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ORIGINAL PAPER

GLAST But Not Least—Distribution, Function, Genetics and Epigenetics of L-Glutamate Transport in Brain—Focus on GLAST/EAAT1

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Abstract Synaptically released L-glutamate, the most important excitatory neurotransmitter in the CNS, is removed from extracellular space by fast and efficient transport mediated by several transporters; the most abundant ones are EAAT1/GLAST and EAAT2/GLT1. The review first summarizes their location, functions and basic characteristics. We then look at genetics and epigenetics of EAAT1/GLAST and EAAT2/GLT1 and perform in silico analyses of their promoter regions. There is one CpG island in *SLC1A2* (EAAT2/GLT1) gene and none in *SLC1A3* (EAAT1/GLAST) suggesting that DNA methylation is not the most important epigenetic mechanism regulating EAAT1/GLAST levels in brain. There are targets for specific miRNA in *SLC1A2* (EAAT2/GLT1) gene. We also note that while defects in EAAT2/GLT1 have been associated with various pathological states including chronic neurodegenerative diseases, very little is known on possible contributions of defective or dysfunctional EAAT1/GLAST to any specific brain disease. Finally, we review evidence of EAAT1/GLAST involvement in mechanisms of brain response to alcoholism and present some preliminary data showing that ethanol, at concentrations which may be reached following heavy drinking, can have an effect on the distribution of EAAT1/GLAST in cultured astrocytes; the effect is blocked by baclofen, a GABA-B receptor agonist and a drug potentially useful in the treatment of alcoholism. We argue that more research effort should be focused on EAAT1/GLAST, particularly in relation to alcoholism and drug addiction.

Keywords Alcoholism · Glutamate transport · Polymorphisms · Epigenetics · DNA methylation · Ethanol · miRNA

Introduction

The most important neurotransmitter at excitatory synapses in the central nervous system is an acidic (dicarboxylic) amino acid L-glutamate (L-Glu, review: [1]; historical overview: [2]; recent review: [3]). According to a classical view, most of the synaptic L-Glu is synthesised in glutamatergic nerve terminals by conversion of L-glutamine via phosphate-activated glutaminase (PAG) and then actively transported into and stored in, synaptic vesicles. Several vesicular glutamate transporters (VGLUT's) driven by H^+-dependent ATPase assist the accumulation of L-Glu by the vesicles [4]. L-Glu is released from the synaptic nerve endings when the glutamate-containing vesicles discharge their content by a stimulus- (depolarization-) coupled Ca^{2+}-dependent mechanisms into the synaptic cleft. L-Glu interacts with L-Glu receptors [3] on the surface of the postsynaptic neuron (or on other cells in the vicinity) and diffuses away or is taken up by a very efficient "high affinity" Na^+-dependent L-glutamate transport (GluT) into surrounding cellular structures; these are mostly, but not exclusively, adjacent astrocytes.

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GluT is mediated by distinct protein molecules derived from five genes (reviews: [5, 6]). In some species such as humans and guinea pigs they are referred to as excitatory amino acid transporters (EAATs) and marked EAAT1-5, while in rats and mice they are called GLAST (GLutamate and ASpartate Transporter; homologous to EAAT1), GLT-1 (or GLT1; GLutamate Transporter One; homologous to EAAT2), EAAC1 (excitatory amino acid carrier one; homologous to EAAT3). There are no alternative designations for the rodent EAAT4 and EAAT5. As all EAATs are members of the solute carrier family SLC1A, the complete designations should be given as EAAT1/GLAST/SLC1A3, EAAT2/GLT1/SLC1A2, EAAT3/EAAC1/SLC1A1, EAAT4/SLC1A6, EAAT5/SLC1A7. Here we shall use EAAT1/GLAST and EAAT2/GLT1 designations when referring to the actual transporters (proteins) with the corresponding *SLC1A3* and *SLC1A2* terms reserved for the respective genes (see the following section for full details).

EAATs transport glutamate against very high concentration gradients [5] thus constantly "sweeping" any residual L-Glu from the extracellular space; this is held to be very important in preventing build-up of excess L-Glu in and around glutamatergic synapses which could act in an "excitotoxic" manner and trigger off, in extreme situations, death of the surrounding neurons [7].

