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SODIUM-COUPLED NEUROTRANSMITTER TRANSPORT: STRUCTURE, FUNCTION AND REGULATION

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Summary

The removal of neurotransmitters by their transporters – located in the plasma membranes of nerve terminals and glial cells – plays an important role in the termination of synaptic transmission. In the last 3 years, many neurotransmitter transporters have been cloned. Structurally and functionally they can be divided into two groups: glutamate transporters, of which to date three have been cloned, couple the flow of glutamate to that of sodium and potassium. The second group of transporters includes those for GABA, glycine, taurine, norepinephrine, dopamine and serotonin. They are sodium- and chloride-dependent, but do not require potassium for function. One of these, the $GABA_A$ transporter, encoded by GAT-1, is perhaps the best characterized. It has been purified and reconstituted and has a molecular mass of around 80 kDa, of which 10–15 kDa is sugar. Amino and carboxyl termini (around 50 amino acids each) are not required for function. The transporter is protected against proteolysis at multiple sites by GABA, provided that the two cosubstrates – sodium and chloride – are present. Several amino acid residues that are critical for function have been identified in the GABA transporter. These include arginine-69 and tryptophan-222 located in the first and fourth putative transmembrane helices, respectively. The first is possibly involved in the binding of chloride. The tryptophan appears to serve as a binding site for the amino group of GABA.

Introduction

High-affinity sodium-dependent transport of neurotransmitters from the synaptic cleft appears to terminate the overall process of synaptic transmission (Iversen, 1975; Kuhar, 1973). Such a termination mechanism operates with most transmitters, including γ-aminobutyric acid (GABA), L-glutamate, glycine, dopamine, serotonin and norepinephrine. Another termination mechanism is observed with cholinergic transmission. After dissociation from its receptor, acetylcholine is hydrolysed into choline and acetate. The choline moiety is then recovered by sodium-dependent transport as described above. As the concentration of the transmitters in the nerve terminals is much higher than in the cleft – typically by four orders of magnitude – energy input is required. The transporters that are located in the plasma membranes of nerve endings and glial cells obtain this energy by coupling the flow of neurotransmitters to that of sodium (Fig. 1). The Na^+/K^+-ATPase generates an inwardly directed electrochemical sodium

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gradient which is utilized by the transporters to drive 'uphill' transport of the neurotransmitters (reviewed in Kanner, 1983, 1989; Kanner and Schuldiner, 1987).

Neurotransmitter uptake systems have been investigated in detail by using plasma membranes obtained upon osmotic shock of synaptosomes. It appears that these transporters are coupled not only to sodium but also to additional ions such as potassium or chloride (Fig. 1).

These transporters are of considerable medical interest. Since they function to regulate neurotransmitter activity by removing it from the synaptic cleft, specific transporter inhibitors can potentially be used as novel drugs for treating neurological disease. For instance, attenuation of GABA removal will prolong the effect of this inhibitory transporter, thereby potentiating its action. Thus, inhibitors of GABA transport could represent a novel class of anti-epileptic drugs. Well-known inhibitors that interfere with the functioning of biogenic amine transporters include antidepressant drugs and stimulants such as amphetamines and cocaine. The neurotransmitter glutamate – at excessive local concentrations – causes cell death, by activating *N*-methyl-D-aspartic acid (NMDA) receptors and subsequent calcium entry. The transmitter has been implicated in neuronal destruction during ischaemia, epilepsy, stroke, amyotropic lateral sclerosis and Huntington's disease. Neuronal and glial glutamate transporters may have a critical role in preventing glutamate from acting as an exitotoxin (Johnston, 1981; McBean and Roberts, 1985).

