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REVIEW

Transporters for L-glutamate: An update on their molecular pharmacology and pathological involvement

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L-Glutamate (Glu) is the major excitatory neurotransmitter in the mammalian CNS and five types of high-affinity Glu transporters (EAAT1–5) have been identified. The transporters EAAT1 and EAAT2 in glial cells are responsible for the majority of Glu uptake while neuronal EAATs appear to have specialized roles at particular types of synapses. Dysfunction of EAATs is specifically implicated in the pathology of neurodegenerative conditions such as amyotrophic lateral sclerosis, epilepsy, Huntington's disease, Alzheimer's disease and ischemic stroke injury, and thus treatments that can modulate EAAT function may prove beneficial in these conditions. Recent advances have been made in our understanding of the regulation of EAATs, including their trafficking, splicing and post-translational modification. This article summarises some recent developments that improve our understanding of the roles and regulation of EAATs.
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Keywords: L-Glutamate; transport; EAAT; trafficking; neurodegeneration; molecular pharmacology; splicing; glia; neurons

Abbreviations: ALS, amyotrophic lateral sclerosis; CNS, central nervous system; DHK, dihydrokainate; EAAT, excitatory amino-acid transporter; ETB-TBOA, (2*S*,3*S*)-3-{3-[4-ethylbenzoylamino]benzyloxy}aspartate; GLAST, L-glutamate/L-aspartate transporter; GLT-1, glutamate transporter 1; Glu, L-glutamate; GTRAP, glutamate transporter-associated protein; 4MG, (2*S*,4*R*)-4-methylglutamate; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate; SLC1, solute carrier family 1; TBOA, L-*threo*-β-benzyloxyaspartate; TNF, tumor necrosis factor

Introduction

All amino-acid and monoamine neurotransmitters possess specific, high-affinity transport mechanisms that have evolved to terminate the synaptic actions of the transmitter and to recycle the molecules involved. For the major excitatory transmitter of the mammalian central nervous system (CNS), L-glutamate (Glu), this transport is vital as high concentrations of Glu in the synaptic milieu can cause neuronal injury through a multifaceted process termed excitotoxicity. Transporters for Glu functionally play a role in preserving the local integrity of excitatory synaptic transmission (Marcaggi and Attwell, 2004). Extracellular levels of Glu are regulated by a family of transporters, which are quite distinct from those for the monoamine (noradrenaline, dopamine and 5-hydroxytryptamine) and other amino-acid (4-aminobutyric acid or glycine) transmitters. High-affinity transporters for Glu (*e*xcitatory *a*mino-*a*cid *t*ransporters (EAATs)) represent a unique family of proteins (reviewed by Kanai and Hediger, 2004) that display considerable homology (50–60% at the amino-acid level) and there has been considerable recent progress in our understanding of their pharmacology, although unlike the monoamine transporters, their physiological regulation is poorly understood. While many clinically effective drugs act via monoamine transporters (Torres *et al.*, 2003), molecules displaying selectivity for the various subtypes of EAATs are only just beginning to emerge. Moreover, there are likely to be considerable advances in our knowledge of the structure–activity relationships governing EAAT function with the recent description of the crystal structure of a bacterial EAAT, which resembles the mammalian proteins (Yernool *et al.*, 2004). Many membrane-associated proteins are internalized quickly and trafficked via intracellular compartments, but such mechanisms are poorly understood for EAATs and likely to be complex as the vast majority of Glu transport occurs into glia, which undergo extensive cytoskeletal reorganization during development and injury of the brain (Pekny and

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Nilsson, 2005). This minireview focuses on these emerging issues and more extensive reviews can be found elsewhere (Bridges *et al*., 1999; Danbolt, 2001; Balcar, 2002; O'Shea, 2002; Campiani *et al*., 2003; Kanai and Hediger, 2004; Shigeri *et al*., 2004; Bridges and Esslinger, 2005).

Characteristics, function and localization of EAATs

Over the past 15 years, five major subtypes of EAAT have been identified (nomenclature in human EAAT1-5; for simplicity, this paper will use this terminology regardless of the species being investigated). EAATs are members of the solute carrier family 1 (SLC1) that also includes the two neutral amino-acid transporters, ASCT1 and ASCT2 (Kanai and Hediger, 2004). EAATs are distinct from the family of proteins responsible for vesicular Glu transport, which have been reviewed elsewhere (Hisano, 2003; Fremeau *et al*., 2004; Shigeri *et al*., 2004). EAATs possess distinct localizations at both the cellular and regional level as well as distinct molecular and pharmacological characteristics. EAAT1 (SLC1A3, the human homologue of L-glutamate/L-aspartate transporter (GLAST)) is present in glial cells throughout the CNS and at high levels in Bergmann glia of the cerebellum (reviewed by Danbolt, 2001; O'Shea, 2002; Kanai and Hediger, 2004). EAAT2 (SLC1A2, human homologue of glutamate transporter 1 (GLT-1)) is almost exclusively glial, and is widespread and highly abundant throughout the CNS (reviewed by Danbolt, 2001; O'Shea, 2002; Kanai and Hediger, 2004). The transporters EAAT3 (SLC1A1, human homologue of EAAC1) and EAAT4 (SLC1A6) are present predominantly in neurons; EAAT3 is abundant throughout the CNS, whereas EAAT4 is predominantly localized to cerebellar Purkinje cells (reviewed by Danbolt, 2001; O'Shea, 2002; Kanai and Hediger, 2004), with low levels of expression also present in the forebrain (Dehnes *et al*., 1998; Massie *et al*., 2001). EAAT5 (SLC1A7) is present in rod photoreceptor and bipolar cells of the retina (Arriza *et al*., 1997; Pow and Barnett, 2000).

