

PORTERS AND NEUROTRANSMITTER TRANSPORTERS

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Summary

Uptake of neurotransmitters involves multiple transporters acting in different brain locations under different physiological conditions. The vesicular transporters are driven by a proton-motive force generated by a V-ATPase and their substrates are taken up *via* proton/substrate exchange. The plasma membrane transporters are driven by an electrochemical gradient of sodium generated by a Na⁺/K⁺-ATPase. Two distinct families of transporters were identified in this group. One cotransports sodium with glutamate and other amino acids and requires additionally an outwardly directed potassium gradient. The second cotransports sodium, chloride and a variety of neurotransmitters, including γ -aminobutyric acid (GABA), glycine and monoamines. Genes and cDNA encoding several members of the latter family have been cloned and studied in detail. The structure and function as well as the evolutionary relationships among these neurotransmitter transporters are discussed.

Introduction

Synaptic transmission involves the release of a neurotransmitter into the synaptic cleft, interaction with the postsynaptic receptor and subsequent removal of the transmitter from the cleft. The majority of transmitters are removed from the cleft by a rapid sodium-dependent uptake system in the plasma membrane of the presynaptic cells and their surroundings. These re-uptake systems are catalyzed by transporters specific for the various neurotransmitters in the brain. Two distinct transporters have been identified so far. One belongs to a family of transporters that primarily deals with glutamate re-uptake and the other belongs to a family of sodium-dependent re-uptake systems for GABA, amino acids and catecholamines (Kanner, 1993; Amara and Kuhar, 1993; Rudnick and Clark, 1993; Nelson, 1993; Worrall and Williams, 1994). The gene products of the latter family are highly conserved and can be grouped into three subfamilies of GABA transporters, catecholamine transporters and amino acid transporters (Liu *et al.* 1992*b*). All transporters in this family show a common structure of presumably 12 transmembrane helices with a single large loop in the external face of the membrane with potential glycosylation sites. The mechanism of action of most of these neurotransmitter transporters involves cotransport of the neurotransmitter, sodium and chloride. Therefore,

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it is anticipated that common amino acid sequences will be shared in these transporters for the transport of sodium and chloride and that the specificity of each of the transporters will come from a specific neurotransmitter binding site. An orphan subfamily (NTT4), somewhat remote from the subfamily of sodium- and chloride-dependent transporters, was identified by sequencing cDNA from mammalian sources (Uhl *et al.* 1992; Liu *et al.* 1993c). This subfamily of neurotransmitter transporters has no known function, but is highly expressed in different parts of the brain. Their structure deviates from the norm by having two potential glycosylation loops outside the membrane. Their identity and mode of action may shed light on some enigmas in neurotransmitter uptake and in the termination of neurotransmission.

The subfamily of glutamate transporters has no sequence homology with the remaining neurotransmitter transporters (Kanner, 1993). So far three members of these families have been identified, sequenced and localized. Two of them transport glutamate and one transports neutral amino acids, such as alanine, serine and cysteine (Pines *et al.* 1992; Kanai and Hediger, 1992; Arriza *et al.* 1993; Shafqat *et al.* 1993). Uptake by these transporters is driven by both sodium and potassium gradients operating in opposite directions. Therefore, the neurotransmitter is cotransported with respect to sodium and antiported with respect to potassium. The mechanistic relationships among the various neurotransmitter transporters as well as their evolutionary origin will be discussed.

Termination of neurotransmission

Neurotransmission in mammals is primed by the synthesis of a specific neurotransmitter that is accumulated into synaptic vesicles or granules. Following a specific signal, the transmitter is secreted from the vesicles into the synaptic cleft (Kanner, 1989; Nelson, 1993). The neurotransmitter interacts with a specific receptor and is removed soon thereafter. Sodium-dependent neurotransmitter transporters are the principal means by which neurotransmitters in the synaptic cleft are inactivated. These carriers transport the neurotransmitters across the plasma membrane of neuronal cells and, in some cases, surrounding glial cells. The necessary energy for the neurotransmission cycle is provided by two distinct ATPases. Synaptic vesicles and granules contain a vacuolar H⁺-ATPase (V-ATPase) that provides the proton-motive force that is utilized for the accumulation of neurotransmitters into the vesicles (Nelson, 1992). The plasma membrane is energized by a Na⁺/K⁺-ATPase that generates electrochemical gradients of sodium and potassium. Consequently, the transporters that reside in the vacuolar system are driven by a proton-motive force and the transporters that are present in the plasma membrane are driven by a sodium gradient, a potassium gradient or both. Although the principal driving force is a sodium gradient generated by this Na⁺/K⁺-ATPase, several variations on this theme can be envisaged. The transporter can utilize both the sodium and potassium gradients that are essentially in opposite directions (Rudnick and Clark, 1993). Some of them can utilize a chloride gradient that is usually present across the plasma membrane of these cells. By the differential utilization of these driving forces, the transporter can be controlled and act in a specific manner in every synaptic cleft or glial cell in which it is present. Since most of the neurotransmitters that

we are dealing with are amino acids, the general transport system of amino acids must take part in this process. Therefore, we expect multiple processes to be involved in the termination of neurotransmission in the synaptic cleft.

