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BRAIN RESEARCH REVIEWS

Review

Neurotransmitter transporters expressed in glial cells as regulators of synapse function

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ABSTRACT

Synaptic neurotransmission at high temporal and spatial resolutions requires efficient removal and/or inactivation of presynaptically released transmitter to prevent spatial spreading of transmitter by diffusion and allow for fast termination of the postsynaptic response. This action must be carefully regulated to result in the fine tuning of inhibitory and excitatory neurotransmission, necessary for the proper processing of information in the central nervous system. At many synapses, high-affinity neurotransmitter transporters are responsible for transmitter deactivation by removing it from the synaptic cleft. The most prevailing neurotransmitters, glutamate, which mediates excitatory neurotransmission, as well as GABA and glycine, which act as inhibitory neurotransmitters, use these uptake systems. Neurotransmitter transporters have been found in both neuronal and glial cells, thus suggesting high cooperativity between these cell types in the control of extracellular transmitter concentrations. The generation and analysis of animals carrying targeted disruptions of transporter genes together with the use of selective inhibitors have allowed examining the contribution of individual transporter subtypes to synaptic transmission. This revealed the predominant role of glial expressed transporters in maintaining low extrasynaptic neurotransmitter levels. Additionally, transport activity has been shown to be actively regulated on both transcriptional and post-translational levels, which has important implications for synapse function under physiological and pathophysiological conditions. The analysis of these mechanisms will enhance not only our understanding of synapse function but will reveal new therapeutic strategies for the treatment of human neurological diseases.

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1. Introduction

In the central nervous system (CNS), neurotransmission proceeds with high temporal and spatial resolutions. At chemical synapses, transmitters presynaptically released upon arrival of an action potential diffuse through the synaptic cleft where they bind and activate postsynaptic receptors. Termination of this signal requires the released neurotransmitters to be rapidly removed or inactivated on a millisecond timescale. At cholinergic synapses, e.g. at the neuromuscular junction, this is achieved by rapid degradation of acetylcholine by the enzyme acetylcholinesterase. Other transmitters, like glutamate, glycine and γ-amino butyric acid (GABA), are not degraded extracellularly, but termination of neurotransmission and prevention of neurotransmitter spreading into the extrasynaptic space are achieved by active transport of transmitter molecules into the surrounding neuronal and glial cells through complex transport systems.

Different studies have highlighted the important role of glial cells on the functional status of the CNS. Glia modulates clearance, availability and release of both excitatory and inhibitory neurotransmitters, therefore operating as key elements for controlling brain homeostasis. The necessity for involving glia in the clearance and/or recycling of transmitters released at high frequency is most likely due to the limited membrane surface present at the neuronal synaptic terminals. This physical constriction excludes the positioning of a high number of neuronal transporter molecules close to the transmitter release zone. Surrounding glial cells supply additional surface that allows the positioning of increased uptake capacity through high-affinity transporters in the perisynaptic region. This finally results in efficient recycling pathways through glia and prevents constant loss of neurotransmitters from the synapses. Thus, e.g. in the GABAergic system, synaptically released GABA is transported into astrocytes where it is either degraded to CO_2 or converted to glutamine in order to be subsequently transferred to GABAergic neurons and re-transformed to GABA. Moreover, several steps required for efficient synthesis of some neurotransmitters, e.g. glutamate, occur through enzymes exclusively located in the astrocytes. In addition to their contribution to recycling and synthesis of neurotransmitters, glial cells have been found to release neurotransmitters in specific CNS regions, e.g. GABA in rat dorsal root ganglia (Minchin and Iversen, 1974; Roberts, 1974). This action is possibly mediated by a non-vesicular transporter mediated release, as transporters are expected to reverse under circumstances such as local cell depolarization or low extracellular neurotransmitter concentrations (Richerson and Wu, 2003). Thus, glial cells may play a key role in directly regulating the basal transmitter concentration at extrasynaptic sites. Conversely, neurotransmitters known to have a relatively low turn-over rate, like dopamine, serotonin or norepinephrine, use exclusively neuronal transporters for reuptake and termination of neurotransmission. In summary, glial cells have been shown to play an active role in the maintenance and modulation of fast neurotransmission in tight cooperativity with neuronal cells.

The significance of glial transport in the regulation of neurotransmitter levels in the synaptic cleft is underscored by the fact that alterations in the expression of predominantly glial transporters cause severe perturbations of neurotransmission at respective synapses. This demonstrates that glial cells function as an efficient buffering system or sink for presynaptically released neurotransmitter. Additionally, it has become clear that transporter proteins expressed by glial cells play a crucial role in shaping synaptic transmission. In this review we discuss our present understanding of the function of these transporters.