In the last few years, major advances in the cloning of these neurotransmitter transporters have been made. After the GABA transporter had been purified (Radian *et al.* 1986), the ensuing protein sequence information was used to clone it (Guastella *et al.* 1990). Subsequently, the expression cloning of a norepinephrine transporter (Pacholczyk *et al.* 1991) provided evidence that these two proteins are the first members of a novel superfamily of neurotransmitter transporters. This result led – using polymerase chain reaction (PCR) and other technologies relying on sequence conservation – to the isolation of a growing list of neurotransmitter transporters (reviewed in Uhl, 1992; Schloss *et al.* 1992; Amara and Kuhar, 1993). This list includes various subtypes of GABA transporters as well as those for all the above-mentioned neurotransmitters, except glutamate. All of the members of this superfamily are dependent on sodium and chloride and, by analogy with the GABA transporter (Keynan and Kanner, 1988), are likely to cotransport their transmitter with both sodium and chloride. Interestingly, sodium-dependent glutamate transport is not chloride-dependent, but rather sodium and glutamate are countertransported with potassium (Fig. 1, Kanner and Sharon, 1978; Kanner and Bendahan, 1982). Recently, three distinct but highly related glutamate transporters have been cloned (Storck *et al.* 1992; Pines *et al.* 1992; Kanai and Hediger, 1992). These transporters represent a distinct family.

Here we describe the current status on two prototypes of these distinct families; the GABA and glutamate transporters.

Stoichiometry

The GABA transporter cotransports the neurotransmitter with sodium and chloride in

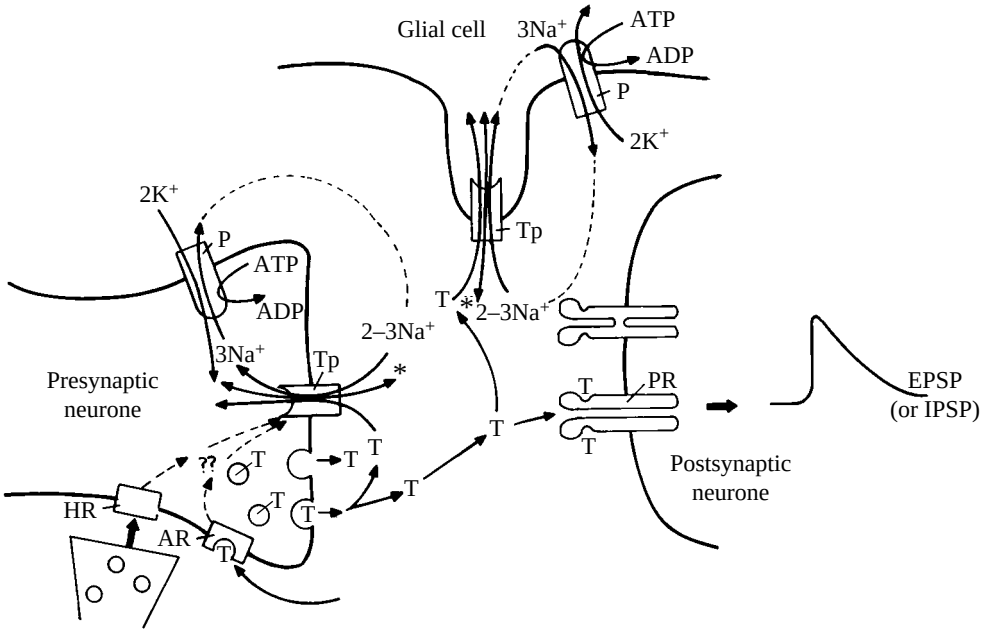

Fig. 1. The role of plasma membrane neurotransmitter transporters in synaptic transmission. Neurotransmitter (T), stored in synaptic vesicles, is released by fusion of the vesicles with the synaptic plasma membrane. After its diffusion across the synaptic cleft, it binds to postsynaptic receptors (PR), resulting in the opening of channels often present in the same structure as the receptor. Channel opening may lead to excitatory or inhibitory postsynaptic potentials (EPSP or IPSP). Transmitter is removed from the cleft by re-uptake mediated by electrogenic sodium-coupled transporters (Tp), which also translocates other ions (∗). In the case of the transporters of GABA and other members of the large superfamily, ∗ is chloride, which moves in the same direction as sodium and the neurotransmitter. In the case of the glutamate transporters, ∗ represents potassium, which moves in the opposite direction to sodium and glutamate. The main driving force for this process is the electrochemical gradient of sodium ions, which is maintained by the sodium pump (Na^+/K^+-ATPase, P). The transporters are located in the synaptic plasma membrane and also in the processes of glial cells, which are in close contact with the synapse. Initial results indicate that the activity of these transporters may be the subject of physiological regulation. This may be mediated by receptors for the same neurotransmitter (autoreceptors, AR) or by those of others (heteroreceptors, HR).

an electrogenic fashion (Kanner, 1983; Keynan and Kanner, 1988). The available measurements include tracer fluxes (Keynan and Kanner, 1988) and electrophysiological approaches (Kavanaugh *et al.* 1992; Mager *et al.* 1993).

