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Review

Structural and functional dynamics of Excitatory Amino Acid Transporters (EAAT)

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Abstract: Glutamate transporters control the glutamate homeostasis in the central nervous system, and, thus, are not only crucial for physiological excitatory synaptic signaling, but also for the prevention of a large number of neurodegenerative diseases that are associated with excessive and prolonged presence of the neurotransmitter glutamate in the extracellular space. Until now, five subtypes of high-affinity glutamate transporters (excitatory amino acid transporters, EAATs 1–5) have been identified. These 5 high-affinity glutamate transporter subtypes belong to the solute carrier 1 (SLC1) family of transmembrane proteins: EAAT1/GLAST (SLC1A3), EAAT2/GLT1 (SLC1A2), EAAT3/EAAC1 (SLC1A1), EAAT4 (SLC1A6) and EAAT5 (SLC1A7). EAATs are secondary-active transporters, taking up glutamate into the cell against a substantial concentration gradient. The driving force for concentrative uptake is provided by the co-transport of Na^+ ions and the counter-transport of one K^+ in a step independent of the glutamate translocation step. Due to the electrogenicity of transport, the transmembrane potential can also act as driving force. Glutamate transporters are also able to run in reverse, resulting in glutamate release from cells. Due to these important physiological functions, glutamate transporter expression and, therefore, the transport rate, are tightly regulated. The EAAT protein family are structurally expected to be highly similar, however, these transporters show a functional diversity that ranges from high capacity glutamate uptake systems (EAATs 1–3) to receptor-like glutamate activated anion channels (EAATs 4–5). Here, we provide an update on most recent progress made on EAAT's molecular transport mechanism, structure-function relationships, pharmacology, and will add recent insights into mechanism of rapid membrane trafficking of glutamate transporters.

Keywords: glutamate transporter; excitatory amino acid transporter; EAAT; SLC1A; neurotransmission; rapid kinetics; molecular dynamics simulation; glutamate transporter regulation; intracellular trafficking; glutamate transporter pharmacology

1. Introduction

L-Glutamate is the major excitatory neurotransmitter of the mammalian central nervous system (CNS) and it mediates synaptic signal transmission by activating an array of ionotropic and metabotropic glutamate receptors. Excessive activation of glutamate receptors results in excitotoxic neuronal cell death which is the cause of a large number of neurodegenerative diseases.

Glutamate transporters control the glutamate homeostasis in the CNS, using the free energy stored in the transmembrane concentration gradient of co/counter-transported ionic species for uphill transport of their specific amino acid substrates [1] and, thus, are not only crucial for physiological excitatory synaptic signaling, but also for the prevention of exitotoxicity. Glutamate transporters maintain extracellular glutamate concentrations within physiological levels (reviewed in [2]), and they initiate the recycling cascade (*glutamate-glutamine cycle,* Figure 1B), which eventually restores released glutamate in synaptic vesicles [3,4].

Bulk glutamate uptake in the CNS is facilitated by glial glutamate transporters [2,3,5,6,7,8,9]. Consequently, it is not surprising that the first glutamate transporters cloned in the early nineties were glial transporters; Glutamate Transporter 1 (GLT1) and Glutamate-Aspartate-Transporter (GLAST) from rat brain [10,11] and their human orthologs Excitatory Amino Acid Transporter 1 and 2 (EAAT1 and EAAT2 [12]). It has been suggested that GLT1/EAAT2 accounts for about 90% of total glutamate uptake in the brain, and thus, is considered as the most important glutamate transporter subtype in the CNS in preventing glutamate neurotoxicity [2,13,14].

With the cloning of the neuronal glutamate transporter subtypes Excitatory Amino Acid Carrier 1 (EAAC1; [11]), and Excitatory Amino Acid Transporters 4 and 5 (EAAT4 and EAAT5), a standardized nomenclature was introduced using the acronym EAAT (Excitatory Amino Acid Transporter) [12]. Based on sequence similarities, EAAT1 is the human homologue of GLAST1, EAAT2 of GLT1, and EAAT3 of EAAC1, whereas the acronyms EAAT4 and EAAT5 are conserved. These 5 high-affinity glutamate transporter subtypes belong to the solute carrier 1 (SLC1) family of transmembrane proteins: EAAT1/GLAST (SLC1A3), EAAT2/GLT1 (SLC1A2), EAAT3/EAAC1 (SLC1A1), EAAT4 (SLC1A6) and EAAT5 (SLC1A7).

EAAT1 and EAAT2 are predominantly but not exclusively expressed in glial cells (astrocytes, microglia, Bergmann glia cells, retinal Müller glia cells and oligodendrocytes) [9,15,16,17]. However, during early stages of brain development and in disease [2,18], EAAT2 is also expressed in neurons. A prominent example for neuronal expression of EAAT2 in adult and healthy CNS is the mammalian retina [15,19,20]. EAAT3 is present in the cell bodies and dendrites of most, if not all, neurons in the brain, in both glutamatergic and GABAergic neurons, indicating that EAAT3/EAAC1 is a uniquely neuronal transporter [21,22]. EAAT4 functions not only as high-affinity/low-capacity glutamate transporter but also as glutamate-gated anion channel and is predominantly expressed in cerebellar Purkinje cells [23,24,25,26].

EAAT5, which exhibits a large anion conductance [27], is primarily expressed in bipolar cells and photoreceptors of the retina [27,28]. Like EAAT4, it acts as a slowly-gated glutamate receptor and/or glutamate buffering system [28,29], rather than a classical high-capacity glutamate transporter.