L-Glu is co-transported with Na^+ and it should be understood that the efficiency (or even the direction) of GluT by EAATs is conditional on existing transmembrane Na^+ gradients. GluT should really be recognized as Na^+- and K^+-dependent because the gradients of both ions are required for a full GluT cycle to function: Na^+ is co-transported with L-Glu into the cell while K^+ is counter-transported (i.e. leaving the cell) by the L-Glu-free EAAT molecule. Additionally, pH and electrical gradients also contribute to the free energy driving the transport (for a review see [6]). Furthermore, EAAT1/GLAST, EAAT3-5 but not EAAT2/GLT1 act as activity dependent Cl^- channels (reviews: [5, 6, 8]). The precise stoichiometries of the transport for EAAT1/GLAST and EAAT2/GLT1 have been well established: $3Na^+$, $1H^+$, 1 L-Glu and 1 K^+ [9, 10, 11; review: 6]. While the Na^+ and K^+ gradients provide the bulk of the driving force for the system and the ions interact directly with the transporter protein molecule, Cl^- passage is not thermodynamically coupled to GluT and follows slope of the Cl^- gradient (review: [6, 8]).

Location of EAATs and Their Possible Roles in Brain Functions

As mentioned above, the role of GluT most often mentioned in the literature is to limit the spread of synaptically released L-Glu and prevent uncontrolled excitation and neurotoxicity. EAATs may, however, be involved in other physiological processes as indicated by their cellular location.

The majority of the EAAT molecules expressed in brain tissue have been located to astrocytes. This is particularly the case for EAAT1 (GLAST) and EAAT2 (GLT1) which both account for most of the EAAT protein expressed in brain [5, 6, 8]. EAAT2/GLT1 protein seems to be distributed in two pools, one enriched in the plasma membrane of astrocytic endfeet apposed to glutamatergic synapses and the other distributed over the whole surface of the cells [12, 13]. GLAST appears in some studies to be preferentially localised to the astrocytic regions close to excitatory synapses [14] but in other studies has been shown to be expressed by the cell bodies of astrocytes [15]. Again, like in the case of GLT1 this may indicate two pools of GLAST. EAAT2/GLT1 accounts, for at least 1 % of all protein in the CNS [8]. EAAT1/GLAST is about 4-times less abundant, based on assays of whole brains. EAAT2/GLT1 appears to be the principal EAAT in the forebrain, particularly in the cerebral cortex and to a lesser extent in the rest of the CNS. GLAST is the main EAAT in the cerebellar cortex where it is located in Bergman glia, which are a type of radial glial cells (reviews: [5, 8, 16]). In addition, GLAST is strongly expressed in other radial-like glial cells including the Müller cells of the retina and in tanycytes in the circumventricular organs [8]. Elsewhere there is evidence for expression in populations of astrocytes and oligodendrocytes. There are intriguing exceptions in the literature, though. For example, Roberts et al. [17] reported that EAAT1 labelling in human brain was located on the plasma membrane of astrocytes but also in the soma and nucleus. Furthermore, they detected EAAT1 protein in neurons, where it was present in the soma, all parts of the axon, dendritic spines and in the postsynaptic density (PSD). This is at variance with most of the other studies which have located EAAT1 in astrocytes; the presence of an apparent EAAT1 antigen in neurons might have been caused by changes taking place in the tissue during the post-mortem delay [17, 18] but there are other ways to explain the observed discrepancies, including the occurrence of splice variants. EAAT1 is now known to be subject to alternate splicing and to give rise to multiple variant proteins. At least one of these is known to be strongly expressed by neurons, especially when they have been subject to stressors such as ischaemia [18].

While EAAT1/GLAST, at least in the healthy CNS, is regularly expressed by glial cells, the location of EAAT2/GLT1, even in healthy brain, is less clear. In developing nervous tissue, particularly in developing cultured brain cells, EAAT2/GLT1 is present, at least transiently, in neurons [19, 20] where L-Glu may be involved in the process of differentiation [21]. Moreover, in the adult brain there is evidence of significant glutamate transport in

axonal nerve endings, possibly mediated by a form of EAAT2/GLT1 [22–24] that is expressed by neurons. A neuronal localisation for some form of EAAT2 is supported at least in part by in situ hybridisation studies [25] but the precise role and importance of neuronal EAAT2 (or a related protein) remains contentious [26–28]. Recent data suggest that the contribution of neuronal GLT1 to glutamate transport may have been underestimated, particularly when using synaptosomal preparations [29].

