Structure and Function of Sodium-coupled GABA and Glutamate Transporters

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Abstract. Neurotransmitter transporters are key elements in the termination of the synaptic actions of the neurotransmitters. They use the energy stored in the electrochemical ion gradients across the plasma membrane of neurons and glial cells for uphill transport of the transmitters into the cells surrounding the synapse. Therefore specific transporter inhibitors can potentially be used as novel drugs for neurological disease. Sodium-coupled neurotransmitter transporters belong to either of two distinct families. The glutamate transporters belong to the SLC1 family, whereas the transporters of the other neurotransmitters belong to the SLC6 family. An exciting and recent development is the emergence of the first high-resolution structures of archeal and bacterial members belonging to these two families. In this review the functional results on prototypes of the two families, the GABA transporter GAT-1 and the glutamate transporters GLT-1 and EAAC1, are described and discussed within the perspective provided by the novel structures.

Key words: GABA — Glutamate — Transporter — Structure — Function

Introduction

Sodium-coupled neurotransmitter transporters are located in the plasma membranes of neurons and glia, where they are present at high density in those areas of the cell membrane that are facing the synapse. They serve to keep the extracellular transmitter concentrations below neurotoxic levels. These transporters are key elements in the termination of the synaptic actions of the neurotransmitters. One of the major pieces of evidence for their importance comes from the study of knock-out mice. In the dopamine transporter knock-out mice the decay of extracellular dopamine is about 100 times longer than normal (Giros et al., 1996). The study of glutamate transporter knock-out mice indicates that glutamate transporters, in particular GLT-1 (Pines et al., 1992), play a central role in preventing both hyperexcitability and excitotoxicity (Tanaka et al., 1997). Termination of synaptic transmission by transporters takes place with most neurotransmitters, including L-glutamate, γ-aminobutyric acid (GABA), glycine, dopamine, serotonin and norepinephrine. Another termination mechanism is observed with cholinergic transmission. After dissociation from its receptor, acetylcholine is hydrolyzed into choline and acetate. Even then, the choline moiety is subsequently recovered by sodium-dependent transport. As the concentration of the transmitters in the nerve terminals is orders of magnitude higher than in the synaptic cleft, energy input is required for this process. The transporters located in the plasma membranes of nerve endings and glial cells obtain this energy by coupling the flow of neurotransmitters to that of sodium. The $(Na^+ + K^+)$-ATPase generates an inwardly directed electrochemical sodium gradient, which is utilized by the transporters to drive "uphill" transport of the neurotransmitters (reviewed in (Kanner, 1983, 1989; Kanner & Schuldiner, 1987; Nelson, 1998). Neurotransmitter uptake systems have been investigated in detail by using plasma membranes obtained upon osmotic shock of synaptosomes. These studies show that these transporters couple the flow of neurotransmitters not only to that of sodium, but also to that of additional ions such as potassium or chloride (Kanner, 1983, 1989; Kanner & Schuldiner, 1987).

Sodium-coupled neurotransmitter transporters are of considerable medical interest. Since they function to regulate neurotransmitter activity by removing the transmitters from the cleft, specific transporter inhibitors can be potentially used as novel drugs for neurological disease. For instance, attenuation of GABA removal will prolong the effect of this inhibitory transmitter. Thus, inhibitors of

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GABA transport could represent a novel class of anti-epileptic drugs. Well-known inhibitors, which inhibit the biogenic amine transporters, include antidepressant drugs such as Prozac and stimulants such as amphetamines and cocaine. The neurotransmitter glutamate causes cell death, at excessive local concentrations, by activating N-methyl-D-aspartic acid receptors and subsequent calcium entry. The transmitter has been implicated in neuronal destruction during ischemia, epilepsy, stroke, amyotropic lateral sclerosis and Huntington's disease. Therefore the glutamate transporters are key players in preventing glutamate from acting as an excitotoxin.

Sodium-coupled neurotransmitter transporters belong to either of two distinct families. The glutamate transporters belong to the SLC1 family, whereas the transporters of the other neurotransmitters belong to the SLC6 family. An exciting and recent development is the emerging of the first high-resolution structures of archeal and bacterial members belonging to these two families (Yernool et al., 2004; Yamashita et al., 2005). The following describes the current status on two prototypes of these distinct families: the GABA and glutamate transporters, with emphasis on the structural basis of function.

