., 1997). Glutamate transporters working under physiological conditions have the capacity to establish concentration gradients of 10^6 across the plasma membrane, theoretically resulting in a glutamate concentration of 10 nM extracellularly and 10 mM intracellularly (Zerangue and Kavanaugh, 1996a). They also allow the maintenance of gradients with subcellular compartments, as in synaptic vesicles where glutamate has been estimated to be present at concentrations up to 60 mM due to highly efficient vesicular glutamate transporters (Burger et al., 1989; Shupliakov et al., 1992; for review see El Mestikawy et al., 2011). In this review,

SIGNIFICANCE

This review is aimed at summarizing our present knowledge on astrocytes and their role in glutamate clearance. Astrocytes are non-neuronal cell present in our brain where they ensure several key fonctions. One of the most important role played by these glial cells is to clear the neurotransmitter glutamate from the extracellular space. This is essential to maintain an appropriate cerebral communication. Such clearance is made possible by highly-selective transporters that are expressed on astrocytes and which are endowed with specific properties and expression profile. They could represent a target of choice for therapuetic strategies in the context of neurodegenerative diseases.

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we discuss how these unique properties allow glutamate transporters to fulfil critical functions such as ensuring the kinetics and specificity of synaptic transmissions or preventing neurotoxicity associated with excessive activation of glutamate receptors (Tzingounis and Wadiche, 2007).

Distribution and Functional Properties of Glutamate Transporters

The family of brain glutamate transporters is composed of five members – namely excitatory amino acid transporters 1 to 5 (EAAT1-5)—which have been extensively reviewed elsewhere (Danbolt, 2001). EAAT1 (GLAST) and EAAT2 (GLT-1) are ubiquitously expressed in the brain, with GLT-1 being particularly abundant in the hippocampus and the cortex whereas GLAST is highly concentrated in the cerebellum (Rothstein et al., 1994; Lehre et al., 1995; Chaudhry et al., 1995; Schmitt et al., 1997; Furuta et al., 1997). EAAT3 (EAAC1) is also ubiquitous in the central nervous system and is particularly concentrated in the hippocampus where it is targeted to somatic and dendritic locations on neurons (Rothstein et al., 1994; Shashidharan et al., 1997; Holmseth et al., 2012). To note, EAAT3 is also expressed on GABAergic nerve terminals where it provides glutamate as a precursor for GABA synthesis (Sepkuty et al., 2002; Mathews and Diamond, 2003; Stafford et al., 2010). EAAT4 expression is essentially found in the cerebellum on the dendrites of Purkinje cells (Fairman et al., 1995; Dehnes et al., 1998). Finally, EAAT5 is very weakly expressed in the central nervous system and is preferentially found in the retina (Arriza et al., 1997; Barnett and Pow, 2000; Lee et al., 2012).

The EAAT family can be roughly divided based on cell-specific expression patterns, with EAAT1 (GLAST) and EAAT2 (GLT-1) mainly located on astrocytes while EAAT3-5 are exclusively neuronal. However, it is important to note that there is evidence supporting GLT-1 expression on cortical and hippocampal neurons, either from in situ hybridization or immunodetection approaches ex vivo and in cultured neurons (Schmitt et al., 1996; Wang et al., 1998; Mennerick et al., 1998; Melone et al., 2009). In the hippocampal CA1 area for example, glutamatergic axon terminals from CA3 pyramidal cells express functional GLT-1 with full glutamate transport ability which are believed to represent 5-10% of total hippocampal GLT-1 (Chen et al., 2004; Berger et al., 2005; Furness et al., 2008). While neuronal GLT-1 allow noticeable glutamate uptake within hippocampal nerve terminals, evidence is still lacking on whether this population of transporters is involved in the regulation of glutamate timecourse in the synaptic cleft. Recently, such neuronal transporters have been shown to contribute significantly to synaptosomal uptake activity (Furness et al., 2008; Petr et al., 2015; for review, see Danbolt et al., 2016).

Based on crystallisation data from a bacterial homologue of glutamate transporters, EAATs are believed to adopt a homotrimeric configuration, although there may be differences between subtypes (Haugeto et al., 1996; Yernool et al., 2003, 2004). All five EAATs transport L-glutamate (and DL-aspartate) with high affinities (~30 μM for GLT-1; Arriza et al., 1994, 1997; Fairman et al., 1995), using energy stored in the concentration gradients of sodium and potassium. During one individual transport cycle, each transporter binds one extracellular molecule of glutamate as well as three sodium ions and one proton. The transporter then undergoes a conformational change towards an inward facing conformation where these substrates are released into the cytoplasm. This step is followed by the binding of one internal potassium ion and a switch back to an outward facing state, completing the transport cycle (Zerangue and Kavanaugh, 1996a; Reyes et al., 2009). To note, EAAT3 also transports cysteine (Zerangue and Kavanaugh, 1996b). Moreover, uncoupled fluxes can occur and EAATs can function as chloride channels, particularly EAAT4 and EAAT5 which display considerable chloride conductance and may thus be alternatively envisioned as inhibitory glutamate receptors (Dehnes et al., 1998; Veruki et al., 2006).

