REVIEW ARTICLE

Sodium ion-dependent transporters for neurotransmitters: a review of recent developments

D. Margaret WORRALL* and D. Clive WILLIAMS†

*Department of Biochemistry, University College, Dublin 4, and †Department of Biochemistry, Trinity College, Dublin 2, Ireland

INTRODUCTION

Sodium-dependent neurotransmitter uptake systems or transporters are the principal means by which neurotransmitters in the synaptic cleft are inactivated. These uptake systems transport the neurotransmitters across the plasma membrane of neuronal cells and in some cases surrounding glial cells. The exception to this is acetylcholine, which is enzymically inactivated by post-synaptic acetylcholinesterase giving choline, which is then transported by a pre-synaptic high-affinity transporter before re-acetylation. Once inside the cell, neurotransmitters are further transported into synaptic vesicles by apparently unrelated proton-driven transport systems.

The high-affinity plasma-membrane uptake systems are dependent on Na^+ for activity, and some have also shown a requirement for Cl^- and/or K^+. Several neurotransmitter transporters, including those for γ-aminobutyric acid (GABA), noradrenaline, 5-hydroxytryptamine (5-HT), dopamine, glutamate, choline and glycine, have been studied over the years, with attention being paid to their ionic gradient coupling requirements, substrate specificities and inhibitor/modulator specificities and potencies. This latter property has much importance for therapeutic drug design and elucidation of the mechanism of action of interfering substances such as cocaine. Thus GABA uptake inhibitors may be anticonvulsants; noradrenaline and 5-HT uptake inhibitors are antidepressants; and glutamate uptake blockers may influence excitotoxicity.

Recently, progress on the elucidation of the structure of these transporters has been extensive due to cloning, sequencing and model building studies. Most of these transporters are similar in structure and are classified as a gene super-family, although very recent studies have demonstrated an exception to the rule in that the glutamate transporter appears to be derived from a second gene family. These families are different in structure from other plasma-membrane transporters and from the neuronal storage vesicular amine transporter.

Some progress has been made on the elucidation of functional domains on the transporters and on the regulation and mechanisms of action, although these studies of necessity have lagged behind the sequencing work.

This review describes the progress to date in this field, which undoubtedly will become vastly more extensive within the next few years.

THE GABA TRANSPORTER: A PROTOTYPE

GABA is the predominant inhibitory neurotransmitter and is widely distributed in the mammalian brain. Its uptake is catalysed by a Na^+- and Cl^--dependent transporter (reviewed by Kanner, 1983) with a stoichiometry of 2 Na^+ and 1 Cl^- for 1 GABA zwitterion (Radian and Kanner, 1983; Keynan and Kanner, 1988). There is much interest in agents that block GABA uptake for their potential as anticonvulsive drugs (see review by Krogsgaard-Larsen et al., 1987) and in selective inhibitors of either glial or neuronal uptake. GABA uptake inhibitors in theory would be 'cleaner' therapeutic agents than GABA receptor agonists, because inhibition of GABA uptake would occur only when GABA was released physiologically.

Two major subtypes of GABA transport have been identified in rat brain using biochemical studies: the $GABA_A$ uptake system, which is inhibited by diaminobutyric acid or *cis*-3-aminocyclohexanecarboxylate (ACHC) and is thought to be neuronal (Bowery et al., 1976; Neal and Bowery, 1977), and $GABA_B$ transport, which is inhibited by β-alanine (Schon and Kelly, 1975) and is thought to be of glial origin.

The GABA transporter is relatively abundant throughout the nervous system (0.1 % of membrane protein) and was the first transporter to have been purified to apparent homogeneity. This was achieved by Radian et al. (1986), in spite of an absence of high-affinity inhibitors for use in binding assays, affinity chromatography and/or labelling. Instead, a reconstitution assay was used in which detergent-solubilized protein fractions were incorporated into liposomes of asolectin and brain lipids, and then radiolabelled GABA uptake was determined (Radian and Kanner, 1985). An ACHC-sensitive transport protein was purified using ammonium sulphate fractionation, DEAE-cellulose chromatography and wheat germ agglutinin affinity chromatography. A protein of apparent molecular mass of 80 kDa was isolated, and polyclonal antibodies raised against this protein were able to immunoprecipitate GABA transport activity from crude transporter preparations. The 80 kDa band could be decreased to 60 kDa on deglycosylation by endoglycosidase F (Kanner et al., 1989), and to 60 and 66 kDa on treatment with papain; in both cases the protein was found to retain transport activity. Further studies using peptide-specific antibodies to several regions of the transporter combined with limited proteolysis with Pronase suggested that the N- and C-terminal regions, possibly including transmembrane helices 1, 2 and 12, are not required for transport activity (Mabjeesh and Kanner, 1992). More recent studies using site-directed mutagenesis and functional expression suggest that only the N- and C-terminal regions are unessential for activity (see later).

Internal amino acid sequence data were obtained from cyanogen bromide fragments of the purified GABA transporter and used to design oligonucleotide probes to isolate the cDNA encoding the transporter (Guastella et al., 1990). A clone designated GAT-1 had 4054 nucleotides in an open reading frame which could code for a protein of molecular mass 67 kDa and 599 amino acids. Expression of this cDNA in *Xenopus*

Abbreviations used: GABA, γ-aminobutyric acid; 5-HT, 5-hydroxytryptamine (serotonin); ACHC, *cis*-3-aminocyclohexanecarboxylate; NMDA, *N*-methylaspartate.

