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REVIEW

Glutamate transporter EAAT2: regulation, function, and potential as a therapeutic target for neurological and psychiatric disease

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Abstract Glutamate is the predominant excitatory neurotransmitter in the central nervous system. Excitatory amino acid transporter 2 (EAAT2) is primarily responsible for clearance of extracellular glutamate to prevent neuronal excitotoxicity and hyperexcitability. EAAT2 plays a critical role in regulation of synaptic activity and plasticity. In addition, EAAT2 has been implicated in the pathogenesis of many central nervous system disorders. In this review, we summarize current understanding of EAAT2, including structure, pharmacology, physiology, and functions, as well as disease relevancy, such as in stroke, Parkinson's disease, epilepsy, amyotrophic lateral sclerosis, Alzheimer's disease, major depressive disorder, and addiction. A large number of studies have demonstrated that up-regulation of EAAT2 protein provides significant beneficial effects in many disease models suggesting EAAT2 activation is a promising therapeutic approach. Several EAAT2 activators have been identified. Further understanding of EAAT2 regulatory mechanisms could improve development of drug-like compounds that spatiotemporally regulate EAAT2.

Keywords Glutamate transporters · Astrocytes · Neurodegenerative disease · Glutamic acid · GLT-1 · Depression · Ceftriaxone · LDN/OSU-0212320

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Abbreviations

NF-kB	Nuclear factor kappa B
Sp1	Specificity protein 1
NFAT	Nuclear factor of activated T-cells
YY1	Yin Yang 1
EGF	Epidermal growth factor
TGF-alpha	Transforming growth factor alpha
EGR	Early growth response protein

Introduction

Glutamate is the predominant excitatory neurotransmitter in the central nervous system (CNS). Glutamate is released from pre-synaptic terminals and diffuses across the synaptic cleft where it binds glutamate receptors. Glutamatergic transmission is terminated once excitatory amino acid transporters (EAATs) take up synaptic glutamate. Five mammalian EAAT isoforms (EAAT1-5) have been characterized (for review see: [1–5]) with each having different nomenclatures, expression patterns, and uptake kinetics (Table 1). EAAT1, 2, and 3 are widely expressed in the CNS, whereas the expression of EAAT4 and 5 is predominately limited to the cerebellum and retina, respectively. The expression of EAAT2 is higher than that of EAAT1 in the forebrain while the inverse is true in the cerebellum. Under normal conditions, EAAT1 and 2 are mainly expressed in astrocytes and localized to the cellular membrane while EAAT3, 4, and 5 are mainly expressed in neurons [6–12]. EAAT2 is the most abundant EAAT and is primarily responsible for glutamate homeostasis in the forebrain [6, 13–16]. Therefore, dysfunction or dysregulation of EAAT2 can lead to excessive glutamate-mediated toxicity [16]. This review describes what is currently known about EAAT2 basic biology and role in disease state.

Table 1 Nomenclature, expression pattern, and uptake kinetics of EAATs

Gene name	Symbol	Alternative name	Cell type	Uptake turnover rate (ms)	Steady state *K*m, (μM)	References
EAAT1	SLC1A3	GLAST	Astrocytes	60	20	[7, 8, 70, 93]
EAAT2	SLC1A2	GLT-1	Mainly in astrocytes	70	18	[7, 8, 70, 97]
			Minority in neurons and oligodendricytes			
EAAT3	SLC1A1	EAAC1	The dendrites of neurons	9–11	28	[6, 7, 70, 121]
EAAT4	SLC1A6		Post-synaptic neuons in Purkinje cells	>300	0.6–2.5	[9, 10, 12, 71, 122]
EAAT5	SLC1A7		Retinal rod photoreceptor and bipolar cells	>1000	61–64	[11, 123]

Structure and pharmacology

EAAT2 splice variants

The EAAT2 gene is composed of 11 exons which form multiple splice variants. EAAT2 transcripts contain a long 3′ UTR (11–12 kb transcript; 3′UTR of ~9.5 kb) and multiple terminal variants (at least two types of N-terminal and three types of C-terminal) which yield a protein product composed of eight transmembrane (TM) domains [3, 17, 18]. EAAT2 also has two *N*-glycosylation sites located in the extracellular loop between TM domains 3 and 4. For the splice variants, the two N-terminal start sequences are MASTEG- and MVSANN-, while the three C-terminal sequences are -PWKREK (-a type), -DIETCI (-b type), and EYQSWV (-c type). EAAT2b (GLT1b) and EAAT2c (GLT1c) contain a PDZ-binding domain (the last three amino acids as indicated by the underline) whereas EAAT2a (GLT1a) does not. The PDZ-binding domain of EAAT2b is involved in an interaction between EAAT2b and the PDZ domain protein, PICK1 [19, 20]. The interaction modulates EAAT2 functions but it has a minor effect on [^{3}H]glutamate uptake [19, 20]. These results are consistent with a previous report that the pharmacological properties of EAAT2a and EAAT2b are indistinguishable [21].

EAAT2 proteins are almost exclusively expressed in astrocytes under normal conditions and rarely in neurons [7, 8, 22, 23]. EAAT2a in CA1 of the hippocampus is expressed in neuronal axon terminals (as well as in astrocytes) but accounts for approximately 10 % of total EAAT2a hippocampal expression [23]. The percentage of total EAAT2 protein in the adult rat hippocampus is 90 % (EAAT2a), 6 % (EAAT2b), and 1 % (EAAT2c) [24]. Results for EAAT2b protein distribution are inconsistent among researchers. In the early study, some results indicate that the EAAT2b protein in the brain is preferentially expressed in neurons [21, 25, 26], while others show that the expression is restricted to astrocytes [27, 28]. The inconsistencies may be attributed to the following reasons: (1) EAAT2b protein levels are much lower than EAAT2a protein levels and (2) the unique splice region is only 11 amino acids between the two variants. Therefore, the antibodies for EAAT2b may have insufficient affinity to distinguish between isoforms [29]. Recently, the verified EAAT2b antibody, with EAAT2 knockout mice as a negative control, shows EAAT2b protein is preferentially expressed in astrocytes [24]. Interestingly, in situ hybridization demonstrates that both EAAT2a mRNA and EAAT2b mRNA are expressed in neurons and astrocytes in the brain implicating cell-specific translational regulation [30]. For the subcellular mRNA distributions in astrocytes, EAAT2a mRNA is predominant in perisynaptic processes, whereas EAAT2b mRNA is distributed in the cell body [30]. EAAT2c protein is expressed in rat and human retinal neurons [31].