The mechanism of sodium-dependent L-glutamate transport has been studied initially using tracer flux studies employing radioactive glutamate. These studies indicated that the process is electrogenic, with positive charge moving in the direction of the glutamate (Kanner and Sharon, 1978). This observation suggested that it would be possible to monitor L-glutamate transport electrically using the whole-cell patch-clamp technique (Brew and Atwell, 1987). This latter technique has the advantage that the membrane

potential can be controlled throughout the transport experiments. In addition to L-glutamate, D- and L-aspartate are transportable substrates with affinities in the lower micromolar range. The system is stereospecific with regard to glutamate, the D-isomer being a poor substrate. Glutamate uptake is driven by an inwardly directed sodium ion gradient and at the same time potassium moves outwards. The potassium movement is not a passive movement in response to the charge carried by the transporter. Rather, it is an integral part of the translocation cycle catalyzed by the transporter. Its role is further described below. Recently, evidence has been presented that another ionic species is countertransported (in addition to potassium), namely hydroxyl ions (Bouvier *et al.* 1992).

The first-order-dependence of the carrier current on internal potassium (Barbour *et al.* 1988), together with the well-known first-order-dependence on external L-glutamate and the sigmoid dependence on external sodium, suggest a stoichiometry of $3Na^+$:$1K^+$:1 glutamate (Kanner and Sharon, 1978; Barbour *et al.* 1988). This stoichiometry implies that one positive charge moves inwards per glutamate anion entering the cell. If a hydroxyl anion is countertransported as well (Bouvier *et al.* 1992), the stoichiometry could be $2Na^+$:$1K^+$:1glutamate:$1OH^-$, and transport would still be electrogenic. A stoichiometry of $2Na^+$:1glutamate is also favoured by direct experimental evidence obtained by kinetic (Stallcup *et al.* 1979) and thermodynamic (Erecinska *et al.* 1983) methods.

The study of the ion-dependence of partial reactions of the glutamate transporter has revealed that glutamate transport is an ordered process. First, sodium and glutamate are translocated. After their release inside the cell, potassium binds to the transporter and is translocated outwards so that a new cycle can be initiated (Kanner and Bendahan, 1982; Pines and Kanner, 1990).

Reconstitution, purification and localization

Using methodology that enables one to reconstitute many samples simultaneously and rapidly, one of each of the subtypes of the GABA (Radian *et al.* 1986) and the L-glutamate (Danbolt *et al.* 1990) transporters have been purified to apparent homogeneity. Both are glycoproteins and both have an apparent molecular mass of 70–80 kDa. The two transporters retain all the properties observed in membrane vesicles. They are distinct not only because of their different functional properties. Antibodies generated against the GABA transporter (Radian *et al.* 1986) react (as detected by immunoblotting) only with fractions containing GABA transport activity and not with those containing L-glutamate transport activity (Danbolt *et al.* 1990). The opposite is true for antibodies generated against the glutamate transporter (Danbolt *et al.* 1992). Recently, the glycine transporter has also been purified and reconstituted. Interestingly, it appears to be a larger protein than the GABA and glutamate transporters – about 100 kDa in size (Lopez-Corcuera *et al.* 1991). The serotonin transporter has also been purified, but these preparations, containing a band around 70 kDa, have been shown to be active only in the binding of [^{3}H]imipramine but not in serotonin transport (Launay *et al.* 1992; Graham *et al.* 1992). Immunocytochemical localization studies of the GABA transporter reveal that in most

brain areas it is located in the membranes of nerve terminals (Radian *et al.* 1990) although, in some areas, such as substantia nigra, glial processes were labelled.