The GABA Transporter GAT-1 I

Mechanism

In GAT-1, GABA is transported together with two sodium ions and a chloride ion in an electrogenic process (Keynan & Kanner, 1988; Kavanaugh et al., 1992; Mager et al., 1993; Lu & Hilgemann, 1999b), even though there still is some dispute on the involvement of chloride. One study suggests that, even though two sodium ions are transported with GABA, exchange of internal with external chloride takes place during the translocation cycle, but there is no net transport of chloride (Loo et al., 2000). The measurements used to study the stoichiometry include radioactive flux experiments in various experimental systems, such as brain plasma membrane vesicles (Kanner, 1978), liposomes inlaid with the detergent-solubilized transporter (Keynan & Kanner, 1988), and *Xenopus* oocytes (Loo et al., 2000), and also electrophysiological measurements (Kavanaugh et al., 1992; Mager et al., 1993). The evidence in favor of co-transport of chloride with GABA and with sodium is based on thermodynamic and kinetic arguments. The reversal potential changes not only as a function of the sodium gradient but also depends on the chloride gradient (Lu & Hilgemann, 1999b), indicating a coupling of the fluxes of sodium, chloride and GABA. Moreover, direct sodium- and GABA-dependent transport of radioactive chloride has been demonstrated in liposomes inlaid with a partially purified GABA transporter preparation (Keynan & Kanner, 1988). On the other hand there is the well-documented observation that at very negative potentials the dependence of the GABA-induced currents on extracellular chloride is not absolute (Lu & Hilgemann, 1999b; Mager et al., 1993). At the present time it is not clear what the mechanistic interpretation of this result is. One possibility is that under these conditions, another ion – such as hydroxyl – may take over the role of chloride. However, the outward GAT 1 current ("reverse transport mode") strictly requires the presence of sodium, GABA, as well as chloride on the cytoplasmic side (Lu & Hilgemann, 1999b). A recent study suggests an interesting explanation for the chloride-independent GABA currents (Krause & Schwarz, 2005). The data by these authors suggest that the GABA-induced currents can be seperated into a stoichiometric current, reflecting the coupled translocation of GABA with two sodium ions and one chloride ion, and a chloride-independent GABA-gated conduction of sodium ions. In addition to steady-state transport currents, a sodium-dependent transient current can be observed in the absence of GABA. This transient can be blocked by bulky GABA analogues, which can bind to the transporter but are not translocated by it (Mager et al., 1993). These transients reflect a charge-moving conformational change, occurring after sodium has bound. The transients can be blocked by internal chloride and it appears that cytoplasmic chloride and external sodium bind to the transporter in a mutually exclusive fashion (Lu & Hilgemann, 1999a), providing strong support for an alternating access mode (Hilgemann & Lu, 1999). A major role for external chloride is to increase the affinity for external sodium (Mager et al., 1996). The only evidence against chloride coupling comes from a study in oocytes where the charge/ flux ratio determination indicates two charges per GABA transported, irrespective of the presence of chloride (Loo et al., 2000).

In contrast to sodium, lithium can not support transport of [³H]-GABA (Bennett, Su & Kanner, 2000; MacAulay, Zeuthen & Gether, 2002), but it can stimulate GABA-induced currents at reduced sodium concentrations, suggesting that during transport one of the two sodium ions can be replaced by lithium (MacAulay et al., 2002). In the absence of GABA, leak currents are observed when lithium replaces sodium. These leak currents have a much steeper voltage dependence than the GABA-induced transport currents observed in the presence of sodium (Bismuth, Kavanaugh & Kanner, 1997; Mager et al., 1996). These lithium-leak currents are inhibited by low concentrations of sodium ions and this inhibition is not competitive. These observations indicate that when sodium binds to GAT-1, the leak mode is converted into a conformation that enables coupled transport (Kanner, 2003; MacAulay et al., 2002). The

turnover number of the transporter can be estimated from the comparison of the GABA-induced steady-state currents and the transient currents. The result is a few cycles per second (Mager et al., 1993) and this is in good agreement with estimates from the transport rates of the purified and reconstituted transporter (Radian, Bendahan & Kanner, 1986).

Even though there is considerable homology between the various SLC6 members, this does not mean that the stoichiometry of transport is the same. An example is the well-studied serotonin transporter SERT. This transporter mediates electroneutral transport, but serotonin (5–HT) induces a transport-associated current because under some conditions this transporter may act as a channel (Mager et al., 1994). This transporter channel appears to be less sodium-selective than the transporter mode (Mager et al., 1994). SERT translocates Na^+, Cl^- and $5–HT^+$ in a 1:1:1 stoichiometry and subsequently a potassium ion is translocated in the opposite direction, resulting in an electroneutral transport cycle (Nelson & Rudnick, 1979). Another example is the bacterial homologue $LeuT_{Aa}$, a leucine transporter from *Aquifex aeolicus*, which mediates sodium-dependent but chloride-independent leucine transport (Yamashita et al., 2005).

TOPOLOGY

The primary sequence of GAT-1, like of most SLC6 members, predicts twelve transmembrane domains (TM) connected by hydrophilic loops with the amino and carboxyl termini residing inside the cell (Guastella et al., 1990). Studies on the serotonin transporter SERT indicate that the theoretical topological model is correct (Chen, Liu-Chen & Rudnick, 1998) and it is also in agreement with the high-resolution crystal structure of $LeuT_{Aa}$ (Yamashita et al., 2005). However, the crystal structure revealed several topological features that could not have been predicted. The first is that there is an internal structural repeat: TM1-TM5 and TM6-TM10 can be superimposed on each other by rotation around a pseudo twofold axis located in the plane of the membrane (Yamashita et al., 2005). The second is the unwinding of membrane-spanning domains, a feature first observed in the calcium pump (Toyoshima et al., 2000). In $LeuT_{Aa}$, TM1 and TM6, which are antiparallel to each other, have breaks in their helical structure approximately halfway across the membrane (Fig. 1). These breaks expose main-chain carbonyl oxygen and nitrogen atoms for hydrogen bonding and ion binding. Residues on TM3, TM7 and TM8 also contribute to the binding of sodium and leucine (Yamashita et al., 2005). Some of these residues had already been implicated in ion and/or substrate binding by functional studies of mutants of several of the neurotransmitter transporters (*see* the next section,

Structure and Function). Therefore it appears that the $LeuT_{Aa}$ structure is a physiologically relevant conformation of the transporter, which probably represents a good model for the study of GAT-1 and other SLC6 members.