Even though the re-uptake systems are as important for neurotransmission as the receptors, they have attracted much less attention because they provide fewer targets for known drugs in comparison with the receptors. This receptor superiority may now change because the 'designer' drug of the 1990s, Prozac, interacts with the serotonin transporter that is a member of the family of sodium and chloride neurotransmitter transporters (Barondes, 1994). Therefore, in the future, we expect more attention to be paid to the system of neurotransmitter transporters. So now, with a clear conscience, we can turn to the more important and interesting aspects of these transporters and attempt to answer the question of how they are acting. It includes revealing the mechanism of neurotransmitter uptake and answering questions such as the following. How are these transporters assembled in the membrane? What is their precise localization and how do they function differentially in different parts of the brain? Since the cloning of the first neurotransmitter transporter (Guastella *et al.* 1990; Nelson *et al.* 1990), several of these questions can be answered in a more precise way. However, we have a long way to go in order to unravel the mechanism of their action.

Structure and origin of neurotransmitter transporters

The transporters belonging to the two families of vesicular transporters and the chloride-dependent transporters may contain 12 transmembrane helices (Guastella *et al.* 1990; Pacholczyk *et al.* 1991; Liu *et al.* 1992*b,d*; Erickson *et al.* 1992). Similar structures have been reported for several other families of transporters, including bacterial, plant and mammalian sugar transporters (Kaback, 1987; Hediger *et al.* 1987; Kwon *et al.* 1992). The mechanistic significance of this structure is not understood and there are no apparent common motifs in all of these transporters. However, a dimeric structure of six helices was proposed as a common structure for membrane transporters (Maloney, 1990). Fig. 1 depicts the general structure of some transporters containing 12 transmembrane helices. Although the number 12 has been maintained for transmembrane helices, there is no apparent rhyme or 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 not recognized.

Neurotransmitters are transported across membranes by at least four distinct families of transporters: (1) vesicular transporters that function in uptake into synaptic vesicles and granules (Schuldiner, 1994); (2) sodium- and chloride-dependent transporters that operate on the plasma membrane of neuronal and glia cells (Nelson, 1993); (3) sodium/potassium-dependent transporters that function on the plasma membranes, especially in glutamate transport (Kanner, 1993); and (4) general amino acid transport systems that participate in controlling the availability of neurotransmitters outside the cells (McGivan and Pastor-Anglada, 1994).