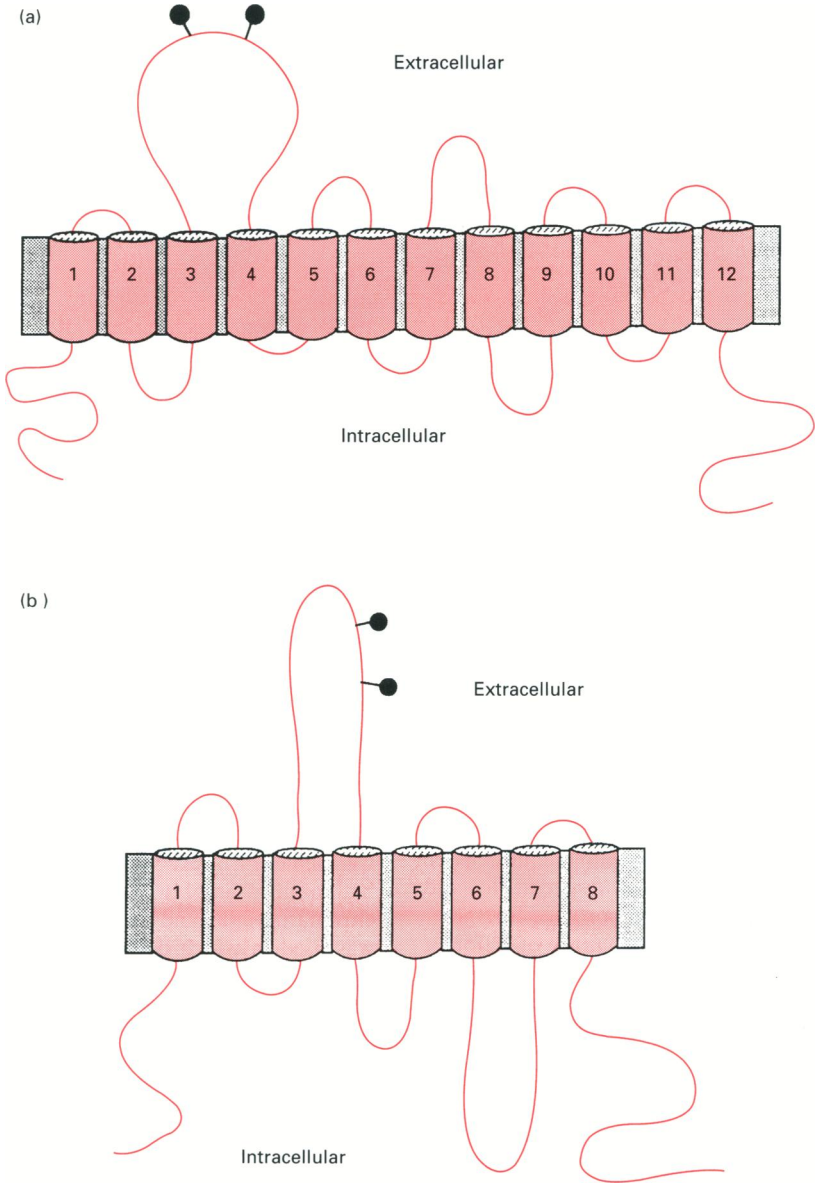

Figure 1 Schematic representation of neurotransmitter transporters

(**a**) Schematic representation of the GABA transporter gene family of proteins. Hydropathicity analysis reveals 12–13 hydrophobic regions of 18–25 amino acids which have been interpreted to form transmembrane domains. The absence of an identifiable signal sequence suggests that the N-terminus is cytoplasmic. The large extracellular loop contains two to four potential N-linked glycosylation sites and there are several potential intracellular phosphorylation sites. (**b**) Schematic representation of the glutamate transporter as depicted by Pines et al. (1992). Kyte–Doolittle analysis predicts eight transmembrane domains, whereas the other cloned glutamate transporters predict six and ten. One similarity with the GABA transporter family is the large extracellular loop between transmembrane regions 3 and 4, which also contains the putative glycosylation sites.

oocytes generated GABA uptake activity with properties similar to the neuronal high-affinity GABA transporter, and the expressed protein could be immunoprecipitated with a polyclonal antiserum raised against the rat brain transporter. The predicted protein contained 12 putative transmembrane regions, three potential glycosylation sites, three potential protein kinase C phosphorylation sites and one potential protein kinase A phosphorylation site. There being no apparent signal sequence and only a moderately hydrophobic N-terminus, the proposed model incorporates cytoplasmically facing N- and C-termini (Figure 1). The human GABA transporter was subsequently cloned and sequenced (Nelson et al., 1990), and found to be almost identical to the rat protein, with just 17 amino acid differences, mainly near the N- (4 residues) and C- (11 residues) termini. Several membrane proteins are predicted to contain 12 transmembrane regions, including the Na^+/ glucose co-transporter as described by Hediger et al. (1987), the passive facilitative glucose transporter and voltage-dependent K^+ channels. However, no overall sequence identity appears to exist between the GABA transporters and these proteins. Subsequent sequencing studies on other neurotransmitter transporters support the concept that they comprise a new super-family of proteins.

Site-directed mutagenesis studies (Pantanowitz et al., 1993) have shown that of the five conserved charged amino acids found in membrane helices, only one of them, arginine-69, is absolutely essential for transport activity. The three other positively charged

amino acids and the single negatively charged glutamate-467 are not essential. Arginine-69 is found in the highly conserved region, amino acids 66–86, which comprises parts of helices 1 and 2.

Using expression studies of deletion mutants of the GAT-1 clone, it has been shown (Bendahan and Kanner, 1993) that transporters truncated at either end of the coding sequence up to the N-terminal side of helix 1 and the C-terminal side of helix 12 are active as transporters.

Reconstitution studies on the GABA transporter have revealed that cholesterol is required for transport activity in synthetic liposomes (Shouffani and Kanner, 1990). In this study cholesterol stimulated transport 20-fold, but optimal reconstituted transport also required one of several phospholipids. The steroid specificity and potency of this cholesterol stimulation suggested a direct interaction of cholesterol with the transporter rather than an indirect effect on membrane properties.

Selective and differential localization of GABA transporters has been shown in GABAergic neurons and glial cells using polyclonal antisera raised against the rat brain GABA transporter (Radian et al., 1990). Immunocytochemical studies localized the transporter to various brain regions including cerebellum, hippocampus, substantia nigra and cerebral cortex. The transporter was co-localized with GABA in the same types of axons and terminals, with some variation in relative contents. Neuronal and glial cell bodies were not stained, but staining was found in glial processes.

The rat brain cDNA clone GAT-1 has been expressed both transiently and stably in mammalian cells (Keynan et al., 1992). This clone expresses a 70 kDa transporter which was concluded to be the neuronal- or A-type transporter, being inhibited by ACHC but not β-alanine, having a similar K_m value for GABA transport, and being Na^+- and Cl^--dependent. Tunicamycin sensitivity of the expression of the transport activity suggested N-linked glycosylation to be important in this respect.

Clark et al. (1992) have recently cloned and expressed a β-alanine-sensitive GABA transporter cDNA from rat midbrain, designated GAT-B. This sequence was 50% identical to the GAT-1 (or GAT-A) transporter and 63% identical to the kidney BGT-1 betaine/GABA transporter (Yamauchi et al., 1992). Surprisingly, *in situ* hybridization studies indicated that this protein is located in the neuronal and not the glial cells. This finding, and a recent report (Saransaari and Oja, 1993) that cerebellar granule cells in culture have a high-affinity Na^+-dependent β-alanine uptake system, suggests that the previous distinction between neuronal and glial GABA transporters based on inhibition studies should be reconsidered.

Liu et al. (1992b) have reported the sequence of genomic and cDNA clones of a mouse brain GABA transporter. The genomic clone contained 12 introns which were in hydrophilic sequences, and thus divided exons for eight of the potential transmembrane helices and for the hydrophilic glycosylated loop between helices 3 and 4. This finding is consistent with the proposed mechanism of 'exon shuffling' for rapid evolution of gene families. Interestingly, one intron located in the highly conserved region between helices 1 and 2 was found to occur at identical positions in five genomic clones and in related sequences in *Drosophila melanogaster* and *Caenorhabditis elegans* proteins, suggesting a specific function in gene organization, RNA processing or differential RNA expression.

An increasing number of GAT clones are being identified and sequenced, which has led to the usual problem of different nomenclatures. Some of these clones are identical, whereas some correspond to distinct gene products. The original clone, GAT-1 (GAT-A), is ACHC-sensitive, whereas GAT-B [nomenclature of Amara (1992)] is β-alanine-sensitive but is neuronal in location and probably corresponds to GAT-3 [nomenclature of Uhl and Hartig (1992)] and GAT-4 [nomenclature of Nelson et al. (1990)]. GAT-2 [nomenclature of Uhl and Hartig (1992)] and GAT-2 and GAT-3 [nomenclature of Liu et al. (1992b)] are also β-alanine-sensitive and similar to the canine betaine transporter.

THE NORADRENALINE TRANSPORTER

The Na^+-dependent noradrenaline transporter is widely distributed throughout the mammalian brain, and is inhibited by cocaine, tricyclic antidepressants (the most potent being desipramine and nortryptiline) and amphetamines (which are alternative substrates). Purification of this protein has proved difficult due to a low abundance of the transporter in mammalian tissue.