A number of elegant studies have shown that effective EAATs are essential for the maintenance of normal excitatory synaptic transmission and thus, for example, Glu clearance from the synaptic milieu determines the time course of Glu receptor activation (Diamond and Jahr, 1997; Takayasu *et al*., 2006) and its dysfunction may result in excitotoxicity (reviewed by Danbolt, 2001; O'Shea, 2002; Kanai and Hediger, 2004). The majority of synapses in the CNS are in close apposition with glia, and glial EAATs are responsible for the bulk of Glu uptake (reviewed by Danbolt, 2001; O'Shea, 2002; Kanai and Hediger, 2004), whereas neuronal EAATs appear to have more specialized roles, some of which are discussed below. Presumably, the existence of multiple EAATs (with subtypes and splice variants distributed both in the same and different cells) reflects the evolutionary development of tightly controlled regulatory processes needed to ensure the efficient maintenance of synaptic transmission for the major mammalian excitatory transmitter. Numerous *in vitro* and *in vivo* studies have demonstrated that transgenic ablation, antisense downregulation or pharmacological inhibition of glial EAATs results in increased extracellular Glu and neuronal death. Furthermore, immunocytochemical and physiological studies on the distribution and function of different EAAT subtypes confirm the functional dominance of the glial transporters (reviewed by Danbolt, 2001). Glial EAATs also serve another vital role in the CNS by supplying Glu for metabolic processes such as the glutamate–glutamine cycle. Thus, a range of evidence suggests that alterations in Glu metabolism or glial viability resulting from impaired Glu transport may also contribute to neuronal damage (Rae *et al*., 2000; Had-Aissouni *et al*., 2002; Ré *et al*., 2003; Aoyama *et al*., 2006). As well as reducing the potentially toxic build-up of extracellular Glu, it is likely that Glu uptake by glial EAATs also signals the energy needs of nearby neurons via activation of the Na^+/K^+ ATPase and glucose transporters, and changes in the levels of ATP and lactate (Voutsinos-Porche *et al*., 2003). This growing body of evidence suggests an interactive role between glial EAATs and intermediary metabolism that has both physiological and pathological relevance.

The existence of Glu transporters on presynaptic neuronal terminals has been suspected for at least 30 years (Beart, 1976; Storm-Mathisen and Iversen, 1979), but the subtype of EAAT responsible for this uptake has remained elusive. Recent evidence suggests the presence of a variant form of EAAT2 in neurons (Schmitt *et al*., 2002; Chen *et al*., 2004) that may account for presynaptic Glu uptake. The difficulty of demonstrating such presynaptic localizations could relate to various issues, including the relatively low abundance of EAATs on these terminals (compared to nearby glial cells) and problems of antibody access and geometry in a relationship that is tipped heavily in favour of astrocytes tightly juxtaposed to nerve terminals (cf. Danbolt, 2001).

While astrocytic EAATs are generally responsible for the majority of Glu uptake (see above), neuronal transporters assume greater significance at certain specialized synapses, particularly in the cerebellum, where the spatial relationship between EAATs and Glu receptors is altered and where many peri- or extrasynaptic Glu receptors are present (Huang and Bergles, 2004). Although physiological roles for neuronal EAATs are poorly documented, the localization of EAAT3 and EAAT4 at these synapses allows the selective modulation of Glu signalling at particular Glu receptors. For example, in the cerebellum where EAAT4 is abundant on Purkinje neurons, signalling at metabotropic glutamate receptors (mGluRs) on these cells is limited by Glu uptake (Wadiche and Jahr, 2005). Similarly, at parallel fibre and climbing fibre synapses on Purkinje neurons, EAATs limit mGluR responses under resting conditions (Otis *et al*., 2004). Furthermore, inhibition of neuronal EAATs in the cerebellum facilitates mGluR-mediated long-term depression (Brasnjo and Otis, 2001). Although EAATs play only a minor role in regulating the synaptic activation of Glu receptors at most central synapses (Chen and Diamond, 2002), their role is more prominent at specialized types of synapses. In the hippocampus, inhibition of glial (but not neuronal) EAATs potentiates activation of postsynaptic mGluRs, resulting in enhanced inhibition of pyramidal neurons (Huang *et al*., 2004). A further example of EAAT inhibition promoting the recruitment of extrasynaptic receptors occurs at *N*-methyl-D-aspartate (NMDA) receptors on retinal ganglion cell

synapses, where EAATs play a critical role in limiting the synaptic activation of NMDA receptors by Glu (Chen and Diamond, 2002).

Modulation of EAAT activity occurs via both short-term (due to altered trafficking and post-translational modifications such as phosphorylation) (Robinson, 2002; Vermeiren *et al*., 2005) and longer-term (due to altered expression and abundance) mechanisms (Rothstein *et al*., 2005; Ganel *et al*., 2006; O'Shea *et al*., 2006). A further mechanism for the regulation for EAAT expression, particularly for the glial transporters, exists in the form of alternative splicing of transcripts. Numerous splice variants of EAAT2 have been identified, showing differential (tissue- and species-dependent) patterns of expression. Despite their existence, functional differences in transport activity have not been identified in the major EAAT2 splice variants that are functional (Utsunomiya-Tate *et al*., 1997; Chen *et al*., 2002; Sullivan *et al*., 2004), suggesting that this alternative splicing may be more important in the differential targeting of transporters to specific cell types or localizations than in regulating the properties of Glu uptake. Interestingly, Sullivan *et al*. (2004) have suggested the differential localization of an EAAT2 splice variant relative to EAAT2 itself could indicate its role in regulating Glu spill over in proximal processes of astrocytes. The presence of an EAAT2 splice variant in neurons (Schmitt *et al*., 2002; Chen *et al*., 2004) may go some way towards explaining earlier difficulties in identifying the EAAT subtype responsible for presynaptic Glu uptake, although some debate remains as to the identity of this transcript (Chen *et al*., 2004; Sullivan *et al*., 2004). A much smaller number of studies have identified splice variants in EAAT1 (Huggett *et al*., 2000; Vallejo-Illarramendi *et al*., 2005a) and neuronal EAAT3 (Matsumoto *et al*., 1999), whereas a variant form of EAAT3 ('EAAC2'), transcribed from an independent promoter, has also been reported (Jin *et al*., 2002). While little is yet known about these transcripts, the form of human EAAT1 lacking exon 9 appears to be retained in the endoplasmic reticulum and exert a dominant-negative effect on full-length EAAT1 (Vallejo-Illarramendi *et al*., 2005a). The involvement of EAAT splice variants in pathological conditions is discussed later in this paper.

