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Role of glutamate excitotoxicity and glutamate transporter EAAT2 in Epilepsy: opportunities for novel therapeutics development

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Abstract

Epilepsy is a complex neurological syndrome characterized by seizures resulting from neuronal hyperexcitability and sudden and synchronized bursts of electrical discharges. Impaired astrocyte function that results in glutamate excitotoxicity has been recognized to play a key role in the pathogenesis of epilepsy. While there are 26 drugs marketed as anti-epileptic drugs no current treatments are disease modifying as they only suppress seizures rather than the development and progression of epilepsy. Excitatory amino acid transporters (EAATs) are critical for maintaining low extracellular glutamate concentrations and preventing excitotoxicity. When extracellular glutamate concentrations rise to abnormal levels, glutamate receptor overactivation and the subsequent excessive influx of calcium into the post-synaptic neuron can trigger cell death pathways. In this review we discuss targeting EAAT2, the predominant glutamate transporter in the CNS, as a promising approach for developing therapies for epilepsy. EAAT2 upregulation via transcriptional and translational regulation has proven successful *in vivo* in reducing spontaneous recurrent seizures and offering neuroprotective effects. Another approach to regulate EAAT2 activity is through positive allosteric modulation (PAM). Novel PAMs of EAAT2 have recently been identified and are under development, representing a promising approach for the advance of novel therapeutics for epilepsy.

Graphical Abstract

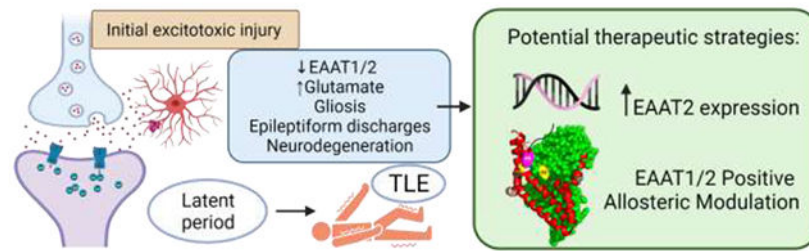
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Keywords

Astrocytes; EAAT2; epilepsy; excitotoxicity; glutamate; glutamate transporter

Epilepsy

Epilepsy was first described in Mesopotamia in 2000 B.C. Although this syndrome has affected important people in history such as Julius Caesar, Vladimir Lenin, and Fyodor Dostoyevsky, it is still a disabling disease for most patients, so prevention and cure are still great unmet needs [1]. According to CDC data, in 2015 1.2% of the total US population had active epilepsy, meaning approximately 3.4 million people were either currently taking anti-seizure drugs (ASDs) or had a seizure within a year [2]. Worldwide, epilepsy has a prevalence of 1–2 % [3,4].

Epilepsy is a complex neurological syndrome characterized by seizures caused by hyperexcitability of neurons and sudden and synchronized bursts of electrical discharges. These seizures present as peculiar sensations, emotions, behavior, convulsions, muscle spasms, and/ or loss of consciousness [5–7].

Seizures can be classified into partial, or focal, and generalized[9]. Partial epilepsies represent the most common type of adult-onset epilepsy and account for more than 60% of all adult cases of temporal lobe epilepsy (TLE) [10]. For reviews on the pathophysiology of epilepsy, see [7,11,12].

Epileptogenesis

An initial injury leading to seizures may be head trauma, childhood seizures, hypoxia, or *status epilepticus* (SE), a single seizure that either lasts longer than 5 minutes or is a series of seizures that occur close together with no recovery of consciousness between seizures [13–15]. The intense seizure activity seen in SE causes excessive glutamate release resulting in overstimulation of glutamate receptors leading to massive influxes of Ca^{2+} , subsequently triggering mass neuronal death via glutamate excitotoxicity mechanisms [7,16]. Following the initial insult or injury there is a latent period that may last up to several years in which complex molecular, biochemical, and structural changes occur, including changes in synaptic plasticity and neuronal connectivity reorganization of neuronal networks. Within minutes to days following the initial insult, acute early changes include rapid alterations to ion channel kinetics, post-translational modifications to existing functional proteins, and activation of immediate early genes. Hours to weeks after the insult, subacute changes occur,

including neuronal death, activation of inflammatory cascades and several transcriptional events. In the following weeks to months, several chronic changes will take place, including anatomical changes, such as neurogenesis, gliosis, mossy fiber sprouting and network reorganization [17,18]. These changes ultimately lead to neuronal networks being more susceptible to hyperexcitability and synchronous firing of these excitatory neurons, leading to more seizures and eventually spontaneous recurrent seizures [19,20]. This results in the emergence of chronic epilepsies such as TLE [8] (figure 1). Confirming this idea, an important recent study using an *in vivo* model of pentylenetetrazole (PTZ) insult, established that a single episode of clonic–tonic seizures affected neuronal viability in the hippocampus, up to 1 week after PTZ treatment. This suggests that even a single seizure episode is a powerful event that causes stress to hippocampal neurons and can cause intricate alterations in neuronal plasticity [21].

A recent study investigated whether glutamate and GABA are linked to the formation of epilepsy networks and the triggering of spontaneous seizures, by infusion of a glutamine synthetase inhibitor into rat hippocampi to create a seizure focus, a translationally rodent model of mTLE. Their results suggest that changes in extracellular brain levels of glutamate and GABA play important roles in epilepsy network formation and in the initiation and propagation of spontaneous seizures [22].

Collectively, these studies suggest that neuronal death due to glutamate excitotoxicity may be both a cause and consequence of epileptic seizures, as abnormal glutamate release by astrocytes is involved in the molecular mechanisms underlying the initial insults during the process of epileptogenesis [23]. Developmental processes play a role in the regulation of epileptogenesis, suggested by differences observed in epileptogenic processes between developing and adult brains. However, age-specific mechanisms of epileptogenesis, as well as specific factors associated with increased seizure susceptibility in the developing brain are not well known. This knowledge is needed for development of proper biomarkers that would allow for the identification of risk factors for developing epilepsy. Further research is also needed for development of therapies for the prevention of epileptogenesis, as well as for monitoring of the progression of epilepsy [24].

Current treatments for epilepsy

Since the early 1990s, many newer ASDs with better tolerability, reduced possibility for drug-drug interactions and satisfactory pharmacokinetics were approved for clinical use [25]. Currently there are 26 drugs marketed as ASDs, with various mechanisms of action including the inhibition of sodium channels to reduce the ability of neurons to fire at high frequencies (phenytoin, carbamazepine, valproate, and lamotrigine, among others), inhibition of T type and L type calcium channels (ethosuximide and gabapentin, respectively), inhibition of voltage-gated potassium channels (Kv7, retigabine/ezogabine) inhibition of glutamate release and receptors (topiramate), and enhancement of GABA action via activation of GABA_A receptors (phenobarbital and benzodiazepines), inhibition of GABA transaminase (vigabatrin), and inhibition of GABA reuptake (tiagabine), and modulation of synaptic release, as examples modulators of the $\alpha 2\delta$ subunit of voltage-

gated calcium channels (gabapentin, pregabalin) and the synaptic vesicle protein (SV) 2A (brivaracetam, levetiracetam) [26–28].

Unfortunately, current pharmacotherapies are not efficacious in approximately one third of patients with epilepsy [29]. Additionally, current treatments have proven successful in suppressing seizures, however, they do not treat the underlying epileptic syndrome, i.e., they are not disease modifying as they do not halt the progression of epileptogenesis. Moreover, many of these treatments, especially the GABA_A receptor activators, can have serious adverse events and tolerance associated with their use [30–33].

Approximately 20% of epilepsy cases are triggered by acute injuries such as stroke, traumatic brain injury, and bacterial, virus and other types of brain and spinal cord infections. There are several opportunities for pharmacological treatment for epilepsy: the injury onset that triggered the epileptogenic process, medical care at the early phases of presentation, and intervention a latent phase between the injury and the development of clinical epilepsy. Unfortunately, even though there are no available treatments, no extensive clinical research seeking novel therapies has been conducted on the last 20 years. In rare cases, certain “self-limiting” childhood and adolescence epilepsy syndromes have remitted spontaneously. One type of treatment that seems to result in “cure” is resective surgery, however, this is sparsely provided. Some patients benefit of medications or dietary treatments, however, once the treatment is discontinued, the seizures tend to persist. At the present time, there is not enough knowledge on the mechanisms behind such “cures”; impeding the development of translational treatment paradigms to “cure” epilepsy [34].

So, despite being a common neurological disorder, epilepsy remains a serious and unmet medical need. Thus, novel treatments for epileptic syndromes are needed, and research suggests that the glutamatergic system, and in particular the glutamate transporter GLT-1/EAAT2 (rodent/human homologues), may provide novel targets for ASDs. Several studies suggest that dysregulation of astrocytic glutamate transporters could be a factor to the development of epilepsy. The development of approaches that modulate the glial excitatory amino acid transporters EAAT1 and EAAT2, and glutamine synthetase could result in fundamental changes on the way epilepsy is treated, by providing a potential prevention of the epileptogenesis processes [35].

