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Short Review

# The Family of $Na^+/Cl^-$ Neurotransmitter Transporters

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**Abstract:** The termination of neurotransmission is achieved by rapid uptake of the released neurotransmitter by specific high-affinity neurotransmitter transporters. Most of these transporters are encoded by a family of genes ($Na^+/Cl^-$ transporters) having a similar membrane topography of 12 transmembrane helices. An evolutionary tree revealed five distinct subfamilies: γ-aminobutyric acid transporters, monoamine transporters, amino acid transporters, "orphan" transporters, and the recently discovered bacterial transporters. The bacterial transporters that belong to this family may help to develop heterologous expression systems with the aim of solving the three-dimensional structure of these membrane proteins. Some of the neurotransmitter transporters have been implicated as important sites for drug action. Monoamine transporters, for example, are targeted by major classes of antidepressants, psychostimulants, and antihypertensive drugs. Localization of individual transporters in specific cells and brain areas is pertinent to understanding their contribution to neurotransmission and their potential as targets for drugs. The most important questions in the field include resolving the mechanism of neurotransmitter transport, the structure of the transporters, and the interaction of each transporter in complex neurological activities.
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Communication between separate cells requires the release of extracellular messengers and specific receptor mechanisms on or in the target cells. Signaling substances include hormones, neurotransmitter substances, trophic factors, and other diffusible substances. In higher organisms, chemical transmission is the principal way of communication between cells, especially in the nervous system. Nerve cells mediate fast signaling between sensory systems and the CNS and between the CNS and effector systems. Within the CNS, nerve cells form complex circuits that serve the integration of inputs and the generation of specific activity patterns. In many cases, synaptic transmission is chemical and involves the secretion of signaling substances. In most systems, the termination of the chemical transmission is achieved by rapid uptake of the released neurotransmitter, by specific high-affinity neurotransmitter transporters, into the synaptic terminal or the surrounding glial cells (Kuhar, 1973; Iversen and Kelly, 1975; Kanner, 1983, 1989; Kanner and Schuldiner, 1987). It has been known for many years that neurons and glia can accumulate neurotransmitters by a $Na^+$-dependent transport process. Neurotransmitters are cotransported with $Na^+$ using the energy stored in transmembrane electrochemical gradients generated by primary ion pumps (Kanner, 1983).

Studies on neurotransmitter uptake indicated the existence of multiple uptake systems, each relatively selective for a specific neurotransmitter (Iversen and Johnston, 1971). Neurotransmitters are transported across membranes by at least four distinct families of transporters: (a) vesicular transporters that function in the uptake of neurotransmitters into synaptic vesicles and granules (Schuldiner, 1994); (b) $Na^+$- and $Cl^-$-dependent ($Na^+/Cl^-$) transporters that operate on the plasma membrane of neuronal and glia cells (Schloss et al., 1992; Uhl, 1992; Amara and Kuhar, 1993); (c) $Na^+/K^+$-dependent transporters that function on the plasma membranes, especially in glutamate transport (Kanner, 1993); and (d) general amino acid transport systems that participate in controlling the availability of neurotransmitters outside the cells (McGivan and Pastor-Anglada, 1994).

A defining moment in the advancement of our knowledge of neurotransmitter transporters came in 1990 when the first cDNAs encoding the γ-aminobutyric acid (GABA) transporters were cloned and sequenced (Guastella et al., 1990; Nelson et al., 1990).

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*Abbreviations used:* ACHC, *cis*-3-aminocyclohexanecarboxylate; GABA, γ-aminobutyric acid; GAT1–4, GABA transporters 1–4; GLYT1 and GLYT2, glycine transporters 1 and 2; $Na^+/Cl^-$, $Na^+$- and $Cl^-$-dependent; NMDA, *N*-methyl-D-aspartic acid; NTT4, neurotransmitter transporter 4; PCP, phencyclidine; TM, transmembrane helix/domain.

Subsequently, several cDNAs encoding these plasma membrane transporters have been cloned, either by independent expression cloning or by using sequence conservation between GABA and nonadrenaline transporters, with the resulting promise of rapid new advances in understanding the mechanism of neurotransmitter reuptake (Blakely et al., 1991; Hoffman et al., 1991; Kilty et al., 1991; Pacholczyk et al., 1991; Shimada et al., 1991; Usdin et al., 1991). This review will deal exclusively with the family of $Na^+/Cl^-$ transporters.

## STRUCTURE OF $Na^+/Cl^-$ NEUROTRANSMITTER TRANSPORTERS

The gene products of the $Na^+/Cl^-$ transporter family are highly conserved. In mammals, they can be grouped into four subfamilies: GABA transporters, monoamine transporters, amino acid transporters, and "orphan" [typified by neurotransmitter transporter 4 (NTT4)] transporters (Nelson and Lill, 1994; Uhl and Johnson, 1994). The transporters of the first three subfamilies show a common structure of presumably 12 transmembrane helices/domains (TM) with a single large loop in the external face of the membrane with potential glycosylation sites (Fig. 1). The orphan subfamily (NTT4), somewhat remote from the other family members, was identified by sequencing cDNA from mammalian sources (Uhl et al., 1992; Liu et al., 1993*c*). Their structure deviates from the norm by having two potential glycosylated loops outside the membrane. Knowing the limitations of hydropathy plots as predictors for the existence of transmembrane helices and the topography of membrane proteins (Eisenberg, 1984), it was proposed that the GABA transporter GAT1 contains 12 transmembrane helices (Guastella et al., 1990; Nelson et al., 1990). This assumption was accepted as a likely working hypothesis by the groups that cloned and sequenced cDNA encoding members of this family of transporters (Amara and Kuhar, 1993). Similar structures have been reported for several other families of transporters, including bacterial, plant, and mammalian sugar transporters (Hediger et al., 1987; Kaback, 1992; Caspari et al., 1994). The mechanistic significance of this structure is not understood, and there are no apparent common motifs in all transporters with the 12-transmembrane topology. However, a dimeric organization of six helices was proposed as a common structure for membrane transporters (Maloney, 1990). Although the number 12 has been maintained for transmembrane helices, there is no apparent reason for the position of short and extended loops between the helices. Some transporters contain charged residues inside their hydrophobic membrane spanning segments, but several have no charges in their transmembrane helices. Therefore, if there is a common mechanism for transport of substrate across the membrane, it is hiding behind motifs that are as yet unrecognized.

The proposed general structure of the $Na^+/Cl^-$ transporters was verified by experimental evidence and logical arguments (Nelson, 1993; Pantanowitz et al., 1993). The evidence includes the observations that there is no cleavable N-terminal sequence, suggesting that the N-terminal part of the transporter is situated in the cytoplasmic face of the plasma membrane (see Fig. 1). Studies using peptide-specific antibodies to several regions of the GABA transporter (GAT1) combined with limited proteolysis with pronase suggested that the N- and C-terminal regions are present inside the membrane (Mabjeesh et al., 1992; Mabjeesh and Kanner, 1993). Three transmembrane segments are required to position the large glycosylated loop at the external surface of the plasma membrane. In addition, in the orphan (NTT4) transporter subfamily, an extended hydrophilic loop with potential glycosylation sites exists between the proposed TM 7 and 8. Taking this together with the experimental evidence on the *E. coli* lactose permease, which withstood the scrutiny of numerous molecular studies (Kaback et al., 1997), it is likely that the structure of $Na^+/Cl^-$ transporters contains 12 transmembrane helices.

A direct experimental approach to identify the position and boundaries of the various transmembrane helices was performed by the use of site-directed antibodies and selective cleavage of representative transporters. An immunofluorescent study with antibodies raised against peptide sequences from the N- and C-termini and the large extracellular loop of the noradrenaline transporter, provided data to support the proposed orientation of these domains (Melikian et al., 1994; Bruss et al., 1995). An electron microscopic immunochemical study provided support for the cytoplasmic location of the dopamine transporter N-terminus (Nirenberg et al., 1996). Fragments with apparent different molecular masses were generated by digestion of GAT1 in native or reconstituted vesicles with pronase (Mabjeesh and Kanner, 1993). The fragments were recognized by site-specific antibodies, and the resulting fragments were in good fit with the proposed membrane structure of GAT1.