Importance of Glutamate Transporters: Lessons From Knock-Out Models and Human Mutations

It was not until the 1990's that the different glutamate transporters were identified and cloned (Storck et al., 1992; Kanai and Hediger, 1992; Pines et al., 1992; Arriza et al., 1994, 1997; Fairman et al., 1995), which eventually led to the generation of subtype-specific glutamate transporter knockout mice. Overwhelming evidence from glutamate transporter knockout models demonstrated that glial glutamate transporters are critical for normal brain function (see Table I). Indeed, impaired GLT-1 activity causes a massive increase in extracellular glutamate levels in GLT-1 knockouts (Tanaka et al., 1997; Mitani and Tanaka, 2003; Takasaki et al., 2008). While mice appear to be normal at birth, they show lower body weight and start to suffer from hyperactivity as well as severe epileptic seizures after two to three weeks, a time-window when synaptic clearing of neurotransmitters shifts from passive diffusion to uptake by transporters (Ullensvang et al., 1997; Furuta et al., 1997; Thomas et al., 2011). About half of GLT-1-deficient animals die within the first month of life (Tanaka et al., 1997; Matsugami et al., 2006). Interestingly, cell-specific knock-outs models recently allowed to distinguish the relative importance of astrocytic versus neuronal GLT-1 (Petr et al., 2015) and confirmed that deletion of astrocytic GLT-1 (induced by expression of human GFAP-CreERT2) recapitulated the deficits observed in global knock-out models, whereas deletion of neuronal GLT-1 (induced by expression of synapsin-Cre) did not generate any behavioral alterations (Petr et al., 2015), although neuronal GLT-1 knock-out mice showed decreased glutamate content in synaptosomes (Petr et al., 2015). Associations of GLT-1 mutations in humans with amyotrophic lateral sclerosis (Trotti et al., 2001), addictive behaviors (Sander et al., 2000; Foley et al., 2004; dos Santos et al., 2012),

TABLE I. Summary of the Relevant Literature Regarding the Effects of Pharmacological Inhibition and Knock Out of Glutamate Transporters

Reference	KO or Drug	Target	Brain region	Preparation	Effect
Mennerick and Zorumski, 1994	THA and Lithium	Glutamate transporters	Hippocampus	Cultured neurons and glia	Increased decay of EPSCs
Barbour et al. 1994	D-aspartate, L-PDC, ACTD, D-th-asp	Glutamate transporters	Cerebellum	Cerebellar brain slices	Increased decay of EPSCs
Tong and Jahr, 1994	THA and Lithium	Glutamate transporters	Hippocampus	Cultured neurons and glia	Increased amplitude of AMPA EPSCs at 34°C but not at 24°C (no effect on kinetics)
Takahashi et al., 1996	D-aspartate	Post-synaptic glutamate transporters	Cerebellum	Cerebellar brain slices	Increased decay of EPSCs
Tanaka et al., 1997	Global GLT-1 knock out	GLT-1 (EAAT2)	Brain-wide	In vivo behavioral observations and hippocampal brain slices	Increased susceptibility to cortical injury; death from spontaneous seizures
Watase et al., 1998	Global GLAST knock out	GLAST (EAAT1)	Brain-wide	In vivo behavioral observations and cerebellar brain slices	Motor discoordination; susceptibility to cerebellar injury
Overstreet et al., 1999	L-PDC	Glutamate transporters	Cerebellum	Cerebellar brain slices	Increased decay of EPSCs
Matsugami et al. 2006	GLT-1 / GLAST double knock out	GLT-1 and GLAST	Brain-wide	Brain-wide fixed tissue imaging	Impairment of neuronal development, differentiation, and stem cell proliferation
Petr et al., 2015	Cell-specific (astrocytic / neuronal) conditional knock out of GLT-1	Astrocytic or neuronal GLT-1	Brain-wide	3H-glutamate uptake	Astrocytic KO: 80% impairment in glutamate uptake, seizures, abnormal weight and premature death; Neuronal KO: reduction in synaptosomal uptake, normal behavior

