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MECHANISMS OF GLUTAMATE TRANSPORT

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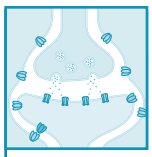

Vandenberg RJ, Ryan RM. Mechanisms of Glutamate Transport. *Physiol Rev* 93: 1621–1657, 2013; doi:10.1152/physrev.00007.2013.—L-Glutamate is the predominant excitatory neurotransmitter in the mammalian central nervous system and plays important roles in a wide variety of brain functions, but it is also a key player in the pathogenesis of many neurological disorders. The control of glutamate concentrations is critical to the normal functioning of the central nervous system, and in this review we discuss how glutamate transporters regulate glutamate concentrations to maintain dynamic signaling mechanisms between neurons. In 2004, the crystal structure of a prokaryotic homolog of the mammalian glutamate transporter family of proteins was crystallized and its structure determined. This has paved the way for a better understanding of the structural basis for glutamate transporter function. In this review we provide a broad perspective of this field of research, but focus primarily on the more recent studies with a particular emphasis on how our understanding of the structure of glutamate transporters has generated new insights.

I. INTRODUCTION

L-Glutamate is the predominant excitatory neurotransmitter in the central nervous system and is involved in a wide variety of brain functions, but it is also a key player in the pathogenesis of many neurological disorders. Glutamate transporters play the important role of regulating extracellular glutamate concentrations to maintain dynamic synaptic signaling processes. The study of glutamate transporters has undergone three distinct phases. The first phase, which started in the late 1960s to early 1970s, was part of the appreciation of the role of glutamate as an excitatory neurotransmitter. This work relied on the use of pharmacological substrates and blockers to define the roles of transporters and also gave the first insights into the diversity of glutamate transporter subtypes and their roles in regulating synaptic transmission. The second phase followed the molecular biology revolution with the cloning of cDNAs encoding glutamate transporters in the early 1990s. This development provided powerful tools to begin to understand the functional properties of transporters, the locations of transporter subtypes, and their specific roles in physiological and pathological states. The third phase involved the determination of the crystal structure of a prokaryotic homolog of glutamate transporters. This breakthrough provided a structural framework to be able to understand and predict the molecular basis for transporter functions. This has been followed up with computer simulations of transporter conformational changes to better understand the transport process.

Because glutamate is the predominant excitatory neurotransmitter, it is not surprising that it is involved in most brain functions and also many neurological disorders, but it also means that it is difficult to provide a comprehensive summary of all aspects of the regulation of glutamate concentrations in a single review. Many aspects of glutamate transporter function and dysfunction have been reviewed in the past (see Ref. 49 for a general review), and throughout this review we highlight the key specialist reviews of the topic. In this review we focus on the latest developments in the field, with a particular emphasis on how the recent knowledge of the structural basis of transporter function has provided important insights into transporter structure and function. In the first section we provide a summary of how glutamate transporters can influence the dynamics of synaptic transmission. In the second section we discuss the structure of the prokaryotic homolog Glt_{Ph} and how it has been used to understand the functions and pharmacological properties of the mammalian counterparts. In the third section we review how glutamate transporter functions can be manipulated by pharmacological agents. We also discuss how transporter functions can be influenced by various endogenous compounds and how other cellular processes impact on transporter functions. In the final section we provide a summary of how glutamate transporter functions are altered under pathological conditions.

There are a number of transporter families that transport glutamate and include the plasma membrane excitatory amino acid transporters (EAATs), the vesicular glutamate

transporters (VGLUTs), and the glutamate-cysteine exchanger. This review focuses on the EAATs, but interested readers should consult some excellent reviews on the other types of glutamate transporters (31, 64, 76).

II. THE FUNCTIONAL PROPERTIES OF GLUTAMATE TRANSPORTERS

Glutamate is the predominant excitatory neurotransmitter in the central nervous system and is directly or indirectly involved in most brain functions. Stimulation of a glutamatergic neuron will cause release of glutamate into the synapse where it has been estimated that glutamate concentrations will transiently rise to low millimolar concentrations and activate ionotropic and metabotropic glutamate receptors **(FIGURE 1)** [see reviews by Mayer (173) and Traynelis et al. (265)]. Excessive glutamate receptor stimulation is toxic to neurons, and it is the role of glutamate transporters to rapidly clear glutamate from the synapse. Glutamate receptors differ considerably in their kinetics of activation, and also by the glutamate concentrations to which they are exposed. In this section of the review we will discuss the functional capacity of glutamate transporters (also called EAATs) and how they can influence synaptic glutamate concentrations. Readers that are interested in a more in-depth discussion of this aspect of glutamate transporters should consult excellent reviews by Marcaggi and Attwell (170) and also Tzingounis and Wadiche (270). The EAATs have the capacity to influence glutamatergic synaptic transmission in a number of ways. The different EAAT subtypes have subtly different properties that will influence their capacity to regulate glutamate levels. Some transporter characteristics that will impact on synaptic neurotransmission include the concentrating capacity of the transporters, glutamate binding affinity of the transporters, rates of transport, the intrinsic chloride conductance, expression levels of the different transporter subtypes, and synapse architecture.

A. Glutamate Transporters Belong to the SLC1 Family

The EAATs belong to the SLC1 family of transporters that also includes two mammalian neutral amino acid transporters (ASCT1, 2) (8, 233, 271) as well as a large number of prokaryotic neutral and acidic amino acid transporters. There are five subtypes of EAATs (EAAT1–5) (6, 7, 70) with the rodent versions of EAAT1 and EAAT2 referred to as GLAST1 (249) and GLT1 (196), respectively, and the rabbit version of EAAT3 referred to as EAAC1 (136). The SLC1 designations are as follows: EAAT1, SLC1A3; EAAT2, SLC1A2; EAAT3, SLC1A1; EAAT4, SLC1A6; EAAT5, SLC1A7. In this review we focus on the acidic amino acid transporters of the family using the EAAT terminology and where appropriate refer to the rodent or rabbit equivalent.

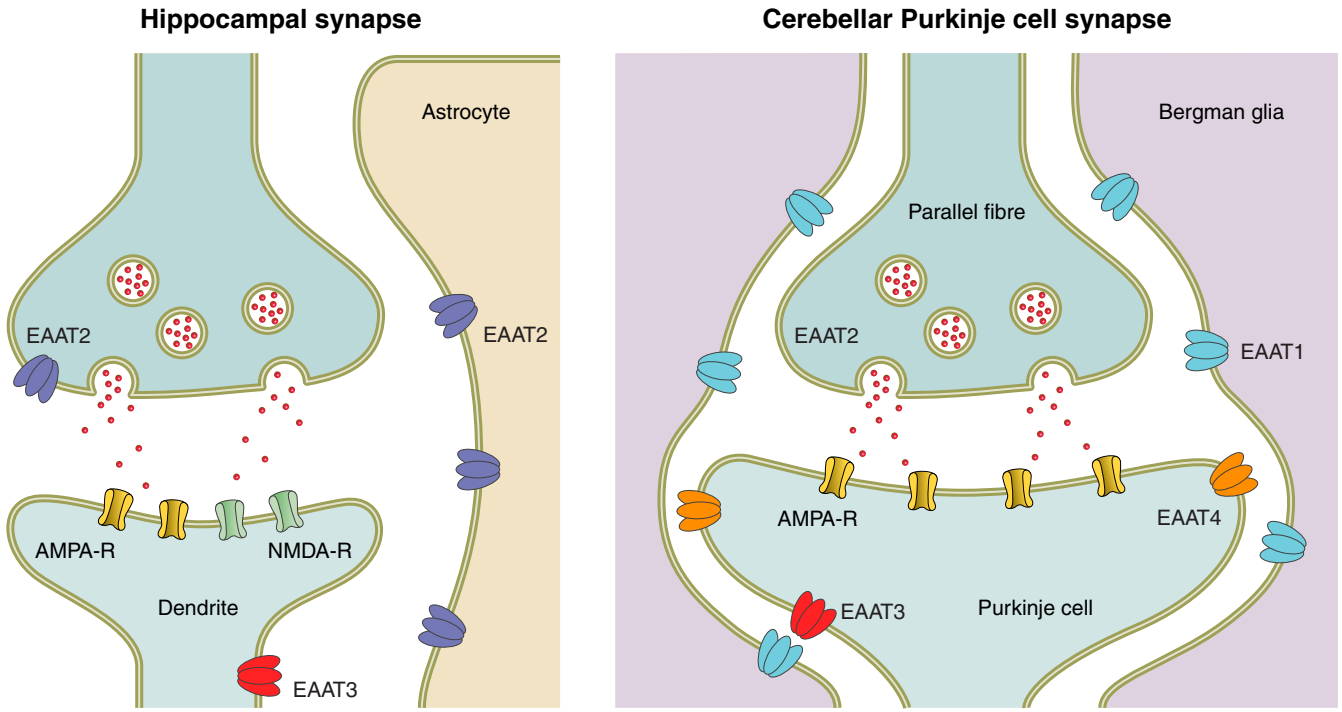

FIGURE 1. Schematic diagrams of the glutamatergic CA1 hippocampal synapse and the cerebellar Purkinje cell synapse showing the predominant pre- and postsynaptic locations and glial cell locations of the EAAT subtypes. See the section II*C* for more details concerning the expression levels and distributions of the various transporter subtypes.

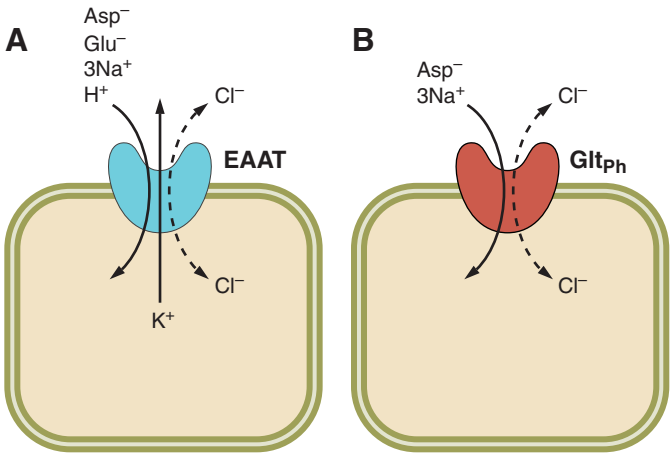

FIGURE 2. Stoichiometry of ion-flux coupling. *A*: glutamate transport by the EAATs is coupled to the cotransport of 3 Na^+ and 1 H^+ followed by the countertransport of 1 K^+. *B*: aspartate transport by Glt_{Ph} is coupled to the cotransport of 3 Na^+. Binding of substrates and Na^+ to the transporters also activates an uncoupled anion conductance through the transporter.

1. The stoichiometry of ion flux coupling: the concentrating capacity of transporters

Glutamate transport by EAAT1, 2, and 3 is coupled to the cotransport of 3 Na^+ and 1 H^+ followed by the countertransport of 1 K^+ (156, 192, 302) **(FIGURE 2, *A* AND *C*)**. The stoichiometry of coupling has not been determined for EAAT4 and EAAT5, but we assume that they have the same coupling ratios. From these ratios, the concentrating capacity of the transporter can be estimated using a modified version of the Goldman-Hodgkin-Katz equation: $[Glu]_o/[Glu]_i = RT/ZF \ln \{([Na^+]_o/[Na^+]_i)^3([H^+]_o/[H^+]_i)([K^+]_i/[K^+]_o)\}$, where $[X]_o$ and $[X]_i$ refer to the outside and inside concentrations of the various ionic species, R is the gas constant, T is temperature (in °K), F is Faraday's constant, and $Z = 2$ because there is a net transfer of 2 positive charges. Note that the term referring to the Na^+ gradient is to the power of 3 because 3 Na^+ are coupled to the transport process.

At equilibrium under standard physiological conditions, this coupling ratio is able to support a 10^6 fold gradient of glutamate across the cell membrane (302). Theoretically, this coupling ratio should ensure that the resting extracellular glutamate concentration should be in the low nanomolar range. This theoretical value contradicts a number of estimates of resting extracellular glutamate concentrations using in vivo dialysis, which predict resting extracellular glutamate concentrations to be as high as 1–4 μM (154, 188). This conundrum was resolved by Herman and Jahr (111) using the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors expressed by CA1 pyramidal cells in acute hippocampal slices. The activity of NMDA receptors was used as a probe for the glutamate concentration. Under resting conditions, it was estimated that the glutamate concentration was as low as 25 nM, which is closer to the theoretical estimates based on the stoichiometry of ion-flux coupling of the EAATs (302).

2. EAATs also have an uncoupled chloride conductance

In addition to the coupled glutamate ion fluxes, substrate binding to the EAATs generates a thermodynamically uncoupled chloride (Cl^-) flux through the transporter (23, 70, 272, 284), reviewed by Vandenberg and colleagues (224, 274) **(FIGURE 3*A*)**. The extent of channel activity varies between transporter subtypes. The neuronal transporters EAAT4 and EAAT5 behave predominantly as Cl^- channels **(FIGURE 3*C*)**, while for EAAT1, EAAT2, and EAAT3 the channel activity represents a much smaller proportion of the ion fluxes associated with transporter function (6, 70, 284) **(FIGURE 3*B*)**. The properties of the Cl^- conductance have been investigated in detail by Wadiche and Kavanaugh

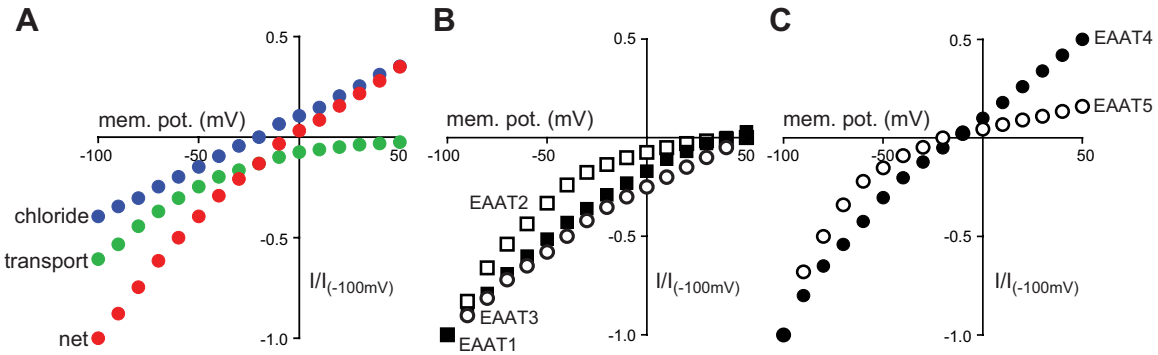

FIGURE 3. Current-voltage plots of currents associated with glutamate transporters. *A*: schematic of the current-voltage relationship for the transport current (green), the chloride current (blue), and the combined or net current (red) for transporters expressed *in Xenopus laevis* oocytes. EAAT1–5 differ in the proportion of current due to transport and chloride channel. *B*: the net currents observed for EAAT1–3 have greater contributions from the transport component than the chloride channel, which results in more positive reversal potentials. *C*: the chloride channel component is greater in EAAT4 and EAAT5 leading to a reversal potential that is closer to the chloride reversal potential.

(286). A Q_{10} value (a change in activity for a 10 degree temperature change) can be used as a measure of the energy required for the two functions of the transporters. The Q_{10} for the transport component is 3.2 ± 0.2, which is significantly greater that the Q_{10} of 1.0 ± 0.1 for activation of the Cl^- conductance component. This difference in Q_{10} values indicates that the energy required for activation of the Cl^- conductance is less than that for the coupled transport process. The Q_{10} for activation of the Cl^- conductance is what may be expected of a channel mechanism rather than a transport process.

Although Cl^- is the most common physiological anion, other anions can also permeate the anion channel of the EAATs. The anion premeabilities relative to Cl^- are: SCN^- (67-fold more permeant than Cl^-) > NO_3^- (17) > I^- (12) > Br^- (3) > Cl^- (1) >> F^-, gluconate, methanesulfonate (285, 286). Based on the size of the largest permeant anion, the pore diameter of the channel is ~5 Å. It is not possible to measure single-channel events from patches pulled from *Xenopus leavis* oocytes expressing EAAT1, but using noise analysis, Wadiche and Kavanaugh (286) have estimated the single-channel conductance in Cl^- to be ~1 fS, with an open probability of <<1.

It should be noted that the above transport equation does not take into account the chloride conductance. While chloride ions will not influence the coupling ratio, they will change the charge transfer associated with the transport process and influence the membrane potential, which will then impact on the concentrating capacity of the transporter. EAAT4 and EAAT5 have significantly greater intrinsic chloride conductances, so the chloride flux will have a greater impact on the cells expressing these transporters than for cells expressing EAAT1–3.

In a study by Veruki et al. (283), the role of the EAAT-mediated chloride conductance in retinal neurons was investigated. After glutamate is released from the presynaptic terminal, it can bind to presynaptic glutamate transporters which results in activation of the intrinsic chloride channel of the transporter. This causes hyperpolarization of the neuron and acts as a negative feedback loop to reduce subsequent glutamate release. Thus the transporter not only removes glutamate but also prevents subsequent release of glutamate.

Although the roles of the chloride conductance mediated by EAAT1–3 have not been clearly established, it may be inferred that the channel activity may serve to maintain ionic homeostasis. Ion-coupled glutamate transport results in the transfer of two positive charges across the membrane, and if this is associated with activation of chloride ion movement into the cell, the depolarization of the membrane will be minimized and the rate of transport will be maintained at optimal levels. Glt_{Ph}, a prokaryotic aspartate transporter that is also a member of the SLC1 family of transporters (see sect. III), has been purified and reconstituted into liposomes (222). This system permits a direct measure of the influence of the chloride channel activity on transporter function. In the absence of any permeant anions, the rate of aspartate transport slows down very rapidly presumably due to the accumulation of positive charges in the liposome as a result of the transport process. In contrast, if the uptake experiment is performed in the presence of permeant anions, the optimal rate of transport is maintained for considerably longer periods (222). If this phenomenon were to influence EAAT function in neurons and glial cells, it would be reliant upon high expression levels of the EAATs to generate sufficient charge transfer to influence the membrane potential. Indeed, EAAT1 and EAAT2 are highly abundant (see sect. IIC), and it is feasible that such an ion homeostatic mechanism is important in regulating EAAT function.