Molecular structure and new functional implications

A major advance in late 2004 was the successful determination of the crystal structure of a Glu transporter homologue from *Pyrococcus horikoshii* (Yernool *et al*., 2004). Although our interpretation of these landmark observations should be tempered with some caution as this transporter shares only 37% homology with human EAAT2, the predictions arising from these findings are especially exciting. The authors employed sequence alignments involving EAAT1–3, reflecting the significant relationships throughout the entire polypeptides of prokaryotic and eukaryotic Glu transporters, and the analyses were in the context of the commonly accepted topology of EAATs (reviewed by Kanner and Borre, 2002). Although earlier observations based on freeze fracture electron microscopy suggested a pentamer (Eskandari *et al*., 2000), recent molecular and biophysical evidence (Gendreau *et al*., 2004; Yernool *et al*., 2004; Grewer *et al*., 2005) favours a functional transporter with a stoichiometry of three identical subunits. Yernool *et al*., (2004) suggest that the oligomeric transporter is bowl-shaped with a solvent-filled extracellular basin extending halfway across the membrane. The structure of this large aqueous basin fits with the idea that the activity of EAATs, and other Na^+/Cl^- co-transporters (Hilgemann and Lu, 1999; Ryan *et al*., 2004), is likely to be exemplified by the alternating-access model allowing access of the substrate to either intracellular or extracellular solution. Based upon recent fluorescence resonance energy transfer analysis with fluorescently labelled human EAAT3, relatively small conformational changes may occur in each subunit in the coupled transport of substrate – here a hybrid version of the 'rocker-switch model' of alternating access was proposed for EAAT3 (Koch and Larsson, 2005). Other work with EAAT3 shows that the transporter undergoes three conformational changes during a cycle and that important Na^+-dependent conformational changes precede Glu binding (Larsson *et al*., 2004). It is particularly interesting that evidence supports the concept that each subunit of the trimer functions independently (Yernool *et al*., 2004; Grewer *et al*., 2005). Of course, much now remains to be resolved about the fine details of the molecular events that subserve each step in the transport process. Key issues to be addressed here include where and how subunit oligomerization is regulated, and how assembly responds to synthesis, intracellular compartmentalization and trafficking events regulating EAAT activity.

Transport of Glu by EAATs is well documented to involve the co-transport of three Na^+ and one H^+, and the counter transport of one K^+ enabling EAATs to maintain a large concentration gradient across the cell membrane (Danbolt, 2001; Kanai and Hediger, 2003; Shigeri *et al*., 2004). There now seems a general consensus from several laboratories that EAATs are likely to subserve at least dual functions both as a transporter and ion channel, whereby residues in the carboxyl-terminal and amino-terminal portions, respectively, are the molecular determinants critical for these different functions (Slotboom *et al*., 2001; Amara and Fontana, 2002; Kanner and Borre, 2002). Put simply, these two functions are Glu transport (i.e. substrate recognition, binding, transport and ion coupling) and chloride flux. A recent elegant study by Vandenberg and co-workers (Ryan *et al*., 2004) has shown this convincingly for EAAT1. Results over some 10 years have demonstrated through the use of site-directed mutagenesis and chimeric transporters that mutations that disrupt chloride permeation do not alter transport, and *vice versa*. Furthermore, while Glu can be transported in the absence of a change in chloride conductance, the latter cannot occur without activation of the transporter, with other evidence suggesting an inverse relationship between transporter rate and anion conductance (Grewer and Rauen, 2005). This functional duality has been best described for the dopamine transporter where presynaptic excitability is regulated by substrate-activated chloride conductance (Mortensen and Amara, 2003). This phenomenon is likely to have physiological relevance for EAAT4/5, which possess greater chloride conductance than

EAAT1–3 (Grewer and Rauen, 2005). For example, Arriza *et al*. (1997) suggest that the channel-like properties of EAAT5 may indicate a role in retinal physiology distinct from neurotransmitter clearance. Whether EAATs play such roles at other specialized synapses (vide supra) requires further investigation. However, as most Glu transport is into astrocytes, which possess EAATs displaying relatively low chloride conductance (Grewer and Rauen, 2005), the functional relevance of this duality in astrocytes remains to be determined.

EAATs, targeting and trafficking

By analogy to monoamine transporters (Torres *et al*., 2003), a variety of evidence indicates that the intrinsic activity of EAATs and cell surface trafficking are regulated, not only by kinases/phosphatases and scaffolding proteins, but also by diverse influences (e.g. endothelin B, vasoactive intestinal peptide, pituitary adenylate cyclase-activating peptide, platelet-derived growth factor, thyroid hormone and trophic factors) that appear to affect trafficking to and from the plasma membrane to intracellular pools involving lipid rafts and caveolae (Figure 1). In general, the transport of oligomeric membrane proteins from intracellular pools to the cell surface seems to be highly organized and subject to quality control involving the endoplasmic reticulum and/or the Golgi apparatus – presumably this is also the case for Na^+/Cl^- co-transporters as cells have inherent systems for minimizing errors and maintaining efficiency (Ellgaard and Helenius, 2003). For EAATs, the story in this area is only just beginning to emerge, with the initial series of investigations implicating various kinases (protein kinases C and B, phosphoinositol-3-kinase, mitogen-activated protein kinase, serum and glucocorticoid inducible kinase 1) and calcineurin (Aronica *et al*., 2003; González *et al*., 2003; Boehmer *et al*., 2006; Li *et al*., 2006), with this phosphatase perhaps linked to the involvement of the immunophilin, FK506 (Labrande *et al*., 2006) (Figure 1). Indeed, based upon early observations, the cell-surface expression and trafficking of EAATs may display appreciable heterogeneity between subtypes of transporter with ~80% of EAAT2 expressed at the cell surface, whereas ~70% of EAAT3 is found in the cytosol (Sheldon *et al*., 2006). Apparently a C-terminal motif in EAAT3 is important for its trafficking and constitutive sorting (Sheldon *et al*., 2006).