The hypothetical presynaptic EAAT2/GLT1 could directly help to replenish the presynaptic neurotransmitter stores. In contrast, L-Glu taken up by astrocytes is, at least in part, metabolized into L-glutamine, which may be passed via specific transport back to neurons including the excitatory glutamatergic nerve terminals, where it is converted to the neurotransmitter L-Glu (see e.g. [30]). Which process—active re-uptake by presynaptic EAAT or the conversion of L-glutamine to L-Glu by phosphate activated glutaminase—is more important to maintain presynaptic L-Glu stores may be a subject of debate [26–28, 31]. Experiments with brain cortical tissue in vitro have indicated that inhibition of GluT (this would include GluT in glia, neurons as well as the "presynaptic" GluT) depended somewhat on the type of inhibitor used but, those compounds which are not transported themselves but clearly inhibit at least one of the main EAATs [32], appeared to potentiate glutamatergic activity [33, 34]. This would seem to suggest that GluT has a primarily moderating effect on glutamatergic neurotransmission and would be consistent with the predominant role of GluT being that of limiting the spread of synaptically released L-Glu and preventing "runaway" excitation; if it was important mainly for replenishing presynaptic stores thus maintaining the potency of glutamatergic synapses, the effect of GluT inhibitors would not be expected to cause an increase in the overall intensity of glutamatergic excitation over a period of many minutes [33, 34].

Other experiments studying glutamate and energy metabolism in brain tissue pointed to a strong metabolic relationship between astrocytes and glutamatergic neurons; possibly favouring L-glutamine transport as being important for maintaining the normal glutamatergic neurotransmission in brain tissue [26, 30] but not necessarily ruling out a significant role of "re-used" L-Glu, i.e. L-Glu released and taken up back to the nerve terminals. Identification and characterization of the hypothetical presynaptic EAAT would help to resolve these questions.

Another potential role of the hypothetical presynaptic transporter is a feedback inhibition of L-Glu release. The neurotransmitter L-Glu has no known inhibitory ionotropic receptor which could play the role of the negative feedback at glutamatergic synapses. In general, it seems difficult to imagine that a powerful and ubiquitous process such as glutamatergic synaptic excitation would function in a system of the size and complexity of the mammalian brain without such a feedback control. More specifically, some models actually suggest that GluT, as it is thought to exert its influence in and around synapses (mainly the EAATs in astrocytes), can adequately protect the postsynaptic neurons from excess L-Glu but will not prevent access of L-Glu to the presynaptic terminal [35]. EAATs can act as Cl^--permeable channels, opening in the presence and during the transport of L-Glu ([36]; review: [37]). Therefore, if placed presynaptically, they could reduce the depolarization of the presynaptic terminals thus effectively exerting presynaptic feedback inhibition. Whilst this presynaptic inhibition model has been demonstrated in retinal bipolar neurons that express EAAT5 [38, 39] this chloride conductance is virtually absent in EAAT2/GLT1 which is currently the most prominent candidate for the brain presynaptic EAAT. However, the Cl^- permeability is not a trait rigidly imposed by the type of EAATs; it could be altered by small structural changes in the particular EAAT molecule [40, 41]; for a review see [37]. Thus it remains a possibility that an as yet unidentified EAAT2/GLT1 variant with structural characteristics capable of assuming suitable conformation and displaying Cl^- permeability may act as the hypothetical putative "inhibitory" glutamate receptor. As to the other EAATs, it is unlikely that GLAST, EAAT3 or EAAT4 can be involved in such process as there is little evidence for presynaptic GLAST (see [17] above, though) but it cannot be ruled out that the highly Cl^--permeable EAAT5 might contribute to the negative feedback control at glutamatergic synapses in some brain regions where it is present in significant amounts [38, 39, 42]. EAAT3 (EAAC1) is located in neurons but, overall, it is expressed at levels about two orders of magnitude lower then EAAT2/GLT1 [8] and is frequently expressed in postsynaptic locations rather than presynaptic elements. EAAT4 is a minor transporter in the cerebellar cortex, located on dendritic spines of Purkinje neurons [5]. Currently, the role of the presynaptic GluT remains hypothetical and will continue to be such at least until the nature of the transport system i.e. which form(s) or variant(s) of EAAT2/GLT1 or any other EAATs, is/are adequately described.

EAATs have also been widely reported outside the CNS. For example, GluT with properties similar to those observed in the CNS was found to be present in cultured 3T3 fibroblasts [43]. Analogous GluT systems were subsequently characterized in fibroblasts derived from several types of human tissue; although various EAATs were identified there by EAAT-selective antibodies, GLAST, or, possibly, GLT would seem to be the two most active EAATs in such preparations [44].