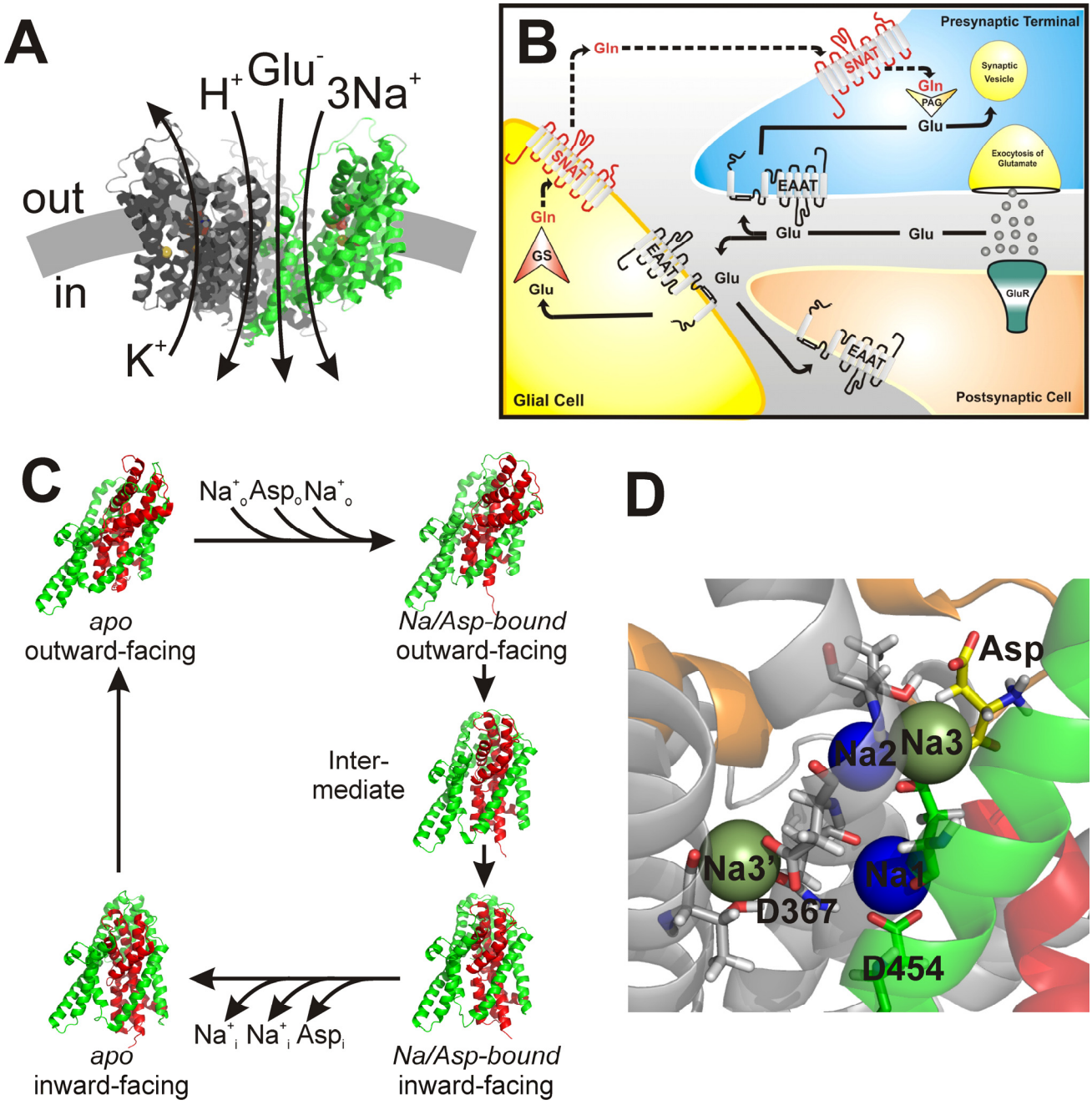

Figure 1. (A) Proposed stoichiometry of the coupling of substrate transport to the co-transport of three sodium ions, one proton, and the counter-transport of one K^+. (B) Glutamate release, activation of glutamate receptors, uptake, and recycling through the glutamate-glutamine cycle at the glutamatergic synapse. Glutamate (circles) binding to glutamate receptors (GluR) mediates the signal to postsynaptic neurons. Rapid removal of glutamate from the synaptic cleft is achieved by glutamate transporters (EAATs), which are located in glial cells and neurons. Once glutamate is transported into the cells (solid arrows), it can be sequestered in the presynaptic neuron in synaptic vesicles, ready for a new cycle of neurotransmission (solid arrow in the presynapse) or, in glial cells, it is degraded by the glia-specific enzyme glutamine-synthetase (GS) to glutamine (Gln), which is released into the

extracellular space, and subsequently taken up by sodium-coupled neutral amino acid transporters (SNAT) in glutamatergic neurons for a new cycle of transmitter synthesis (broken arrows; PAG: phosphate activated glutaminase). (C) Molecular mechanism of glutamate transport based on structural data. Known crystal structures of several states in the transport cycle, including an intermediate on the translocation pathway, are shown (description of state is given below each figure). The transport domain is highlighted in red, the trimerization domain in green. (D) Structural evidence predicts two Na^+ binding sites in mammalian EAATs, Na1 and Na2 (blue). Computational analysis of Na^+ binding by MD simulations, as well as mutational studies, provided evidence for two other potential Na^+ binding sites, termed Na3 and Na3'. The substrate, Asp, is shown in yellow in stick representation. RL2 is shown in orange and RL1 in red.

Considering the almost ubiquitous expression of glutamate transporters in the CNS as well as in other organs such as the intestine [30], the kidney [31], it is not unexpected that EAAT dysfunction has been implicated in a wide range of diseases, such as Alzheimer's disease (AD) [32,33], amyotrophic lateral sclerosis (ALS) [34], depression [35], epilepsy [13,36], Huntington's disease [37], ischemia [38,39,40], stroke [41], multiple sclerosis [42], schizophrenia [43,44] and Tourette syndrome [45].

Although the members of the EAAT protein family are structurally expected to be highly related, these transporters show a functional diversity that ranges from high capacity glutamate uptake systems (EAAT1–3) to receptor like glutamate activated anion channels (EAAT4–5) [18].

Here, we provide an update on the most recent progress made on EAATs molecular transport mechanism, structure-function relationship, pharmacology, and we will add recent insights into mechanism of rapid membrane trafficking of glutamate transporters.

2. Molecular transport mechanism

Information on the molecular transport mechanism has been obtained not only through functional studies, but also through the determination of the structures of the archeal aspartate transporter from *Pyrococcus horikoshii*, GltPh [46,47,48]. GltPh was crystallized in the outward-facing conformation, as well as in a cross-linked state that was interpreted as the inward-facing configuration. In this section, we will focus on evidence from functional studies.

The alternating access hypothesis [49] has been applied to secondary-active transporters in general, and in particular to glutamate transporters. The alternating access mechanism is based on the assumption that the transporter cycles through at least two discrete conformational states, one of them allowing access of substrate to its binding site from the extracellular side (outward-facing), the other one allowing access from the cytoplasm [49] (inward-facing). For Na^+-dependent glutamate transporters, alternating access is controlled by the coupling of substrate transport to that of Na^+ ions. Na^+ regulates external/internal accessibility of the substrate binding site based on the Na^+ concentration gradient across the membrane. Due to the co/counter-transport of several ionic species, the transport cycle consists of multiple individual reaction steps, many of which have been isolated and characterized experimentally. A hypothesized transport mechanism is illustrated in Figure 1C.

A critical parameter for any Na^+-driven transporter is the substrate/Na^+ coupling stoichiometry.

Using reversal-potential measurements, the transporter subtypes EAAT1–3 were shown to co-transport one glutamate anion with three Na^+ ions and one proton, in exchange for one K^+ ion, which is counter-transported in the outward direction [1,50] (Figure 1A). This coupling stoichiometry is consistent with the Hill coefficients of > 2 determined from glutamate dose response curves. Therefore, a total of two positive charges enter the cell for each transporter glutamate molecule, suggesting that transport is electrogenic (associated with charge transport). This expectation is consistent with experimental results, showing glutamate-induced inward currents in *Xenopus* oocytes as well as transporter-expressing mammalian cells [51].

Much information has been obtained about glutamate transporter function under steady-state conditions. However, defining individual steps in the transport cycle is not straightforward at steady-state, but should be rather done under pre-steady-state conditions. Here, a pre-existing steady state is perturbed by changing the glutamate concentration (or that of one of the co- or counter-transported cations), or the transmembrane potential. With both approaches, the system can be perturbed rapidly, on the sub-millisecond time scale.

Glutamate concentration jumps were applied by rapid solution exchange [52,53,54,55], as well as sub-millisecond photolysis of caged glutamate [56,57]. Both approaches have yielded valuable insight into transporter function, in particular regarding the mechanism of the glutamate-dependent half-cycle. For example, it was found that partial reactions associated with glutamate translocation are associated with charge movement [57,58], indicating that translocation itself, or cation binding reactions linked to it, are electrogenic (see below). In addition, these experiments showed that glutamate translocation is rapid, occurring on a millisecond time scale, and is not rate limiting for steady-state turnover of the transporter [52,53,56]. Finally, glutamate binding was found to be rapid, close to the diffusion-controlled limit [52,54,56], in agreement with the hypothesis of buffering of glutamate by transporters after synaptic release on a millisecond time scale [59].

Further analysis of currents following rapid glutamate concentration jumps demonstrates the existence of a two-exponential decay [53,57,60], suggesting that the translocation process consists of at least two separable steps [60]. These results are consistent with the existence of intermediate/s along the translocation pathway (Figure 1C) [60]. This intermediate could potentially be an occluded state. The significant temperature dependence (activation energies of 100–120 kJ/mol) of these two relaxation processes would be in agreement with conformational changes as the underlying factor [60]. Important information was also obtained by rapidly removing extracellular glutamate from the transporter when locking the transporter in the Na^+/glutamate-dependent half-cycle [53]. Here, voltage dependence of the decay rate was opposite to that found upon application of glutamate, suggesting that the current decay was rate-limited by electrogenic outward transitions through the glutamate translocation step(s) [53].