Glutamate transporters

Glutamate is the primary excitatory neurotransmitter in the mammalian CNS and is vital for normal brain functioning [36]. Glutamate is stored in synaptic vesicles at presynaptic terminals, that, in response to an action potential and increase in intracellular Ca²⁺, fuse with the plasma membrane of the presynaptic neuron and glutamate is released into the synaptic cleft and can then activate these receptors. Glutamate receptors fall into two different classes, ligand gated ion channels (ionotropic receptors) which include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, kainate receptors, and N-methyl-D-aspartic acid (NMDA) receptors, and G-protein coupled receptors (metabotropic receptors) [37,38]. Upon activation of post-synaptic glutamate receptors, Ca²⁺ channels are

opened and Ca^{2+} influxes into the post-synaptic neuron trigger an action potential in the post-synaptic neuron and further downstream signaling in physiological conditions.

Glutamate neurotransmission is terminated upon the uptake of extracellular glutamate by a family of five structurally distinct subtypes of high-affinity sodium dependent excitatory amino acid transporters (EAATs, rat/human homologue): GLAST/EAAT1, GLT-1/EAAT2, EAAC1/EAAT3, EAAT4 and EAAT5 [39].

The EAATs are secondary-active transporters that couple the movement of one glutamate with the symport of three Na^+ ions and one H^+ and the counter transport of one K^+ [40]. The Na^+ gradient generated by $\text{Na}^+ \text{K}^+$ -ATPase activity drives glutamate transport under physiological conditions [41]. Glutamate taken up by astrocytes is converted into glutamine via glutamine synthetase and is then transported to the extracellular space, where it is taken up by the presynaptic neuron and converted back into glutamate via glutaminase [42]. Glutamate is then packaged into synaptic vesicles via vesicular glutamate transporters (vGLUTs) [43]. Table 1 shows the differences in the nomenclature of glutamate transporters between human and rodent, their main biological activity and predominant expression in the CNS. This review will focus on EAAT2, the most abundant subtype of EAATs [44], that is expressed throughout the brain and in the spinal cord. EAAT2 is present primarily in astrocytes, and in neurons and oligodendrocytes, accounting for about 95% of the total glutamate transport activity and 1% of total brain protein in the CNS [45–49]. Thus, EAAT2 plays a central role in the CNS for maintaining the homeostasis of glutamate and therefore preventing excitotoxicity [37,50–52].

EAATs function is essential for fast removal of glutamate from the extracellular space upon its release into the synaptic cleft. It is key that extracellular glutamate concentrations are kept low (25–600 nM), as elevated concentrations to approximately 2–5 μM result in excitotoxic injury [53–55]. Glutamate excitotoxicity is the process in which failure to remove extracellular glutamate from the synaptic cleft results in sustained extracellular glutamate levels and excessive activation of NMDA receptors [55]. This results in excessive Ca^{2+} influx into the post-synaptic neuron leading to downstream activation of a cascade of phospholipases, endonucleases, and proteases like calpain that leads to cell apoptosis [56]. Additionally, the excessive Ca^{2+} influx and subsequent depolarization of the post-synaptic neuron may lead to the efflux of excessive glutamate spillage promoting subsequent excitotoxicity [39]. Thus, the EAATs, and in particular EAAT2, are crucial for maintaining low extracellular glutamate concentrations to prevent NMDA receptor (NMDAR) and AMPA receptor (AMPA) mediated excitotoxicity. EAATs are also present on GABAergic terminals, where they take up glutamate that is converted into GABA by glutamic decarboxylase (GAD). This function contributes to limiting spill-over of glutamate of active synapses, limiting the activation of extra-synaptic and neighboring synapses glutamate receptors [58].

In addition to the EAAT family of transporters and vesicular glutamate transporters, there are also glutamate-cystine exchangers (SLC7A11, xCT) present in neurons and glia that exchange intracellular glutamate for extracellular cystine to provide a cystine source for the synthesis of glutathione [59]. Extracellular accumulation of glutamate can also cause

toxicity through interaction with these exchangers as they become blocked and glutathione cannot be synthesized, leading to oxidative stress and downstream cell death [57]. These exchangers are relevant for epileptic seizures in the context of gliomas, as non-vesicular secretion of glutamate via these exchangers constitutes the main mechanism contributing to high extracellular glutamate concentrations [60].

Astrocytes and Epilepsy

Historically, the focus of epilepsy research has been *neurocentric*, since epilepsy is caused by aberrant synchronized firing of neuronal populations, primarily due to imbalance between excitatory and inhibitory neurotransmission. However, in the past two decades we have seen more research looking further into astrocytes and their role supporting and modulating neuronal activity, which has provided compelling evidence that glial cells are involved in pathophysiology of epilepsy [4]. Astrocytes can release many neuroactive molecules, including glutamate, D-serine, ATP, adenosine, GABA, TNF α (tumour necrosis factor α), prostaglandins, proteins and peptides that may affect neuronal activity and synaptic physiology [61,62]. Astrocytes regulate and respond to extracellular glutamate levels in the CNS via astrocytic glutamate transporters and the metabotropic glutamate receptors (mGluRs) 3 and 5 [63]. In epileptic tissue, reactive astrocytes utilize several mechanisms that regulate seizure development, which include dysregulation of astrocytic transporters and mGluRs [63,64]. Moreover, epileptic seizures cause neuronal cell loss and astrogliosis, a hallmark of epilepsy [65,66]. Inflammatory processes concomitant to SE result in changes in morphology and protein expression in astrocytes, including decreased expression of EAAT proteins, resulting in decreased glutamate uptake [67–69]. These inflammatory changes can also result in TNF α R and AMPA and NMDA receptors activation, further promoting excitotoxicity [70]. IL-1R activation by IL-1 β can also promote glutamate release from the astrocytes [71,72].

It is strongly suggested that mTOR signaling is altered in SE [73], but just recently evidence of upregulation of the vascular endothelial growth factor-3 (VEGF-3) -mediated mTOR activation in reactive astrocytes after the onset of SE was associated with the regulation of GLT-1 expression. This suggests that this pathway could be involved in preventing hyperexcitability induced by repeated seizure activity [74].

Another area that has gathered increased recognition in the pathogenesis of epilepsy is energy homeostasis. Proper energy sources available to neurons are needed for excessive neuronal discharges to happen. On the other hand, an endogenous mechanism for seizure termination is energy depletion during seizures. In this sense, astrocytes play a very important role as they can control neuronal energy homeostasis through neurometabolic coupling, hence, astrocyte dysfunction in epilepsy leads to bias of key metabolic and biochemical mechanisms, and evidence suggests that dysfunctional glutamate metabolism in astrocytes contribute directly to neuronal hyperexcitability [75–77]. In early epileptogenesis, closure of astrocyte intercellular gap junction coupling limits activity-dependent trafficking of energy metabolites, resulting in impaired clearance of glutamate and K⁺ from the extracellular space. Accumulation of extracellular K⁺ leads to neuronal hyperexcitability, which can result in gap junction uncoupling, a recent finding demonstrated in a study

using a transgenic mouse with astrocyte specific expression of a pH sensor (Lck-E2GFP), that established that astrocytes react to epileptiform activity with intracellular alkalization [78]. Additionally, the metabolism of adenosine, a metabolic product of ATP degradation that largely inhibits energy-consuming processes as an evolutionary adaptation to conserve energy, is increased by dysfunctional astrocytes.

Metabolic therapeutic approaches that prevent the utilization of glucose could represent an effective therapy for epilepsy, as astroglia energy homeostasis play a critical role in the control of neuronal excitability. In this regard, high fat low carbohydrate “ketogenic diets”, as well as inhibitors of glycolysis and lactate metabolism are of increasing interest as antiepileptic strategy [76,79].

Additionally, loss of glutamine synthetase in the human epileptogenic hippocampus was suggested as possible mechanism for raised extracellular glutamate in mTLE [80], as well as an important causative factor for glioblastoma-associated epilepsy [81]. Indeed, a recent study reported that selective deletion of gene for glutamine synthetase in the mouse cerebral cortex induces glial dysfunction with decreased levels of EAAT1 and EAAT2, and vascular impairment that precede epilepsy and neurodegeneration. This study suggests that abnormal glutamate metabolism could cause epilepsy by initially affecting glia which then results in disruption of the neurovascular coupling [82].

For further information on the general role of glia and neuron-glia interactions in epilepsy, see [4,63,64,76,83,84].