An important breakthrough was made by Pacholczyk et al. (1991), who employed an expression cloning strategy to isolate the cDNA for the human noradrenaline transporter. This was achieved by transfecting HeLa cells with pooled clones from a human neuroblastoma cDNA library, and screening the transfected cells for intracellular uptake of a radiolabelled noradrenaline analogue, m-iodobenzylguanidine, with rescreening and subdivision of pools until a single clone was obtained. The cloned cDNA gave an open reading frame of 1851 bases, predicting a protein of 617 amino acids and molecular mass 69 kDa. Hydropathy analysis predicted 12 transmembrane domains, cytoplasmic N- and C-termini and a large extracellular loop containing three potential glycosylation sites. The sequence was found to be very similar to that of the GABA transporter, particularly in the transmembrane regions. The absolute amino acid sequence identity is 48%, increasing to 68% when conservative substitutions are included.

Expression of the cDNA clone in HeLa cells allowed the uptake and pharmacological properties of the transporter to be studied. Noradrenaline uptake was saturable, Na^+-dependent and sensitive to inhibition by tricyclic antidepressants, with specificity and potencies identical to those of the native membrane-bound transporter. The expressed transporter was also inhibited by cocaine with a potency comparable to that expected from *in vitro* transport and binding studies, and by the transporter substrate d-amphetamine. All of the fundamental properties of this uptake system are found coded by this single cDNA species.

A recent report (Brüss et al., 1993) mapped the human noradrenaline transporter to the long arm of chromosome 16 (16q12.2).

THE 5-HT TRANSPORTER

The neurotransmitter 5-HT (serotonin) is thought to play an important role in depression, sleep, obsessive compulsion disorder, bulemia and obesity. In addition to serotonergic neurons, 5-HT is also stored in blood platelets, where through its release it is involved in platelet activation, shape change and aggregation during blood coagulation. In both platelets and neurons 5-HT is transported by a high-affinity uptake system which co-transports Na^+ and Cl^-, and counter-transports K^+ (Rudnick, 1977).

The transporter is specifically inhibited by tricyclic antidepressants, but with a different order of potency from that of the noradrenaline transporter, the most effective inhibitors being imipramine, citalopram and paroxetine. The binding of radiolabelled inhibitors can be used as an effective assay for the 5-HT transporter, and affinity chromatography of the transporter with

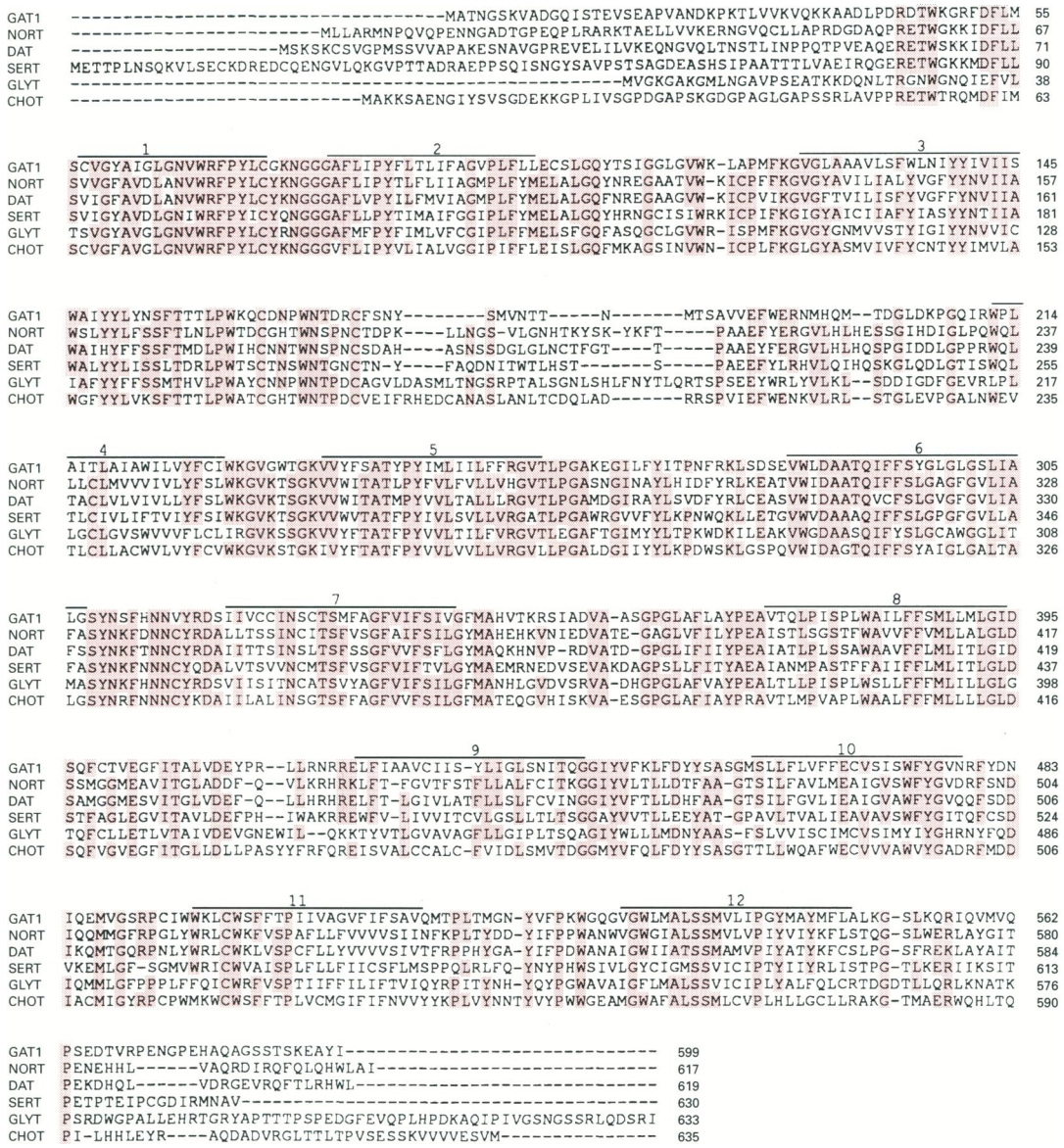

Figure 2 Sequence alignment of the major Na^+- and Cl^--dependent neurotransmitter transporters

Shown are the sequences of the transporters for GABA (GAT) (Guastella et al., 1990), noradrenaline (NORT) (Pacholczyk et al., 1991), dopamine (DAT) (Shimida et al., 1991), 5-HT (SERT) (Mayser et al., 1991), glycine (GLYT) (Guastella et al., 1992) and choline (CHOT) (Mayser et al., 1992). Conserved residues shared by at least four transporters are highlighted in red. Putative transmembrane spanning domains are indicated by bars.

a citalopram derivative has also been developed. A 10000-fold partial purification of the digitonin-solubilized platelet transporter was achieved by Biessen et al. (1990) using wheat germ agglutinin and aminomethylcitalopram affinity chromatography, resulting in SDS/PAGE protein bands of 78 and 55 kDa. The digitonin-solubilized rat cerebral cortex 5-HT transporter was partially purified (2000-fold) using a similar strategy (Graham et al., 1992). More recently, a one-step purification of the transporter from fresh human platelets has been described by Launay et al. (1992), using affinity chromatography with 5-HT or 6-fluorotryptamine as ligands. A single band of 68 kDa was obtained, which exhibited [^{3}H]paroxetine binding and was shown to be a glycoprotein containing two sialic acid residues.

The sequence identity between the GABA and noradrenaline transporters led a number of workers to surmise that other transporters might belong to this gene family, particularly the 5-HT transporter, since it shares some drug-binding specificity with the noradrenaline transporter. Using a degenerative oligonucleotide probe corresponding to the region of greatest identity between the GABA and noradrenaline transporters (residues 66–86 in the former and residues 78–98 in the latter), a cDNA clone encoding the 5-HT transporter was isolated from rat brain by Blakeley et al. (1991a). Simultaneously the expression and cloning of the 5-HT transporter from rat basophilic leukaemia cells was reported by Hoffman et al. (1991). Expression of both the transporter clones in non-neuronal cells was achieved, generating Na^+-dependent 5-HT uptake ability which was sensitive to antidepressants.