essential tremor (Thier et al., 2012; Yu et al., 2013), schizophrenia and bipolar disorders (Deng et al., 2004; Spangaro et al., 2012; Dallaspezia et al., 2012; Poletti et al., 2014b, 2014a; O'Donovan et al., 2015; Fiorentino et al., 2015; McCullumsmith et al., 2015) have been reported. GLAST knockout mice develop properly but exhibit a mild phenotype with impaired motor coordination and increased susceptibility for cerebellar injury consistent with the predominant role of GLAST in glutamate uptake in the cerebellum (Watase et al., 1998). Furthermore, while GLAST-deficient mice do not show increased susceptibility to seizures, they display enhanced seizure duration when seizures are experimentally induced (Watanabe et al., 1999). Consequently, human mutations in the EAAT1 gene (SLC1A3) are associated with both ataxia and seizure (Jen et al., 2005; de Vries et al., 2009).

Deletion of neuron-specific glutamate transporters has demonstrated that these proteins are not vital for survival and mice lacking neuronal transporters display no marked neurodegeneration but can present behavioral abnormalities (Peghini et al., 1997) as suggested by association between mutations in the gene coding for EAAT3 (SLC1A1) and obsessive compulsive disorder in humans (Shugart et al., 2009; Dallaspezia et al., 2014). Overall, the normal phenotype of EAAT3 knockouts is in good accordance with its relatively low expression levels (Holmseth et al., 2012). However, because putative EAAT3 expression on GABAergic terminals—which yet remains an open debate—has been proposed to favour inhibitory transmission by providing extracellular glutamate for GABA synthesis, downregulation of EAAT3 has often been associated with hippocampal hyper-excitability (Sepkuty et al., 2002; Mathews and Diamond, 2003; Stafford et al., 2010).

Roles of Glutamate Transporters in Shaping Excitatory Transmission

It was initially hypothesised that the brief duration of synaptic events could result from the rapid diffusion of neurotransmitters away from the synaptic cleft (Eccles and Jaeger, 1958). Since then, evidence has come to light that

diffusion alone cannot account for quick synaptic transients and that synaptic transmission termination involves the action of specific neurotransmitter uptake and/or degradation systems. Indeed, while diffusion is the prominent mechanism by which the synaptic activity of glutamate is curtailed during the first two post-natal weeks, the brain undergoes a rapid shift after this period towards specific transporters as the predominant means to terminate glutamate signalling (Thomas et al., 2011). Glial glutamate transporters appear to be central actors in the regulation of glutamate timecourse at the synapse (Barbour et al., 1994), as highlighted by their high expression levels (1% of total brain proteins; Lehre and Danbolt, 1998) and location close to synapses. During a synaptic event, glutamate release results in an excitatory postsynaptic current (EPSC), i.e., a flux of cations across the membrane, mediated by activation of postsynaptic glutamate receptors, leading to a depolarisation of the cell. The kinetics of these EPSCs is a product of two main factors: (i) the concentration and timecourse of the neurotransmitter at the synapse and (ii) the properties of the postsynaptic receptors activated by the neurotransmitter. Under physiological conditions, the concentration and timecourse of glutamate in the synaptic cleft is short and sharp, reaching about 1 mM and lasting approximately 1.2 ms (Clements et al., 1992). AMPA- and NMDA-type glutamate receptors—the main contributors to glutamate-mediated excitatory neurotransmissions—become desensitised with prolonged exposure to glutamate (Mayer and Westbrook, 1985; Hestrin, 1992; Colquhoun et al., 1992; Trussell et al., 1993). Whereas desensitisation of NMDARs occurs relatively slowly (hundreds of milliseconds; Forsythe and Westbrook, 1988), AMPARs desensitise rapidly and saturating concentrations of glutamate result in EPSCs of about 10 ms (Hestrin, 1992; Colquhoun et al., 1992; Lawrence et al., 2003). Thus, it appears that receptor desensitisation does not play a major role in controlling the timecourse of postsynaptic receptor activation during low neurotransmitter release events as, according to Clements et al. (1992), the synaptic glutamate transient is shorter than the maximum potential EPSC. Rather, the desensitisation of glutamate receptors is believed to play a more important role in curtailing postsynaptic excitation during multivesicular release of glutamate (Trussell et al., 1993) where the timecourse of glutamate at the synaptic cleft is prolonged.