The powerful concentrating capacity of glutamate transporters, combined with the uncoupled chloride fluxes, means that glutamate transport is associated with large fluxes of osmolytes. To compensate for these osmolyte movements, the EAATs also appear to allow a considerable amount of water flux, with the rates of flux being ~10% of that of the aquaporins (165–168). Water can permeate through both the transport pathway and the chloride channel, but during active transport the majority of water flux appears to be via the chloride channel (273).

B. Kinetic Properties of Glutamate Transporters

When glutamate is released from the presynaptic terminal, it will diffuse across the synapse and then bind to glutamate receptors to generate a postsynaptic signal. The ability of transporters to influence this process will depend on a number of variables. Over 40 years ago, Jardetsky (128) proposed an alternating access model for transporter function **(FIGURE 4)**. This model still provides a useful concept in understanding the various transporter states that have the capacity to influence the way that the EAATs can regulate glutamate concentrations. In brief, the alternating access model proposes that substrates bind to the extracellular face of the transporter and then the transporter undergoes conformational changes which cause the substrate binding site to become exposed to the intracellular surface leading to the subsequent release of the substrate inside the cell. Further conformational changes are required to reorientate the transporter such that the substrate binding site is exposed to the extracellular solution to allow the transport cycle to start again. Within this scheme, the key events that will determine the kinetic parameters of transport are as follows: the initial binding rates for substrates; the likelihood that the bound glutamate will be transported into the cell rather than being released back into the extracellular solution; the turnover rate of the transporter, which is the

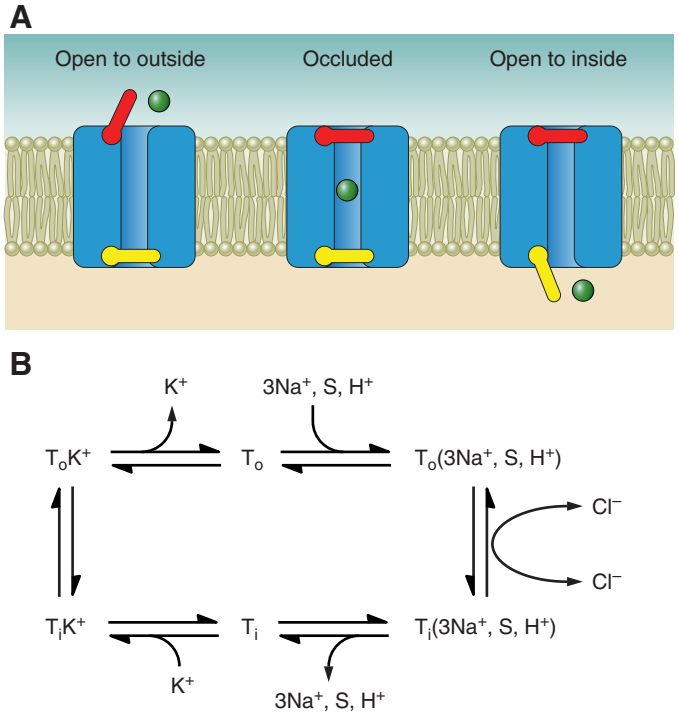

FIGURE 4. Alternating access model. The mechanism of glutamate transport can be modeled using the concept developed by Jardetzky in 1966 (128). Substrates (glutamate/Na^+/H^+) bind to the extracellular face of the transporter, and then the transporter undergoes conformational changes to form an occluded state (*A*, *B*). Further conformational changes will expose the substrate to the intracellular solution leading to the release of the substrate inside the cell. Conformational changes are then required to reorientate the transporter (K^+ countertransport) such that the substrate binding site is exposed to the extracellular solution to allow the transport cycle to start again.

time required for a complete transport cycle; and the density of glutamate transporters in close proximity to the synapse. The different EAAT subtypes vary in these parameters and thereby provide a range of speeds with which they regulate glutamate concentrations.

Early studies of glutamatergic neurotransmission (41, 155) suggested that the time course of neurotransmission was determined by the kinetics of ionotropic glutamate receptor activation, with glutamate transporters playing a passive role in clearing up glutamate after receptor activation was complete. However, it appears that the effect of glutamate transporters depends on which glutamate receptors are being used as a measure of glutamate concentration dynamics. AMPA receptors have faster activation rates than NMDA receptors, and Diamond and co-workers (54, 55) have used the rate of AMPA receptor activation as a measure of glutamate concentration dynamics. In this case, glutamate transport inhibitors can slow the time course of AMPA receptor responses, suggesting that transporters can affect the concentration of glutamate within the first 100 μs after release. NMDA receptor activation under physiological Mg^{2+} concentrations is considerably slower than AMPA receptor activation, and Sun et al. (250) have studied the rate of NMDA receptor activation as a measure of glutamate concentration dynamics. Under these conditions, glutamate transport blockers have no effect on the rate of NMDA receptor activation generated by a single electrical pulse. However, with short bursts of firing, transporters begin to influence the rate of NMDA receptor activation. Thus it appears that transporters will play a role in clearing glutamate from the synapse under conditions of high synaptic activity and also influence the extent of glutamate spillover from the synapse (250).

The turnover rate for transport (285) is considerably slower than the rate of synaptic transmission, which raises the question: How can a relatively slow transport process influence fast synaptic transmission? The transfer of two positive charges per transport cycle together with the glutamate-activated chloride channel makes it possible to use a range of electrophysiological techniques to study transporter functions. Fast application of glutamate allows high temporal resolution of the kinetics of the transport process (96, 111). With the use of this technique, it is possible to observe a biphasic response to glutamate. A rapidly rising peak current is followed by a steady-state current (96) **(FIGURE 5)**. EAAT1, -2, and -3 show similar concentration-dependent

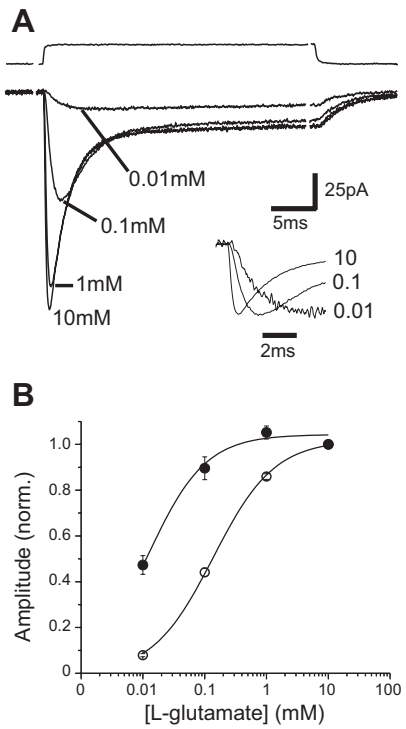

FIGURE 5. Fast and slow responses to glutamate. *A*: rapid application of glutamate to patches from HEK293 cells expressing GLT1 (EAAT2) reveals a peak (open circles) current and a steady-state (closed circles) current. The *inset* shows the scaled responses to demonstrate the concentration dependence of the rise time for the peak current. *B*: concentration dependence of the peak (closed circles) and steady-state currents (open circles) shows differences in binding constants compared with the steady-state rate of transport. [From Bergles et al. (22)].

responses for the steady-state current (EC_{50} in the 10–20 μM range). However, EAAT2 has significantly higher EC_{50} values for the initial peak responses (EC_{50} values ~140 μM; Refs. 22, 270) (see **TABLE 1**). The peak response reflects the initial binding of glutamate to the transporter, whereas the steady-state current is reflective of the turnover rate of the transporter. The EAAT3 transporter shows the fastest turnover rate, and for EAAT4 and EAAT5, the turnover rate is up to 100-fold slower (81, 178). In one study it was suggested that the predominant role of EAAT5 is as a glutamate and Na^+-gated chloride channel, with the transport function playing a very minor role (81).

Another factor that needs to be considered is the capture efficiency of the transporters. After glutamate binds to the transporter, there are two possible outcomes. First, glutamate may unbind and diffuse away from the transporter, and second, the transporter may undergo conformational changes causing glutamate to be transported across the membrane. The capture efficiency for EAAT1–3 is ~0.5, which means that glutamate has an equal chance of unbinding as being transported (22, 286). After glutamate unbinds, the most likely event is rebinding to another transporter in close proximity, and in this way the transporters behave as buffers for the released glutamate. This process ensures that the glutamate can be very rapidly cleared from the synapse preventing prolonged receptor activation. A further corollary of this argument is that the expression levels of glutamate transporters will play a very important role in their buffering capacity and also the dynamics of glutamatergic transmission. With high expression levels in close proximity to glutamate release sites, there will be very rapid buffering of glutamate, whereas with lower or more dispersed expression of transporters, there will be slower removal of glutamate and greater capacity for spillover of glutamate to neighboring synapses.

The reviews by Tzingounis and Wadiche (270) and Marcaggi and Attwell (170) expand on these points to describe how glutamate transporter subtypes, at various well-defined synapses, influence glutamatergic neurotransmission.

Table 1. *Comparison of the kinetic parameters of glutamate transport by EAAT1–5*

Transporter	Turnover Rate, s^{-1}	Steady-State K_m, μM	Pre-Steady-State Affinity, μM
EAAT1	16	7–20	20
EAAT2	14, 41	12–18	140
EAAT3	90–110	8–28	20
EAAT4	<3	0.6–2.5	5
EAAT5	<1	61–64	

Data from References 7, 22, 70, 96, 178, 270, 285, and 286.

C. Expression Patterns of EAAT Subtypes

The five glutamate transporter subtypes are differentially expressed in different regions of the brain and also different cell types. This topic has been comprehensively reviewed by Danbolt (49), and in the following section we will give a brief overview of the cellular and regional distribution of the five EAAT subtypes and also highlight a few of the more recent studies on this topic.

EAAT1 is highly abundant and is the major glutamate transporter in the cerebellum (152) and a number of smaller regions such as the inner ear (80), the retina (204), and circumventricular organs (21). In the cerebellum, EAAT1 is about 6-fold more abundant than EAAT2 and 10-fold more abundant than EAAT4. The highest density of EAAT1 is in the Bergman glia where there are ~18,000 transporters/μm^3. In the CA1 region of the hippocampus, the density of EAAT1 is ~3,200/μm^3 (152). EAAT1 is mostly expressed in astrocytes and often coexpressed with EAAT2, but they do not form hetero-oligomeric complexes (109) (see sect. IIIC).

EAAT2 is the most abundant glutamate transporter in all regions except the brain regions where EAAT1 is more abundant (see above) and is responsible for ~90–95% of glutamate uptake in the forebrain. In the hippocampus there are ~12,000 EAAT2 transporters/μm^3, whereas in the cerebellar layer there are only 2,800 transporters/μm^3 (152). EAAT2 is predominantly expressed in astrocytes, but up to 10% of EAAT2 is expressed in presynaptic neuronal terminals in the hippocampus (79). At this stage, it is not clear whether glutamate is taken up by presynaptic terminals in the cerebellum.

EAAT3 is expressed in neurons with highest concentrations in the hippocampus, cerebellum, and basal ganglia (46, 109, 113, 144, 216), but the level of expression is ~100-fold less than those of EAAT1 and EAAT2 (113). A large proportion of EAAT3 is intracellular, but it is thought that it may be rapidly mobilized to be expressed in the plasma membrane (see sect. IV*D*). Furthermore, EAAT3 is predominantly expressed in the dendrites and soma, which suggest that it plays a homeostatic or cell metabolism role rather than in directly regulating synaptic transmission (113).

EAAT4 expression is highly localized, with most EAAT4 expressed in the Purkinje cells of the cerebellum (53). There are low levels of expression in other regions such as the forebrain, and it should be noted that the orginal EAAT4 cDNA clone was isolated from a human motor cortex cDNA library (70). There is some expression in close proximity to the synapse (10, 190), but the majority of EAAT4 is expressed in dendritic spines that are in close proximity to astroctyes and also low levels in the soma.

The average density of EAAT4 in Purkinje cells is ~1,800/μm^3 (53).

EAAT5 is exclusively expressed in the retina (6) and has been identified in both cone and rod photoreceptor terminals and in axon terminals of rod bipolar cells (200, 292). Thus, in the retina, EAAT5 is well placed to directly influence glutamatergic neurotransmission (283, 292).

Glutamate transporters are also expressed in a number of other organs. The expression of EAAT3 in the kidney is well characterized, where it plays a role in dicarboxylic amino acid reabsorption (12, 136, 194), also see review by Broer (34). EAAT3 is also expressed in the heart (184), enteric neurons (163), and placenta (172). EAAT1 is also expressed in heart (184), placenta (172), bone osteocytes (122), and mammary glands (171), and EAAT2 is expressed in the plancenta (172) and mammary glands (171).

D. Homo- and Hetero-oligomers of Glutamate Transporters

Most glutamate transporters consist of three identical protomers to form a homotrimeric complex (see sect. IIIC for a detailed description of the structure), but there are a few reports of heterotrimeric complexes. The most abundant transporters, EAAT1 and EAAT2, are found as homotrimers, whereas EAAT3 and EAAT4 can form either homotrimers or associate to form mixed EAAT3/EAAT4 trimers (187). When EAAT3 is expressed in Madin-Darby canine kidney cells, the transporters are trafficked to the apical membrane. However, when EAAT4 is coexpressed with EAAT3, some of the EAAT3 is trafficked to the basolateral surface and associates with the EAAT4 (187). Thus it appears that the neuronal glutamate transporters have the capacity to form heterotrimers, which provides another source of diversity in transporter function.

E. Splice Variants

A number of splice variants of the EAAT1, -2, and -3 have been identified, with the EAAT2 variants being investigated in greatest detail. The splice variants can be classified broadly into the functional variants and the nonfunctional variants. The functional variants arise through alternate splicing events of the RNA leading to different NH_2 and COOH termini. Up to four EAAT2 functional variants have been described, and different investigators have used different terminology. The COOH-terminal variants have been termed EAAT2a, EAAT2b, and EAAT2c, with the EAAT2a variant representing the majority of EAAT2 protein. EAAT2a is ~15-fold more abundant than EAAT2b and 100-fold more abundant than EAAT2c (114). The 5′ end of the EAAT2 mRNA can also be alternatively spliced to yield different NH_2 termini (193). The different NH_2 and COOH termini give rise to proteins that have the capacity to differentially associate with different scaffolding proteins leading to differences in expression patterns, but it is interesting to note that there are no obvious functional differences between each of the variants (193). Furthermore, the different splice variants can associate to form mixed EAAT2a/EAAT2b trimers (228).

The nonfunctional variants are generated from aberrant RNA splicing events leading to the loss of exons 3, 7, or 9 (161). The proteins generated are missing important structural and functional regions of the transporters. It is surprising that many of the proteins encoded by mRNAs for these variants are found in various brain regions (149), but as may be expected, none of these aberrant proteins generates proteins that are capable of binding or transporting glutamate (84, 228). Whereas these aberrant variants can associate with "normal" splice variants of the same subtype, they require a 10-fold excess over the normal variant to influence the functional properties (228). These aberrant forms are found at low levels in normal aged human brains, but elevated levels have been detected in Alzheimer's disease (228) and amyotrophic lateral sclerosis (161). It is not clear at this stage whether elevated levels of these aberrant forms are produced as a consequence of the disease process or directly influence the progression of the disease.

III. Glt_{Ph}: A MODEL FOR THE STRUCTURE AND FUNCTION OF EAATs

The cloning of glutamate transporter cDNAs in the 1990s (6, 8, 70, 136, 196, 249) provided the tools to begin to understand transporter protein structure and function. Whereas considerable progress was made, the major steps in understanding the structural basis for transporter function came with the determination of three crystal structures of an archaeal homolog of the EAATs, Glt_{Ph} (26, 205, 299). In the following section we discuss the structures of Glt_{Ph} and how they are likely to relate to the EAATs. We will not provide an exhaustive review of the large number of mutagenesis studies that were carried out prior to determination of the crystal structure, but rather we will highlight the older mutagenesis studies that have provided insight into the structural basis for the functional properties or where there is discrepancy between the mutagenesis studies and the crystal structures obtained.

A. Amino Acid Sequence Comparison With EAATs

The amino acid sequence of Glt_{Ph} shares 37% identity with human EAAT2 (299) **(FIGURE 6)**. This is relatively high

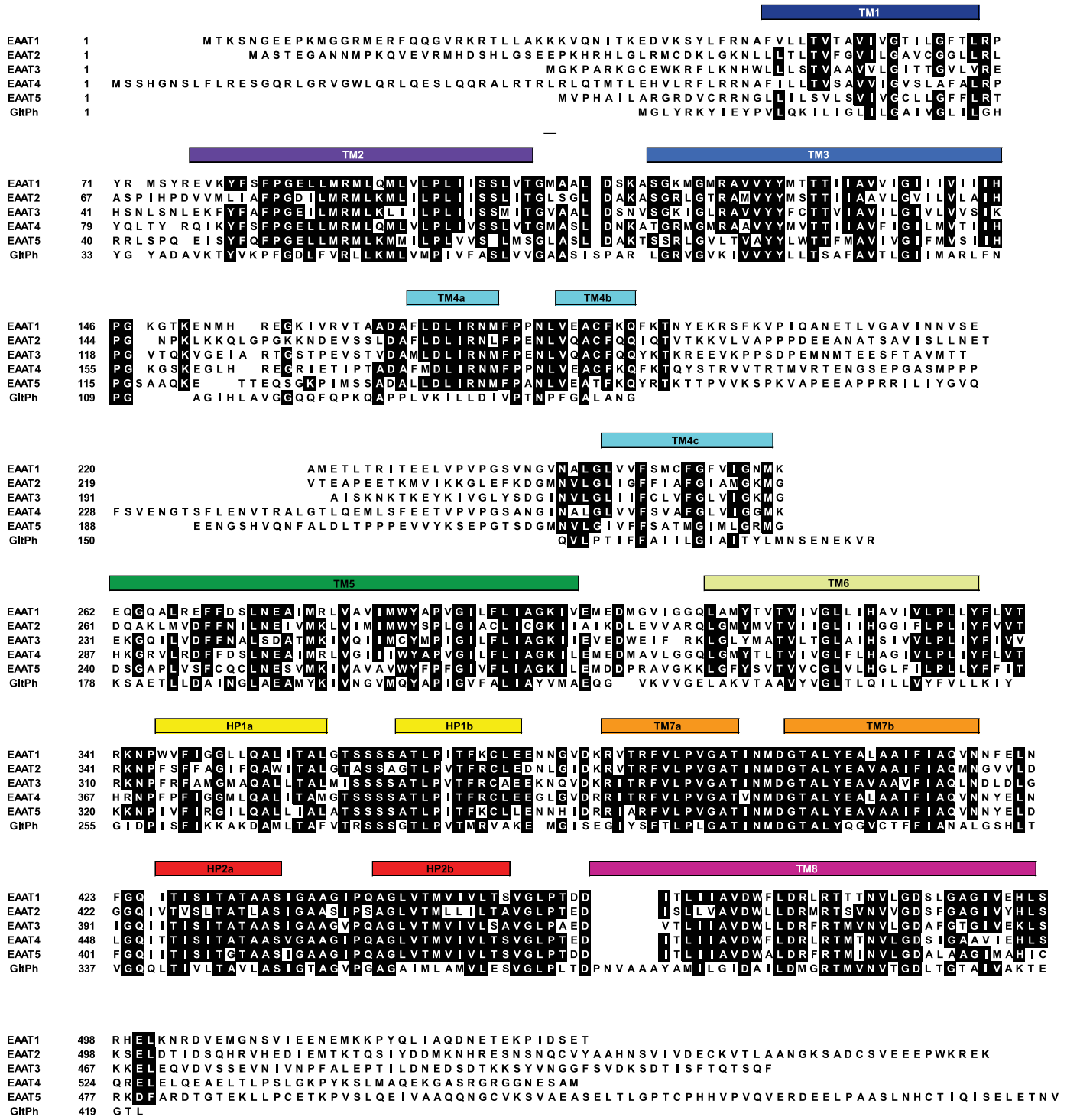

FIGURE 6. Amino acid sequence alignments of EAAT1–5 and Glt_{Ph}. The EAAT1–5 and Glt_{Ph} share ~30–40% amino acid sequence identity. Residues that are conserved in 4 of the 6 transporters are highlighted with black background and white writing. The assignment of structural elements is based on the crystal structure of Glt_{Ph} (PDB 1FXH), and the colors correspond to the structural elements shown in **FIGURE 7, *C* AND *D***.

considering the large evolutionary distance between the two organisms and that the amino acid sequences of the EAATs themselves only share ~45–55% identity. The degree of identity between Glt_{Ph} and the EAATs is considerably higher within the COOH-terminal half of the transporters, and it is this region that forms the binding sites for substrate and the various co- and countertransported ions. On these grounds, it was proposed that the structure of Glt_{Ph} is a good model for the structure of the EAATs. Indeed, many recent mutagenesis studies of the EAATs to investigate structure and function relationships have been designed based on the structure of Glt_{Ph}, and in most cases the results

and conclusions drawn are consistent with the structure of Glt_{Ph} (see following sections of this review).