Although the general structure of the $Na^+/Cl^-$ transporters was verified by experimental evidence, the position of the first three transmembrane helices was debated recently (Bennett and Kanner, 1997; Olivares et al., 1997). One of the hallmarks of $Na^+/Cl^-$ transporters is the conserved amino acid sequence that includes the residue WRFPXXXYXNGGGAF. With very few exceptions, this sequence is highly conserved not only for the amino acid residues, but also in the nucleotide sequences of the mRNA. Moreover, in all the transporter genes, there is an intron within the coding sequence of the three glycine residues (Liu et al., 1992*a*). The position of this intron is conserved not only in the vertebrate genes, but also in the genes encoding $Na^+/Cl^-$ transporters in insects. According to the original proposal for the membrane organization of $Na^+/Cl^-$ transporters, the vicinity of these three

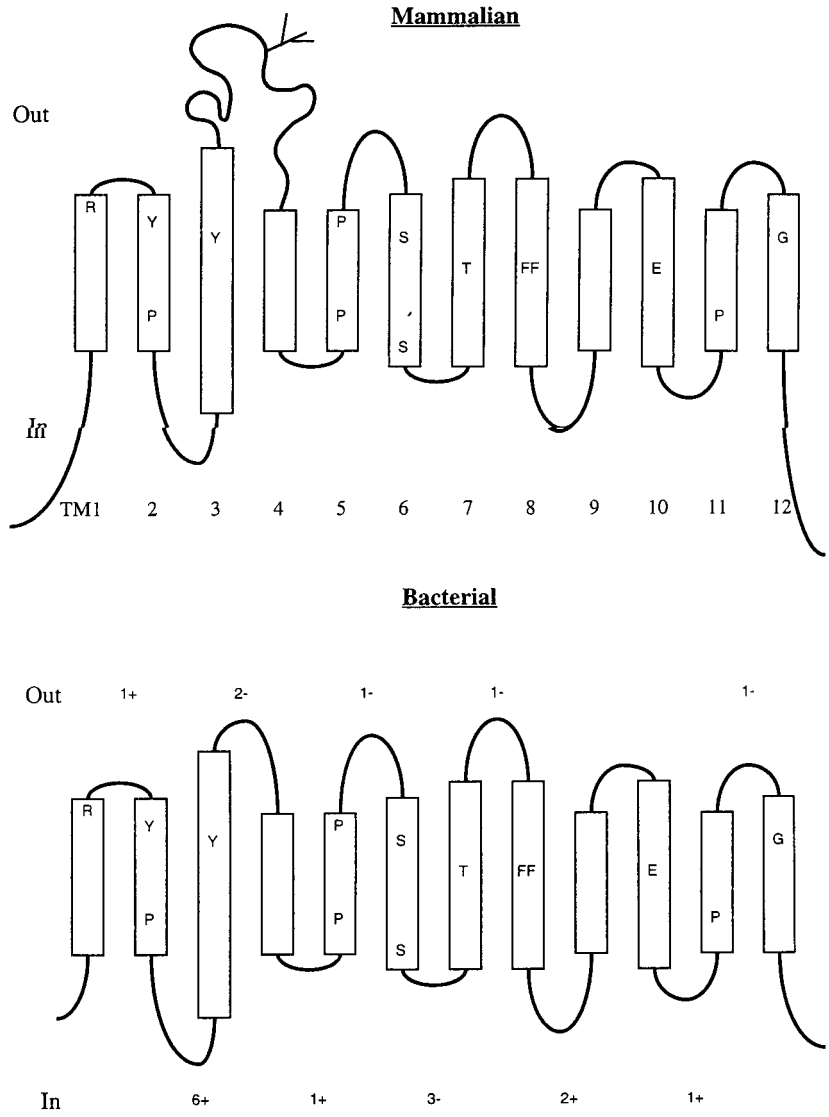


**FIG. 1.** Schematic representation of the membrane topology of mammalian and bacterial transporters that belong to the family of $Na^+/Cl^-$ neurotransmitter transporters. Potential sizes of the various transmembrane helices are depicted, and some highly conserved amino acids potentially situated in the membrane are indicated. The net charge of each loop in STRYP1 (gene of the *Symbiobacterium thermophilum* genome) is indicated.

glycine residues is situated between TM 1 and 2. This assumption was challenged by two recent studies on the membrane topology of GAT1 and the glycine transporter GLYT1 (Bennett and Kanner, 1997; Olivares et al., 1997). *N*-Glycosylation scanning mutagenesis was performed with GAT1 in which the glycosylation sites in the large external loop were inactivated by N to D substitutions. Functional transporters with glycosylation at the predicted external loops 3 and 6 essentially demonstrated the validity of the membrane topography from the large external loop (no. 2) to the end of the transporter (Bennett and Kanner, 1997). Surprisingly, insertion of the sequence NNSST to the predicted internal loop 1 resulted in a glycosylation at this site. However, the mutated transporter was not active in GABA transport. Similar glycosylation scanning was performed with GLYT1, leading to the same conclusion that the predicted membrane topography from the large glycosylated loop to the C-terminus of the transporter is as originally predicted by the hydropathy plot. This work further suggested that the predicated internal loop 1 is essentially facing the outer surface of the membrane (Olivares et al., 1997). To reconcile this with the fact that the N-terminus is at the cytoplasmic side of the membrane, both groups proposed that the rather long TM 3 essentially traverses the membrane twice, generating TM 2 and 3 (see Fig. 1). The original amino acid residues proposed to be TM 2 are the first to cross the membrane. Indeed, in some, but not all, $Na^+/Cl^-$ transporters, the amino acid sequence that was proposed to be comprised of the third transmembrane segment contains up to 38 uncharged amino acids (Nelson, 1993). However, in one of the bacterial transporters that are homologous to the mammalian $Na^+/Cl^-$ transporters, there are only 24 uncharged amino acids in the same position (N. Nelson et al., unpublished observations). Moreover, a recent study on the topography of the serotonin trans-

porter using cysteine-modifying reagents and biotinylated probe scanning endorses the original proposal for the topography of $Na^+/Cl^-$ transporters (Chen et al., 1997*a*; Barker and Blakely, 1998). In any case, the long stretch of uncharged amino acids (124–161 in GAT1) may have an important role in the mechanism of energy coupling and/or substrate translocation across the membrane (Chen et al., 1997*b*). High-resolution structural information on even one of the family members may shed light not only on their membrane topography, but also on their function at a molecular level.

## THE FIVE SUBFAMILIES OF $Na^+/Cl^-$ NEUROTRANSMITTER TRANSPORTERS

Genomic cloning of genes encoding neurotransmitter transporters and the search for sequences without known function in the GenBank revealed that insects and *C. elegans* contain genes encoding potential neurotransmitter transporters (Liu et al., 1992*a*). Therefore, it was assumed that this family of transporters evolved from a common ancestor about 1 billion years ago (Nelson, 1993). Recently, we identified and cloned a bacterial gene with relatively high homology to neurotransmitter transporters (N. Nelson et al., unpublished observations). This gene is present in a potential tryptophanase operon of *Symbiobacterium thermophilum* (Hirahara et al., 1992), which contains the genes encoding the enzyme tryptophanase and a potential tryptophan transporter. Recently, the entire genomic sequences of *Haemophilus influenzae* and *Methanococcus jannaschii* were determined (Fleischmann et al., 1995; Bult et al., 1996). Each genome contains a single gene homologous to the mammalian neurotransmitter transporters. These findings indicate that the family of neurotransmitter transporters is related to the onset of life and is present not only in eukaryotes, but also in eubacteria and archaebacteria. So far, genes encoding transporters of this family have not been identified in plants and fungi. It is also noteworthy that homologous transporters were not found in bacteria, such as *E. coli, Bacillus subtilis,* and cyanobacteria, where most of the genomic sequence is known. Therefore, it appears that plants, fungi, and those bacteria that primarily use protonmotive force for their transport systems gave up the use of these transporters.

An evolutionary tree constructed with different parts of the various transporters suggests that the bacterial transporters are loosely grouped with the orphan (NTT4) transporters or with the amino acid transporters (Fig. 2). The separation into five subfamilies of transporters is quite apparent. The subfamilies as we see them now are GABA transporters, the subfamily of monoamine transporters, the subfamily of amino acid transporters, the subfamily of orphan (NTT4) transporters, and the subfamily of bacterial transporters. The evolutionary tree reveals some interesting relationships between the various transporters. In the subfamily of GABA transporters, GAT1 appears to be quite remote from the other subfamily members. This is in line with its substrate specificity that is exclusive for GABA. Similarly, the creatine transporter is also separated from the other subfamily members. All the other subfamily members transport $\beta$-alanine, and they are grouped together in the evolutionary tree. It is interesting that $\beta$-alanine transport requires a higher degree of amino acid sequence conservation than does GABA transport. The three subfamilies with known substrates are distinguished not only by their sequence homology, but also by their substrate specificity and pharmacology.

### The GABA transporter as a prototype

GABA is the predominant inhibitory neurotransmitter and is widely distributed in the mammalian brain. Its uptake is catalyzed by transporters ubiquitously distributed in the various brain parts. Biochemical and physiological studies revealed two major subtypes of GABA transport systems. The $GABA_A$ uptake system, which is inhibited by diaminobutyric acid or *cis*-3-aminocyclohexanecarboxylate (ACHC), is thought to be neuronal (Bowery et al., 1976), and the $GABA_B$ transport system, which is inhibited by $\beta$-alanine, is thought to be of glial origin (Schon and Kelly, 1975). One of the GABA transporters (GAT1) was the first to have been purified to apparent homogeneity (Radian et al., 1986). A reconstitution assay was used in which detergent-solubilized membrane protein fractions were incorporated into liposomes of asolectin and brain lipids, and then radiolabeled GABA uptake was determined (Radian and Kanner, 1985). A glycosylated protein with an apparent molecular mass of 80 kDa was isolated and, following reconstitution, exhibited ACHC-sensitive GABA uptake into the liposomes. The GABA uptake was not sensitive to $\beta$-alanine, and it was concluded that GAT1 is the neuronal GABA transporter (Kanner et al., 1989). This conclusion was endorsed by immunocytochemical localization of GAT1 in rat brain sections using an antibody that was raised against the purified protein (Radian et al., 1990). However, some of the results indicated that GAT1 may also be present in glial cells.