Glutamate Excitotoxicity and Epilepsy

As described above, epilepsy has been characterized by recurrent spontaneous seizures due to hyperexcitability and hypersynchrony of brain encephalic neurons [4,84]. Seizures occur with a disbalance between excitatory and inhibitory neurotransmission, and glutamate, as the predominant excitatory neurotransmitter in the brain, unquestionably plays a crucial role in both the initiation and proliferation of seizure activity and in epileptogenesis. Studies in the 1980s demonstrated that intracerebral injection of glutamate into experimental animals induced seizures [85]. Later, *in vivo* microdialysis studies revealed increased glutamate levels in the hippocampus following the seizures emergence induced by pilocarpine [86]. Additionally, EEG and microdialysis studies of patients with epilepsy showed significant increases in extracellular glutamate levels in the hippocampus. These studies described excitotoxic glutamate concentrations of 5.9 μM before a seizure, 17.6 μM during a seizure onset, and 11.7 μM up to 16.5 minutes following the termination of a seizure, i.e., 2, 6 and 4-fold increases compared to basal glutamate concentrations, respectively [87,88]. A study observed that SE-induced glutamate release overstimulates glutamate receptors and results in sustained chronic seizure activity and development of seizure-induced brain damage that occurs over a dynamic process [89]. This was further validated in subsequent studies showing that the increase in glutamate concentrations positively correlates with the intensity of epileptic activity [67,90], demonstrating that increased levels of extracellular glutamate play a role in the initiation and spreading of seizures [23]. Other studies established that augmented concentration of glutamate in the synaptic cleft during the

initial insult is associated with excitotoxicity and it remains high during epileptogenesis [91,92]. Furthermore, studies demonstrated that, during SE, astroglial cells are activated by the presence of cytokines and reactive oxygen species. This leads to decreased glutamate clearance, resulting in accumulation in the extracellular space and increased risk for neuronal excitotoxicity [71,93]. Beyond that, it was demonstrated that abnormal release of glutamate through a single astrocyte was able to activate increases in intracellular Ca^{2+} mediated by NMDA receptor in many neighboring neurons [23,67]. This can lead to an increase in intracellular Ca^{2+} in the astrocytes themselves, resulting in a further intensified release of glutamate that promotes excitotoxicity [67,69,94].

Non-NMDA receptor-mediated excitotoxicity is also relevant in the context of glutamate transporter suppression. Studies in cultured organotypic spinal cord slices were very useful to study this matter. In early studies, slow toxicity was achieved by inhibiting glutamate transport continuously, raising the concentration of glutamate concentration in the medium and resulting in degeneration of motor neurons over several weeks. This degeneration was prevented by non-NMDA receptor antagonists, glutamate synthesis or release inhibitors, but not by NMDA receptor antagonists [95,96]. Another study identified motor neurons to be selective sensitive to calcium-permeable AMPA/kainate receptors [97]. Other study investigated topiramate, an anti-convulsant compound that has anti-excitotoxic properties by blocking AMPA-receptor evoked currents, for potential treatment of ALS. The study revealed that topiramate prevented motor neuron degeneration, but it did not increase survival in G93A SOD1 transgenic mice, an animal model of ALS, suggesting that it could be useful as a neuroprotectant, but were not effective in more complex motor injury paradigms such as the mouse model of ALS [98]. Importantly, AMPA receptors mediate fast synaptic excitation in brain regions pertinent to epilepsy, and AMPA receptor antagonists, such as perampone, have been shown to decrease epileptiform activity in *in vitro* and *in vivo* studies [99].

Several studies have also indicated a causal role for excessive glutamate release by astrocytes and activation of mGluRs in the excitotoxic events leading to the synchronous firing of large populations of neurons during seizures [19,64,71,100,101].

In addition to observing that increased extracellular levels of glutamate have been associated with seizures and epilepsy, it is also noted that initiation of glutamatergic hyperactivity and seizures are facilitated by increased expression and activity of NMDA and AMPA receptors [8,102]. A study found that glutamate binding is altered in hippocampus and cortex of rats after pilocarpine-induced SE [103], and a later study using the same model expanded these findings and found an increase in NMDA receptor expression and altered AMPA receptor activity in the epileptic areas of the hippocampus [104]. Other studies demonstrated that an early event in SE in rats leads to an increase in surface expression of the GluN1 subunits of NMDA receptors along with an increase of NMDA synaptic and extra-synaptic currents in both kainate and pilocarpine induced models of epilepsy [102,105]. Additionally, autopsy samples from TLE patients have also shown that there is increased expression of NMDA, AMPA, and kainate receptors in the epileptic foci [106]. To further support this, increased NMDA channel activation in the epileptic region in the frontal cortex and temporal lobe in patients with epilepsy were revealed by positron emission tomography

(PET) analysis *in situ* using an NMDA tracer [105,107]. Genome wide screens have also shown that p38 MAPK, which is activated upon the binding of glutamate to the NMDA receptor and can induce apoptosis [108], is overexpressed in the hippocampus of epileptic rats [109]. Prolonged SE as an initial insult has been shown to result in the internalization of GABA receptors and migration of NMDARs to neuronal synapses [110] resulting in further reduced inhibition and hyperexcitability [23]. Additionally, it has been found that SE induces posttranscriptional modifications and epigenetic changes in NMDAR subunits, which contribute to long-lasting changes in circuit connectivity [111].

In this context, the development of therapeutic approaches targeting NMDARs to reducing glutamate excitotoxicity have been proposed to prevent seizure-induced neuronal death, epileptogenesis, and subsequently spontaneous recurrent seizures. This potential strategy is reasonable, as much of the excitotoxic damage and molecular and synaptic changes that further propagate epileptogenesis are mediated by NMDARs [5,7,102]. Early studies demonstrated that NMDAR antagonist AP-5 ((2R)-amino-5-phosphonovaleric acid (AP-5)) inhibits epileptogenesis in *in vitro* models of epilepsy including the three-chamber and low magnesium models [112]. Additionally, treatment with MK-801, another NMDAR antagonist, was shown to impair epileptogenesis in the kindling model of epilepsy, in which animals are repeatedly stimulated with electrodes to activate neural pathways inducing increased susceptibility to evoked seizures, resulting in the progression to spontaneous recurrent seizures [113]. Similar results with MK-801 have also been observed in the kainic acid model of epilepsy [114]. Also, this treatment was shown to impair mossy fiber sprouting induction, a structural change to neuronal circuits that occurs during the latent period that promotes epileptogenesis [17]. MK-801 and ketamine treatments have also been shown to suppress seizures and be neuroprotective following prolonged SE in preclinical studies [17,115,116].

Another potential strategy to target NMDARs was demonstrated by inhibition of brain-specific microRNA-134, which is necessary for NMDAR-dependent spine remodeling [117]. This resulted in very efficacious prevention of spontaneous recurrent seizures months after the initial SE event in mice [17,118].

However, NMDA receptor antagonists have been found to lead to on-target side effects such as hallucinations and dissociative effects, due to the spectrum of activities that are mediated by excitatory signaling and NMDA activation [119–121]. Additionally, blockage of AMPA-receptor activity also yield in considerable adverse events, such as CNS-depressant side effects [122]. This suggests that other approaches for reducing glutamate-mediated excitotoxicity and prevent epileptogenesis should be explored.

EAAT2 and epilepsy

Many studies provide ample evidence that epileptic seizures influence the expression levels of EAATs (table 2). It is suggested that enhanced glutamate release and neuronal activity during epilepsy may result in modulation of EAATs expression. The regulatory processes of EAATs during epilepsy are not fully understood, but they seem to occur over a dynamic process that involves several factors, including compensatory mechanisms and

changes in ion gradients and therefore electrochemical environment associated with epileptic hyperactivity. Despite decades of research, there still much to know about the functional characteristics of glutamate transporters and their relationship with neuropathology and behavior of epilepsy [123].

Below we discuss knockdown studies that were pivotal for our current understand of the role of EAATs in epilepsy studies, as well as studies demonstrating changes in EAATs expression in several animal models and human epilepsies, including temporal and developmental differences. Table 2 summarizes these findings, and figure 2 depicts the human mutation studied thus far

Knock out/down studies

One of the first studies examined loss of GLAST and GLT-1 in organotypic spinal cord cultures using antisense oligonucleotides, which demonstrated an essential role of these astroglial transporters, as their loss was toxic to motor neurons. Further, this study examined GLAST and GLT-1 knockdown *in vivo* in rats, and observed increased extracellular glutamate concentrations, neuronal degeneration, and progressive paralysis [124]. One year later, remarkable studies by Tanaka's group show that GLT-1 knockout mice experience spontaneous lethal seizures at 6 weeks of age and increased susceptibility to acute cortical injury, directly proving the involvement of astrocytic glutamate uptake in the prevention epileptic seizures and neuronal death [46]. These mutant mice also showed an increase in synaptic concentrations of glutamate and histological analysis of brains taken from these mice revealed neurodegeneration in the CA1 region of the hippocampus, suggesting that GLT-1 plays an important role in the maintenance of low extracellular glutamate concentrations and prevention of excitotoxicity and seizure activity.

A study analyzed conditional deletion of astrocytic or neuronal GLT-1 in mice [125]. Elimination of astrocytic GLT-1 resulted in 80% loss of GLT-1 protein and of glutamate uptake activity, excess mortality, lower body weight, and seizures, suggesting that astrocytic GLT-1 is of major importance. On the other hand, neuronal GLT-1 deletion resulted in normal survival, weight gain, and no seizures. However, the glutamate uptake capacity into synaptosomes was significantly reduced, suggesting a greater contribution to synaptosomal glutamate uptake of neuronal GLT-1 than expected [125].