2. *In vivo* functions of glial GABA transporters

GABA is the principal inhibitory neurotransmitter in the adult mammalian brain, where it activates $GABA_A$, $GABA_B$, and $GABA_C$ receptors. High-affinity GABA transport processes play a key role in controlling the levels of this amino acid in the extracellular space upon its release from the presynaptic terminal. Pioneering studies of GABA transport on bulk-prepared neuronal and glial cells (Henn and Hamberger, 1971), in addition to following studies on cell lines and primary cultures of astrocytes and neurons (Hertz and Schousboe, 1987), have clearly demonstrated that GABA transporter mediated uptake takes place in both cell types. This is thought to have crucial consequences in the regulation of the GABAergic system. First, GABA uptake is believed to be a key event in terminating phasic synaptic currents induced by GABA release via synaptic vesicle exocytosis from presynaptic terminals. Second, GABA transporters expressed by neurons may also play a role in replenishing the supply of presynaptic transmitter by transporting it back into the GABAergic nerve ending. Third, GABA transporters are expected to be involved in regulating the extracellular GABA concentration throughout the brain, thus modulating tonic activation of synaptic and extrasynaptic GABA receptors. This may also involve reverse activity of GABA transporters, thereby mediating non-vesicular release of GABA to the surrounding cerebrospinal fluid (CSF) (Richerson and Wu, 2003). Interestingly, the kinetic characterization of GABA uptake in GABAergic neurons and astrocytes in primary cultures showed that the capacity of GABA transport is much higher in neurons than in astrocytes (Hertz and Schousboe, 1987). Therefore, it was hypothesized that GABA neurotransmission is preferentially based on recycling of GABA in GABAergic neurons (Schousboe, 2003). In addition, a secondary fraction of released GABA would be taken up by astrocytes surrounding the synapse, where it can be metabolized. Thus, the activity of astrocytic GABA

transporters would inevitably lead to a loss of GABA from the overall neurotransmitter pool.

Cloning of GABA transporters has facilitated the elucidation of the precise functional roles of GABA transport processes in neurons and glial cells. To date, four GABA transporters have been identified in the central nervous system: GABA transporter subtype 1 (GAT1) (slc6a1), Betain-GABA transporter-1 (BGT-1) (slc6a12), GAT2 (slc6a13), and GAT3 (slc6a11) (Borden et al., 1992; Guastella et al., 1990; Yamauchi et al., 1992). Molecular characterization of these proteins has revealed that GAT1, GAT2 and GAT3 are high-affinity GABA transporter subtypes whereas BGT1 shows a lower affinity. GABA transporters are mainly energized by the sodium gradient across the membrane. Although Cl^- can significantly enhance the rate of transport, Cl^- alone cannot drive GABA uptake in the absence of Na^+. The stoichiometry for GAT1, GAT2 and GAT3 has been proposed to be 2 Na^+:1 Cl^-:1 GABA, whereas for BGT1 is 3 Na^+:2 Cl^-:1 GABA (Karakossian et al., 2005; Loo et al., 2000; Sacher et al., 2002). Immunohistochemical studies using antibodies raised against recombinant proteins confirmed GAT expression on both neurons and astrocytes. Notably, GABA transporters do not follow a cell type specific expression pattern. Thus, GAT-1 is not only localized to axon terminals forming symmetric synaptic contacts but also to distal astrocytic processes (Conti et al., 1998; Minelli et al., 1995; Ribak et al., 1996). GAT2 is weakly expressed throughout the brain, primarily in arachnoid and ependymal cells, where it has been proposed to have a nutritional role, and to a much lesser extent in neurons and astrocytes (Conti et al., 1999). GAT3 expression is found predominantly on glial cells. BGT-1 is not present at GABAergic synapses but seems to be located extrasynaptically on both neurons and astrocytes (Borden et al., 1995; Zhu and Ong, 2004). Therefore, glial cells mainly express GAT1, GAT3 and, to a lower extent, BGT1 and GAT2, located both extrasynaptically and on neighboring GABAergic synapses, thus raising several issues regarding the relative contribution of each transporter to overall GABA uptake by glial cells and their functional significance.