When voltage jumps were applied to the glutamate-free form of the transporter, Na^+-dependent transient currents were observed in response, relaxing within the millisecond time scale [57,61]. These currents are most likely caused by Na^+ binding to the glutamate-free transporter, or conformational changes associated with this binding, as has been demonstrated for other Na^+-coupled, secondary-active transporters, such as glucose and phosphate transporters [62,63]. Voltage jumps were also applied in the sole presence of K^+ on both the intracellular and extracellular side of the membrane. Under these K^+-exchange conditions, transient currents were observed, indicating that K^+ binding and/or translocation is/are electrogenic [64], however, the currents did not allow conclusions on the sign of the charged species that moves within the transmembrane electric field.

To clarify the direction of charge transport under K^+-exchange conditions, K^+ concentration jumps were employed in the presence of constant internal K^+. Under such single turnover conditions, transient outward current was observed, indicating that the charged species that relocates within the membrane has a negative charge. These results suggest that negatively-charged cation binding sites create the charge movement, rather than the movement of the externally-applied positively charged cation (K^+). This points to the existence of a charge compensation mechanism, in which negative charge of the binding sites overcompensates for the positive charge of the single, bound K^+, but only partially compensates for the positive charges of the three co-transported Na^+ ions [64]. The experimental results are consistent with computational analysis using Poisson-Boltzmann electrostatic calculations based on structures of the inward- and outward-facing configurations of EAAT3 homology models. These calculations predict negative valence of the outward-inward-facing transition in the presence of bound K^+.

The hypothesis of a charge compensation mechanism described in the previous paragraph is consistent with the valence of the charge movement of the fully-loaded, Na^+/glutamate bound transporter obtained from pre-steady-state kinetic analysis in the glutamate exchange mode. Inward charge movement was observed in response to rapid glutamate concentration jumps, with an apparent valence of about +0.45. This valence is much lower than the valence of +3 that would be expected if 3 positive charges (3 Na^+, 1 glutamate$^-$, and 1 H^+) were translocated through the full width of the membrane. The cause of this reduced valence, which reduces the Born-barrier for charge translocation across the membrane, is twofold: 1) Charge compensation and 2) distribution of electrogenic reactions over several steps, thus reducing the width of the transmembrane electric field that has to be traversed by the positive charges.

Taken together, the functional studies point to a sequential, multistep transport mechanism, for which structural evidence is also mounting (Figure 1C). In this mechanism, substrate/cation binding/release steps are coordinated with conformational changes to allow for alternating access of the transporter binding sites for ions/substrate. Voltage dependence of transport is distributed over many different binding/translocation steps [58,64], including Na^+ dissociation on the intracellular side [65], resulting in a relatively shallow voltage dependence of steady state glutamate transport, considering the large number of positive charges co-transported with glutamate (3 Na^+, 1 H^+). If these charges were transported across the membrane in a single step, transport would be strongly inhibited upon depolarization.

3. Anion conductance

Glutamate transporters facilitate anion flux across the membrane [66,67], in addition to charge transport due to electrogenic glutamate movement across the membrane. Transmembrane anion current caused by this flux is kinetically, but not stoichiometrically coupled to glutamate transport [54,56,68]. The anion conductance is a feature of all EAAT subtypes, although its contribution to the total current varies, with EAATs 4 and 5 displaying the largest relative anion conductance [27,69]. The anion conductance displays selectivity for hydrophobic anions [66,68]. While distinct single-channel currents have not been observed in glutamate transporters, noise analysis provided an estimate of unitary conductance, which is in the range of fS-pS [52,70,71], which is low compared to that found for traditional ion channels.

The molecular mechanism of the anion current (permeation and gating) is as yet poorly

understood. It may be mediated by an anion channel [70,72] or by anion uniport. Anions are conducted at specific step(s) in the transport cycle (Figure 2B, a potential anion-conducting state is indicated by the red box [52,56,57,73]). Therefore, the anion current carried by this conductance is a convenient tool to dissect this/these specific transporter reaction step(s), because in the presence of highly permeant anions, such as SCN^-, the anion current is much larger than the transport current [55,67].

Currents measured in response to glutamate concentration jumps showed that transient transport current precedes anion currents [53,57], suggesting that Na^+/glutamate-induced gating occurs before the anion channel is activated. Consistent with this observation, large anion currents were detected in the Na^+/glutamate exchange mode, suggesting that anions are mainly conducted when the transporter resides in Na^+- and/or glutamate-bound states [57,74]. These states are the Na^+-bound empty transporter (in the absence of glutamate), which mediates the leak anion conductance, as well as the fully Na^+/glutamate loaded state (Figure 2B). In a mutant transporter (D439N, EAAC1 numbering), the transported acidic amino acid actually inhibits the anion conductance, as is also found for competitive inhibitors, such as DL-*threo*-β-benzyloxyaspartic acid (TBOA) [74]. However, in contrast to TBOA, which presumably prevents Na^+ binding to the TBOA-bound transporter (most likely to the Na2 binding site, [75]), this site can still be occupied in the mutant transporter, although Na^+ binding is very slow. This delayed Na^+ binding step following glutamate binding results in the activation of the anion conductance, leading to the hypothesis that the anion conductance is Na^+-gated, but not glutamate gated [57].

In a recent report, an intermediate between the outward-facing and inward-facing conformations was identified in an asymmetric trimer of GltPh. This intermediate conformation was proposed to represent the anion conducting state [47], because water permeates into the transporter at the interface between the trimerization domain and the transport domain. Water permeation could be the basis for the formation of an aqueous pathway that allows movement of anion across the membrane domain of the transporter. This interpretation is consistent with previous functional analysis, in which the anion conducting states were linked to steps in the glutamate translocation pathway (Figure 2B, [52,57,60]).

The requirement for bound Na^+ for anion permeation is consistent with the hypothesis of an intermediate translocation state, because this state is expected to be Na^+ bound and, most likely, in a Na^+-occluded form, in which Na^+ cannot dissociate until inward- or outward-facing states are occupied. The required presence of Na^+ is also consistent with the large negative electrostatic potential in the transport domain, as evidenced by the negative valence of the Na^+-free transporter. Thus, binding of Na^+ ions has to neutralize some of the negative charge to prevent repulsion of the negatively-charged anion, explaining why the substrate-free, K^+-bound transporter is unable to conduct anions.

Structure function analysis has been carried out to determine the structural basis for the anion conductance. Vandenberg and colleagues showed that specific mutations in TMD2 of EAAT1 result in alteration of anion conductance properties, without changing the kinetics of glutamate transport. Thus, it was speculated that TMD2 may contribute to the pathway for anion flux across the membrane. Further evidence was obtained recently using a mutagenesis approach at the transport domain interface. Here, Cater et al. [76] could show that mutations to the trimerization-domain/transport-domain interface have substantial effects on anion permeation, while little effect on transporter turnover was observed. Thus, it is possible that this interface is part of the permeation pathway, the formation of which would require transition through an intermediate state along the translocation energy hyper-surface. A hypothetical mechanism is shown in Figure 2B. Other studies have shown that it is possible to inhibit glutamate transport without altering the properties of the anion conductance. Thus,

it is likely that, while kinetically the two transport modes may be linked, they occur through separate pathways.