Tanaka's group recently expanded their studies to examine the consequences of GLT-1 dysfunction in different encephalic brain regions. Region-specific GLT-1 knockout mice were generated by crossing floxed-GLT-1 mice with mice expressing the Cre recombinase in a particular domain of the ventricular zone. Selective deletion of GLT-1 in the diencephalon, brainstem and spinal cord was sufficient to reproduce the phenotypes of the global GLT-1 null mice, including excess mortality, decreased body weight, and lethal spontaneous seizure. On the other hand, dorsal forebrain-specific GLT-1 knockout mice had nonlethal complex seizures including myoclonic jerks, hyperkinetic running, spasm, and clonic convulsions. This suggests that GLT-1 dysfunction in the dorsal forebrain is involved in the pathogenesis of infantile epilepsy and GLT-1 in the diencephalon, brainstem and spinal cord might be very important in the prevention of seizure-induced sudden death [126].

Although this review is focused on glial transporter EAAT2, it is noteworthy to discuss a study on the system x_c^- , a glial transporter that mediates the release of glutamate in the synaptic cleft in exchange for cystine and represents the key source of hippocampal extracellular glutamate. A genetic knockout of xCT (the specific subunit of system x_c^-) in a Theiler's murine encephalomyelitis model of viral-induced epilepsy observed a small decrease in neuronal injury, as well as increases in astrogliosis in the CA1 hippocampal region and in cortical EAAT2 expression, adding to our knowledge for future development of novel anti-seizure therapies [127].

No changes in expression

An early study in a rodent kindling model reported that GLT-1 levels remain unchanged [128]. Two other studies in surgically removed tissue from temporal lobe of patients with epilepsy found no change in EAAT2 expression [80,129].

Further studies (below) provided abundant evidence of decreased transporter expression in epilepsy, and some report transient increased expression of transporters, followed by decreased expression. Explanations for the different observations among studies may include differences in tissue processing, stage of epileptogenesis development or severity of the epileptic disease.

Increased expression

Some studies reported that glial EAATs were increased shortly after seizures, possibly indicating acute activation of astrocytes but decreased in the later chronic phase. In an *in vivo* model of kainic acid-induced seizures and in hippocampal organotypic slice cultures, decreased immunoreactivity of EAAC1 neuronal transporter was observed in hippocampal regions, whereas GLT-1 expression levels were increased, indicating a differential regulation of neuronal and glial glutamate transporters that could be playing a role in seizure induction, neurotoxicity and neuronal plasticity [130]. In a chronic model of epilepsy using PTZ, GLAST and GLT-1 expression in the hippocampus increased within 24 h of kindling, however, their levels returned to baseline 30 days after the last seizure [131]. In a SE model triggered by intrahippocampal application of kainic acid, the levels of GLT-1 significantly increased within 24h post-SE; but decreased to lower levels than in control brains at later timepoints [35].

Collectively, these studies show a temporal upregulation followed by downregulation in these models, providing more evidence that astrocytic glutamate transporter dysregulation contributes to the development of epilepsy.

A recent study implicated GLT-1 dysfunction and increased extracellular glutamate levels in the hippocampus in a mouse model of Lafora disease, a fatal rare disease marked the presence of insoluble polyglucosan accumulation in brain, epilepsy and neurodegeneration.. In this study, the researchers found that the minor isoform of the GLT-1 gene, GLT-1b, was upregulated in the hippocampus, and they suggested this to be associated with compensatory mechanisms triggered by neuronal disturbance. Additionally, glutamate clearance dysfunction was observed in Lafora mice, observed by a challenge with a blockade of GLT-1 that resulted in these animals to be unable to clear glutamate [132].

Decreased expression

Human patients—Several studies have reported impaired glutamate transport function in human epilepsy. Decreased levels of both EAAT1 and EAAT2, increased extracellular glutamate levels and neuron loss were observed in the epileptogenic hippocampus of patients with TLE both during and after seizures [67,133,134]. One study found that decreased levels of EAAT2 protein and alternatively spliced forms of EAAT2 were present in sclerotic, but not the intact parts of the hippocampus in TLE patients [135]. One study reported reduced expression of EAAT1 and EAAT2 in the hippocampus CA1, which the authors suggest may either be an adaptive response to neuronal death or a causative event contributing to neuronal death [67]. Another study demonstrated down regulation of EAAT1 and EAAT2 expression in sclerotic hippocampi, and pronounced neuronal loss in the CA1, whereas in the CA2 there was no decrease or even an increase in areas with less neuronal loss. This suggests compensatory mechanisms of the transporters in response to the pathology [136]. Intriguingly, significant reductions in mRNAs and protein levels of EAAT3 and EAAT4 in human focal epileptic brain regions were observed, with no changes in EAAT2 mRNA, but decreases in EAAT2 protein expression at epileptic foci, suggesting regional reductions in EAAT expression associated with increased local glutamate levels and likely contribution to both hyperexcitability and spontaneous generation of epileptic discharges [137].

Human mutations—In this section we will discuss human mutations identified in EAAT1 and EAAT2 and their relationship to epileptic phenotypes. A study looked for mutations in SLC1A3 (the gene encoding EAAT1, see table 1) in episodic ataxia 6 (EA6), a neurological disease characterized by long attack duration, absent myokymia, nystagmus, tinnitus, and epilepsy. Mutant P290R in EAAT1 was identified, and *in vitro* studies showed decreased EAAT1 protein expression and functionality, which could contribute to neuronal hyperexcitability [138]. Later, the same group identified mutation C186S in a patient with severe episodic and progressive ataxia, migraine headache and seizures. This mutant had a significant reduction in glutamate uptake [139]. Another study confirmed that P290R mutation resulted in reduced EAAT1 cell surface expression and decreased glutamate uptake, and add that it resulted in increased anion currents, even though the transporter expression were decreased, suggesting a gain-of-function in anion conduction as a pathological process in EA6 [140]. A later study also determined this mutation to cause glial apoptosis [141]. In a *Drosophila* model of ataxia, this mutation disrupted EAAT1-mediated chloride channel, and caused astrocyte malformation and paralysis episodes, suggesting that the chloride channel properties of EAAT1 is correlated to the pathophysiological mechanisms causing dysfunction of neural circuit, which could be an important and novel mechanism for epilepsy [142]. A meticulous study on several mutations (M128R, C186S, P290R, T3128A, A329T, V393I, R499Q) expressed in heterologous cells observed impairments of multiple EAAT1 properties ranging from changes in transport function, impaired trafficking to increased protein expression [143]. Collectively, these genetic and functional assays studies depict a crucial role of cerebellar transporters (as EAAT1 is mainly expressed in the cerebellum), and therefore cerebellar function, in epileptogenesis, although much still needs to be unraveled.

Regarding EAAT2 mutants, a study reported *de novo* mutations in SLC1A2 (gene that encodes EAAT2) as an important cause of epileptic encephalopathies, a group of severe early-onset epilepsies. These mutations include G82R and L85P, and individuals with such mutations had a very severe phenotype, marked by seizures beginning in the first week of life and profound developmental impairment, which could be associated with dysregulation of EAAT2 and glutamate excitotoxicity [144,145]. A recent study further examined recurrent *de novo* L85P EAAT2 variant, which was associated with early onset developmental and severe epileptic encephalopathy, through a dominant negative mechanism that decreases the expression of EAAT2 [146]. Very intriguingly, this study suggests that there is a critical amount of functional EAAT2 protein (somewhere between 0 and 50% of wild type) that correlated to seizures development. These considerations could be crucial in the development of therapeutics.

The mutation sites described here are also shown in the figure 2, in which the glutamate transporter structure was drawn based on the structure of human EAAT1 [147]. Figure 2A shows the 2-D structure, and 2B the three-dimensional protein structure. Transmembrane domains 3 and 6-8 and two hairpin loops (HP1 and HP2) form the transport domain, shown in green, which contains the substrate binding sites (or orthosteric site). Transmembrane domains 1, 2, 4 and 5 and parts of the N-terminal surrounding the central core transport domain form a peripheral rigid scaffold, shown in red. This is designated the scaffolding domain, which provides the interaction interface between three subunits of the trimer and is thought to facilitate “the elevator-like movement” of the transport domain. Figure 2B also shows an allosteric site, which was essential for the identification of allosteric modulators, discussed below.

Animal studies—Animal studies have also been pivotal in examining changes in EAAT expression in several epilepsy models. A study on chronic seizures induced by injection of FeCl₃ into the amygdala found that glial transporters GLAST and GLT-1 were downregulated at 60 days in the hippocampal tissue, indicating a disturbance of glutamate regulation caused by the downregulation of these glutamate transporters [148]. In a model of amygdala-kindled rats, levels of GLAST protein were initially downregulated in the piriform cortex/amygdala region, and later increased in this region and in the hippocampus, whereas GLT-1 levels remained unchanged, suggesting differential regulation in kindling [149]. Downregulation of GLT-1 was also shown in pilocarpine models [104] and correlated with thalamic neuronal death in kainic acid-induced models of SE as well [150]. In an intrahippocampal kainic acid model of SE, it was observed an initial increase in GLT-1 immunoreactivity in the hippocampus, followed by a downregulation of GLT-1 at 4- and 7-days post-insult, suggesting a differential transporter regulation in the pathophysiology of epileptogenesis [65,81]. Recently, a microarray analysis on differentially expressed genes in the pilocarpine induced mTLE model determined 3,232 potential genes highly correlated with epilepsy. The SLC1A2 gene, that encodes the human EAAT2 protein, was shown to be related to brain development and its expression is significantly decreased in the hippocampus of epilepsy patients, suggesting EAAT2 as potential biomarker of epilepsy [151].