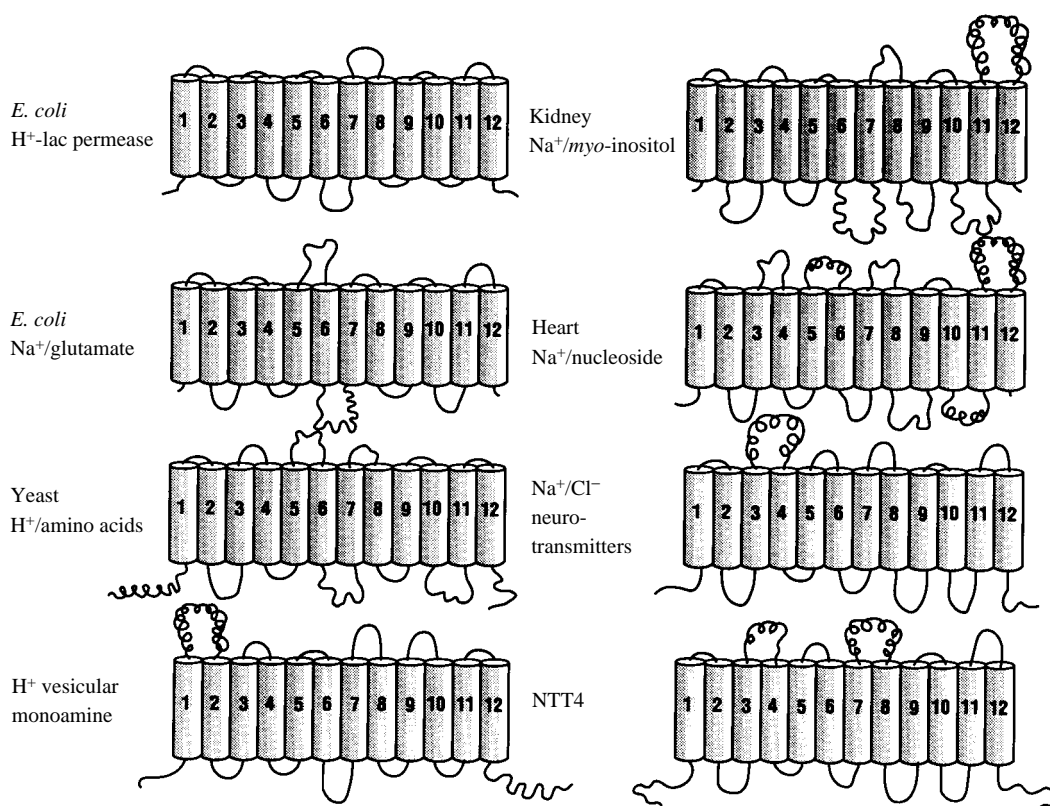


Fig. 1. Proposed organization of transmembrane helices and peripheral loops in transporters with 12 transmembrane helices. The examples depicted in the figure are: lactose permease of *Escherichia coli* (Kaback, 1987); Na⁺/glutamate symport carrier (Deguchi *et al.* 1990); general amino acid permease of *Saccharomyces cerevisiae* (Jauniaux and Grenson, 1990); vesicular amine transporter (Liu *et al.* 1992d); Na⁺/myo-inositol cotransporter (Kwon *et al.* 1992); Na⁺/nucleoside cotransporter (Pajor and Wright); Na⁺/Cl⁻ neurotransmitter transporters (Liu *et al.* 1992b,c) and NTT4 orphan transporters (Liu *et al.* 1993c).

General amino acids and glutamate transporters

Amino acid transport in bacteria and eukaryotic cells is carried out by numerous transporters with different specificities. They include neutral, basic and acidic amino acid transport systems as well as several other systems specific for particular amino acids (Christensen, 1990; McGivan and Pastor-Anglada, 1994). Recently, cDNAs encoding glutamate transporters that are members of the acidic amino acid transport system X⁻ were cloned and sequenced (Storck *et al.* 1992; Pines *et al.* 1992; Kanai and Hediger, 1992). Expression of these cDNAs in *Xenopus* oocytes or mammalian cells revealed that they encode the Na⁺/K⁺/glutamate transporters that were implicated in glutamate reuptake in glutamatergic synapses (Kanner, 1993). Subsequently, cDNAs encoding neutral amino acid transporters structurally related to the glutamate transporters were cloned and expressed (Arriza *et al.* 1993; Shafqat *et al.* 1993). These transporters

exhibited significant sequence homology with bacterial nutrient uptake systems (Jiang *et al.* 1989; Tolner *et al.* 1992). The structure of the mammalian transporters of this family includes 6–10 transmembrane helices and an extended loop with potential glycosylation sites between transmembrane helices III and IV (Kanner, 1993). Although they are distributed not only in brain but also in several peripheral tissues, it was proposed that they were the transporters functioning in the termination of neurotransmission in glutamatergic neurons. Glutamate uptake by these glutamate transporters is driven by opposed sodium and potassium gradients and they fail to operate without the presence of sodium outside and potassium inside the cells. It was suggested that uptake of the anion glutamate is accompanied by the inward transport of two Na^+ and that one K^+ and one OH^- (or HCO_3^-) are transported out (Bouvier *et al.* 1992). The mechanism of such a complicated transport system is not understood and even the substrate binding site is not known. In order to understand neurotransmission, the mechanism of action of the transporters should be elucidated. In addition, one of the main questions that has to be answered is whether these transporters are sufficient for the daunting tasks of rapid removal of glutamate from the synaptic cleft and of preventing glutamate toxicity when excess glutamate is secreted from the cells. We think that more transporters may be involved in this process and NTT4 is one of the candidates for this task (Liu *et al.* 1993c; El Mestikawy *et al.* 1994; Jursky *et al.* 1994).

