STRUCTURE, FUNCTION AND BRAIN LOCALIZATION OF NEUROTRANSMITTER TRANSPORTERS

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Summary

We studied four different cDNAs encoding GABA transporters and three different cDNAs encoding glycine transporters in mouse and rat brains. A genomic clone of two of the glycine transporters (GLYT1a and GLYT1b) revealed that they derive from differential splicing of a single gene. The third glycine transporter (GLYT2) is encoded by a separate gene. Antibodies were raised against seven of these neurotransmitter transporters and their cytochemical localization in the mouse brain was studied. In general, we observed a deviation from the classical separation of neuronal and glial transporters. It seems that each of the neurotransmitter transporters is present in specific places in the brain and is expressed in a different way in very specific areas. For example, the GABA transporter GAT4, which also transports \( \beta \)-alanine, was localized to neurons. However, GAT1, which is specific for GABA, was localized not only to neurons but also to glial cells. The recently discovered glycine transporter GLYT2 was of particular interest because of its deviation from the general structure by a very extended N terminus containing multiple potential phosphorylation sites. Western analysis and immunocytochemistry in frozen sections of mouse brain demonstrated a clear caudal–rostral gradient of GLYT2 distribution, with massive accumulation in the spinal cord and brainstem and less in the cerebellum. Its distribution is typically neuronal and it is present in processes with varicosities. A correlation was observed between the pattern we obtained and that observed previously from strychnine binding studies. The results indicate that GLYT2 is involved in the termination of glycine neurotransmission at the classical inhibitory system in the hindbrain. The availability of four different GABA transporters made it possible to look for specific binding sites upon the neurotransmitter transporters. An extensive program of site-directed mutagenesis led us to identify a potential neurotransmitter binding site on the GABA transporters.

Introduction

Amino acids are known to function as neurotransmitters in the brain. The most important amino acids in this respect are glutamic acid, acting as a stimulatory neurotransmitter, and glycine, acting as an inhibitory neurotransmitter (Kanner, 1989, 1994). Termination of neurotransmission by these amino acids proceeds via removal of the secreted amino acids from the synaptic cleft by a rapid sodium-dependent uptake system located not only in the presynaptic cells but also in glial cells and perhaps even in

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the postsynaptic cells. Therefore, the termination of neurotransmission is a complicated mechanism involving several transporters with different pharmacologies. The transporters involved in neurotransmission may also be present in peripheral tissues and may function in uptake processes not related to signal transduction. Conversely, neurotransmitter transporters may function in a variety of processes including (1) termination of neurotransmission, (2) detoxification, (3) protection from reactive substances, (4) nutrition, and (5) modulation of receptor activity. Cloning and expression of cDNAs encoding neurotransmitter transporters enabled us to investigate their function as well as their precise localization in different parts of the brain.

A cDNA encoding a GABA transporter in rat brain was the first to be cloned, sequenced and expressed (Guastella et al. 1990; Nelson et al. 1990). Subsequently, a cDNA encoding a noradrenaline transporter was obtained by expression cloning (Pacholczyk et al. 1991). The stretches of amino acids conserved between the two transporters were utilized for cloning of numerous other neurotransmitter transporters of this family; these could be divided into three distinct subfamilies, consisting of monoamine, GABA and amino acid transporters (Shimada et al. 1991; Kilty et al. 1991; Usdin et al. 1991; Hoffman et al. 1991; Blakely et al. 1991; Clark et al. 1992; Borden et al. 1992; López-Corcua et al. 1992; Liu et al. 1992a,b,c, 1993a,b; Smith et al. 1992; Guastella et al. 1992; Fremeau et al. 1992; Uchida et al. 1992; Yamauchi et al. 1992; Maysen et al. 1992; Guimbal and Kilimann, 1993). In addition, a subfamily of orphan transporters was identified (Uhl et al. 1992; Liu et al. 1993c). We examined members of the four subfamilies and raised specific antibodies that were utilized for localizing the transporters in various parts of the brain. We also searched for specific substrate binding sites by site-directed mutagenesis.