B. Differences and Similarities in the Functional Properties of Glt_{Ph} and the EAATs

The choice of Glt_{Ph} for crystallization attempts was made based on a number of factors. These include the high degree of amino acid sequence similarity between Glt_{Ph} with the EAATs; proteins derived from hyperthermophilic organisms, such as *Pyrococcus horikoshii,* are likely to be stable which may facilitate crystallization; and it was possible to purify sufficient quantities of stable protein. However, the functional properties of Glt_{Ph} were not established prior to the original structure determination (299). Therefore, it was important to establish the similarities and differences in function between Glt_{Ph} and the EAATs so as to validate Glt_{Ph} as a model for the EAATs. The first crystal structure of Glt_{Ph} was determined with a resolution of 3.5 Å (299), and at this resolution it is difficult to discern the precise conformational states of amino acid residue side chains, the identity of various ions bound to the transport or the native substrate. In the crystal structure of Glt_{Ph}, a nonprotein electron density was observed in close proximity to residues that had been implicated from older mutagenesis studies in forming the glutamate binding site (20, 44) or being important for transporter function (230), and from this it was presumed that the electron density was due to a bound substrate molecule that was most likely glutamate. However, subsequent studies of the functional properties of Glt_{Ph} found that L-glutamate is not a good substrate and that the smaller acidic amino acid L-aspartate was the preferred substrate of Glt_{Ph} (26, 220). There are three other important functional differences between the EAATs and Glt_{Ph}. First, L-aspartate transport by Glt_{Ph} is coupled to the cotransport of 3 Na^+, but does not appear to be coupled to the cotransport of 1 H^+ or the countertransport of 1 K^+ as observed for the EAATs (98, 220, 302) **(FIGURE 2*B*)**. Second, the affinity of L-aspartate for Glt_{Ph} is much higher than for the EAATs (~120 nM for Glt_{Ph} compared with 3–30 μM for the EAATs) (7, 8, 70, 220). Third, the rate of substrate transport by Glt_{Ph} is considerably slower than for the EAATs (one L-aspartate molecule transported every ~3 min for Glt_{Ph} at 30°C compared with 80 ms for EAAT2) (220, 285). Despite these differences in the kinetics of the transport process and substrate selectivity, Glt_{Ph} is a good model for understanding EAAT structure and function. After the description of the structure of Glt_{Ph}, we highlight some approaches that have been used to understand the differences between Glt_{Ph} and the EAATs.

C. Structure of Glt_{Ph}

Glt_{Ph} consists of three identical protomers that associate within the membrane to form a bowl-shaped structure. The central cavity of the bowl is ~50 Å in diameter and 30 Å deep and faces the extracellular solution **(FIGURE 7, *A* AND *B*)**. Each protomer forms an unusual wedge-shaped structure consisting of eight transmembrane α-helical domains (TM 1–8) and two helix-turn-helix motifs or hairpin (HP) loops termed HP1 and HP2 **(FIGURE 7*C*)**.

The Glt_{Ph} complex can be viewed as consisting of three transport domains that are stabilized by a central core or scaffold domain. The central scaffold domain consists of TMs 1, 2, 4, and 5 from each of the three protomers, and these associate to stabilize the complex. The three transport domains are formed by TMs 3, 6, 7, and 8 and HP1 and 2 from each of the three protomers (205, 299) **(FIGURE 8, *A* AND *B*)**. Functional studies on both EAAT3/4 and Glt_{Ph} have confirmed that each protomer is capable of substrate transport and also support the uncoupled chloride conductance (94, 140, 148, 222). The design of the trimer is particularly well suited for achieving efficient and rapid transport. Glutamate can rapidly diffuse halfway across the membrane before coming in contact with the hydrophilic surface at the bottom of the bowl structure. This will minimize the number of binding and translocation events required to ferry the glutamate molecule across the membrane. When glutamate comes in contact with its binding site, one of two events will happen: either the glutamate will be transported, or the glutamate will unbind and diffuse away from the site. With the bowl-shaped structure of the transporter that contains three independent transport domains, the likelihood of glutamate diffusing away in the bulk solution before coming in contact with another binding site is greatly diminished. Indeed, a study by Leary and co-workers (147) demonstrated that the unbinding rate of substrates was substantially slowed in the wild-type trimeric complex compared with a trimeric complex which contains two transport incompetent protomers and a single functioning protomer. Thus the trimeric bowl-shaped structure is ideally designed to facilitate rapid and efficient transport. The trimeric structure of the human glutamate transporter, EAAT2, and the bacterial glutamate transporters from *Escherichia coli*, *Bacillus caldotenax,* and *Bacillus stearothermophilus* have also been confirmed using biochemical techniques (86, 300). Interestingly, an oligomeric structure for the EAATs had previously been proposed, but the number of subunits required for the complex was controversial with dimers through to pentamers being proposed (68, 109).

The protomer forms an unusual wedge-shaped structure with 8 TM regions that vary in length and some of which deviate from perpendicular by as much as 45° as well as two HP loops **(FIGURE 7*C*)**. It is interesting to note that when the mammalian glutamate transporters were first cloned, there was controversy about the assignment of TM domains, with 6, 8 and 10 TM domains proposed (136, 196, 249). Subsequent studies on EAAT1, GLT-1 (rat EAAT2), and the bacterial glutamate transporter

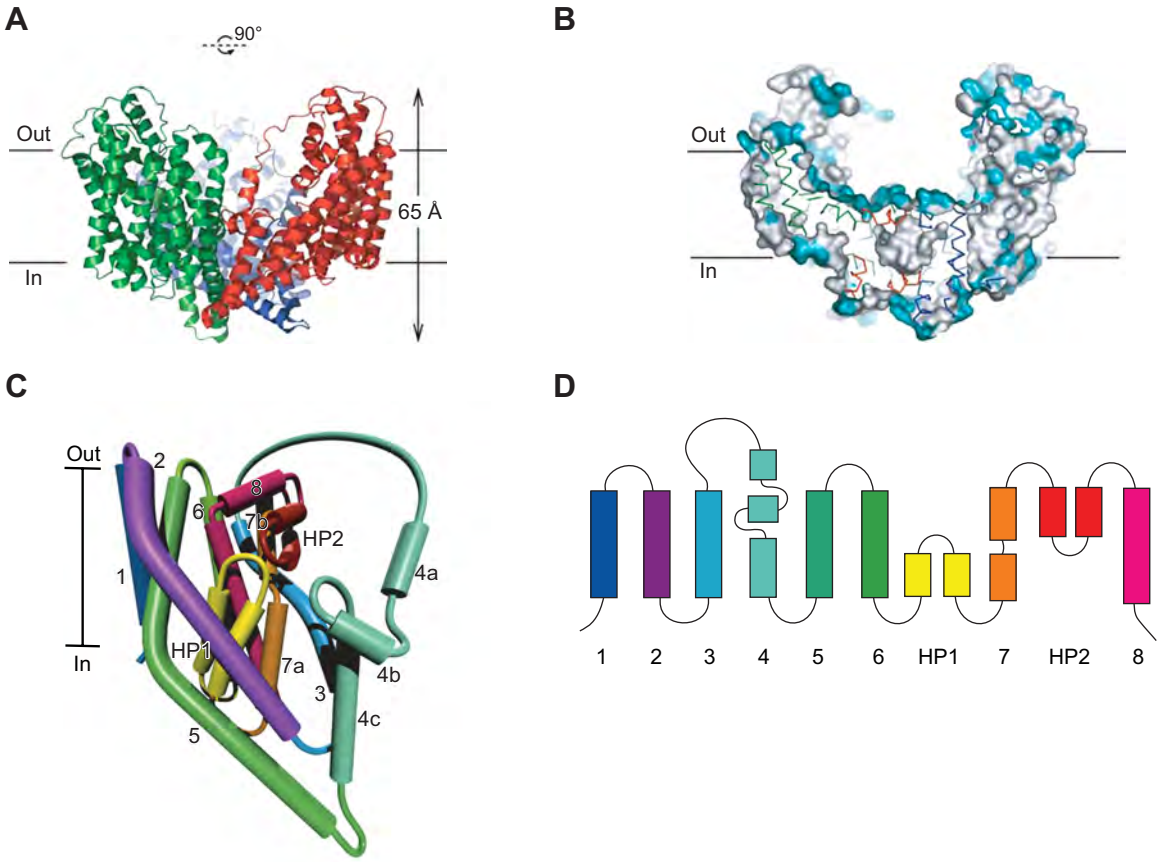

FIGURE 7. Structure of Glt_{Ph}. *A*: the Glt_{Ph} trimer viewed in the plane of the membrane with each protomer colored red, blue, and green. *B*: the aqueous bowl shown by surface representation of the trimer sliced through the center of the basin. Polar and apolar residues are colored cyan and white, respectively. *C*: schematic representation of a single protomer of Glt_{Ph}. *D*: a two-dimensional topology diagram of a single protomer colored as in *C*. PDB 1XFH. [From Yernool et al. (299). Reprinted by permission from Macmillan Publishers Ltd.]

from *B. stearothermophillus* postulated the existence of HP or reentrant loops, based on the patterns of exposure of cysteine mutants to the extracellular solution, the intracellular solution, and those that were inaccessible (100, 102, 103, 229–231, 243, 245). The proposed locations of the reentrant loops correspond reasonably well with the HP loops identified in the structure of Glt_{Ph} (**FIGURE 7*D***), with the discrepancy of one versus two reentrant loops being explained by the alternate accessibility of A385C (132). In addition, the results of several studies that have investigated proximity relationships between pairs of introduced cysteine residues provide further support that the structure of the Glt_{Ph} protomer is conserved in the human glutamate transporters (33, 47, 153, 201, 223).

D. Substrate, Blocker, and Ion Binding Sites in Glt_{Ph} and Comparisons with the EAATs

The transport domain for each protomer is formed by TM3, 6, 7, and 8 and HP1 and 2 (26, 205, 299), and within these TM regions, the central unwound portion of TM7, the center of TM8 together with the loop regions of HP1 and HP2, form the substrate binding site (**FIGURE 9*A* AND TABLE 2**). The amino group of the substrate forms contacts with the side chain of D394 (TM8) and the backbone carbonyl groups of residues R276 (HP1) and V355 (HP2). The α-carboxyl group of the substrate forms contacts with the side chains of N401 (TM8) and T398 (TM8) and the main chain N of S278, while the γ-carboxyl interacts with the side chains of T314 (TM7) and R397 (TM8). In earlier mutagenesis studies of the EAATs, it was demonstrated that the specificity for acidic amino acids over small neutral amino acids was due to the γ-carboxyl group interactions with the arginine residue in TM8 (equivalent to R397 in Glt_{Ph}). In the neutral amino acid transporters, ASCT1/2, there is a threonine or cysteine residue at the corresponding site, and a R447C mutation in EAAT3 alters substrate selectivity such that small neutral amino acids are transported and there is reduced acidic amino acid transport (20, 44). In addition, D444 in EAAT3 (equivalent to D394 in Glt_{Ph}) has been shown to be important for acidic amino acid binding and productive transport (261).

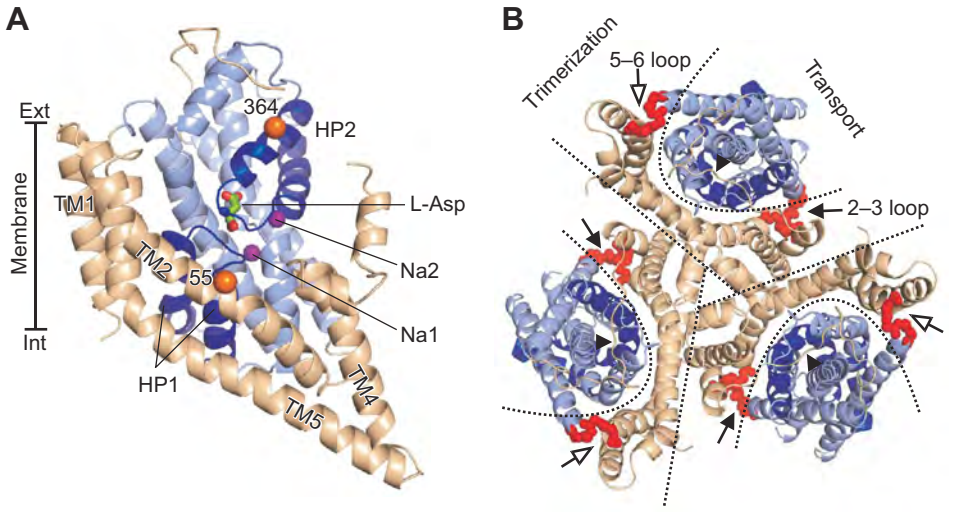

FIGURE 8. Trimerization and transport domain of Glt_{Ph}. A single protomer shown in the plane of the membrane (*A*) and the trimer viewed down the center from the extracellular side of the membrane (*B*). The protomer is divided into the "trimerization domain" (TM1, TM2, TM4, and TM5, colored in light brown) and the "transport domain" (TM3, TM6, TM7, and TM8 in light blue, HP1 and HP2 in dark blue). Bound aspartate is shown in stick representation, and Na1 and Na2 are shown as purple spheres. PDB 3KBC. [From Reyes et al. (205). Reprinted by permission from Macmillan Publishers Ltd.]

Glt_{Ph} is highly selective for L- and D-aspartate over L-glutamate, whereas the EAATs transport L- and D-aspartate as well as L-glutamate with similar efficacy. At present, there is little understanding of the structural basis for this difference in substrate selectivity. The substrate binding domain is highly conserved between Glt_{Ph} and the EAATs, and there are no obvious amino acid residue differences that could explain why Glt_{Ph} is so highly selective for aspartate over glutamate. Furthermore, there are several pharmacological substrates of the EAATs, such as L-serine-*O*-sulfate, 3-MG, and 4-MG (see sect. IV*A1*) that are not transported by Glt_{Ph}. This wider range of substrates for the EAATs can also be extended to nontransportable blockers. Threo-β-benzyloxyaspartate (TBOA) is a nonselective inhibitor of the EAATs and also Glt_{Ph}, but other EAAT subtype selective nontransportable blockers, such as kainate and dihydrokainate, do not inhibit transport by Glt_{Ph} (220). The role of the highly conserved methionine residue in the NMDG motif of TM7 has been investigated in EAAT3 (212). While the affinity for L-aspartate is unchanged for the M367L mutant

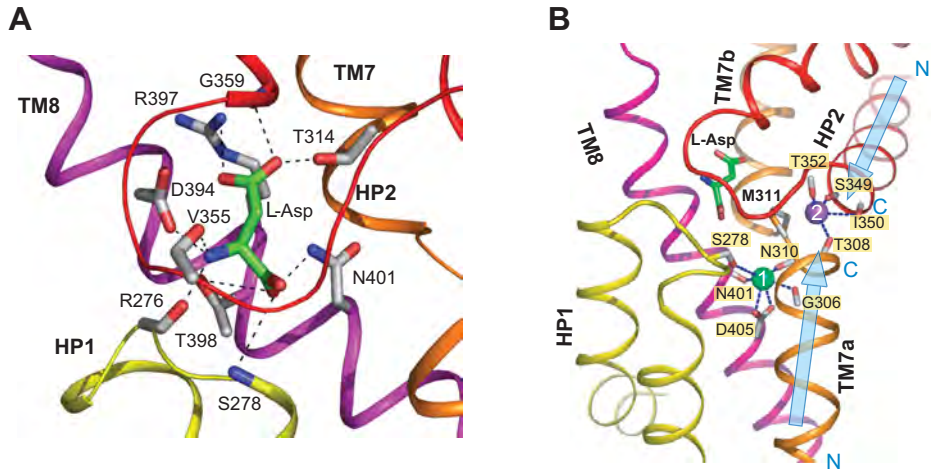

FIGURE 9. Aspartate and Na^+ binding sites (Na1 and Na2) in Glt_{Ph}. *A*: view of the aspartate-binding site showing HP1 (yellow), TM7 (orange), HP2 (red), and TM8 (magenta). A remarkable number of polar contacts solvate the highly charged substrate and include interactions with D394, main-chain carbonyls of R276 (HP1) and V355 (HP2), the amide nitrogen of N401 (TM8), the hydroxyl of T398 (TM8), the main-chain nitrogen of S278 (HP1), the guanidinium group of R397 (TM8), the hydroxyl of T314 (TM7), and the main-chain nitrogen of G359 (HP2). *B*: oxygen atoms that are within 3.5?Å of the sodium ions are labeled and connected to the sodium ions by dashed lines. Light blue arrows represent the dipole moments of helices TM7a and HP2a. PDB 2NWX. [From Boudker et al. (26).]