GLAST was found to be present and probably functional at the mammalian neuromuscular junction [45], in the

choroid plexus [46] and in other tissues, for example in pancreas [47] and in the heart muscle cells where it may have an important function [48]. GLT and possibly other EAATs exist in testes [49, 50] and EAAT5 is present in many peripheral tissues [42, 51]. In general, GluT outside the CNS is likely to be involved in a variety of specific physiological processes that may or may not be directly related to the activity of peripheral nerves or any other excitable tissue [52].

At this point, it should be emphasized that the reliability of all data concerning the presence or absence of EAATs, whether in the CNS or in the rest of the body, is only as good as the quality and reliability (i.e. specificity) of antibodies (review: [5]), or, if mRNA is detected, on the selection of hybridizing oligonucleotides. EAATs tend to be present in many splice variants [53, 54] and are susceptible to post-translational modification such as phosphorylation and glycosylation so some forms of EAAT may easily escape attention. It is, therefore, not advisable to draw final conclusions as to the presence/absence and/or changes in the expression of any EAAT on the basis of data from a single antibody that may not recognise the rich diversity of EAAT splice variants and their post-translationally modified forms.

EAAT1, EAAT2, EAAT3, EAAT4, and EAAT5 transporters in humans and their mouse and rat homologues GLAST, GLT1, EAAC1, EAAT4 and EAAT5 are encoded by *SLC1A3*, *SLC1A2*, *SLC1A1*, *SLC1A6*, and *SLC1A7* genes, respectively. In the remainder of this article, we shall look at and compare what is known of the genetics and epigenetics of the two most abundant EAATs: EAAT1/GLAST (*SLC1A3*) and EAAT2/GLT1 (*SLC1A2*) in human brain. We shall also briefly review short-term (non-genetic, non-epigenetic) regulation of EAAT1/GLAST and, when relevant, we shall include involvement of EAATs malfunction in disease, particularly in alcoholism.

Genetics of Human EAATs; Possible Involvement in Disease

Human SLC1A3 *(encoding EAAT1) gene* is located on chromosome 5 (5p13.2). The entire gene comprises 81980 bp and has 10 exons. To date, at least 16 splice variants of *SLC1A3 gene* have been described (e.g. [53–55]) but additional splice variants are likely to be discovered.

There are 1701 polymorphic sites that have so far been discovered in *SLC1A3* gene. Two different mutations of *SLC1A3* gene have been reported in patients with episodic ataxia (EA). The mutation rs137852619 in the *SLC1A3* -gene, resulting in Pro290Arg amino acid change was described by Jen et al. [56] in a juvenile patient displaying EA type 2 (EA2) with seizures, migraine, and alternating hemiplegia. MRI showed cerebellar atrophy, and neurologic examination showed mild interictal truncal ataxia. Functional expression studies showed decreased expression of the mutant EAAT1 protein with a markedly reduced capacity for GluT. This partial "human knockout" exhibits a phenotype which suggests that any potential compensatory up-regulation of EAAT2 is not sufficient to prevent damage or developmental perturbation of the brain. De Vries et al. [57] identified mutation rs137852620 in three members of a Dutch family with EA resulting in a Cys186Ser substitution in EAAT1. In these patients no mutations were found in the CACNA1A gene even though they showed symptoms of EA usually characteristic of EA2 and including interictal nystagmus (but no myokymia), attacks of mild ataxia with a duration of several hours and a positive response to acetazolamide. Additional associated symptoms such as vertigo, nausea/vomiting and photophobia were also present. The syndrome was designated as EA, type 6; in studies in vitro, this mutation produced an 18 % decrease in GluT compared to the normal (wild type) transporter [57].

Several other DNA polymorphisms of *SLC1A3* gene have been studied in association studies of ADHD, schizophrenia, Gilles de la Tourette syndrome, glaucoma; mostly with negative results [58–61]. Murphy et al. [62] noted a weak association between suicidal behaviour and rs2269272 polymorphism. Turic et al. [63] described association between ADHD and rs2269272 polymorphism. Walsh et al. [64] published results of copy number variation (CNV) analysis of whole genome in patients with schizophrenia (150 cases) and controls (268 cases). He described microdeletion or microduplication in *SLC1A3* gene in four cases of schizophrenic patients but none in controls. This could suggest a possible relationship between *SLC1A3* gene mutation and schizophrenia. In this context, it may be of interest to mention that Shan et al. [65] reported decreased expression of EAAT1 protein in the superior temporal gyrus of patients with schizophrenia.

Human SLC1A2 *(encoding EAAT2) gene* is located to chromosome 11 (11p13-p12). The entire gene comprises 168859 bp and 17 exons. So far, 8 splicing variants of *SLC1A2* have been described. To date, 3767 DNA polymorphisms have been discovered in this gene.

