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REVIEW

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The role of neurotransporters in excitotoxicity, neuronal cell death, and other neurodegenerative processes

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Abstract Neurotransporters are high-affinity transport proteins located in the plasma membrane of both presynaptic nerve and glial cells that mediate the removal of neurotransmitters from the synaptic cleft or represent intracellular transport systems that concentrate neurotransmitters in synaptic vesicles. They comprise three subgroups, Na^+/Cl^-- or Na^+/K^+-dependent cell surface transporters and H^+-dependent transporters associated with synaptic vesicles. The new insights into neurotransporter diversity provide the means for novel approaches of studying neurotransmitter uptake processes at the molecular level, such as substrate translocation, antagonist binding, and regulation of gene expression, intracellular trafficking, and posttranslational modification. Moreover, modeling neurotransporter-related disorders and therapeutic strategies in genetically engineered animals are now feasible research strategies. Through an improved understanding of the modulation of neurotransporter function in the brain it may be possible to identify the molecular factors underlying the etiopathogenesis and pathophysiology of neurodegenerative disorders. Due to their specificity for distinct neuronal systems neurotransporters and their genes are potential targets for novel therapeutic strategies.

Key words Neurotransmitter uptake mechanisms · Neurotransporters · Serotonin transporter · Molecular structure · Genomic organization · Candidate genes · Neurotoxins · Neurodegenerative disorders

Abbreviations *ChAT* Choline acetyltransferase · *CRE* cAMP response element · *DAT* Dopamine transporter · *GABA* γ-Aminobutyric acid · *GluT* Glutamate transporter · *5-HT* 5-Hydroxytryptamine · *5-HTT* 5-HT transporter · *ILPR* Insulin gene linked polymorphic region · *MDMA* 3,4-Methylenedioxymethamphetamine · *MPP^+* 1-Methyl-4-phenylpyridine ion · *MPTP* 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine · *PD* Parkinson's disease · *TBZOH* Dihydrotetrabenazine · *VAChT* Vesicular ACh transporter · *VMT* Vesicular monoamine transporter

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Introduction

High-affinity transport proteins (neurotransporters) located in the plasma membrane of both presynaptic nerve and glial cells mediate the removal of the neurotransmitter from the synaptic cleft, thereby terminating its action at pre- and postsynaptic receptors and fine-tuning the temporal aspects of neuronal communication. Intracellular transport systems facilitate the reaccumulation of neurotransmitters in synaptic vesicles for additional cycles of release. At both plasma and vesicular membranes neurotransmitter influx is coupled directly to transmembrane ion gradients that provide the energy for transport against a concentration gradient. On the basis of their molecular structures, subcellular distributions, and pharmacological properties neurotransporters have been divided into three main subgroups, Na^+/Cl^-- or Na^+/K^+-dependent cell surface transporters (e.g., serotonin, dopamine, or glutamate transporters) and H^+-dependent transporters associated with synaptic vesicles (e.g., vesicular monoamine and acetylcholine transporters; Fig. 1). The new insights into neurotransporter diversity provide the means for novel approaches of studying neurotransmitter uptake processes at the molecular level. Current research strategies are focusing on functional mechanisms of substrate translocation and inhibitor binding, molecular regulation of neurotransporter gene expression, and posttranslational modification of the neurotransporter protein. Important information will also be derived from the analysis of genomic regulatory elements, modeling neurotransporter-related disorders, and novel therapeutic strategies in genetically engineered animals. Through an improved understanding of the modulation of neurotransporters function in the brain it may be possible to identify the molecular factors underlying the etiopathogenesis of neurodegenerative disorders. This review fo-