Using the antibodies raised against the glutamate transporter, the immunocytochemical localization of the transporter was studied at the light and electron microscopic level in rat central nervous system. In all regions examined (including cerebral cortex, caudato-putamen, corpus callosum, hippocampus, cerebellum and spinal cord), it was found to be located in glial cells rather than in neurones. In particular, fine astrocytic processes were strongly stained. Putative glutamatergic axon terminals appeared to be non-immunoreactive (Danbolt *et al.* 1992). The uptake of glutamate by such terminals (for which there is strong previous evidence) may therefore be due to a subtype of glutamate transporter different from the glial transporter. Using a monoclonal antibody raised against this transporter, a similar glial localization of the transporter was found (Hees *et al.* 1992).

A new superfamily of Na^+-dependent neurotransmitter transporters

Partial sequencing of the purified $GABA_A$ transporter allowed the cloning of the first member of the new family of Na^+-dependent neurotransmitter transporters (Guastella *et al.* 1990). After expression cloning of the noradrenaline transporter (Pacholczyk *et al.* 1991), it became clear that it had significant homology with the $GABA_A$ transporter. The use of functional cDNA expression assays and amplification of related sequences using the polymerase chain reaction (PCR) resulted in the cloning of additional transporters belonging to this family, such as the dopamine (Shimada *et al.* 1991; Kilty *et al.* 1991; Usdin *et al.* 1991) and serotonin (Hoffman *et al.* 1991; Blakely *et al.* 1991) transporters, additional GABA transporters (Clark *et al.* 1992; Borden *et al.* 1992; Lopez-Corcuera *et al.* 1992; Liu *et al.* 1993*a*), transporters of glycine (Smith *et al.* 1992; Liu *et al.* 1992*b*; Guastella *et al.* 1992), proline (Fremeau *et al.* 1992), taurine (Uchida *et al.* 1992; Liu *et al.* 1992*a*) and betaine (Yamauchi *et al.* 1992) and two ‘orphan’ transporters, whose substrates are still unknown (Uhl *et al.* 1992; Liu *et al.* 1993*c*). In addition, another family member, which was originally thought to be a choline transporter (Mayser *et al.* 1992), is probably a creatine transporter (Guimbal and Kilimann, 1993). A novel glycine transporter cDNA encoding for a 799 amino acid protein has recently been isolated (Liu *et al.* 1993*b*). This is significantly longer than most members of the superfamily. If we take into account that part of the mass of these transporters consists of sugar, it could encode the 100 kDa glycine transporter that has been purified and reconstituted (Lopez-Corcuera *et al.* 1992).

The deduced amino acid sequences of these proteins reveal 30–65 % identity between different members of the family. On the basis of these differences in homology, the family can be divided into four subgroups: (a) transporters of biogenic amines (noradrenaline, dopamine and serotonin); (b) various GABA transporters as well as transporters of taurine and creatine; (c) transporters of proline and glycine; and (d) ‘orphan’ transporters. These proteins share some features of a common secondary structure (illustrated in Fig. 2). Each transporter is composed of 12 hydrophobic putative transmembrane α-helices. The lack of a signal peptide suggests that both amino- and carboxy-termini face the cytoplasm. These regions contain putative phosphorylation sites, which may be involved in regulation of the