The functional importance of GABA transporters in the regulation of GABAergic neurotransmission has been initially highlighted by studies on the modulatory action of GAT inhibitors on GABA receptor activation. In different brain areas, GAT1 antagonists increase the decay time of evoked inhibitory postsynaptic currents (IPSCs) without modifying that of spontaneous IPSCs (sIPSCs) (Engel et al., 1998; Keros and Hablitz, 2005; Overstreet and Westbrook, 2003; Thompson and Gahwiler, 1992). In addition, they also enhance the amplitude of tonic GABA mediated currents (Keros and Hablitz, 2005; Marchionni et al., 2007; Nusser and Mody, 2002; Semyanov et al., 2003), thus indicating that GAT1 affects both phasic and tonic GABA receptor mediated inhibition. By using microdialysis techniques, GAT1 inhibitors have been also shown to increase extracellular GABA concentration in the brain (Fink-Jensen et al., 1992; Juhasz et al., 1997; Richards and Bowery, 1996). Altogether, these findings suggest that GAT1 is responsible for controlling extracellular levels of GABA at synapses. Because GAT1 is located both on neurons and glia, these studies do not provide direct information about the relative contribution of glial uptake in this context. In addition, inhibitors of GABA transporters have been shown to exert anti-convulsant activity. In different animal models of epilepsy, highly selective GAT1 blockers like Tiagabine, which is a clinically useful antiepileptic drug, EF1502, which exhibits inhibitory activity at BGT1 and GAT1 (Clausen et al., 2005), and SNAP-5114, a GAT2/3 selective compound (Borden et al., 1994), display a broad anti-convulsant profile following intraperitoneal administration (Madsen et al., 2009). Interestingly, EF1502 combined with tiagabine displayed a synergistic anti-convulsant action, suggesting that increased extracellular concentrations of GABA induced by GAT1 inhibition result in the spill-over of this transmitter into the extrasynaptic space where the subsequent inhibition of extrasynaptically BGT1 facilitates the activation of different populations of GABA receptors. This hints to an important role of BGT1 in regulating GABA levels at extrasynaptic sites. In contrast, combination of SNAP-5114 with tiagabine only produced an additive interaction (Madsen et al., 2009). A likely explanation to this is that inhibition of GAT1 in neurons and glia together with glial GAT3 ultimately causes an increased concentration of GABA at the synapse, which results in an anti-convulsant effect mediated by GABA receptors in close proximity to GAT1 and GAT3. This indicates that inhibitors of astroglial GAT3 are able to increase the availability of GABA in the extracellular neurotransmitter pool (Sarup et al., 2003a,b; Schousboe, 2003) thus suggesting that glial GABA uptake plays a functionally important role in regulating the availability of GABA in the synapse.

Genetic approaches have confirmed GABA transporter functions previously disclosed by pharmacological studies. GAT1 knockout (KO) mice also display increased GABA mediated tonic conductances (Chiu et al., 2005; Jensen et al., 2003), prolonged decay time of evoked IPSCs (Bragina et al., 2008) but no alterations in the frequency, amplitude and kinetics of sIPSCs (Jensen et al., 2003). No major compensatory changes in proteins or structures related to GABA transmission were observed in GAT1 KO mice (Chiu et al., 2005). Notably, no change in GABA content of synaptic vesicles was observed in GAT1 deficient mice (Bragina et al., 2008). Microdialysis studies also showed that spontaneous release of GABA was comparable in wild-type and GAT1 deficient mice, whereas KCl-evoked output of GABA was significantly increased in KO mice (Bragina et al., 2008). These findings demonstrate that ablation of GAT1 chronically elevates ambient GABA without affecting its intracellular levels. Thus, GAT1 mediated uptake plays an essential role *in vivo* controlling extracellular levels of GABA in the synapse with no obvious function on recycling of GABA in GABAergic neurons. In addition, prolongation of evoked IPSC, but not sIPSCs, decay time indicates that in GAT1 KO mice synchronous activity of several synaptic terminals results in the accumulation of extracellular GABA whereas after unitary release events the uptake capacity provided by other GABA transporters is sufficient for the fast clearance of synaptically released GABA. Thus, GAT-1 limits the interaction between closely spaced sites by restricting diffusion beyond the synaptic specializations (Overstreet and Westbrook, 2003). A second GAT KO mouse line has been generated for the glial GAT3 (slc6a11). These animals show early postnatal lethality (Mouse Genome Database (MGD), Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, Maine, (URL: http://www.

informatics.jax.org, June, 2009)) possibly due to GABA mediated overinhibition, although no analyses have been carried out to clarify this remarkable phenotype. Nevertheless, heterozygous mice carrying only one functional allele of GAT3 survive and appear grossly indistinguishable from wild-type animals. Interestingly, these heterozygous mice require a significantly higher dose of the $GABA_A$ receptor antagonist metrazol to reach various seizure stages when compared with wild-type control mice (Deltagen, Inc., NIH initiative supporting placement of Deltagen, Inc. mice into public repositories, MGI Direct Data Submission 2005). This increased resistance to pharmacologically induced seizures is in agreement with observations using GAT3 inhibitors, as mentioned above, and suggests that glial GAT mediated uptake plays an essential *in vivo* role controlling extracellular levels of GABA in the synapse, as hypothesized more than 3 decades ago (Schousboe, 1979).