The two proteins were identical in core sequence but differed in their N- and C-terminal regions, and Snyder (1991) observed

that these differences might reflect isolated base changes in the sequence data between the two laboratories which resulted in a shift in the reading frame. This has since been confirmed by the two groups and a single cDNA encoding a protein of 630 amino acids (Figure 2) is correct. The sequence of an uncharacterized neurotransmitter cDNA isolated from rat spinal cord by Mayser et al. (1991) is also in agreement with this consensus sequence, and so must be the 5-HT transporter.

As with the previous transporters, hydropathy analysis indicated 12 putative transmembrane regions, but there are only two potential glycosylation sites in the large extracellular loop between transmembrane regions 3 and 4. Comparison of the predicted amino acid sequences with those of the GABA and noradrenaline transporters showed 38% and 47% identity respectively. Blakeley et al. (1991a) also examined the sequence for residues which are shared with the noradrenaline transporter but not found in the GABA transporter in an attempt to identify possible tricyclic antidepressant and cocaine binding domains. No striking areas of similarity were apparent, but the transmembrane regions 5 and 7 contained the most shared residues and an acidic residue (Asp-98 in the 5-HT transporter; Asp-75 in the noradrenaline transporter) is conserved in the monoamine transporters in transmembrane domain 1, which was postulated to be involved in cocaine and substrate binding.

Recently the human brain and human platelet 5-HT transporter cDNAs were isolated and sequenced (Lesch et al., 1993a,b). The 630 amino acids in an open reading frame were consistent with a protein of 70 kDa with 12 transmembrane helices, and with 92% sequence identity to the rat clone. One additional cyclic AMP-dependent protein kinase potential phosphorylation site was found in the N-terminal cytoplasmic region. The protein kinase C phosphorylation site in the rat sequence was not conserved in the human sequence. Another human 5-HT transporter cDNA from a choriocarcinoma cell line was cloned and expressed in HeLa cells yielding a high-affinity Na^+- and Cl^--dependent 5-HT transport activity with the correct pharmacology, being blocked by selective 5-HT uptake inhibitors and by cocaine and amphetamines. Once again an open reading frame predicted a 630-amino-acid protein with 92% identity to the rat brain transporter, with 12 transmembrane helices and conserved sites for post-translational modification (Ramamoorthy et al., 1993). Multiple hybridizing RNAs were observed in lung and placental tissues. A single gene was consistent with somatic cell hybrid, and *in situ* hybridization studies suggested a chromosomal location on chromosome 17q11.1–17q12.

Long-term administration to rats of 5-HT-uptake-inhibitor-based antidepressants, but not of monoamine oxidase inhibitors or 5-HT receptor agonists, has been reported (Lesch et al., 1993c) to decrease 5-HT transporter mRNA steady-state concentrations in the brain. This regulatory effect at the level of gene expression may have relevance to the neuroadaptive mechanisms which may be the basis of uptake-inhibitor-based therapy for depression.

THE DOPAMINE TRANSPORTER

Dopaminergic neurotransmission plays an important role in control of movement, hormone release and in disorders such as Parkinson's disease and schizophrenia. Dopamine is inactivated by re-uptake into the presynaptic terminal via a Na^+- and Cl^--dependent transporter which appears to be the most important site for the behavioural effects of amphetamines and cocaine (Iversen, 1976; Kuhar and Zarbin, 1978; Ritz et al., 1987; Bergman et al., 1989; Horn, 1990). Dopamine uptake systems selectively accumulate the *N*-methyl-4-phenylpyridine (MPP^+) metabolite of the neurotoxin *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Javitch and Snyder, 1985), and a number of drugs/ligands have been found to inhibit dopamine uptake, including mazindol, nomifensine and cocaine. In striatal membrane preparations these compounds bind both low- and high-affinity sites (see Boja et al., 1992), which could reflect either different transporter species or separate binding sites or conformational states in the one protein. Photoaffinity labelling of the transporter was achieved by Sallee et al. (1989) using a derivative of GBR-12909, [^{125}I]FAPP {1-(2-[bis-(4-fluorophenyl)-methoxy]ethyl)-4-(2-[4-azido-3-iodophenyl]ethyl)piperazine}. A polypeptide of an apparent molecular mass 62 kDa was visualized following SDS/PAGE, which could be reduced to 48 kDa on treatment with endoglycosidase F.

As in the case of the 5-HT transporter, the identity between the GABA and noradrenaline transporters was utilized to isolate the cDNA clone for the dopamine transporter. Two groups simultaneously published identical sequences for the rat brain transporter (Kilty et al., 1991; Shimada et al., 1991) and the cloning and sequencing of the bovine brain protein was subsequently published (Usdin et al., 1991). The hydrophobicity profile of both proteins is again virtually superimposable with those of the other transporters. The rat brain clone encoded a protein of 619 amino acids (69 kDa) with four putative N-linked glycosylation sites in the large extracellular loop, and three potential protein kinase C phosphorylation sites (Kilty et al., 1991). Expression of the rat brain cDNA in both COS and HeLa cells was achieved, generating cocaine-sensitive dopamine transport activity. In transfected COS cells, two cocaine binding sites were conferred from a single cDNA (Boja et al., 1992), suggesting that a single transporter species codes for both low- and high-affinity binding sites.

The bovine brain dopamine transporter cDNA predicts a protein of 693 amino acids (75 kDa) which has a considerably longer C-terminus than the rat brain protein but is highly similar otherwise. In this protein three potential glycosylation sites are present in the large extracellular loop and several possible protein kinase C phosphorylation sites are present. There are also two potential cyclic AMP-dependent protein kinase A sites on intracellular loops near the C-terminus, neither of which are present in the rat brain protein.

The human dopamine transporter has also recently been cloned and sequenced (Vandenbergh et al., 1992). The human transporter shows greater than 95% amino acid identity to the rat protein and has one less N-linked glycosylation site, and the gene was found on chromosome 5p15.3. Bannon et al. (1992) have reported a large down-regulation of dopamine transporter mRNA with human aging.

Site-directed mutagenesis of the dopamine transporter has recently been carried out by Kitayama et al. (1992), who targeted amino acids analogous to those known to have functional roles in catecholamine receptor proteins. Asp-79 (helix 1), which is shared by the other biogenic amine transporters, was substituted by Ala, Gly or Glu, and a decrease in both transport and cocaine analogue binding was observed. Both Ser-356 and Ser-359 substitutions (to Ala and Gly) in transmembrane helix 7 resulted in reduced transport but had less effect on the cocaine analogue binding.