This purified preparation of GAT1 was used for obtaining cyanogen bromide fragments, and the amino acid sequences of the polypeptides were used to design oligonucleotide probes to isolate the cDNA encoding the rat transporter (Guastella et al., 1990). The open reading frame in the cDNA encodes a hydrophobic protein containing 599 amino acids and having a calculated molecular mass of 67 kDa. The human GABA transporter was found to be almost identical to the rat protein, with just 17 amino acid differences, mainly near the N- (four residues) and C- (11 residues) termini (Nelson et al., 1990). Expression of the GAT1 mRNA in *Xenopus* oocytes generated GABA uptake activity with pharmacology similar to the neuronal high-affinity GABA transporter (Guastella et al.,

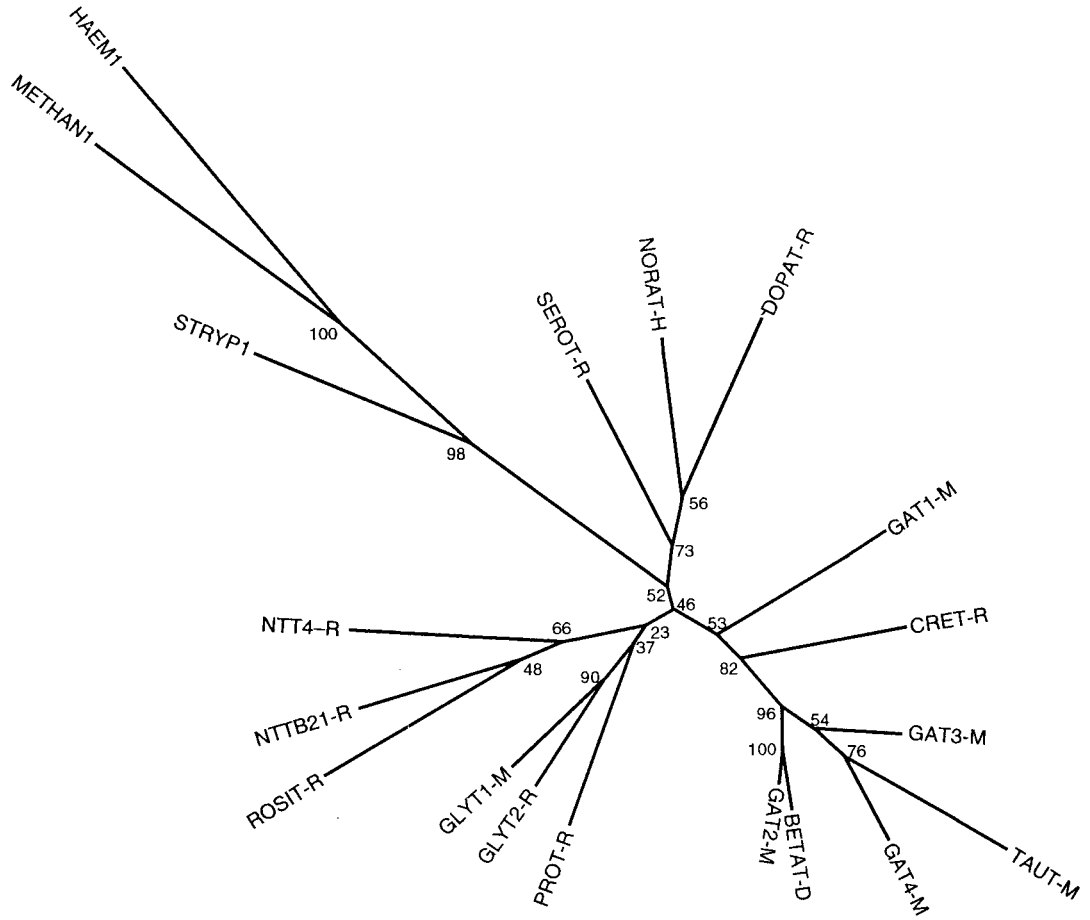


**FIG. 2.** Evolutionary tree constructed from the amino acid sequences of representative neurotransmitter transporters. The tree was constructed using the amino acid sequences of the part corresponding to TM 4–7 (see Lill and Nelson, 1998). The source of some transporters is indicated by the last letter: -D, dog; -H, human; -R, rat; -M, mouse. HAEM1 is a gene of the *Haemophilus influenzae* genome, METHAN1 is a gene of the *Methanococcus jannaschii* genome, and STRYP1 is a gene of the *Symbiobacterium thermophilum* genome. BETAT, betaine transporter; CRETR, creatine transporter; DOPAT, dopamine transporter; NORAT, noradrenaline transporter; PROT, proline transporter; SEROT, serotonin transporter; TAUT, taurine transporter.

| Abbreviation | Name | Substrate | Accession | Reference |
|---|---|---|---|---|
| BETAT-D | Dogncbta | Betaine | M80403 | 1 |
| CRETR-R | Cretr | Creatine | X67252 | 2 |
| DOPAT-R | Ratdoper | Dopamine | M80570 | 3 |
| GAT1-M | Gat1 | GABA | M92378 | 4 |
| GAT2-M | Gat2 | GABA | M97632 | 5 |
| GAT3-M | Gat3 | GABA | L04663 | 6 |
| GAT4-M | Gat4 | GABA | L04662 | 6 |
| GLYT1-M | Glyt1 | Glycine | X67056 | 7 |
| GLYT2-R | Glyt2 | Glycine | L21672 | 8 |
| HAEM1 | | ? | U32703 | 9 |
| METHAN1 | | ? | | 10 |
| NORAT-H | Humnortr | Noradrenaline | M65105 | 11 |
| NTT4-R | Ntt4r | ? | S52051 | 12 |
| NTTB21-R | rB21a | ? | S76742 | 13 |
| PROT-R | Protr | Proline | M88111 | 14 |
| ROSIT-R | ROSIT | ? | U12973 | 15 |
| SEROT-R | Rsertran | Serotonin | X63253 | 16 |
| STRYP1 | Sat1 | ? | — | 17 |
| TAUT-M | Taurt | Taurine | L03292 | 18 |

References: (1) Yamauchi et al., 1992; (2) Guimbal and Kilimann, 1993; (3) Shimada et al., 1991; (4) Liu et al., 1992*a*; (5) López-Corcuera et al., 1992; (6) Liu et al., 1993*a*; (7) Liu et al., 1992*c*; (8) Liu et al., 1993*b*; (9) Fleischmann et al., 1995; (10) Bult et al., 1996; (11) Pacholczyk et al., 1991; (12) Liu et al., 1993*c*; (13) Smith et al., 1995; (14) Fremeau et al., 1992; (15) Wasserman et al., 1994; (16) Blakely et al., 1991; (17) N. Nelson et al., unpublished observations; (18) Liu et al., 1992*b*.

1990). The rat brain cDNA encoding GAT1 has also been expressed in mammalian cells (Keynan et al., 1992). Tunicamycin sensitivity of the expression of the transport activity suggested that the N-linked glycosylation is important for the activity and/or proper assembly of the transporter. The cDNA of GABA transporter GAT1 is the only neurotransmitter transporter that was cloned based on its amino acid sequence. Most of the other cDNAs encoding members of this transporter family were cloned based on the initial sequence information obtained for the GABA and noradrenaline transporters.

A multitude of $Na^+$-dependent GABA uptake systems with $K_m$ values ranging from 1 $\mu M$ to 4 m$M$ were reported (Krogsgaard-Larsen et al., 1987). A closer look at the distribution of one of the GABA transporters (GAT1) revealed a much more complicated picture, suggesting the presence of more GABA transporters localized in specific parts of the brain (Kanner and Bendahan, 1990). Moreover, different types of GABA transporters could be present not only in different brain areas, but also in different stages during nervous system development (Cherubini et al., 1991). Therefore, it was not surprising that four pharmacologically distinct GABA transporters were cloned from the mouse brain library (Liu et al., 1993*a*). Homologous cDNAs were cloned from rat brain and other mammalian sources (Borden et al., 1992; Clark et al., 1992). We termed the transports GAT1 to GAT4, and we will use this nomenclature in this review (mouse GAT3 = rat GAT2 and mouse GAT4 = rat GAT3 or GAT-B; see Borden et al., 1992; Clark et al., 1992; Liu et al., 1993*a*). The GABA uptake activities of the four transporters were measured in mRNA-injected *Xenopus* oocytes (Liu et al., 1993*a*). The $K_m$ values for GABA uptake by the expressed GAT1 to GAT4 were 6, 79, 18, and 0.8 $\mu M$, respectively. GAT2 is also an effective betaine transporter with a $K_m$ of ~200 $\mu M$. GAT3 and GAT4 also transport $\beta$-alanine with $K_m$ values of 28 and 99 $\mu M$, respectively. They also transport taurine with $K_m$ values of 99 $\mu M$ and 1.4 m$M$. Whereas GAT1 and GAT4 gene expression is brain-specific, GAT2 and GAT3 mRNAs were detected in other tissues, such as liver and kidney, in which GAT3 mRNA was especially abundant (López-Corcuera et al., 1992; Liu et al., 1993*a*; Jursky et al., 1994). The expression of GAT3 mRNA in mouse brain is developmentally regulated, and its mRNA is abundant in neonatal brain, but not in adult brain.

Pharmacological studies of the four GABA transporters revealed that $\beta$-alanine inhibited GABA uptake by the expressed GAT3 and GAT4, and to much lesser extent by GAT2 (López-Corcuera et al., 1992; Liu et al., 1993*a*). Betaine inhibited GABA transport only by GAT2. GABA transport by GAT1 and GAT4 was more sensitive to diaminobutyric acid, guvacine, and nipecotic acid than that by GAT2 and GAT3. GAT2 was the most sensitive to phloretin and quinidine, whereas GAT3 and GAT4 were the most sensitive to guanidinopropionic acid, diaminopropionic acid, and hypotaurine. It is apparent that even though GAT2, GAT3, and GAT4 exhibit high sequence homology, they have different substrate and inhibitor specificities. Moreover, GAT1 and GAT4 have the highest affinity for GABA and are more sensitive to nipecotic acid.