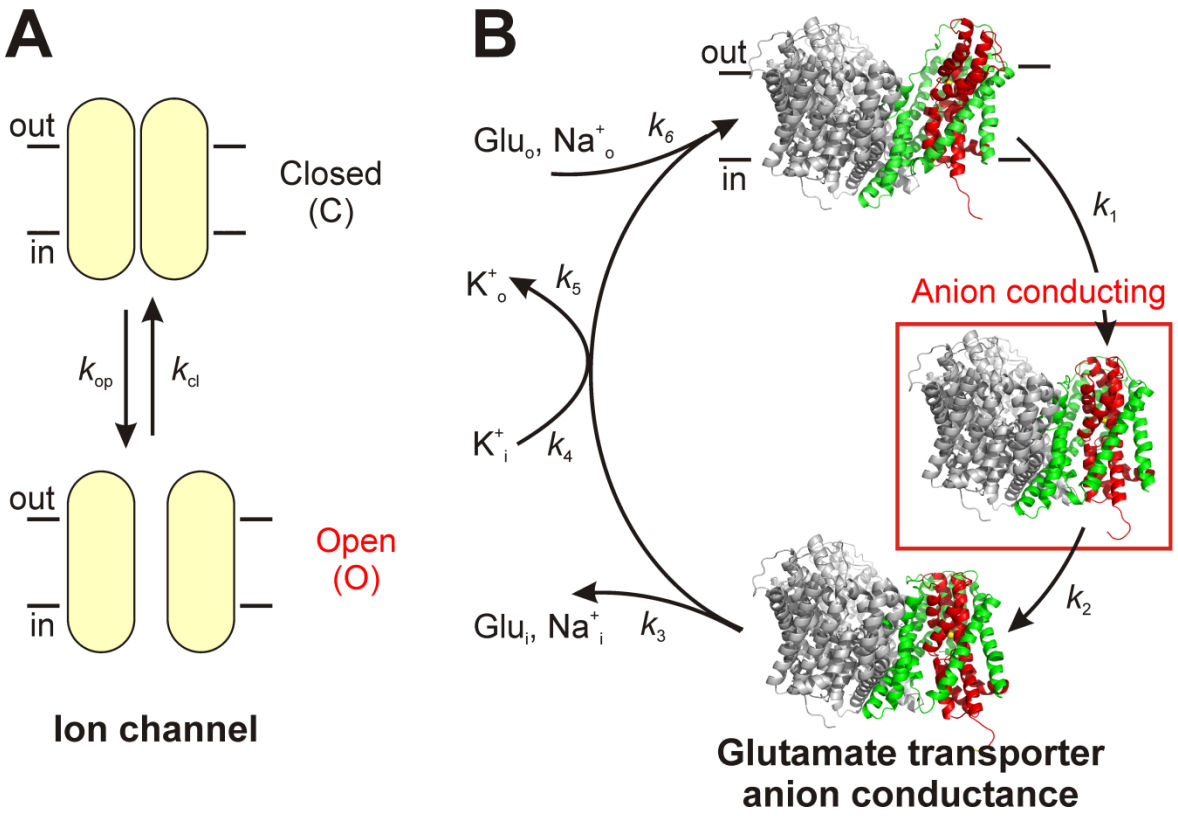

Figure 2. Mechanism of anion conductance activation. (A) Channel opening equilibrium of a traditional ion channel, in which open and closed states are in dynamic equilibrium, determining the channel open probability. (B) The anion conducting state of glutamate transporters (red box) is hypothesized to be an intermediate on the translocation pathway, in-between inward- and outward-facing states. Channel open probability is determined by the relative rate constants, k_i, that prescribe the population of states as the transporter cycles at steady state, far from equilibrium.

The current data suggest a model, in which the anion conductance is activated transiently each time the transporter moves through a full transport cycle (Figure 2B). Activation occurs when intermediate anion conducting state(s) is/are populated, which are linked to Na^+ and Na^+/glutamate-bound conformations. Thus, channel open probability is controlled by the population of the anion conducting state(s) under conditions of steady-state cycling. This mechanism differentiates the transporter anion conductance from traditional ion channels, in which open probability is determined by the equilibrium between open and closed states (Figure 2A). Due to these complex properties of the anion conductance, additional research will be necessary to fully understand the molecular mechanism of this interesting property of glutamate transporters, as well as of other secondary-active transporters that exhibit anion conducting behavior.

4. Insight from molecular dynamics simulations

Membrane transporters present ideal model systems for molecular dynamics (MD) studies, because no covalent bonds are formed/broken during substrate transport. Thus, MD simulations have been performed with a variety of transport systems, including the archeal glutamate transporter GltPh, yielding important insight into the mechanism of substrate and ion binding/dissociation events, as well as the conformational dynamics of the transport process.

Initially, simulations focused on the dynamics of the outward-facing configuration. Predictions from structural analysis as to the function of re-entrant loop 2 (RL2) as the extracellular gate were confirmed by MD, showing rapid formation of an open-loop form upon removal of the substrate from its binding site. These simulations also confirmed the intimate link between substrate binding and Na^+ binding to the Na2 sodium binding site, which does not exist in the open-loop structure [77,78].

Simulations were also performed after adding a third Na^+ ion to the GltPh structure, in order to identify the location of the third Na^+ binding site (termed Na3 site) on the transporter. A sodium ion was stable when placed in a site formed by oxygens in the side chains of D312, N310 and T92 (Figure 1D). This binding site is probably occupied first when Na^+ binds to the *apo*-form of the transporter, but is only accessible through transient occupation of the Na1 site [79]. Binding of Na^+ to this site has been confirmed in MD simulations from other groups as well, although a slightly different structural configuration of the Na3 site was found [80], and is also consistent with results from mutagenesis studies, in which amino acid residues analogous to D312 and T92 in a mammalian glutamate transporter were investigated. It should be noted that there is no evidence for the Na3 site from structural studies. However, experimental determination of the coupling stoichiometry indicated that aspartate transport is driven by co-transport of three sodium ions, similar to the mammalian glutamate transporters [81].

An alternative proposal places the location of the third Na^+ binding site in the vicinity of the bound aspartate molecule (Na3' site, Figure 1D). MD simulations showed that a Na^+ ion is also stable in this site, which is formed by T314 and N401 in GltPh, as well as oxygens from the α and β-carboxylate of the substrate [82]. This proposal is attractive, because it could explain the tight coupling between substrate and Na^+ transport: Aspartate and Na^+ need one another for binding and, thus, act in a cooperative manner. Functional analysis of mutant transporters supports the importance of the analog of T314 in a mammalian glutamate transporter for Na^+-driven transport and binding [82]. Final proof of the location of the third sodium binding site, however, will have to come from structural analysis.

MD simulations were also performed on the basis of the structure of the inward-facing conformation. Structural models were used to predict that RL1 acts as the intracellular gate that must open to allow substrate release to the cytoplasm. Initial MD simulations on a 50 ns time scale were consistent with this prediction, although movement of RL1 was slower and of less magnitude than that of the external gate in the outward-facing conformation, RL2 [83]. Subsequent computations using a microsecond time scale, however, demonstrated that motions of RL2 are even more important for substrate release, indicating that RL2 serves as both external and internal gate. Here, motion of the RL2 tip away from the transport core destabilizes the substrate, which leads to further motions of RL1 and substrate release [84,85]. These conclusions were confirmed by another group, who also observed motions of both RL1 and RL2 involved in intracellular gating [84,85]. Overall, a picture emerges, in which extracellular and intracellular gating are quite distinct in kinetics and mechanism, consistent

with the asymmetric kinetic properties found experimentally for forward and reverse glutamate transport [65].

Finally, we discuss results from simulations that were designed to model the substrate translocation reaction pathway. Since the transitions associated with translocation are expected to be slow, simplified coarse graining models have been used based on elastic or anisotropic normal mode (NM) analysis [86,87,88]. Generally, these models reproduce the alternating-access model predicted from the structures. In addition, intermediates on the translocation pathway are predicted that include several non-native contacts between the transport domain and the trimerization domain [88]. Those predictions of NM analysis may be useful for future experimental validation.