Recently, Vaughan et al. (1993) verified the cDNA sequence as a brain dopamine transporter by raising anti-peptide antibodies from 14 different regions of the dopamine transporter, and showed that four of these antisera caused immunoprecipitation of striatal transporter photolabelled with 1-[2-(diphenylmethoxy)ethyl]-4-[2-(4-azido-3-[^{125}I]iodophenyl)]-piperazine

Table 1 Properties of transporter proteins that have been cloned and sequenced

Transport	Tissue	Amino acids	Molecular mass (kDa)	Glycosylation sites	References
GABA					
GAT-1	Rat brain	599	67.0	3	Guastella et al. (1990)
	Human brain	599	67.0	3	Nelson et al. (1990)
GAT B	Rat brain	627	70.0	3	Clark et al. (1992)
NAT	Human brain	617	69.0	3	Pacholczyk et al. (1991)
5-HT	Rat brain	630	70.17	2	Blakeley et al. (1991a)
	RBL cells	630	70.17	2	Hoffman et al. (1991)
	Rat spinal cord	630	70.17	2	Mayser et al. (1991)
	Human placenta	630	70.32	2	Ramamoorthy et al. (1993)
	Human brain	630	70.32	2	Lesch et al. (1993)
Dopamine	Rat brain	619	69.0	4	Shimada et al. (1991)
	Bovine brain	693	75.0	3	Usdin et al. (1991)
Choline	Rat spinal cord	635	70.63	2	Mayser et al. (1992)
Glycine	Rat brain	633	70.0	4	Guastella et al. (1992)
Taurine	Rat brain	621	70.0	2	Smith et al. (1992)
	MDCK cells	655	72.0	2	Uchida et al. (1992)
Glutamate	Rat brain	573	64.0	2	Pines et al. (1992)
	Rat brain	543	60.0	2	Storck et al. (1992)
	Rabbit intestine	524	57.0	2	Kanai and Hediger (1992)

(DEEP). The antisera recognized the transporter from three species but were tissue-specific.

THE CHOLINE TRANSPORTER

The neurotransmitter acetylcholine is inactivated by the action of acetylcholinesterase, which cleaves it to produce acetate and choline (see Silver, 1974). However, the rate-limiting step in the synthesis of acetylcholine appears to be the transport of choline into cholinergic neurones (Kuhar and Murrin, 1978; Tucek, 1978) which occurs via a specific high-affinity transporter coupled to an electrochemical sodium ion gradient. Choline transport activity has been solubilized from synaptosomal membranes and incorporated into synthetic liposomes (King and Marchbanks, 1980), and this activity is blocked by hemicholinium-3, which is a specific inhibitor of choline transport. Monoclonal antibodies have also been isolated which block the high-affinity uptake of choline (Knipper et al., 1989). Using a reconstitution assay for transport activity, extensive purification of the choline transporter has been achieved by Mono-Q anion-exchange chromatography followed by immunoaffinity chromatography with this monoclonal antibody (Knipper et al., 1991). One major glycoprotein of apparent molecular mass 90 kDa was visualized on SDS/PAGE which gave an isoelectric point at pH 4.7. This molecular mass is consistent with earlier reports of a 90 kDa protein on photoaffinity labelling and Western blotting (Knipper et al., 1989), although Rylett (1988) reports a polypeptide of 42 kDa on affinity labelling with [^{3}H]choline mustard. The purified 90 kDa protein (Knipper et al., 1991) required Na^+ in order to accumulate choline into synthetic liposomes, could be inhibited by hemicholinium-3, and treatment with endoglycosidase F reduced the apparent molecular mass to 65 kDa.

These properties and molecular mass are consistent with those of the previously described transporters, and indeed the subsequent cloning and sequencing of the rat spinal cord choline transporter has shown it to be a member of the same gene family (Mayser et al., 1992). The cDNA encodes for a protein of 635 amino acids with two potential glycosylation sites, and the transcribed mRNA generated Na^+-dependent choline uptake when expressed in *Xenopus* oocytes. Interestingly, this protein is more similar to the kidney betaine transporter described below (51% identity) than to the other known neurotransmitter transporters (37–49% identity).

THE GLYCINE TRANSPORTER

Glycine is an inhibitory neurotransmitter in the brain and spinal cord of vertebrates, the activity of which is terminated via high-affinity uptake into presynaptic terminals and neighbouring glial cells (see references cited in López-Corcuera et al., 1989). Glycine also modulates excitatory neurotransmission by regulating the activity of glutamate at *N*-methylaspartate (NMDA) receptors (Johnson and Ascher, 1987). The glycine transporter is dependent on the co-transport of Na^+ and Cl^- (Mayor et al., 1981), with an estimated stoichiometry of 2 Na^+: 1 Cl^-: 1 glycine (Aragón et al., 1987). Depolarization studies on the transporter have also led to speculation that this protein could be involved in the release of glycine (Aragón and Gimenez, 1986). There are no high-affinity ligands or inhibitors for the glycine transporter, but an assay involving reconstitution into synthetic liposomes has been developed (López-Corcuera and Aragón, 1989) and used to partially purify the transporter. The protein was solubilized with 2% cholate and purified 6–7-fold on wheat germ agglutinin affinity chromatography (López-Corcuera et al., 1989).

The rat brain glycine transporter has been cloned and sequenced by Smith et al. (1992) and by Guastella et al. (1992), and belongs to the GABA transporter gene family, or superfamily as it has now become. There is a difference in the length and sequence of the first 10–14 amino acids of the N-terminus between these two clones, which are otherwise identical. Unlike the case of the 5-HT transporter, this difference cannot be accounted for by single base alterations or a reading frame shift, and it remains to be seen whether a single transporter species accounts for both clones. Expression of Na^+- and Cl^--dependent glycine transport activity was achieved by both groups in spinal cord and brain stem regions in which glycine is a putative neurotransmitter. *In situ* hybridization studies by Smith et al. (1992) revealed co-localization of the transporter with NMDA receptors in the pyramidal layer of the hippocampus where glycine receptors are absent, thus supporting a regulatory role for glycine at these synapses.