3. Glycine transporters

The glycinergic system is the second important inhibitory transmitter system within the mammalian CNS. In contrast to GABA that is used throughout all major brain regions as an inhibitory neurotransmitter, glycinergic synapses are found predominantly in caudal regions of the CNS, i.e. brainstem and spinal cord. Upon vesicular release, glycine binds to the predominantly postsynaptically localized glycine receptors (GlyRs), resulting in the opening of an intrinsic chloride channel which causes inhibition of the postsynaptic cells via an influx of chloride ions causing a hyperpolarisation of the cell or alternatively shunting inhibition. In addition to its inhibitory function at glycinergic synapses, glycine is a high-affinity coagonist for ionotropic glutamate receptors of the N-methyl-D-aspartate (NMDA) subtype (Dingledine et al., 1990; Johnson and Ascher, 1987). This heterotetrameric receptor is assembled from two NR1 subunits that have been shown to contain the glycine binding side (Kuryatov et al., 1994) and two glutamate binding NR2 subunits (Schorge and Colquhoun, 2003). The binding of a ligand, glycine, or alternatively D-serine, to the NR1 subunit of the receptor is a prerequisite for the activation of the receptor by glutamate (Kleckner and Dingledine, 1988). The affinity of the NR1 subunit for its ligand, however, appeared to be determined by the coassembled NR2 subunit (Laurie and Seeburg, 1994). More recently, NMDA receptors that contain two different glycine binding subunits, NR1 and NR3, were identified (Chatterton et al., 2002). These heteroreceptors are activated by glycine alone and thus constitute a novel class of excitatory glycine receptor. Their *in vivo* functions, however, remain elusive.

The dual function of glycine at both inhibitory as well as excitatory synapses underscores the importance of a precise regulation of the extracellular glycine concentration. Similar to GABA, the CSF glycine concentration is regulated by both neuronal and glial transporters. Up to now, two different high-affinity transporters, GlyT1 (Slc6a9) and GlyT2 (Slc6a5), have been discovered (Guastella et al., 1992; Liu et al., 1992, 1993). Both transporters are encoded by single genes, although multiple splice variants have been described (Adams et al., 1995; Ebihara et al., 2004). Glycine transporters are structurally related to the previously discussed GABA transporters and use predominantly the sodium gradient across the membrane as an energy source for the intracellular accumulation of glycine (Gether et al., 2006). Interestingly, GlyT2 requires 3 Na^+ and 1 Cl^- for the import of one glycine molecule whereas GlyT1 takes only 2 Na^+ and 1 Cl^- per transport cycle (Roux and Supplisson, 2000; Supplisson and Roux, 2002). As a consequence of this stoichiometry difference, it is widely accepted that under physiological conditions GlyT2 can only mediate import of glycine into the cytosol maintaining millimolar intracellular versus submicromolar extracellular glycine levels, whereas GlyT1 might reverse transport directionality after cell depolarization or at low extracellular glycine concentrations, thus facilitating non-vesicular release of glycine from the cytosol into the surrounding extracellular space. Immunohistochemical analysis of glycine transporters has revealed that GlyT2 is exclusively expressed by glycinergic neurons in caudal regions of the CNS, representing the only known reliable marker for these neurons (Poyatos et al., 1997; Zeilhofer et al., 2005). In contrast to GlyT2, GlyT1 shows a broader expression pattern in the CNS. Although GlyT1 is predominantly expressed in glial cells of brain stem and spinal cord (Zafra et al., 1995), i.e. regions which are rich in glycinergic neurotransmission, it can be also found in brain regions like cortex or hippocampus (Jursky and Nelson, 1996; Zafra et al., 1995) both in glial cells and in a subset of presumptive glutamatergic neurons (Adams et al., 1995; Cubelos et al., 2005). Altogether, these differences both in their ionic stoichiometry and cellular location raise several issues regarding the relative contribution of each transporter to the regulation of the extracellular glycine concentration throughout the brain and their physiological function.