Table 2. *Amino acid residues that contribute to the formation of substrate and ion binding sites in Glt_{Ph} and the EAATs*

Ligand Site	Residues in Glt_{Ph}	Residues in EAATs	Location	Comments	Reference Nos.
Substrate	R276		HP1	Amino group of substrate	26
	S278		HP1	α-Carboxyl group of substrate	
	T314		TM7	γ-Carboxyl group of substrate	
	V355		HP2	Amino group of substrate	
	D394		TM8	Amino group of substrate	
	R397	D444 (EAAT3)	TM8	γ-Carboxyl group of substrate	261
	T398	R447 (EAAT3)	TM8	α-Carboxyl group of substrate	20
	N401		TM8	α-Carboxyl group of substrate	262
Na1 site	G306		TM7	Identified by thallium binding in Glt_{Ph} crystals	26
	N310		TM7		
	N401		TM8		
	D405		TM8		
		L303	TM5	Identified by simulation and mutagenesis experiments	241
Na2 site	T308		TM7	Identified by thallium binding in Glt_{Ph} crystals	26
	S349		HP2		
	I450		HP2		
	T352		HP2		
Na3 site	Y89		TM3	Proposed from simulation and mutagenesis experiments	15, 121, 146, 210, 241, 259, 260
	T92	T101 (EAAT3)	TM3		
	S93		TM3		
	N310		TM7		
	D312		TM7		
	T314		TM8		
	G404		TM8		
	N401	N451 (EAAT3)	TM8		262
K^+ site		Y403 (EAAT2)	TM7	Proposed from electrostatic mapping and mutagenesis experiments	20, 44, 112, 138, 221, 257, 263, 304
		E404 (EAAT2)	TM7		
		D367 (EAAT3)	TM7		
		D444 (EAAT3)	TM8		
		R447 (EAAT3)	TM8		
		D454 (EAAT3)	TM8		
		R477 (EAAT1)	TM8		
H^+ site		E373 (EAAT3)	TM7	From mutagenesis experiments	95
Cl^- channel	S65	S103 (EAAT1)	TM2	Anion selectivity	116, 119, 143, 222, 223
		D112 (EAAT1)	TM2	Cl^-channel gating	
		D272 (EAAT1)	TM5	Cl^- channel gating	
		K384 (EAAT1)	TM7	Cl^- channel gating	
		R385 (EAAT1)	TM7	Cl^- channel gating	

(equivalent to M311 in Glt_{Ph}), both D-aspartate and L-glutamate affinity were reduced by 10- to 20-fold. Although these observations do not explain the differential substrate selectivity between Glt_{Ph} and the EAATs, they do suggest that the methionine residue influences they way substrates fit into the binding pocket.

E. The Location of the Na^+ Binding Sites: Mutagenesis Studies

Transport via Glt_{Ph} and the EAATs is coupled to the cotransport of 3 Na^+ (98, 302), but at the moderate resolution of the Glt_{Ph} crystal structures (~3.5 Å), it is not

possible to distinguish Na^+ from bound water molecules. To probe cation binding sites on Glt_{Ph}, protein was crystallized in the presence of the heavy atom thallium (Tl^+) which has a strong anomalous signal. This method revealed that each protomer contained two Tl^+ binding sites, and competition studies showed that these sites were selective for Na^+ over K^+ and Li^+ (26). Na^+ #1 (Na1) sits below the bound substrate and is coordinated by elements of TM7 and TM8, while the site for Na^+ #2 (Na2) is formed by HP2 and TM7 **(FIGURE 9*B*)**. Most of the interactions for Na1 and Na2 are with main chain carbonyl oxygen atoms, which occur due to their proximity to nonhelical TM segments. This is also seen in other Na^+-coupled transporters such as the Na^+/Cl^--dependent neurotransmitter transporter family homolog ($LeuT_{Aa}$) (297), the Na^+-K^+-ATPase (181), and the Na^+/H^+ exchanger (123). The site for the third coupled Na^+ (Na3) has not been identified using crystallographic methods, but several additional Na^+ sites have been proposed using MD simulations and mutagenesis which will be discussed below.

1. Na1

There is only a single side chain, of D405 in TM8, that coordinates Na1 **(FIGURE 9*B*)**, and mutation of this residue to an asparagine in Glt_{Ph} (D405N) reduces the coupling of Na^+ to aspartate binding and also leads to the loss of this binding site in the crystal structure (26). MD simulations support the existence of the Na1 site in Glt_{Ph} (15, 92, 110, 121), with one study identifying an additional leucine residue (L303) that coordinates Na1. Mutation of the equivalent residue in EAAT1 resulted in an ~20-fold decrease in Na^+ affinity compared with wild-type EAAT1, suggesting a role for this residue in Na^+ binding (241).

Currently, there is some debate as to whether the Na1 site is conserved in the human glutamate transporters. A study that investigated the equivalent mutation to D405N in EAAT3 (D454N) found that this mutant transporter was not capable of transporting glutamate, but glutamate binding could be measured as it blocks a leak chloride conductance. With the use of this measure, it was found that the Na^+ dependence of glutamate binding is not affected by the D454N mutation, which led the authors to conclude that this residue did not form part of the Na1 site (260). In contrast, another study by Kanner and colleagues (263) found that mutations of the same aspartate residue in GLT-1 (EAAT2) and EAAC1 (EAAT3) to Asn, Ser, or Cys resulted in transporters that were capable of functioning in the exchange mode but had altered cation selectivity and suggest that this aspartate residue participates in an overlapping Na^+ and K^+ binding site. A more recent study proposes that D454 in EAAT3 is protonated at physiological pH and does participate in Na^+ binding, presumably of Na1, and thus the D454N mutation does not affect Na^+ binding but disrupts the K^+-dependent relocation step (182). In contrast, the D405N mutation does affect Na^+ and aspartate coupling in Glt_{Ph}, and this transporter is not coupled to the countertransport of K^+ (26, 220), suggesting that this residue may play a different role in Glt_{Ph} compared with the human glutamate transporters.

2. Na2

The second Na^+ identified in the crystal structure of Glt_{Ph} (Na2) is coordinated by four carbonyl oxygens from HP2 and TM7 with the COOH termini of HP2a and TM7a pointing toward this site, suggesting that the dipole moment of these broken helices may play a role in the formation of this cation binding site **(FIGURE 9*B*)** (26). Although no side chains were identified in the crystal structure of Glt_{Ph} that directly coordinate Na2, mutagenesis studies of residues in HP2 have shed light on the cation dependence of transport and blocker interactions with the EAATs.

Early studies on the cation dependence of transport by the mammalian glutamate transporters revealed that Li^+ could support substrate transport by EAAT3 (25) but at least one Na^+ could not be replaced by Li^+ in EAAT2 (101). Two serine residues (S440 and S443) that are unique to EAAT2 and located in HP2 were found to be responsible for this difference in the cation dependence of transport (25, 305). In addition, S440 is important for determining whether compounds such as MPDC and 4MG are substrates or inhibitors of EAAT2, and the coupling cation also influences the properties of these compounds. Together with other studies that have demonstrated that the affinity of Na^+ for the transporter is dependent on the substrate being transported (176, 278), these findings suggest that S440, which is located between bound substrate and Na2, may play a key role in substrate/cation coupling in the EAATs.

3. Na3

The third Na^+ binding site in Glt_{Ph} has not been identified using crystallographic methods, but several sites have been proposed based on evidence from site-directed mutagenesis experiments, electrostatic calculations, and molecular dynamics (MD) simulations. One of the proposed sites is defined by residues N310 and D312, which are part of a highly conserved motif (NMDGT) in the nonhelical region of TM7 and also three residues from TM3 (Y89, T92, and S93) (15) **(FIGURE 10, *A* AND *B*)**. A site very similar to this one has been proposed by Tajkhorshid and colleagues (121) that includes an additional water molecule but does not include the backbone carbonyl oxygen from Y89. Mutagenesis studies of two of the proposed coordinating ligands in Glt_{Ph} (T92 and S93) and EAAT1 (T130 and T131) reduce the ability of Na^+ to support transport, suggesting these residues are important for Na^+ binding/coupling (15). The existence of a Na^+ binding site at this position is also supported by other mutagenesis studies in EAAT3 where T101 (T92 in Glt_{Ph}) was shown to be important for Na^+

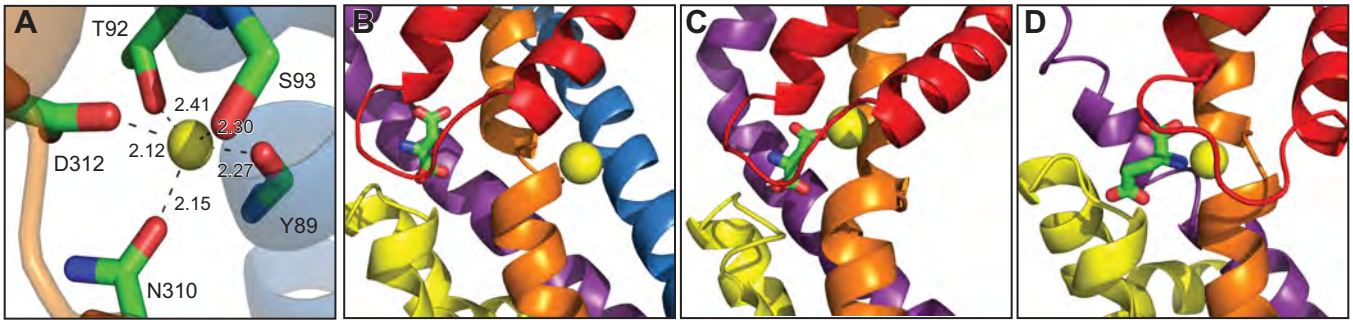

FIGURE 10. The third Na^+ binding site in Glt_{Ph}. *A*: the coordination of the Na3 binding site identified in Bastug et al. (15). TM3 (blue) and TM7 (orange) are shown in the cartoon, and the coordinating residues are shown in stick representation and labeled. Na3 is shown as a yellow sphere, and all distances are in Å. *B–D*: a close-up view of the substrate binding site with Na3 (yellow sphere) as predicted by MD simulations from Bastug et al. (15) (*B*), Larsson et al. (146) (*C*), and Shirivasta et al. (241) (*D*). HP1 (yellow), TM7 (orange), HP2 (red), and TM8 (magenta) are shown in cartoon representation, and bound substrate is shown in stick representation. PDB files of MD simulations were kindly supplied by Serdar Kuyucak (*B*), Sergei Noskov (*C*), and Ivet Bahar (*D*). Figures were made using Pymol (226).

binding (259), D368 (D312 in Glt_{Ph}) has been suggested to bind Na^+ prior to substrate (260), and the interaction between N366 and D368 (N310 and D312 in Glt_{Ph}) has been shown to be important for transporter function (210).

Two other Na^+ binding sites have been proposed that are formed by elements of TM7 and TM8 and are closer to the substrate binding site. Larsson et al. (146) have proposed an alternative site for the third Na^+ ion that is formed by T314 (TM7), A353 (HP2), N401 (TM8), and the bound Na^+ ion appears to form part of the substrate binding site **(FIGURE 10*C*)**. Another study demonstrated that mutation of N401 (N451 in EAAT3) changed the cation selectivity and substrate affinity of transport supporting the existence of a cation binding site which involves N401 (262). In addition, Shirivasta et al. (241) identified a site that is formed by N310 and D312 from TM7 and G404 from TM8 **(FIGURE 10*D*)**.

4. Evidence for other Na^+ binding sites in the EAATs

Several other mutagenesis studies have proposed a Na^+ binding role for residues in the transport domain that are not directly involved in any of three Na^+ sites in Glt_{Ph} described above. Mutation of a conserved tyrosine residue in TM7 of EAAT2 to phenylalanine (Y403F) increases the affinity for Na^+ and also allows other cations, such as Li^+ and Cs^+, to support transport (304). An adjacent aspartate residue in TM8 (D440) affects both Na^+ and K^+ affinities, and it was proposed that this binding pocket may serve as a transient or stable cation binding site (211). In addition, it has been proposed that an aspartate residue in TM8 of EAAT3, in combination with bound substrate, determines the affinity of Na^+ for this transporter (258).

The precise location of the three coupled Na^+ in Glt_{Ph} and the EAATs may only be resolved by a higher resolution structure of Glt_{Ph} and/or a high-resolution structure of a human glutamate transporter. In addition, there may be several sites in the protein where Na^+ transiently interact on their path into and/or out of their binding sites which may explain the number of residues implicated in Na^+ binding from mutagenesis studies and also the various sites predicted from MD simulations.

5. Order of substrate and Na^+ binding

The stoichiometry of ion coupling of glutamate transport by the EAATs has been known for over 15 years (156, 192, 302), but the order of ion and substrate binding has only recently become clear. Several kinetic studies of EAAT function predict that at least two of the coupled Na^+ bind before substrate, and the third coupled Na^+ binds after (141, 259, 306). The crystal structure of Glt_{Ph} revealed two Na^+ binding sites, and it was proposed that Na1 bound first, followed by substrate and Na2 (26). The recent identification of the Na3 binding site in Glt_{Ph} together with data measuring the free energy of binding of the coupled Na^+ and aspartate, supports the order predicted from the EAAT studies where Na3 binds first followed by Na1, substrate, and then finally Na2 which serves to lock HP2 down over the substrate binding site **(FIGURE 11)** (15, 110). The binding of Na2 and the closure of HP2 is required for transport as the nontransportable blocker TBOA, which prevents the closure of HP2 and the formation of the Na2 site, arrests the transporter in an open-to-out state **(FIGURE 12*A*)** (26).

F. H^+/K^+ Binding Sites in the EAATs

Glutamate transport by the EAATs is coupled to the cotransport of a H^+ and the countertransport of a K^+. This additional coupling, particularly to the outwardly directed K^+ gradient, increases the concentrating capacity of the EAATs but can also be detrimental under ischemic conditions (2). Glt_{Ph} transport is not coupled to H^+ or K^+; there-

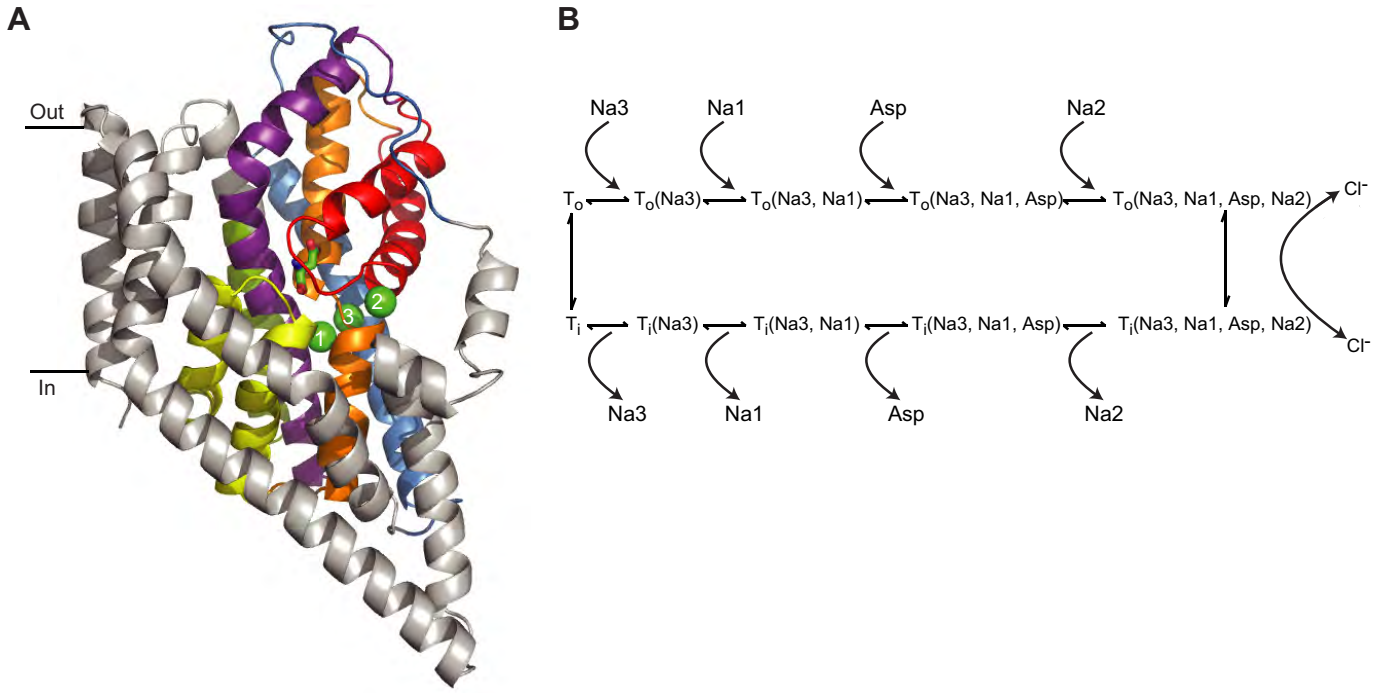

FIGURE 11. Order of binding for aspartate and Na^+ to Glt_{Ph}. *A*: a single protomer of Glt_{Ph} is shown in the plane of the membrane with bound aspartate, Na1 and Na2 (PDB 2NWX) and Na3 as identified in Bastug et al. (15). The trimerization domain is shown in gray, and the transport domain is colored (TM3, blue; TM6, green; HP1, yellow; TM7, orange; HP2, red; TM8, magenta). The 3 Na^+ are shown as green spheres and numbered. *B*: the proposed order of binding for substrate and ions to Glt_{Ph}. Figure was made using Pymol (226). [From Bastug et al. (15).]

fore, the structures of Glt_{Ph} shed little light on how the EAATs are coupled to these ions, but several mutagenesis studies have identified residues that appear to play a role in K^+ or H^+ coupling in the EAATs.