We have performed a guided computer analysis [66] of *SLC1A2* mRNA and we have found several binding sites for miRNAs. We have found hsa-miR-19b-3p that has been reported to be upregulated in serum of children with autism [67]; in fact, hsa-miR-19b-3p has been suggested as a possible candidate for circulating miRNA-based prediction-marker of autism. Among other miRNA's of interest here, hsa-miR-5585-5p was found to be associated with glioblastoma [68], hsa-miR-5095 and hsa-miR-1273a were

detected in microvesicles released from glioma cells [69] and hsa-miR-136-5p was reported as associated with Alzheimer's disease [70].

Association studies bewteen *SLC1A2* gene and essential tremor yielded ambiguous results as they seemed to be reliant on the studied populations, diagnostic criteria, sample sizes etc. [71]. Fiorentino et al. [72] suggested that rare variants in the *SLC1A2* gene may confer susceptibility to schizophrenia and bipolar disorder.

Mechanisms of EAATs involvement in neurological disease given the high abundance of EAATs in brain tissue coupled with the ubiquity of glutamatergic excitatory synapses, where EAATs appear to be indispensable, it would seem almost obvious that any changes in the expression, location or functional characteristics would have far-reaching consequences and could underlie pathologies of many neurological disorders. Indeed, it follows from what has been discussed so far that any deficiency in GluT could lead to chronically increased concentrations of L-Glu at the synapse and in its immediate environment. Such "hyperglutamatergic" state could profoundly influence the brain function and even result in L-Glu "excitotoxicity" producing neuronal death. The hyperglutamatergic excitotoxicity associated with the dysfunction of GluT may not be at work only in the "obvious" neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer disease or Huntington's chorea [73, 74; reviews: 6, 7, 75]. For example, Ye et al. [76] analysed glioma cell lines from human brain tumours and this revealed up to 100-fold lower glutamate reuptake in comparison with normal astrocytes. There was both lower expression and change in subcellular distribution of EAAT1 in glioma cells compared to healthy astrocytes. Ye et al. [76] hypothesized that the brain necrosis and seizures typical for patients with glioblastoma could be influenced by unusual glutamate release from glioma cells; the effect could be directly linked to a deficient GluT by the pathologically transformed glia. The lack of reuptake (perhaps in addition to "leaky" plasma membranes in such glia or regulated release of glutamate by the glia, i.e. "gliotransmission") would lead to higher extracellular concentrations of L-Glu and result in overexcitation and neurotoxicity associated with glioblastomas.

Interestingly, it has been suggested that a hyperactive GluT could contribute to the etiology of schizophrenia (characterised at least in part as exhibiting features of reduced glutamatergic neurotransmission) and this has been supported by several studies. Neuroleptics such as clozapine and haloperidol have been shown to down-regulate GluT, GLT1 in particular [77, 78]. In addition, there is evidence from human studies that EAAT2/GLT1 may be significantly upregulated in parts of brain which display deficiencies in schizophrenia ([79]; review [80]). It is plausible that increased EAAT expression could lead to the hypoglutamatergic transmission state in such patients.

Complete knockout of GLT1 has initially no apparent serious consequences but the (-/-) GLT1 animals eventually develop severe seizures and tend to die prematurely with only about 50 % surviving at 6 weeks of age [81]. More recent studies, however, using a conditional knockout [82], show that there is only modest impact on adult animals when 80 % or more of the GLT1 is knocked out, suggesting that the lethality of GLT1 knockout in younger animals might be a consequence of a developmental event such as the known transient overexpression of NMDA receptors that is observed in the developing rodent brain. Accordingly, GLT1 may not be as essential for the adult brain as previously thought. Complete knockout of GLAST produced less serious defects than the complete knockout of GLT1 [83]. Obviously, there was a degree of compensation with the missing EAAT being, to some extent, substituted by the other most abundant one. Double knockout (both GLT and GLAST), however, resulted invariably in a sudden death in utero at about E17/E18 [84], illustrating the absolute necessity of GluT, at the very least, in brain development. The timelines for death appear to coincide approximately with the timeline for expression of GLT1 in the brain, but somewhat later than the initial timeline for expression of GLAST [85]. Interestingly, the pathology of the double knockout is complex and includes, for example, deficits in callosal formation, which argue for glutamate acting on multiple targets and against death being due solely to an excitotoxicity event.