Genetic inactivation of GlyT expression revealed different but complementary functions of both transporters in the neonatal animal. Inactivation of each of the GlyT genes caused severe perturbation of glycinergic neurotransmission that finally resulted in premature death of the mutant animals (Gomeza et al., 2003a,b). Electrophysiological analysis of the glycinergic neurotransmission in the brainstem of GlyT2 deficient mice revealed that neuronal GlyT2 is essential for the replenishment of glycine within the presynaptic terminal for vesicular release. Thus, loss of GlyT2 from the presynaptic nerve terminal results in a decrease of the vesicular glycine content and thereby a reduction in glycinergic IPSC amplitude. The extracellular concentration of glycine within the CSF, more specifically at synaptic sites, however, appeared not to be controlled by this neuronal transport system, since no changes in GlyR mediated tonic conductances were observed in these animals. Therefore, the efficient removal of glycine from the synaptic cleft of glycinergic synapses in neonatal animals was expected to be mediated by glial GlyT1. Consistently, ablation of GlyT1 expression by gene inactivation resulted in a strong facilitation of GlyR activity in the brainstem and spinal cord and consequently overinhibition of the animal. Moreover, recordings from hypoglossal motoneurons of GlyT1 deficient mice revealed strong GlyR mediated tonic currents and prolonged mIPSC decay (Gomeza et al., 2003a) kinetics. This indicates that the removal of glycine from the synaptic cleft by predominantly glial expressed

GlyT1 is the rate limiting step in the off kinetic glycinergic neurotransmission.

More recently, these results have been verified and extended by pharmacological analyses. In agreement with observations on GlyT1-deficient mice, systemic inhibition of GlyT1 by ALX5407 or LY2365109 in adult rats resulted in respiratory depression and impaired motor performance (Perry et al., 2008). Likewise, recordings from lamina X neurons from spinal cord revealed that acute inhibition of GlyT1 resulted in an extracellular accumulation of glycine at synaptic sites (Bradaia et al., 2004). However, *in vivo* application of both the GlyT1 specific inhibitor Org24598 or the GlyT2 blocker Org25543 into the dorsal spinal cord of rats resulted in a significant increase in extracellular glycine levels (Whitehead et al., 2004), suggesting that in the mature spinal cord the extracellular glycine concentration is not exclusively controlled by GlyT1 but also by GlyT2. Similar analyses from lamina X neurons showed that inhibition of GlyT2 also caused an extracellular accumulation of glycine at synaptic sites (Bradaia et al., 2004). Moreover, inhibition of GlyT2 *in vivo* resulted in a significant impairment of motor performance as well as respiratory activity in adult rats (Hermanns et al., 2008), symptoms previously observed in GlyT1 deficient mice due to extracellular accumulation of glycine (Gomeza et al., 2003a). After long lasting inhibition of GlyT2, however, a rundown of presynaptically released glycine has been monitored (Bradaia et al., 2004) in agreement with the symptoms observed in GlyT2 KO mice. In summary, these observations suggest that in the mature nervous system, neuronal GlyT2 is not only essential for the replenishment of presynaptic glycine for vesicular release but also contributes to the regulation of the synaptic glycine concentration.

Together, these findings suggest that in caudal regions of the CNS, glial GlyT1 and neuronal GlyT2 closely cooperate in the regulation of extracellular glycine at inhibitory synaptic sites. After presynaptical release of glycine, both transporters contribute to the fast removal of glycine, thus ensuring the rapid termination of glycine dependent neurotransmission. In addition, glycine neurotransmission is based on recycling of glycine in glycinergic neurons by GlyT2. Thus, a significant extent of released glycine is subsequently accumulated into glycinergic nerve endings, enabling its incorporation into synaptic vesicles and subsequent release upon depolarization. A secondary fraction of the transmitter is incorporated into glial cells via GlyT1. At later timepoints, when glycine concentration at glycinergic synapses is too low for GlyR activation, GlyT1 might reverse transport directionality, releasing glycine from the cytoplasm of glial cells and thus providing additional substrate for neuronal accumulation of glycine by GlyT2.