EAAT2 regulation

EAAT2 expression is regulated at the level of transcription (including epigenetic modification), translation, trafficking, transport, and degradation. For regulation at the transcriptional level, endogenous and pharmacological modulators can induce activation or repression, such as EGF, cAMP, PACAP TGFbeta, TNFalpha, ceftriaxone, and estrogen related compounds as well as co-culturing astrocytes with neurons [32–37]. The EAAT2 promoter contains several transcription factor-binding sequences, including NF-κB, Sp1, N-myc, CREB, EGR, and NFAT [37, 38]. Several lines of study have demonstrated that NF-κB plays an important role in the transcriptional regulation of EAAT2 by functioning as an intrinsic activator. EGF-induced EAAT2 transcriptional activation is mediated by NF-κB activation, but not by the canonical enhancement of IκB degradation and nuclear accumulation of the p65 isoform of NF-κB [36, 39]. On the other hand, TNFalpha-mediated

EAAT2 repression also utilizes NF-κB activation but also requires N-myc activation which leads to the conversion of NF-κB to a transcriptional repressor [36]. Tamoxifen (antagonist of the estrogen receptor), raloxifene (selective estrogen receptor modulator), and 17beta-estradiol increase EAAT2 expression via activation of both NF-κB and CREB [33–35]. Ceftriaxone, a beta-lactam antibiotic, increases EAAT2 expression via the conventional NF-κB pathway with IκB degradation and p65 nuclear accumulation [40, 41]. It is also notable that basal EAAT2 protein expression in primary astrocyte culture is maintained at a low level, but co-culturing with neurons strongly enhances EAAT2 expression [42, 43]. This induction involves binding of NF-κB to the EAAT2 promoter and activation of kappaB-motif binding phosphor-protein (KBPP) [38, 44]. These results indicate that the NF-κB pathway serves as an important modulator of EAAT2 expression at the transcription level. On the other hand, riluzole, a neuroprotective drug for the treatment of amyotrophic lateral sclerosis (ALS), has many effects which include enhanced EAAT2 uptake activity and protein expression [45, 46]. EAAT2 transcriptional activation by riluzole is mediated by heat shock factor 1 (HSF1) which regulates heat shock proteins (HSPs) that are essential for proper protein folding, trafficking, and degradation in cellular stress responses [46]. EAAT2 expression is also regulated epigenetically. Histone deacetylase (HDAC) inhibitors (valproic acid and trichostatin A) enhance EAAT2 expression in primary astrocytes [47]. Yin Yang 1 (YY1), a ubiquitous transcription factor, decreases EAAT2 promoter activity by recruiting HDACs as co-repressors in primary astrocytes [48]. In addition, the EAAT2 promoter exhibits hypomethylation in cortex relative to the cerebellum, suggesting a potential explanation for why EAAT2 expression is higher in cortex than in cerebellum [49]. The methylation on CpG sites of the EAAT2 promoter is reduced in astrocytes isolated from astrocyte–neuron co-cultures when compared to astrocytes cultured alone [50]. These results indicate that the acetylation and methylation state of the EAAT2 promoter is strongly involved in the regulation of EAAT2 expression.

EAAT2 expression and function are regulated at the post-transcriptional level. EAAT2 translation is controlled by corticosterone in primary astrocyte cell lines, primary cortical neuron–astrocyte mixed cultures, and mice [51]. This regulation involves the 5′-UTR of EAAT2. Neuronal exosomes have been shown to use miR-124a, via a translational regulation mechanism, to increase EAAT2 protein [52]. In addition, EAAT1 and EAAT2 protein, but not mRNA, are increased in ephrin-A3-knockout mice and accompany synaptic changes [53, 54]. Recently, we have developed drug-like, small-molecule pyridazine derivatives which can activate EAAT2 translation [55–57]. One of these compounds increases EAAT2 protein within only 2 h in vivo which is very rapid when compared with transcription activators which require 24–48 h. This translational activation mechanism involves PKC activation and subsequent YB-1 phosphorylation [56]. At the level of post-translational modification, EAAT2 protein undergoes palmitoylation at cysteine 38 (C38) which is required for normal glutamate uptake function [58]. EAAT2 is also constitutively sumoylated in the CNS in vivo. The sumoylated form of EAAT2 is localized to intracellular compartments while non-sumoylated EAAT2 is primarily found at the plasma membrane [59]. Desumoylation in primary astrocytes causes increased EAAT2-mediated glutamate uptake. In addition, EAAT2 is ubiquitinated at the C-terminal which mediates PKC-induced internalization and degradation [60–62]. In addition, the glutamate uptake function of EAAT2 is decreased by reducing membrane cholesterol levels by dissociation from lipid rafts which are microdomains of organized glycosphingolipids, cholesterol, and protein receptors. This indicates that plasma membrane organization of lipid rafts regulate EAAT2 activity [63]. Moreover, the activity of EAAT2 is associated with clustering at the astrocyte perisynapse which is mediated by neuronal activity [64, 65]. There are many potential EAAT2 regulators at the post-transcriptional level.

There are pharmacological agents that directly modulate the transport function of EAAT2. Parawixin1, purified from the spider *Parawixia bistriata* venom, directly enhances the glutamate uptake function of EAAT2 [66, 67]. On the other hand, there are many glutamate transporter inhibitors. The pharmacological properties of these compounds are described in recent review articles [68, 69]. In this review, inhibitors are categorized into two classes: (1) competitive inhibitors (binding to the substrate binding site) and (2) noncompetitive inhibitors (interacting with a site that is different from the substrate binding site) (Fig. 1). The competitive inhibitors (i.e., substrate analogues) include the cyclic molecules L-*trans*-2,4-pyrrolidinedicarboxylic acid (PDC; nonselective EAATs affinity) [11, 70–73], and Dihydrokainic acid (DHK; a selective inhibitor for EAAT2) [70]. To reduce affinity for the glutamate receptor, a bulky substituent was added to the hydroxyl group of hydroxyl-aspartate, e.g. L-*threo*-beta-benzyloxyaspartate (TBOA) and (3*S*)-3-[[3-[[4-(Trifluoromethyl)benzoyl]amino]phenyl]methoxy]-L-aspartic acid (TFB-TBOA). While TBOA is a nonspecific subtype inhibitor, TFB-TBOA has higher affinity for EAATs than TBOA [74, 75]. More recently, *N*-[4-(2-Bromo-4,5-difluorophenoxy)phenyl]-L-asparagine (WAY-213613) has been developed. WAY-213613 has higher potency and selectivity for EAAT2 over EAAT1 and EAAT3 [76, 77]. HIP-B is a noncompetitive EAATs inhibitor which binds at