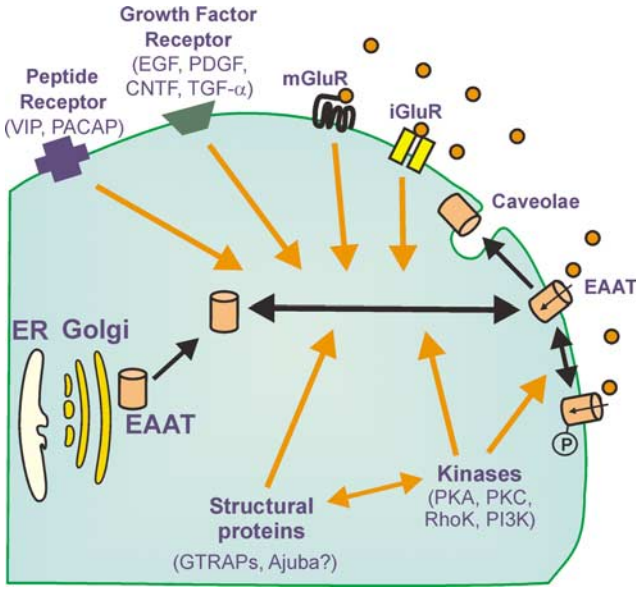

Figure 1 Mechanisms contributing to short- and long-term regulation of EAAT activity. Cell-surface expression and activity of EAATs is regulated by diverse influences including various receptors, phosphorylation ('P'), trafficking to and from intracellular pools and interactions with lipid rafts and structural proteins. This figure should be considered as a general schema only, in that it reflects the various processes involved. Whether individual effects occur independently of transcription, via various intracellular pools or purely at the level of the cell surface, has not always been elucidated.

Thus far all the scaffolding proteins clearly linked to EAATs (Glu transporter-associated proteins (GTRAPs) and Ajuba) (Jackson *et al*., 2001; Lin *et al*., 2001; Marie *et al*., 2002) seem to have some association with actin systems. Diverse molecules (e.g. integrins, tropomysosin, gelsolin and cofilin) are involved in regulating actin dynamics (Fass *et al*., 2004) and linkages to EAAT function remain to be fully elucidated. Given that the majority of Glu uptake is into astrocytes, rather than neurones, and these cells possess remarkable potential for cytoskeletal re-arrangement in response to diverse stimuli arising locally (Hughes *et al*., 2004; Pekny and Nilsson, 2005; Zagami *et al*., 2005), via the circulation or the cerebrospinal fluid, then Rho family small GTPases (Rho, Rac, etc.) may play a key role in regulating their actin organization (Hall, 1998; Abe and Misawa, 2003). A number of G-protein-coupled receptors including those for protease-activated receptors, lysophosphatidic acid, sphingosine-1-phosphate and mGluRs also influence astrocytic phenotype (Aronica *et al*., 2003; Sorensen *et al*., 2003), but possible links to EAAT function require further clarification. Moreover, astrocytes are known to possess caveolae, which have been implicated in cell migration, and structures positive for caveolin-1 are known to co-align with stress fibres (Navarro *et al*., 2004), which are well documented in astrocyte biology. Much remains to be discovered about the functional involvement of caveolae, but they are crucial regulators of signalling cascades and cholesterol-rich lipid-raft domains, and have a remarkable propensity to form supramolecular complexes (Parton, 2003).

Cholesterol-rich lipid rafts have recently been associated with EAAT2 (Butchbach *et al*., 2004), although cholesterol was found to be important for Glu uptake more than 15 years ago (Shouffani and Kanner, 1990). Treatment of mixed cortical cultures with the cholesterol-depleting agent methyl-β-cyclodextrin rapidly decreases Glu uptake and membrane cholesterol (<5 min) predominantly by an EAAT2 mechanism rather than an EAAT1 mechanism (Butchbach *et al*., 2004). Immunostaining revealed dispersion of the normal clustering of EAAT2 on the plasma membrane of astrocytes (Butchbach *et al*., 2004). EAAT3 staining within neurites of neurones in these cortical cultures was less sensitive to this treatment, although *in vivo* methyl-β-cyclodextrin reduces EAAT3-mediated Glu transport and induces the expression of GTRAP3–18 (Butchbach

et al., 2003). EAAT3 too is probably associated with such lipid microdomains, as polarized distributions have been noted in other model systems (Cheng *et al*., 2002). Interestingly, protein kinase Cα, which is implicated in the redistribution of EAAT3 (González *et al*., 2003), regulates caveolae dynamics (Parton, 2003). Clustering of EAATs has been recognized for some time, although its significance remains unclear (Zhou and Sutherland, 2004), but with these recent insights, dynamic changes in the localization of Glu transporters are likely to reflect altered trafficking from intracellular compartments in concert with overall alterations in cytoskeletal motility. Recently, a further level of complexity has been described whereby EAAT1/2 in astrocytes undergo glycosylation in response to ciliary neurotrophic factor in a manner that effects transporter assembly and localization in raft microdomains (Escartin *et al*., 2006).