A large body of evidence implicating glutamate transporters in synaptic transmission has come from the use of specific uptake inhibitors (Table I), leading to a build-up of synaptic glutamate as witnessed by increased glutamate receptor activation (Mennerick and Zorumski, 1994; Barbour et al., 1994; Tong and Jahr, 1994; Takahashi et al., 1996; Overstreet et al., 1999). Although documented in several brain areas including the hippocampus (Bergles and Jahr, 1997) and the cerebellum (Clark and Barbour, 1997), this involvement of transporters in the synaptic timecourse of glutamate has remained a matter of debate due to experimental variability which is likely to result from the brain preparation used, as astrocytic coverage of synapses is not uniform (Ventura and Harris, 1999), is highly plastic (Genoud et al., 2006; Ostroff et al., 2014; Bernardinelli et al., 2014), and astrocytic proximity to the synapse is known to have an impact upon glutamate uptake (Oliet et al., 2001; Melone et al., 2009; Pannasch et al., 2014). In the cerebellum where astrocytes are believed to completely ensheath synapses, pharmacological blockade of glutamate transporters using the competitive uptake inhibitors (i.e., competing for the glutamate binding site) D-aspartate or L-trans-pyrrolidine-2,4-dicarboxylate (PDC) results in prolonged AMPAR activation as evidenced by slower decay kinetics of AMPAR-mediated EPSCs (Barbour et al., 1994; Takahashi et al., 1996). Within the mossy fibre – granule cell glomerulus of the cerebellum, Overstreet and colleagues (1999) also observed prolonged AMPAR-mediated EPSCs upon application of the glutamate uptake inhibitor PDC, as well as glutamate spillover between synapses adjacent to the glomerulus during sustained activity. Importantly, the impact of glutamate transporter inhibition on the timecourse of synaptic glutamate strongly depends on experimental design. Indeed, while blocking glutamate transporters seems to have no impact at 24°C, it is associated with an increase in the amplitude of AMPAR EPSCs at 34°C (Tong and Jahr, 1994), suggesting that glutamate transport is a temperature-dependent process and that most studies carried out at room temperature may have underestimated the role of transporters in the control of synaptic glutamate receptor activation.

In contrast to the cerebellum, astrocytic ensheathment of hippocampal synapses has been reported to be incomplete with 60% coverage only (Ventura and Harris, 1999), which could possibly suggest a physiological role of glutamate spillover in this brain region. As such, findings from the hippocampus have been rather mixed with studies reporting delayed kinetics of AMPAR EPSCs following transporter blockade—either through the action of competitive uptake blockers or by replacing sodium with lithium in the aCSF to prevent the occurrence of full transport cycles—as in the cerebellum (Mennerick and Zorumski, 1994), whereas others discounted the role of transporters in the hippocampus, either because of incomplete astrocytic coverage of synapses in this brain region or due to the observation that glutamate can diffuse out of the cleft and act on adjacent synapses (Kullmann et al., 1996). To note, it seems that experimental conditions can dramatically impinge on experimental estimations of the role of glutamate transporters in this brain region and may explain this apparent discrepancy. In particular, Asztely and colleagues reported that spillover of neurotransmitter between synapses is greatly reduced at physiological temperatures since diffusion of neurotransmitters, receptor kinetics and neurotransmitter transport are temperature-dependent processes (Asztely et al., 1997). It is also worth mentioning that competitive uptake inhibitors potentially yield higher concentrations of extracellular glutamate in comparison to non-competitive inhibitors, therefore leading to greater glutamate spillover and increased receptor desensitisation. In

addition, astrocyte process mobility (Genoud et al., 2006; Ostroff et al., 2014; Bernardinelli et al., 2014) as well as glutamate transporter expression and surface localisation (Benediktsson et al., 2012; Murphy-Royal et al., 2015; Al Awabdh et al., 2016) are dynamically regulated by neuronal as well as their intrinsic activity. Thus, careful attention to experimental design must be paid when interpreting and comparing individual findings.