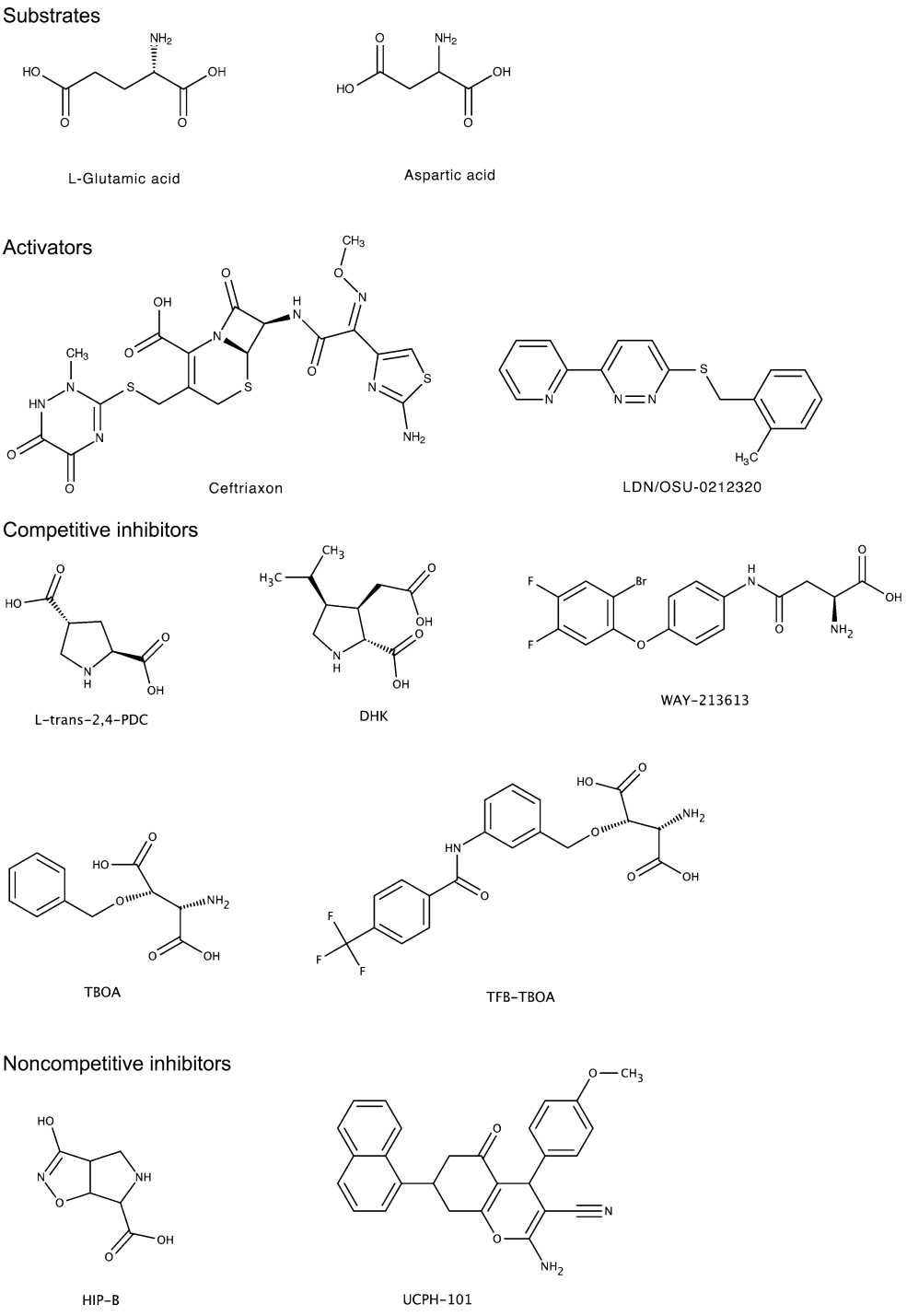

Fig. 1 Structure of substrate, activators, and inhibitors

an allosteric binding site [78, 79]. UCPH-101 is an EAAT1-selective noncompetitive inhibitor that targets a predominantly hydrophobic crevice in the trimerization domain of EAAT1 [80–82]. In sum, these studies show that EAAT2 expression is dynamically regulated at the transcription and post-transcriptional level and that EAAT2 function can be pharmacologically modulated with moderate specificity.

Mechanisms of glutamate transport

Extracellular glutamate transport is achieved by the co-transportation of 3 Na^+ and 1 H^+ for the antiport of 1 K^+. The Na^+ gradient drives glutamate transport. The mechanisms of glutamate transport have been assessed by mutagenesis and crystal structure studies (for review see: [5, 83]) based on the archaeal homolog of the EAATs from