1. K^+ binding site

A glutamate residue in TM7 is conserved in the EAATs but is replaced with a glutamine residue in the K^+-independent transporters ASCT1/2 and Glt_{Ph}. Mutation of E404 in EAAT2 to a glutamine residue results in a transporter that is no longer coupled to the countertransport of K^+ and instead acts as a Na^+-dependent exchanger (138). Mutation of the adjacent residue in EAAT2 (Y403) has a similar effect (304), as does removing the positive charge of R477 in EAAT3, a residue that interacts with the γ-carboxyl group of bound glutamate (20, 44). Another study investigated the position of an arginine residue in EAAT1 and Glt_{Ph} and found that when an arginine residue (R477, EAAT1 numbering) is removed from TM8 and transferred to HP1 (which mimics Glt_{Ph}), the resulting double mutant transporter is no longer K^+ dependent and has an increased affinity for substrate. The opposite mutation in Glt_{Ph} resulted in a transporter with reduced affinity for substrate and an increased turnover rate, but this double mutant transporter was not dependent on intracellular K^+ (221). It has also been shown that Tl^+ can partially replace intracellular K^+ to support glutamate transport in EAAT3 and that mutation of the residue equivalent to the Na1 ligand (D405) in Glt_{Ph} has a greater effect on Tl^+ affinity compared with Na^+ affinity, which led the authors to suggest that the Na1 site identified in Glt_{Ph} may actually be a K^+ binding site in EAAT3 (257). These results are supported by a study described above that suggests D405 participates in an overlapping Na^+ and K^+ binding site in EAAT2 (263).

The mutations described above result in a similar phenotype, but these residues are not close in three-dimensional space and are unlikely to form a single K^+ binding site. In addition, another K^+ binding site was proposed using electrostatic mapping of an EAAT3 homology model (112). This site partially overlaps with the substrate binding site and also contains a conserved aspartate residue (D444) that is important for glutamate binding (261), which means that glutamate and K^+ binding are mutually exclusive in this model, which agrees with functional evidence. Further information is required to understand if and how these residues contribute to K^+ binding and how this coupling mechanism drives the reorientation of the glutamate transporters.

2. H^+ binding site

Intracellular acidification as a result of glutamate transport was observed as early as 1983 (66), yet there was some

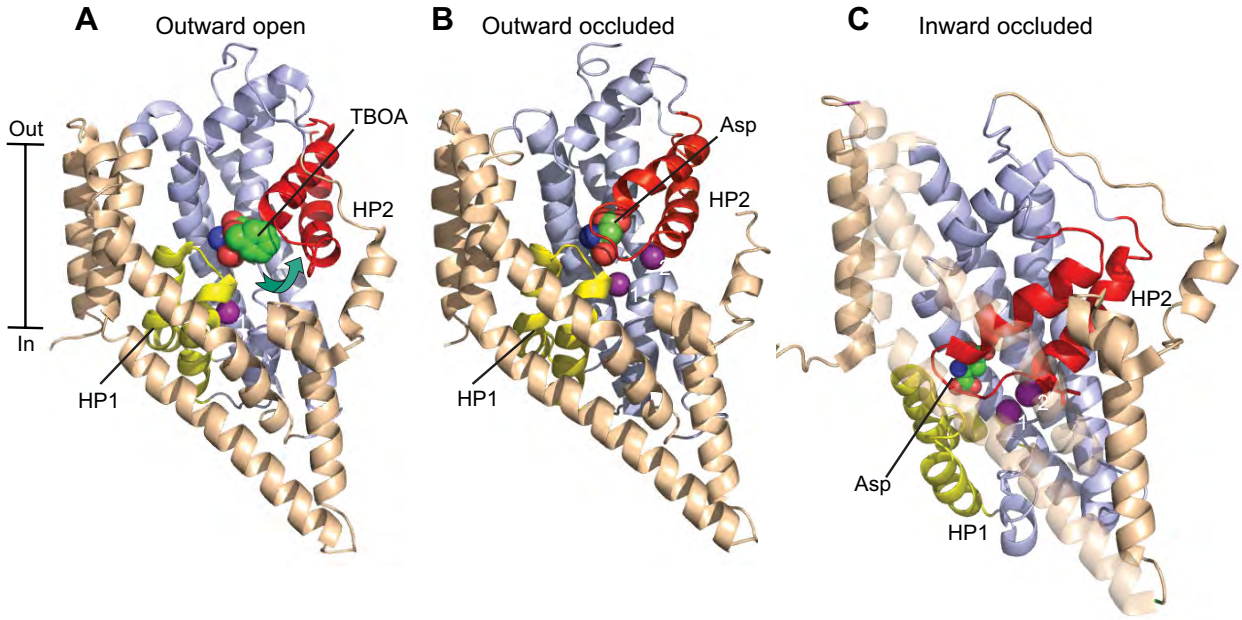

FIGURE 12. The different conformational states of Glt_{Ph}. A single protomer of Glt_{Ph} is shown in the plane of the membrane in three different conformational states that have been observed in crystal structures. The "trimerization domain" (TM1, 2, 4, 5; light brown) and the "transport domain" (TM3, TM6, TM7, TM8; light blue; HP1, yellow; HP2, red). Bound TBOA (*A*) and aspartate (*B* and *C*) are in space filling representation; Na1 and Na2 are shown as purple spheres. Please note movements of HP2 (red) and HP1 (yellow). *A*: the benzyl ring of TBOA props open HP2 (indicated by green arrow). PDB 2NWW. *B*: HP2 is closed over bound aspartate. PDB 2NWX. *C*: Asp and Na^+ are ready to be released into the cell, but their path is blocked by HP1. PDB 3KBC. Figures made using PyMol (226).

controversy as to whether a H^+ was cotransported with glutamate or a OH^- countertransported along with K^+ in the relocation step of the transport cycle (28, 66, 227). In 1996, Zerangue and Kavanaugh (302) clearly demonstrated that glutamate transport via EAAT3 was coupled to the cotransport of 1 H^+. A follow-up study confirmed this finding in EAAT2 (156), and it was suggested that protonation of the substrate glutamate could be the process by which H^+ was coupled to transport (244). Two subsequent studies revealed that protonation of EAAT3 was required to form a high-affinity substrate binding site, suggesting that protonation of the substrate itself was not likely to be the mechanism by which protons were coupled to glutamate transport (232, 290). A conserved glutamate residue in EAAT3 (E373), which corresponds to E404 in EAAT2 discussed in relation to K^+ coupling above, was suggested to be the H^+ acceptor (95). Mutation of E373 to glutamine results in a transporter that cannot catalyze net flux, but can support electroneutral Na^+-dependent exchange that is pH independent. In addition, the introduction of this glutamate residue into the H^+-independent ASCT2 transporter renders the transporter pH sensitive, demonstrating that this reside plays an important role in H^+ coupling (95).

G. Conformational Changes and the Mechanism of Transport

The first information about movements of Glt_{Ph} came from the structure of Glt_{Ph} in complex with the nontransportable inhibitor TBOA (26). The aspartate moiety of TBOA binds to the substrate binding site in a similar manner to aspartate, but the additional benzyl ring attached to the β-carbon props HP2 in an open conformation, which exposes the substrate-binding site to the extracellular solution and disrupts the binding of Na2 **(FIGURE 12*A*)**. This movement of HP2 has also been observed in a fluorescent-based ligand binding assay (26) and in computational studies (120, 241) and suggests that HP2 may serve as an extracellular gate of the transporter. Both structures of TBOA- and aspartate-bound Glt_{Ph} likely represent so-called outward facing states of the transporter, in which the substrate-binding site is near the extracellular solution and either "open" to or "occluded" from the extracellular solution **(FIGURE 12, *A* AND *B*)**.

The structure of an inward facing state of Glt_{Ph} in complex with aspartate was solved by crystallizing Glt_{Ph} with two introduced cysteines in the presence of Hg^{2+} (205). The cysteine residues at positions K55 (TM2) and A364 (HP2) had previously been shown to form a spontaneous disulfide bond in EAAT1 (223), but these residues are over 20 Å apart in the "occluded out" structure. This inward-facing structure demonstrates that the trimerization domain remains relatively rigid, which agrees with other cross-linking data (99), while the transport domains moves ~18 Å toward the cytoplasm across the lipid bilayer resulting in the substrate and ion binding sites facing the cytoplasm **(FIGURE 12*C*)**. This inward oc-

cluded conformation was also predicted by the examination of the inverted structural repeats that are present in Glt_{Ph} (47). These are structural elements that are approximately related by a twofold symmetry and are positioned in an anti-parallel orientation in the membrane (27). The bound substrate molecule in the "inward occluded" structure is still occluded from the intracellular solution under the tips of HP1 and HP2, and it remains unknown how aspartate is released into the cytoplasm. Hints from MD simulations suggest that ions and substrate unbind in the reverse order (see sect. III*E5*); Na2 unbinds first, followed by aspartate and Na1 (52, 92), but further functional and structural studies of the "inward open" state of Glt_{Ph} and the EAATs are needed to confirm these predictions.

The structures of the outward- and inward-facing states not only provide a mechanism by which the substrate and bound ions are carried across the membrane, but also illustrate how each protomer within the trimer can function independently, which is supported by both functional (94, 140, 148, 222) and computational (133, 248) studies. At present, there is little information about how the transporter returns to the "outward-facing" state, either empty in the case of Glt_{Ph} or K^+ bound for the EAATs. This step is predicted to be the rate-limiting step in the transport cycle, which may make it difficult to study using MD simulations because of the longer simulation times required.

Large-scale conformational changes have also been predicted on the basis of further cross-linking studies with EAAT1 (133). Single cysteine substitutions of residues within HP2 are able to form inter-protomer crosslinks with corresponding residues in neighboring protomers. In the crystal structure of Glt_{Ph}, these residues are more than 40 Å apart, which suggests that the individual protomers must undergo considerable movements within the trimeric complex. This observation was followed up by simulation studies using an anisotropic network model, which predict that such large-scale movements within the trimeric complex are possible (133). At this stage, it is not clear how these large-scale movements influence the smaller scale intra-protomer movements required for the transport process. In addition, there is some controversy as to the existence of the inter-protomer crosslinks. Two other studies investigating one of the residues described in the study above (V452/A364) did not observe any inter-protomer crosslinking within the trimer of EAAT1 (223) or Glt_{Ph} (205) under similar experimental conditions.

H. Formation and Gating of the Chloride Channel

Traditionally, transporters and ion channels have been thought of as functionally and structurally distinct, but there are a growing number of membrane proteins, including the glutamate transporters, that appear to contain the dual functions of a transporter and a channel. The first hints as to the structural elements that form the channel of glutamate transporters came from a series of studies using site-directed mutation studies of the EAATs. The groups of Susan Amara, Baruch Kanner, and our group identified residues in HP2 that when mutated to cysteine and modified with MTSET caused the block of glutamate transport without affecting glutamate activation of the chloride channel (26, 214, 221). These studies demonstrated that it was possible to selectively manipulate one function without affecting the other, which suggests that there were distinct molecular determinants for the two functions. A similar conclusion was drawn from a study of EAAT3, where it was demonstrated that Li^+ could be used to partially support transport but not allow activation of the chloride channel (25). These studies were followed up by an investigation of the role of TM2 in EAAT1 in the formation of the channel (223). Three lines of evidence support the conclusion that TM2 plays an integral role in channel formation. First, cysteine mutants of TM2 residues showed greater sensitivity towards negatively charged MTS reagents than positively charged MTS reagents, demonstrating that this region is aqueous accessible and has a preference for negatively charged molecules (223). Second, mutations of residues in TM2 alter the extent of activation of the chloride channel without affecting the rate of glutamate transport (223). Third, the S103V mutation changes the relative anion permeability sequence of the channel such that Br^- is more permeant than NO_3^- (223). This last point indicates that the S103 residue is likely to form part of a Cl^- selectivity filter within the channel. Glt_{Ph} also allows a substrate-activated Cl^- conductance through the transporter, and the equivalent residue in TM2 is S65. The S65V mutant Glt_{Ph} also shows impaired Cl^- channel activity (222), which reenforces the conclusion that residues in TM2 form part of a Cl^--selective channel through the transporter.

The intracellular loop between TM2 and TM3 contains a series of charged residues. Mutation of D112A in EAAT1 generated a transporter with altered Cl^- channel properties. Application of the transport blocker TBOA reveals a large constitutive Cl^- leak conductance through the mutant transporter. The mutant transporter supports normal levels of glutamate transport, but the transport process does not activate the Cl^- channel any further than the background constitutive activity. These observations suggest that the mutation locks the Cl^- channel of the transporter in an open state that is insensitive to glutamate (223). Further studies by Fahlke and colleagues have investigated the equivalent residue in EAAT3 (116) and EAAT4 (143) and suggest that neutralization of this conserved aspartate residue in these isoforms results in more complex changes where anion conduction, anion gating, and substrate transport are affected.

The initial work on TM2 mutants was carried out prior to knowledge of the crystal structure of Glt_{Ph}. To further characterize the roles of regions in close proximity to TM2, a homology model of EAAT1 was used to identify charged residues that may influence channel function (119). Charged residues in TM5 (D272) and TM7 (K384 and R385) are likely to be in close proximity to D112. Mutations of these residues increased the amplitudes of both the leak and glutamate transport-activated conductances observed. However, none of the mutations altered the relative anion permeability of the channel (119). These results suggest that D112, D272, K384, and R385 are unlikely to form part of the selectivity filter for the channel, but may regulate the rate and extent of channel opening and closing. We have attempted to rationalize how the functional properties of these mutant transporters, together with what we know of the conformational states of Glt_{Ph}, can be explained to provide a structural model for Cl^- channel gating and ion permeation. We proposed that the Cl^- channel of the transporter would open as the "transport domain" passes through the "trimerization domain," and these mutations at the intracellular edge of these domains may affect the ability of these domain movements and thus the gating of the Cl^- conductances **(FIGURE 13*A*)** (274, 279). This model has gained further support from the "inward occluded" structure which shows the large movement the "transport domain" undergoes during transport (205) **(FIGURE 12*C*)** and also a more recent intermediate structure from Boudker and colleagues (282) that reveals the formation of an aqueous pore in the vicinity of S103 (S65), which has been suggested to be the beginning of a Cl^- channel **(FIGURE 13*B*)**.

IV. EXOGENOUS AND ENDOGENOUS MANIPULATION OF GLUTAMATE TRANSPORTER FUNCTIONS

The activity of glutamate transporters can be manipulated in a number of ways by pharmacological agents, endogenous compounds, and also through a variety of cellular regulatory systems. In this section we review the different types of pharmacological agents and how they alter the functional properties of EAATs and then discuss how various endogenous compounds can regulate EAAT function and expression levels. Finally, we discuss how the activity of glutamate transporters can be regulated by protein kinase C and through association with a variety of enzymes, scaffold proteins and other membrane proteins.

A. Pharmacology of Glutamate Transporters

Pharmacological inhibitors can be broadly classified into two groups: substrate inhibitors and blockers **(FIGURE 14)**. Substrate inhibitors often mimic the structures of the endogenous substrates L-glutamate and L-aspartate and can be

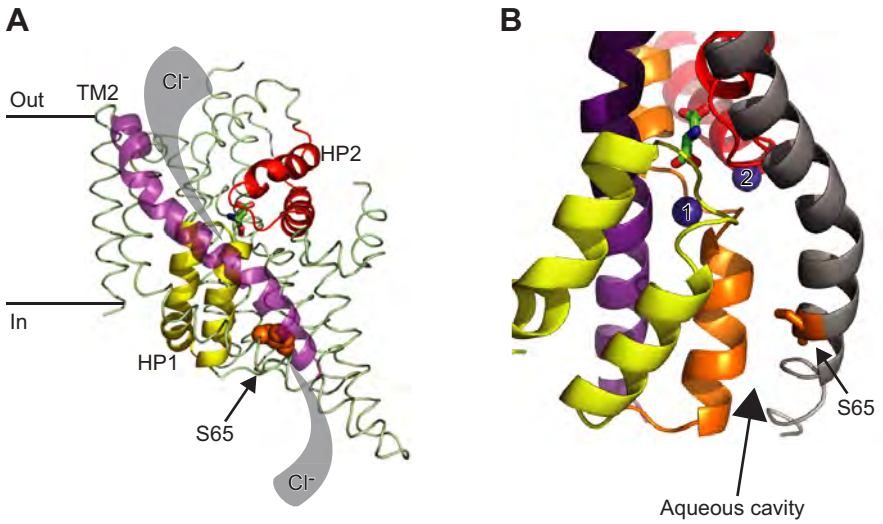

FIGURE 13. A possible Cl^- permeation pathway through a Glt_{Ph} protomer. *A*: the aspartate-bound protomer viewed in the plane of the membrane, PDB 2NWX. HP1 (yellow), HP2 (red), and TM2 (purple) are shown in cartoon representation while the rest of the protomer is shown in ribbon representation. S65 in TM2 of Glt_{Ph} (highlighted in orange), which is equivalent to S103 in TM2 of EAAT1, is likely to form part of the anion selectivity filter. TM2 is in the stable "trimerization domain," and movements of the inner "transport domain" relative to the "trimerization domain" may open a pore allowing Cl^- to pass through the transporter. The proposed Cl^- pathway is depicted in gray. [From Vandenberg et al. (274).] *B*: a close-up of the region around S65 in the intermediate outward facing structure (iOFS), PDB 3V8G. An aqueous cavity is seen surrounding S65 (orange stick) lined by residues from HP1 (yellow) and TM7 (orange). Bound aspartate is shown in stick representation, and Na1 and Na2 are shown as blue spheres, TM2 (gray), HP2 (red), and TM8 (magenta). Figures were made using PyMol (226).