**Structure and function of GABA and glycine transporters**

All members of the sodium- and chloride-dependent neurotransmitter transporters maintain a similar general structure. Most transporters are constructed from about 600 amino acids arranged in 12 transmembrane helices, with short N- and C-termini on the cytoplasmic side of the membrane and a large potentially glycosylated loop between transmembrane helices III and IV. Exceptions to this general structure are the members of the subfamily of NTT4, which contain an extra-large glycosylated loop between helices VII and VIII, and the glycine transporter GLYT2, which contains an exceptionally long N terminus with several potential phosphorylation sites (Liu et al. 1993a,c). The extensive homology among the different members of the family gave little, if any, indication of the function of some stretches of amino acids in substrate binding and transport or in the pathways of sodium and chloride movements across the membrane. The only conserved charged amino acid is an arginine residue situated in the middle of transmembrane helix I. This arginine (Arg-69) was found to be essential for the activity of GAT1 and substitutions by glutamine, histidine, lysine, threonine, methionine or glycine inactivated the transporter (Pantanowitz et al. 1993). It was suggested that arginine-69 functions in the binding of negatively charged chloride ions. Tryptophan residues were also substituted by site-directed mutagenesis into serine, leucine, tyrosine or phenylalanine.
(Kleinberger-Doron and Kanner, 1994). Only tryptophan-222 and tryptophan-230 were essential for the activity or the assembly of the transporter. It was suggested that tryptophan-222 functions in the binding of substrate to the transporter in a manner analogous to the proposed function of a tryptophan residue in acetylcholine esterase (Sussman et al. 1991). Substitutions of tryptophan-230 prevented the proper sorting of the transporter into the plasma membrane. A similar effect was observed for the assembly of glucose transporter, in which mutation of tryptophan-388 caused a defective translocation through the secretory pathway (García et al. 1992). Is this sorting defect specific for tryptophans? Probably not. We find severe reduction in the assembly of membrane proteins from a variety of eukaryotic sources after substitutions of single amino acid inside transmembrane helices. This sensitivity contrasts with the insensitivity to changes in the assembly of transporters into the bacterial plasma membrane (Kaback, 1988, 1992). It is conceivable that the correct conformation of transmembrane helices of eukaryotic proteins is detected by the proofreading system of the secretory pathway and that this correct conformation is a prerequisite for the transfer of the transporter to the plasma membrane.

The potentially glycosylated loop between transmembrane helices III and IV is not well conserved and even its size varies among the different transporters. However, its first 30 amino acids are highly conserved and its strategic position in the transporters suggests possible involvement in substrate binding. The conserved positions according to GAT1 are W146, W161, W168, Y150, S154, T156, L159, P160, N169, C164 and C173. In addition to the latter two cysteines, a cysteine corresponding to C74 in GAT1 is conserved in all the transporters. We substituted each one of the corresponding cysteine residues in GAT3 with different amino acids. As shown in Table 1, substitution of C62 with serine diminished the GABA uptake activity of the transporter. However, substitution of the same residue with valine did not affect the activity of the expressed transporter in *Xenopus* oocytes. Substitution of C153 or C162 with serine or valine resulted in the loss of GABA uptake by the expressed transporter mutants. It is possible that the cysteine residues at positions 153 and 162 form a covalent bond that is important for the activity of the transporter. We are exploring the possibility that this structure is involved in the binding of substrate to the various transporters. The availability of four different GABA transporters prompted us to look for the location of substrate binding sites by site-directed mutagenesis. By swapping amino acid sequences between GAT1,

<table>
<thead>
<tr>
<th>Substitution</th>
<th>Rate of GABA uptake (pmol oocyte h(^{-1}))</th>
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<tbody>
<tr>
<td>Control</td>
<td>9.5</td>
</tr>
<tr>
<td>Cysteine-62 to serine</td>
<td>0.3</td>
</tr>
<tr>
<td>Cysteine-62 to valine</td>
<td>9.3</td>
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<tr>
<td>Cysteine-153 to serine</td>
<td>0</td>
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<tr>
<td>Cysteine-153 to valine</td>
<td>0</td>
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<tr>
<td>Cysteine-162 to serine</td>
<td>0.1</td>
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<tr>
<td>Cysteine-162 to valine</td>
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which is specific for GABA transport, and GAT3, which transports GABA and \( \beta \)-alanine, we identified the short loop between helices IX and X as a potential \( \beta \)-alanine binding site (S. Tamura, H. Nelson, A. Tamura and N. Nelson, unpublished results).