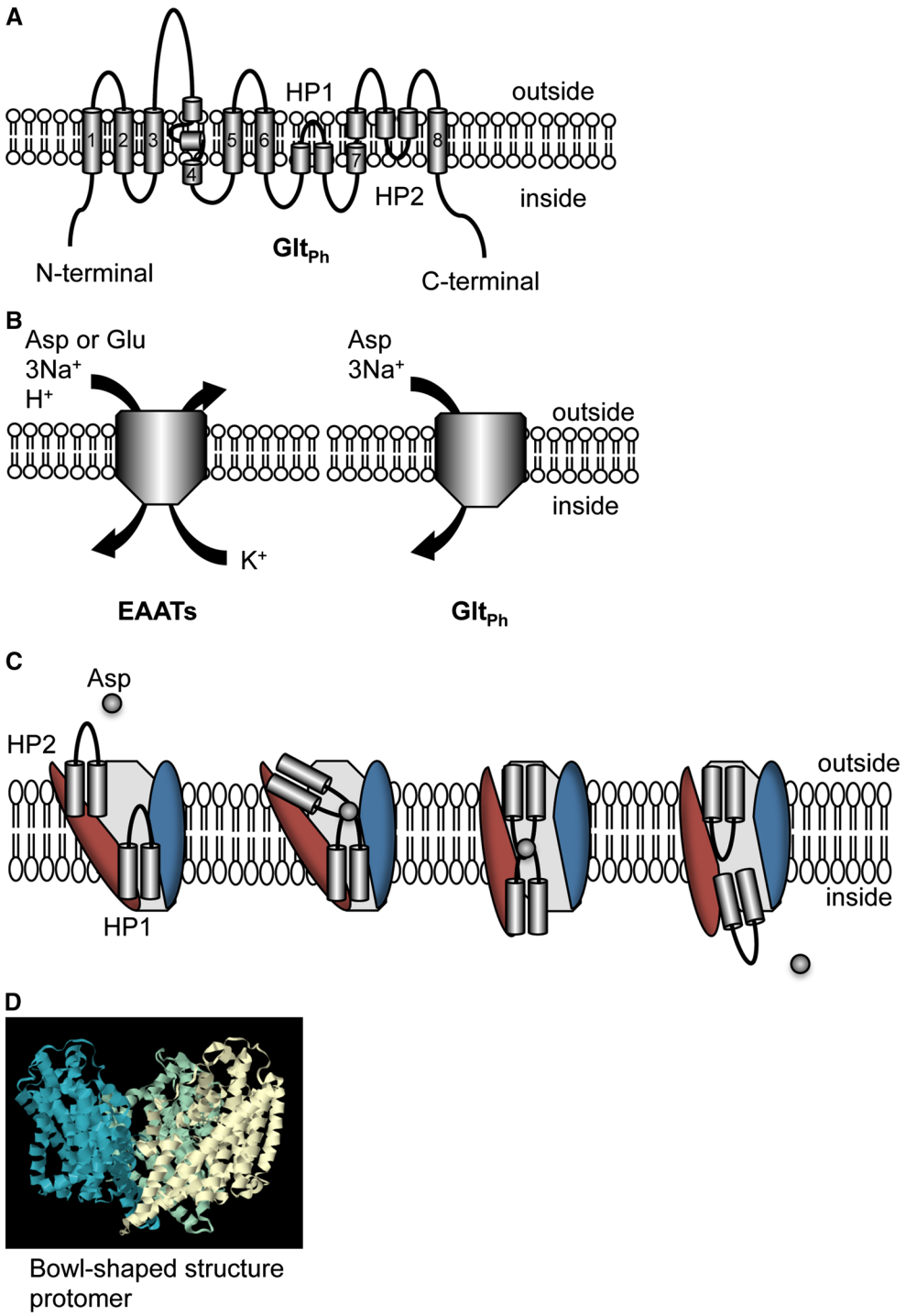

Fig. 2 Transport mechanism and structure of glutamate transporter. There are eight transmembrane (TM) domains and two helical hairpins (HP1 and HP2). **a** The topology model of an archaeal homolog of the EAATs from pyrococcus horikoshii, Glt_{Ph}, is shown. TM 1, 2, 4, and 5 are included in the scaffold domain (trimerization domain) while TM 3, 6, 7, and 8, and HP1-2 are included in the core transport domain. **b** The stoichiometry of transport. EAATs exhibit influx of glutamate/aspartate (Glu), 3 Na^+ and 1 H^+, and outflux of 1 K^+. Glt_{Ph} utilizes influx of aspartate (Asp) and 3 Na^+. **c** The hypothetical transport mechanism of Glt_{Ph}. Reyes et al. proposed that the tips HP1 and HP2 contribute to substrate binding and transport [85] and are accompanied with rotation of the core transport domain (*red*). The trimerization domain is indicated in *blue*. **d** The bowl-shaped structure of the Glt_{Ph} trimer. Each subunit is represented as *blue*, *green*, and *white*. The crystal structure is based on Glt_{Ph} binding with TBOA (PBD 2NWW, http://www.rcsb.org/pdb/home/home.do)

pyrococcus horikoshii, Glt_{Ph} [84–86]. The Glt_{Ph} protein has 37 % conserved identity with human EAAT2. Glt_{Ph} contains the 8 TM domains and two re-entrant loops (Fig. 2a). Although, there are some differences between the archaeal and the mammalian transporter, including substrate preference (high affinity of L-aspartate when compared with EAATs transport as well as similar efficiency for aspartate and glutamate), the cotransport and countertransport molecules (without 1 H^+, 1 K^+), and slow transport turnover (Fig. 2b). However, the Glt_{Ph} structure provides important information for understanding the transport mechanism. The first six TM domains of N-terminal form a scaffold domain. The C-terminal domain contains two helical hairpins (HP1 and HP2), and two TM domains, including the core transport domain. Initiation of transport requires substrate binding on the extracellular side. The tips HP1 and HP2 are proposed to contribute to the substrate binding and transport (Fig. 2c, [85]). The structure of Glt_{Ph} shows a homotrimeric assembly of subunits containing the substrate-binding site to form a bowl-shaped structure (Fig. 2d). The cavity of the bowl faces the extracellular side with a solvent-filled extracellular basin extending halfway across the membrane bilayer. This trimeric formation is also observed in EAATs protein from biological studies. EAAT1 and EAAT2 are homotrimeric, while EAAT4 forms heterotrimers [15, 87, 88]. Understanding underlying details of the mechanisms of EAAT2 transport will facilitate drug development for EAAT2 modulators.

Physiology and functions

Glutamate uptake

Pre-synaptic nerve terminals release glutamate by synaptic-vesicle exocytosis. The glutamate release elevates glutamate concentration in the synaptic cleft, and the glutamate binds to glutamate receptors (NMDA and AMPA receptors) on the post-synaptic neurons. This binding stimulates the neurons via Ca^{2+} or Na^+ influx. The glutamate is then quickly removed from the synaptic cleft by EAAT2 in astrocytes. EAAT2 is responsible for up to 80–90 % of total extracellular glutamate uptake activities [6, 14–16, 89]. The roles of glutamate uptake by EAAT2 are to modulate glutamate transmission, prevent excitotoxicity, supply glutamate to adjacent neuron via conversion to glutamine, and energy production.