In an all-atom approach, a combination of motion planning, to predict intermediates along the translocation pathway, and relaxation of the predicted intermediates with MD was used to obtain information of structural transitions during translocation. During translocation, water penetrates the interface between transport and trimerization domain, generating accessibilities for amino acid residues that are in agreement with experimental observations [89].

The mechanism of proton co-transport is not fully understood. Structure-function studies indicate that protonation of the transporter occurs at the acidic glutamate side chain in position 373 (EAAC1 numbering) [90]. This conclusion is consistent with results from MD simulations of an EAAT3 homology model built on the basis of the GltPh structure. In agreement with the experimentally-determined binding sequence, protonation of E373 is essential for substrate interaction in the absence of protonation the substrate becomes unstable in its binding site [91]. Deprotonation of E373 is expected to be essential for K^+ interaction, which provides a positive charge that would keep the total charge balance preserved between the mammalian transporters and GltPh, which does not exchange H^+ for K^+. Consistently, E373 is replaced by a glutamine residue in GltPh. Thus, it is likely that the H^+ and K^+ co-counter-transport functions are intimately linked in the mammalian transporters and have co-evolved.

The results from MD simulations described above continue to provide important insight into glutamate transporter function and provide evidence for an “elevator-like” mechanism of transport, in which the transport domain moves up and down through the trimerization domain in an elevator-like fashion, allowing access to the binding sites either from the intracellular or extracellular sides of the membrane.

5. Intracellular trafficking of excitatory amino acid transporters

Glutamate transporters are fast in removing their substrate from the synaptic cleft [53,92]. However, the impact of glutamate transporters on synaptic transmission generally does not depend on rapid substrate turnover rates, but on their high density expression at synapses (for review see [93]).

In fact, glutamate transporters are expressed at high densities of 15.000–20.000/μm² in the vicinity of glutamatergic synapses [2]. Thus, the large number of transporters present at the synapse may significantly and rapidly “buffer” glutamate that binds to and dissociates from the transporter binding sites multiple times before it is either translocated across the membrane or diffuses out of the synaptic cleft. Due to the rapid buffering of glutamate, excitatory amino acid transporters may shape the temporal profile of the synaptic glutamate concentration, keep the signal local, prevent spillover, such that low-affinity AMPA (2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid) receptors experience only a very short burst of high glutamate concentration, and high-affinity NMDA

(*N*-methyl-D-aspartic acid) receptors are then activated by a prolonged low glutamate concentration within the synaptic cleft. In other words, the density of glutamate transporters in synaptic membranes and its regulation plays a crucial role in regulating synaptic transmission in the CNS.

Glutamate transporter plasma membrane expression appears to be as dynamic [94] as that of AMPA and NMDA receptors [95,96]. A typical example for regulated plasma membrane expression among the different glutamate transporter subtypes is EAAT3/EAAC1 and this process also called *membrane trafficking* has been extensively reviewed in [94,97,98,99]. As summarized in Figure 3, cell surface trafficking of glutamate transporters is generally independent of protein synthesis, and is not only activated by kinases/phosphatases and scaffolding proteins, but also regulated by a variety of other factors including e.g. endothelin B, vasoactive intestinal peptide, pituitary adenylate cyclase-activating peptide, platelet-derived growth factor, thyroid hormone and trophic factors that appear to affect trafficking to and from the plasma membrane to intracellular pools involving lipid rafts and caveolae; see for review [100].

Poitry-Yamate et al. [101] showed that EAAT2/GLT1 and EAAT1/GLAST are clustered on astrocytic processes and that their distribution is regulated in the time scale of minutes by neuronal activity. EAAT2/GLT1 and EAAT1/GLAST function was activated by neurons and this effect was mimicked by pre-incubation of astrocytes with micromolar concentrations of glutamate or by the synaptic release of glutamate from neurons. Thus, increase in transport activity is dependent on neuronal release of glutamate, is associated with the local redistribution (clustering) of EAAT2/GLT1 and EAAT1/GLAST, but is independent of transporter synthesis and of glutamate receptor activation. In line with this observation, blockage of neuronal activity reduces the density and alters the perisynaptic localization of EAAT2/GLT1 clusters *in situ* (organotypic hippocampal slice cultures). Vice versa, enhanced neuronal activity increases the size of EAAT2/GLT1 clusters and their proximity to synapses [102]. The mechanisms underlying these effects may be dependent on EAAT2/GLT1 ubiquitination [103,104]. As shown in Figure 3A, EAAT2/GLT1 is constitutively endocytosed into the recycling endosome via a clathrin-dependent pathway, a process that is dependent on the ubiquitination of lysines located in the cytoplasmic C-terminus (lysines 517 and 526) of the transporter [105], suggesting the involvement of cytoplasmic C-terminal domains in the trafficking process of different glutamate transporter subtypes are about to appear (EAAT2/GLT1 alternative splicing: see [106] and [18]). The return translocation of EAAT2/GLT1 (Figure 3A) from the recycling endosome to the plasma membrane was blocked by specifically inhibiting the de-ubiquitinating enzyme (DUB) ubiquitin C-terminal hydrolase-L1 (UCH-L1), supporting the existence of an ubiquitination/de-ubiquitination cycle that ensures the correct concentration of EAAT2/GLT1 at the cell surface [105]. Garcia-Tardon et al. identified the ubiquitin ligase Nedd4-2 as a mediator of the EAAT2/GLT1 endocytosis that is triggered by protein kinase C (PKC) activation [107]. PKC promotes the phosphorylation of Nedd4-2, its association with EAAT2/GLT1, and the subsequent ubiquitination of the transporter that precedes its endocytosis (Figure 3A).

Similar to EAAT2/GLT1 and EAAT3/EAAC1, the trafficking process of EAAT1/GLAST depends apparently on specific cytoplasmic C-terminal domains. In the case of EAAT3/EAAC1 the cell-surface density of EAAT3 is regulated by interaction with post-synaptic density-95/Discs large/Zonula occludens (PDZ) proteins [108] and a tyrosine-based internalization signal [109] in the C-terminus of EAAC1. EAAT1/GLAST possesses a similar, well-conserved internalization motif in the C-terminal region and the PDZ (PSD-95/Drosophila discs-large protein/zonula occludens protein) domain-containing proteins Na^+/H^+ exchanger regulatory factor 1 (NHERF1) and NHERF2 interact

with the EAAT1/GLAST. Sato et al. suggest that NHERF1 interacts with EAAT1/GLAST during endoplasmic reticulum (ER) export, while NHERF2 interacts with EAAT1/GLAST in the secretory pathway from the ER–Golgi intermediated compartment (ERGIC) to the plasma membrane (Figure 3A), thereby modulating the cell surface expression of EAAT1/GLAST [110]. Thus, the organelle-specific interactions of EAAT1/GLAST with NHERF1 or NHERF2 play significant roles in regulating the intracellular trafficking of EAAT1/GLAST and modulate the cell surface expression level of EAAT1/GLAST by accelerating its ER–Golgi trafficking.