STRUCTURE AND FUNCTION

Some of the functionally important GAT-1 residues in the context of the topological model based on the $LeuT_{Aa}$ structure (Yamashita et al., 2005) are depicted in Fig. 1. Cysteine-74 is highly conserved between the neurotransmitter transporters in the SLC6 family. This residue is not important for transport activity, but is the only endogenous cysteine that can be impacted by impermeant MTS reagents. Therefore, in most studies the C74A mutant is employed, because this mutant is fully functional and is a suitable background for studies using the cysteine scanning accessibility method (Kanner, 2003; Zomot & Kanner, 2003; Zhou, Bennett & Kanner, 2004; Zomot, Zhou & Kanner, 2005). Many additional cysteines are not impacted by impermeant sulfhydryl reagents, because they are buried in the interior of the transporter or are accessible only from the cytoplasmic aqueous space. The latter cysteines can react with permeant sulfhydryl reagents such as MTSEA and it has been shown that cysteine-399, located in the cytoplasmic part of TM8, is a major determinant of inhibition of GAT-1 by membrane-permeant sulfhydryl reagents (Golovanevsky & Kanner, 1999).

Many residues that are critical for transport are located in TM1 and in particular in TM1b (Fig. 1). This is in agreement with the $LeuT_{Aa}$ structure, where mostly main-chain atoms of TM1 are seen to interact directly with the two sodium ions and the leucine substrate, which are occluded in the binding pocket of this transporter (Yamashita et al., 2005). Of the liganding groups provided by TM1 only asparagine-27, which is equivalent to asparagine-66 of GAT-1, contributes a side-chain oxygen that interacts with Na1 (Yamashita et al., 2005). Indeed, the N66C mutant is inactive in transport even though the mutant transporter is expressed at the plasma membrane at levels similar to wild-type GAT-1 (Zhou et al., 2004) and the same is true for N66D and N66Q mutants (Zhou, Y. and Kanner, B.I., unpublished observations).

The G65C mutant is also defective in transport but normally expressed at the plasma membrane (Zhou et al., 2004) and the amide nitrogen of the equivalent glycine of $LeuT_{Aa}$ interacts with the carboxyl group of the transported leucine (Yamashita et al., 2005). The same is true for the equivalent of leucine-64 (Yamashita et al., 2005), but in GAT-1 the L64C mutant still has measurable transport activity (Zhou et al., 2004). Apparently the introduction of a cysteine at position 65 results in a larger perturbation in the orientation of its amide nitrogen than at position 64.
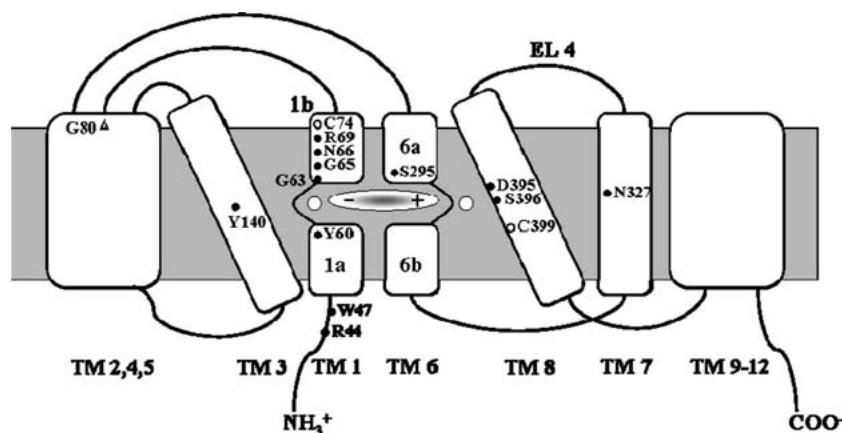
Fig. 1. Topological model of the GABA transporter GAT-1. The model is based on the LeuT$_{Aa}$ structure (Yamashita et al., 2005). Indicated are the GABA molecule (*large oval*) and two sodium ions (*large empty spheres*). The position of chloride is not known, since LeuT$_{Aa}$ transport does not require chloride and the chloride ion observed in the LeuT$_{Aa}$ structure is unlikely to be relevant for GAT-1. The positions of two endogenous cysteine residues (positions 74 and 399), which are sites of action of MTS reagents (*small empty circles*) and of functionally important residues (*small filled circles*), are indicated by their number in the GAT-1 sequence and their single letter code. The position of glycine-80, shown to be critical for one of the conformational changes during transport (Zhou & Kanner, 2005), is indicated as a small open triangle. Further details are described in the text.

Mutations at tyrosine-60 result in the reduction of the apparent affinity for sodium (Kanner, 2003). In the three other GABA transporters, GAT-2, -3 and -4, there is a glutamate at the equivalent position of tyrosine-60 and in the taurine transporter TAUT, glycine occupies this position. The Y60E mutant is inactive and the same is true for the reciprocal GAT-4 mutant E61Y (Melamed & Kanner, 2004). Interestingly, the GAT-4-E61G mutant has an increased apparent affinity for taurine (Melamed & Kanner, 2004). The equivalent of tyrosine-60 in LeuT$_{Aa}$ is asparagine-21, which is close but not at hydrogen-bonding distance from the substrate and the sodium ions (Yamashita et al., 2005). However, it is possible that one of these distances becomes smaller in one of the other, yet to be crystallized, conformations. Alternatively, the observed effects of mutations at this position in the GABA transporters may be due to indirect effects.