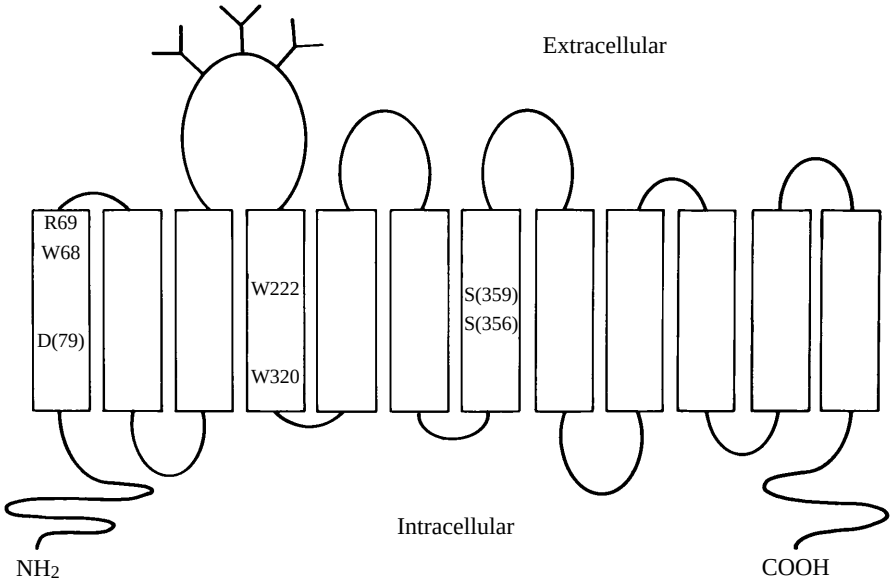

Fig. 2. Schematic representation of the GABA transporter GAT-1 and the location of critical amino acids. Putative transmembrane segments are shown as rectangles. The potential glycosylation sites are indicated by branched lines. Critical residues of GAT-1 are indicated using the one-letter code and their positions are also given. Also indicated are residues important for the optimal functioning of the dopamine transporter DAT-1. In this case, the positions are indicated in parentheses using the numbering of DAT.

transport process. The second extracellular loop between helices 3 and 4 is the largest, and it contains putative glycosylation sites.

Alignment of the deduced amino acid sequences of 13 different members of this superfamily, whose substrates are known (subgroups a–c) revealed that some segments within these proteins share a higher degree of homology than others. The most highly conserved regions (>50 % homology) are helix 1, together with the extracellular loop connecting it with helix 2, and helix 5, together with a short intracellular loop connecting it with helix 4 and a larger extracellular loop connecting it with helix 6. These domains may be involved in stabilizing a tertiary structure that is essential for the function of all these transporters. Alternatively, they may be related to a common function of these transporters, such as the translocation of sodium ions. The region stretching from helix 9 onwards is far less conserved than the segment containing the first 8 helices. Possibly, this domain contains some residues that are involved in translocating the different substrates. The least conserved segments are the amino and carboxy termini. As was mentioned above, these areas may be involved in regulation of the transport process. The ‘orphan’ transporters differ from all other members of the family in three regions. They contain much larger extracellular loops between helices 7–8 and helices 11–12.

Molecular cloning and predicted structure of glutamate transporters

Transporters for many neurotransmitters were cloned on the assumption that they were

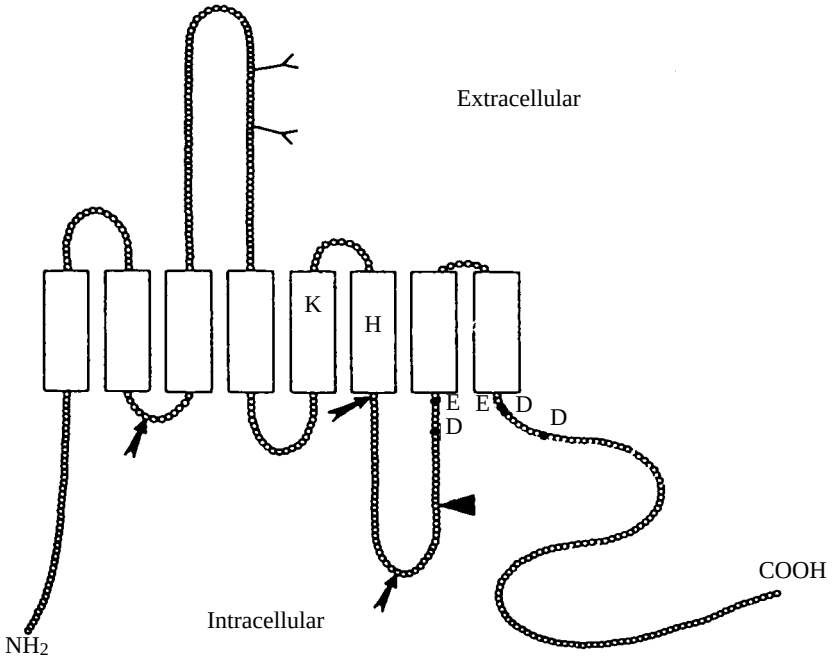

Fig. 3. Schematic representation of the L-glutamate transporter GLT-1, showing its proposed orientation in the plasma membrane. Putative transmembrane segments are shown as rectangles. One putative protein kinase A and three putative protein kinase C phosphorylation sites are indicated by an arrowhead and arrows, respectively. The remaining two potential protein kinase C sites in the putative extracellular loop between helices 3 and 4 are not shown. The two putative glycosylation sites are also located on this loop and are indicated as branched lines. The locations of the conserved negatively charged amino acids that may be located in the membrane are also indicated in this model (see text).