**Localization of the neurotransmitter transporters in mouse brain**

A typical feature of neuronal transporters, such as GAT1, GAT4, GLYT2 and NTT4, is that they are localized in processes and nerve endings without detectable presence in cell bodies. Little is known about the sorting mechanism of these transporters, but in heterologous polarized epithelial cells the expressed transporters GAT1, GAT4 and GAT3 sorted into the apical and basolateral membranes, respectively (Pietrini et al. 1994; J. Ahn, G. Rudnick and M. J. Caplan, in preparation). Since there are four different GABA transporters and three variants of glycine transporters, we decided to follow the distribution of individual transporters using specific antibodies. To prevent crossreactivity among the various transporters, the least homologous parts of the transporters (the N and C termini and the potential glycosylated loop) were expressed as fusion proteins with maltose-binding proteins in *Escherichia coli* cells. Antibodies were raised in guinea pigs and affinity-purified on Affigel to which the specific fusion proteins were covalently bound. For immunocytochemistry, mice or rats were perfused with a solution containing 4% paraformaldehyde in phosphate-buffered saline or 4% paraformaldehyde in zinc salicylate, pH 6.5 (Mugnaini and Dahl, 1983). Brain slices were decorated with the affinity-purified antibodies and detected by the VECTOR Elite kit with 3,3'-diaminobenzidine (DAB) (F. Jursky and N. Nelson, unpublished results).

**Noradrenaline transporter**

A bovine adrenal noradrenaline transporter was one of the first cDNAs encoding a neurotransmitter transporter to be cloned in our laboratory (S. Mandiyan, H. Nelson and N. Nelson, unpublished results). Fig. 1 shows the amino acid sequence of this transporter; it is highly homologous to the one cloned from human brain (Pacholczyk et al. 1991). We raised an antibody against the loop between helices III and IV of the bovine transporter. On Western blots with membranes derived from mouse and rat brains, the antibody stained a single protein band of about 70kDa. Immunocytochemistry revealed crossreactivity in all noradrenergic rat brain cell groups (Fig. 2) but no crossreactivity in striatum and nucleus accumbens, which are innervated by dopamine. We did not find a significant difference in crossreactivity between the locus coeruleus and other noradrenergic-projecting cells differing in sensitivity to DSP4 (Lyons et al. 1989). Distribution of the noradrenergic cells and axons was studied using antibodies against enzymes of the catecholamines biosynthetic pathways (for a review, see Moore and Bloom, 1979). The distribution of noradrenaline transporter correlates very well with the distribution of noradrenergic neurons, and the function of this transporter is likely to be in the termination of neurotransmission by noradrenaline at the relevant synapses.

**Glycine transporters**

The amino acid glycine acts as an inhibitory neurotransmitter in the caudal mammalian
There are also indications that glycine does not play a purely metabolic role in the forebrain (Pycock and Kerwin, 1981; Malosio et al. 1991). Three different cDNAs encoding glycine transporters have been cloned. Two of them, GLYT1a and GLYT1b, are transcribed by differential splicing in a single gene. According to the results of in situ hybridization, they are abundant in most of the brain areas and GLYT1a mRNA is also present outside the central nervous system (Borowsky et al. 1993; Liu et al. 1993a). The third transporter, GLYT2, is encoded by a separate gene and is present in the spinal cord, the brainstem and to a lesser extent in the cerebellum and the thalamus. As shown in Fig. 3, the clear caudal–rostral distribution gradient of GLYT2 in mouse brain reflects the distribution of glycinergic neurons and follows closely the distribution of the glycine receptor (Araki et al. 1988). Weak immunoreactivity is present in the cerebral cortex, hippocampus and basal forebrain. Immunoreactivity is present in gray matter slightly protruding into the white matter. Because the staining patterns are almost identical with brain. There are also indications that glycine does not play a purely metabolic role in the forebrain (Pycock and Kerwin, 1981; Malosio et al. 1991). Three different cDNAs encoding glycine transporters have been cloned. Two of them, GLYT1a and GLYT1b, are transcribed by differential splicing in a single gene. According to the results of in situ hybridization, they are abundant in most of the brain areas and GLYT1a mRNA is also present outside the central nervous system (Borowsky et al. 1993; Liu et al. 1993a). The third transporter, GLYT2, is encoded by a separate gene and is present in the spinal cord, the brainstem and to a lesser extent in the cerebellum and the thalamus. As shown in Fig. 3, the clear caudal–rostral distribution gradient of GLYT2 in mouse brain reflects the distribution of glycinergic neurons and follows closely the distribution of the glycine receptor (Araki et al. 1988). Weak immunoreactivity is present in the cerebral cortex, hippocampus and basal forebrain. Immunoreactivity is present in gray matter slightly protruding into the white matter. Because the staining patterns are almost identical with...
the binding of the glycine receptor antagonist strychnine, GLYT2 is thought to accompany the glycine receptor in the caudal brain. The other two variants of glycine transporters, GLYT1a and GLYT1b, differ only in their N-terminal parts, which are the first 10 amino acids in GLYT1a and the first 15 amino acids in GLYT1b (Liu et al. 1993a). This difference can be exploited to obtain specific antibodies against GLYT1a