Additionally, in agreement with findings from pharmacological epilepsy models, decreased expression and function of GLAST and GLT-1, associated with increased levels of extracellular glutamate and excitotoxic neuronal death were observed in a mouse model of tuberous sclerosis, a genetic disorder associated with epilepsy [152,153]. Rats subjected to traumatic brain injury (TBI), a condition that can also lead to the development of epileptic seizures, also had decreased brain expression of EAAT2 [154]. Furthermore, the spontaneously epileptic rat (SER, a double mutant *zi/zi*, *tm/tm*), presents both tonic convulsions and absence-like seizures from 8 weeks of age. Decreased expression of EAAT1 and mGluR1 were shown in the SERs hippocampus, whereas EAAT2 is increased, suggesting that epileptogenesis in SER is associated with regulation of EAAT1, EAAT2 and mGluR1 [155]. Another study found that a conditional astrocytic knock-out of mGluR5 during epileptogenesis impairs high-frequency glutamate uptake mediated by EAAT1 and EAAT2, suggesting a compensatory response to glutamate dysregulation [156].

The role of astrocytes in epileptogenesis has been investigated in a study in two models: blood-brain barrier (BBB)-breakdown with treatment with deoxycholic acid, and direct exposure of the neocortex to serum-derived albumin. These insult models result in fast upregulation GFAP (glial fibrillary acidic protein, a marker of astrocytes), followed by the development of an epileptic focus within 4-7 days, and decreased clearance capacity for both extracellular glutamate and potassium. Frequency-dependent synaptic facilitation leading to seizure-like activity were confirmed by *in vitro* electrophysiological recordings during epileptogenesis. These findings suggested that during early epileptogenesis there was a transcription-mediated astrocytic transformation, which was accompanied by downregulation of EAAT2 [157]. Recently, another study reinforced the notion that BBB dysfunction plays an important role in epilepsy. In an endothelial *Cdk5*-deficient mouse model, the authors observed spontaneous seizures, decreased glutamate reuptake through GLT-1, and increased glutamate synaptic function. Ceftriaxone, a β -lactam antibiotic that was previously shown to increase GLT-1/EAAT2 protein expression levels [158], was shown to restore GLT-1 function and to inhibit seizures, revealing a previously unknown link between cerebrovascular factors and epileptogenesis [159].

Recently, a study explored sex differences in a model of pilocarpine-induced-SE in mice. It was found that males had shorter latency to seizures and higher prevalence of SE, which was accompanied by more hippocampal neuronal death, glial activation, higher levels of inflammatory markers, and higher levels of GABA transporter 3 (GAT-3). On the other hand, mRNA levels of GLAST and GLT-1 were higher in female mice. These findings suggest that male mice are more vulnerable to SE than female mice, and importantly, these observed sex differences correspond to the sex differences seen in GLAST and GLT-1 mRNA expression. These findings can contribute to development of tailored therapies for both sexes [160].

Collectively, these studies suggest that the involvement of downregulated or dysfunctional GLT-1/EAAT2 in seizure onset and epileptogenesis seems to be dependent on the specific epileptic syndrome or animal model. Taken together, it appears that the expression and functional activity of glial EAATs are decreased in the chronic phase of epilepsy in experimental rodent models and in patients, possibly contributing to brain damage and epileptogenesis. However, further studies are needed to accurately determine

whether transporters regulation correspond to causative or compensatory changes during epileptogenesis.

Upregulation of EAAT2 expression as potential therapeutics

Many studies discussed above demonstrated the involvement of downregulated or dysfunctional GLT-1/EAAT2 in seizure onset and epileptogenesis. In the converse situation, several studies show that upregulation of GLT-1/EAAT2 seems to be neuroprotective in epilepsy models. Table 3 summarizes the studies demonstrating potential therapeutic strategies involving modulation of GLT-1/EAAT2 transporter.

A study successfully demonstrated that EAAT2 transgenic mice with a 1.5-2-fold overexpression of EAAT2 protein levels, had decreased chronic seizure frequency, mortality, reduced neuronal degeneration in the hippocampus after SE, and decreased SE-induced neurogenesis and mossy fiber sprouting in a pilocarpine-induced model of SE. This suggests that this strategy can be neuroprotective against SE-induced neuropathological changes, chronic seizure development and SE-induced death [161]. This group later showed that treatment with compound LDN/OSU-0212320, a pyridazine derivative they previously discovered that increases EAAT2 expression through translational activation, had neuroprotective properties in primary cultured neurons, as well in an *in vivo* pilocarpine-induced TLE model [162].

Moreover, several studies examined the effects of ceftriaxone, an GLT-1/EAAT2 protein enhancer that acts in the transcriptional level [158,163], in epilepsy models. Ceftriaxone treatment restored the levels of GLT-1 in a mouse model of tuberous sclerosis, which was correlated with decreased hippocampal extracellular glutamate levels and reduced neurodegeneration [153,164]. Similarly, ceftriaxone had protective effects by reducing seizure activity in a traumatic brain injury-induced epilepsy [154], in a *Cdk5*-knockout model that leads to spontaneous seizures in mice [159] and in an acute mortality in PTZ models of epilepsy [165–167]. A combination of treatment with ceftriaxone and valproic acid in the PTZ rat model dose-dependently reduced seizures and enhanced motor and cognitive functions [168]. A recent study reported that ceftriaxone administration in a pilocarpine model of mTLE led to a reduction of glutamate levels, and elevation of GS activity and GLT-1 expression in the acute phase. However, 4 weeks after SE glutamate levels and GLT-1 expression were decreased, and impairment of learning and memory ability were attenuated of in the chronic phase of epileptogenesis [169]. It remains to be determined whether ceftriaxone treatment for human epilepsy is clinically translatable.

Additionally, riluzole, an FDA- approved drug for amyotrophic lateral sclerosis (ALS) [170], was also investigated as potentially antiepileptic. Previously, riluzole has been reported to decrease presynaptic glutamate release [171,172], to increase EAAT2 levels [173–176], and also acts as non-specific ion channel blocker [177]. A study demonstrated that riluzole inhibited the development of behavioral seizures in kindling acquisition [178]. Riluzole was also shown to enhance the anti-seizure action of conventional ASDs, namely valproate, phenobarbital and ethosuximide, in an mice model of PTZ-induced convulsions [179]. Conversely, one study raised concerns about the repurposing of riluzole for epilepsy, as it

was shown to be toxic when combined with neuronal hyperexcitation, in a model of infusion of the K⁺ channel blocker 4-aminopyridine (4-AP) in the hippocampus [180]. Also, riluzole treatment inhibited pre-ictal spikes and spike-wave discharges in the models of gamma-hydroxybutyrate lactone (GBL)-induced absence seizure model and in the pilocarpine-induced limbic seizure model. These findings suggest that riluzole could be developed as antiepileptic treatment against limbic seizure and absence seizure [181]. Collectively, these studies suggest that, even though riluzole has promising neuroprotective actions in some studies, and this action seems to be accomplished primarily by inhibiting excitatory neurotransmission by modulation of presynaptic release, uptake and postsynaptic activities of glutamate, the mechanisms behind these actions are not well understood. Therefore, further studies are needed before riluzole can be proposed as candidate supplementary therapy for epilepsy.

Another strategy to increase GLT-1 levels *in vivo* is through inhibition of Hsp90, which was shown to prevent the degradation of this transporter and to suppress spontaneous recurrent seizures in a rodent TLE model [182,183]. These studies suggest the possibility of developing therapeutics with Hsp90 inhibitors that would prevent GLT-1/EAAT2 degradation. Such a strategy, both for TLE and other excitotoxicity-related disorders, warrants future investigations.

Lastly, a study reported that inhibition of p38 MAPK signaling results in increased GLT-1 expression in the brains of rats subjected to a lithium chloride-pilocarpine epilepsy model, which resulted in decreased latency of the first epileptic seizure and attenuated severity of seizures [184]. This study suggests a potential mechanism by which GLT-1/EAAT2 expression is upregulated, adding to our current understanding of the regulation of this transporter in epilepsy.

The findings described in experimental models suggest that inducing expression of EAAT2 could be developed as a therapeutic approach to treat epilepsy and be a disease modifying course of treatment. However, this approach raises a concern about unknown off target effects in other regions of the brain due to the prevalence of EAAT2 in the brain and spinal cord, as well as possible regulation of various other proteins. Thus, enhancement of the function of EAAT2 through a direct activation of EAAT2 emerges as an alternative approach. In the next section, we will discuss positive allosteric modulation of EAAT2 to enhance transport activity, a mechanism that may serve as a novel therapeutic to provide neuroprotection and prevent spontaneous recurrent seizures and epileptogenesis.

Positive Allosteric Modulation of EAAT2 as potential therapeutics

While NMDA and AMPA receptor antagonists and calcium channel blockers may reduce glutamate excitotoxicity in epilepsy, neither approach has been sufficient in addressing the unmet medical need in treating epilepsy without causing serious adverse events [5,107,119].