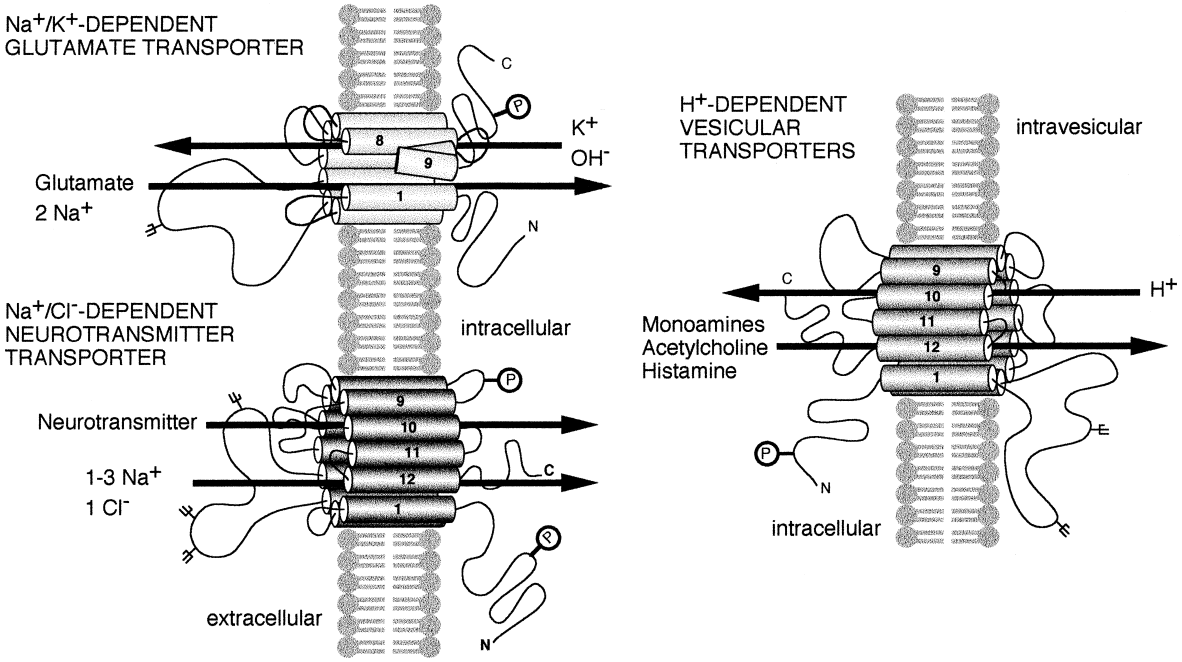


Fig. 1 Structural diversity and ion dependency of neurotransmitter uptake proteins (neurotransporters) [56]. High-affinity neurotransporters located in the plasma membrane of both presynaptic nerve and glial cells mediate the removal of the neurotransmitter from the synaptic cleft, thereby terminating its action at pre- and postsynaptic receptors and fine-tuning the temporal aspects of neuronal communication. Intracellular transport systems facilitate the reaccumulation of neurotransmitters in synaptic vesicles for additional cycles of release. At both plasma and vesicular membranes, neurotransmitter influx is coupled directly to transmembrane ion gradients that provide the energy for transport against a concentration gradient. On the basis of their molecular structures, subcellular distributions, and pharmacological properties, neurotransporters have been divided into three main subgroups, Na^+/Cl^--dependent [e.g., norepinephrine, dopamine, serotonin, GABA (four subtypes), glycine, proline, taurine, and several orphan transporters], Na^+/K^+-dependent cell surface transporters (glutamate/aspartate transporter, at least four subtypes) and H^+-dependent transporters associated with synaptic vesicles (e.g., vesicular monoamine and acetylcholine transporters)

cuses on the molecular biology and pharmacology of neurotransporters and in particular their relevance for excitotoxicity and neurodegeneration. While the majority of cloned neurotransporters correspond to known, functionally identified subfamilies, novel transporter sequences that had escaped detection by radioligand binding have emerged. In all cases molecular biology is providing a more complete picture of the unexpectedly large number of neurotransporters, their distribution, substrate specificity, drug-binding profiles, molecular structure, and gene organization. In addition to defining uptake mechanisms as candidate genes for neurodegenerative disorders, this review specifically emphasizes those neurotransporters that are potential targets for novel drug development.