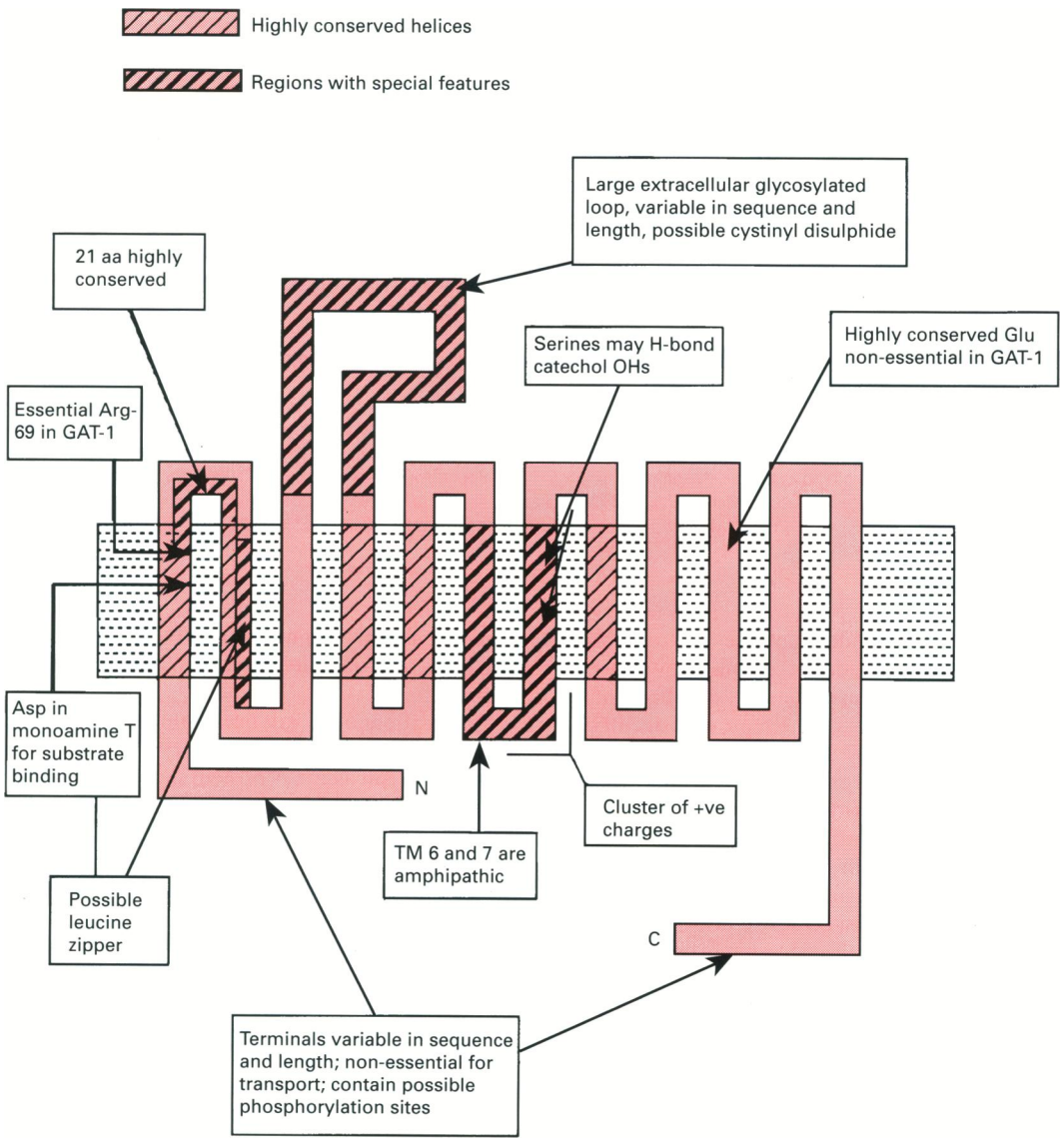

Figure 3 Common and essential features of the twelve-transmembrane-helix family

THE TAURINE TRANSPORTER

Taurine is a sulphated analogue of β-alanine and is a putative neurotransmitter, the exact role of which has not yet been determined. High-affinity Na^+-dependent uptake of taurine has been demonstrated and appears to be closely linked with GABA uptake, as each compound will competitively inhibit the uptake of the other (Miyamoto et al., 1991). However, Sivakami et al. (1992) characterized the uptake of both GABA and taurine in bovine retinal pigment epithelium cells and concluded that a separate taurine transporter exists which has a much lower affinity for GABA than the GABA transporter. Three groups have cloned cDNA species for the taurine transporter from MDCK cells (Uchida et al., 1992), rat brain (Smith et al., 1992) and mouse brain (Liu et al., 1992a) using probes based on the now established transporter sequence similarities. As might be expected, the taurine transporter sequence is most similar to that of the β-alanine-sensitive GABA transporter (62% identity), and it shows 52% identity with the neuronal GABA transporter.

The mouse brain clone predicts 590 amino acids with the now established 12 transmembrane helix motif and sequence identity. The amino acid content suggests an acidic protein of pI 5.98, which is suggested to be much lower than that of other cloned neurotransmitter transporters (pI ~8.0). However, at a protein level, the purified choline transporter (pI 4.7) and the 5-HT transporter (pI 5.6–6.2) have been shown to have low pI values. This clone, when expressed in *Xenopus* oocytes, showed uptake of taurine (K_m 4.5 μM) and β-alanine (K_m 56 μM). A single transcript was found enriched in kidney, and *in situ* hybridization showed specific localization to the corpus callosum, striatum and anterior commissure in brain, suggesting a potential role for these amino acids as neurotransmitters or osmoregulators depending on tissue expression.

THE L-PROLINE TRANSPORTER

Proline has not previously been classified as an amino acid neurotransmitter, although it is released from brain slices in a Ca^{2+}-dependent manner following depolarization (Balcar et al.,

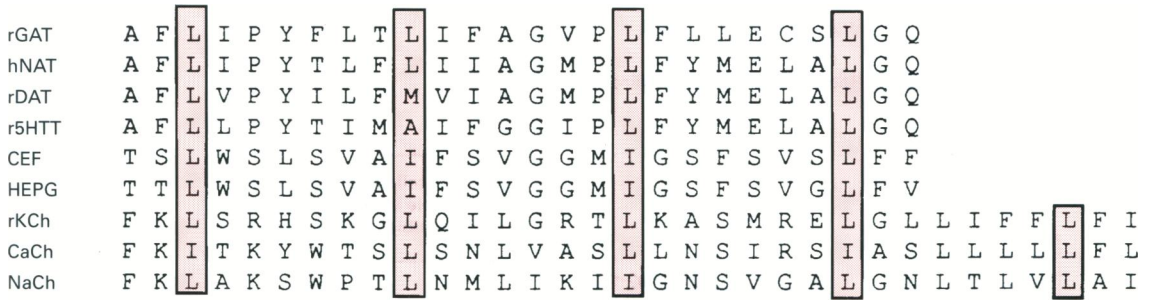

Figure 4 Comparison of the proposed leucine zipper motif between the neurotransmitter transporter proteins, facilitated glucose transporter proteins (CEF and HEPG), and K^+, Na^+ and Ca^{2+} channel proteins

Interestingly, the Na^+-dependent glucose transporter does not contain any leucine zipper motif. This site is present in the second transmembrane domain of all the transporter proteins and adjacent to the S4 region in the relevant subunit of the ion channel proteins. Note that there is also a highly conserved glycine residue between the second and third leucines in the transporter sequences and the K^+ channel sequence. This motif has been proposed as a possible means of dimerization of the transporters (White and Weber, 1989). However, these hydrophobic transmembrane domains naturally have a high content of leucine and isoleucine, which may undermine the significance of such a motif. Also, the second leucine/isoleucine is not present in the 5-HT, dopamine or choline transporter sequences. Abbreviations: rGAT, rat GABA transporter; hNAT, human noradrenaline transporter; rDAT, rat dopamine transporter; r5HTT, rat 5-HT transporter; rKCh, CaCh and NaCh, rat K^+, Na^+ and Ca^{2+} channel proteins respectively.

1976), and high-affinity Na^+-dependent uptake has also been described (Bennett et al., 1972). Based on the homology between the GABA and noradrenaline transporters, a specific proline transporter cDNA has also been isolated (Fremeau et al., 1992). The cDNA predicts a protein of 637 amino acids with 44–45% identity with other transporters, and expression of proline uptake in HeLa cells was achieved. Hybridization studies showed a 4.0 kb RNA species which is concentrated in subpopulations of putative glutamatergic neurones. In view of such clear localization of this transporter, Fremeau et al. (1992) have postulated a synaptic role for L-proline in these specific excitatory pathways. The proline transporter has been localized to human chromosome 5q32–33.

THE BETAINE TRANSPORTER

Betaine is a non-perturbing osmolyte which protects cells such as those in marine organisms and mammalian kidney from high extracellular electrolyte concentrations. Accumulation of betaine is Na^+-dependent, and the cloning and sequencing of this protein has revealed it to be a member of the neurotransmitter transporter family (Yamauchi et al., 1992). The expressed protein was Na^+- and Cl^--dependent, could transport betaine and GABA, and had a low affinity for proline. Northern hybridization studies showed high levels of RNA in kidney medulla, but no transcripts were detected in several other tissues, including brain. A high level of expression in renal cells was dependent on osmolarity. There are reports, as yet unpublished, of brain betaine transporter cDNA, suggesting that this transporter might play different roles with the two different substrates in brain and kidney.

The properties of the transport proteins discussed in the previous sections are summarized in Table 1.

GLUTAMATE TRANSPORTERS: A NEW GENE FAMILY

Glutamate is the major excitatory neurotransmitter in the brain, and both high- and low-affinity uptake of glutamate occurs in neuronal, glial and peripheral tissues. The high-affinity transporter will also transport aspartate, and is dependent on Na^+, but unlike the previously described transporters it does not require Cl^- ions. The glutamate transporter protein has a vital physiological role in limiting the neurotoxic effects of high synaptic glutamate concentrations. Such effects have been implicated in epilepsy, brain ischaemia and amyotrophic lateral sclerosis, where glutamate transport is impaired (Rothstein et al., 1992).