The subfamily of GABA transporters contains several transporters exhibiting high sequence homology to each other (Liu et al., 1993*a*). It includes the transporters GAT1 (Guastella et al., 1990; Liu et al., 1992*a*), GAT2 (López-Corcuera et al., 1992), GAT3 (Liu et al., 1993*a*), GAT4 (Liu et al., 1993*a*), creatine (Guimbal and Kilimann, 1993), and taurine (Liu et al., 1992*b*; Smith et al., 1992*b*; Uchida et al., 1992). Although GAT2, GAT3, GAT4, and the taurine transporters exhibit >60% identity in their amino acid sequences, they are only ~50% identical to GAT1 and ~40% identical to members of the other subfamilies of $Na^+/Cl^-$ transporters. Despite the high sequence identity between the GABA and taurine transporters, the latter is not capable of GABA transport and GABA does not inhibit its taurine uptake activity (Liu et al., 1992*b*; Uchida et al., 1992). In contrast, the taurine transporter is also an efficient $\beta$-alanine transporter. *Xenopus* oocytes injected with mRNA of the cloned taurine transporter expressed uptake activities with $K_m$ values of 4.5 $\mu M$ for taurine and 56 $\mu M$ for $\beta$-alanine (Liu et al., 1992*b*). This value is close to the reported affinity for $\beta$-alanine uptake into neurons or astrocytes and synaptosomal preparations (Kanner and Bendahan, 1990). Competition between GABA and $\beta$-alanine, as well as between taurine and $\beta$-alanine, was reported. It was concluded that one of the GABA uptake systems, presumably the glial transporter, transports $\beta$-alanine (Kanner and Bendahan, 1990). The cloned high-affinity GABA transporter (GAT1) does not transport $\beta$-alanine, but GAT2 transports $\beta$-alanine at very low rates (Borden et al., 1992; Liu et al., 1993*a*). Therefore, it can be concluded that $\beta$-alanine transport is shared by both GABA and taurine transport systems.

The precise function of each individual GABA transporter is not entirely clear. It is apparent that GAT1 and GAT4 are the principal players not only in the termination of GABA neurotransmission, but also in maintaining the homeostasis of GABA concentrations in the various brain parts. The principle of coupled transporters, one neuronal and one glial, that function in the termination of GABA neurotransmission also holds for the termination of glycine neurotransmission (Jursky and Nelson, 1996*a*,*b*). The expression of the various transporters during the development of the various brain parts is modulated to fulfill the neurological function required for each developmental stage. For example, it was demonstrated that GABA acts as an excitatory transmitter in the early postnatal stage of the developing hippocampus (Ben-Ari et al., 1997). At about postnatal day 5, the GABA switches to its adult role as an inhibitory neurotransmitter. Although

this phenomenon was attributed to functional changes in the $GABA_A$, *N*-methyl-D-aspartic acid (NMDA), and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) ionotropic receptors, it may be meaningful for the process that at postnatal day 0, GABA transporters are almost absent from the hippocampus, and at postnatal day 5 GAT1 is present at relatively high amounts in this brain region (Jursky and Nelson, 1996*b*). Therefore, the presence of specific transporters in the various brain parts is not only vital for the correct neurotransmission, but also for the proper development of the CNS.

**Monoamine transporters**

This subfamily of neurotransmitter transporters contains the transporters of noradrenaline, serotonin, and dopamine (Rudnick and Clark, 1993). An important breakthrough in the study of these transporters was made by cloning a human cDNA encoding the noradrenaline transporter (Pacholczyk et al., 1991). Not only did it give an exciting opportunity to study the transporter in depth, but it also helped to define the family of $Na^+/Cl^-$ neurotransmitter transporters. The sequence of this transporter was found to be very similar to that of the GABA transporter, particularly in the transmembrane regions. Expression of the noradrenaline transporter cDNA in HeLa cells allowed noradrenaline uptake that was saturable, $Na^+$-dependent, and sensitive to inhibition by tricyclic antidepressants, with specificity identical to those of the native membrane-bound transporter (Pacholczyk et al., 1991). All the fundamental properties of noradrenaline uptake in the brain were found to be coded by this single cDNA species. The transporter is widely distributed throughout the brain and is inhibited by cocaine, tricyclic antidepressants, and amphetamines. Therefore, the cloning of cDNA encoding noradrenaline transporter circumvented the necessity to purify the protein that had proved difficult due to its low abundance in mammalian brain.

The sequence identity between the GABA and noradrenaline transporter led a number of workers to assume that other transporters might belong to this gene family. By using a degenerative oligonucleotide probe corresponding to the region of greatest identity between the GABA and noradrenaline transporters, a cDNA clone encoding the dopamine transporter was isolated from rat brain (Kilty et al., 1991; Shimada et al., 1991), and the cloning and sequencing of the bovine brain cDNA was reported subsequently (Usdin et al., 1991). Expression of the rat brain cDNA in both COS and HeLa cells was achieved, generating cocaine-sensitive dopamine transport activity. It was shown in transfected COS cells that two cocaine binding sites were conferred from a single cDNA, suggesting that a single transporter species codes for both low- and high-affinity binding sites (Kilty et al., 1991; Shimada et al., 1991; Usdin et al., 1991; Vandenbergh et al., 1992). The cloned human dopamine transporter shows >95% amino acid identity to the rat protein and has one less N-linked glycosylation site.

In both platelets and neurons, serotonin is transported by a high-affinity uptake system that cotransports $Na^+$ and $Cl^-$ and countertransports $K^+$ (Rudnick and Clark, 1993). By using affinity chromatography with serotonin or 6-fluorotryptamine as ligands, the transporter was purified to an apparent homogeneity (Launay et al., 1992). However, here too, in the cloning attempts for this neurotransmitter transporter, the identity between the GABA and noradrenaline transporters was used for designing degenerative oligonucleotide probes corresponding to the regions of greatest identity. The attempt resulted in the isolation of a cDNA clone encoding the serotonin transporter in rat brain (Blakely et al., 1991). Simultaneously, by using expression cloning, the serotonin transporter from rat basophilic leukemia cells was also cloned (Hoffman et al., 1991). Expression of each cDNA in nonneuronal cells generated $Na^+$-dependent serotonin uptake ability that was sensitive to antidepressants. Comparison of the predicted amino acid sequences with those of the GABA and noradrenaline transporters showed 38% and 47% identity, respectively. However, several regions in the protein shared relatively high homology with the noradrenaline and dopamine transporters, defining these three transporters as a tight subfamily in the family of $Na^+/Cl^-$ neurotransmitter transporters (Fig. 2). Monoamine transporters, as well as other family members, are regulated by phosphorylation and dephosphorylation by protein kinases and phosphatases. This subject was discussed recently in an excellent review and will not be covered in this review (see Blakely et al., 1997).

Because of its relevance to drug addiction and highly advanced pharmacology, the subfamily of monoamine transporters attracted significant attention. Numerous excellent reviews were written on this subject (Rudnick and Clark, 1993; Blakely et al., 1997). Therefore, it is not surprising that the first knockout mice lacking genes encoding neurotransmitter transporters were obtained for members of this subfamily (Giros et al., 1996; Bengel et al., 1998). Disruption of the mouse dopamine transporter gene resulted in a mouse phenotype that is reminiscent of the wild-type mouse under the influence of cocaine (Giros et al., 1996). Disruption of the mouse serotonin transporter gene resulted only in minor locomotor changes (Bengel et al., 1998; Sora et al., 1998). These observations stress the main obstacle of using global knockout of genes and the unexpected effects of such intervention. It was expected that the serotonin gene knockout would result in a wide range of physiological and behavioral alterations, yet the mutant mice were able to cope with such a drastic intervention with no major handicap. The dopamine gene knockout resulted in one of the most definite phenotypes, yet it causes major adaptive changes, such as decreases in neurotransmitter and re-

ceptor levels (Bosse et al., 1997; Gainetdinov et al., 1997; Jaber et al., 1997).

There are several obstacles to analyzing the effect of gene interruption through conventional gene knockout techniques. Unrestricted genetic deletion may lead to severe developmental defects or premature death (Joyner, 1994). This approach can preclude analysis of postdevelopmental gene functions. Furthermore, such an approach may sometimes cause one to overlook many important effects or to obtain misleading results due to developmental circumvention of the activity that is missing due to gene knockout. If mutant mice complete development, interpretation of the experimental results often runs into two types of difficulties. First, global gene knockout makes it difficult to attribute abnormal phenotypes to a particular system in the CNS. Second, it is often difficult to exclude the possibility that the abnormal phenotype observed in adult animals is caused directly by a developmental defect. Recently, a system of *Cre/loxP* was developed to circumvent these obstacles (Lakso et al., 1992; Tsien et al., 1996). This system is based on a phage P1-derived site-specific recombination in which the *Cre* recombinase catalyzes recombination between two 34-bp *loxP* recognition sequences (Lakso et al., 1992). The *loxP* recognition sequences are introduced in introns of genes encoding neurotransmitter transporters. Transgenic mice are generated by introducing these engineered sequences into the original genes by homologous recombination (Tsien et al., 1996). This gene manipulation should result in a modified gene that is competent to transcribe and translate intact neurotransmitter transporter. The homozygous mutant mice are then crossed with homozygous transgenic mice that express the *Cre* gene in specific brain regions. This is achieved by cloning the *Cre* gene after a promoter that expresses the downstream gene only in the desired parts of the brain. Several types of mutant mice containing region- and cell-specific *Cre* expression are already available, and it is likely that in a short period of time the repertoire of mutant mice will increase tremendously. Therefore, it is anticipated that, in the future, mutant mice with neurotransmitter transporters containing *loxP* will be able to mate with several mutant mice containing the *Cre* expression system, and will therefore allow gene deletion in many cell- and system-specific parts of the CNS.