Fig. 2. Rat locus corullereus immunostained with antibody raised against the recombinant loop fusion protein of the bovine noradrenaline transporter. A cDNA fragment encoding the amino acid sequence of residues 150–234 (see Fig. 1) of the bovine noradrenaline transporter was cloned in-frame into pMAL vector (New England Biolabs). The fusion protein between the maltose-binding protein and the loop of the noradrenaline transporter was overexpressed in *Escherichia coli*, purified by electrophoresis on SDS-containing gels and electro-eluted as previously described (Nelson, 1983). The antibody raised in guinea pigs was affinity-purified and used for immunocytochemistry in brain slices. Scale bar, 100 μm.
and GLYT1b. Peptides corresponding to these amino acid stretches were synthesized and antibodies against them were raised in guinea pigs. In addition, a common antibody against the two variants was raised by injecting a fusion protein containing the C-terminal part and N terminus corresponding to the GLYT1a polypeptide fused to the maltose-binding protein. The antibody raised against the specific GLYT1b polypeptide, or the common N-terminal and C-terminal parts, gave the same patterns in Western analysis and in brain sections (Fig. 4). This may be due to the presence of GLYT1a in all the places where GLYT1b is present (Borowsky et al. 1993). GLYT1 immunoreactivity is present in every brain region. In addition to the gray matter, immunoreactivity is visible in all white matter fiber tracts, cortical radial fibers and round-shaped cells, suggesting that at least one of the subtypes, most probably GLYT1b, is localized in glial cells. No staining of the cell bodies or processes of the pyramidal or granule cells was detected in the hippocampus, where the in situ hybridization experiments suggested the presence of GLYT1a (Borowsky et al. 1993). The antibody against the C terminus showed a signal mostly in the hilus stratum lacunosum moleculare and only a weak signal in stratum pyramidale around the cell bodies. As in the cerebral cortex, staining of non-principal cell bodies was observed. It was proposed that GLYT1 may play a role in the modulation of

Fig. 3. Distribution of GLYT2 immunoreactivity in the rat brain stained with antibody against the N-terminal recombinant fusion protein of the rat GLYT2 transporter. The antibody was raised against the unique N terminus of this transporter. Similar results were obtained with antibody raised against the potentially glycosylated loop. The morphology consists of processes with frequent 2 μm varicosities. cb, cerebellum; cx, cerebral cortex; ob, olfactory bulb; sc, spinal cord; th, thalamus. Scale bar, 2 mm.
N-methyl-D-aspartate (NMDA) receptors in various parts of the brain (Liu et al. 1992c; Smith et al. 1992). We cannot yet substantiate this hypothesis; detailed electron microscopy should be utilized for this purpose.

GABA transporters

We cloned and expressed four distinct GABA transporters of mouse brain (Liu et al. 1993b). Antibodies against their N and C termini as well as their glycosylated loops were raised and analyzed by Western blots and immunocytochemistry in brain sections. Two members of this subfamily, GAT1 and GAT4, were found to be brain-specific. In accordance with previous observations, GAT1 shows a relatively even distribution in all parts of the brain (Mabjeesh et al. 1992). Its largely indiscriminate distribution parallels the distribution of GABA. However, this transporter may be present not only in neurons but also in glial cells (Radian et al. 1990). In contrast, GAT4 is strongly expressed in the spinal cord, brainstem, thalamus, hypothalamus and the palidal aspect of the basal forebrain and is very weakly expressed in the cerebellum, hippocampus, cerebral cortex and striatum. Both transporters are localized in the neuropil but are not detectable in cell bodies. The distribution patterns makes GAT1 a better candidate than GAT4 for the GABA transporter that functions in established GABAergic synapses. The major pattern of distribution of GAT4 is similar to that of the glycine transporter GLYT1a (Figs 4, 5).