Purification of a rat brain glutamate transporter has been achieved by Danbolt et al. (1990), using reconstitution of transport as an assay. A protein showing a major band of 80 kDa on electrophoresis was isolated by ion-exchange, hydroxyapatite and lectin chromatography. A polyclonal antibody was subsequently raised against this transporter and immunochemical staining demonstrated that the protein is located in glial processes (Danbolt et al., 1992).

A peptide-specific monoclonal antibody (9C4) was used (Levy et al., 1993) to show that the apparent heterogeneity in molecular mass indicated by wide bands on electrophoresis is due to a single antigenic epitope identified as residing within the peptide TQSVYDDTKNHRESNSNQC (residues 518–536; C-terminal to transmembrane helices in all subsequent models).

Three groups have recently published distinct sequences for glutamate transporters using different approaches. Pines et al. (1992) used the antibody which blocked glial glutamate transport (Danbolt et al., 1992) to screen a rat brain cDNA library, and succeeded in isolating and expressing a glutamate transporter cDNA. This was designated GLT-1 and encoded a protein of 573 amino acids with eight predicted transmembrane regions using Kyte–Doolittle analysis (Figure 1b) (Kyte and Doolittle, 1982).

Kanai and Hediger (1992) isolated a clone from rabbit small intestine by expression cloning in *Xenopus* oocytes. This glutamate transporter was named EAAC1 and the predicted protein is 524 amino acids in length and 57 kDa in molecular mass. Sequence analysis using the Eisenberg algorithm predicts 10 transmembrane regions (Eisenberg et al., 1984), but due to a large hydrophobic stretch near the C-terminus (residues 357–444), Kanai and Hediger (1992) concede the possibility of alternative structures.

The third group of Storck et al. (1992) isolated a cDNA encoding a protein which co-purified with galactosyltransferase and which demonstrated glutamate and aspartate high-affinity uptake upon expression in *Xenopus* oocytes. This transporter was named GLAST 1, and encoded a protein of 543 amino acids, which predicted a six transmembrane domain structure on hydrophobicity analysis.

The internal identity between the three proteins is approximately 50% and the sequences predict many similar structural

characteristics, although they differ somewhat in hydrophobicity plots. The location of the first six transmembrane helices is agreed for all three proteins but the hydrophobicity analyses then differ towards the C-terminal half, with no (GLAST 1), two (GLT-1) or four (EAAC1) additional predicted helices. The three proteins all contain two putative extracytosolic glycosylation sites between transmembrane regions 3 and 4, but show no sequence identity with the 12-transmembrane-domain GABA gene family. However, significant identity was found with the *Escherichia coli* GltP proton glutamate transporter (30%) and with the DctA dicarboxylate transporter from *Rhizobium meliloti* (27%). In particular the sequence AAIFIAQ is highly conserved, although predicted to be either transmembrane or C-terminal and intracellular in the various glutamate transporter models, and may have some functional significance (Pines et al., 1992).

Amara (1992) has suggested that, in view of the doubt and variability existing in the numbers of transmembrane regions (11–13 for the GABA family and 6–10 for the glutamate transporters), it might be more appropriate to classify the gene families according to their ion dependency, i.e. Cl^--dependent or -independent.

A recent report describes the first direct demonstration of the regulation of neurotransmitter transporter by phosphorylation, namely phosphorylation of the purified pig brain L-glutamate transporter by protein kinase C, predominantly at serine residues (Casado et al., 1994). C6 glioma exposed to phorbol ester showed a 2-fold stimulation of glutamate transport with a concomitant increase in phosphorylation. Phorbol ester also stimulated glutamate transport in pT7-GLT-1-transfected HeLa cells. However, mutation of serine-113 to asparagine, although not affecting levels of expression, abolishes stimulation by phorbol ester.

GENERAL CONSIDERATIONS AND CONCLUSIONS

Until late 1992 we would have concluded that there was evidence for only one gene super-family of Na^+-dependent transporters, based on predicted amino-acid sequence similarities. However, the recent work on glutamate transporters now supports at least two families, one family with 12 putative transmembrane regions and one with 6–10 transmembrane regions. Features of the 12-transmembrane-region receptor family are described in Figure 3.

Membrane GABA-T family: domains and functional groups

Hydropathy plot analyses suggest that for the GABA-T family the protein is folded into 12 transmembrane helices, with both N- and C-terminal extremities being cytoplasmic, and with a large extracellular domain between transmembrane helices 3 and 4. It was possible to sub-divide the super-family into two major subtypes, the cousins being grouped into the monoamine substrate family and the amino acid substrate family on the basis of not so much sequence identity but rather the presence of specific amino acid residues such as aspartate or arginine in transmembrane domain 1.

The use of site-directed mutagenesis to study the functional domains of the cloned transporter proteins is likely to be the next explosion in this area. However, in order to confidently assign functional roles to individual amino acids, it will be important to verify levels of expression and membrane incorporation and topography of mutant proteins, e.g. using peptide-specific antibodies raised against various extracellular parts of the molecule. It will be especially important to experimentally verify the 12-transmembrane-helix motif by protein studies. The recent elucidation (Unwin, 1993) of the nicotinic acetylcholine receptor secondary structure at 9 Å (0.9 nm) resolution using electron microscopy of tubular crystals showed that the subunit secondary structure appeared as a single transmembrane helix, thus allowing a pentameric structure with five helices lining an ion channel surrounded on the lipid-facing side probably by β-sheets made up from the remainder of the bilayer-spanning structures. This contrasts greatly with the model subunit structures from hydropathy analyses predicted as having originally five helices and then subsequently four helices spanning the membranes.

Studies on establishing correct structures, confirming the model folding patterns predicted from hydropathy profiles, lag well behind the cloning, sequencing and expression studies. Little evidence has been presented using topological probes such as chemical modification or sequence-specific antibodies. This probably reflects the relatively low abundance of most of these transporters and in some cases their instability in isolates. The ease with which the structure of expressed transporter cDNAs can be manipulated by deletion, mutation or chimera formation will undoubtedly lead to a massive increase in information on structure essential for function.

For the monoamine transporters, site-directed mutagenesis has shown an aspartate in transmembrane helix 1 (Asp-79 in the dopamine transporter, Asp-75 in the noradrenaline transporter, Asp-98 in the 5-HT transporter) and two serines (356 and 389 in the dopamine transporter) in transmembrane helix 7 to be essential or important for, e.g., dopamine uptake (Kitayama et al., 1992), and has led to the suggestion that these amines could bind through these residues, the negative group binding to the protonated amine and the serine hydroxy group hydrogen bonding to the catechol moiety of the substrates. These residues are conserved in all monoamine transporters but not in GABA and betaine transporters (e.g. Asp-79 → Gly-63). Transmembrane helices 6 and 8 in these transporters also contain high levels of serine and threonine residues and thus this region could be highly involved in catechol moiety binding. It is thus also possible that the tricyclic antidepressants could bind through their terminal amine to this negatively charged residue in transmembrane helix 1. For the dopamine transporter, Asp-79 was essential for cocaine binding whereas the serines in transmembrane helix 7 appeared less essential in this respect.

In the case of the GABA transporter, Arg-69 was found to be essential for activity (Pantanowitz et al., 1993), a result which seems to be in conflict with the earlier proteolytic experiments of Mabjeesh and Kanner (1992) which suggested that transmembrane helices 1, 2 and 12 were not necessary for activity. It is possible that this residue or indeed this helix is important for correct membrane organization of the protein rather than for activity *per se*.