## The subfamily of $Na^+/Cl^-$-dependent amino acid transporters

The subfamily of $Na^+/Cl^-$-dependent amino acid transporters contains genes encoding transporters for glycine and proline (see Fig. 2). The amino acid glycine is a classical inhibitory neurotransmitter localized in the spinal cord, brainstem, and retina (Daly, 1990). Its effects are mediated by the glycine receptor, a ligand-gated $Cl^-$ channel that is competitively antagonized by strychnine (Grenningloh et al., 1987). Glycine also modulates excitatory neurotransmission as an obligatory coagonist with glutamate at NMDA-activated glutamate receptors (Johnson and Ascher, 1987; Kemp and Leeson, 1993). Although a specific proline receptor has not yet been identified, it was argued that this amino acid may function as a neurotransmitter (Fremeau et al., 1992). It was shown that radiolabeled L-proline is released from brain slices and synaptosomes in a $Ca^{2+}$-dependent manner following $K^+$-induced depolarization (Nickolson, 1982). These and other experimental findings supported a synaptic role for L-proline in specific excitatory pathways in the brain. Proline may also be involved in sustaining cytoplasmic glutamate levels and thus modulate glutamatergic neurotransmission (Miller et al., 1997).

By using low-stringency screening and PCR, cDNAs encoding the amino acid transporters of glycine (GLYT1) and proline were cloned and sequenced (Fremeau et al., 1992; Guastella et al., 1992; Liu et al., 1992*c*; Smith et al., 1992*a*). During the course of cloning the first mouse glycine transporter (GLYT1), we obtained a clone that was different from the one that was published previously (Liu et al., 1992*c*, 1993*b*) in its 5′ untranslated region and the first 15 amino acids. From this point to the 3′ end of the cDNA, the two clones were identical. The kinetics and pharmacology of the two expressed glycine transporters named GLYT1a and GLYT1b were indistinguishable. Genomic cloning and sequencing revealed that GLYT1a and GLYT1b are coded by a single gene (Liu et al., 1993*b*). The first exon encodes the 5′ untranslated sequence and the N-terminal 10 amino acids of GLYT1a. The second exon encodes the 5′ untranslated sequence and the N-terminal 15 amino acids of GLYT1b. Therefore, the GLYT1 variants may be generated by alternative splicing. It was also pointed out that GLYT1b may be transcribed from a promoter that is localized in the intron between the first and second exons. This gene organization and differential splicing may also exist in the rat genome. The two reported glycine transporters cloned from cDNA rat brain libraries are homologous to the mouse GLYT1a (Guastella et al., 1992) and GLYT1b (Smith et al., 1992*a*), respectively. Recently, a third splicing variant of GLYT1 (GLYT1c) was found in human brain (Kim et al., 1994).

Cloning and expression of cDNAs encoding GLYT1 left several unanswered questions regarding glycine reuptake in the CNS (Guastella et al., 1992; Liu et al., 1992*c*; Smith et al., 1992*a*). The distribution of GLYT1 mRNA did not correlate with the glycine receptor, and its predicted amino acid sequence was considerably shorter than expected from the determined molecular weight of the purified brainstem transporter (López-Corcuera et al., 1991). Therefore, we sought a novel glycine transporter that would be associated with the glycine receptor in the glycinergic neurons. A novel glycine transporter (GLYT2) was cloned from a rat brain cDNA library (Liu et al., 1993*b*). GLYT2 is ~48% and 50% homologous to the previously cloned

mouse glycine transporter (GLYT1) and rat proline transporter (PROT), respectively. GLYT2 differs from GLYT1 in molecular structure, tissue specificity, and pharmacological properties. The cDNA of GLYT2 encodes for 799 amino acid residues with an extended N-terminal peptide containing 200 amino acids before the first transmembrane domain. The calculated molecular weight of GLYT2 was in agreement with that of the isolated glycine transporter from spinal cord (López-Corcuera et al., 1991). Potential phosphorylation sites for protein kinase C, cyclic AMP-dependent kinase, and calmodulin-dependent kinase were identified in the extended N-terminal region. *Xenopus* oocytes injected with GLYT2 cRNA transport glycine had a $K_m$ of 17 $\mu M$, and the uptake of glycine is resistant to inhibition by sarcosine. These data are also in line with those obtained with the purified and reconstituted glycine transporter (López-Corcuera et al., 1991). The experimental data suggested that GLYT2 may play a major role in the termination of the inhibitory effect of glycine in the brainstem and spinal cord of vertebrates. This assumption was substantiated by in situ hybridization and immunocytochemical studies (Borowsky et al., 1993; Jursky et al., 1994; Jursky and Nelson, 1995; Luque et al., 1995). The distribution of GLYT2 correlated very well with that of the strychnine-sensitive glycine receptors in most CNS regions, except cerebellum (Jursky and Nelson, 1995; Luque et al., 1995). It was concluded that GLYT2 is the principal transporter that mediates the termination of glycine neurotransmission.

The mRNAs of GLYT1 and the proline transporter were more abundant near excitatory pathways in the CNS, suggesting a neuromodulatory role for proline and glycine in glutamatergic cells. It was proposed that one of the functions of both transporters is to remove glycine and proline from the environment of glutamate receptors (Liu et al., 1992*c*; Smith et al., 1992*a*). This possibility has important pharmacological implications that will be discussed later.

The subfamily of amino acid neurotransmitter transporters contains only glycine and proline transporters. The extensive screening of brain cDNA libraries in several laboratories left almost no stones unturned. Yet low abundant transporters are emerging (N. Nelson et al., unpublished observations).

### The orphan (NTT4) subfamily of transporters with no known substrate

This subfamily of transporters consists of four gene products that appear to encode transporters that differ structurally from the three other subfamilies. They display large second and fourth extracellular domains with sites for N-linked glycosylation in both large domains (Fig. 2). Substrates for all of these gene products, which are highly expressed in the brain or kidney, have never been identified. During the cloning of several neurotransmitter transporters, we encountered a cDNA clone structurally related to the other members of the family of $Na^+/Cl^-$-dependent transporters (Liu et al., 1993*c*). We denoted this alleged transporter as NTT4 and observed that it exhibits ~35% identity with the other members of this family of transporters. Two other closely related members of this subfamily were cloned and their predicted amino acid sequences are ~65% identical with NTT4 (Uhl et al., 1992; El Mestikawy et al., 1994). They have been termed orphan transporters because their substrates have not yet been identified. Recently, cDNA encoding another member of this subfamily was cloned from a kidney cell line (Wasserman et al., 1994). It expresses quite specifically in the kidney, and once again the substrate for this transporter could not be identified. Northern blot analysis of peripheral and neural tissues demonstrated that the expression of NTT4 mRNA is restricted essentially to the nervous system (Liu et al., 1993*c*). In situ hybridization demonstrated a broad, but discrete, localization of the NTT4 message in the CNS, particularly in the cerebellum (Purkinje and granule cell layers), hippocampus (pyramidal and granular cell layers), and thalamus and throughout the cerebral cortex (El Mestikawy et al., 1994; Luque et al., 1996). This distribution parallels that of the neurotransmitters glutamate and aspartate. One noticeable exception to the distribution of mRNA for NTT4 and these excitatory neurotransmitters is the cerebellar Purkinje cell layer, in which GABAergic neurons are localized. NTT4 was found to codistribute widely with the subset of specific variants of the NMDA receptor subunit 1, namely, NMDAR1 1–4b. Polyclonal antibodies obtained by immunizing guinea pigs with fusion proteins were used to identify and characterize NTT4 in the rat CNS. Overall, the immunochemical localization of NTT4 correlates with the distribution of NTT4 mRNA. For example, although prominent hybridization signals are found in the CA3 hippocampal neurons and Purkinje cell layer, correspondingly high levels of immunostaining are present in the mossy fibers of the hippocampal region, as well as in the cerebellar molecular layer, into which both cell populations extend their respective processes.

Identification of the substrate for NTT4 is difficult, because in situ hybridization and immunohistochemistry indicate that this transporter is synthesized by phenotypically different neuronal populations, e.g., glutaminergic, GABAergic, catecholaminergic (locus ceruleus), histaminergic (mammillary nuclei), and serotoninergic neurons (raphe nuclei). The failure to obtain any uptake with >100 different substances tested in our and other laboratories may be the result of special properties of this transporter: (a) this subfamily of transporters may require a $\beta$ subunit that might be necessary for their uptake activity; (b) unknown or unappreciated cofactors or heterooligomers may be involved in the functional expression of this transporter; (c) they may require other facilitative ions besides $Na^+$, $Cl^-$, and $K^+$; (d) they may be expressed in the vacuolar system; and (e) this subfamily of trans-

porters may transport an unknown neurotransmitter. As the NTT4 transporter appears to be present in CNS regions with specific glutaminergic innervation, it is likely to be involved in excitatory pathways. It is noteworthy that the glutamate transporters, discovered so far, fail to explain the termination of glutamatergic neurotransmission (Luque et al., 1996).