Glutamate transporters can further actively control the duration of excitatory transmissions by competing for glutamate in the synaptic cleft and thereby curtailing glutamate receptor activation. This hypothesis emerged following the demonstration that transporters have an affinity for glutamate comparable to that of glutamate receptors, with the Km of glutamate transporters roughly 30 μM (Arriza et al., 1994), and was further supported by evidence from transporter currents recordings in astrocytes indicating that transporters can bind glutamate as rapidly as AMPARs (Wadiche et al., 1995; Bergles and Jahr, 1997). However, how transporters carry out this role remains an open question, especially when considering that the duration of a complete transport cycle is comprised between 11 to 70 ms depending on experimental conditions (Wadiche et al., 1995; Otis and Jahr, 1998; Bergles and Jahr, 1998). It was first hypothesised that glutamate transporters are able to remove glutamate on a synaptic time scale through glutamate binding, thus shielding receptors from the neurotransmitter (Diamond and Jahr, 1997). This hypothesis was strengthened by manipulations of the resting membrane potential of astrocytes to block transport but not binding of glutamate to the transporters, which allowed to show that glutamate binding and translocation across the astrocyte membrane play a strong role in controlling the timecourse of synaptic glutamate (Mennerick et al., 1999). These observations are in agreement with previous work on glutamate transporter kinetics which proposed that it is not the glutamate translocation but rather translocation of the potassium ion which is the rate limiting step of the transport cycle (Wadiche et al., 1995; Otis and Jahr, 1998). Other studies further elucidated the role of transporters in synaptic transmission using more physiological preparations, taking advantage of the structural changes in astrocytic coverage of the synapse known to occur during lactation (Theodosis and Poulain, 1984). Using this preparation, Oliet and colleagues (2001) observed an increase in presynaptic metabotropic glutamate receptor (mGluR) activation when glutamate transporter function was reduced by morphological changes or blocked pharmacologically, thereby decreasing the probability of neurotransmitter release (fig. 1). This physiological model definitively demonstrated that astrocytic coverage of synapses regulates synaptic efficacy by controlling presynaptic glutamate receptor activation through glutamate uptake.

Strong evidence indicates that another primary role of glutamate transporters is to control spillover of glutamate between synapses and maintain the specificity of synaptic transmissions. Recent studies have suggested that glutamate transporters play a limited role in glutamate uptake during single synapse/low activity events, but that transporter activity is crucial during high activity/multi-synapse events to control glutamate receptor activation and prevent spillover to maintain input specificity, both in the hippocampus and the cerebellum (Arnth-Jensen et al., 2002; Marcaggi et al., 2003). Under specific conditions, spillover of glutamate can play a physiological role. In the mossy fibre glomerulus of the cerebellum, spillover from excitatory mossy fibre synapses onto inhibitory granule cell interneurons activates presynaptic mGluRs, which triggers a decrease of neurotransmitter release (Mitchell and Silver, 2000). Importantly, activation of mGluRs reduces the GABAergic tone from these interneurons resulting in feedforward disinhibition, thereby increasing the efficacy of excitatory mossy fibres. The architecture of mossy fibre glomerulus excludes a role for astrocytic glutamate transporters in this synaptic spillover inside the glomerulus, as astrocytes are only found on the periphery. Rather, astrocytes surround this structure to limit glutamate spillover onto extra-glomerular synapses. In the hippocampus, glutamate spillover is also believed to play a role in synaptic unsilencing through the recruitment of AMPARs to silent synapses initially containing NMDAR only (Kullmann et al., 1996; Isaac et al., 1997; Malinow

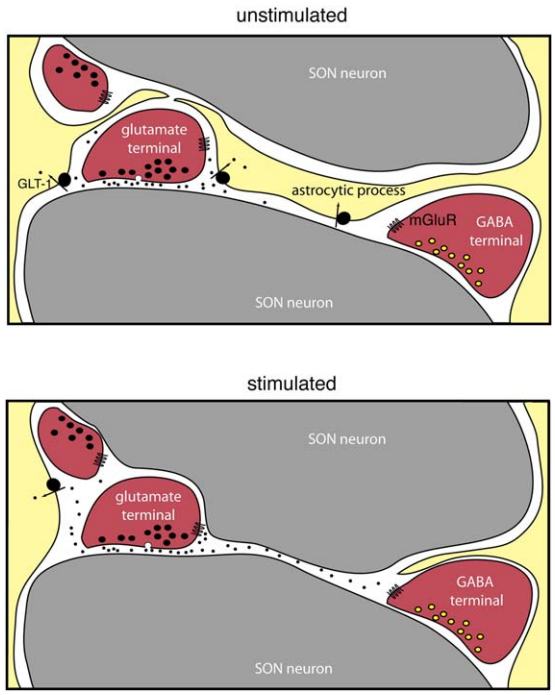

Fig. 1. Under unstimulated conditions (upper panel), the synaptic elements are enwrapped by thin astrocytic processes in the supraoptic nucleus. Glutamate release from excitatory terminals is rapidly cleared from the extracellular space by GLT-1 transporters expressed on astroglia, thereby controlling the concentration of the transmitter in the cleft while preventing its diffusion outside the synapses. Under stimulated conditions such as lactation (lower panel), the remodelling that affect the astrocytic processes enables glutamate to build up locally and to diffuse away, thereby increasing the activation of auto-receptors and favouring heterosynapic modulation respectively.

and Malenka, 2002). As previously mentioned, spillover of glutamate in this brain area is tightly regulated by transporters in order to regulate efficiently heterosynaptic plasticity (Asztely et al., 1997). Therefore it is likely that high levels of activity are needed to induce synaptic unsilencing.