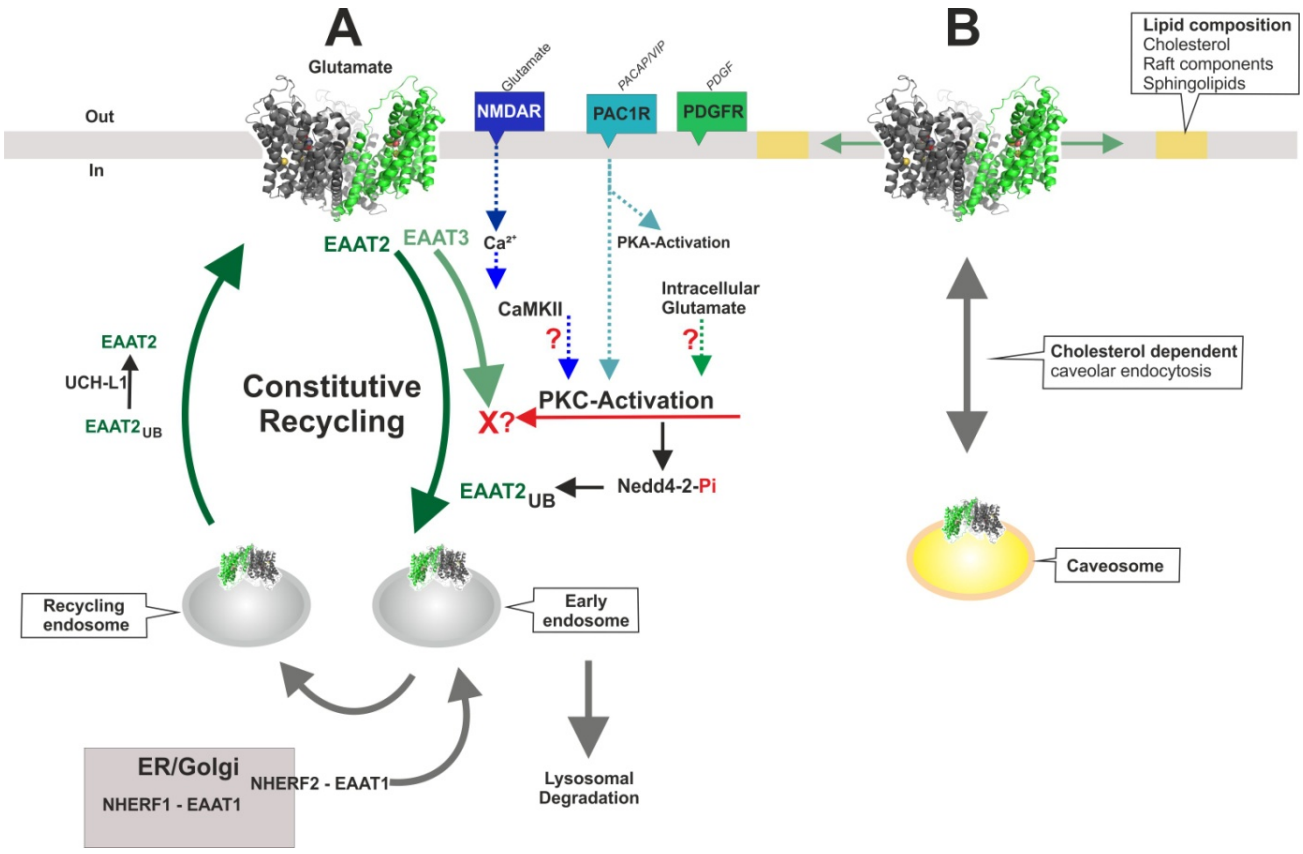

Figure 3. Regulated membrane trafficking of EAATs. Glutamate transporters traffic either via clathrin-dependent pathway (A) and/or via cholesterol-caveolin-rich microdomains (B) constitutively from the plasma membrane to intracellular pools and are recycled back to the cell surface. This process is regulated by a variety of factors including the activation of NMDA-receptors (NMDAR), PACAP-receptors (PAC1R) and/or PDGF-receptors (PDGFR), however, until now most of the interacting partners are unknown. Protein kinase activation appears to be central in regulating the endocytosis of EAATs. In the case of EAAT3, protein kinase C (PKC) activation blocks endocytosis of EAAT3 and as a result the unaffected exocytosis of EAAT3 causes its accumulation in the plasma membrane. However, the underlying molecular mechanism is still unsolved. In contrast to EAAT3, PKC-activation supports endocytosis of EAAT2. Here the ubiquitin ligase Nedd4-2 was identified as mediator of EAAT2 endocytosis that is triggered by PKC activation. PKC promotes the

phosphorylation of Nedd4-2, its association with EAAT2 and the subsequent ubiquitination of the transporter that precedes endocytosis. The return translocation of EAAT2 from the recycling endosome to the cell surface is induced by the action of the de-ubiquitinating enzyme UCH-L1. NHERF1 interacts with EAAT1 during ER export while NHERF2 interacts with EAAT1 in the secretory pathway from ER-Golgi intermediated compartment to the plasma membrane thereby regulating the intracellular trafficking of EAAT1 and modulating the cell surface expression level of EAAT1 by accelerating its ER-Golgi trafficking. Besides endocytosis and exocytosis EAATs traffic through surface lateral diffusion in the plasma membrane. Lipid rafts, enriched in cholesterol and glycosphingolipids (B, highlighted in yellow), appear to support glutamate transporter function. Caveolae, a special type of lipid raft, appear to be the locus of a clathrin-independent raft-dependent endocytosis of EAAT3. Caveolin facilitates both the constitutive delivery and internalization of EAAT3.

Generally, endocytosis and exocytosis of glutamate transporters have initially been thought to account alone for the cell surface trafficking process. However, membrane proteins also traffic through surface lateral diffusion in the plasma membrane (Figure 3B). Proteoliposome reconstitution experiments have shown that cholesterol is required for Na^+-dependent [^{3}H]-glutamate uptake [111] and Na^+-dependent [^{3}H]-glutamate uptake is significantly reduced by long term treatment with a cholesterol-depleting agent [112], suggesting plasma membrane organization in or outside of lipid rafts may be another means of regulating glutamate transporter function.

Lipid rafts are lipid-protein microdomains of the plasma membrane that are enriched in cholesterol and glycosphingolipids [113]. These lipid raft microdomains have been implicated in regulating the trafficking and clustering of membrane-associated proteins as well as their intracellular signaling molecules. Lipid rafts are present on the surfaces of neurons as well as glial cells and Butchbach et al. suggest that membrane cholesterol is important for the function of glutamate transporters (Figure 3B, [114]), whereas EAAT1/GLAST, EAAT3/EAAC1 and EAAT4 are less strongly associated with lipid raft-containing microdomains than EAAT2. EAAT2/GLT1 appears to be located in non-caveolar lipid rafts [115]. Gonzales et al., however, conclude that caveolin facilitates both the constitutive delivery and internalization of EAAT3/EAAC1 and that caveolin interacts with EAAT3/EAAC1, suggesting that this interaction may participate in the regulation of EAAT3/EAAC1 trafficking by caveolin (Figure 3B) [116].

It has been reported that the organization of lipid rafts is disrupted in Alzheimer's disease brains because Cholesterol-24S-Hydroxylase (CYP46), a key enzyme in the maintenance of cholesterol homeostasis in the brain, is markedly increased in astrocytes, but decreased in neurons [117]. Tian et al. demonstrated that increased expression of CYP46 in primary astrocytes results in a reduction of membrane cholesterol levels and leads to the dissociation of EAAT2 from lipid rafts and the loss of EAAT2 including glutamate uptake function [118] suggesting a glutamate transporter cholesterol interaction that promotes forward glutamate transport in the presence of cholesterol (Figure 3B).

6. Pharmacology of glutamate transporters

One of the pathophysiological conditions, in which glutamate transporters are important, is stroke.

Here the transporters are functioning in the reverse mode, releasing glutamate into the extracellular space instead of taking it up. The availability of inhibitors that can block this mode of transport would be useful therapeutically, because glutamate release that subsequently leads to damage of neurons may be prevented, or at least delayed, potentially leading to a more favorable outcome of the condition.