Glutamate uptake by EAATs plays an important role in reducing the glutamate concentration in the extracellular space as there is no strong evidence of a specific enzyme for glutamate degradation here. The baseline concentration of extracellular glutamate is as low as 25 nM in hippocampal slice [90]. The concentration of released glutamate from the pre-synaptic terminal reaches approximately 1 mM, and is rapidly decreased by binding to glutamate transporters (rate constant: 10^7 M^{-1} s^{-1}) [91–93]. EAATs then transport glutamate into the intracellular space slowly ($\sim$30 glutamate/s) [4, 94–97]. A growing body of evidence shows that EAAT2 effects synaptic transmission. Blocking of EAAT2 with a specific inhibitor, DHK, shows extended NMDA-receptor mediated excitatory post-synaptic current [98]. EAAT2 is responsible for increased glutamate uptake during late-LTP (long-term potentiation) and may also play an ongoing role in hippocampal circuitry to encode and store information [99]. These results indicate that EAAT2 contributes to glutamatergic signal transmission as well as maintenance of synaptic glutamate concentration at a low level.

Excess glutamate diffuses from the synaptic cleft to the extrasynaptic (outside of the synapse) space. The activation of synaptic NMDA receptors promotes cell survival while extrasynaptic NMDA receptor activation promotes cell death which is termed excitotoxicity (for review see: [100, 101]). The primary synaptic NMDA receptor subunit GluN2A (NR2A) and the primary extrasynaptic NMDA receptor GluN2B (NR2B) contribute to neuronal survival and death, respectively [102]. Because of this differential distribution of NMDA receptor subunits, the synaptic and extrasynaptic NMDA receptors have different intracellular signaling pathways. The activation of synaptic NMDA receptors induces anti-apoptotic genes and many transcription factors, including cyclic-AMP response element binding protein (CREB) via nuclear $Ca2^+$ signaling. The activation of CREB subsequently increases BDNF production which has neuroprotective properties [100]. On the other hand, the activation of extrasynaptic NMDA receptors inhibits the neuroprotective signaling promoted by synaptic NMDA receptors which lead to excitotoxicity. Excitotoxicity is postulated to contribute to a myriad of acute and chronic diseases. Acute increased glutamate is involved in many diseases associated with severe neuronal damage such as stroke and traumatic brain injury. Chronic excitotoxicity and/or hyperexcitability are/is related to chronic neurological disease as well as psychiatric disease, including Alzheimer's disease, Parkinson's disease, ALS, major depressive disorder, and addiction. Therefore, reduction of glutamate could prevent excitotoxicity in numerous neurological and psychiatric diseases.

Once glutamate is taken up by the astrocyte, it is converted into glutamine via glutamine synthetase. Glutamine is then transported back to the pre-synaptic neuron and then converted back into glutamate via glutaminase. This completes the glutamate–glutamine cycle which was first proposed in the 1970's [103, 104]. It is notable that these canonical biochemical pathways are not essential for supplying glutamate for neurotransmitter production or release

[2]. Blocking the glutamate–glutamine cycle failed to suppress glutamatergic synaptic transmission in organotypic brain slices [105] indicating that the glutamate–glutamine cycle contributes little to glutamate transmission. However, a local astrocyte-dependent glutamate–glutamine cycle is required to maintain glutamate transmission in active neurotransmission at excitatory terminals [106].

Glutamate taken up by EAAT2 can also be oxidized for energy by astrocytes [107]. Glutamate oxidative metabolism occurs at high rates in astrocytes [108–110] and produces ATP. The oxidation of one glutamate is estimated to produce 24–27 ATPs via the TCA cycle and pyruvate-recycling pathway. The sodium–potassium ATPase spends one ATP to maintain membrane sodium–potassium gradients to drive glutamate uptake. Therefore, one glutamate can then net produce 23–26 ATPs [107]. Although EAAT2 is not localized to mitochondria, mitochondria are densely localized at perisynaptic astrocytic processes where EAAT2 is expressed. EAAT2, therefore, has the potential to interact with hexokinase which is a mitochondrial protein and is the first step of glycolysis [111]. These results indicate that some glutamate transported via EAAT2 is taken up by local mitochondria for oxidative energy metabolism.

Reverse transport

EAATs can release glutamate into the extracellular space via reverse transport (for review see: [2, 112]). A driving force (Na+ influx) for the inward transport of glutamate into astrocytes by EAATs is generated by the sodium–potassium ATPase under normal physiological conditions. When the driving force is reduced, such as in a membrane-depolarized condition, EAATs can reverse glutamate transport outward [113]. It is known that sodium–potassium ATPase activity is decreased in an energy deprivation condition due to the lack of ATP synthesis which results in disruption of the sodium and potassium gradients. Indeed, ischemia-induced depolarization causes glutamate release via reverse transport of neuronal EAATs [114]. Interestingly, extrasynaptic NMDA receptors on dendrite membranes are closely apposed to astrocytic processes [115]. Therefore, reversed astrocytic glutamate transport may stimulate extrasynaptic NMDA receptors in disease condition to induce excitotoxicity [116].

Heteroexchange

EAATs can also facilitate substrate exchange of external and internal substrate in a 1:1 ratio (for review see: [2]). It is notable that transportable inhibitor, like PDC, induce release of internal endogenous substrates (i.e., glutamate), thus, exacerbating excitotoxicity [117, 118]. Recently, the results of EAAT2 reconstituted in liposomes shows that heteroexchange is electroneutral but is voltage dependent [119].

Anion conductance

EAATs also exhibit anion conductance that is not coupled to glutamate transport [91, 120–123]. The magnitude of this conductance is inversely related to the pattern of glutamate transport rates (the glutamate transport rate are EAAT1/EAAT2/EAAT3 $\gg$ EAAT4/EAAT5). EAAT4 and EAAT5 have a high chloride conductance which inhibits glutamate transmission. EAAT4 and EAAT5 are expressed in cerebellar and retinal neurons, respectively. EAAT4 and EAAT5 predominantly serve to inhibit glutamate transmission in these neurons rather than to transport glutamate [124]. EAAT2 exhibits weak anion conductance and mainly plays a role in glutamate uptake.