Because each transport cycle requires the consumption of one molecule of ATP (Danbolt, 2001), astrocytic glutamate uptake may seem to be a major energetic drain. However, the brain has evolved efficient neurotransmitter recycling systems allowing to avoid additional energy expenses (for review, see Harris et al., 2012). Indeed, astrocytic glutamate uptake represents the major pathway by which glutamate is cleared from the extracellular space and recycled. Once glutamate is removed from the synaptic cleft by astrocytic transporters, it is converted into the non-biologically active glutamine. Glutamine is then exported back to the extracellular space through system N transporters and imported by the SLC38A1 and SLC38A2 Na^+-coupled transporters into presynaptic terminals where it is converted back into glutamate (for review, see Bhutia and Ganapathy, 2016). Although complex, this system demands less energy compared to the production of glutamate through the citric acid cycle, thus lowering the energetic costs of excitatory neurotransmissions. Importantly, part of the glutamate imported by astrocytes can also be used for energy production in the TCA cycle. Glutamate can be readily converted into alpha-ketoglutarate by transamination reactions or by reaction with the enzyme glutamate dehydrogenase, which was shown to be highly enriched in astrocytes (Lovatt et al., 2007). The complete oxidation of one glutamate molecule results in a vastly higher yield of ATP for astrocytes (from 24 up to 27 ATP per glutamate molecule) compared to the single ATP consumed to import glutamate inside the cell (for review see McKenna, 2013). Evidence suggests that the majority of glutamate taken up by astrocytes could be consumed in energy production as opposed to glutamine production and glutamate recycling (Sonnewald et al., 1993). Finally, as mentioned above and although it remains controversial, hippocampal GABAergic terminals have been proposed to express EAAT3 glutamate transporters which could be responsible for providing inhibitory neurons with glutamate as a precursor for GABA production (Rothstein et al., 1994; Mathews and Diamond, 2003; Stafford et al., 2010).

Together, these reports illustrate the diversity of functions achieved by glutamate transporters in regulating neurotransmitter homeostasis and recycling, controlling the kinetics of synaptic transmission, ensuring input specificity, and allowing heterosynaptic plasticity or synaptic unsilencing.

Terminating Synaptic Transmission: Strength in Numbers and Surface Dynamics

Although it appears that glutamate transporters precisely tune the timecourse of glutamate in the synaptic cleft, their uptake performances remain limited by an affinity for glutamate lower than NMDA receptors and almost equal to that of AMPA receptors (Arriza et al., 1994), and a transport probability of 50-70% only once a molecule of glutamate is bound to the transporter (Otis and Jahr, 1998). This raises the question of how these proteins maintain strict extracellular levels of glutamate and keep up with the pace of synaptic glutamate release. One means by which transporters efficiently clear glutamate from the synaptic cleft is by sheer strength in numbers. Immunoblot assays have estimated GLT-1 to account for 1% of total brain proteins (Lehre and Danbolt, 1998). This staggering concentration of transporters in the brain is believed to be necessary to efficiently remove glutamate from the cleft on a millisecond timescale, due to the rather slow uptake cycle of the transporter itself. The lifetime of glutamate in the synaptic cleft is less than 10 ms (Clements et al., 1992), however one individual transporter removes glutamate from the synaptic cleft at a rate of one molecule every 11 ms at its optimal rate (Bergles and Jahr, 1998). Therefore, considering that a single synaptic vesicle contains roughly 4000 molecules of glutamate (Clements et al., 1992), it is necessary to have more transporters than molecules of glutamate present at the synaptic cleft in order to rapidly terminate the action of the neurotransmitter. The few studies which have investigated the concentration of transporters in the brain confirmed this assumption by demonstrating that hippocampal brain tissue contains GLT-1 at a concentration of 8,500 transporters/μm^2 as well as GLAST at 2,500 transporters/μm^2 (Lehre and Danbolt, 1998), which alongside simple diffusion of glutamate from the synaptic cleft to the extrasynaptic space should be sufficient to clear glutamate rapidly.