Vesicular transporters

Synthesis of neurotransmitters and/or their accumulation into synaptic vesicles are the priming steps for neurotransmission. Vesicular transporters function in neurotransmitter accumulation into the vesicles driven by the proton-motive force. Distinct transporters may function in the uptake of different substrates and they may differentially utilize the proton gradient or membrane potential generated by the V-ATPase in the various organelles (Nelson, 1992). cDNAs encoding monoamine transporters were the first to be cloned and sequenced (Liu *et al.* 1992d; Erickson *et al.* 1992; Krejci *et al.* 1993). Recently, cDNAs encoding vesicular acetylcholine transporters in *Caenorhabditis elegans* and *Torpedo marmorata* electric lobe were cloned, sequenced and shown to have a high degree of homology with the monoamine transporter (Alfonso *et al.* 1993; Varoqui *et al.* 1994). Their open reading frames encode hydrophobic proteins with 12 potential transmembrane helices and a large hydrophilic loop between helices I and II that faces the lumen and contains three potential glycosylation sites. The expressed transporters are quite promiscuous with substrate and will transport not only the various catecholamines but also indolamines, *N*-methyl-4-phenylpyridinium and several biogenic amines and their precursors. An interesting connection between catecholamine uptake and bacterial multidrug transport has been pointed out (Schuldiner, 1994). Indeed, the amino acid sequences of the mammalian catecholamine transporters are homologous to those of several bacterial transporters that induce multidrug resistance. Like the mammalian vesicular transporters, the mechanism of bacterial drug transport involves substrate/proton exchange. This similarity suggests that the vesicular transporters evolved from bacterial

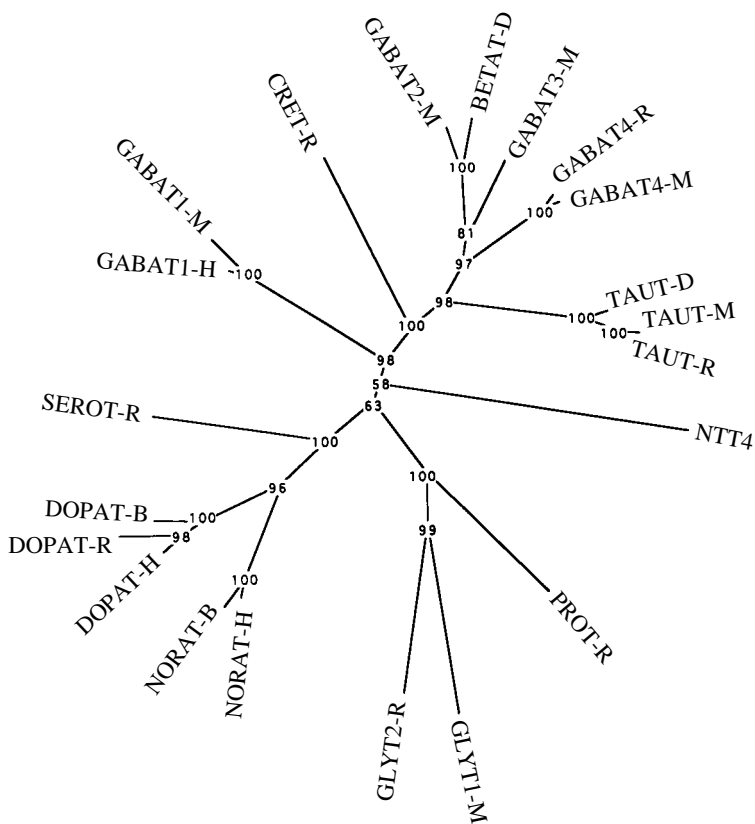


Fig. 2

transporters that confer drug resistance and, in turn, that the vesicular transporter may function in drug secretion from mammalian cells.

Evolution of the sodium- and chloride-dependent family of transporters

The family of sodium- and chloride-dependent neurotransmitter transporters is more unique than that of the vesicular and glutamate transporters and as yet no homologous transporters have been described in bacteria or fungi. The most primitive organisms that contain these transporters are insects and worms (Liu *et al.* 1992*b*). Therefore, this family of transporters may have diverged about 0.5 billion years ago. However, we recently identified a highly homologous gene in thermophilic bacteria that may or may not have emerged by convergent evolution from eukaryotes (N. Nelson and H. Nelson, unpublished results). Even though they are not restricted to the nervous system, the Na⁺/Cl⁻ transporters may have evolved concomitantly with the emergence of neuronal cells. This family of transporters contains three subfamilies of monoamine, GABA and amino acid transporters that share similar structures with 12 transmembrane helices and a large loop between helices III and IV containing 2–4 potential glycosylation sites (Amara

Fig. 2. Evolutionary relationships among the family members of Na⁺/Cl⁻ neurotransmitter transporters. Alignments of amino acid sequences as well as DNA sequences were carried out by means of the PILEUP program of the GCG 7.2 package (Devereux *et al.* 1984), running under VMS on a μ VAX 3800. The evolutionary tree shown here was then constructed by the DNAML program of the PHYLIP 3.5 package (Felsenstein, 1989), running on a DECStation 3000 under Open VMS. After running DNAML, a maximum likelihood program, which calculates trees figuring the evolutionary distances of species as well as their grouping, the identical alignments were used by the program SEQBOOT to generate 100 bootstrapped samples. These samples were used to build another set of 100 trees by means of the parsimony program DNAPARS. The trees constructed by DNAPARS were fed into CONSENSUS, a program that joined them into a single consensus tree and provided the additional information of how many times a specific grouping occurred during the construction of the consensus tree. Since the trees constructed by the two different methods were grouped completely identically, we were able to provide the percentages of grouping within the DNAML tree displayed by the figure. Values given at a single node show how many times out of 100 trials the species grouped towards the end of the two branches emerging from that node were placed in that particular position.