**Bacterial transporters of unknown function**

Genomic cloning of genes encoding neurotransmitter transporters and a search for sequences without known function in the GenBank revealed that insects and *C. elegans* contain genes encoding potential neurotransmitter transporters (Liu et al., 1992*a*). Recently, we identified and cloned a bacterial gene with relatively high homology to neurotransmitter transporters (S. Mandiyan et al., unpublished observations). This gene (STRYP1) is present in a potential tryptophan operon of *Symbiobacterium thermophilum* that contains the gene encoding the enzyme tryptophanase (Hirahara et al., 1992). In many other bacteria, a similar operon exists and includes a gene for tryptophanase followed by the gene encoding a tryptophan transporter (Deeley and Yanofsky, 1981; Kamath and Yanofsky, 1992). However, the tryptophan transporter in these bacteria has no homology whatsoever to neurotransmitter transporters. Therefore, we suspect that, in those thermophilic bacteria, the gene that follows the tryptophanase and is homologous to neuronal transporters functions as tryptophan permease. Recently, the entire genomic sequences of *Haemophilus influenzae* and *Methanococcus jannaschii* were determined (Fleischmann et al., 1995; Bult et al., 1996). Each genome contains a single gene homologous to the mammalian family of neurotransmitter transporters. It is highly likely that, in light of the high sequence identity between the bacterial protein and neurotransmitter transporters, their mechanism of action may be similar, if not identical.

The complete sequence of the yeast genome and the extensive sequencing of plant genomes revealed no genes with homology to neurotransmitter transporters. This raises an interesting evolutionary question, namely, why have fungi and plants avoided the use of these transporters? One possible cause is that $Na^+/Cl^-$ transporters strictly require $Na^+$ gradients for their action and they could not be adjusted to protonmotive force, which is the main driving force for transport in fungi and plants. Bacterial proteins are usually not glycosylated, and indeed the bacterial homologues of neurotransmitter transporters are missing the large external loop (no. 2) (N. Nelson et al., unpublished observations). This configuration may provide important structural information not only for the bacterial, but also for the mammalian, transporters. Hydropathy plots suggest 12 transmembrane helices for the membrane topography of these transporters. Figure 1 depicts the proposed arrangement of the transporters in the bacterial membrane. Like the mammalian ones, both the N- and C-termini may be situated at the cytoplasmic face of the membrane. Figure 1 also indicates the charge balances of the proposed internal and external loops. Like most other membrane proteins, the rule of net positive charges in internal loops is maintained. It is interesting to note that the third transmembrane helix contains a long stretch of uncharged amino acids, 24 in *Symbiobacterium thermophilum* and 38 in *Methanococcus jannaschii.* However, it is not apparent how these stretches of amino acids can fold into two transmembrane helices. Thus, the original proposal of the membrane topography for the $Na^+/Cl^-$ transporters is supported by the sequence analysis of the bacterial transporters. The function of these transporters is not known, but their existence opens up new and interesting research avenues.

## TRANSPORTERS AS PHARMACOLOGICAL TARGETS

In the normal brain, transporters maintain low intrasynaptic and extracellular neurotransmitter levels, and by this action they (a) regulate synaptic efficacy, (b) ensure synaptic fidelity, and (c) reduce the potential neurotoxicity of excessive excitatory neurotransmitters. Deviation from a well controlled state might cause neurological disease, and modulation of an aberrant activity may remedy malfunctioning systems.

Many of the neurotransmitter transporters have been implicated as important sites for drug action. The function of transporters in terminating the synaptic activities of released neurotransmitters makes them an important target for several drugs. It was demonstrated that transporters may be involved in the action of numerous drugs and specific toxins that affect the nervous system (Snyder and D'Amato, 1986; Bergman et al., 1989; Kuhar et al., 1991). Monoamine transporters, for example, are targeted by major classes of antidepressants, psychostimulants, and antihypertensive drugs (Blakely et al., 1997). They are also necessary for the actions of several classes of neurotoxins that selectively poison specific neuronal targets (Snyder and D'Amato, 1986). As a result of these activities, the members of these four gene subfamilies have become candidate genes for involvement in several neuropsychiatric disorders. The subfamily of monoamine transporters is a target for important addictive drugs, such as cocaine. Several lines of evidence suggest that cocaine's rewarding and reinforcing actions in the brain are due to activities of dopamine transporters. The drug also possesses a reasonable affinity to the other $Na^+$- and $Cl^-$-dependent plasma membrane monoamine transporters, as well as to some $Na^+$ channels (Bergman et al., 1989; Kuhar et al., 1991). Effective antidepressants share the ability to inhibit the serotonin transporter (Blakely et al., 1997). Transporter-mediated accumulation and/or sequestration of neurotoxins plays a key role in several current experimental models of the specific dopaminergic neurodegenerative processes

found in Parkinson's disease (Snyder and D'Amato, 1986). For example, accumulation of $MPP^+$ can be rendered toxic in cells expressing the dopamine transporter (Kitayama et al., 1992). Conversely, cells become resistant to this poison if they overexpress the vesicular monoamine transporter. The presence of high transporter levels results in the accumulation of $MPP^+$ into their vacuolar system (Schuldiner, 1994). There is a good fit between the onset of Parkinson's disease and the expression levels of transporter mRNA in rat and human brains (Uhl et al., 1994). Moreover, the "designer" drug of the 1990s, Prozac, interacts with the serotonin transporter that is a member of the family of $Na^+/Cl^-$ neurotransmitter transporters (Vandenbergh et al., 1992; Ramamoorthy et al., 1993).

Human studies indicated that GABA transporter function is reduced in epileptic hippocampi (During et al., 1995; Williamson et al., 1995). This may result from a reversed GABA transport due to membrane depolarization. This action reduces the risk of hyperactive glutamatergic neurons that, when they pass a certain threshold, cause seizures. Therefore, there is much interest in agents that block GABA uptake. These agents may be used as anticonvulsive drugs by acting as selective inhibitors of either glial or neuronal uptake. In theory, GABA uptake inhibitors would be "cleaner" therapeutic agents than GABA receptor agonists, because inhibitors of GABA uptake would be present only when GABA was released physiologically. The way by which certain drugs exhibit unique selectivity is not apparent. There are many receptor subtypes for every neurotransmitter, most of which have a distinctive distribution. If the duration of neurotransmitter action of all of them is regulated by reuptake, a given drug would be expected to affect all of them. The action of Prozac, for example, speaks against such a sweeping effect. Prozac is quite selective in blocking serotonin transporters. It is now widely prescribed not only for depression, but also for less serious prevalent behavioral symptoms. The question why one blocker of a given transport system is a better drug than another with similar inhibitory properties is not easy to answer. The selectivity of reuptake blockers may result from the different accessibility of different brain parts to the drug. Therefore, the drug augments the action of those brain synapses where the neurotransmitter is already being released. Thus, the elevated neurotransmitter concentration in the various synapses may affect differently various brain cells or developmental stages (Ben-Ari et al., 1997). The therapeutic effect of a transporter blocker may take weeks to develop. It is logical to assume that their primary action in prolonging neurotransmitter effects is to set up a series of molecular changes in the brain that change the homeostasis of several metabolic processes. This possibility is supported by recent studies with knockout mice lacking the dopamine transporter (Giros et al., 1996). The lack of dopamine transporter affected not only the duration in which the neurotransmitter was present in the synapse, but also its biosynthesis and several other metabolic processes. Understanding these changes should help in the development of rational new drugs that are based on the modulation of neurotransmitter transporter activity.

Due to the special structure of glycine, which is the smallest amino acid and has no L- and D-isomers, there is no pharmacology for glycine transporters. This pharmacology can now be developed using expression systems for the various glycine transporters. This search should consider the finding that even alanine, which is structurally related to glycine, fails to inhibit glycine transport, as well as the observation that sarcosine inhibits GLYT1 isoforms, but not GLYT2 (Liu et al., 1993*b*). These properties suggest a compact structure of the substrate binding site that would not allow even slight alteration of the glycine structure. Nevertheless, a hydrophobic pocket may exist near the binding site of glycine transporters. This site may enable the design of inhibitory drugs for glycine uptake. An alternative avenue may reveal an allosteric site for antagonists of glycine uptake. The importance of such an antagonist is apparent from the important function of glycine receptors in the CNS and the possibility of modulating the glutamate (NMDA) receptors. The glycine site of NMDA receptors has generated enormous interest since it was first described 10 years ago (Johnson and Ascher, 1987). Potent pharmacological agents that interact selectively with this site were discovered, and they include both agonists and antagonists that modulate the activity of the receptor. Glycine is a coagonist of this receptor, and the receptor is inactive in the absence of glycine (Kleckner and Dingledine, 1988). It was demonstrated that both the glutamate and the glycine recognition sites reside on the same protein (Moriyoshi et al., 1991). The affinity of the NMDA receptor to glycine is in the low micromolar concentration. The glycine concentration in brain fluids is orders of magnitude higher. This raised some doubts about the possible physiological role of glycine, because presumably the glycine binding site is saturated at all times. In this respect, glycine transporters may play a crucial role in the modulation of the synaptic response mediated by NMDA receptors (Liu et al., 1992*c*; Smith et al., 1992*a*). The presence of glycine transporters in the general area of the NMDA receptors may lower the glycine concentration in these areas. Moreover, placement of glycine transporters in the vicinity of an NMDA receptor may completely alter its response to glutamate. There is evidence suggesting that the glycine site on NMDA receptors is not saturated (Wood and Sidhu, 1986). The modulatory role of the glycine site makes it an attractive target for therapeutic anticonvulsant and neuroprotective agents (Priestley et al., 1990; Singh et al., 1990). It is apparent that modulating the activity of the glycine transporters in the vicinity of the NMDA receptor may achieve similar pharmacological effects with less side

effects than the use of agonists and antagonists that interact with the glycine site in the NMDA receptor.