As many studies propose that glutamate excitotoxicity is a key event in both the initial insult and epileptogenic process, reducing extracellular glutamate concentrations by increasing glutamate uptake via glutamate transporters offers promise in preventing neuronal death

after initial insult and preventing epileptogenesis with treatment during the latent period. EAAT2, as the predominant glutamate transporter in the brain [48], appears as a promising therapeutic target. Our lab has pursued the development of compounds that selectively target EAAT2. Previous studies have demonstrated that the venom *P. bistriata* spider stimulates glutamate uptake in rat brain synaptosomes [185] and the purified compound Parawixin 1 selectively and directly enhances the activity of EAAT2 [186]. Further, mutagenesis studies were performed to identify the region in EAAT2 involved in transport stimulation, these investigations identified an allosteric site [187]. Using this knowledge, an *in-silico* approach for screening was performed using a model based on the structure of a glutamate transporter homologue from *Pyrococcus horikoshii*. This study identified molecules that were characterized as positive allosteric modulators (PAMs) of EAAT2 upon evaluation in glutamate uptake in transfected COS-7 cells [188]. We propose that the molecules interact with the interface between transport and scaffolding domains (shown in figure 2B), to alter the equilibrium between different conformations of the transporter and result in altered transition dynamics, ultimately resulting in increased glutamate transport. Current studies using functional, structural, and biophysical approaches are aimed at understanding the specific mechanisms that are involved in allosteric transporter modulation. Importantly, these compounds were subsequently shown to have neuroprotective properties *in vitro* [189]. Medicinal chemistry efforts continue to develop further lead series based on these compounds to optimize their drug-like properties.

Further studies with this class of compounds in *in vitro* and *in vivo* models of epilepsy are needed to validate their therapeutic potential in epilepsy. Nonetheless, preliminary experiments suggest that this class of compounds are neuroprotective and decrease calcium influx in primary hippocampal cultures subjected to low magnesium insults, an *in vitro* epilepsy model.

Recently, our group elucidated the chemical structure of another purified compound isolated from *P. bistriata* venom, Parawixin10. This compound is an acylpolyamine that was determined to be a non-selective positive allosteric modulator of glial transporters EAAT1 and EAAT2, with *in vitro* neuroprotective properties [190]. In previous studies, Parawixin10 was demonstrated to be neuroprotective and to have anticonvulsant properties in an *in vivo* model of intrahippocampal injection of NMDA [191]. In another study, pretreatment with Parawixin10 prevented the onset of seizures induced by kainic acid, NMDA, and PTZ [192]. Another study demonstrated that chronic administration of Parawixin10 in the pilocarpine-induced rat model of epilepsy resulted in neuroprotection, increased the latency of recurrent seizures, and decreased duration and severity of seizures, with no behavioral deficits analyzed by the Morris water maze approach [193]. Even though it is predicted that Parawixin10 does not yield well to drug development, these studies served as proof of concept that allosteric modulation of glial transporters EAAT1 and EAAT2 may offer neuroprotection and anticonvulsant properties.

Further studies with selective EAAT2 PAM in several epilepsy models, and other models that involve glutamatergic dysfunction, will further determine whether this class of compounds can be developed as therapy to provide neuroprotection, to prevent spontaneous recurrent seizures, and to halt epileptogenesis.

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Abbreviations:

ALS

amyotrophic lateral sclerosis

AMPA

α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPAR

AMPA receptor

APV

(((2*R*)-amino-5-phosphonovaleric acid; (2*R*)-amino-5-phosphonopentanoate)

ASDs

anti-seizure drugs

BBB

blood-brain barrier

CDC

Centers for Disease Control and Prevention

Cdk5

Cell division protein kinase 5

CNS

central nervous system

EAAC1

excitatory amino acid carrier 1

EAATs

excitatory amino acid transporters

EAAT1-3

human excitatory amino acid transporter subtypes 1-3

EAAT2

human glutamate transporter 2

EA6

episodic ataxia 6

EEG

electroencephalogram

4-AP

4-aminopyridine

GAT-3

GABA transporter 3

GFAP

glial fibrillary acidic protein

GAD

glutamate decarboxylase

GLAST

glutamate and aspartate transporters

GLT-1

rat glutamate transporter 1

GluN1

subunit of NMDA receptor

HP

hairpin loop

Hsp90

heat shock protein 90

IL-1 β

Interleukin 1 beta

IL-1R

Interleukin-1 receptor

i.p.

intraperitoneal

LDN/OSU-0212320

3-[[[2-Methylphenyl)methyl]thio]-6-(2-pyridinyl)-pyridazine

NMDA

N-methyl-D-aspartic acid

NMDAR

N-methyl-D-aspartic acid receptor

mTLE

mesial temporal lobe epilepsy

MRI

Magnetic Resonance Imaging

PAMs

positive allosteric modulators

PTZ

pentylentetrazole

p38 MAPK

p38 mitogen-activated protein kinases

SE

status epilepticus

SER

spontaneously epileptic rat

TNF α R

tumor necrosis factor α receptor

VEGF-3

vascular endothelial growth factor-3

vGluT

vesicular glutamate transporter

xCT

glutamate-cystine exchangers

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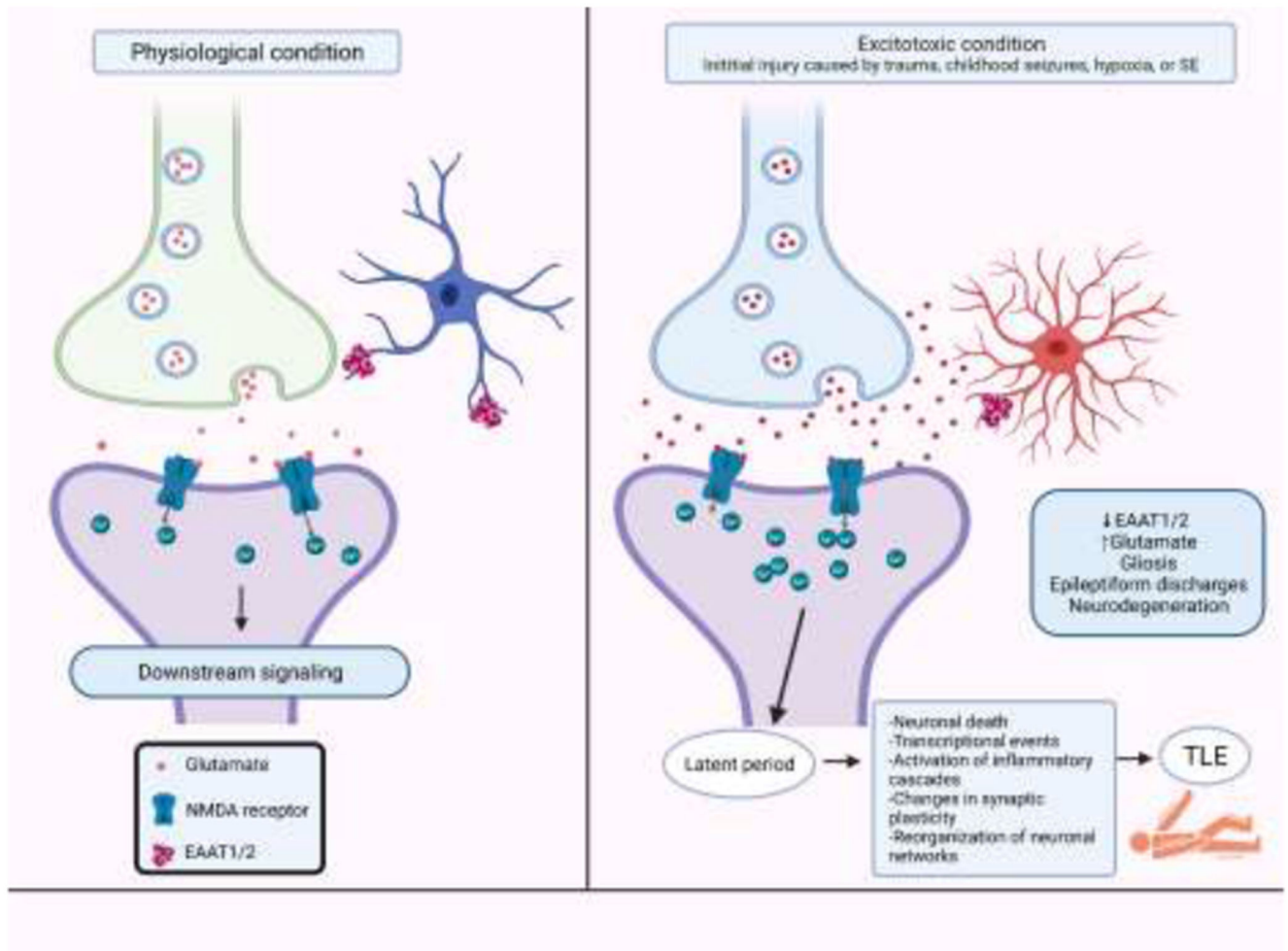


Figure 1. Representation of a tri-partite glutamatergic synapse, under physiological and excitotoxicity conditions, leading to the development of epilepsy.