Name	Synonym	Substrate	Reference
Humdoptra	DOPAT-H	Dopamine	Vandenberg <i>et al.</i> (1992)
Ratdoper	DOPAT-R	Dopamine	Shimada <i>et al.</i> (1991)
Bovdopatr	DOPAT-B	Dopamine	Udin <i>et al.</i> (1991)
Humnortr	NORAT-H	Noradrenaline	Pacholczyk <i>et al.</i> (1991)
Ntt1	NORAT-B	Noradrenaline	Pacholczyk <i>et al.</i> (1991)
Rsertran	SEROT-R	Serotonin	Blakely <i>et al.</i> (1991)
Glyt2	GLYT2-R	Glycine	Liu <i>et al.</i> (1993a)
Glyt1	GLYT1-M	Glycine	Liu <i>et al.</i> (1992c)
Ntt4r	NTT4	?	Uhl <i>et al.</i> (1992); Liu <i>et al.</i> (1993c)
Gat1	GABAT1-M	γ -Aminobutyrate	Liu <i>et al.</i> (1992b)
Hsgat1mr	GABAT1-H	γ -Aminobutyrate	Nelson <i>et al.</i> (1990)
Ratttrnsr	TAUT-R	Taurine	Smith <i>et al.</i> (1992)
Taurt	TAUT-M	Taurine	Liu <i>et al.</i> (1992a)
Dognac1tau	TAUT-D	Taurine	Uchida <i>et al.</i> (1992)
Gat4	GABAT4-M	γ -Aminobutyrate	Liu <i>et al.</i> (1993b)
Rat3gat	GABAT4-R	γ -Aminobutyrate	Borden <i>et al.</i> (1992)
Gat3	GABAT3-M	γ -Aminobutyrate	Liu <i>et al.</i> (1993b)
Gat2	GABAT2-M	γ -Aminobutyrate	López-Corcuera <i>et al.</i> (1992)
Dognacbt	BETAT-D	Betaine	Yamauchi <i>et al.</i> (1992)
Protr	PROT-R	Proline	Freneau <i>et al.</i> (1992)
Cretr	CRET-R	Creatine	Mayser <i>et al.</i> (1992)

and Kuhar, 1993; Nelson, 1993). A fourth subfamily of orphan transporters (NTT4) was shown to have 12 transmembrane helices with two large loops between helices III and IV and helices VII and VIII, both containing potential glycosylation sites (Uhl *et al.* 1992; Liu *et al.* 1993c). Fig. 2 shows an evolutionary tree constructed from most of the known sequences of sodium- and chloride-dependent neurotransmitter transporters. The subfamilies of monoamine, amino acid, GABA and NTT4 transporters are separated to different extents. The tightest subfamily is that of the monoamine transporters, with the serotonin transporter diverging somewhat earlier than the noradrenaline and dopamine

transporters. The glycine and proline transporters are grouped together, but all diverged early during the onset of the subfamily. The subfamily of GABA transporters is quite diverse where the GAT1 and creatine transporters were separated earlier in the branched tree, and the taurine, betaine and the remaining GABA transporters are grouped together. NTT4 and its related transporters (not shown) are branched as a separate subfamily that is also reflected by their unique structure (Liu *et al.* 1993c).

Genomic clones and exon shuffling

Cloning of genes encoding neurotransmitter transporters revealed some interesting properties and evolutionary trends of this gene family (Liu *et al.* 1992b). A mouse gene encoding the GABA transporter GAT1 was cloned first and shown to have 14 introns, one of them situated before the initiator methionine codon. The position of the first intron inside the reading frame was strictly maintained not only in all the mammalian genes cloned so far, but also in a *Drosophila* gene (Liu *et al.* 1992b). The positions of the introns in the mouse gene encoding the GAT1 transporter are shown in Fig. 3. We have cloned and partially sequenced several genes encoding neurotransmitter transporters and observed that their introns are situated at similar, if not identical, positions to those in the gene encoding the GABA transporter (N. Nelson, Q.-R. Liu, S. Mandiyan and H. Nelson, unpublished). Most of the introns define protein modules that contain one out of the twelve transmembrane helices. It was proposed that genes evolve *via* intron-mediated recombination of exon modules that code for functional or structural elements (Gilbert,