Realization that deficient GluT could be, if not a cause, than at least a contributory factor, in the neuronal death as it occurs in neurodegenerative diseases resulted in several studies aimed at finding how to increase and restore the normal expression of EAAT (for reviews see [86, 87]). Several mechanisms capable of (up)regulating EAATs have been identified but neither of them helped to satisfactorily explain aetiology of any neurological disorder nor have they been shown to lead to a development of any truly useful therapies which would prevent or at least significantly slow down the neuronal death [88, 89]. One remarkable aspect of these studies is that they have been targeting, in almost all cases, EAAT2/GLT1 rather than EAAT1/GLAST. Given the already highly abundant expression of GLT1 by astrocytes, there is potentially only a limited capacity for the cells to further increase GLT1 expression, whereas there may be lesser impediments to the up-regulation of GLAST.

Epigenetics and Transcription Regulation of EAATs

Expression of EAATs is regulated by several mechanisms; this topic has been researched and reviewed extensively in recent years. In short, there are mechanisms capable to upregulate or downregulate glutamate transporters and they can operate at the level of transcription, or translation, via epigenetic mechanisms or in response to changes in the immediate environment (reviews: [6, 90, 91]).

Unger et al. [90] performed software analysis of the human EAAT1 promoter and identified 13 potential target sites for transcription factors. Furthermore, in her experimental studies in vitro, Unger et al. [90] described stimulatory effects of dbcAMP, PACAP, EGF, and TGFα on EAAT1 gene expression. Additional transcription regulators potentially active at the promoter (CART, CEBP, GATA, HAML, MZF1, NFjB, NKXH, Oct1, SP1, and YY1F) are yet to be evaluated.

Interestingly, in an earlier study, brain derived neurotrophic factor (BDNF) was found to have no effect on EAAT1/GLAST expression by cultured astrocytes [92]. This is in contrast with more recent investigation showing that BDNF can up-regulate GLAST, at least in the retina [93]. The GLAST upregulation would result in a more rapid clearance of L-Glu and this mechanism in concert with the concommitant increase in glutamine synthase which catabolises any accumulated glutamate, could contribute to the neuroprotective effects of BDNF [93]. Furthermore, the BDNF effect on EAAT1/GLAST expression might explain results of an association study which uncovered a relationship between 66Met allele of BDNF (that is associated with an increased BDNF expression) and protection of patients with alcoholism from colour vision deficiency induced by long-term excessive alcohol intake and thought to be linked to an increased NMDA receptor density [94].

Most of the other studies have been focusing on EAAT2/GLT1; this should not surprise as this transporter is not only responsible for much of GluT in brain tissue (perhaps 90 %, [8]; recent review: [95]) but also because it is, as discussed above, the EAAT most often related to neurological and mental diseases [reviews: 5, 7, 8, 75]. For example, the mechanisms of action of the antibiotic ceftriaxone that has been used to upregulate EAAT2/GLT1 in animal experiments and tested in human trials as a potential therapeutic agent for amyotrophic lateral sclerosis [87–89], have been investigated in great detail; ceftriaxone seems to upregulate EAAT2/GLT1 via the transcription factor NFκB signalling pathway [96]. Another case of a EAAT2/GLT1-focused study is a recent identification of a neuronal miRNA which can regulate EAAT2/GLT1 expression in astrocytes [97].

Studies of epigenetic regulation of EAATs expression have barely begun [review: 6]. In our current investigations we have performed CpG island analysis of 5′ end of *SLC1A3* human gene (RefSeq: JQ085380, GenBank). Using the software of Takai and Jones [98] we have found no CpG islands; this would seem to suggest that the methylation of that part of the gene is not an important regulatory mechanism of the human EAAT1 expression.

In the case of human EAAT2, CpG island analysis of 5′ end of *SLC1A3* gene (RefSeq: EF078920, GenBank) by the same method [98] identified a CpG island spanning 1515 bp upstream of the first exon. Interestingly, Zschocke et al. [99] found that the lack of EAAT2 expression in human glioma cell lines was associated with a densely methylated *SLC1A2* promoter. This contrasts with a hypomethylation of the same promoter region of *SLC1A2* gene in normal human brain. It is therefore, probable that EAAT2 expression is, at least in part, regulated by the differential methylation of the *SLC1A2* gene promoter. Perisic et al. [100] have recently extended the earlier studies and provided a more detailed analysis of the methylation site at the *SLC1A2* (EAAT2/GLT1) promoter in the rat brain. They found higher methylation in the cerebellum compared to the cerebral cortex which is in agreement with the differential distribution of EAAT2/GLT1 in the mammalian CNS [reviews: 5, 15].