In higher brain regions, like cortex or hippocampus, where the GlyT2 is only expressed sporadically, the availability of extracellular glycine is thought to be controlled exclusively by GlyT1. As mentioned above, binding of glycine to the NR1 subunit of the NMDA receptor is essential for the activation of the receptor by glutamate and allows thereby the modulation of synaptic plasticity. As expected, application of GlyT1 inhibitors to hippocampal slice preparations resulted in the facilitation of the NMDA receptor component of glutamatergic synaptic transmission. Systemic inhibition of GlyT1 by LY2365109 in rats caused a facilitation of the NMDA induced dopamine release in the prefrontal cortex and hyperactivity (Perry et al., 2008). Inhibition of GlyT1 by NFPS in rat hippocampus resulted in a partial inhibition of long term potentiation (LTP) elicited by tetanic high frequency stimulation (Manahan-Vaughan et al., 2008). Interestingly, the inhibition of GlyT1 ameliorated the effects of prior exposure to potent NMDA receptor inhibitors like MK801 (Manahan-Vaughan et al., 2008). In contrast, application of high concentrations of glycine to a similar slice preparation alone resulted in the induction of a robust GlyT1 dependent LTP that was independent of the NMDA receptor (Igartua et al., 2007). Whether these effects are mediated by glially or neuronally expressed GlyT1 is not clear at present, since both cell types have been shown to express the transporter in this brain region (Adams et al., 1995; Cubelos et al., 2005). First indication that effects caused by GlyT1 inhibitors were associated at least in part with forebrain neuronal GlyT1 comes from the analysis of mice carrying a neuron specific disruption of the GlyT1 gene (Singer et al., 2007; Yee et al., 2006). In these mice, an enhanced NMDA receptor component of glutamatergic neurotransmission was found. Consequently, they were more resistant in pharmacological model-systems for psychosis and showed improved recognition memory (Singer et al., 2007; Yee et al., 2006). This contrasts findings from mice that carried a general deletion of glial and neuronal GlyT1 in the forebrain. Here, no enhancement of the NMDA receptor component of glutamatergic neurotransmission was observed, although these mice display some behavioral changes (Singer et al., 2009) different from those observed in animals deficient for only forebrain neuronal GlyT1. Therefore, genetic approaches have suggested different functions of glial and neuronal GlyT1 in the modulation of glutamatergic neurotransmission that would result in additional mechanisms of GlyT1 mediated synaptic plasticity in the forebrain.

4. Glial expressed glutamate transporters

In contrast to glycine and GABA, which both have predominantly inhibitory functions, glutamate serves as the principal excitatory neurotransmitter in most regions of the CNS. After presynaptic release, glutamate binds to both ionotropic and metabotropic receptors which differ in their affinity and localization. There are two major classes of ionotropic glutamate receptors. First, the non-NMDA receptors, which include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and Kainate receptors. Although both receptor classes are structurally related, they differ in their functional role in glutamatergic neurotransmission. Whereas AMPA receptors are the major receptors mediating fast excitatory synaptic transmission, Kainate receptors appear to have predominantly modulatory functions. Due to the low affinity of these receptors to glutamate, their activation is closely coupled to their localization to synaptic sites. The second major class of ionotropic glutamate receptors is the NMDA receptor. It displays that higher affinity for glutamate and active NMDA receptors can be found both at synaptic sites as well as extrasynaptically. Although the activity of this receptor class is blocked at large by Mg^{2+} under resting

conditions, it has been shown to have important functions as an essential coincidence detector that is involved in a variety of effects like learning and memory. Additionally, glutamate activates metabotropic glutamate receptors (mGluRs), G-protein coupled receptors that modulate the activity of enzymes producing second messengers, such as phospholipase C or adenylyl cyclase, different channels and ionotropic glutamate receptors (Conn and Pin, 1997). They are localized at synaptic sites and within their periphery, and contribute to the regulation of glutamatergic neurotransmission in the brain.

High extracellular glutamate concentrations not only prevent efficient neurotransmission but additionally cause excitotoxicity. Thus, the extracellular concentration of glutamate must be precisely regulated at synaptic sites as well as at extrasynaptic locations. In addition, the high abundance of glutamatergic transmission requires an efficient recycling system for the neurotransmitter to ensure a constant transmitter supply for presynaptic release. It is generally accepted that major proportions of glutamate released by presynaptic terminals are taken up not by neurons but by surrounding astrocytes that encase the presynaptic terminal. In the plasma membrane of these cells, high-affinity glutamate transporters are located that play a key role in the control of glutamate clearance and its availability, thus modulating glutamatergic neurotransmission. Within the astrocytic cytoplasm, glutamate is efficiently converted by the enzyme glutamine synthetase (GS) to glutamine that does not bind to glutamate receptors. This "inactivated" metabolite of glutamate is subsequently returned to neurons to be reconverted back to glutamate by a phosphate activated glutaminase (PAG), being thus reused for vesicular release. Interestingly, PAG is mainly expressed in neurons whereas GS is exclusively localized in astrocytes (Hertz et al., 1999). Therefore, glutamate metabolism in the brain is cell compartmentalized, placing astrocytes in a key position with regard to the regulation of glutamate homeostasis and, consequently, glutamatergic neurotransmission (Hertz et al., 1999; Schousboe and Waagepetersen, 2006). The disadvantage of the high energy cost of this glutamate–glutamine cycle is greatly compensated by the prevention of excitotoxicity caused by elevated levels of extracellular glutamate in the synapses.