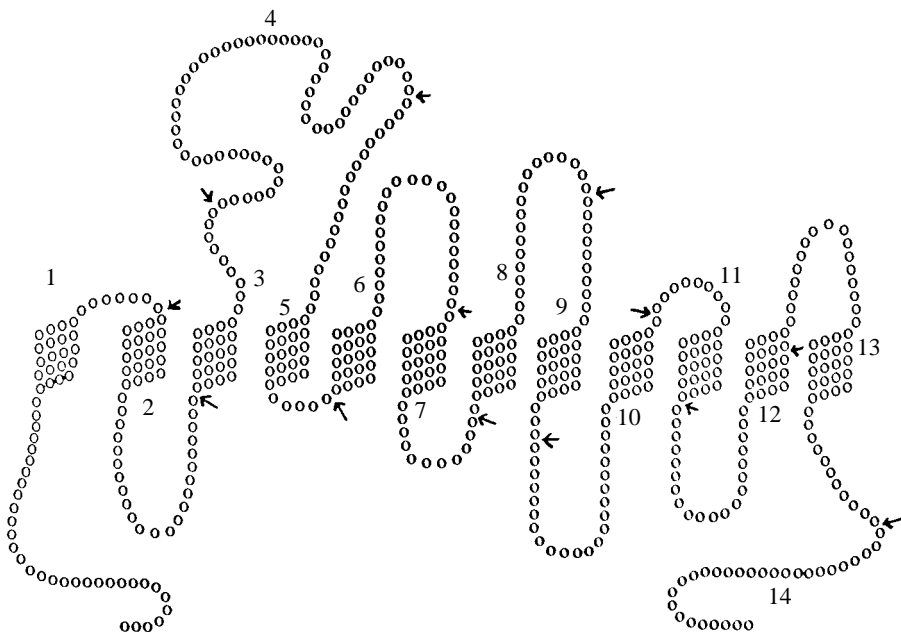


Fig. 3. Proposed membrane topology of GABA transporter and the position of introns in the gene encoding GAT1. The exons of the open reading frame are numbered and the positions of introns are marked by arrows.

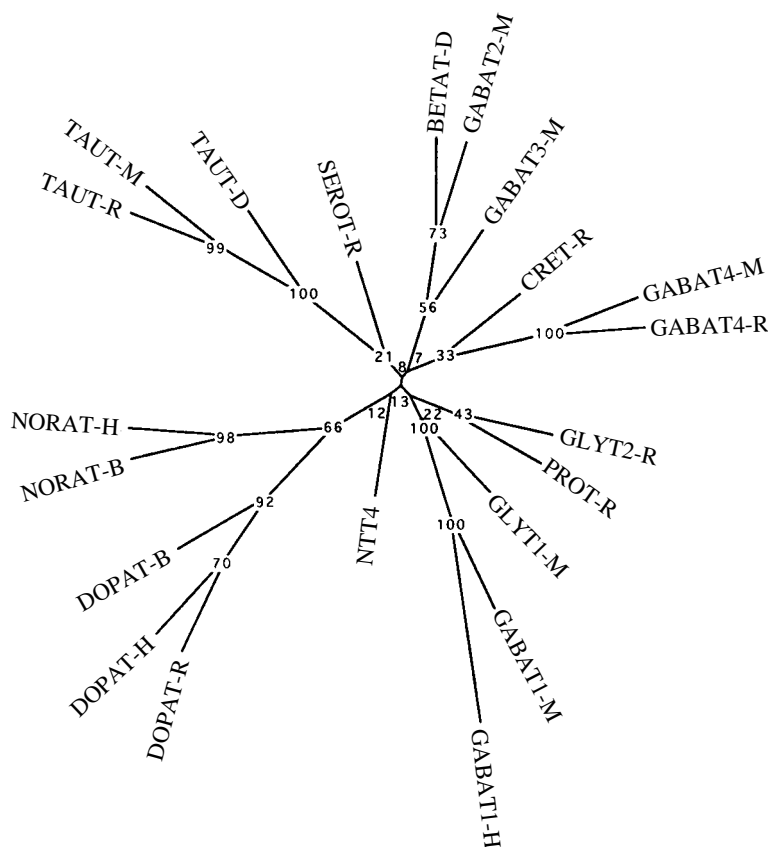


Fig. 4. Partial sequences encoding exon 2 (for numbering and position of exons, see Fig. 3), as defined by the flanking regions given in Liu *et al.* (1992b) for GAT1, were cut out of the complete DNA sequence alignment. The resulting alignment was used to calculate an evolutionary tree from 100 bootstrapped samples as described in the legend to Fig. 2. Note that unlike that in Fig. 2, the tree shown here does not have meaningful branch lengths, but only displays the grouping of species.