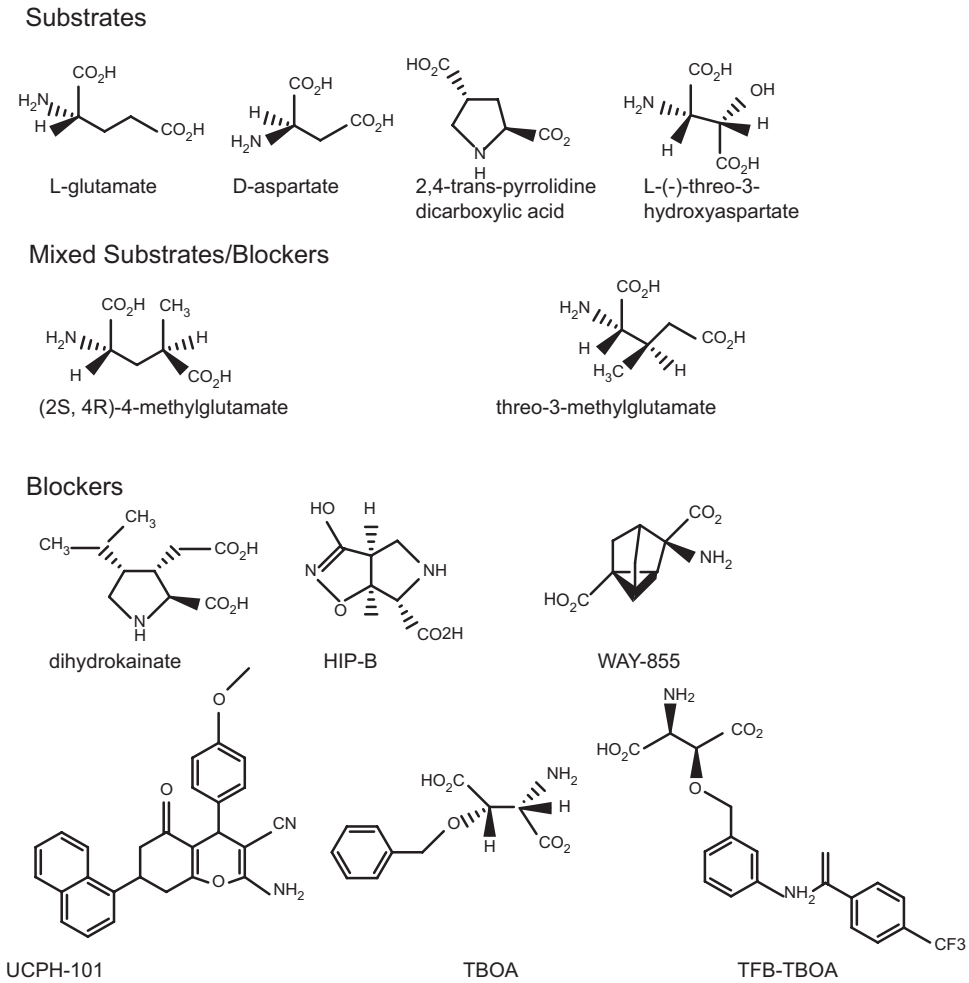

FIGURE 14. Structures of substrates, mixed substrate/blockers, and blockers.

transported by the EAATs and in doing so prevent L-glutamate from being transported. On the other hand, blockers bind to the transporter and prevent L-glutamate from binding, but are not transported themselves. Although there are a few exceptions, most substrates are smaller than blockers. Pharmacological inhibitors of EAATs are unlikely to be of therapeutic value, but the main uses of these compounds have been to use EAAT subtype-selective inhibitors to understand the physiological roles and distributions of the various EAAT subtypes and also to probe different functional states of the transporters.

1. Substrate inhibitors

The first and probably most studied pharmacological substrate of the EAATs is D-aspartate. D-Aspartate is transported with similar efficacy as the endogenous substrates L-glutamate and L-aspartate for the five EAAT subtypes (6, 7, 13, 70). While there is no selectivity for subtypes, ^{3}H-labeled D-aspartate has been particularly useful because it is not metabolized once it enters the cell (13). Other nonselective substrates that have also been useful include *threo*-β-hydroxy-aspartic acid, L-cysteic acid, and L-cysteine-sulfinic acid (7, 13). Two substrates that shows subtype selectivity are L-cysteine and L-serine-*O*-sulfate. L-Cysteine is a substrate for EAAT3 with kinetic properties similar to that of L-glutamate and L-aspartate, but has very low affinity for the other transporters subtypes (303). L-Serine-*O*-sulfate has ~10-fold higher affinity for EAAT1 and EAAT3 compared with EAAT2, and also shows an intriguing voltage dependence of uptake by EAAT2 (7, 179, 278). The various pharmacological substrates also show differences in transport efficiency. For example, the maximal transport rate for threo-β-hydroxy-aspartic acid by EAAT1 is 55% of that of glutamate, whereas for EAAT2 the maximal rate is 33% of that of glutamate (7).

Another class of substrates used for the study of transporters has been "caged glutamate." Caged glutamate is a glutamate molecule with a nitrobenzyl group attached through the α-carboxyl group. Upon ultraviolet irradiation, the nitrobenzyl group is released to leave glutamate. The advantage of this style of compound is that it is possible to achieve very rapid application of glutamate to transporters. With this method,

Grewer and colleagues (96, 289) have made very precise measures of the early stages of the glutamate transport cycle.

2. Mixed substrate inhibitors/blockers

The methyl-glutamate series of compounds are particularly interesting from a mechanistic point of view. *2S,4R*-4-methylglutamate is a substrate of EAAT1 but is a blocker of EAAT2 (276). Threo-3-methylglutamate is inactive at EAAT1 but is a reasonably potent blocker of EAAT2 (276), a weak blocker of EAAT3, and a substrate of EAAT4 (65). In a recent study it was demonstrated that the difference in substrate versus blocker actions for 2*S*,4*R*-4-methylglutamate at EAAT1 and EAAT2 is related to the ion coupling capacity and also a Gly for Ser residue difference in the HP2 domain that forms part of the substrate recognition site (118).

Another class of compounds that also shows different actions at the EAAT subtypes are the pyrrolidine dicarboxylic acids (PDCs). These compounds consist of a pyrrolidine ring with two carboxyl groups attached at various locations around the ring. In contrast to the methylglutamates, the PDC compounds do not have any appreciable activity at glutamate receptors, which makes them more useful in studying EAATs in vivo. L-*Trans*-2,4-PDC acts on all five subtypes but is a substrate for EAAT1–4 and a blocker of EAAT5 (6, 7, 32, 70, 97). L-*Trans*-2,3-PDC has a slightly different profile; it is 10-fold more potent at EAAT2 than the other subtypes, and it is also a blocker of EAAT2 whereas L-*trans*-2,4-PDC is a substrate of EAAT2 (30, 142, 293).

One of the consequences of using a substrate inhibitor is that the compound accumulates inside the cell. This may also then trigger release of the endogenous substrate, L-glutamate, in a hetero-exchange process. In some applications, this can lead to quite different observations than the study of nontransportable blockers, which has prompted the development of EAAT blockers.

3. Nontransportable blockers

The first transport blocker identified was kainate and its closely related compound dihydrokainate. Kainate is related to the pyrrolidine dicarboxylic acids by having a constrained analog of aspartate imbedded into the compound **(FIGURE 14)**. The pyrrolidine ring serves to fix the conformation of the imbedded aspartate and thereby limits the range of sites that it can bind to (see review by Bridges et al., Ref. 29).

These compounds are selective for EAAT2 over the other transporter subtypes (8), but they do show activity on glutamate receptors, especially the kainate receptors. Dihydrokainate shows somewhat higher selectivity for transporters over receptors (134). The poor selectivity of these compounds for transporters over receptors prompted the development of compounds using a different strategy. *Threo*-β-hydroxy-aspartic acid is a substrate of the EAATs, but shows some activity at NMDA receptors. Shimamoto et al. (237) reasoned that by adding extra groups via the β-hydroxyl group that the extra bulk at this position would prevent NMDA receptor binding. The extra bulk at this position would still allow binding to the transporter, but it would prevent the compound from being transported (237). The first compound developed using these ideas was TBOA (237). TBOA binds to all five EAAT subtypes with affinities varying from low to mid micromolar range (see Refs. 235, 237, 240 for all reported values), but has little if any affinity for any glutamate receptors. This provided the first compound capable of blocking all EAAT subtypes with little cross-reactivity with other receptors and allowed transporter function to be studied in vitro and in vivo with greater accuracy. The study of TBOA interactions with the archaeal homolog of the EAAT family, Glt_{Ph}, has also allowed a greater understanding of the mechanism of inhibition and the gating mechanism of the transporter (see sect. III*G* for more details). The remaining limitation in the use of TBOA is its comparatively low affinity for the transporters. With an affinity in the low micromolar range, it is not particularly well suited for ligand binding assays. This shortcoming prompted the development of higher affinity blockers based on the same principles used for the development of TBOA. Addition of larger groups to the β-hydroxyl group achieves this aim. TFB-TBOA contains an extra benzyl derivative and has affinity for the EAATs in the low to mid-nanomolar range (239), which is much better suited for ligand binding assays (238).

The in vivo effects of TBOA have been studied, and as may be expected, TBOA causes severe convulsive behaviors following intracerebroventricular injection. This is likely to be due to increased levels of extracellular glutamate. TFB-TBOA has higher affinity for the EAATs in in vitro preparations and is also more potent in inducing convulsions (239). For further information on TBOA and related analogs, see the review by Shimamoto (236).

A new class of EAAT1-selective transport blockers has recently been developed, which are considerably larger and do not bear any obvious structural similarity to glutamate or aspartate. The most potent of these compounds UCPH-101 inhibits glutamate transport by EAAT1 with an IC_{50} of 0.6 μM and has little or no inhibitory effects at EAAT2 and EAAT3 at concentrations up to 300 μM (67, 124, 131). UCPH-101 is a noncompetitive inhibitor that appears to interact with the transporter within a hydrophobic pocket of the trimerization domain (1). Amino acid residues within TM3, TM4c, and TM7a appear to influence the activity of UCPH-101, but it appears that the actions of UCPH-101 are limited to the protomer to which it binds rather than inhibiting the whole trimeric complex. This style of inhibitor is quite different from that of other pharmacological inhibitors in that it is an allosteric noncompetitive inhibitor. At present, there have been few studies using this new inhibitor, but it

should prove to be very useful in distinguishing the roles of the different transporter subtypes and also for developing allosteric modulators of transporters.

Researchers at Wyeth have also developed a series of EAAT2 selective inhibitors (61). The first of these compounds, 3-amino-tricycloheptane-1,3-dicarboxylic acid (WAY-855), shows up to 30-fold selectivity for EAAT2 over other transporter subtypes and with no activity at AMPA receptors or NMDA receptors, but it does show some activity at kainate receptors (61, 62). The more recently developed compound, *N*4-[4-(2-bromo-4,5-difluorophenoxy)phenyl]-L-asparagine (WAY-213613), is more potent (IC_{50} of 80 nM for EAAT2) and also shows 30-fold selectivity for EAAT2 over EAAT1 and EAAT3 with little if any activity at ionotropic and metabotropic glutamate receptors (63, 93). This compound is likely to provide considerable advantages over some of the other EAAT2 blockers due to its lack of activity at other receptors and transporters.

Another constrained analog of glutamate that inhibits EAAT3 is (+)-HIP-B (38, 78). This compound behaves quite differently than most other blockers in that it is not a competitive blocker. Although the compound has a free carboxyl group, it does not appear to interact with the glutamate binding site of the transporter. (+)-HIP-B binds to an allosteric site on the transporter and has a mixed mechanism of inhibition. (+)-HIP-B can bind to multiple conformational states of the transporter, but has a preference for the inward facing configuration (38).

B. Endogenous Modulators of Glutamate Transport

The activity of glutamate transporters can also be modulated by a variety of endogenous compounds that are structurally unrelated to glutamate or aspartate. Furthermore, there are a number of membrane proteins that associate with EAATs that have the capacity to influence transporter functions. In the next section we review the actions of endogenous modulators of EAAT function, small ionic species such as Zn^{2+}, lipid compounds such as arachidonic acid, as well as protein kinases which alter the phosphorylation state of transporters, and then how particular proteins such as glutamate transporter associated proteins (GTRAPs) and the Na^+-K^+-ATPase influence transporter function.

1. Zn^{2+}

Zn^{2+} play many different roles in living organisms. It has been estimated that up to 10% of all proteins utilize Zn^{2+} and include catalytic roles in many enzymes, and key structural roles in DNA binding proteins that regulate gene expression (3). In particular brain regions of the central nervous system, such as the mossy fibers of the hippocampus, Zn^{2+} is stored in glutamatergic vesicles and is coreleased with glutamate upon presynaptic stimulation. The released Zn^{2+} can then modulate the activity of glutamate receptors, various ion channels, and also glutamate transporters (75, 275).

Zn^{2+} concentrations vary over a considerable range. Resting Zn^{2+} is likely to be of the order of 10 nM, but upon electrical stimulation Zn^{2+} concentrations may rise to 100–300 μM (75). This provides a considerable concentration range in which to modulate the activity of the synapse. NMDA receptors respond to low to mid nanomolae Zn^{2+} (57), whereas glutamate transporters are modulated by Zn^{2+} at concentration in the low to mid micromolar range (246, 277).

The actions of Zn^{2+} were first studied on glutamate transporters of the salamander retina (246). In Müller cells, the predominant glutamate transporter is a homolog of the human EAAT1, whereas in cone cells the predominant transporter has properties that are similar to human EAAT5. Zn^{2+} appears to have a direct effect on the transporters, which is readily reversible upon washout of Zn^{2+} from the preparation. In Müller cells, low μM Zn^{2+} noncompetitively inhibits glutamate transport and also stimulates the anion current associated with the transport process. In contrast, Zn^{2+} stimulates the anion current associated with glutamate transport in cone cells.

The actions of Zn^{2+} on the human glutamate transporters have also been investigated. In EAAT1, Zn^{2+} inhibits glutamate transport with minimal or no effect on the anion conductance. For EAAT2 and EAAT3, Zn^{2+} has no effect and for EAAT4, Zn^{2+} inhibits the anion conductance (180). The Zn^{2+} binding site on EAAT1 and EAAT4 has been studied using site-directed mutagenesis (180, 277). Switching His146 or His156 in extracellular loop 2 to Ala abolishes Zn^{2+} sensitivity, and equivalent mutations to corresponding residues in EAAT4 also abolish Zn^{2+} sensitivity. His156 is not conserved in EAAT2, but if this residue is introduced, EAAT2 becomes sensitive to Zn^{2+}.

The Zn^{2+} binding sites on EAAT1 and EAAT4 are located in the second extracellular loop (3–4 loop) of the transporters, which is distinct from the substrate binding sites (26, 180, 277). In the "apo" structure of Glt_{Ph}, this 3–4 loop is in a slightly different conformational state than in the aspartate-bound form of the transporter (26), and this loop undergoes significant conformational changes during the translocation process (43, 205). If Zn^{2+} were to bind to this loop, it may restrict or alter the movements of the loop and thereby cause a reduction in the rate of transport. At this stage, it is difficult to provide a more complete answer as to how Zn^{2+} inhibits the transporters because the second extracellular loop is not well conserved between transporter subtypes (8, 70, 299), so it is difficult to predict the structure or the conformational changes that take place for the human glutamate transporters.

2. Lipids

Arachidonic acid is a 20-carbon *cis*-polyunsaturated fatty acid, which is produced by astrocytes and neurons upon stimulation by glutamate. Arachidonic acid is freely diffusible in biological membranes and may modulate the activity of membrane proteins via direct interactions with the protein, but it can also be converted to a wide variety of related biologically active compounds, including prostaglandins, leukotrienes, and endocannabinoids (reviewed by Attwell and Mobbs, Ref. 9). Although it is not possible to measure free arachidonic acid concentrations in extracellular spaces of the central nervous system, it has been estimated that concentrations up to 30 μM may be generated under normal physiological conditions. Under ischemic conditions or excessive glutamatergic signaling, arachidonic acid concentrations may increase to 100 μM (14) (see sect. V*B*). Another important consideration is that arachidonic acid readily forms micelles at concentrations greater than ~30–50 μM, which places an upper limit on the likely free arachidonic acid concentration available for directly modulating membrane proteins.

Arachidonic acid modulates the activity of glutamate transporters under a number of conditions. A 3-min application of 30 μM arachidonic acid to salamander retina Müller cells causes prolonged inhibition glutamate transport, which suggests that arachidonic acid binds and inhibits the transporter via the membrane lipid-protein interface (14). The most abundant glutamate transporter subtype in salamander Müller cells is a homolog of EAAT1. Arachidonic acid also causes similar levels of inhibition and rates of onset and reversal of inhibition when applied to human EAAT1 expressed in *Xenopus laevis* oocytes (301). The actions of arachidonic acid on GLT1 (EAAT2) have also been investigated using proteoliposomes enriched with the transporter, and in this case, arachidonic acid appears to inhibit glutamate transport via an aqueous phase interaction (268). In a separate study of EAAT2 expressed in *Xenopus laevis* oocytes, arachidonic acid stimulates glutamate transport, but at substantially higher arachidonic acid concentrations, which are unlikely to be physiological or even pathological (301).

Arachidonic acid also has a very unusual effect on glutamate transport by EAAT4. Application of arachidonic acid to oocytes expressing EAAT4 in the presence of glutamate or aspartate induces a novel H^+ current through the transporter (69, 199, 269). At this stage, the physiological significance of the H^+ current is not clear. However, it is intriguing that niflumic acid, a cyclooxygenase inhibitor that bears no structural similarity to arachidonic acid, also induces a similar H^+ current in EAAT4 (199). Thus it is possible to pharmacologically mimic the actions of arachidonic acid on EAAT4.

In the original crystal structure of Glt_{Ph} there are a series of external crevices between subunits that are exposed to the lipid membrane, but also provide access for lipids to interact with regions that are close to the glutamate/aspartate binding site (**FIGURE 8*B***). Yernool et al. (299) have speculated that these cavities may have the capacity to bind lipid modulators of transporters. In a more recent structure of Glt_{Ph} trapped in an early transport intermediate state, another lipid exposed cavity between TM1 and TM7 was identified (282). It remains to be tested whether these cavities in Glt_{Ph}, and similar cavities in the EAATs, may form lipid binding sites.

The functions of glutamate transporters are also influenced by the lipid components of the cell membrane (reviewed by Divito and Amara, Ref. 58). Cholesterol regulates the activity of EAAT2 and also EAAC1 (EAAT3). EAAT2 appears to associate with lipid raft structures of the membranes of primary cortical cultured neurons, and disruption of these structures by the depletion of cholesterol results in a reduced rate of glutamate transport. The effect is also observed for EAAT1, EAAT3, and EAAT4 but not to the same degree as for EAAT2 (37). Furthermore, in a study of primary neuronal cultures from rat cortex, it has been demonstrated that cholesterol derived from cocultured astrocytes can increase the rate of neuronal EAAC1 (EAAT3)-mediated glutamate transport (39).