The efficient clearance of glutamate from the extracellular space is achieved by a whole family of high-affinity transporters, the excitatory amino acid transporters (EAATs). In contrast to the GATs and GlyTs, the EAATs do not belong to the Slc6 family of neurotransmitter transporters but form a family on their own. In total, 5 different EAATs have been characterized in the mammalian nervous system: EAAT1 (also named Glast), EAAT2 (Glt1), EAAT3 (EAAC1), EAAT4 and EAAT5 (Danbolt, 2001). In addition to their main substrate glutamate, these transporters mediate L-aspartate and D-aspartate uptakes. EAAT3 also accepts L-cysteine as a substrate. Similar to the transporters for glycine and GABA, the EAATs use predominantly the sodium gradient across the membrane as the main energy source for the intracellular accumulation of glutamate. The transport stoichiometry for this family of transporters has been proposed to be 3 Na^+ and one proton per transport cycle of one molecule of glutamate, while one potassium ion is concurrently extruded from the cell (Danbolt, 2001). Consequently, activity of these transporters results in an increase of the intracellular sodium concentration. In glial cells it has been reported that this augmentation causes increased activity of the Na^+/K^+ ATPase (Rose and Ransom, 1996; Voutsinos-Porche et al., 2003) and upregulation of the astrocytic glucose metabolism (Loaiza et al., 2003). This mechanism is considered to couple astrocytic energy metabolism to the synaptic activity in its surrounding.

Initially, Glast1 (EAAT1) and Glt1 (EAAT2) have been described as glial transporters, whereas EAAT4 and EAAC1 (EAAT3) were assumed to be exclusively expressed in neurons (Rothstein et al., 1994). More recently, however, it has been demonstrated that most transporters are expressed at different levels by both neuronal and glial cells (Chen et al., 2002, 2004; Hu et al., 2003). Thus, the contribution of individual transporters and/or cell types to the total glutamate transport activity depends on the expression of EAATs in the respective brain regions.

The major glutamate transporter present in regions that are rich in glutamatergic neurotransmission is the predominantly glially expressed transporter Glt1 (EAAT2). In the forebrain regions, Glt1 alone accounts for more than 95% of the total high-affinity glutamate uptake capacity (Tanaka et al., 1997). Mice carrying genetically inactivated Glt1 alleles displayed a marked neurological phenotype including spontaneous epileptic seizures and neuronal degeneration as well as an increased mortality of homozygous mutant mice. Consistently, the synaptic concentration of glutamate was found to be increased in these mice. These findings confirm that Glt1 is involved in the maintenance of a low extracellular glutamate concentration. There were, however, no differences in the decay kinetic of AMPA or NMDA receptor mediated postsynaptic responses, showing that this glutamate transporter does not determine the postsynaptic decay rates. Additionally, lowering of the extracellular glutamate concentration by Glt1 restricts mGluR activation within hippocampal interneurons and thus contributes to the control of their activity (Huang et al., 2004b). Whether the effects seen in Glt1 deficient mice are caused by the loss of neuronal or glial expressed Glt1 is not clear at present. The contribution of Glt1 to the regulation of glutamatergic transmission, however, appears to depend on a tight encasement of the synapse by glial cells (Oliet et al., 2001). At synapses tightly wrapped by glial membranes, as found in the supraoptic nucleus of rats, inhibition of Glt1 leads to a buildup of glutamate in the synaptic cleft that results in enhanced activation of presynaptic mGluRs. Loosening of the glial encasement of the synapse, as found in lactating animals, reduced this effect, thus demonstrating that control of the synaptic glutamate concentration depends on the intimate contact between glial cells and the respective synapse. In contrast to the strong phenotype seen in Glt1 deficient mice, mice deficient for the second predominantly glial expressed glutamate transporter Glast (EAAT1) display only a very subtle motor coordination phenotype (Watase et al., 1998), which is most likely caused by cerebellar dysfunction. Additionally, Glast seems to cooperate with Glt1 in controlling mGluR function in the interneurons of the hippocampus (Huang et al., 2004b). Deficiency for the predominantly neuronal expressed glutamate transporters EAAC1 (EAAT3) and EAAT4 causes only very moderate

phenotypes. Even animals deficient for both transporters (EAAC1$^{-/-}$/EAAT4$^{-/-}$) survived until adulthood, were fertile and did not show any obvious motor coordination phenotype (Huang et al., 2004a). Together, these data suggest that the predominant glial expressed transporters Glt1 and Glast are the major players in regulating extracellular glutamate concentration in most brain regions, although the loss of one specific transporter can be compensated by other transporters at least in case of low frequency activity.