No positive identification of the ion-binding sites has yet emerged, Kanner's group having shown (Pantanowitz et al., 1993) that the single negatively charged glutamate-467 (in GAT-1) was not essential for activity; nor were the four positively charged amino acids found in membrane helices.

The large predicted extracellular region between transmembrane regions 3 and 4 is common to all of these Na^+-coupled transporters and has two or three predicted N-linked glycosylation sites. Its sequence varies greatly between transporters and this may suggest an important role in substrate binding. Removal of carbohydrate moieties has been reported to reduce transport of both dopamine (Zalecka and Erecinska, 1987) and 5-HT (Launay et al., 1992). The tunicamycin sensitivity of expression of the GAT-1 clone also supports a role for glycosylation state in transport (Keynan et al., 1992).

The two highly conserved cysteines that are found nine residues apart in this extracellular domain in all of these transporters might form an intracellular disulphide bond as found in some

neurotransmitter receptors. This would obviously be a major constraint on flexibility of tertiary structure in this extracellular loop. However, in the case of the 5-HT transporter, treatment with thiol-reducing reagents increased both 5-HT transport and imipramine binding suggesting that a disulphide, if present, is not essential for activity (Biassoni and Vaccari, 1985; Biessen et al., 1988).

Amara and Kuhar (1993) have commented on the distribution of proline residues in transporter proteins, there being conserved proline residues in five of the twelve transmembrane helices, and on their suggested roles of (1) induction of conformational change by realigning amphipathic helices after *cis–trans* isomerization of proline-containing peptide bonds, (2) binding pocket formation due to bending of transmembrane helices, and (3) hydrogen bonding through the more negative nature of carbonyl groups in proline-containing peptide bonds.

Monomers or oligomers?

Cloning studies have as yet produced no evidence for heterologous subunits for transporters and it is reasonably assumed that the transporters are either monomeric or homo-oligomeric in their native state. As yet no evidence has been produced for regulatory/accessory proteins being associated with these transporters. Some transporters show large apparent molecular sizes after detergent solubilization, e.g. 5-HT transporters have been reported to have an apparent molecular mass of 300–500 kDa (Talvenheimo and Rudnick, 1980; O'Riordan et al., 1986; Graham et al., 1992) although irradiation-inactivation studies have given a target size of 67–86 kDa (Mellerup et al., 1985; Plenge et al., 1990). Multiple sites for two ligands have been reported on transporters, e.g. 5-HT transporters having two sites for 5-HT and two sites for tricyclic antidepressants (Langer et al., 1983; Phillips and Williams, 1984; Wennogle and Meyerson, 1983, 1985; Humphreys et al., 1988) even in the solubilized state (O'Riordan et al., 1990), suggesting an oligomeric structure. There are several reports of aggregated complexes on SDS/PAGE for GABA, 5-HT and glutamate transporters (Radian et al., 1986; Kanner et al., 1993) but of course these may be artefactual. By far the most intriguing suggestion is that these transporters contain a 'leucine-zipper' structure in the second transmembrane helix. However, this structure is not present in all members of the family (Figure 4) and is perhaps overemphasized considering the high incidence of leucine and isoleucine in membrane proteins. Nonetheless, this region of the molecule is an obvious target for site-directed mutagenesis. Expression of the cDNA for a single polypeptide conferring transport activity suggests that the transporter is either monomeric or homo-oligomeric, and this seems a reasonable conclusion in the absence of any highly homologous cDNAs from cloning work.

Little work has yet been done on the lipid requirements for transporter function or stabilization. In the case of the GABA and glutamate transporters a highly specific and potent requirement for cholesterol has been demonstrated (Shouffani and Kanner, 1990). Whether this is true of the other family members is as yet unknown. In view of the differences between glutamate and GABA transporter structures this similarity in lipid requirement would seem to be an important feature worthy of further study.

Regulation: post-translational modification

Regulation of transporter activity or stability is obviously of importance in determining transport abnormalities, drug interference with transport and adaptation of transport activity. The extent of transporter regulation by phosphorylation is as yet undetermined. The members of the transporter family have potential sites for phosphorylation by protein kinase C and in some cases by protein kinase A. No general principles or common localization of phosphorylation sites and their specificity have as yet been established. There have been several reports of phosphorylation-dependent regulation of neurotransmitter uptake, but whether this is due to direct phosphorylation of the transporter proteins is as yet unknown, with the exception of the report by Casado et al. (1994) of phosphorylation of the glial glutamate transporter at serines causing an increase in glutamate transport. Casado et al. (1991) have also reported that phorbol esters increase glutamate transport into cultured rat brain cortical neurons but not glial cells. Anderson and Horne (1992) have reported that protein kinase C activators decrease 5-HT transport into human blood platelets. Cool et al. (1991) have reported a cyclic AMP-dependent activation of 5-HT transport in a human placental choriocarcinoma cell line. Kadowaki et al. (1990) reported cyclic AMP-dependent stimulation of dopamine transport in hypothalamic primary cultures.

Biotechnological implications

Obviously there is and will be more interest from the pharmaceutical industry in elucidating the structures and substructures (such as drug binding sites) of these neurotransmitter transporters, their mechanism(s) of transport and their sub-types for the design of selective drugs which target these proteins and for the elimination of side effects mediated through them. The elucidation of the regulation of transporter function and expression will be of great importance in intervention strategies for therapy of several syndromes which have their basis in abnormal neurotransmission. 5-HT transporters may be important targets for antidepressants, anti-obesity drugs and therapy of compulsive-aggressive behaviour. Reversal of 5-HT transport has been implicated (Bourgoin et al., 1988) in the anti-nociceptive actions of the peptide calcitonin and thus may play a role in the treatment of pain in cancer and migraine. The amphetamines, including the neurotoxin 3,4-methylenedioxymethamphetamine (MDMA; 'Ecstasy') have been shown to cause Ca^{2+}-independent 5-HT release by reversal of the 5-HT transporter (Rudnick and Wall, 1992). Glutamate transport may be a potential target for minimization of excitotoxicity in ischaemia, epilepsy and neurodegenerative diseases such as Huntingdon's chorea. However, the opposite may also be true in that the electrogenic nature of glutamate transport suggests that in depolarized states neurons might reverse glutamate uptake to Ca^{2+}-independent release via these transporters, contributing to more cell toxicity. Selective inhibition of glial GABA uptake over neuronal GABA uptake may result in better anticonvulsant drug design. Elucidation of the binding sites on transporters for drugs such as cocaine and the neurotoxic metabolite MPP^+ may establish not only cleaner therapeutics but also help elimination or therapy of addiction in some cases. The use of stably transfected cell lines containing transporter genes could allow rapid inexpensive selective screening methods *in vitro* for identification both of toxic compounds and of new pharmaceutical agents.

Mechanisms of transport: the next step?

Little work has as yet been performed on the transport mechanism(s) of neurotransmitters beyond establishing the substrate specificities, ion specificities and dependence of transport, and identifying alternative substrates and inhibitors. Undoubtedly the structural information being derived from and predicted by

the molecular biological studies will lead to studies which establish whether the transport mechanism(s) involve substrate channelling, step-wise transfer of substrate using several amino acid residues, and the nature of protein conformational changes which may mediate transfer. The design of peptides which can be tested either as neurotransmitter-selective transporting agents or using anti-peptide antisera for selective blockade of substrate, inhibitor or ion binding and/or transport is now feasible as we know the predicted amino acid sequences of several of the transporters.

In 4 years our understanding of the structure of the plasma-membrane Na^+-coupled neurotransmitter transporters has greatly increased. However, major questions such as structure–function properties, sub-types, mechanisms of transport and regulation of transport remain to be answered.

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