C. Stimulation of EAAT2 Expression and Function

1. Ceftriaxone

In a number of neurodegenerative conditions, reduced expression levels of EAAT2 have been implicated as a causative factor in the pathogenesis of the condition (see sect. V). These observations have prompted the search for compounds that may elevate EAAT2 expression levels and thereby reduce exposure to high levels of glutamate. The group of Rothstein et al. (217) used a novel approach of screening 1,040 FDA-approved drugs for their ability to increase EAAT2 expression levels. A number of drugs caused increases in EAAT2, and the drug with the most robust effects on expression levels was the β-lactam antibiotic ceftriaxone. Furthermore, ceftriaxone also provides a neuroprotective effect in animal models of disease (217). Two groups have followed up this study and found similar results (19, 151); however, other groups have been unable to reproduce these results. Lipski et al. (162) and Melzer et al. (175) found that ceftriaxone does provide neuroprotection in excitotoxic inflammatory CNS damage, but that it does not cause any changes in EAAT2 expression or EAAT2 function. Although the reasons for differences between these studies and those of the Rothstein group are not clear, it is possible that the different cell and animal models used by the various investigators lead to different EAAT2 expression pattern changes. It should be noted that many of the cell-based assays rely on primary cell cultures from fetal or newborn rats or mice. Further work will be required in

animal models that faithfully reproduce the pathological features of the various neurodegenerative disease states.

The mechanism of ceftriaxone stimulation of EAAT2 expression has been investigated in human fetal astrocytes. Ceftriaxone stimulates EAAT2 transcription through a NF-κB signaling pathway. Ceftriaxone stimulates the translocation of p65 and activates NF-κB, which then binds to the EAAT2 promoter and increases transcription (151).

2. Thiopyridazine derivatives

The groups of Lin and Cuny (42) have developed a high-throughput screening approach to identify compounds that increase expression levels of EAAT2. Approximately 140,000 compounds were screened, and 11 of the compounds were investigated in detail. These compounds showed up to fourfold increases in expression of EAAT2 with EC_{50} values in the range of 100 nM to 20 μM. These compounds provided a starting point for the synthesis of novel compounds that elevate EAAT2 expression (296). A series of thiopyridazine derivatives were found to elevate EAAT2 levels by up to sixfold and with EC_{50} values in the the low micromolar range. These studies have been conducted using a primary astrocyte cell line stably transfected with EAAT2. It will be of great interest to see how effective these compounds are in brain slices and also in pathological conditions such amyotrophic lateral sclerosis (see sect. VC).

3. Riluzole

Riluzole is a drug that is currently approved for the treatment of amyotrophic lateral sclerosis (see sect. VC). Riluzole shows anti-excitoxicity activity, but it is likely to mediate these effects via a number of distinct targets (59). One group of the proposed targets are the glutamate transporters EAAT1–3 (77). At 100 μM, riluzole decreases the K_m for glutamate transport by each of the three subtypes by twofold and has no effect on the V_{max} for glutamate transport by EAAT2 and EAAT3 and a marginal increase in V_{max} for EAAT1.

4. Parawixia bistriata toxin

The venom of the spider *Parawixia bistriata* contains a particularly interesting toxin. The venoms of many spiders, snakes, and venomous cone shells contain large mixtures of toxins, many of which inhibit the functions of ion channels, transporters, and receptors. The purified toxin, Parawixin1, is unusual in that it stimulates the rate of glutamate transport by EAAT2 (71, 72). The toxin has no effect on the apparent affinities of glutamate or Na^+, nor does it influence reverse glutamate transport or glutamate exchange in the absence of internal K^+. It was concluded that the toxin facilitates the reorientation of the K^+-bound transporter. This is the rate-limiting step in the transport process, so facilitating this step will increase the turnover rate of the transporter. One of the intriguing questions that arises from this study is how can this toxin bind to the transporter in such a way as to stimulate its function? Does it bind within the membrane to facilitate transmembrane domain movements? Does it bind at an extracellular site that transmits a conformational change to facilitate reorientation? Does it bind to an intracellular site? If so, can it cross the cell membrane to bind to an intracellular site? It will be of great interest to see if some of these questions can be answered and if these observations can lead to the development of novel compounds that speed up the rate of glutamate transport.

D. Protein Kinase C

The strength and dynamics of glutamatergic neurotransmission is influenced by the geometry of the synapse, and the number and distribution of receptors and transporters. At synapses with a high density of transporters, glutamate is rapidly buffered and cleared. Conversely, at synapses with lower density of transporters, glutamate can diffuse from the synapse to other synapses and influence their activity. Protein kinase C (PKC) is a well-studied regulator of glutamate transporter expression and activity (reviewed by Gonzalez and Robinson, Ref. 90). The actions of PKC on glutamate transporters vary between transporter subtypes and are also dependent on the cell type expressing the transporter. Prior to the cloning of glutamate transporters, it was well recognized that phorbol esters (PKC activators) stimulate the V_{max} of glutamate transporter by astrocytes, but had no effect on transport by neurons (40).

PKC has different effects on GLT1 (EAAT2) depending on the cell type in which it is expressed. Human retinoblastoma cells only express EAAT2, and in these cells, PKC decreases glutamate transport by increasing the K_m for transport (82). However, in cells transfected with GLT1 (EAAT2), activation of PKC has no effect on glutamate transport (254). Furthermore, in primary cultures of a mixture of neurons and astrocytes derived from embryonic rat tissue, activation of PKC causes a rapid decrease in cell surface expression without affecting the total GLT1 (EAAT2) expression (135). In this study, the site of action of PKC on GLT1 (EAAT2) was localized to a 43-amino acid residue region of the COOH-terminal domain. Mutation of S486 partially abolishes the effect of PKC.

PKC also has different effects on GLAST1 (EAAT1) function depending on the cell type in which it is expressed. When expressed in *Xenopus laevis* oocytes, PKC reduces glutamate transport currents, but this does not correlate with a reduction in cell surface expression, and thus we may conclude that this effect is not related to internalization of the transporter (45). In cerebellar glial cells, PKC decreases GLAST1 (EAAT1)-mediated uptake through a reduction in the V_{max}, and a reduction in cell surface expression (89). However, in primary cultures of

forebrain astrocytes, PKC increases the V_{max} (40, 60), while at the same time causing a loss in GLAST1 (EAAT1) immunoreactivity which may result from modification of intracellular epitopes such that they are no longer recognized by anti-GLAST antibodies (252).

PKC has been shown to increase cell surface expression of EAAC1 (EAAT3) in C6 glioma cells and in neuron-enriched cultures (50, 88). However, in transfected Madin-Darby cells and in *Xenopus laevis* oocytes, PKC activation causes a decrease in cell surface expression (266).

From these apparently contradictory observations, it is clear that the regulation of glutamate transport activity by PKC is complex and likely to differ from cell type to cell type and also by the way that PKC is stimulated. There are multiple subtypes of PKC, which differ in their mechanisms of activation, their activity on various targets, and their distribution. PKC subtypes may be classified into three groups. The first group includes the β and γ subtypes and is activated by diacylglycerol and phorbol esters and also requires Ca^{2+}. Members of the second group do not require Ca^{2+}, but are activated by phorbol esters and diacylglycerol and include the δ, ε, θ, and η subtypes. The last group includes the ζ and λ subtypes, which are insensitive to diacylglycerol, Ca^{2+}, and phorbol esters (185). The groups of Robinson and Rothstein have collected a large body of data demonstrating important roles for PKC in regulating glutamate transporter function. We will highlight some of the key studies below. Through the use of a series of selective and nonselective PKC inhibitors, it has been possible to isolate the effects of PKC subtypes on transporter functions. C6 glioma cells have been a widely used cell line for the study of glutamate transporters, and in these cells the PKC α, ε, and δ subtypes are expressed. PKC-α mediates the increase in cell surface expression of EAAC1 (EAAT3) in these cells, while PKC-ε also increases glutamate transporter function by possibly increasing the catalytic efficiency of the transporter (88). In Müller cells of the retina, PKC-δ plays the predominant role in maintaining the expression of GLAST1 (EAAT1) at the cell surface (36). In C6 cells, transfected with GLT1 (EAAT2), PKC stimulation results in the association of ubiquitin with GLT1 (EAAT2). This complex is then targeted for endocytosis and degradation (234).

E. Proteins That Associate With or Regulate Glutamate Transporters

Glutamate transporters are enriched in presynaptic and postsynaptic membranes and in astrocytic processes, which implies that there are proteins that facilitate the enrichment of transporters in these locations. In the following section we will discuss the various scaffolding proteins and signaling molecules that influence transporter function and expression.

1. Na^+-K^+-ATPase and glycolytic enzymes

Glutamate transport requires the cotransport of 3 Na^+, 1 H^+ and the countertransport of 1 K^+ and with the high expression levels of glutamate transporters there is considerable flux of cations associated with the transport process. In recent years it has become apparent that the glutamate transporters, GLAST1 (EAAT1) and GLT1 (EAAT2), form macromolecular complexes with the Na^+-K^+-ATPase (85, 206). Presumably this provides a ready source of Na^+ and K^+ or at least to maintain the local ion gradients at optimal levels to facilitate the transport process. At present, it is not clear how the two proteins associate or the degree of cooperativity that they confer upon each other.

Maintaining the Na^+ and K^+ ion gradients required for glutamate transport consumes a considerable amount of energy (see reviews in Refs. 107, 170), but glutamate can also be a source of energy. Robinson's group (85) has investigated the association of glutamate transporters with various intracellular proteins using immunoprecipitation followed by mass spectrometry and proteomic analysis to identify additional proteins that form complexes with glutamate transporters. A large number of proteins have been identified, and they can be classified into the following classes of proteins: plasma membrane proteins; chaperones, trafficking, and adaptor proteins; signaling molecules; glycolysis enzymes; and mitochondrial proteins. It is intriguing that many of these proteins may be classified as being involved in energy metabolism because this suggests that this cocompartmentalization provides the means to spatially coordinate glutamate transport with the energy demands of the cell (16, 85, 87). Glutamate transport may also be involved in regulating protein synthesis. Glutamate transport by EAAT1 expressed in retinal Müller cells has been shown to trigger a metabolic signaling pathway leading to phosphorylation of mTOR (mammalian target of rapomycin) and AP-1 binding to DNA (164). These observations suggest that transporters may also form an integral component of extra- and intracellular signaling pathways mediated by glutamate.

2. Glutamate transporter associate proteins

Rothstein's group identified a series of glutamate transporter associated proteins (GTRAPs) that regulate the cell surface expression of the EAATs (160, 219). The first of these proteins, GTRAP3–18, slows the trafficking of EAAT3 from the endoplasmic reticulum (ER) to the cell surface (160, 169, 219). GTRAP3–18 is found exclusively in the ER, and while it can regulate EAAT3 expression/trafficking, it also regulates other trafficking events through interactions with the small GTPase protein Rab1 (169). GTRAP41 and GTRAP48 associate with EAAT4 and appear to stabilize the transporter at the cell surface, making it less likely to be internalized or degraded (126).

V. GLUTAMATE TRANSPORTERS IN PATHOLOGICAL STATES

As glutamate transporters play a fundamental role in regulating the activity of glutamatergic synapses, it is not surprising that there are many examples of neurological conditions that are associated with altered glutamate transporter function. In most instances, disruptions to transporter functions cause elevated glutamate levels. These increases may be dramatic as in the case of ischemia following a stroke, or more subtle and prolonged as in the case of amyotrophic lateral sclerosis (ALS) or Alzheimer's disease (AD), which can lead to excitotoxicity and cell death. Dysfunctional transport can arise through a number of mechanisms. Reduced energy supply to the brain and disruptions to ion gradient homeostasis will alter the concentrating capacity of transporters causing elevations in extracellular glutamate. Altered expression levels of various transporter subtypes will change the capacity of transporters to efficiently clear extracellular glutamate. The production of aberrant splice variants will generate nonfunctional transporters, which may then reduce the function of normal variants. Some of the transporter subtypes are particularly susceptible to oxidizing conditions that can reduce their functional capacity. Finally, a number of point mutations in transporters have been identified, and some of these significantly reduce or abolish transporter function. A note of caution should be made in interpreting EAAT expression levels in pathological states. In a recent study, Li et al. (157a) demonstrated that the COOH-terminal and NH_2-terminal regions of GLT1 (EAAT2) and to some extent GLAST1 (EAAT1) are particularly susceptible to degradation in post mortem tissue. As many antibodies used to detect EAAT expression levels are directed against the terminal regions of the transporters, measured reductions in expression levels may be exaggerated (157a). In the next section we first review what we have learned from gene knockout and knockdown studies in mice and then how glutamate transporter functions are impaired in various human neurological disorders. In many instances, it is not clear whether altered glutamate transporter function is a primary contributing factor in the pathogenesis of the condition or the altered function is generated as a consequence other factors.

A. Gene Knockout and Knockdown Studies

EAAT2/GLT1 is responsible for the majority of glutamate uptake, and in cortex of EAAT2/GLT1 knockout mice <10% of glutamate transport activity is maintained. These mice only survive a couple of weeks after suffering from spontaneous seizures and acute cortical injury. Analysis of synaptic transmission shows that synaptically released glutamate levels are increased and remain elevated for longer periods in the knockout mice (255). Furthermore, the mice are significantly more susceptible to acute brain injury. Heterozygotes of the GLT1 knockouts do not show any overt pathological features, but they do show moderate sensorimotor impairment, hyperlocomotion, lower anxiety, and altered fear conditioning (139a). It has been suggested that these mice may provide interesting models for studying neurodegenerative disorders with mild hyperglutamatergic activity (139a).

EAAT1/GLAST1 knockouts do not produce an overt phenotype. Although EAAT1 is enriched in the cerebellum, it was quite surprising that EAAT1 knockouts do not cause any marked changes in cerebellar anatomy or electrophysiological characteristics of glutamate neurotransmission. The EAAT1 antisense oligonucleotide-treated mice do show greater degrees of edema volume when subjected to traumatic brain injury and also show subtle changes in gait and coordination (287).

EAAT3/EAAC1 knockout mice do not show any initial overt symptoms but do show reduced spontaneous motor activity and elevated glutamate levels (194). However, older EAAT3 knockout mice show characteristics of dicarboxylic amino aciduria (elevated acidic amino acids secreted in the urine; see sect. V*G*). Furthermore, neuronal glutathione levels are reduced, which may be responsible for age-dependent neurodegeneration. It appears that EAAT3 is one of the major routes for the uptake of cysteine (see sect. IV*A1*), and the reduced cysteine uptake may be responsible for reduced glutathione production leading to oxidative stress and degeneration (4). EAAT3 antisense oligonucleotide treatment leads to reduced EAAT3 protein expression and also makes the mice more susceptible to clonic seizures (215).

B. Ischemia and Stroke

In ischemia there is a reduction in blood supply, which deprives the brain of oxygen and glucose. This causes a reduction in ATP production, which will then have a myriad of consequences. One of the immediate critical disruptions that occurs is a rundown in the activity of the Na^+-K^+-ATPase, with a subsequent diminution of the Na^+ and K^+ gradients across neuronal and glial cell membranes. As glutamate transport is coupled to the cotransport of 3 Na^+, 1 H^+ and the countertransport of 1 K^+, disruption to the Na^+ and K^+ gradients will reduce the concentrating capacity of the transporter, leading to an elevation in extracellular glutamate concentrations **(FIGURE 15)**. In a review by Allen et al. (2), the impact of a modest increase of extracellular K^+ from 3 to 23 mM has been calculated based on the transport equation in section I of this review and also a few simplifying assumptions (see Ref. 2 for details). The minimal extracellular glutamate concentration would rise more than 1,000-fold from 0.3 nM to 0.4 μM. Experimentally measured resting synaptic glutamate concentrations are not as low as 0.3 nM with one estimate of the resting concentration being 25 nM (111), but nevertheless, an increase to 0.4 μM will have a significant impact on the function of

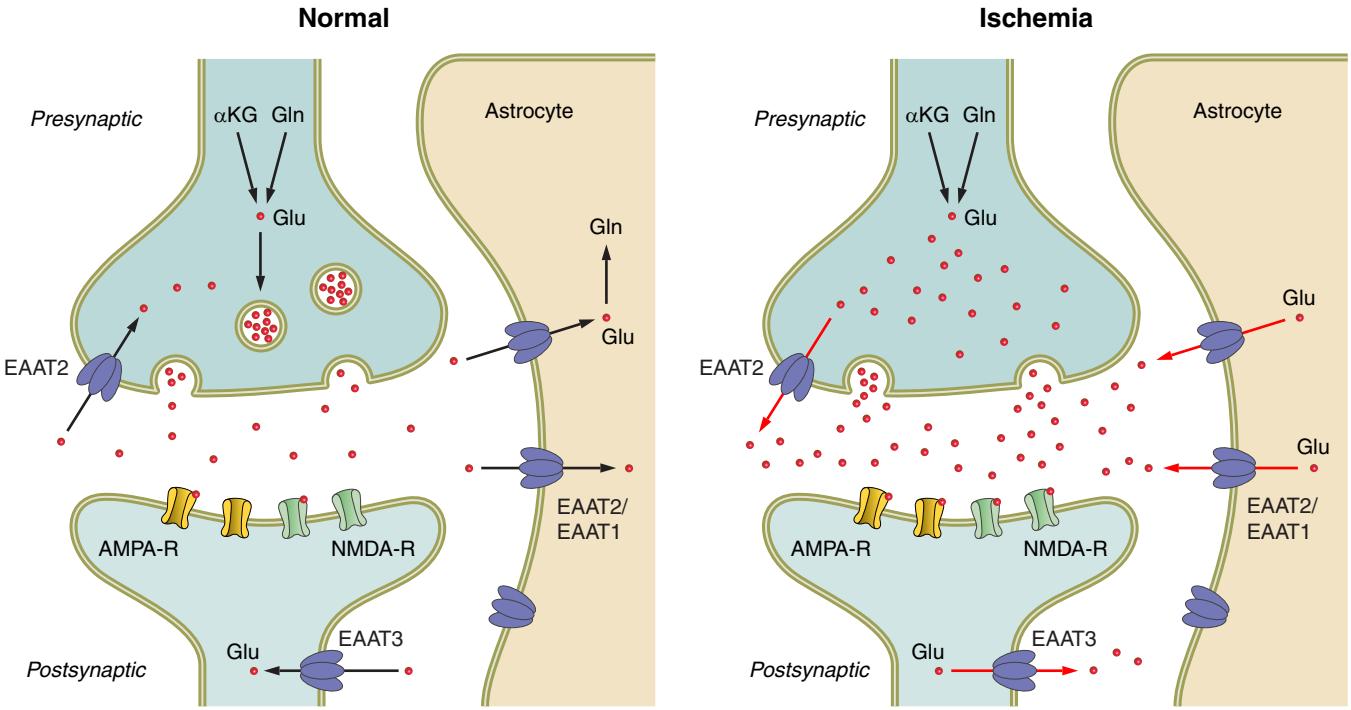

FIGURE 15. Schematic diagram of a glutamatergic synapse under normal conditions and under ischemic conditions. Under normal conditions, glutamate can be synthesized from glutamine or from α-ketoglutarate (108, 174), and after glutamate is released into the synapse, it is removed by glutamate transporters on the pre- and postsynaptic membranes and also glial cells. EAAC1 (EAAT3) is located on the postsynaptic membrane, whilst GLAST (EAAT1) and GLT1 (EAAT2) are located on glial cell membranes. Under ischemic conditions, disruptions to Na^+, K^+, and pH gradients will cause transporters to function in reverse, leading to elevated extracellular glutamate concentrations (see text for details). [Figure concept adapted from Allen et al. (2).]

glutamate receptors. The EC_{50} for glutamate activation of NMDA receptors is of the order of 0.5–3.3 μM (see review by Traynelis et al., Ref. 265) and so a sustained concentration of 0.4 μM may generate significant glutamate receptor activity. Under ischemic conditions, elevations of K^+ may be as high as 60 mM (105, 213), which would cause an even greater elevation in extracellular glutamate.