It has to be stressed that the precise localization as well as the expression levels of these transporters, and consequently their contribution to the regulation of glutamate dependent neurotransmission, might not be the same in different brain regions. In cortical regions, i.e. regions where synapses are tightly surrounded by astrocytic processes, most likely glial glutamate uptake is responsible for controlling extracellular glutamate concentration. In other brain regions, like e.g. the hippocampus, where synapses are only partially encased by astrocytic processes, a major proportion of glutamate uptake might be accomplished by the neurons themselves. Up to now, the precise analysis of these questions has been hampered by the lack of suitable mouse models that allow the inactivation of transporter expression in specific cell types. More information, however, is available about the regulation of EAAT levels and their influence on overall glutamate uptake. Although some interacting proteins have been identified recently that stabilize EAAT localization at the plasma membrane (Jackson et al., 2001; Lin et al., 2001) or facilitate their internalization (and subsequent degradation) (Gonzalez et al., 2003, 2005), bulk EAAT activity is thought to be regulated predominantly on the level of expression. Several lines of evidence suggest that unknown secreted neuronal factors contribute to the regulation of the Glast and Glt1 gene expressions (Schlag et al., 1998; Zelenaia et al., 2000) in the brain. Interestingly, dysregulation of glutamate transporter expression is discussed to be causal and/or associated with several neurodegererative diseases like amyotrophic lateral sclerosis (ALS), ischemia/stroke and epilepsy (Maragakis and Rothstein, 2004). Moreover, hypoxia resulted in a massive downregulation of Glast and glial Glt1 expressions both *in vitro* (Dallas et al., 2007) and *in vivo* (Pow et al., 2004) whereas neuronal expression of Glt1 was upregulated (Pow et al., 2004). This differential regulation of EAAT expression in neurons and glial cells might enhance excitotoxicity induced by elevated glutamate concentration after strong hypoxia (Rossi et al., 2000). Similarly, reduced Glt1 expression is thought to be causal for glutamate induced neurodegeneration of motoneurons in genetic models for ALS (Howland et al., 2002). Recently, astrocytic Glt1 expression has been shown to be induced by the presence of axons, a process depending on the Kappa-B motif binding phosphoprotein (KBBP) (Yang et al., 2009). Notably, dysregulation of KBBP expression is found in genetic models of ALS, suggesting that this pathway contributes to the pathogenesis of this disease. The involvement of EAATs in the pathogenesis of neurodegenerative and/or neurological syndromes suggests that the manipulation of their expression might constitute a new strategy for the treatment of these diseases. Indeed, the partial replacement of astrocytes by transplantation of "healthy" precursor-cells into SOD^{G93A} rats that develop ALS, resulted in marked improvement of the disease progression (Lepore et al., 2008). Interestingly, the expression of Glt1 can be increased by antibiotics of the β-lactam family, and this upregulation appears to be neuroprotective both in neurotoxicity induced by oxygen/glucose deprivation and in animal models for ALS (Rothstein et al., 2005). The precise mechanism how β-lactam antibiotics regulate Glt1 expression however still remains elusive.

5. Summary and perspectives

In the last couple of years the analysis of transporter deficient mice together with pharmacological approaches has greatly enhanced our understanding on how glial cells and neurons cooperate in the fast removal of transmitter from the synaptic cleft of active synapses. This has resulted in new insights into the physiology and pathophysiology of the nervous system. Nevertheless, the complexity of the expression pattern of many of these transporters has prevented deeper insights in the destiny of neurotransmitters after presynaptic vesicular release. The development of new mouse models that allow specific inactivation of the respective transporter genes in individual cell types would greatly enhance the progress in this field. Findings from the glutamate transporters, but also from other neurotransmitter transporters, have suggested that transporter expression levels in neurons and glial cells might be differentially regulated. The precise analysis of these regulatory mechanisms will not only enhance our understanding of the function of neuron/glia interaction but might additionally result in the development of new therapeutic strategies for the treatment of human neurological diseases like epilepsy, ALS or even psychiatric diseases like schizophrenia.

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