Disease relevancy

Neurodegenerative disease is primarily characterized by the pathology of neuronal death which occurs in different regions and cell types in a disease specific pattern. Neuronal death is predominant in excitotoxicity. The current therapeutic strategy is to prevent neuronal death (e.g., the prevent excitotoxicity) and delay disease-related symptom progression. Excess glutamate plays an important role in excitotoxicity, and, therefore, glutamate uptake enhancement is one of the most promising drug targets for the prevention of excitotoxicity. On the other hand, psychiatric diseases are characterized by no obvious classical pathological phenomenon. Recently, advances in brain imaging techniques have revealed dysregulation in the glutamate pathway that make it a candidate therapeutic target for psychiatric disease. Here, we review how the glutamate pathway is involved in select neurological (stroke, Parkinson's disease, epilepsy, ALS, and Alzheimer's disease) and psychiatric diseases (major depressive disorder and addiction) as well as accumulating evidence that EAAT2 activators are a potential therapeutic strategy for drug development in these diseases.

Stroke

Stroke is the second leading causes of death in both men and women (12 % of total death, WHO data in 2012) and the third leading cause of disability-adjusted life-years (DALYs) worldwide [125, 126]. DALYs indicate how many years of "healthy" life are lost. Stroke patients typically experience a long period of disability associated with the disease. Stroke is caused by either ischemia (80 %

of cases) or hemorrhage (20 %) [127]. Ischemic stroke causes a reduction in oxygen and glucose supplies which in turn prevents ATP synthesis. The deprivation of ATP leads to reduced sodium–potassium ATPase activity, disruption of sodium and, potassium gradients, and, in turn, increased synaptic glutamate concentrations via reverse transport of glutamate by neuronal EAATs [102, 114]. Neuronal EAATs involvement here is confirmed by the finding that ischemia-induced glutamate release is blocked by PDC (non-specific glutamate transporter inhibitor) but not DHK (EAAT2 specific inhibitor). EAAT2 uptake function is preserved in the ischemic condition. This is possibly due to the fact that astrocytes are able to metabolize glycogen and glucose [128], as well as produce ATP by oxidation of glutamate to obtain energy for maintenance of glutamate uptake in an acute ischemic condition. Several studies of ischemia animal models show glutamate levels in the brain are increased which subsequently cause excitotoxicity [129–134]. The detailed mechanisms of excitotoxicity in the context of stroke are described in a current review article [102]. The strategy of reducing glutamate by blocking neuronal reverse transport and/or enhancing EAAT2 glutamate uptake is attractive here. Several studies demonstrate that increased EAAT2 provides neuroprotection in ischemia. GFAP-driven EAAT2 expression in astrocytes enhances neuroprotection after moderate oxygen–glucose deprivation in rat hippocampal slice cultures [135]. Ceftriaxone-induced EAAT2 expression shows protective effects in several ischemia animal models [136–141]. These results indicate that EAAT2 is a potential therapeutic target for stroke.

Parkinson's disease

Parkinson's disease (PD) is characterized by motor symptoms, including akinesia, bradykinesia, rigidity and tremor. The neuropathological hallmark of PD is the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc). The development of PD motor symptoms is believed to be due to a loss of dopaminergic neurons here. This leads to loss of modulatory input and results in glutamatergic hyperexcitability of the subthalamic nucleus (STN) which projects to the medial globus pallidus and the substantia nigra pars reticulata—the output regions of the basal ganglia [142, 143]. In addition, overactivation of the glutamatergic neurons in the STN can result in excitotoxicity and degeneration of surviving neurons in the SNc. Blocking-enhanced glutamate transmission in this circuit could both alleviate symptoms and delay progression. Both preclinical and clinical studies demonstrate that glutamate receptors are a therapeutic target for PD (see review [142, 144]). Recently, several studies demonstrated that EAAT2 expression is involved in PD. Decreased EAAT2 expression has been reported in animal models of PD, including the 6-hydroxydopamine-lesioned PD model and the acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treated mouse model [145, 146]. Recently, growing evidence shows ceftriaxone has beneficial effects in PD animal model, including the prevention of motor dysfunction, neuronal death, and PD related memory deficits [147–152]. EAAT2 has the potential as a target for PD therapy.

Epilepsy

Epilepsy is a chronic neurological disease characterized by seizures and has a prevalence of 1–2 % worldwide. Epileptic seizures generally fall into two major categories: generalized seizures and partial seizures. Generalized seizures exhibit a widespread electrical discharge in both brain hemispheres and are typically associated with genetic factors. The partial seizures are a local electrical discharge in the brain and are typically caused by brain injury, stroke, or tumor. The molecular mechanisms underlying the development of epilepsy during initial insults (epileptogenesis) are not well understood. The progression of epileptogenesis during the latent period involves differential release of several neurotrophic factors, neuronal sprouting, altered synaptic plasticity, neurogenesis, and excitotoxicity [153, 154] which subsequently result in the development of spontaneous recurrent seizures. Enhanced synaptic glutamate concentration during the initial insult is related to excitotoxicity and is maintained during epileptogenesis [155, 156]. EEG and microdialysis studies in epilepsy patients show that the basal glutamate concentration in epileptogenic areas is 4.7 times higher than in nonepileptogenic areas of the hippocampus [157]. The concentration of glutamate in epileptogenic areas reaches neurotoxic concentration under basal conditions and increases further during seizures [157, 158]. Dysfunction of glutamate transport may contribute to high extracellular glutamate in the epileptogenic hippocampus. Impaired glutamate transport function has been reported in human epilepsy but remains controversial [159–163]. Excessive glutamate released by astrocytes plays a role in the synchronous firing of large populations of neurons during seizures [164–167]. Reducing glutamate-mediated excitotoxicity may prevent epileptogenesis and, subsequently, spontaneous-recurrent seizures. Ceftriaxone shows protective effects by reducing seizure activity and the acute mortality in a pentylenetetrazole model of epilepsy [168], a model of tuberous sclerosis [169], and a traumatic brain injury-induced epilepsy model [170]. We have investigated pilocarpine-induced status epilepticus in EAAT2 transgenic mice and the pyridazine-derivative EAAT2 translational-activator-treated mice [56, 171]. Enhanced

EAAT2 expression significantly prevents seizure-induced epileptogenesis and subsequent spontaneous recurrent seizures. Overall, these studies indicate that enhanced EAAT2 protein expression is a potential therapeutic approach for epilepsy.