However, most of the known inhibitors prevent uptake of glutamate. Therefore, the use of inhibitors has mainly been directed at understanding EAATs biophysical properties, cellular distribution and physiological roles, instead of directly treating disease. In addition to inhibitors, developing compounds that can up-regulate the expression of transporters should be beneficial, since the reduction of excessive extracellular levels of glutamate is expected to be neuroprotective.

Based on their mode of action, glutamate transporter inhibitors that interact with the substrate binding site can be grouped into two categories. The first category includes transportable substrate inhibitors (Figure 4A). These substrate-like inhibitors compete with glutamate for transport and, depending on their concentration, can prevent substrate transport to varying degrees. Secondly, non-transportable, competitive blockers were developed, which competitively bind to the transporter, thus preventing the endogenous substrate from binding and being transported (Figure 4B). Some compounds display differential effects based on the transporter subtype used, resulting in subtype specificity. An example is the pyrrolidinedicarboxylic acid (PDC) group of compounds (Figure 4B). They are conformationally restricted substrate analogues that have a pyrrolidine ring with two carboxylic acid groups. The positions of the carboxylic acid groups yield different molecules like L-trans-2, 4-PDC, which acts as a substrate for EAAT1-4, but is a blocker for EAAT5 [69,119,120,121,122]. In addition, 2-S, 4R-4-methyl glutamate acts as a transported substrate for EAAT1/GLAST, but shows non-transportable blocker activity for EAAT2/GLT1 [123]. Structural differences between EAAT1 and EAAT2, that is a glycine for serine substitution in the RL2 domain, which is a component of the substrate recognition site, have been implicated in the differential effects of 2S,4R-4 methyl glutamate for both subtypes [124]. Finally, *threo*-3-methyl glutamate is another example for subtype-selective effects [123,125]

The physiologically-relevant glutamate transporter substrates are L-glutamate (Figure 4A) and the L and D-forms of aspartate [126,127], which are thought to be transported in their negatively-charged protonation state. Other studies have shown that cysteine can also be transported, in both the deprotonated or protonated form [128].

7. Substrate inhibitors

Initial studies were aimed at molecules with structures mimicking those of the physiological substrates, glutamate and aspartate. Substitutions at the amino acid side chain and different modifications of the side-chain carboxylate yielded a variety of novel compounds. Compounds of this category include L-glutamate-ω-hydroxamate (Figure 4A, [129]), cysteic acid, cysteine sulfinic acid [130], serine-o-sulfate [131] and threo-β-hydroxy-aspartic acid (Figure 4A, [132]). Transporter subtype selectivity was seen for L-Cysteine, displaying high affinity for EAAT3/EAAC1 [128], and L-Serine-O-sulfate, favoring EAAT1/GLAST and EAAT3/EAAC1 with about 10-fold higher affinity compared to EAAT2/GLT1 [120,133,134]. Unlike many glutamate transporter substrates, which also act at glutamate receptors, some potent and selective substrate-like compounds show negligible affinity for glutamate receptors. Their weak affinity for glutamate receptors was attributed to a favorable spatial arrangement of the longer alkyl chain [135].

Because substrate inhibitors are transported into the cell, intracellular glutamate may subsequently be released in a hetero-exchange mechanism. This is a disadvantage for physiological applications and has led to on the development of non-transportable blockers [136], as discussed in the next paragraph.

8. Non-substrate inhibitors (transport blockers)

Kainate, a pyrrolidinedicarboxylate that shows selectivity for EAAT2/GLT1, was one of the first non-transportable glutamate transporter inhibitors (blockers) identified (Figure 4B). However, kainate is also a substrate for glutamate receptors. In contrast, the kainate derivative, dihydrokainate interacts with ionotropic and metabotropic glutamate receptors to a lesser extent [120], although interaction with ionotropic receptors is still not negligible [137].

To obtain blockers with a higher specificity for glutamate transporters, Shimamoto and colleagues modified DL-*threo*-β-hydroxyaspartate (THA), a transportable substrate, by adding a bulky substituent to the hydroxy group to generate DL-*threo*-β-Benzyloxyaspartate (TBOA, Figure 4B) [138]. TBOA is a potent competitive blocker that inhibits all EAATs with varying affinity from the low to mid micromolar range [139,140,141]. TBOA did not show significant effects at both the inotropic and metabotropic glutamate receptors.

The structure of TBOA provided important insight into the transport blocking mechanism, with its bulky, hydrophobic substituent being implicated in non-polar interaction with a hydrophobic part of the binding pocket [142]. The binding mode of TBOA has been clarified through X-ray crystallography, which demonstrated that the bulky benzyl group interacts with the open-loop configuration of RL2, preventing this loop from closing (Figure 4C). In the absence of loop closing, the outward-to-inward-facing transition (Figure 1C) cannot take place, and, thus, the compound is not transportable.

The Shimamoto group subsequently characterized a series of TBOA analogs, and from these (2*s*,3*s*)-3-{3-[4-(trifluoromethyl)benzoylamino]benzyloxy}aspartate (TFB-TBOA, Figure 4B) proved most potent with IC50 values below 300 nM in EAATs1–3. All of the analogs tested blocked transport-induced currents in all EAAT subtypes, as well as the leak currents of EAAT5 [143]. Because of its high affinity, TFB-TBOA is very suitable for ligand binding assays [144].

Another new derivative of TBOA has been synthesized recently, [(2S,3S)-3-(3-(6-(6-(2-(2-(2-(2-(2-aminoethoxy)ethoxy)-ethoxy)ethoxy) acetamido)hexanamido)-hexanamido)-5-(4-(trifluoromethyl)benzamido)benzyloxy) aspartic acid] (LL-TBOA). This compound is a non-transportable competitive inhibitor of the EAATs and is thought to preferentially block the reverse transport mode. LL-TBOA inhibited glutamate uptake with IC50 values in the low micromolar range (less than 10 μM) in EAATs 1–3. The compound showed cardioprotective effects on cardiomyocytes in rat hearts, when perfused before global ischemia [145].

Efforts directed at attaining novel inhibitors for EAAT2 yielded 3-amino-tricyclo [2.2.1.0(2.6)] heptane-1, 3-dicarboxylic acid (WAY-855, Figure 4B), a conformationally restricted analogue of glutamate, which is a competitive inhibitor. WAY-855 has a high affinity for EAAT2/GLT1. It also inhibits transport in EAAT1/GLAST and EAAT3/EAAC1, but with about 50-100-fold lower affinity. WAY-855 showed no activity on ionotropic glutamate receptors and, unlike substrate inhibitors, did not trigger heteroexchange [146]. Following the development of WAY-855, the same group characterized a series of compounds that varied in their potency towards EAATs 1–3. WAY-213613

was the most selective with 59 and 45-fold selectivity towards EAAT2/GLT1 in comparison to EAAT1/GLAST and EAAT3/EAAC1 respectively [147].