Sodium/chloride-dependent transporters

A flurry of recent cloning endeavors has led to the molecular characterization of a large number of Na^+/Cl^--dependent transporters, including the transporters for the biogenic amines (norepinephrine, dopamine, and serotonin), γ-aminobutyric acid (GABA), glycine, proline, taurine, betaine, choline, and several orphan carriers (for review see [1, 6–8, 82, 81]). Moreover, several subtypes of the GABA [16, 38, 63] and glycine transporter [39, 64, 94] have been identified. Although some variations exist, the structure of proteins in this gene family is best described by a model with 12 transmembrane segments, intracellular amino and carboxyl termini, and an extended extracellular loop with several potential glycosylation sites between transmembrane segments III and IV (Fig. 2). This loop is most divergent among family members and may therefore participate in substrate recognition and inhibitor binding. On the basis of their remarkable amino acid identity of 69–80% and their properties as targets of tri- and heterocyclic antidepressants as well as amphetamine, cocaine, and their analogs, the carriers for the biogenic amines serotonin, dopamine, and norepinephrine constitute a subfamily. Several neurotransmitter transporters (e.g., serotonin, dopamine, GABA, and glutamate transporter) are electrogenic and possess several unexpected ion channel-like properties.

Serotonin transporter

Mood, cognition, and motor functions as well as circadian and neuroendocrine rhythms including food intake, sleep, and reproductive activity are modulated by the midbrain raphe serotonin (5-hydroxytryptamine, 5-HT) system. The 5-HT transporter (5-HTT) plays a central