Glycine-63 is critical for GAT-1 function (Kanner, 2003). All amino acid transporting members of the SLC6 family have a glycine at this position, but in the biogenic amine transporters of SLC6 an aspartate occupies this position. One of the most exciting features of the LeuT$_{Aa}$ structure is a direct contact between Na1 and the carboxyl group of the transported leucine (Kanner, 2005; Yamashita et al., 2005). The substrates of the biogenic amine transporters obviously do not have a carboxyl group to contribute to the binding of sodium. However, modeling of an aspartate at glycine-24 of LeuT$_{Aa}$, which is the equivalent of glycine-63 of GAT-1, shows that the β-carboxyl group of the aspartate can be positioned to within 1 Å of the substrate leucine and within 3 Å of the sodium ion (Yamashita et al., 2005). Therefore it has been proposed that in the

biogenic amine transporters the aspartate's carboxy group supplants the carboxy group of amino acid substrates and coordinates a sodium ion (Yamashita et al., 2005). Even though no GABA transport can be measured in GAT-1 mutants at position 63, the lithium leak currents are still intact. The membrane-impermeant MTSET inhibits these lithium leak currents in the G63C but not in the G63S mutant, indicating that this position, which apparently is at the binding pocket, can be reached from the external aqueous medium (Kanner, 2003).

Arginine-69 is another residue that is critical for the activity of GAT-1, and even the R69K mutant is inactive (Pantanowitz, Bendahan & Kanner, 1993; Kanner, 2003). The R69K mutant exhibits transient currents which behave as if the apparent affinity for sodium is increased and the concentrations of sodium required to inhibit the lithium leak currents by the R69K mutant are lower than in the wild type. Moreover GABA can bind to the mutant transporters, yet is not transported (Kanner, 2003). In the LeuT$_{Aa}$ structure, this conserved arginine is seen to participate in the formation of the external gate of the transporter (Yamashita et al., 2005). In an alternating access mechanism the closing of the "outer" (or extracellular) gate is coupled to the opening of the "inner" (or intracellular) gate. If the outer gate cannot close, the inner gate can probably not open and this can explain why, although external GABA can bind to R69K transporters, it can not be transported. This is in harmony with the important functional role of arginine-69 of GAT-1, but does not offer a satisfactory explanation of the increased apparent affinity for sodium of this mutant. It is likely that this arginine residue plays an additional, as yet not understood role, in one of the other confor-

mations of the transporter. The LeuT$_{Aa}$ counterparts of two other critical GAT-1 residues, arginine-44 and tryptophan-47, appear to be involved in the formation of the "inner" gate (Yamashita et al., 2005). The GAT-1 mutants at these two positions seem to be more strongly affected in the return of the unloaded transporter than in the translocation of the substrate-loaded transporter (Bennett et al., 2000), an observation which is currently difficult to explain.

Tyrosine-140 is located in TM3 of GAT-1 (Fig. 1). Even when this tyrosine is replaced by related residues, such as phenylalanine or tryptophane, transport activity is abolished. Yet sodium still can bind to these mutants, as judged by their ability to perform the sodium-dependent transient currents (Bismuth et al., 1997). However, unlike wild-type GAT-1, these transients cannot be suppressed by GABA or its non-transportable analogues, leading to the conclusion that tyrosine-140 is involved in the binding of GABA (Bismuth et al., 1997). It is satisfying to see that the hydroxyl of the corresponding tyrosine in LeuT$_{Aa}$, tyrosine-108 directly interacts with the carboxy group of leucine (Yamashita et al., 2005).

Even though lithium by itself does not support GABA transport (Bennett et al., 2000; MacAulay et al., 2002), it has been proposed that lithium can replace sodium at one of the binding sites, but not at the other (MacAulay et al., 2002). In order to identify putative lithium selectivity determinants, the five GAT-1 residues corresponding to those whose side-chains participate in the sodium-binding sites, Na1 and Na2, of the bacterial leucine-transporting homologue LeuT$_{Aa}$, have been mutated (Zhou, Zomot & Kanner, 2006). In GAT-1 and in most other neurotransmitter transporter family members, four of these residues are conserved. Three of these correspond to Na1 residues, asparagine-66 in TM1, serine-295 in TM6 and asparagine-327 in TM7 and the fourth corresponds to a Na2 residue, serine-396 of TM8 (Fig. 1). However, aspartate-395 in TM8 replaces the Na2 residue threonine-354 of LeuT$_{Aa}$. At varying extra-cellular sodium, lithium stimulates sodium-dependent transport currents as well as [^{3}H]-GABA uptake in wild-type GAT-1 and the extent of this stimulation is dependent on the GABA concentration (Zhou et al., 2006). In mutants where aspartate-395 is replaced by threonine or serine, the stimulation of transport by lithium is abolished (Zhou et al., 2006). Moreover, these mutants are unable to mediate the lithium leak currents. Even though their transport properties are severely impacted, this phenotype is not observed in mutants at the four other positions. Thus, at saturating GABA, the site corresponding to Na2 behaves as a low-affinity sodium binding site where lithium can replace sodium. Probably GABA participates in the other sodium binding site, just like leucine does in the Na1 site, and at limiting GABA this site determines the apparent sodium affinity of GABA transport (Zhou et al., 2006).