Some of these considerations may only be answered by use of caveolin knockdown (Ge and Pachter, 2004) or of appropriate knockout mice. At present, there are many unknowns in this area, as cyclodextrins do not distinguish between lipid rafts and caveolae (Sowa *et al*., 2001), lipid rafts are difficult to study in isolation and data emerging from investigations in heterologous expression systems may not adequately reflect the true biology of intact physiological cells. Certainly, more complexities will emerge here as there is preliminary evidence that EAAT2 clustering may be influenced by a clathrin- and dynamin-dependent pathway (Zhou and Sutherland, 2004) and EAAT3 may interact with caveolin-1/2 (Gonzalez *et al*., 2004).

New molecules targeting EAATs

For over 30 years, virtually all drugs acting at EAATs were structural analogues of Glu or L/D-aspartate (Balcar and Johnston, 1972), and included various cyclic molecules such as L-*trans*-2,4-pyrrollidinedicarboxylate, kainate and dihydrokainate (DHK) (Bridges *et al*., 1999; Balcar, 2002). Many of the early analogues studied proved to be transportable blockers (i.e. substrates) and produced release of radiolabelled substrates. Moreover, among these many compounds, very little pharmacological selectivity existed across EAAT1–5,

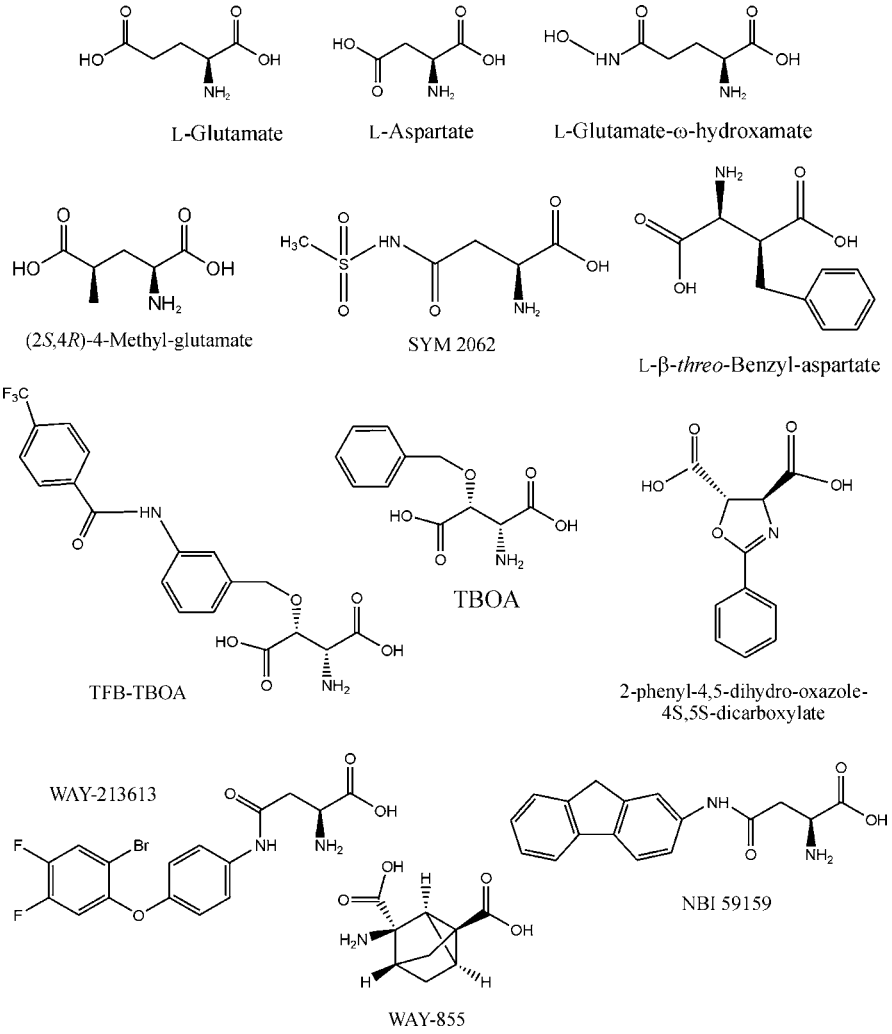

Figure 2 Structure of EAAT ligands. TBOA, L-*threo*-β-benzyloxyaspartate; TFB-TBOA, (2*S*,3*S*)-3-{3-[4-(trifluoromethyl)benzoylamino]benzyloxy}aspartate.

although DHK and L-serine-*O*-sulphate displayed some selectivity for EAAT2 and EAAT1, respectively. Studies of L-*threo*-β-benzyloxyaspartate (TBOA) (Shimamoto *et al*., 1998), L-*anti*-*endo*-3,4-methanopyrollidinedicarboxylate (Chamberlin *et al*., 1998) and their analogues revealed non-transportable blockers providing some of the first clues to the design of blockers versus substrates (Figure 2). More recent studies have revealed potent analogues of TBOA such as the blocker (2*S*,3*S*)-3-{3-[4-(trifluoromethyl)benzoylamino]benzyloxy}aspartate (Shimamoto *et al*., 2004) and caged derivatives that can be released by photolysis (Takaoka *et al*., 2004). Bridges and co-workers (Bridges *et al*., 1999; Koch *et al*., 1999) undertook pharmacophore modelling and more recently these ideas have been extended by the rational design and proof-of-principle syntheses of blockers active at EAAT3 (Campiani *et al*., 2001). These guidelines for SAR can be summarized as follows: (1) steric excess favours inhibitors versus substrates, (2) among constrained molecules, generally folding results in blockers, while unfolded molecules tend to be substrates and (3) conformational restriction *per se* does not differentiate transportable from non-transportable inhibitors. There have been a number of advances in this area driven by the premise that EAAT blockers, rather than substrates, would not release Glu into the extracellular space by heteroexchange (facilitated exchange–diffusion) exacerbating excitotoxicity and thus might have therapeutic potential (Dunlop *et al*., 1999).