Rapid Regulation of GluT Activity by Other Than Genetic or Epigenetic Mechanisms

EAATs are not only subject to genetic or epigenetic regulation; there are important mechanisms which can activate GluT on the scale of seconds, minutes and hours and are independent of the mechanisms such as transcription, translation or DNA methylation. It was shown many years ago that GluT could be "activated" in the presence of its substrate [101]. It was later demonstrated that, in particular in the case of EAAT1/GLAST, there can be a shift from an "inactive" pool of the transporter in the cytoplasm to the "active" location in the plasma membrane in response to the presence of L-Glu (or indeed any other transportable EAAT substrate) in the extracellular environment (for a review and discussion see [102]).

The movements of GLAST in response to D-aspartate and other transportable substrates have been visualised in cultured astrocytes and it has been shown that they are sensitive to inhibitors of enzymes mediating phosphorylation (protein kinase C-delta [103, 104]) and dephosphorylation (okadaic acid; inhibitor of protein phosphatases [104, 105]). Use of rottlerin as an inhibitor of PKC-δ in these studies has, however, been questioned and alternative explanations for its

actions have been advanced including an inhibitory effect of rottlerin on Na^+,K^+-dependent ATPase [105–107]. Indeed, there is evidence that glutamate transporters, including EAAT1/GLAST, are colocalized in the plasma membrane with Na^+/K^+-dependent ATPase [14, 108–110], which drives the GluT by generating the necessary ionic gradients directly into a local subcellular microdomain. Other enzymes and even mitochondria have been suggested to exist in a tight complex [110] driving and regulating GluT. In fact, combined effects of subtoxic doses of metabolic inhibitors and compounds which interfere with GluT will cause neuronal death both in mixed cultures of neurons and glia and in vivo [111, 112] thus further illustrating the tight functional relationship between energy supplying metabolic enzyme systems and GluT.

EAAT1/GLAST: Possible Role in Alcoholism?

Rimondini et al. [113] reported an increase in the expression of EAAT1/GLAST in the cingulate cortex of rats intermittently exposed to ethanol vapours. Furthermore, similar or higher overexpression of EAAT1/GLAST has been reported in post mortem brains of patients suffering from alcoholism [114]. In fact the increase in EAAT1/GLAST, studied by Western blotting using a custom-prepared antibody was among the quantitatively largest neurochemical changes reported in alcoholism [114]. At about the same time, it was noted [115] that mutant mice transfected with a mutant gene *Per2* (Period2) that had been shown to associate with alcoholism in humans displayed a lower level of EAAT1/GLAST (but not that of EAAT2/GLT which might have even shown a modest compensatory increase [115]). Significantly, the affected mutant mice were more prone to drinking alcohol than the wild type mice, when given a choice. The overall lower activity of EAAT1/GLAST appeared to be a result of a disturbed diurnal cycle in EAAT1/GLAST expression which might have lead to "hyperglutamatergic" states i.e. increased concentration of L-Glu in the extracellular space, overexcitation of neurons which would then be compensated by the intake of alcohol and ensuing inhibition of the NMDA-type of glutamate receptors (review: [116]). This mechanism, however, does not directly explain the observed increase in EAAT1/GLAST apparently caused by long-term excessive drinking; one might surmise though that the increase in EAAT1/GLAST is a part of a compensatory mechanism responding to the excessive extracellular levels of L-Glu [117]. In order to further explore mechanisms by which alcohol may influence the activity of GluT we initiated experiments testing alcohol effects on the distribution of GLAST in astrocytes derived from neonatal rats.

Presence of D-aspartate and other substrates of GluT can produce a shift of EAAT1/GLAST from a cytoplasmic compartment (possibly in the form of small vesicles) to the plasma membrane which may be accompanied by an increase in GluT [103–106]. We have carried out a series of preliminary experiments testing a hypothesis that ethanol interferes with this mechanism thus perhaps increasing the need for expression of additional GLAST protein to keep GluT functioning. In fact, we found that ethanol itself, at concentrations which can occur during heavy drinking [118], caused a shift of GLAST from cytoplasm to the plasma membrane. Some of the results are shown in Fig. 1.

We do not yet know whether the effect of alcohol is accompanied by an increased GluT, however, we noticed that baclofen, a GABA(B) agonist that has been proposed as a drug alleviating pathological alcohol-craving in alcohol-dependent humans [119, 120] reversed this process (Fig. 1). The mechanism of baclofen actions on craving is not clear but it may be related to a GABA(B) receptor mediated regulation of one of the fibre tracts from midbrain to ventral striatum which are involved in actions of psychoactive drugs such as alcohol [121].