CONFORMATIONAL CHANGES

The LeuT$_{Aa}$ structure represents a conformation with two sodium ions and leucine occluded in the binding pocket. This conformation, where the binding pocket is closed to either side of the transporter, probably represents the substrate-loaded transition state of the transporter (Yamashita et al., 2005). Obviously, the transporter must undergo many conformational transitions during the transport cycle so that the transporter is sequentially accessible to the outside and the inside and these states are separated by the transition state. There are several pieces of evidence for the existence of conformational changes by GAT-1. The well-known sodium-dependent transient currents by GAT-1 are very slow and thus can not represent the binding and unbinding of sodium itself, which should be a very fast process. Rather, they are believed to reflect a slow, charge-moving, conformational change which occurs after the transporter binds (or releases) sodium (Mager et al., 1993, 1996; Lu & Hilgemann, 1999a; Kanner, 2003). The leak mode, observed in the absence of sodium and in the presence of lithium (Mager et al., 1996), represents a distinct conformation of the transporter (MacAulay et al., 2001). In the presence of low concentrations of sodium, the lithium-leak currents are inhibited, apparently because sodium mediates the transition from the leak mode to the coupled mode of the transporter (MacAulay et al., 2002; Kanner, 2003). There is also biochemical evidence for such conformational transitions. It has been found that GABA can protect against the proteolytic cleavage of GAT-1, provided that the two cosubstrates, sodium and chloride, are also present (Mabjeesh & Kanner, 1993).

The accessibility of engineered cysteines at many positions of GAT-1 has been inferred from the functional impact on the activity of these cysteine mutants by sulfhydryl reagents. Often this reactivity is influenced by the presence of sodium, chloride, GABA and non-transportable GABA analogues. In several cases these positions are far away from the substrate binding site, as defined by the LeuT$_{Aa}$ structure (Yamashita et al., 2005). Therefore it is unlikely that these residues are directly occluded by substrate or ion binding, but the changes in reactivity are likely to reflect conformational changes in response to occupation of the binding pocket. One of the examples is the fourth extracellular loop which connects TM7 with TM8. Inhibition of transport of A364C in the background of the C74A mutation (so that the engineered cysteine is the only one that can be functionally impacted by membrane-impermeant MTSET), is potentiated by the presence of sodium and chloride, whereas GABA protects (Zomot & Kanner, 2003). This protection is apparently not due to the ability of GABA to enable transport, because the protection is also observed at 4°C, where the rate

of transport is markedly slowed down. Moreover, protection is also seen with the non-transportable substrate analogue SKF 100330A (Zomot & Kanner, 2003). Interestingly, in the absence of sodium, SKF 100330A actually potentiates the inhibition of transport of A364C/C74A by MTSET, indicating that this blocker can also bind to the mutant GAT-1 in the absence of sodium but that this results in a different conformation of the transporter (Zomot & Kanner, 2003).

Also many of the accessibility changes inferred from studies on TM1b apparently reflect conformational changes, even though this structural element is close to the binding pocket. In several positions sodium potentiates the inhibition while at other nearby positions sodium protects, as if sodium binding to its more intracellular site results in a rotation of TM1b (Zhou et al., 2004). The accessibility of the endogenous cysteine at position 399 also appears to be dependent on the conformation of the transporter (Golovanevsky & Kanner, 1999). This position was originally anticipated to be on the intracellular loop connecting TM8 with TM9, but according to the LeuT$_{Aa}$ structure, is probably near the Na2 site.

The ability to undergo multiple conformational changes requires the presence of elements that permit flexibility of the transporter's structure. Glycines can introduce considerable flexibility to proteins, and recently a glycine residue has been implicated to act as a hinge in potassium channels (Jiang et al., 2002). Moreover, evidence has been presented suggesting that glycine residues, engineered into the proton-coupled lactose transporter, confer conformational flexibility to it (Weinglass, Smirnova & Kaback, 2001). In order to determine if glycine residues play a role in the conformational changes during neurotransmitter transport, site-directed mutants of GAT-1, in a domain at the top of TM2 (Fig. 1) containing three consecutive glycines, have been analyzed. These glycine residues are conserved throughout the sodium- and chloride-dependent neurotransmitter transporter family. Only cysteine replacement of glycine-80 results in the complete loss of [³H]-GABA uptake, but oocytes expressing this mutant exhibit the sodium-dependent transient currents, which are thought to reflect a charge-moving conformational change. When sodium is removed and subsequently added back, the transients by G80C do not recover, as opposed to wild type where recovery is almost complete. Remarkably, the transients by G80C can be restored after exposure of the oocytes to either GABA or a depolarizing pre-pulse. These treatments also result in a full recovery of the transients by the wild type. Whereas in wild type, lithium-leak currents are observed after prior sodium depletion, this is not the case for the glycine-80 mutants, unless GABA is added or the oocytes are subjected to a depolarizing pre-pulse. Thus glycine-80 appears essential for conformational transitions in GAT-1. When this residue is mutated, removal of sodium results in "freezing" the transporter in one conformation from which it can only exit by compensatory changes induced by GABA or depolarization. These results can be explained by a model invoking two outward-facing states of the empty transporter and a defective transition between these states in the glycine-80 mutants (Zhou & Kanner, 2005).