1978; Go and Nosaka, 1987; Dorit *et al.* 1990). The genes encoding neurotransmitter transporters may be fine examples of such an evolutionary process. Assuming that the position of the introns is conserved, we analyzed the evolutionary relationship among the homologous exons in several transporters in which their cDNA sequence is available. This procedure may indicate a relationship among transporters of different subfamilies not revealed by the evolutionary trees constructed with the full-size transporters. The identification of the putative splicing sites in the cDNAs included in one analysis was made easy by the highly homologous sequences that were aligned to the splicing sites of GAT1. The restricted length of the analyzed regions gave rise to a decrease in the certainties of the groupings. Nevertheless, a score of interesting links could be filtered out of the relationships of exons. Some of the analyzed exons gave an evolutionary tree similar to that

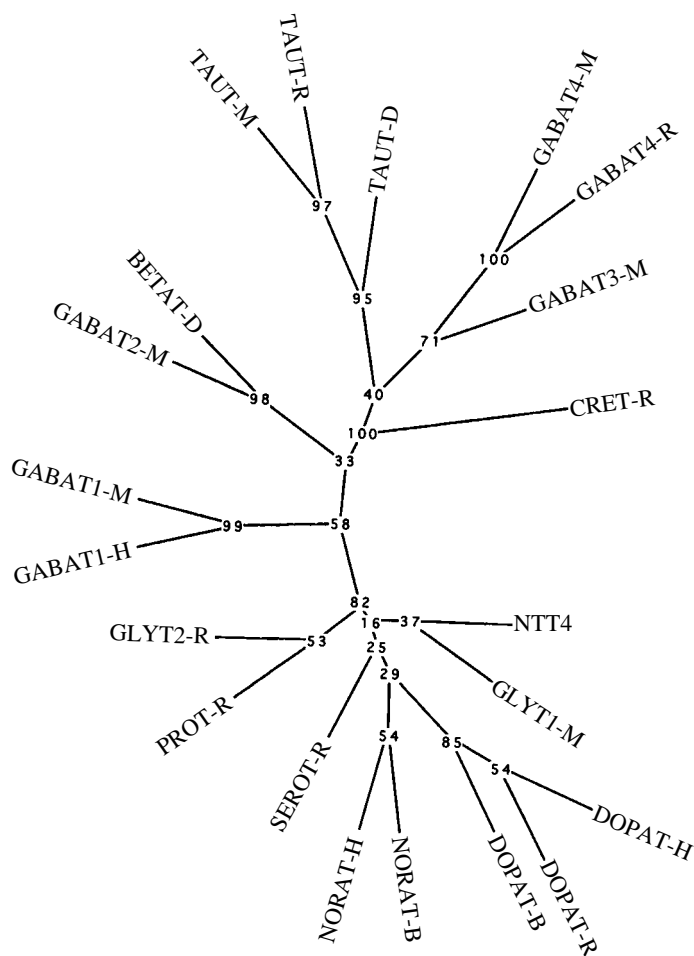


Fig. 5. Partial sequences encoding exon 3 cut out of the complete DNA sequence alignment. The resulting set of partial sequences was used to calculate an evolutionary tree from 100 bootstrapped samples as described in the legend to Fig. 2. As in Fig. 4, the tree shown here does not have meaningful branch lengths, but only displays the grouping of species.

described in Fig. 2, which resulted from the analysis of the entire proteins. Others that deviated from this pattern are discussed below.

The evolutionary tree constructed from sequences of exon 2 (see Fig. 3) is shown in Fig. 4. In this tree, the glycine, proline and GABA (GAT1) transporters are grouped together, and NTT4 takes a close position but with a low degree of certainty. Creatine and GAT4 transporters are branched separately from GAT2 and GAT3 and the taurine and betaine transporters. With exon 3 (Fig. 5), NTT4 branched, together with GLYT1, and is separate from GLYT2 and the proline transporters. Exon 4 represents the external loop with potential glycosylation sites. An evolutionary tree constructed from this part of the transporters revealed strong relationships between GAT1 and NTT4 that are branched