The activation of EAAT1/GLAST by ethanol is consistent with the "hyperglutamatergic" hypothesis of alcoholism, particularly as it applies to the mechanism of "craving" and its relief by ethanol. Ethanol would not only antagonize the effect of the high extracellular L-Glu by inhibiting NMDA receptors but could further relieve the stressed/overexcited neurons by activating EAAT1/GLAST which would then move excess L-Glu away from the extracellular space into the surrounding astrocytes. This mechanism could reinforce the chronic upregulation of EAAT1/GLAST thus facilitating the rapid relief of hyperglutamatergic states by ingested ethanol.

As there are few or no data on the effect of baclofen during heavy alcohol intoxication, at least not at the cellular/molecular level, and we have not yet tested an effect of baclofen on EAAT1/GLAST in astrocytes previously chronically exposed to ethanol, we cannot unequivocally relate the current observations to the "anti-craving" effect of baclofen. The above finding, however, represents the first identification of a possible common molecular target of ethanol and baclofen in brain and further underscores the central position of GluT in regulating synaptic functions in brain. It also potentially expands the "glutamatergic" hypothesis of alcoholism perhaps providing an additional therapeutic target [122, 123].

Concluding Remarks

We have presented a brief summary of the most important information on the location and function of excitatory amino acid transporters (EAATs) in brain. Additionally, we have surveyed recent progress in genetics and epigenetics

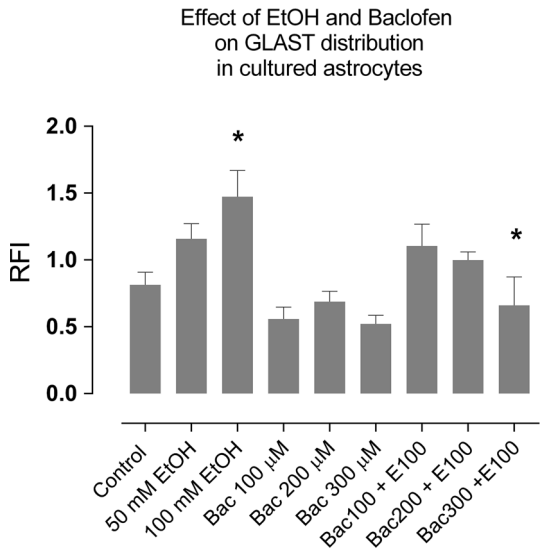

Fig. 1 Effect of alcohol on the distribution of GLAST in the cultured rat astrocytes. Cultured astrocytes were prepared from foetal rat brains and cultured as described previously [104]. Distribution of GLAST antibody (same antibody as used before [104–106]) is expressed as relative fluorescence index defined as earlier [104]. Essentially, when RFI = 1, the fluorescence (GLAST) is distributed approximately equally between the plasma membrane and cytoplasm, while RFI >1 signifies shift of the fluorescence (GLAST) to the membrane. The results are means SEM from 5 to 11 randomly selected cells. The value of RFI in the presence of 100 mM ethanol is significantly different from control ($P < 0.05$) and RFI under 100 mM ethanol plus 300 μM baclofen is significantly lower ($P < 0.05$) than the RFI observed with after 100 mM ethanol only (both marked with an *asterisk*; not all statistically significant differences are marked). Statistics were computed by ANOVA multiple comparisons using Tukey's test. Baclofen alone produced no significant effect. The cells were incubated in the presence of drugs for 45 min

of EAAT1/GLAST and EAAT2/GLT and extracted ("in silico") some new information on the corresponding genes, particularly with respect to the transcriptional and epigenetic regulation of their expression. We have also addressed the question of rapid regulation of EAAT1/GLAST in response to extracellular environment and presented preliminary data which, together with existing data in the literature, support the role of glutamate transport (GluT)—particularly that which is mediated by EAAT1/GLAST—in the brain cells exposed to alcohol.

We conclude that, in general, the role of EAAT1/GLAST has not been given sufficient attention; there is evidence that GLAST can be functionally upregulated to a significant extent and potentially targeted to functional compartments, such that it can reduce excitotoxicity. Obviously the desired pharmacological tool to evoke this type of upregulation to deal with disease states such as ALS or stroke is probably not alcohol, but the mechanisms underpinning this upregulation indicate tremendous scope for manipulating GLAST expression to minimise excitotoxicity. There is a scope for a great expansion of research into the regulation of EAAT1/GLAST at transcriptional, translational, epigenetic and cellular level in both health and disease.

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