ALS

Amyotrophic lateral sclerosis (ALS) is a selective motor neuron degenerative disease which exhibits rapid progression in the brain and the spinal cord. The incidence is 2–3/100,000. Sporadic ALS accounts for approximately 90 % of cases and hereditary (familial ALS) accounts for the other 5–10 %. SOD1 mutation accounts for 20 % of familial ALS [172]. While differing or currently unknown causes result in ALS, a similar pathogenesis occurs for all ALS cases, including: oxidative damage, aberrant RNA metabolism, accumulation of intracellular aggregates, growth factor deficiency, defects in axonal transport, mitochondrial dysfunction, glial cell pathology, and glutamate excitotoxicity [173]. A 30–95 % loss of the EAAT2 protein in the motor cortex and spinal cord is observed in approximately 60–70 % of ALS patients [174]. The loss of EAAT2 protein is also observed in a transgenic animal model of mutant SOD1-mediated familial ALS [175–177]. These results suggest that the EAAT2 protein decline is correlated with neuronal loss. Enhanced EAAT2 expression by genetic manipulation and pharmacological treatment in SOD1 mice has shown some beneficial effects [41, 56, 178]. However, ceftriaxone treatment in patients with ALS did not show clinical efficacy [179]. There are many complex conditions in the disease stage that may have led to the lack of efficacy in this trial. It is notable that this study did not include pharmacodynamic results, such as measuring EAAT2 expression pre- and post-treatment. EAAT2 PET imaging is required in future clinical trials to confirm EAAT2 is upregulated as expected.

Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by declarative memory impairments and progressive dementia. The cognitive deficits are more significantly correlated with reduced glutamatergic pre-synaptic-bouton density than with neurofibrillary tangles or amyloid-β burden [180]. In addition, deficiencies in the glutamatergic system (e.g., reduced glutamate uptake) have been observed in AD and are correlated with cognitive decline [181–186]. Reduced glutamate uptake function may result in increased extracellular glutamate levels which, in turn, potentially increase amyloid-β production over time [187–189]. Amyloid-β can directly further enhance glutamate release thus creating a positive feedback cycle that synergistically increases glutamate at the synaptic cleft [190–192]. In addition, amyloid-β has been reported to prevent induction of long-term potentiation (LTP) and promote long-term depression (LTD) [193–196]. This amyloid-β-facilitated LTD is inhibited by addition of an extracellular glutamate scavenger [193]. Overall, homeostatic regulation of extracellular glutamate levels may play a crucial role in the pathogenesis of AD.

EAAT2 plays an essential role in cognitive functions [99, 197, 198]. The loss of EAAT2 protein and function are observed in AD patients [181, 184, 185] and constitutes an early event in disease pathology. This EAAT2 protein decline is likely due to disturbances at the post-transcriptional level because EAAT2 mRNA is not decreased in AD patients [199]. Mice that lack one allele for EAAT2 and crossed with $A\beta PP_{swe}$/PS1ΔE9 mice, an animal model of AD, exhibit accelerated cognitive deficits when compared to $A\beta PP_{swe}$/PS1ΔE9 mice. These results suggest that decreased EAAT2 levels may contribute to AD pathogenesis. We have investigated whether restored EAAT2 protein could benefit cognitive functions and pathology in $APP_{Sw,Ind}$ mice, an animal model of AD. We conducted this investigation using both a transgenic mouse approach by crossing EAAT2 transgenic mice with $APP_{Sw,Ind}$ mice and a pharmacological approach using a novel EAAT2 translational activator (LDN/OSU-0212320) [200]. Both approaches resulted in restored EAAT2 protein function which attenuated premature death, memory loss, and AD-like pathology (amyloid β deposition and loss of synaptic integrity) in $APP_{Sw,Ind}$ mice. It is notable that LDN/OSU-0212320 could (1) reverse cognitive deficits after a short treatment period, (2) sustain the cognitive benefits even after 1 month of treatment cessation, (3) restore synaptic integrity, and (4) increase EAAT2 expression via the translational, rather than the transcriptional, activation mechanism which resolves the central problem of reduced EAAT2 protein expression. LDN/OSU-0212320 or its derivatives may have therapeutic potential as a drug for AD.

Major depressive disorder

Major depressive disorder (MDD) is estimated to have a lifetime prevalence of 12.8–16.6 % and is predicted to be the 2nd leading cause of disease burden by 2030 [201–203]. Symptoms of depression are characterized by a depressed mood or a loss of interest or pleasure in daily activities consistently for at least 2 weeks. Brain imaging studies have demonstrated abnormalities in the prefrontal–limbic circuit, specifically decreased top-down connectivity and increased bottom–up connectivity. MDD patients show decreased activity in the prefrontal cortex (associated

with executive function) while activity in the amygdala (involved in emotional response) and the anterior cingulate cortex (related to decision-making and emotional regulation) is increased [204, 205]. These network changes are postulated to contribute to the clinical phenotypes of depression, but it is not clear what causes these changes. One possibility may be related to a loss of astrocytes in the depressed brain. Astrocyte loss is a prominent feature of mood disorders [206, 207] although there is no unique pathology for depression. Postmortem studies of patients with depression have demonstrated that the density of glial cells is decreased in the amygdala as well as prefrontal, orbitofrontal, and cingulate cortices [208–213]. Astrocyte-related genes, glial fibrillary acidic protein (GFAP), EAAT1, EAAT2, and glutamine synthetase, are reduced in cortical and amygdalar regions of MDD patients [211, 214–217]. In preclinical studies, a reduction in astrocyte number in the hippocampus and frontal cortex region is observed after chronic unpredictable stress [218, 219]. Moreover, the local treatment of the astrocyte toxin, L-alpha-aminoadipic acid (L-AAA), but not the neurotoxin ibotenate, in the prefrontal cortex produces an anhedonia-like behavior (a core clinical feature of depression) and anxiety behavior [218]. These results suggest that dysregulated glutamate transmission, due to pathological astrocytic functional changes, may be involved in depression symptom development. Indeed, the anti-glutamatergic agent, riluzole, has antidepressant effects in depressed patients and animal models of depression [220–223].