Recently, it was suggested that the primary disturbance in glutamatergic function may be etiological in schizophrenia (Tsai et al., 1995). Dopaminergic agents, such as amphetamines, induce a psychotic state by elevating dopamine in the synapses. In contrast, phencyclidine (PCP) induces a schizophrenia-like psychotic state by blocking the NMDA receptor (Javitt et al., 1994, 1996). Experimental evidence suggests that elevation of glycine concentrations in the brain reverses PCP-induced behaviors in rodents (Toth and Lajtha, 1986). Moreover, a significant improvement in negative symptoms was observed when a glycine transporter inhibitor glycyldodecylamide was given to PCP-treated animals (Javitt et al., 1997). These initial findings stress the importance of improving the pharmacology of glycine transporters. The availability of cDNAs encoding glycine transporters may facilitate the discovery of efficient agonists and antagonists of their activity.

The few examples described above are only part of a long list of the potential actions of neurotransmitter transporters in regulating the activity of neurotransmission. New drugs can now be synthesized and assayed in systems that express specific neurotransmitter transporters. By following the activity of the new drugs, one can predict their possible effects on certain neurotransmission pathways in the brain. It is apparent that experimental animal models in which specific neurotransmitter transporters are knocked out or genetically altered will be very helpful in assessing the results obtained with the newly developed drugs.

## MECHANISM OF TRANSPORT

At the end of his life, King Solomon admitted that some things were unknown to him, such as the way of a man with a woman (in spite of being married to a thousand wives). There are about a thousand transporters, yet their mechanism of action is still an enigma. The lactose permease of *E. coli* is the closest to being understood (Kaback et al., 1997). The known part of the mechanism of lactose transport includes an elusive substrate binding site and a conformational-driven twisting helix. To reach a similar position in $Na^+/Cl^-$ neurotransmitter transporter mechanisms, much more work is still needed. There are a few cardinal questions that have to be answered. Does the 12 transmembrane helix structure indicate a similar mechanism of action in all such transporters? Is there a general transporter fold? Are twisting helices a common denominator in all transporters? How is the electrochemical gradient coupled with the vectorial transport of substrates? To answer these questions, distinct steps in substrate transport have to be addressed: (a) What is the nature of the substrate-binding site and which part(s) of the transporters are involved in substrate binding? (b) Where do the different inhibitors bind and how much do their binding sites overlap with the substrate binding site? (c) Which are the amino acid residues involved in the $Na^+$ and $Cl^-$ binding? (d) How does energy, expressed as electrochemical gradient of $Na^+$, drive the substrate transport across the membrane? (e) How does the translocation machinery operate? (f) What is the significance of the transporter channel activities?

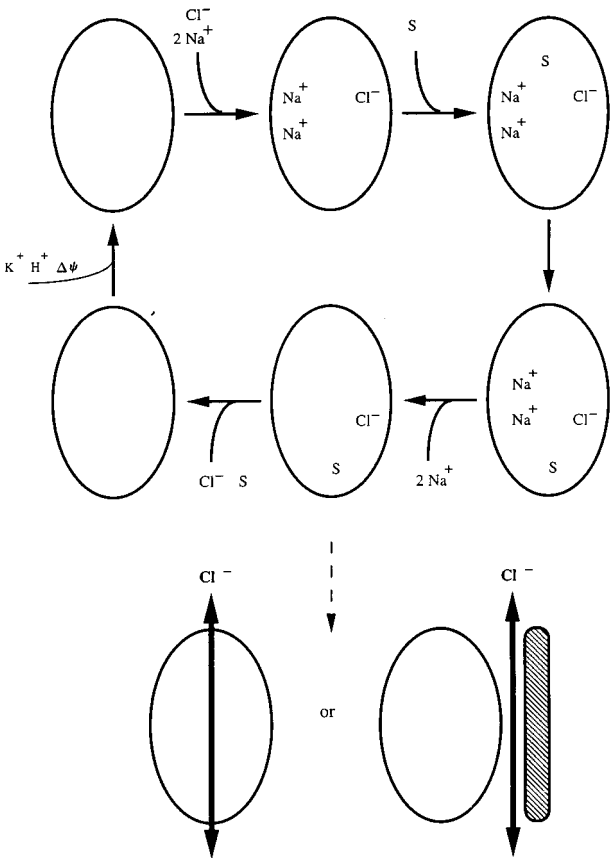


**FIG. 3.** Schematic representation of the minimal steps in the uptake cycle and the possibility of channel formation with and without the involvement of additional protein. The scheme describes the kinetic coupling and binding order of GABA transporters. Serotonin transport is stimulated by internal $K^+$, but the ion is not absolutely required. The thermodynamic coupling for dopamine transport is not clear. Two $Na^+$ ions may be involved, but one of them may not be transported across the membrane. S, substrate.

Having stated the main open questions, we will examine some of the available information that is pertinent to answering them. Like the other transporters, the catalytic cycle of neurotransmitter transporters can be described by a conventional representation of substrate- and ion-induced conformational changes (Rudnick, 1997). Figure 3 depicts a schematic representation of the minimal requirements for the transport of neurotransmitters across the membrane. The driving force for transport is a $Na^+$ electrochemical gradient that is used through cotransport of $2Na^+$, $1Cl^-$, and

1S (substrate). These ions bind first, and the conformational change induced by this binding enables the substrate binding. Following the transport step, the $Na^+$ ions are released first, and then the substrate and $Cl^-$ are also released. The transporter is rendered competent for the next cycle by returning to the original conformation spontaneously, or through facilitation by $K^+$ or $H^+$ gradients (see Cao et al., 1997), or by a membrane potential ($\Delta\Psi$). Each transporter has a variation on the above theme. The serotonin transporter represents one of the extreme deviations, because it has an electroneutral transport that is dependent on an outward $K^+$ gradient (Rudnick, 1997). We are all looking forward to the surprises that the orphan transporters have in store for us.

**Substrate binding and translocation**

The sequence homology of all the neurotransmitter transporters in this family suggests a common mechanism for transport. However, even in the subfamily of monoamine transporters, significant differences in the mode of ion transport and stoichiometry were observed. Dopamine may be cotransported with $2Na^+$ and $1Cl^-$, noradrenaline is cotransported with $1Na^+$ and $1Cl^-$, and serotonin is also cotransported with $1Na^+$ and $1Cl^-$, but its transport is also dependent on the presence of $K^+$ in the cytoplasmic side of the transporter (Rudnick and Nelson, 1978; Gu et al., 1996; Rudnick, 1997). Strategies based on protein modification have provided some information on some probable residues involved in transport activity. Unlike conventional methods, such as analyses of site-directed or deletion mutants, chimeras can provide phenotypes that allow the delineation of functions associated with particular protein domains. Chimeric proteins have been constructed from domains of the dopamine and noradrenaline transporters (Buck and Amara, 1994, 1995; Giros et al., 1994) and human or rat serotonin transporter (Barker et al., 1994). The chimeric proteins were constructed using two experimental strategies: (a) exchanging domains by exploiting unique restriction sites that are already present in the sequence or engineered into the sequence by PCR; and (b) using an in vivo method that generates chimeras within bacteria transformed with linear plasmid DNA containing a single copy of each parental cDNA in a tail-to-head configuration (Buck and Amara, 1994, 1995; Moor and Blakely, 1994; Barker and Blakely, 1998). The results suggest that a region spanning TM 5–8 in the noradrenaline and dopamine transporters is important for conferring inhibitor sensitivity to a variety of inhibitors, including imipramine (Buck and Amara, 1995). The domain in the noradrenaline transporter spanning TM 1–3 also contributes to the affinity for antidepressants. Determinants responsible for the substrates' affinity were localized in the region spanning TM 1–3, as well as TM 11 and 12 (Buck and Amara, 1994). In another study, it was reported that the region of the TM 8–12 has a predominant influence on the affinity and selectivity for different substrates (Giros et al., 1994). Similarly, chimeras constructed between the rat and human serotonin transporter indicated that determinants in the region of TM 11 and 12 are responsible for the species-specific differences in the binding of the imipramine and amphetamine to the transporter (Barker et al., 1994). Mutations in F586, in TM 12 of the human serotonin transporter, revealed this residue as responsible for high-affinity interaction of tricyclic antidepressants with the transporter (Barker and Blakely, 1996). Chimeric serotonin transporters from human and *Drosophila* were used recently for elucidating their antagonist binding sites (Barker et al., 1998). Domains in TM 1 and 2 were identified as being important for mazindol and citalopram binding. Eight amino acids that differ between the two transporters were substituted. A single mutation (Y95F) in TM 1 of the human serotonin transporter shifted the antagonist binding to the *Drosophila* transporter (Barker et al., 1998).