The intense seizure activity seen in SE causes excessive glutamate release resulting in overstimulation of glutamate receptors leading to massive influxes of Ca^{2+} , subsequently triggering mass neuronal death via glutamate excitotoxicity mechanisms. Following the initial insult or injury there is a latent period that may last up to several years in which complex molecular, biochemical, and structural changes occur, including changes in synaptic plasticity and neuronal connectivity reorganization of neuronal networks. Within minutes to days following the initial insult, acute early changes include rapid alterations to ion channel kinetics, post-translational modifications to existing functional proteins, and activation of immediate early genes. Hours to weeks after the insult, subacute changes occur, including transcriptional events, neuronal death, and activation of inflammatory cascades. The chronic changes that follow over weeks to months include anatomical changes, such as neurogenesis, mossy fiber sprouting, network reorganization, and gliosis. These changes ultimately lead to neuronal networks being more susceptible to hyperexcitability and synchronous firing of these excitatory neurons, leading to more seizures and eventually

spontaneous recurrent seizures This results in the emergence of chronic epilepsies such as TLE. Figure created with BioRender.com.

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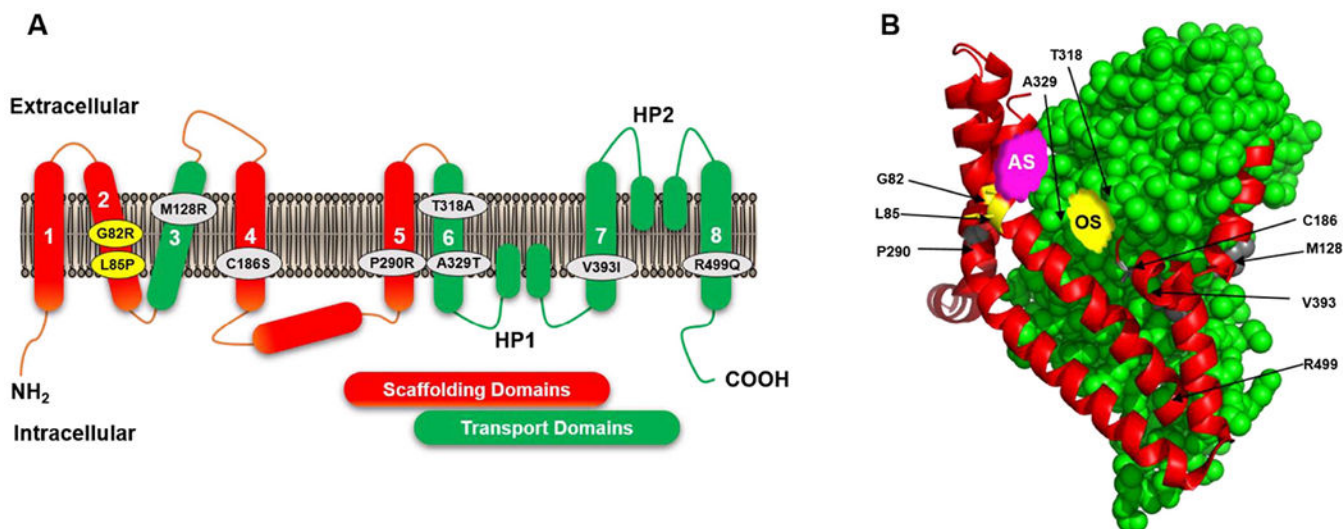


Figure 2.

A. Two-dimensional membrane topology diagram of a single protomer of glutamate transporter, depicting eight transmembrane domains and hairpin loops, and showing the spatial distribution of EA6 and epileptic encephalopathies associated missense mutations: M128R, C186S, P290R, T3128A, A329T, V393I, R499Q in EAAT1 (in grey circles) and G28R and L85P in EAAT2 (in yellow circles). The scaffolding and transport domains are shown in red and green, respectively.

B. The tertiary structure of a single glutamate transporter protomer shown in the plane of the membrane, depicting scaffolding and transport domains in red and green, respectively, substrate and sodium binding sites (orthosteric site or OS, in yellow), a proposed allosteric site (AS in pink, from reference [188]), and positions of the EA6 and epileptic encephalopathies mutations. Structure was modelled using the crystal structure of EAAT1 in complex with L-aspartate (PDB 5LLM, ref [147]) as template, using PyMol Molecular Graphic Systems, version 2.4.1, Schrodinger, LLC.

Note: Residues G82, L85 (EAAT2 numbering), M128, C186, P290 and V393 (EAAT1 numbering) are conserved between EAAT1 and EAAT2 transporter subtypes. However, T318 in EAAT1 is M317 in EAAT2, A329 in EAAT1 is G328 in EAAT2 and R499 in EAAT1 is K498 in EAAT2.

Nomenclature of glutamate transporters, main biological activity, and predominant expression in the CNS.

Table 1.

Protein name (human)	Protein name (rodent)	Gene	Main biological activity	Predominant expression in mature brain	References
EAAT1	GLAST	SLC1A3	Glutamate transporter	Astrocytes, oligodendrocytes in cerebellum, cortex, spinal cord (perisynaptic)	[194–196]
EAAT2	GLT-1	SLC1A2	Glutamate transporter	Astrocytes (perisynaptic), axon terminals (presynaptic), oligodendrocytes in whole brain and spinal cord	[44,196]
EAAT3	EAAC1	SLC1A1	Glutamate and cysteine transporter	Neurons (postsynaptic, cell soma and dendrites) in whole brain	[196,197]
EAAT4	EAAT4	SLC1A6	Glutamate transporter, glutamate-gated chloride channel	Neurons (postsynaptic, dendritic spines) in cerebellum	[198]
EAAT5	EAAT5	SLC1A7	Glutamate-gated chloride channel	Neurons (presynaptic) in retina	[199]
vGLUT1, 2 and 3	vGLUT1, 2 and 3	SCL17A7, 6 and 8	Glutamate transporter	Mainly presynaptic throughout the brain	[200–204]
xCT	xCT	SLC7A11	Cystine-glutamate antiporter	Astrocytes, microglia retinal Muller cells, immature cortical neurons, and glioma in the CNS	[205–207]

Table 2.

Involvement of astroglial transporters in genetic/pharmacological models of epilepsy syndromes. GLAST and GLT-1 are the rodent homologues, EAAT1 and EAAT2 are the human homologues.

Transporter	Model/epilepsy syndrome	Effects	References
<i>Knock down studies</i>			
GLAST and GLT-1	Knock down rats	Increased extracellular glutamate concentrations Neuronal degeneration Progressive paralysis	[124]
GLT-1	Knockout mice	Spontaneous lethal seizures at 6 weeks Increased susceptibility to acute cortical injury Increased glutamate synaptic concentrations Neurodegeneration in the hippocampus CA1	[46]
GLT-1	Conditional deletion of astrocytic or neuronal GLT-1 in mice	Astrocytic GLT-1 deletion: - 80% loss of GLT-1 protein and of glutamate uptake activity - excess mortality - lower body weight - seizures Neuronal GLT-1 deletion: - normal survival - weight gain - no seizures - reduction of synaptosomal glutamate uptake capacity	[125]
GLT-1	Region-specific GLT-1 knockout mice	Selective deletion of GLT-1 in the diencephalon, brainstem and spinal cord reproduced global GLT-1 null mice phenotypes: - excess mortality - decreased body weight - lethal spontaneous seizure Dorsal forebrain-specific GLT-1 knockout mice resulted in nonlethal complex seizures: - myoclonic jerks	[126]

Transporter	Model/epilepsy syndrome	Effects	References
System x_c^-	Genetic knockout of xCT in murine model of viral-induced epilepsy	<ul style="list-style-type: none"> - hyperkinetic running - spasm - clonic convulsion Slight decrease in neuronal injury Increase in astrogliosis in the hippocampal CA1 Increase in GLT-1 expression in the cortex	[127]
No changes in expression			
GLT-1	Rodent kindling model	GLT-1 levels remain unchanged	[128]
EAAAT2	Resected tissue from mTLE patients	EAAAT2 levels remain unchanged	[80,129]
Increased expression			
GLT-1	Kainic acid-induced seizures and hippocampal organotypic slice cultures	↑ GLT-1 expression ↓ EAAAT1 expression	[130]
GLAST and GLT-1	Chronic model of epilepsy with PTZ kindling	↑ expression of EAAAT1 and EAAAT2 in the hippocampus within 24 h of PTZ kindling Levels returned to basal Levels 30 days after the last seizure	[131]
GLT-1	SE model with intrahippocampal kainic acid	↑ GLT-1 levels within 24h post-SE At later timepoints, GLT-1 levels dropped to lower levels than in control brains	[35,65]
GLT-1	Mouse model of Lafora disease	↑ GLT-1b associated with compensatory mechanisms activated in response to neuronal disturbance	[132]
Decreased expression			
EAAAT1 and EAAAT2	Epileptogenic hippocampus of mTLE patients	↓ levels of EAAAT1 and EAAAT2 Increased extracellular glutamate levels Neuron loss observed during and after seizures	[67,133,134]
EAAAT2	Epileptogenic hippocampus of mTLE patients	↓ levels of EAAAT2 in sclerotic, but not the intact parts of the hippocampus in TLE	[135]
EAAAT1 and EAAAT2	Temporal lobe and hippocampus in intractable temporal lobe epilepsy	↓ expression of EAAAT1 and EAAAT2 in hippocampal CA1 Neuronal death	[67]
EAAAT1 and EAAAT2	Epileptogenic hippocampus of mTLE patients	↓ EAAAT1 and EAAAT2 expression in sclerotic hippocampi with neuronal loss in CA1	[136]
EAAAT2, EAAAT3 and EAAAT4	Human focal epileptic brain	↓ EAAAT3 and EAAAT4 mRNAs and protein No changes in EAAAT2 mRNA, but ↓ EAAAT2 protein at epileptic foci	[137]