In the past decade accumulating evidence demonstrated that neuronal proteins, such as receptors and transporters, were not static structures in the plasma membrane but were rather mobile and dynamic in their cycling between intracellular and extracellular compartments as well as between compartments on the cell surface (Choquet and Triller, 2013; Ladépêche et al., 2014). Not surprisingly, this is not a strictly neuronal phenomenon and several studies have now shown evidence for surface diffusion of receptors, transporters and water channels in the plasma membrane of astrocytes (Crane et al., 2010; Arizono et al., 2012; Toulme and Khakh, 2012; Murphy-Royal et al., 2015; Al Awabdh et al., 2016). With regards to surface diffusion of transporters, it was recently demonstrated that surface diffusion of GLT-1 is instrumental in the control of the glutamate timecourse at the synapse. Although the concentration of transporters expressed at the plasma membrane is undoubtedly critical in the rapid removal of glutamate from the cleft, astrocytic ensheathment of hippocampal synapses is believed to be incomplete (Ventura and Harris, 1999). Indeed as discussed above, electron microscopy evidence shows that only 60 % of each individual hippocampal synapse is ensheathed by astrocytes (Ventura and Harris, 1999), suggesting that the effective number of astrocytic transporters is limited by the size of the

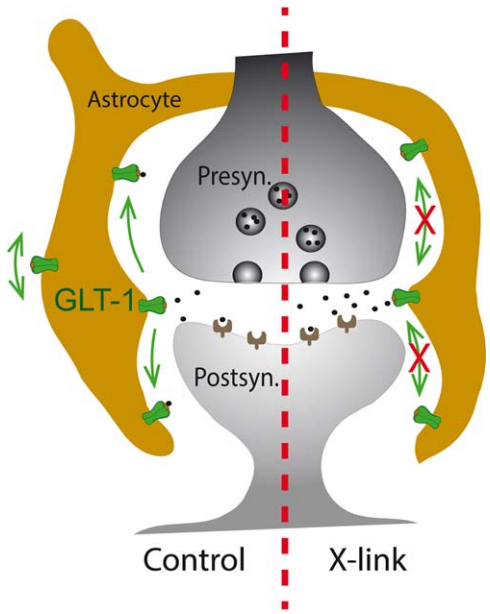

Fig. 2. Schematic diagram illustrating the membrane mobility of GLT-1 at the surface of astrocytic processes under control conditions (left) and under conditions were the ability has been reduced through cross-linking (X-link; right). Under control conditions, binding of glutamate to GLT-1 transporters will cause them to be displaced away from the vicinity of synaptic contacts, thereby favouring the turnover of unbound GLT-1 at the site of glutamate release. Under X-link conditions, the reduced mobility impaired such a turnover, resulting in accumulation of glutamate within the synaptic cleft (From Murphy-Royal et al., Nat Neurosci 2015).

astrocytic membrane opposing the synaptic cleft. To note, astrocytic processes appear to be highly mobile (Genoud et al., 2006; Ostroff et al., 2014; Bernardinelli et al., 2014) and thus insights on astrocytic ensheathment of synapses gained from fixed-tissue electron microscopy studies are limited by the fact that this technique does not allow access to the dynamic movements of astrocytic processes over time and may impact upon astrocytic morphology (Heller and Rusakov, 2015), which may both lead to over- or underestimations. However, full coverage of hippocampal synapses by astrocytes is not likely. It has been demonstrated that GLT-1 diffuses slowly when opposed to the synaptic cleft, likely supporting accumulation of transporters at the vicinity of synapses (Murphy-Royal et al., 2015; Al Awabdh et al., 2016). Once exposed to glutamate, GLT-1 shows increased surface mobility and exits astrocytic membrane areas in the vicinity of synapses through Brownian diffusion (fig. 2), allowing glutamate-bound transporters to exchange for naive ones reaching this confined space (Murphy-Royal et al., 2015; Al Awabdh et al., 2016). Therefore, surface diffusion of transporters along the astrocytic membrane from 'synapse-facing' to extrasynaptic compartments is likely to aid in increasing total numbers of transporters on a short timescale. It would be very interesting to see the role of surface diffusion of transporters in other brain regions, particularly in the cerebellum, where Bergmann glia completely ensheath Purkinje cell synapses. Perhaps this more constrained synaptic environment, which impedes glutamate diffusion out of the synapse, may favour a greater role for surface trafficking of glutamate transporters on the astrocytes in clearance of the synaptic cleft.