The observations using chimeric transporters are in line with conclusions reached from site-directed mutagenesis experiments. Substitution of D79 in TM 1 of the dopamine transporter resulted in some alteration to the binding of a cocaine analogue and a reduction of the apparent affinity to dopamine (Kitayama et al., 1992). Recently, it was shown that the correspondent amino acid residue in TM 1 of the noradrenaline transporter is critical for its function (Barker and Blakely, 1998). Further studies with mutations in TM 7 and TM 11 indicated that both domains contain determinants that influence substrate translocation and the affinity for $MPP^+$, respectively (Kitayama et al., 1993). The large external loop between TM 3 and TM 4 is unlikely to have a direct role in the transport mechanism. However, TM 3 and the beginning of the large loop are quite likely to be involved in transport. The three cysteines in the external loops of the rat serotonin transporter were substituted with alanine or serine and the mutants were analyzed for sensitivity to methanethiosulfonate reagents (Chen et al., 1997*a*,*b*; Stephan et al., 1997). Although the mutant C109A located between TM 1 and 2 had no effect on transport activity, substitution of C200 or C209 inflicted severe effects on the expression and activity of the transporter. The results support the possibility that C200 and C209 may be linked by a disulfide bond in the second external loop of the serotonin transporter and that C109 is situated on the external face of the transporter. Assuming the original model proposed for the membrane architecture, TM 3 is unique in its length (Fig. 1). It contains from 24 up to 37 uncharged amino acids. This structure may be important for the transport activity of the family members. One of the possible mechanisms for solute transport across membranes is a sliding $\alpha$-helix that changes its position in the membrane by substrate binding and/or electrochemical potential (Racker, 1976). If such a mechanism exists, TM 3 is a very good candidate to fulfill this function. TM 3

contains a tyrosine residue that is conserved in all the family members, including the bacterial transporters. Recently, it was shown that this tyrosine residue (Y140) is critical for GABA recognition and transport by GAT1 (Bismuth et al., 1997). Even substitution to the other aromatic amino acids, phenylalanine (Y140F) and tryptophan (Y140W), results in completely inactive transporters. Electrophysiological characterization reveals that both mutants exhibit the $Na^+$-dependent transient currents associated with $Na^+$ binding, as well as the Cl -dependent lithium leak currents characteristic of GAT1. Both mutants are inactive in GABA-induced steady-state transport currents. It was concluded that Y140 may be involved in the ligand binding of the amino group of GABA and other neurotransmitters (Bismuth et al., 1997). Electrophysiological studies also indicate that the conserved residue W68 in the first putative TM 1 of GAT1 may be involved in the binding of $Na^+$ and/or $Cl^-$ (Mager et al., 1996).

The availability of four cDNAs encoding homologous GABA transporters presented some opportunities to analyze the substrate binding sites of these transporters. The importance of short external loops for the activity of GAT1 was demonstrated by their high sensitivity to changes. Insertion of glycosylation sites into some of the hydrophilic loops of GAT1 resulted in its inactivation, suggesting high sensitivity to amino acid replacements in those sites (Bennett and Kanner, 1997). This is in line with the observations showing the importance of hydrophilic loops for transport and substrate specificity (Kanner et al., 1994; Tamura et al., 1995). Inspection of amino acid sequences revealed that the putative short external loops of GAT2, GAT3, and GAT4 are nearly identical, but are significantly different from that of GAT1. Site-directed mutagenesis was used to generate GAT1 transporters in which the amino acid sequences of these loops were identical to those of GAT3 (Tamura et al., 1995). When the loop structure of GAT3 was introduced into loop 4 of GAT1, the apparent $K_m$ was lowered to 2.0 $\mu M$, which was similar to the $K_m$ value reported for the high-affinity GABA transporter GAT4 (Liu et al., 1993*a*; Tamura et al., 1995). This $K_m$ value was about fourfold lower than that reported for GABA uptake by GAT1 ($K_m = 8.7\ \mu M$). When the amino acid sequence of loop 6 of GAT1 was substituted with that of GAT2, an apparent $K_m$ of 35 $\mu M$ was observed, which is ninefold higher than the value for GAT1. When amino acids in loop 5 of GAT1 were substituted to give an amino acid sequence identical to that in the corresponding loop of GAT3, $\beta$-alanine inhibited the GABA uptake activity of the mutated GAT1 transporter. To determine whether the three amino acids in loop 5 of GAT3 were important for $\beta$-alanine binding, the three residues of GAT3 were replaced by the corresponding amino acids in GAT1. This reverse mutation of GAT3 exhibited less sensitivity in its GABA uptake to $\beta$-alanine. This result suggested that three amino acids in loop 5 participated in the $\beta$-alanine binding domain of GAT3 (Tamura et al., 1995). The pharmacology of the mutated transporters followed the changes in the substrate binding specificity and affinity. These reciprocal effects suggest that the short external loops 4, 5, and 6 take part in the substrate binding of $\beta$-alanine and GABA. It was suggested that these three external loops form a pocket on the transporter into which the substrate binds.

The mechanism of GABA transport was analyzed by tracer fluxes and electrophysiological approaches (Kanner, 1983; Mager et al., 1993). It was demonstrated that the transporter cotransports the neurotransmitter with $Na^+$ and $Cl^-$ in an electrogenic fashion. Although the transport is absolutely dependent on the presence of $Na^+$, the $Cl^-$ dependency is not absolute. It is not clear whether this phenomenon is a result of a loose coupling for $Cl^-$ ions or the replacement of the $Cl^-$ by another anion, such as hydroxyl. The function of the substrate in the electrogenicity of the transporter was analyzed by following transient currents generated across the plasma membrane by expressing GAT1 in *Xenopus* oocytes (Kavanaugh et al., 1992; Mager et al., 1993) or HeLa cells (Risso et al., 1996). Although the transport of positive charge across the membrane was GABA-dependent, the transient currents generated by $Na^+$ ions were independent of the substrate. This phenomenon probably reflects a conformational change of the transporter that takes place following the binding of $Na^+$ to the protein. Mutational analysis of negatively charged amino acids in GAT1 revealed that the conserved residue E101 is critical for the function of the transporter (Keshet et al., 1995). Even its replacement by aspartate left only ~1% of the transport activity. It was concluded that E101 is critical for one or more of the obligatory conformational changes during the transport cycle. The mechanism of substrate translocation is not clear, and it may involve movements and twists of $\alpha$-helices or a novel mechanism of channel activity.

## Transporters as ion channels

Recently, it became apparent that several transporters are involved in additional activities, such as water and ion transport, that may have significant effects on the osmolarity and the membrane potential of certain cells and membranes (Mager et al., 1994; Loo et al., 1996; Panayotova-Heiermann et al., 1997; Sonders et al., 1997). This bulk transport of water and/or ions is characteristic of channels, and one of the main open questions is whether the apparent channel activity is also involved in the mechanism of substrate transport. This subject was discussed recently in several excellent and thoughtful reviews (DeFelice and Blakely, 1996; Lester et al., 1996; Sonders and Amara, 1996; Su et al., 1996). Several of the $Na^+/Cl^-$ neurotransmitter transporters were shown to have channel activity associated with their transport activity (Sonders et al., 1997; see Fig. 3). Two major implications are to be

drawn from these observations. It was argued that transporters preceded channels as sensors of the external environment (Nelson, 1993; Rudnick, 1997). Accordingly, it is logical to assume that channel activity was evolved for sensing the changes in the composition of the external milieu. The second implication is mechanistic and may contribute to the understanding of transport activity. From the work performed with several transporters, it appears that not all of them exhibit channel activity. Even those transporters that were shown to have channel activity manifested different properties of this extrinsic activity. The uncoupled ion flux is likely to result from events in which the transporter transiently operates as an ion channel instead of like a carrier. The physiological and mechanistic significance of this phenomenon depends on the probability and the duration of this event. The turnover of the carrier mode is ~10/s, and the ion flux through channels is >10,000/s. Hence, the existence of a channel activity at 0.1% of the time would have high mechanistic and physiological implications. It was possible to demonstrate that serotonin transporter channels are open very rarely relative to the number of catalytic cycles, and it is likely that this uncoupled current is probably not part of the transport process. However, it is important to note that even though serotonin transport is electroneutral, inward currents were recorded in oocytes expressing the serotonin transporter (Mager et al., 1994). As the uncoupled current is influenced by substrate binding or transport, it is difficult to estimate the contribution of each of these processes to the steady-state current during transport. Fluctuation analysis with *Drosophila* serotonin transporter, expressed in *Xenopus* oocytes, showed that ~500 serotonin molecules are transported per channel opening (Galli et al., 1996, 1997). At the same time, at −20 mV, ~10,000 electronic charges were translocated. This observation can be explained by assuming that one channel opening takes place every 500 transport cycles or that 500 serotonin molecules and 10,000 ions pass through a common channel. Even though serotonin transporters from *Drosophila* and mammals are highly homologous, the recorded channel events in the two transporters are not entirely compatible. The detection of channel activities in transporter molecules are very interesting, but more work is required to reveal their significance for the transport mechanism.

Another unsettled issue is the possible involvement of additional protein(s) in the uncoupled ion transport. The results of site-directed mutagenesis (Lin et al., 1996) and the observed correlation between their effects on transport and channel activities favor the possibility that the neurotransmitter transporter gene products by themselves can fulfill a dual function. This assumption is also supported by the detection of channel activity in two different heterologous expression systems. However, there is no direct evidence to prove this point.

## EPILOGUE

The field of neurotransmitter transporters has just entered a turning point. Most of the cDNAs encoding these transporters have been cloned and expressed in heterologous systems to reveal the transport and pharmacological properties of individual transporters. The few that are missing are likely to be cloned in the near future, and the function of the orphan transporters will be solved sooner or later. A three-dimensional structure of even one of the neurotransmitter transporters is highly desired. The main tools to achieve this goal are being developed. These include the expression of the bacterial homologue in proper bacterial expression systems and/or the expression of the mammalian transporters in yeast. Achieving these goals may help large amounts of crystallizable transporter to be obtained and may provide a system in which second-site suppressors of inactive transporters may be studied. Recent structural studies revealed a pattern of common folds for many proteins without sequence similarity. The membrane transporter fold is one of the most important folds that is still completely unknown. We hope that this fold will be understood soon.

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