Transporter	Model/epilepsy syndrome	Effects	References
EAAAT2	Whole exome sequencing followed by transfection in HEK293 cells	Identification of a recurrent <i>de novo</i> Leu85Pro EAAAT2 variant which causes: <ul style="list-style-type: none"> - severe, early onset developmental epileptic encephalopathy via a dominant negative mechanism that modulates EAAAT2 localization and function 	[146]
GLAST and GLT-1	Chronic seizures induced by FeCl ₃ amygdalar injection in rats	↓ GLAST and GLT-1 at 60 days in the hippocampal tissue	[148]
GLAST	Amygdala-kindling in rats	↓ GLAST in the piriform cortex/amygdala region as early as 24 h after one stage 3 seizure and persisting through multiple stage 5 seizures ↑ EAAC1 in same region and hippocampus once animals reached stage 5 level GLT-1 levels remained unchanged	[149]
GLT-1	Induction of SE by i.p. injection of pilocarpine and kainic acid in rats	↓ GLT-1, correlated with thalamic neuronal death	[104,150]
GLT-1	Intrahippocampal kainic acid model of SE in rats	↑ GLT-1 immunoreactivity in the hippocampus (initially) ↓ GLT-1 at 4- and 7-days post-insult	[65,81]
GLT-1	Microarray analysis on differentially expressed genes in the pilocarpine induced mTLE model in rats	↓ GLT-1 in the hippocampus	[151]
GLAST and GLT-1	Tuberous sclerosis mouse model	↓ GLAST and GLT-1 expression and function: <ul style="list-style-type: none"> - increases in extracellular glutamate levels - excitotoxic neuronal cell death 	[152,153]
GLT-1	TBI model in rats	↓ GLT-1 expression	[154]
GLAST and GLT-1	Spontaneously epileptic rat (SER)	↓ GLAST and mGluR1 in the hippocampus ↑ GLT-1, associated with: <ul style="list-style-type: none"> - tonic convulsions - absence-like seizures from 8 weeks 	[155]
GLAST and GLT-1	Conditional knock-out of mGluR5 from astrocytes during epilepsy development	↓ glutamate uptake mediated by GLAST and GLT-1	[156]
GLT-1	BBB-breakdown with deoxycholic acid and serum-derived albumin exposure to neocortex in rats	↓ GLT-1, with reduced clearance capacity for both extracellular glutamate and potassium ↓ GFAP, followed by delayed development of an epileptic focus	[157]
GLT-1	Endothelial <i>Cdk5</i> -deficient mouse model	↓ GLT-1, decreased glutamate reuptake, increased glutamate synaptic function and spontaneous seizures	[159]

Transporter	Model/epilepsy syndrome	Effects	References
GLAST and GLT-1	Pilocarpine-induced-SE in male and female mice	↑ GLAST and GLT-1 in females ↑ GAT-3 in males, accompanied by more hippocampal neuronal death, glial activation, higher levels of inflammatory markers, shorter latency to seizures and higher prevalence of SE in males	[160]

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Table 3.

Potential therapeutic strategies for epilepsy involving modulation of astrocytic glutamate transporters. Administration routes for compounds are included.

Compound/ strategy	Model/study/administration route	Effects	References
<i>Upregulation of GLT-1/EAAT2 expression as potential therapeutics</i>			
Genetic overexpression of EAAT2	EAAT2 transgenic mice Pilocarpine-induced model of SE	1.5-2-fold overexpression of EAAT2 protein levels in the hippocampus after SE: - decreased chronic seizure frequency - decreased mortality - reduced neuronal degeneration - decreased SE-induced neurogenesis and mossy fiber sprouting	[161]
LDN/OSU-0212320, pyridazine derivative ↑ GLT-1/EAAT2 expression through translational activation	Pilocarpine-induced TLE mouse model Administration route: i.p. injection 3 h before pilocarpine insult	Treatment protects cultured neurons from glutamate excitotoxic injury and death via GLT-1 activation Reduces mice mortality, neuronal death, and spontaneous recurrent seizures	[162]
Ceftriaxone, β-lactam antibiotic ↑ GLT-1/EAAT2 expression in the transcriptional level	<i>In vitro</i> studies	Restores GLT-1 function	[158,163]
	Mouse model of tuberosclerosis Administration route: i.p. injections either at postnatal day 21 or at six weeks of age until death or other pre-defined endpoint of experiment	Inhibits seizures associated with decreased extracellular glutamate levels in the hippocampus Reduces neuronal death	[164]
	TBI-induced epilepsy in rats Administration route: i.p. injections for 7 days after TBI	Restores GLT-1 expression in the injured cortex Reduces seizure activity	[154]
	Endothelial <i>Cdk5</i> -deficient mouse model Administration route: i.p. injections for 5 days before PTZ-induced convulsions in 4-wk-old <i>Cdh5-Cre;Cdk5^{f/f}</i> mice	Restores GLT-1 function Inhibits seizures	[159]
	Rat model of PTZ-induced convulsions Administration route: i.p. injections for 4 or 7 days before PTZ-insults [165] i.p. injections for 7 days before PTZ-insults [167] i.p. injections of ceftriaxone, valproic acid or the combination daily for 7 days after PTZ- insults [168]	Reduces acute mortality In combination with valproic acid reduces seizures and enhances motor and cognitive functions	[165,167] [168]
	Pilocarpine-induced TLE rat model Administration route: i.p. injections for 5 days 48 hours before pilocarpine administration	In the acute phase of epileptogenesis (first 72 hours after SE): - reduces glutamate levels	[169]

Compound/ strategy	Model/study/administration route	Effects	References
Riluzole, FDA - approved drug for ALS	<i>In vitro</i> studies Bipolar electrode stimulation in the right amygdala of rats to induce kindling Administration route: i.p. injection 30 min before kindling stimulation. Mice model of PTZ-induced convulsions Administration route: i.p. injection 30 min before PTZ insults 4-AP infusion in the hippocampus in rats Administration route: i.p. 30 min before 4-AP perfusion Pilocarpine-induced limbic seizure model and in GBL-induced absence seizure model in rats Administration route: i.p. after pilocarpine-insult, or 2 h before GBL-induced absence seizure Mouse model of TLE Administration route: i.p. injection of Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AG) 35-45 days after KA-induced SE [164] oral gavage of Hsp90 inhibitor HSP990 4-5 weeks KA-induced SE [165] Pilocarpine-induced limbic seizure model in rats Administration route: i.p. injection of specific inhibitor of p38 MAPK, SB203580, 30 min before pilocarpine insults	- reduces GS activity - reduces GLT-1 expression In the chronic phase of epileptogenesis (4 weeks after SE): - increases GLT-1 expression - attenuates impaired learning and memory ability ↑ EAAT2 levels, stimulates glutamate uptake, prevents glutamate release from presynaptic terminals, acts as non-specific ion channel blocker Inhibits kindled seizures and the development of behavioral seizures in kindling acquisition Enhances the anti-seizure actions of valproate, phenobarbital and ethosuximide Showed toxicity when combined with neuronal hyperexcitation Inhibits pre-ictal spikes and spike-wave discharges ↑ GLT-1 levels Prevents GLT-1 degradation Suppresses spontaneous recurrent seizures ↑ GLT-1 levels Reduces time to first epileptic seizure Attenuates seizure severity	[170–177] [178] [179] [180] [181] [182,183] [184]
	Positive Allosteric Modulation of GLT-1/EAAT2 as potential therapeutics		
GT949 and GT951 PAMs of EAAT2	<i>In vitro</i> studies	↑ glutamate uptake through EAAT2 Do not change EAAT2 expression Offer neuroprotection <i>in vitro</i>	[188,189]

Compound/ strategy	Model/study/administration route	Effects	References
Parawixin10 Acylpolyamine PAM of EAAT1 and EAAT2	Seizures induced by intrahippocampal injection of kainic acid, NMDA, and PTZ in rats Administration route: right lateral ventricle injections for 1 week, 1 h or 24 h after NMDA injection [185] Intracerebroventricular injections 10 min before insults [186]	Offers neuroprotection and anticonvulsant properties and prevents onset of seizures	[190–192]]
	Pilocarpine-induced limbic seizure model in rats Administration route: right lateral ventricle injections 60 min after induction of SE, and then daily for four days	Chronic administration of Parawixin10 results in neuroprotection, increased latency of recurrent seizures, and decreased duration and severity of seizures	[193]