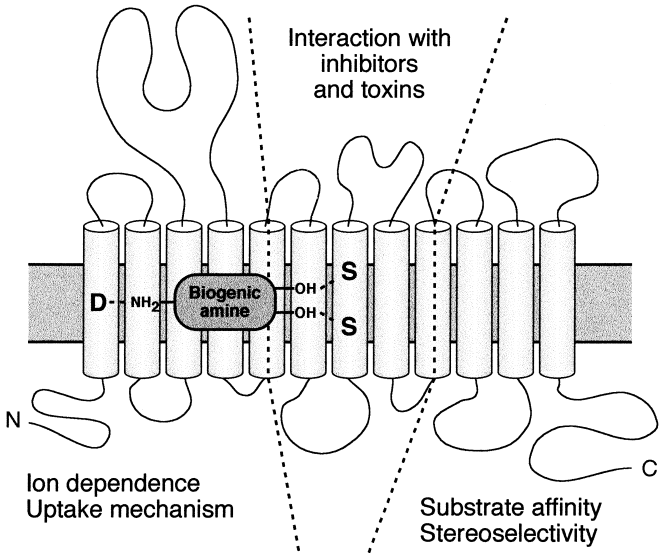


Fig. 2 Hypothetical functional domains of Na^+/Cl^--dependent neurotransporters

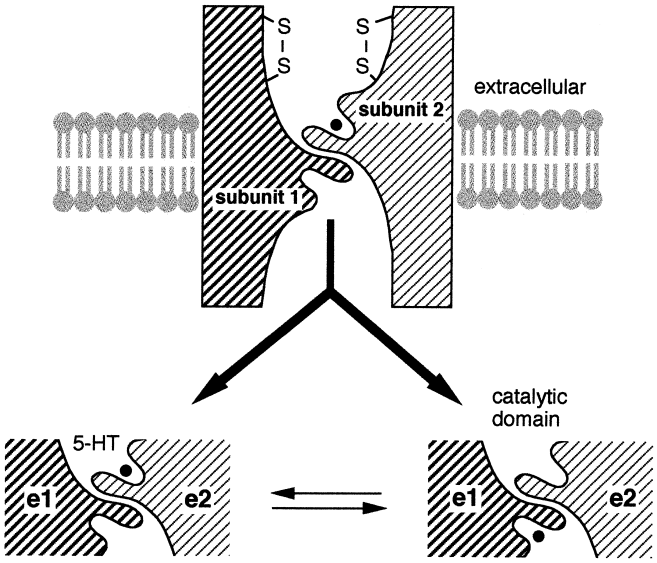


Fig. 3 Hypothetical quarternary structure of the serotonin transporter (*5-HTT*). The section is across two or four subunits that form the 5-HTT. The complex forms a cavity that extents across the bilayer, and the catalytic domain is at the center of the cavity. Each subunit contributes one transport site to the complex. Subunit 1 is shown in an e1 state while subunit 2 is in an e2 state binding extracellular 5-HT. When subunit 2 translocates bound 5-HT across the membrane, subunit 1 must undergo the antiparallel conformational change and vice versa. The structure of each subunit may be stabilized by an extracellular, internal disulfide bridge. (Adapted from [107])

role in the termination of serotonergic neurotransmission by Na^+-driven uptake of 5-HT into the presynaptic neuron and is regarded as an initial site of action of antidepressant drugs [14] and several neurotoxic agents. Interest in the mechanism of environmental factor-, disease-, and therapy-induced modification of 5-HTT function and its impact on early brain development, event-related synaptic plasticity, and neurodegeneration is widespread and intensifying [14, 56, 101].

Autoradiographic studies using [^{3}H]cyanoimipramine and [^{3}H]citalopram have demonstrated highest densities of 5-HTT on serotonergic cell bodies in the midbrain raphe complex and in its projection areas including cortical areas, entorhinal cortex, CA_3 region of the hippocampus, amygdala, substantia nigra, caudate putamen, and hypothalamus [19, 24, 43]. While in adult life 5-HTT expression appears to be restricted to raphe neurons, it has been detected in the cingulate cortex and thalamus during postnatal development of the brain (B. Hoffman, personal communication), and astrocytes may participate in the 5-HT clearance [25]. Tricyclic antidepressants such as imipramine and the selective 5-HT uptake inhibitors fluoxetine, paroxetine, citalopram, and sertraline occupy several pharmacologically distinct sites overlapping at least partially the substrate binding site [88] and are widely used in the treatment of depression, anxiety, impulse control disorders, and substance abuse including alcoholism [55, 56]. Neurotoxins, including the amphetamine derivatives 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") and nonstimulant fenfluramine [62], and the novel 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) analog 2′-NH_2-MPTP [2], are concentrated in serotonergic neurons by this transporter. Twin studies have suggested that 5-HT uptake is genetically controlled [70], and dysregulation of 5-HTT function has been reported in a variety of complex behavioral traits and disorders such as depression, bipolar, anxiety, obsessive-compulsive, schizophrenic, and neurodegenerative disorders, substance abuse, and eating disorders [29, 47, 69, 73, 76, 96, 97].

Paralleling the recognition of these clinical links, research has increasingly focused on the structure and function of the human 5-HTT. Cloning of the rat and human 5-HTT [13, 44, 57, 67, 80] has identified a protein with 12 putative transmembrane domains, and studies using site-directed mutagenesis and deletion mutants indicate that an aspartate residue and possibly a serine cluster in transmembrane I and VII, respectively, participate in substrate translocation and competitive antagonist binding (Fig. 2). Evidence from studies with deletion mutants and from computational analyses indicate that 5-HTT function may depend on the formation of quarternary structures, such as dimers and tetramers (Fig. 3). Several conducting states have been reported for 5-HTT: (a) during gating a transport-associated current caused by a flux of ions which are not involved in the transport but can pass through the channel, (b) a transient current triggered by extreme negative potentials, and (c) a leakage current [66]. 5-HTT function is acutely modulated by posttranslational modification including phosporylation/dephosporylation through protein kinase C and calmodulin-dependent protein kinase A as well as via nitric oxide and cGMP [46, 71]. Several intracellular signal transduction pathways converge on the transcriptional apparatus of the 5-HTT gene and expression is regulated