Generally, progress in the development of pharmacological agents targeting EAATs has been hampered by the absence of new subtype-selective radioligands for EAATs as the development of [^{3}H]D-aspartate in 1976. (2*S*,4*R*)-4-methylglutamate (4MG) displayed some differences in its interactions with EAAT1/2 (Vandenberg *et al*., 1997) encouraging Beart and O'Shea to employ [^{3}H]4MG in a high-throughput assay (Apricò *et al*., 2001). Screening of analogues of 4MG revealed a number of active, new molecules ($\sim IC_{50}$ values 150–400 μM) with aromatic hydrophobic substituents (phenyl, benzyl, etc.) in the 4-position of Glu. Rather surprisingly, a new family of sulphamido analogues (e.g. SYM 2062) was revealed with replacement of the ω- or α-carboxyl group of Glu and aspartate, allowing retention of activity ($\sim IC_{50}$ values 5–100 μM; Beart *et al*., 2002) (Figure 2). These sulphamido analogues question the need for two acidic biosteres to bind to the transporters (Bridges *et al*., 1999; Campiani *et al*., 2003). However, we should recall that many years ago Roberts and Watkins (1975) demonstrated that the ω-hydroxamates of Glu and L-aspartate possessed activity versus Glu uptake. In a synthetic chemistry programme directed at EAATs, Wyeth firstly reported a conformationally restricted Glu analogue (WAY-855) that was an EAAT2-preferring non-substrate inhibitor (Dunlop *et al*., 2003). More recently, Dunlop and co-workers found a series of amido and ureido derivatives of aspartate that possessed high affinities for Glu uptake, and appreciable selectivity for EAAT2 (Dunlop *et al*., 2005) and EAAT3 (Coon *et al*., 2004), respectively. These studies represent major advances as not only were both families of molecules non-substrate inhibitors, but the most potent members (WAY-213613 and NBI 59159, respectively) had affinities of $\sim$100 nM (Figure 2). Importantly, distal lipophilic pockets were hypothesized to be key contributors to the resultant pharmacological activity, suggesting that existent pharmacophore models need updating. Clearly, these new lead structures offer great opportunities for the development of novel subtype-selective radioligands. A positive development here has been the recent synthesis and characterization of the non-transportable ligand [^{3}H]ETB-TBOA (2*S*,3*S*)-3-{3-[4-ethylbenzoylamino]benzyloxy}aspartate (Shimamoto *et al*., 2006) (Figure 2).

The strategy of enhancing Glu transport to expedite its removal from the synaptic milieu during a possible excitotoxic event has also received some attention because, in theory, such drugs could prove beneficial in ischaemic stroke injury and amyotrophic lateral sclerosis (ALS). Hypothetically, such enhancement of EAAT activity could be achieved by drugs acting either directly on the transporter proteins or indirectly on trafficking mechanisms. One such molecule is MS-153 (Shimada *et al*., 1999), which acts by a kinase-related mechanism to potentiate Glu uptake and to provide benefit in experimental stroke injury (Umemura *et al*., 1996). A purified component of the venom from the spider, *Parawixia bistriata*, provides neuroprotection via a non-competitive mechanism in a retinal model of injury (Fontana *et al*., 2003). Enhanced EAAT activity could also be achieved by increasing transcription or altering the stability of existent transcripts. Thus, a particularly exciting development is the recent report by Rothstein *et al*. (2005) that β-lactam antibiotics (e.g. ceftriaxone) were found in a screen of more than a thousand Food and Drug Administration-approved drugs and nutritionals to selectively elevate EAAT2 expression. Ceftriaxone appeared to be cytoprotective both *in vitro* and *in vivo* in a mouse model of ALS (Rothstein *et al*., 2005), and further insights into the mechanism underlying the apparent two- to five-fold increase in EAAT2 gene activation will be eagerly awaited. A further recent study demonstrated that the neuroimmunophilin ligand GPI-1046 selectively induced expression of EAAT2 *in vitro* and *in vivo*, as well as protecting motor neurons in cell culture and in a mouse model of ALS (Ganel *et al*., 2006). Taken together, these two recent studies provide strong support for the hypothesis that enhancement of EAAT activity could provide clinical benefit in neuropathological conditions involving excitotoxicity. Additional recent evidence suggests the potential for modulation of EAATs by pathways involving growth factors, neurotrophic factors and peptides (Figure 1) (Figiel *et al*., 2003; Escartin *et al*., 2006). As yet, allosteric modulators of EAAT function have not been identified that might produce therapeutic beneficial effects, but given the success in this area with many other proteins (Christopoulos, 2002), the site for Zn^{2+} binding distinct from the substrate binding site (Mitrovic *et al*., 2001) may be such a target.