Fig. 4. Distribution of GLYT1a and GLYT1b immunoreactivity in the rat brain stained with antibody raised against the recombinant fusion protein of the C-terminal part of the mouse transporter common to GLYT1a and GLYT1b. Antibody concentration was adjusted to show the rostral–caudal gradient. cb, cerebellum; cx, cerebral cortex; ob, olfactory bulb; sc, spinal cord; th, thalamus. Scale bar, 2 mm.
This similarity suggests a possible function of GAT4 in neurons where GABA terminals were found to be apposed to glycine receptors (Triller et al. 1985, 1986).

**Orphan transporter NTT4**

The function of this orphan subfamily of transporters is not known and they may even act in an ectopic function, such as in signal transduction, that has no relationship to transport processes. *In situ* hybridization with a probe for NTT4 mRNA revealed co-distribution with glutamatergic neurons (Mestikawy et al. 1994; J. M. Luque, G. Richards, F. Jursky and N. Nelson, unpublished results). We also raised antibodies against recombinant fusion proteins corresponding to the N terminus, the C terminus and the first extended loop of the transporter. The first two antibodies are suitable for Western analysis, whereas the antibody against the first extended loop can be used for immunocytochemistry. In Western blot analysis, we detected a single 80kDa protein band in all the main brain regions. In immunocytochemistry, two morphologically different immunoreactivities were observed. One stains neuropils distributed throughout the brain (Fig. 6). This pattern was abundant in cerebral cortex, hippocampus, striatum, ventral palidum, thalamus, the central gray area and cerebellum. The second pattern appeared in pericellular synapses, mainly in the ventral cochlear nucleus, the vestibular nuclei, the trapezoid body and the spinal trigeminal nucleus and spinal cord. Antibody staining was detected exclusively in gray matter, with no detectable presence in cell bodies or processes. Injection of ibotenic acid with subsequent *in situ* hybridization demonstrated the neuronal character of NTT4 (Mestikawy et al. 1994). Since, in the

Fig. 5. Distribution of GAT4 immunoreactivity in rat brain stained with antibody raised against the recombinant loop fusion protein of the rat GAT4 transporter. *cb*, cerebellum; *cx*, cerebral cortex; *ob*, olfactory bulb; *sc*, spinal cord; *th*, thalamus. Scale bar, 2 mm.
hippocampus, strong staining is observed in mossy fiber terminals and most probably in the terminal CA1 field corresponding to the strong in situ hybridization signal in the granule cell layer and the CA3 pyramidal cell layers (Luque et al. 1994), one can conclude that the function of NTT4 is probably presynaptic. Its distribution suggests a function in excitatory neurons, and its differential distribution in the hippocampus indicates a function in long-term potentiation. An interesting feature of the immunoreactivity in the olfactory bulb is the differential labeling in the glomerular layer, which indicates a different state of activity in the individual glomeruli.

**Perspective**

Now that the race to clone genes encoding neurotransmitter transporters is practically over, we can turn to answering the important question concerning the mechanism of action of these transporters. The initial step in this direction is to demonstrate the site of action of the various transporters. In situ hybridization gave some indications of the function of the transporters by localizing their site of synthesis. Studies with specific antibodies should reveal the site of action of the various transporters. By employing electron microscopy, the co-localization of individual transporters with specific receptors can be followed. The mechanism of action of neurotransmitter transporters is very
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complicated. It involves cotransport of substrate, sodium and chloride. The function of ion gradients is not clear and the way in which substrates cross the membrane is not known. Since transporters are not likely to be crystallized, so that their structure can be determined at high resolution by X-ray analysis, two-dimensional crystalline arrays and resolution by electron diffraction may be a more useful way to shed light on their structure. Until this is achieved, rational site-directed mutagenesis should be undertaken. This study will be facilitated by the expression of neurotransmitter transporters in yeast cells. Neurotransmitter transporters may serve as a future target for drugs designed to correct neurological disorders. Some disorders are likely to be linked with mutations in the genes encoding transporters.

References