related to the GABA (Guastella *et al.* 1990) and norepinephrine (Pacholczyk *et al.* 1991) transporters (reviewed in Uhl, 1992; Schloss *et al.* 1992; Amara and Kuhar, 1993). This approach was unsuccessful for the glutamate transporter. Recently, three different glutamate transporters have been cloned using different approaches: GLAST (Storck *et al.* 1992), GLT-1 (Pines *et al.* 1992) and EAAC1 (Kanai and Hediger, 1992). The former two appear to be of glial (Storck *et al.* 1992; Danbolt *et al.* 1992), the latter of neuronal (Kanai and Hediger, 1992), origin. Indeed, the three transporters are not related to the above superfamily (Storck *et al.* 1992; Pines *et al.* 1992; Kanai and Hediger, 1992). However, they are very similar to each other (Fig. 3), displaying approximately 50 % identity and approximately 60 % similarity. They also appear to be related to the proton-coupled glutamate transporter from *Escherichia coli* and other bacteria (gltP, Tolner *et al.* 1992) and the dicarboxylate transporter (dct-A, Jiang *et al.* 1989) of *Rhizobium meliloti*. In these cases, the identities are around 25–30 %. Thus, they form a distinct family. They contain between 500 and 600 amino acids. Recently, it has been shown that this family also encodes sodium-dependent transporters that do not use dicarboxylic acids as substrates, but rather neutral amino acids (Shafqat *et al.* 1993; Arriza *et al.* 1993).

GLT-1, which encodes the glutamate transporter that was purified (Pines *et al.* 1992; Danbolt *et al.* 1990, 1992), has 573 amino acids and a molecular mass of 64 kDa, in good agreement with the value of 65 kDa of the purified and deglycosylated transporter (Danbolt *et al.* 1992). Hydropathy plots are relatively straightforward at the amino-terminal side of the protein and the three different groups have predicted six transmembrane α-helices at very similar positions (Storck *et al.* 1992; Pines *et al.* 1992; Kanai and Hediger, 1992). In contrast, there is much more ambiguity at the carboxyl side where zero (Storck *et al.* 1992), two (Pines *et al.* 1992) or four (Kania and Hediger, 1992) α-helices have been predicted. However, all three groups note uncertainty in assigning transmembrane α-helices in this part of the protein, taking into account alternative possibilities, including membrane spanning β-sheets (Storck *et al.* 1992). It is clear that experimental approaches to delineate their topology are badly needed.

One of the proposed models (GLT-1, Pines *et al.* 1992) is shown here (Fig. 3) to point out some other structural features. These include potential glycosylation sites in the large extracellular loop between helices 3 and 4 and some of the conserved charged amino acids. These include a conserved lysine located in helix 5 and a histidine in helix 6. Preliminary site-directed mutagenesis studies indicate that the histidine is critical for the activity of GLT-1 (Zhang *et al*. 1994). Other conserved negatively charged amino acids are also marked. They are all located in the part of the transporter where the hydropathy plot is ambiguous. Thus, it is possible that one or more of these amino acids reside in the membrane, possibly on β-sheets traversing it. Site-directed mutagenesis studies of these amino acids are in progress. Also indicated are the putative protein kinase A and C phosphorylation sites. It has been shown that phorbol esters activate glutamate transport in glial, but not in neuronal, cells (Casado *et al.* 1991). It appears that at least one of these sites – located in the loop connecting putative helices 2 and 3 – is involved in this (Casado *et al.* 1993).