EAAT2 expression may play a role in depression-like symptoms. Glutamate release and uptake in the frontal cortex and hippocampus are increased by acute stress [224–226]. Chronic mild, predictable stress also causes increased glutamate release but also results in EAAT2 upregulation [227–230]. This likely accounts for the fact that predictable stress has beneficial effects on depressive- and anxiety-like behaviors [231, 232]. On the other hand, chronic, unpredictable stress reduces EAAT2 expression which is a similar phenomenon observed in MDD patients [215, 216, 233, 234]. A growing body of evidence suggests that loss of EAAT2 function causes depression-like behaviors. DHK treatment in rat prefrontal cortex or cerebral ventricle induces anhedonia behavior as measured by intracranial self-stimulation [197, 235]. DHK treatment in the amygdala reduces social interaction [236]. The inhibition of EAAT2 in the lateral habenula increases susceptibility to chronic stress, including increased anxiety and disinhibition during rapid eye movement sleep [237]. These results indicate that loss of EAAT2 expression contributes to depression-like behavior. In addition, chronic antidepressant treatments increase EAAT2 expression in the hippocampus [233]. Ceftriaxone reduces helplessness behavior in the forced swim and tail suspension tests [238]. It is notable that animal models can reflect only a single or limited range of symptoms of depression. In humans, the symptoms of depression are complex and arise from multi-phenomena. Thus, to determine whether enhanced EAAT2 expression is a target for depression treatment, several models of depression must be examined.

Addiction

Addiction is a behavioral state characterized by altered reward processing, disrupted emotional responses and poor decision-making. The motivationally relevant circuitry involved here is the cortico-striato-thalamic circuit, including prelimbic cortex, nucleus accumbens, and ventral tegmental area. Brain imaging studies have demonstrated that relapse in drug seeking is related to activation of the prefrontal and anterior cingulate cortices which project to the nucleus accumbens [239]. During the late withdraw period, low activities in the prefrontal and anterior cingulate cortices are observed. Animal studies demonstrate that the reinstatement of drug seeking occurs due to an imbalance in glutamate transmission from the prelimbic cortex to the nucleus accumbens core [240]. These impairments lead to disruption of cortico-striato-thalamic processing. Reinstatement of drug seeking in animal models is commonly associated with increased extracellular glutamate levels in the nucleus accumbens [241–244]; however, extracellular glutamate is not increased by the reinstatement of food seeking [244]. Thus, the enhanced glutamate levels in the nucleus accumbens play an important role in drug addiction [240, 245].

Importantly, the expression of EAAT2 is altered in animal models of addiction. Animal studies of cocaine self-administration exhibit reduced glutamate uptake [246, 247] and heroin-reduced EAAT2 expression causes the spillover of synaptic glutamate that leads to the activation of extrasynaptic NMDA receptors in the nucleus accumbens core [248]. Ethanol withdrawal reduces EAAT2 expression in striatum [249]. Ceftriaxone attenuates relapse-like ethanol intake [250–252]. Ceftriaxone was also shown to prevent cue-induced heroin seeking through increased glutamate uptake and a subsequent reduction in synaptic glutamate spillover [248]. Ceftriaxone also prevents morphine physical dependence [253]. In addition, ceftriaxone reduces the reinstatement of drug seeking in animals trained to self-administer cocaine [247, 254–256]. Propentofylline, an adenosine uptake and phosphodiesterase inhibitor, prevents cue-primed cocaine seeking via restored expression of EAAT2 in the nucleus accumbens [257]. Ceftriaxone attenuates the reinstatement of methamphetamine seeking in a condition place preference paradigm [258]. Ceftriaxone also reduces nicotine

withdrawal and nicotine-seeking behaviors [259]. These results strongly suggest that enhanced EAAT2 protein expression is a potential therapeutic approach for addiction treatment.

Other diseases

Schizophrenia is associated with dysregulation of the glutamatergic system, including EAAT2 expression (for review see [260, 261]). Briefly, a glutamate receptor blocker, phencyclidine, induces schizophrenic-like behavior. Although the results of EAAT2 expression changes in schizophrenia remain controversial, antipsychotic drug treatments decrease EAAT2 expression. Ceftriaxone exacerbates phencyclidine-induced prepulse inhibition impairment [262]. Traumatic brain injury (TBI), which typically results in glutamate excitotoxicity at and near the lesion site, is expected to be a therapeutic target for enhanced EAAT2 (for review see [263, 264]). Ceftriaxone treatment shows beneficial effects for TBI including neuroprotection [170, 265]. The Huntington's disease animal model exhibits both decreased EAAT2 protein and mRNA expression; however, dysregulation of EAAT2 expression in patients with Huntington's disease is controversial (for review see [1, 266, 267]). Ceftriaxone attenuates the Huntington's disease phenotype in the R6/2 mouse [268]. EAAT2 expression in the spinal cord is decreased in animal models of pain (for review see [261, 269]). Ceftriaxone has anti-nociceptive effects in pain models [270–272]. EAAT2 expression is altered in malignant gliomas (for review see [273]). Multiple sclerosis (MS) exhibits both excitotoxicity and oxidative stress (for review see [263, 265]). Ceftriaxone dampens excitotoxic inflammatory CNS damage in a mouse model of MS, but this may not be due to increased EAAT2 expression [274]. Finally, EAAT2 has also been linked to autism [275, 276]. Certainly, the evidence is overwhelming that EAAT2 dysregulation plays an important role in several neurological and psychiatric diseases.

Future perspectives

Glutamate transmission is essential for normal brain functions, inducing learning and memory. Reduced glutamate transmission affects normal brain functions and produces negative side effects through decreased glutamate receptor occupancy. Several neurological and psychiatric diseases exhibit excess extracellular glutamate and loss of EAAT2 expression. Restored EAAT2 expression levels and function may provide therapeutic benefit. Here, we have described three types of EAAT2 activators at different levels; (1) transcriptional (2) translational, and (3) functional. Each type has advantages and disadvantages. Certain diseases demonstrate decreased EAAT2 protein but not EAAT2 mRNA. In such a case, the transcriptional activator type, such as ceftriaxone, is unlikely to increase EAAT2 at a therapeutic level as EAAT2 dysregulation occurs post-transcriptionally. The translational activator type, such as pyridazine-derivative, is unlikely to increase EAAT2 when there is no EAAT2 mRNA; but this type is a putative fit for an immediate EAAT2 requirement disease such as stroke. The EAAT2 functional activator, such as spider extracts, is unlikely to modulate disease symptoms when there is little EAAT2 protein remaining. A better understanding of disease mechanisms will be essential to design and select therapy types for disease with EAAT2 dysregulation. Accumulation of knowledge in the EAAT2 variant, structure, localization, expression regulation, trafficking, and degradation could produce a therapeutic drug that provides higher spatiotemporal EAAT2 regulation. In addition EAAT2 function can now be monitored by PET probe in human subjects. This may pave the way for tailor-made medicine for disease associated with EAAT2 dysregulation.

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