The Glutamate Transporters

MECHANISM

Sodium-dependent glutamate transport is an electrogenic process (Kanner & Sharon, 1978; Brew & Attwell, 1987; Wadiche et al., 1995b), consisting of two distinct half-cycles: first, glutamate is co-transported with three sodium ions and a proton and subsequently the binding sites reorient upon counter transport of potassium (Kanner & Bendahan, 1982; Pines & Kanner, 1990; Kavanaugh et al., 1997). The stoichiometry of the process has been determined to be $3Na^+:1H^+:1K^+$:glutamate$^-$(Zerangue & Kavanaugh, 1996; Levy, Warr & Attwell, 1998). Under physiological conditions, the inwardly directed sodium gradient and outwardly directed potassium gradient as well as the interior negative membrane potential promote the accumulation of the transmitter into the cell against its concentration gradient (Zerangue & Kavanaugh, 1996; Levy et al., 1998). but at elevated external potassium concentrations the transporters mediate its efflux out of the cells (Kanner & Bendahan, 1982; Kanner & Marva, 1982; Szatkowski, Barbour & Attwell, 1990; Zhang et al., 1998). According to the transport cycle, elevated external potassium concentrations will render an increased proportion of transporters in the inward-facing conformation.

In addition to the ion-coupled glutamate translocation, glutamate transporters mediate a thermodynamically uncoupled chloride flux activated by two of the molecules they transport, sodium and glutamate (Fairman et al., 1995; Wadiche, Amara & Kavanaugh, 1995a). The physiological relevance of the uncoupled anion conductance is not clear, but an attractive proposal is that the chloride conductance may serve to clamp the membrane potential at a level enabling efficient glutamate accumulation (Billups, Rossi & Attwell, 1996; Eliasof & Jahr, 1996). The two positive charges that move into the cell with each transport cycle will depolarize the cell membrane and reduce the driving force for glutamate transport. Entry of chloride ions via the uncoupled chloride conductance is expected to offset the depolarization, enabling the maintenance of low extracellular

glutamate concentrations. In EAAC1 (also termed EAAT-3 (Arriza et al., 1994)), lithium can replace sodium in coupled glutamate uptake, but not in its capacity to gate the glutamate-dependent uncoupled anion conductance (Borre & Kanner, 2001), and additional studies have reinforced the idea that the conformation gating the anion conductance is different from that during substrate translocation (Borre, Kavanaugh & Kanner, 2002; Ryan & Vandenberg, 2002; Seal et al., 2001). Recent evidence indicates multiple transitions between the coupled transport cycle and anion conductance states (Shachnai, Shimamoto & Kanner, 2005). In addition, the uncoupled anion flux can be altered by substituting some of the amino acid residues of transmembrane (TM) domain 2, without significantly affecting the properties of coupled glutamate translocation (Ryan, Mitrovic & Vandenberg, 2004). In spite of these insights, little is known of the mechanism of glutamate-induced anion permeation, but it has been suggested that glutamate itself may gate the anion permeation (Wadiche et al., 1995a).

Topology

Glutamate transporters have a non-conventional topology (Fig. 2) containing two re-entrant loops and two transmembrane domains (7 and 8) in their carboxyl terminal half (Grunewald, Bendahan & Kanner, 1998; Grunewald & Kanner, 2000; Slotboom et al., 1999). Moreover, the two re-entrant loops are in close proximity (Brocke et al., 2002). The recently solved crystal structure of the glutamate transporter homologue GltPh shows a bowl-shaped trimer with a solvent-filled extracellular basin extending halfway across the membrane bilayer (Yernool et al., 2004). The monomer is the functional unit and its structure beautifully confirms and refines both the topology and the proximity of the re-entrant loops. Moreover, as will be detailed in the next section, the equivalent amino acid residues from the eukaryotic glutamate transporters, which are involved in potassium binding (Kavanaugh et al., 1997; Zhang et al., 1998), sodium specificity (Zhang & Kanner, 1999; Borre & Kanner, 2001) and the liganding of the γ-carboxyl group of glutamate (Bendahan et al., 2000), are located at the binding pocket of GltPh (Yernool et al., 2004). Therefore, the GltPh structure appears to be a good model for the study of its eukaryotic counterparts.
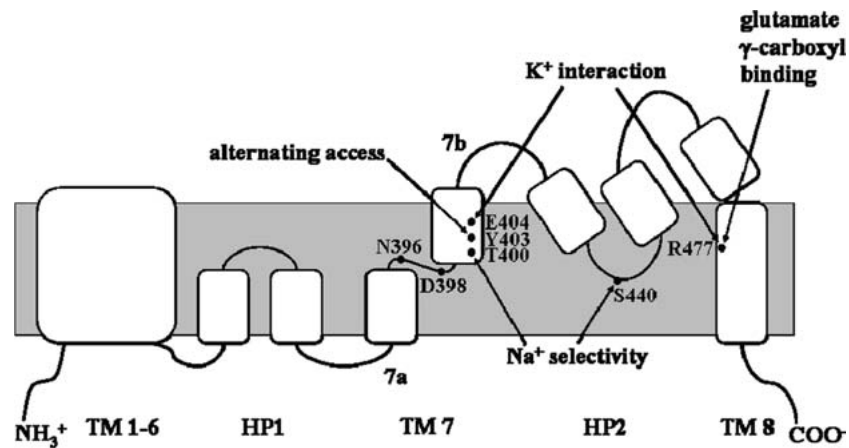
Structure and Function

Some of the functionally important GLT-1 residues in the context of the topological model based on the GltPh structure (Yernool et al., 2004) are depicted in Fig. 2. Also indicated in this Figure are residues critical for transport whose role in the transport cycle has been pinpointed. These include threonine-400