It appears that EAAT3, located on neuronal membranes, is largely responsible for the elevation of extracellular glutamate (83). The reason for the selective role of EAAT3 is that the cytosolic neuronal glutamate concentration is higher than the cytosolic glial cell glutamate concentration (191). With a higher internal glutamate concentration, the transporter is not able to maintain such a low external concentration (substitution of 10 mM for 1 mM internal glutamate concentration into the above transport equation will translate into higher extracellular glutamate concentration).

Transient ischemia can also lead to prolonged changes in glutamate transporter expression patterns. A number of studies have reported reductions in EAAT2 in the cortex and hippocampus following transient ischemia, while studies on EAAT3 and EAAT1 have yielded conflicting results, with both increases and decreases observed (91, 137, 139, 202). Nonetheless, as EAAT2 is the predominant glutamate transporter, reductions in EAAT2 are likely to reduce the clearance rate of glutamate and contribute to a prolonged excitotoxic response.

C. Amyotrophic Lateral Sclerosis

ALS is a progressive loss of motor neuron function, which causes a rapid decline in motor function, while leaving intelligence and awareness relatively unaffected. It has an incidence of 1–2/100,000, and in 90% of cases, the causes are unknown. In the remaining 10%, a genetic component has been identified. There are a number of theories for the cause of ALS, and one idea that has gained some traction is disrupted regulation of extracellular glutamate leading to excitotoxicity and cell death. Increased levels of glutamate in the cerebrospinal fluid of ALS patients compared with age-matched controls have been detected by various groups (125, 197, 198, 218), and reduced levels of glutamate uptake have been observed (214). It appears that there is a selective loss of EAAT2, with EAAT1 and EAAT3 relatively unaffected (214). One of the genetic factors that has been associated with ALS is a point mutation in the enzyme superoxide dismutase 1 (SOD1) (35). SOD1 is required for the inactivation of oxygen free radicals, and the mutant protein has impaired activity leading to elevated oxygen free radicals. The EAAT2 subtype is particularly susceptible to elevated levels of the free radicals, which may be due to the

larger number of redox-sensitive cysteine residues in EAAT2 compared with other transporter subtypes. Free radical modification of EAAT2 may then lead to reduced EAAT2 function and impaired glutamate clearance (267). ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions (35). In Sprague-Dawly rats that overexpress the ALS-associated SOD1 G93A mutant, EAAT2 expression levels are reduced in the early stages of disease progression. These changes occur prior to neuronal cell loss, which suggests that the reduced EAAT2 are contributing factors in cell loss (117).

Another potential mechanism for reduced EAAT2 levels in ALS is through aberrant splicing events in the maturation of mRNAs encoding EAAT2. Elevated levels of EAAT2 splice variants that are missing exons 3, 7, and 9 have been detected in ALS patients (161). These variants give rise to nonfunctional transporters and have been reported to also cause downregulation of functional variants, which could explain the reduced glutamate clearing capacity in ALS patients (161). It should also be noted that these nonfunctional aberrant splice variants are also found in age-matched controls, and there is an ongoing debate as to the relative amounts of the variants in different conditions (115, 149, 177, 183).

D. Alzheimer's Disease

AD is characterized by senile plaques surrounded by dystrophic neurites, neurofibrillary tangles, and regional atrophy caused by neuronal loss. While the well-known β-amyloid and tau proteins have roles in AD etiology, the mechanisms responsible for the regional specificity of pathological change have yet to be resolved. Glutamatergic neurons are preferentially lost in AD, and reductions in glutamate transport have been reported in brain regions affected in AD and also astrocytes derived from AD patients and transgenic animals with tau protein abnormalities (48, 73, 74, 106, 158). One alternate hypothesis to explain neuronal loss is reduced glutamate transporter function leading to excitoxicity and cell death (17, 228). Consistent with these suggestions are observations of altered glutamate transporter expression levels and patterns of expression (127, 157). However, in other studies, changes in EAAT2 expression in AD have not been observed (18).

Aberrant splice variants of EAAT2 mRNA and the translated protein have been detected in AD brains in post mortem tissue, which are elevated compared with age-matched controls. Furthermore, in one study where elevated nonfunctional splice variants are detected, the levels of the functional EAAT2 variants are reduced in AD brains compared with controls. These results suggest that EAAT2 function may be impaired in AD. However, a cautionary note should be taken into account when interpreting whether these changes can explain the reduced EAAT2 function. When the aberrant variants were expressed in *Xenopus laevis* oocytes, a 10-fold excess of aberrant splice variant RNAs over wild-type RNA levels were required to reduce the functional capacity of wild-type EAAT2 transporters (228). While there may be regions of the AD brain with greatly elevated aberrant splice variant production, most estimates of these levels are not sufficient to explain the reduced EAAT2 functional capacity. Thus, at this stage, it is not clear whether aspects of the pathological features of AD are caused by a loss in EAAT function or the changes in EAAT function are a consequence of other more fundamental changes in neuronal function.

E. Chronic Pain

Under normal physiological states, intrathecal injection of the glutamate transport inhibitor TBOA induces significant dose-dependent nociceptive behavioral responses (159). This is not unexpected because inhibition of transport will elevate glutamate levels and cause significant excessive stimulation of glutamate receptors on spinal cord sensory neurons. Furthermore, in chronic pain states, glutamate uptake is reduced, which suggests that elevated glutamate may be a contributing factor in generating the behavioral responses (24, 251). However, a very intriguing response is observed for intrathecal injection of TBOA in animal models of neuropathic pain and inflammatory pain. In contrast to physiological conditions where TBOA induces pain, in chronic pain models, TBOA alleviates pain responses (298). Why do the responses to TBOA change depending on the conditions in which it is used? At present there are no clear answers, but there are some very interesting possibilities that may lead to the development of alternate therapeutic approaches for the treatment of pain. If we assume that TBOA causes an increase in extracellular glutamate, a number of possibilities arise. First, elevated glutamate may induce toxicity of glutamatergic neurons and thereby diminish the capacity for sensory transduction. Second, elevated glutamate levels may stimulate metabotropic glutamate receptors to inhibit subsequent neurotransmitter release and diminish signaling. Third, a prolonged small elevation of glutamate levels may initially cause excessive glutamate receptor activation, but may be followed by desensitization and diminished sensory transduction. An alternative possibility is that TBOA may in fact reduce extracellular glutamate levels under pathological conditions. Under chronic pain states there may be increased energy utilization associated with excessive sensory neuron activity. This may reduce the supply of ATP and the ability to maintain efficient glutamate transporter activity (see above discussion on ischemia). Under this pathological state, glutamate transporters may function in reverse leading to elevated glutamate concentrations. TBOA is a transport blocker and is equally effective at blocking uptake of glutamate as blocking the reverse operation of the transporter. Thus TBOA

may in fact act to limit the pathological elevation in glutamate levels. One of the key observations lacking that is required to address these possibilities is being able to measure resting and TBOA-induced glutamate levels in both normal physiological states and in chronic pain states. For a more detailed discussion of these possibilities, see the review by Tao et al. (256).

F. Obsessive Compulsive Disorder

Obsessive compulsive disorder (OCD) is a complex neurological condition that affects 1–3% of the population and appears to have a genetic component to its etiology (203). A number of imaging studies and animal behavior studies have implicated altered glutamate neurotransmission as a contributing factor (186, 195, 207–209). In addition, a number of genetic linkage studies have been conducted which reveal an association between OCD and mutations in the gene encoding EAAT3 (SLC1A1) (5, 56, 104, 145, 242, 247, 291, 294). Nineteen different SNPs have been identified in the SLC1A1 gene with the minor allele varying in frequency from 0.09 to 0.46 (242, 247). The SNPs are located in exons, introns, and both the coding and noncoding regions of the protein. There have been few follow-up studies on the consequences of the mutations, with one exception. A T164A mutation in one individual with OCD has been detected, and this mutation has been reported to cause a reduction in the maximal velocity of transport and a small decrease in K_m (281). However, subsequent studies have not detected any significant differences in transporter function (Ryan and Vandenberg, unpublished results).

G. Dicarboxylic Amino Aciduria

Dicarboxylic amino aciduria is a metabolic disorder characterized by the excretion of glutamate and aspartate in the urine due to incomplete reabsorption of these amino acids from the glomerular filtrate in the kidney. It is a relatively rare autosomal recessive disease with an estimated frequency of 1:36,000 (11). Recently, two mutations were identified in EAAT3, which is the main acidic amino acid transporter in the kidney and intestine, in two individuals with dicarboxylic amino aciduria. The 1333 C-T mutation and the 1184–1186 deletion result in a R445W substitution and a I395 deletion, respectively (12). The mutant transporters were expressed in *Xenopus laevis* oocytes and also a canine kidney cell line (MDCKII) to characterize their functional properties. The EAAT3 del395 is not expressed at the cell surface, and no function was detected (12). The R445W mutant is expressed at the cell surface, but alters the phenotype of the transporter. The expression levels achieved were ~10% of that of wild-type EAAT3, and the affinity of glutamate for the transporter was increased by 15-fold. The mutation also decreased the proportion of current generated by the transport process compared with the uncoupled chloride conductance (see sect. II*A3*). One of the patients with the R445W mutation presented with kidney stones and upon subsequent investigation was found to have greatly elevated urinary glutamate and aspartate. Further investigation of a sibling identified similar elevated glutamate and aspartate levels and the same mutation. As mentioned throughout this review, glutamate homeostasis in the brain is critical to normal brain function, and EAAT3 is a widespread neuronal glutamate transporter. Interestingly, two of the four cases of dicarboxylic amino aciduria reported previously were associated with mental retardation (253, 264), and the two siblings with the R445W mutation have admitted to behaviors that are consistent with OCD, but have declined further psychological testing. If the diagnosis were to be confirmed, it may provide a link between dicarboxylic amino aciduria and OCD, or other neurological disorders, and could provide a simple method for identifying potential candidates for these diseases.

H. Episodic Ataxia

Episodic ataxia (EA) is a rare neurological condition characterized by periods of incoordination and imbalance associated with progressive ataxia and other neurological conditions such as epilepsy and hemiplegic migraine (129). There are six known subtypes of EA, and most of the known mutations that cause EA types 1, 2, and 5 are found in voltage-gated K^+ and Ca^{2+} channels (129). Two mutations in *SLC1A3*, the gene encoding EAAT1, have been identified in patients with EA type 6. A proline for arginine substitution in TM5 (P290R) was identified in a patient with EA, seizures, and migraine which was not found in his asymptomatic parents. Expression and functional studies of this mutant showed a significant decrease in cell surface expression and minimal glutamate transport activity (130). A follow-up study examined the P290R mutant using electrophysiological methods and found that in addition to low levels of glutamate transport activity and reduced cell surface expression, this mutant transporter also displays an increase in the opening of the uncoupled Cl^- conductance (295). Another EAAT1 mutation identified in three episodic ataxia affected family members is C186S (51). This conservative mutation resulted in only a minor reduction in glutamate uptake activity, and cell surface expression was not investigated further. Indeed, two groups have shown that mutating all three native cysteine residues in EAAT1 to serine or alanine has no functional impact on the transporter (225, 232), and further investigation is required to determine if this modest decrease in activity has any pathological implications or is the cause of episodic ataxia in this family.

I. Glioma

Astrocytic gliomas are particularly aggressive brain tumors, and glutamate appears to play a key role in their pathogenesis. Whilst most cancers spread and grow through the vas-

culature and lymphoid systems, the growth of gliomas is restricted by the physical barriers of the skull. To grow, the gliomas appear to induce the death of surrounding cells to make space for growth. These changes are associated with significant elevations in extracellular glutamate concentrations, which have been implicated in the cell death process through excitotoxic processes. As part of this process, the expression levels of EAAT2 are markedly reduced (150, 280, 307), and the glutamate/cystine transporters are elevated (189). The consequence of this combination is an elevation of extracellular glutamate via exchange with cystine, and a lack of glutamate uptake due to reduced EAAT2. For a more thorough review of this topic, see the review by Watkins and Sontheimer (288).

VI. CONCLUSIONS AND OUTLOOK

The study of glutamate transporters and their roles in brain function under physiological and pathological conditions has made considerable progress in the last decade. This has come about through a combination of molecular biology methods, the use of pharmacological tools, electrophysiology techniques, and more recently through X-ray crystallography and computer simulations of transporter functions. However, there remain many aspects of transporter function that are poorly understood. In the final section of this review, we highlight areas of research that require further work to fully appreciate how glutamate transporters work. We then discuss how this understanding may be applied to provide a better understanding of pathological conditions and the potential for pharmacological manipulation of transporter functions in the treatment of pathological conditions.

A. How Do Glutamate Transporters Work?

The crystal structures of Glt_{Ph} have provided very detailed information about how aspartate and two Na^+ bind to the transporter, and further work using molecular dynamics simulations are, for the most part, consistent with the crystal structures. These studies have also led to predictions for how a third Na^+ binds to the transporter and also the order of Na^+ and aspartate binding. However, we are still coming to terms with how the energy derived from Na^+ binding is harnessed by the transporter to drive the cotransport of aspartate against its concentration gradient. One of the important pieces of information required to understand this process is a full description of the conformational states of the transport process. Although crystal structures of three distinction conformational states of Glt_{Ph} have been determined, the ion and substrate binding states are not overly different, and we do not yet understand how these interactions change leading to the release of aspartate and the cotransported Na^+.

The mechanism of K^+ countertransport also remains unresolved. As aspartate transport by Glt_{Ph} is not coupled to K^+ countertransport, the crystal structures have not provided definitive evidence for the K^+ binding site(s). Mutagenesis studies of the EAATs have identified up to three separate potential sites, and at present, it is not clear whether K^+ moves between the three distinct sites or if mutagenesis of a particular residue within one of the proposed sites leads to a secondary conformational state in one of the other proposed sites, leading to a loss of K^+ coupled countertransport.

There has been some progress in defining the structural elements required for the chloride channel activity of the EAATs. Transmembrane domain 2 (TM2) and surrounding structural elements appear to play important roles in chloride permeation, but at this stage the structural changes required for chloride channel opening and closing remain elusive. One of the key missing elements in understanding this process is to identify a conformational state of the transporter in which a chloride ion is bound and then how this changes during the various functional states of the transport cycle. Further work is also required to understand the functional role(s) of the chloride channel function for the various EAAT subtypes. There is growing evidence of the functional role of chloride channel activity of EAAT5 in regulating the activity of retinal neurons but for other transporter subtypes, the evidence for discrete roles of channel activity is limited.

One aspect of transporter function that has received very limited attention is the role of the cell membrane in influencing functional properties. Specific lipid-transporter interactions have been observed for arachidonic acid and EAAT1–4, which suggests that other lipid-transporter interactions may influence transporter function. Lipid interactions may fall into two general categories: direct lipid-transporter interactions and nonspecific effects of different lipid compositions of cell membranes on transporter function. There is considerable scope for a large number of direct lipid-transporter interactions with variation in lipid tail length, different levels of saturation of lipid tails, and different head groups all having the potential to form specific contacts with transporter. Some of these specific interactions may alter the way that the EAATs undergo conformational changes within the cell membrane that are required for the transport process. Cell membranes from different parts of cells have the potential to vary in thickness (through variation in tail length) and also in rigidity (through variation in cholesterol content and the extent of lipid saturation). Both of these factors may also then impact on the rate or extent of conformational changes of the EAATs that are possible.

B. Pharmacological Manipulation of Glutamate Transporters for the Treatment of Neurological Disorders

A large number of compounds have been identified that manipulate EAAT function, but at present there are very

few EAAT-selective compounds that show therapeutic potential for the treatment of neurological disorders. The two main reasons for this deficiency are that most EAAT-selective drugs lead to reduced EAAT activity, which in most scenarios will lead to elevated extracellular glutamate concentrations and excitotoxicity. As the EAATs, and EAAT2 in particular, are highly abundant and expressed in many regions of the central nervous system, any EAAT inhibitors are likely to be toxic. Compounds that cause increased EAAT function, most likely through increased cell surface expression, do have the potential to be of therapeutic value. Ceftriaxone causes elevated EAAT2 levels, and further development of this type of approach may be fruitful. However, this approach also has the potential for toxic consequences. A generalized elevation in EAAT2 expression levels or elevated EAAT2 function may impact on glutamate neurotransmission through reduced glutamate receptor occupancy, or altered glutamate receptor expression levels to compensate for more rapid and efficient glutamate clearance. While this may be of therapeutic value in discreet regions affected by a neurological disorder, in other unaffected regions of the brain the consequences may be more toxic. There is a considerable way to go before we can hope to be able to achieve regional- and subtype-specific upregulation of EAAT expression. Another aspect of regulation of EAAT expression that shows promise is the identification of intracellular proteins that have the capacity to specifically regulate EAAT subtypes. It will be of great interest to see if such an approach may be harnessed to provide the regional and subtype selective enhancement of transporter function.

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