Glutamate Transporters as Potential Therapeutic Targets

As mentioned above, glutamate transporter dysfunction is believed to play a role in many diseases of the central nervous system and in particular in excitotoxicity-related disorders. As such, glutamate transporters make for good potential therapeutic targets. However, our understanding of the complex regulation of glutamate transporter transcription and translation (Ghosh et al., 2011, 2015, 2016), cell surface expression and stabilisation (Stenovec et al., 2008; Yang et al., 2009, 2010; Underhill et al., 2015), and internalisation (González-González et al., 2008; Sheldon et al., 2008; Underhill et al., 2014; Ibáñez et al., 2016) is still rather limited. Deciphering the mechanisms by which glutamate transporters are expressed and stabilised at the cell surface is of utmost importance, as it could reveal innovative targets for the treatment of diseases such as traumatic brain injury or epilepsy where extracellular glutamate is high. In particular, exploring interactions with other proteins or intracellular organelles emerges as important field in further understanding the regulation of glutamate transporters. Indeed, the list of direct and indirect interactors of glutamate transporters is extensive and includes Na/K-ATPase pumps, glycolytic enzymes, mitochondrial proteins (Genda et al., 2011; Bauer et al., 2012; Jackson et al., 2014; Ugbode et al., 2014), Aquaporin-4 (Zeng et al., 2007; Li et al., 2012), discs large homologue 1 (DLG1; Underhill et al., 2015) and multiple glutamate transporter associated proteins (GTRAPs) (Jackson et al., 2001; Lin et al., 2001). The large body of evidence for GLT-1 interaction with Na/K-ATPase pumps, mitochondria, and glycolytic enzymes is suggestive of a macromolecular functional complex which could contribute to the regulation of transporter activity, although in situ imaging evidence of these complexes is still lacking and would require deeper investigations, for example using electron microscopy that can easily resolve large multi-protein complexes.

As many of the neurodegenerative diseases associated with glutamate transporter dysfunctions involve a downregulation of transporter function and/or expression (Trotti et al., 2001; McCullumsmith et al., 2015), research has been directed towards the development of molecules with the potency of enhancing glutamate transporter expression. Interestingly, beta-lactam antibiotics (e.g., ceftriaxone) were found to increase glutamate transporter expression and thereby trigger neuroprotection in ischemic injury and motor neuron degeneration models (Rothstein et al., 2005). Since this pivotal discovery, beta-lactam antibiotics have been repeatedly used by

many laboratories to trigger neuroprotection through transporter expression manipulation in a number of pathological models spanning from multiple sclerosis (Melzer et al., 2008) to alcohol intake in rodents (Sari et al., 2011). Whether beta-lactam antibiotics offer efficient neuroprotection in humans still remains an open question which will require further clinical trials. To note, a recent randomised, double-blind, placebo-controlled study investigated the efficacy of ceftriaxone in treating human patients with ALS (Cudkowicz et al., 2014). While the drug proved promising in the lab, it failed to alleviate the symptoms of ALS with no difference in survival rates between drug- and placebo-treated patients. Unfortunately, the functional binding of ceftriaxone to its target and the action of ceftriaxone on glutamate transporter expression levels were not investigated. Therefore, while ceftriaxone remains a good experimental tool for manipulating glutamate transporter expression in the lab, we are yet to fully understand its action spectrum on the human organism and whether/how it may be used and improved in order to increase its efficacy as a neuroprotective agent.

Future Directions

While the key action of glutamate transporters in synaptic transmission regulation is now well established, evidence is still lacking for potential roles of transporters in higher brain processes. Neurotransmitter receptors undergo dynamic regulations in order to store information in the brain and behave accordingly when further stimulation is received. However, whether glutamate transporters (or transporters for other neurotransmitters) undergo certain forms of plasticity in response to physiological stimuli is unknown. Recent reports have revealed astrocytic morphological plasticity changes following specific learning paradigms such as fear conditioning (Ostroff et al., 2014), but whether glutamate transporter activity or expression is remodelled by experience remains to be investigated. Interestingly, the existence and function of protein multimers and protein-organelle complexes on astrocytes in a physiological setting remains an exciting yet open question. There is now a large body of evidence of protein co-compartmentalization in astrocyte processes which includes Na,K-ATPase pumps, glutamate transporters and mitochondria (Genda et al., 2011; Bauer et al., 2012; Jackson et al., 2014; Ugbode et al., 2014). However, expression of these complexes in situ and precise understanding of their physiological role is lacking. Nevertheless, recent reports suggest that these interactions are activity-dependent (Jackson et al., 2014) and may play an important role in the regulation of astrocytic calcium signalling (Jackson and Robinson, 2015), and further attention will have to be dedicated to their involvement in glutamate transporter function. Finally, glutamate transport is an electrogenic process involving ion fluxes (Na^+, K^+, H^+) across the plasma membrane (for review see Cater et al., 2015). Thus, an open question is whether transporters might act as glutamate receptors relaying information to astrocytes on neuronal activity, and exploring putative glutamate transporter-mediated ion signalling in astrocytes may reveal new mechanisms involved in neuron-glia interactions.

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CONFLICT OF INTEREST STATEMENT

The authors declare no known conflicts of interest.

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