(Borre & Kanner, 2001) and serine-440 from HP2 (Zhang & Kanner, 1999), which are both determinants of sodium specificity, tyrosine-403 (Zhang et al., 1998) and glutamate 404 (Kavanaugh et al., 1997) from TM7b, which are specifically required for the interaction with potassium, and arginine-477 from TM8, which controls the interaction with the γ-carboxyl group of the transported substrate and is also important for the interaction with potassium (Bendahan et al., 2000). The numbering of these conserved residues is from GLT-1 (Pines et al., 1992), one of the five cloned eukaryotic glutamate transporters. The other four are GLAST (Storck et al., 1992), EAAC1 (Kanai & Hediger, 1992) and excitatory amino acid transporters 4 and 5 (Arriza et al., 1997; Fairman et al., 1995). In two of the above mutants the primary data came from EAAC1 (Bendahan et al., 2000; Borre & Kanner, 2001), because this isoform expresses much better in oocytes than GLT-1. The converse is true in HeLa cells. The numbering of the corresponding residues in EAAC1 is lower by 30 than that of their GLT-1 counterparts.

Additional residues, which are critical for transport, have been identified. These residues are located in the boundary between TM7a and TM7b, the so-called NMDGT motif (the asparagine and aspartate residues of this motif are shown in Fig. 2), which has been implied to play an important role in the interaction with sodium (Borre & Kanner, 2001; Zarbiv et al., 1998), in TM8 (Borre & Kanner, 2004), where mutation of arginine-475 (GLT-1 numbering) resulted in a cation leak, and in the tip of HP1 (Grunewald & Kanner, 2000; Slotboom et al., 1999). These results are in very good agreement with the GltPh structure, where, as noted before, TM7 and TM8 as well as the re-entrant loops HP1 and HP2 are seen to form the binding pocket and many of the side-chains of the critical residues point toward the binding pocket (Yernool et al., 2004). HP1 and HP2 appear to control the access of the substrates to the binding pocket from the intra- and extra-cellular side, respectively, and at their tips the re-entrant loops come in close proximity to each other in the structure. This structure apparently represents the substrate-occluded form of the transporter (Yernool et al., 2004). This proximity observed in the structure is in beautiful agreement with functional studies of paired cysteine mutants of GLT-1, which show that when two cysteines are introduced at the tips of these re-entrant loops, the transport activity of the double cysteine mutant can be blocked under oxidative crosslinking conditions (Brocke et al., 2002). Apparently these two cysteines can become so close to each other that a disulfide bond can be generated, which can restrict the conformational changes, which occur during transport (see next section).

A long time ago it was concluded that glutamate transport is a two-step process, in which sodium and

Fig. 2. Topological model of the glutamate transporter GLT-1. The model is based on the GltPh structure (Yernool et al., 2004). Indicated are some of the functionally important residues with their single-letter code and number in the GLT-1 sequence (*filled circles*) together with the functions they participate in. Not indicated is the proximity between the tip of HP2 (S440) and the tip of HP1. Further details are described in the text.

glutamate are transported first, and in a separate subsequent step, potassium is transported in the opposite direction (Kanner & Bendahan, 1982; Pines & Kanner, 1990). The behavior of the GLT-1 mutant E404D strongly supports this idea, because this mutant can not mediate net transport of glutamate, but is capable to mediate obligate exchange of radiolabeled glutamate from one side of the membrane with unlabeled glutamate from the other (Kavanaugh et al., 1997), consistent with the idea that the potassium translocation step is distinct from the glutamate translocation step (Kanner & Bendahan, 1982; Pines & Kanner, 1990). Indeed, using reconstitution experiments, a defective interaction of the E404D transporters with potassium was directly demonstrated (Kavanaugh et al., 1997). It has been proposed that this glutamate residue, which interacts with potassium during the "back-stroke" of the transport cycle, is also the residue that carries the proton during the "forward-stroke" of the transport cycle (Grewer et al., 2003). Glutamate can still activate the anion-conductance in the E404D mutant, indicating that the activation of the anion conductance is associated with the "forward-stroke".

In the R447C mutant of EAAC1 (where the arginine corresponds to arginine-477 of GLT-1), no glutamate or aspartate transport is observed, yet cysteine transport is normal. However, the voltage dependence of the currents induced by L-cysteine is incompatible with net electrogenic transport; it rather reflects the anion conductance activated by L-cysteine (Bendahan et al., 2000). Indeed, this mutant acts like an exchanger because it can not interact with potassium (Bendahan et al., 2000). Thus this arginine residue appears to have a dual role in the transport cycle, interacting with the γ-carboxyl group of the transported substrate during the "forward-stroke" and it somehow is required for the interaction with

potassium during the "back-stroke", perhaps by a role in the positioning of glutamate-404 (Kanner & Borre, 2002). Interestingly, a non-protein electron density was observed in the GltPh structure (Yernool et al., 2004), which possibly could represent bound glutamate or aspartate and the side-chain of the conserved arginine, which corresponds to arginine-477 of GLT-1, points directly to this electron density. Once again this is in nice harmony with the functional studies on the eukaryotic transporter mutants.

In the LeuT$_{Aa}$ structure, the resolution is high enough to see the two sodium ions entrapped in the binding pocket and one of the two sodium ions make direct contact with the carboxyl group of the substrate (Yamashita et al., 2005). In the case of GltPh the resolution is lower and the sodium ions are not visible and thus it is not clear if one of them interacts directly with the bound substrate. Interestingly, in EAAC1 there is evidence for a functional interaction between the driving ions (sodium or lithium) and the substrate (Menaker, Bendahan & Kanner, 2006).