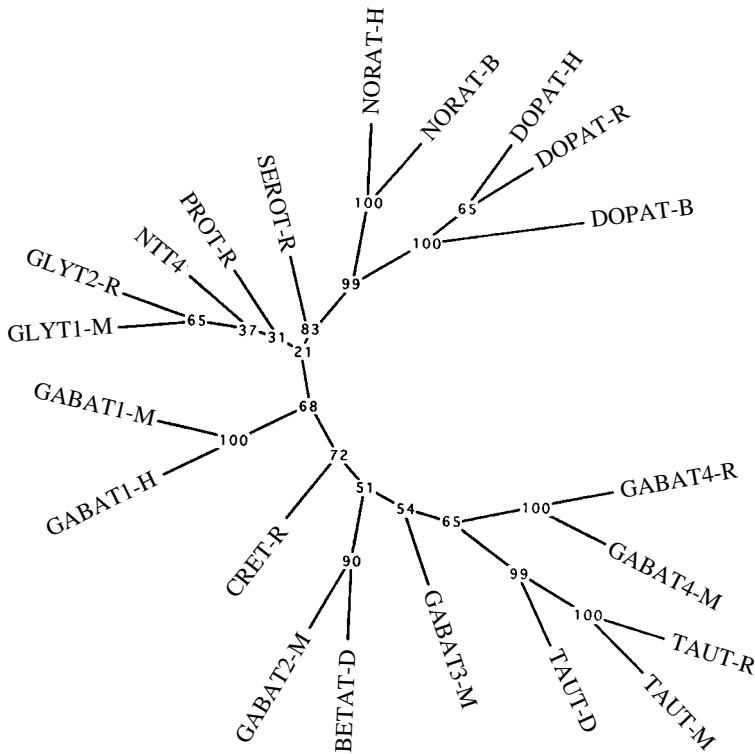


Fig. 6. Exon 7 was cut out of the complete DNA sequence alignment as defined by the flanking sequences given in Liu *et al.* (1992b). The resulting set of partial sequences was used to calculate an evolutionary tree from 100 bootstrapped samples as described in the legend to Fig. 2. Note that as in Figs 4 and 5, the tree shown here does not have meaningful branch lengths, but only displays the grouping of species.

separately with a relatively high score. It is worth noting that, at exon 5, NTT4 is branched with GAT4 (not shown) which, together with GAT1, functions in the termination of GABA transmission in neurons. Fig. 6 depicts the evolutionary tree constructed from sequences of exon 7. In this tree, NTT4 clearly branched together with the glycine and proline transporters and GLYT1 and GLYT2 are grouped close to each other. The phenomenon of the latter two transporters coming together repeated itself in exons 4, 5 and 10. The two transporters have relatively low overall sequence homology and the above-mentioned exons may be involved in the common function of these transporters. Fig. 7 shows the evolutionary tree constructed from exon 8. In this tree, the serotonin transporter is grouped with proline and glycine (GLYT1) transporters and the glycine transporter GLYT2 is on a separate branch. In exon 9, the two glycine transporters are also separated, but here GLYT2 goes with the proline transporter (not shown). In the tree constructed with exon 11, the glycine and proline transporters are together again but NTT4 is branched together with the serotonin transporter. Analysis of this exon revealed that all the neurotransmitter transporters capable of β -alanine transport

neurotransmitter transporters or any other transporter. Crystallization and resolution of the fine structure of a multitransmembrane transporter may help to clarify, but may not necessarily solve, all the problems. Transport across membranes is a dynamic process that may involve subtle conformational changes necessary for transporting the substrate across the membrane. One important aspect of the substrate binding site is likely to be revealed by co-crystallization with the substrate. For example, a crystal structure of 12 transmembrane transporters may show that not all the helices are at the plane of the membrane. Let us assume that helices I, II, IX and X of sodium/chloride-dependent neurotransmitter transporters form a four-helix bundle that is arranged as a dent in the membrane. If the sodium-binding site is situated on the loop between helices I and II and part of the substrate binding site is located on the loop between helices IX and X, this structure may provide the underlying mechanism of sodium-dependent solute transport across the membrane. These helices were chosen because they are connected with the shortest external loops, enabling the formation of a dent in the membrane. However, the positions of short loops are different in the various transporters that have 12 transmembrane domains. Therefore, if short loops have anything to do with the mechanism of transport, each family of transporters may transport its substrate *via* a different combination of helices.

The precise localization of neurotransmitter transporters and their involvement in the regulation of neurotransmission and related processes are likely to come as a windfall of the cloning of their cDNAs. A combination of *in situ* hybridization with immunocytochemical localization should reveal their sites of synthesis as well as their sites of action. We believe that, in addition to the traditional function of transporters in transporting substrates across membranes, they will be implicated in signal transduction across the membrane. Transporters preceded receptors in sensing the environment and there is no reason for discontinuing this function in advanced organs such as the brain. One of the ways by which transporters can function in signal transduction is through conformation-sensitive interactions with second messengers in the cytoplasm. These interactions may play a role in the regulation of the transporters' activity. It would not be surprising if cytoplasmic proteins modulated the activity of the transporters and also if the activity of some of them is totally dependent on the interaction with accessory polypeptides. Finally, we should look forward to the unexpected.

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