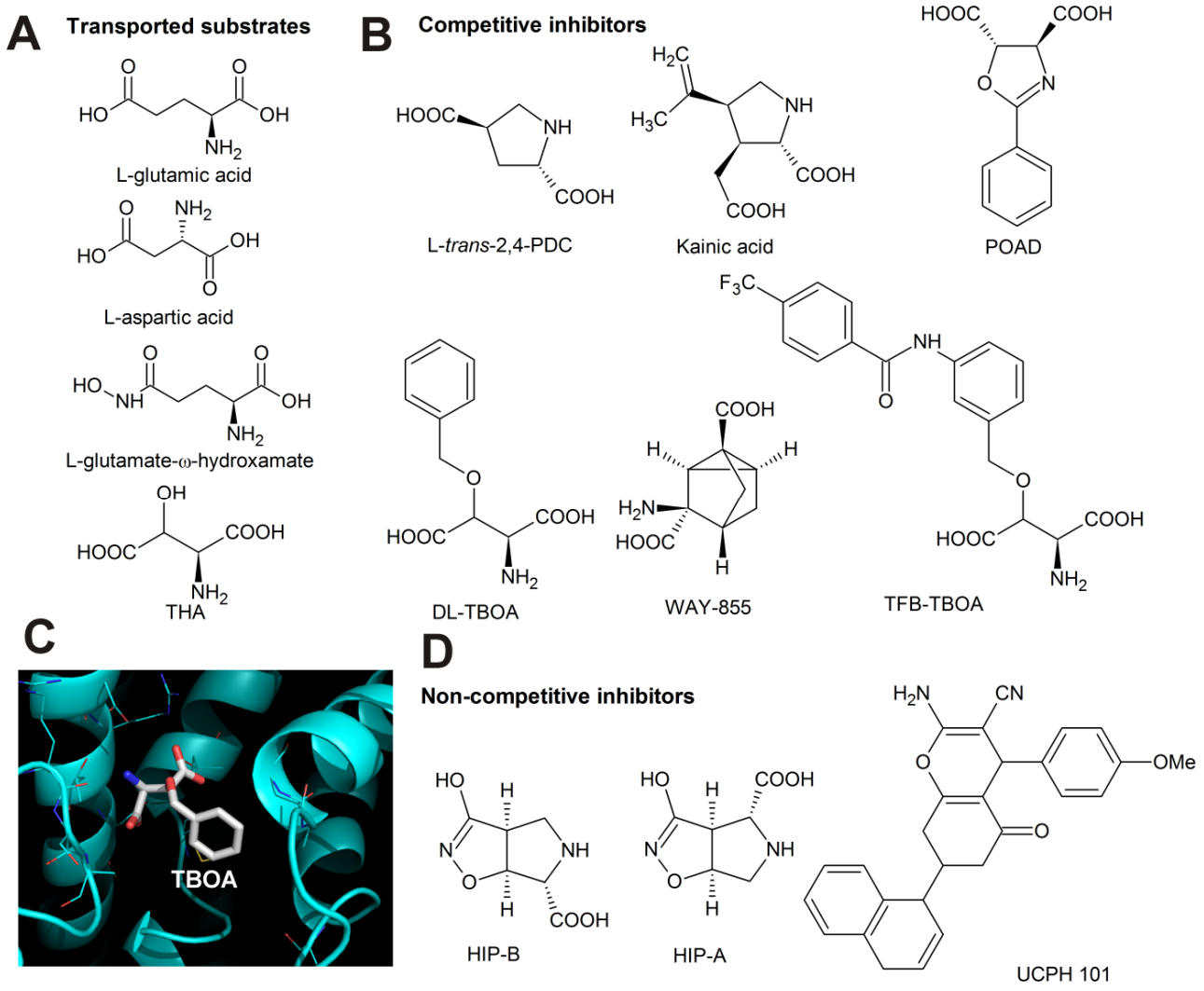

Figure 4. Glutamate transporter pharmacology. (A, B): Representative structures of the most important classes of transported substrates and competitive inhibitors of glutamate transporters. (C) Binding mode of the prototypical inhibitor TBOA to the archeal aspartate transporter GltPh. (D) Structures of non-competitive inhibitors.

A series of compounds based on oxazole and oxazoline rings in their side chain have been investigated by Campiani and colleagues [135]. One of them, which lacked a free amine group, displayed potent and selective blocker activity. Molecular modeling studies of this compound showed that its lack of a protonatable amine in conjunction with steric stress contributed to EAAT selectivity and that protonation of the amino group was not necessary for high affinity interaction with the transporter [135,148].

9. Non-competitive inhibitors

Non-competitive inhibitors modulate substrate uptake by binding to a site that is spatially removed from the substrate binding site. The bicyclic glutamate analogs HIP-A and HIP-B (Figure 4D) belong to this class of uptake inhibitors, whose inhibitory effect cannot be overcome by high substrate concentrations. At low concentrations, HIP-A and HIP-B also displayed an inhibiting activity of substrate-induced [^{3}H] D-aspartate release, an action not seen when the blockers TBOA or dihydrokainate [149] were applied. Further investigation showed that HIP-B did i) not alleviate the effects of TBOA, a competitive blocker, and ii) preferentially interacted with the inward facing conformation of the transporter [150]. HIP-A and HIP-B both potently inhibit glutamate-induced glutamate release [149], rendering the compounds potentially useful for preventing effects of ischemic conditions.

Another group of subtype-selective non-competitive inhibitors for EAAT1 have recently been identified, with UCPH-101 (Figure 4D) being the most potent of these coumarin analogues. The inhibitory activity of UCPH-101 is achieved by binding to a hydrophobic pocket at the subunit interface, which is specific to EAAT1/GLAST [151]. The inhibitor displayed no significant interaction with EAAT2/GLT1 and EAAT3/EAAC1 [151,152]. Results from site directed mutagenesis experiments suggest a hydrophobic pocket located in the trimerization domain as the binding site for UCPH-101 [153].

10. Activators

The development of small-molecule compounds that can up-regulate glutamate transporter function would be an important advancement. Such molecules are expected to be neuroprotective, since several neurological diseases involving glutamatergic dysfunction are associated with loss of glutamate transport. β-lactam antibiotics were among the first activators that were developed. They have been shown to confer neuroprotection by up-regulating glutamate transporter expression through gene activation [154]. Parawixin 1, a compound from spider venom, belongs to a different class of compounds, enhancing EAAT2/GLT1-specific glutamate uptake without altering the transporter's affinities for glutamate or sodium. Since Parawixin 1 had no effect in a mutant transporter lacking the K^+-dependent reorientation step, its effect must be in the reorientation step of the K^+-bound transporter [155,156]. Finally, a thiopyridazine derivative increased EAAT2/GLT1 expression levels in astrocytes. Modifications of this compound yielded several derivatives that enhanced EAAT2/GLT1 levels greater than 6-fold at concentrations lower than 5 μM [157]. Developing activators remains an important goal, due to their potential neuroprotective effects.

11. Conclusions

Interesting results from recent research have expanded our knowledge of glutamate transporter structure, function, and regulation. In particular, the continued determination of structures in various conformations enable us to obtain structural pictures of the transporter as it moves through the alternating access transport cycle (Figure 1C). These structures allow a dynamic view of the transport process, supplemented by results from molecular dynamic simulations and functional studies, highlighting, for example, the effects of electrostatics on the energetics of the major

transitions in the cycle. In addition, we continue to obtain new information on the dynamics of anion channel/transporter dual function, with recent work establishing the role of particular structural domains that control properties of anion permeation. Despite this increase in structural knowledge, functional studies will remain important to further improve our understanding of molecular transport mechanism, which requires not only static, but also dynamic information.

Furthermore, our understanding of processes involved in the regulation of transporter abundance in the membrane has been enhanced by recent advances in the identification of transcriptional regulation (discovery of promoter sequences), as well as regulatory mechanisms that control trafficking and cell surface targeting (Figure 3). Establishing regulatory pathways that can be targeted to increase glutamate transporter expression in the plasma membrane, as well as pharmacological tools to directly up-regulate transporter turnover, may be useful for increasing glutamate uptake in vivo, a desirable intervention under conditions in which glutamate uptake is impaired, which could result in neuroprotection.

Advances have also been made in terms of the pharmacology of glutamate transporters. Not only have blockers been identified that displace glutamate with high affinity in the nM range, but progress has also been made with respect to the development of subtype-selective inhibitors. In addition, non-competitive inhibitors were identified that may allow the establishment of new modes of modulation of transporter function in vivo.

Together, these recent advances in our understanding of glutamate transporter function and dynamics may lead to novel therapeutic strategies that directly target glutamate transporters in a variety of nervous system disorders.

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Conflict of Interest

All authors declare no conflicts of interest in this paper.

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