In the crystal structure of the archeal homologue, the amino acid residues of the NMDGT motif are found to form part of the substrate binding pocket (Yernool et al., 2004). The side-chains of the methionine and threonine point towards it, but intriguingly the side-chains of the asparagine and the aspartate of this motif are pointing away from the binding pocket. These two residues are seen to interact with each other and with additional residues of TM domains 3, 6 and 8. These interactions were suggested to be important for shaping and stabilizing the binding pocket structure (Yernool et al., 2004). In the neuronal glutamate transporter EAAC1, the equivalent residues are asparagine-366 and aspartate-368. Substitution mutants N366Q and D368E, but not N366D and D368N, show glutamate-induced inwardly-rectifying steady-state currents, but their

apparent substrate affinity is dramatically decreased. Such currents, which reflect electrogenic net uptake of substrate, are not observed with the reciprocal double mutant N366D/D368N. Remarkably, the double mutant exhibits slow substrate-induced voltage-dependent capacitative transient currents. These currents apparently reflect the reversible sodium-coupled glutamate translocation step, because the interaction of the double mutant with potassium is largely impaired. Moreover, when the analogous double mutant in the glutamate transporter GLT-1 is reconstituted into liposomes, a slow exchange of radioactive and unlabelled acidic amino acids is observed (Rosental, Bendahan & Kanner, 2006). These results suggest that it is the interaction of asparagine-366 and aspartate-368 that is important during the glutamate translocation step. On the other hand, the side-chains of these residues themselves are required for the subsequent potassium relocation step (Rosental et al., 2006). Moreover, the apparent affinity for sodium of the N366Q mutant was markedly decreased, implying an additional role of the motif (Rosental et al., 2006). In parallel, it was reported that neutralization of the aspartate residue inhibits binding of Na$^+$ to the glutamate-free form of the transporter, implying a role of this residue in the coordination of the first sodium (Tao, Zhang & Grewer, 2006). The multiple effects of the mutations of asparagine-366 and aspartate-368 on the interaction both with sodium and potassium are reminiscent of results obtained by homology modeling of the Na$^+$, K$^+$-ATPase on the structure of the Ca^{2+}-ATPase (Ogawa & Toyoshima, 2002). In the latter case it was found that the binding sites for sodium and potassium are partly overlapping, and this could also be the case for the neuronal glutamate transporter. However, in the absence of structures of this transporter with a resolution high enough to observe sodium and potassium ions directly, the possibility that the effects of the mutations are indirect cannot be excluded. In the case of a direct effect and assuming that the GltPh structure is physiologically relevant, a picture would emerge where the side-chains of the two residues point toward the binding pocket in the glutamate-free form of the transporter. Binding of glutamate would then bring about a conformational change, so that the two side-chains point to each other and away from the binding pocket, which would also result in a redistribution of the bound sodium ions.

Conformational Changes

The GltPh structure represents apparently a conformation with the substrate (glutamate or aspartate) occluded in the binding pocket. This conformation, where the binding pocket is closed to either side of the transporter, probably represents the substrate-loaded

transition state of the transporter (Yernool et al., 2004). Obviously, the transporter must undergo many conformational transitions during the transport cycle so that the transporter is sequentially accessible to the outside and the inside and these states are separated by the transition state. As discussed in the previous section, it is likely that the conformation of the NMDGT motif in the glutamate-free form differs from that of the glutamate-bound form. In fact, there are several pieces of evidence for the existence of conformational changes by GLT-1. First of all, it has been shown that the proteolytic cleavage pattern depends on the presence of sodium and substrate during the exposure to the protease (Grunewald & Kanner, 1995). Second there is a wealth of information on the effects of substrates and driving ions on the accessibility of engineered cysteines. Mutation of tyrosine-403, which is a determinant of potassium interaction (Zhang et al., 1998), to cysteine does not disrupt uptake of radioactive substrate, presumably because exchange is still possible. Transport by Y403C is inhibited by positively charged MTS reagents, but not by negatively charged MTSES, consistent with a cationic binding site near tyrosine-403 (Zarbiv et al., 1998). The external accessibility of the cysteine engineered at position 403 is diminished by transportable substrates, yet this is apparently not due to a physical blockade of the access of the MTS reagents because the non-transportable substrate analogue dihydrokainate has the opposite effect (Zarbiv et al., 1998). Therefore it appears that the accessibility of the cysteine at position 403 is determined by the transport status of GLT-1. The presence of glutamate is expected to increase the proportion of inward-facing transporters, whereas dihydrokainate would trap the transporters in an outward-facing conformation (Zarbiv et al., 1998). It appears that also the accessibility of HP2 (Grunewald, Menaker & Kanner, 2002; Zhang & Kanner, 1999) and of HP1 (Grunewald & Kanner, 2000) depends on the conformation of the transporter. The same appears to be true for the distance between two engineered cysteine pairs, with the one cysteine of one pair in TM7b and the other in HP2, whereas the cysteines of the other pair reside in HP1 and HP2 (Brocke et al., 2002).

Future Directions

The field of neurotransmitter transporters has been transformed by the recently published structures of archeal/bacterial homologues belonging to the SLC1 and SLC6 families. Available functional data on the neurotransmitter transporters indicate that the structures of the homologues are relevant for the study of their eukaryotic counterparts. These structures capture one conformation and therefore

functional studies of the neurotransmitter transporters, which focus on the conformational changes occurring during transport, are needed to close in on the mechanism of neurotransmitter transport. In parallel, high-resolution structures capturing additional conformations will also be of paramount importance towards this long-term goal.

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