Structure–function relationships in the superfamily of neurotransmitter transporters

It has been shown previously that parts of amino and carboxyl termini of the $GABA_A$ transporter are not required for function (Mabjeesh and Kanner, 1992). In order to define these domains, a series of deletion mutants was studied in the GABA transporter (Bendahan and Kanner, 1993). Transporters truncated at either end until just a few amino acids distant from the beginning of helix 1 and the end of helix 12 retained their ability to catalyze sodium- and chloride-dependent GABA transport. These deleted segments did not contain any residues conserved among the different members of the superfamily. Once the truncated segment included part of these conserved residues, the transporter's activity was severely reduced. However, the functional damage was not due to impaired turnover or impaired targeting of the truncated proteins (Bendahan and Kanner, 1993).

Fragments of the Na^+/Cl^--coupled $GABA_A$ transporter were produced by proteolysis of membrane vesicles and reconstituted preparations from rat brain (Mabjeesh and Kanner, 1993). The former were digested with pronase, the latter with trypsin. Fragments with different apparent molecular masses were recognized by sequence-directed

antibodies raised against this transporter. When GABA was present in the digestion medium, the generation of these fragments was almost entirely blocked (Mabjeesh and Kanner, 1993). At the same time, the neurotransmitter largely prevented the loss of activity caused by the protease. The effect was specific for GABA; protection was not afforded by other neurotransmitters. It was only observed when the two cosubstrates, sodium and chloride, were present on the same side of the membrane as GABA (Mabjeesh and Kanner, 1993). The results indicate that the transporter may exist in two conformations. In the absence of one or more of the substrates, multiple sites located throughout the transporter are accessible to the proteases. In the presence of all three substrates – conditions favouring the formation of the translocation complex – the conformation is changed such that these sites become inaccessible to protease action.

The substrate translocation performed by the various members of the superfamily is sodium-dependent and usually chloride-dependent. In addition, some of the substrates also contain charged groups. Therefore, charged amino acids in the membrane domain of the transporters may be essential for their normal function. This was tested using the GABA transporter (Pantanowitz *et al.* 1993). Of five charged amino acids within its membrane domain (see Fig. 2) only one, arginine-69 in helix 1, is absolutely essential for activity. It is not merely the positive charge that is important, as even its substitution with other positively charged amino acids does not restore activity. The functional damage is not due to impaired turnover or impaired targeting of the mutated protein. The three other positively charged amino acids and the only negatively charged one are not critical (Pantanowitz *et al.* 1993). It is possible that the arginine-69 residue may be involved in chloride binding.

The transporters of biogenic amines contain an additional negatively charged residue in helix 1 (aspartate-79 in Fig. 2). Replacement of aspartate-79 in the dopamine transporter with alanine, glycine or glutamate significantly reduced the transport of dopamine and MPP^+ (a Parkinsonism-inducing neurotoxin) and the binding of CFT (a cocaine analogue), without affecting B_{max}. Apparently, aspartate-79 in helix 1 interacts with dopamine's amine during the transport process. Serine-356 and serine-359 in helix 7 (see Fig. 2) are also involved in dopamine binding and translocation, perhaps by interacting with the hydroxyl groups on the catechol (Kitayama *et al.* 1992).

Studies of other proteins indicate that, in addition to charged amino acids, aromatic amino acids containing π-electrons are also involved in maintaining the structure and function of these proteins (Sussman and Silman, 1992). Therefore, tryptophan residues in the membrane domain of the GABA transporter were mutated into serine as well as leucine (Kleinberger-Doron and Kanner, 1994). Mutations at the 68 and 222 positions (in helix 1 and helix 4, respectively) led to a decrease of over 90 % in the GABA uptake.

On the basis of the alignments of the transporters of the superfamily, it was postulated that tryptophan-222 is involved in the binding of the amino group of GABA. Using ^3H-labelled tiagabine, an analogue that binds to GABA transporters but does not appear to be transported (Braestrup *et al.* 1990), we have recently obtained evidence supporting this idea. While mutants at tryptophan-68 bound tiagabine at least as well as the wild type, those at tryptophan-222 were completely deficient in this process (N. Kleinberger-Doron and B. I. Kanner, in preparation).

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