EAATs in pathological conditions

Although a very strong case exists for EAAT function being altered in several neurological conditions, often it proves difficult to discern cause and effect in terms of dysfunction of Glu transporters. Nevertheless, excellent work on ALS (Rothstein *et al*., 2005) and spinocerebellar ataxia (Ikeda *et al*., 2006) has provided persuasive evidence for seminal

Table 1 Involvement of EAATs in neurological disorders

	Human tissue	*Experimental models*
ALS	[Glu] ↑ in CSF.[1] ↓ Uptake in CNS tissue.[2] ↓ GLT-1 in CNS tissue.[3]	ALS mutant SOD1 inactivates EAAT2.[4] Extracellular [Glu] ↑ in the cortex of ALS-SOD1 transgenic mice.[5] Focal EAAT2 loss in ALS-SOD1 transgenic mice.[6]
Epilepsy	↑Plasma [Glu] in epileptic patients.[7] ↑Hippocampal [Glu] in seizures.[8] Temporal lobe epilepsy: EAAT3 ↑ in hippocampal granule cells, EAAT2 ↓ in hilus and CA1, EAAT1 ↑ in CA2/3.[9] ↑Incidence of aberrant EAAT2 splicing in temporal lobe epilepsy patients.[10]	Fatal seizures in EAAT2 knockout mice.[11] ↓ EAAT1, 2 and 3 protein in GLAST, GLT-1 and EAAC1 protein in the brain of genetically epileptic rats.[12]
Huntington's disease	EAAT2 mRNA ↓ in neostriatum, but the number of cells expressing EAAT2 mRNA ↑.[13] Uptake ↓ in the caudate and putamen.[14]	EAAT2 mRNA and uptake down in the striatum and cortex of R6 transgenic mice.[15] Impaired Glu metabolism in R6/2 mouse brains.[16]
Alzheimer's disease	Uptake ↓ in the cortex[17] and astrocytes[18] and EAAT2 protein ↓[19] in AD patients. Aberrant neuronal expression of EAAT1[20] and EAAT2[21] associated with tau accumulation.	Glu uptake and EAAT1, EAAT2 protein ↓ in APP transgenic mice.[22] ↓ EAAT2 and Glu uptake in GFAP-tau transgenic mice.[23] β-amyloid ↓ Glu uptake.[24]
Ischaemic stroke injury	EAAT2 promoter polymorphism associated with ↑ [Glu] and frequency of stroke.[25] Neonatal hypoxic–ischaemic encephalopathy: EAAT1 ↓ in molecular layer, ↑ in Purkinje and inner granule cell layer; EAAT4 ↓ in Purkinje cells.[26]	EAAT reversal in severe ischaemia *in vitro*.[27] EAAT2/3 ↓ in piglet brain and neuronal expression of EAAT2 after hypoxia–ischaemia.[28] EAAT2 ↑ in the cortex, ↓ in the striatum after hypoxia–ischaemia in rats.[29]
White matter injury	↑ Serum [Glu] in relapsing MS patients.[30] ↑ [Glu] in white matter and acute lesions of MS brains.[31] ↓ EAAT1 and EAAT2 in oligodendrocytes in MS lesions.[32] ↓ EAAT1/2/3 in CNS tissue from MS patients.[33] ↑ EAAT1 and EAAT2 mRNA, protein and uptake in MS optic nerve.[34]	TNF-α ↓ EAAT-1 expression and Glu uptake in cultured oligodendrocytes.[32] Depolarization causes EAAT reversal in spinal white matter.[35]
Infection	↑ Plasma [Glu] in HIV patients.[36] Strong expression of EAAT1 in activated macrophages/microglia of HIV-infected brains.[37] Uptake ↓ by >50% in AIDS dementia brains.[38]	TNF-α ↓ uptake in primary human astrocytes.[39] TNF-α, interferon-γ and interleukin-1β ↓ uptake in cultures of rat hippocampus.[40] HIV-1 or gp120 ↓ EAAT2 and uptake in astrocytes.[41] Microglia and macrophages express EAAT2 in SIV-infected primates.[42] LPS ↑ EAAT2 in cultured astrocytes and microglia.[43]
Retinal disease/Glaucoma	↑ [Glu] in vitreous body of glaucoma[44] and diabetic retinopathy patients.[45] ↑ [Glu] in aqueous humor in retinal artery occlusion.[46] ↓ EAAT1 in glaucomatous eyes.[47]	↑ Intraocular [Glu] after optic nerve lesion.[48] ↓ EAAT1 activity after retinal ischaemia.[49]
Neuropsychiatric disorders	↑ EAAT1 and EAAT2 mRNA in the thalamus of schizophrenia.[50] ↓ Striatal EAAT3/4 mRNA in bipolar disorder,[51] ↓ striatal EAAT3 mRNA in schizophrenia,[51] ↓ striatal EAAT4 mRNA in major depression.[51] ↑ mRNA for EAAT3- and EAAT4-interacting proteins in the thalamus in schizophrenia.[52] ↑ mRNA for EAAT2 in prefrontal cortex of untreated schizophrenics, reduced by antipsychotic treatment.[53]	Altered expression of mRNA for EAAT-interacting proteins in clozapine-treated rats.[52] Clozapine and haloperidol ↓ EAAT2/3 mRNA in regions of rat brain.[54] Clozapine ↓ EAAT2 and uptake in cultured astrocytes.[55]

Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; EAAT, excitatory amino-acid transporter; GFAP, glial fibrillary acidic protein; MS, multiple sclerosis; SOD, superoxide dismutase; TNF-α, tumour necrosis factor-α.

[1] Rothstein *et al.* (1990); [2] Rothstein *et al.* (1992); [3] Rothstein *et al.* (1995); [4] Trotti *et al.* (1999); [5] Alexander *et al.* (2000); [6] Howland *et al.* (2002); [7] Janjua *et al.* (1992); [8] During and Spencer (1993); [9] Mathern *et al.* (1999); [10] Hoogland *et al.* (2004); [11] Tanaka *et al.* (1997); [12] Dutuit *et al.* (2002); [13] Arzberger *et al.* (1997); [14] Cross *et al.* (1986); [15] Liévens *et al.* (2001); [16] Behrens *et al.* (2002); [17] Masliah *et al.* (1996); [18] Liang *et al.* (2002); [19] Li *et al.* (1997); [20] Scott *et al.* (2002); [21] Thai (2002); [22] Masliah *et al.* (2000); [23] Dabir *et al.* (2006); [24] Keller *et al.* (1997); Lauderback *et al.* (1999); [25] Mallolas *et al.* (2006); [26] Inage *et al.* (1998); [27] Rossi *et al.* (2000); [28] Martin *et al.* (1997); Pow *et al.* (2004); [29] Cimarosti *et al.* (2005); [30] Westall *et al.* (1980); [31] Srinivasan *et al.* (2005); [32] Pitt *et al.* (2003); [33] Werner *et al.* (2001); [34] Vallejo-Illarramendi *et al.* (2006); [35] Li *et al.* (1999); Li and Stys (2001); [36] Droge *et al.* (1993); [37] Vallat-Decouvelaere *et al.* (2003); [38] Sardar *et al.* (1999); [39] Fine *et al.* (1996); [40] Ye and Sontheimer (1996); [41] Wang *et al.* (2003); [42] Chretien *et al.* (2002); [43] O'Shea *et al.* (2006); Persson *et al.* (2005); [44] Dreyer *et al.* (1996); [45] Ambati *et al.* (1997); [46] Wakabayashi *et al.* (2006); [47] Naskar *et al.* (2000); [48] Yoles and Schwartz (1998); [49] Barnett *et al.* (2001); [50] Smith *et al.* (2001); [51] McCullumsmith and Meador-Woodruff (2002); [52] Huerta *et al.* (2006); [53] Matute *et al.* (2005); [54] Schmitt *et al.* (2003); [55] Vallejo-Illarramendi *et al.* (2005b).

roles for EAAT2 and EAAT4, respectively, in these degenerative conditions. One mechanism attracting attention as likely to contribute to EAAT dysfunction is altered splicing of EAATs and/or altered expression of splice variants. Despite an initial report of aberrant splicing of EAAT2 in ALS patients resulting in non-functional transporters (Lin *et al*., 1998), later studies have identified similar variations in control patients (Nagai *et al*., 1998; Meyer *et al*., 1999; Honig *et al*., 2000; Flowers *et al*., 2001), suggesting that this altered splicing may not be a cause of ALS. A more recent study in the G93A-superoxide dismutase1 mouse model of ALS identified differential distribution of various splice variants of EAAT2 in pre-symptomatic transgenic animals (Münch *et al*., 2002), and a similar result has been reported in human motor cortex from ALS patients (Maragakis *et al*., 2004). Altered splicing of EAAT2 has also been found in brains from mice fed washed cycad flour containing the suspected neurotoxin that induces features like those of the Guamanian disorder, ALS–Parkinson's dementia complex (Wilson *et al*., 2003). An intriguing report by Guo *et al*. (2002) demonstrated that some aberrantly spliced forms of EAAT2 mRNA suppressed expression of normal EAAT2 protein *in vitro*, and this mechanism may explain the reduction in EAAT expression in human gliomas (Ye *et al*., 1999). Aberrant splicing of EAAT2 has also been detected in glial cells infected with an enterovirus that has been detected in the spinal cord of ALS patients (Legay *et al*., 2003).

Recent investigations have also shed light on the expression of EAAT2 splice variants under conditions relevant to other pathological states. Thus, altered splicing of EAAT2 has been reported in human astrocytoma (Münch *et al*., 2001). The expression of EAAT2 splice variants is altered in the brains of mice subjected to chemical hypoxia (Münch *et al*., 2003) and hypoxia was shown to induce the expression of a splice variant of EAAT2 in neurons of pigs (Pow *et al*., 2004). Fluid-percussion injury, a model of traumatic brain injury, was shown to alter EAAT2 splicing in rat brains (Yi *et al*., 2005), reducing the expression of the GLT-1v form in a number of brain regions. A higher frequency of altered EAAT2 splice variants was also detected in brains of epileptic patients (Hoogland *et al*., 2004), whereas the abundance of EAAT2b-immunoreactive protein was found to be reduced in the cortex of Alzheimer's disease patients (Maragakis *et al*., 2004). The overall hypothesis here could be that exaggerated expression of EAAT2 splice variants is a pathological response. At this point, it is notable that alterations in the expression of splice variants of other EAATs in response to pathological stimuli have not yet been reported.

There is an extensive literature on the alterations in Glu transport reported across a range of pathologies that affect the CNS. Some of these reports are more anecdotal in nature and here we have attempted a synthesis of recent or key findings where there is strong evidence for the involvement of EAATs and which is supported by a substantial body of supportive data, either from animal models or *in vitro* systems (Table 1). Interpretation of these data should always be carried out bearing in mind the issue of cause and effect, especially given the remarkable propensity of neurones and glia to display temporally dependent adaptative responses. Thus, for example, an elevation in extracellular Glu might elicit an early rise in EAAT activity by a genomic or trafficking mechanism to normalize synaptic function, or a later rise or fall due to astrogliosis or cell death, respectively. Moreover, these alterations in EAAT function could be in neurons, astrocytes, oligodendrocytes and/or microglia, which are all capable of displaying such responses (Matute *et al*., 2006; O'Shea *et al*., 2006).

While Table 1 specifically refers to neurological and psychiatric conditions where there are multiple reports on the involvement of EAATs, there are a number of other conditions worthy of mention where substantial alterations in Glu transport may have effects determining the clinical progression of the condition. Thus, changes in EAATs are reported to occur in alcoholism (Flatscher-Bader *et al*., 2005), spinal cord trauma (Vera-Portocarrero *et al*., 2002) and cancer (Ye *et al*., 1999; Markert *et al*., 2001).

Conclusions and summary

In summary, it is clear that EAATs play key roles in the maintenance of excitatory synaptic transmission and are variously affected in a range of neuropathological conditions. Advances in our understanding of EAAT regulation suggest a number of therapeutic strategies for the management of CNS disorders, and such strategies may be of a traditional pharmacological or genetic nature. Therapeutic modulation of EAATs will be greatly expedited by a better understanding of the basic regulation of these transporters.

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Conflict of interest

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