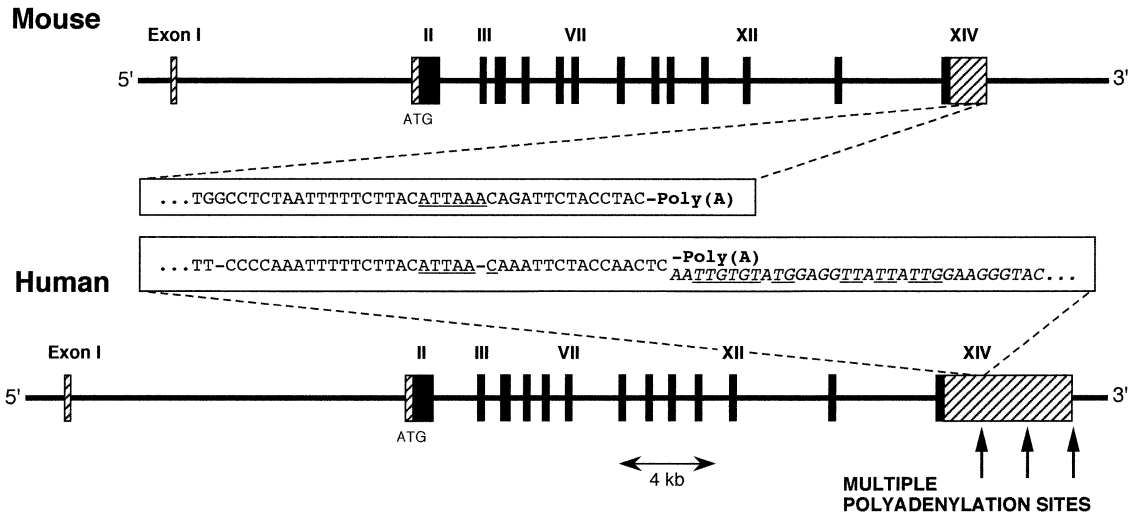


Fig. 4 Genomic organization of the murine and human serotonin transporter (*5-HTT*). Positions of exons (I–XIV; *black boxes*, coding regions; *hatched boxes*, noncoding regions) and translation initiation site (*ATG*) are indicated. Comparison of cDNA and genomic sequences indicate that the mouse signal for mRNA cleavage/polyadenylation is a natural variant which occurs in 12% of the known genes and exhibits 75–80% as much activity as the classical cleavage/polyadenylation signal (*AATAAA*). The proximal polyadenylation signal-like motif in the human gene is considerably divergent, which is likely to lead to cell-specific alternate usage of additional polyadenylation sites resulting in multiple mRNA species in humans

by cAMP, protein kinase C, and tyrosine kinase dependent mechanisms [21, 41, 81, 100].

The primary structure of the human platelet 5-HT uptake site is identical with that of human brain 5-HTT and thus confirms the human platelet 5-HT uptake site as a peripheral model of the brain 5-HTT in molecular pharmacological and neurobiological research [59]. In support of this concept the presence of the brain vesicular monoamine transporter in human platelets has also been demonstrated [58]. The human 5-HTT gene has been mapped to the chromosome 17q11.2 and is composed of 14 exons spanning approx. 35 kb (Fig. 4) [60]. Comparison of the human and murine 5-HTT genes reveals a striking conservation of both the exon/intron organization and the 5′-flanking regulatory sequences [9]. While only a single mRNA species occurs in rats and mice, the most proximal signal for polyadenylation in the human gene appears to be highly degenerate in comparison to the rat and murine motif (Fig. 4). This polyadenylation signal-like motif may lead to alternate usage of additional polyadenylation sites resulting in multiple mRNA species in humans [4, 80]. A TATA-like motif and several potential binding sites for transcription factors including AP1, AP2, SP1, and a cAMP response element (CRE)-like motif are present in the 5′-flanking region (Fig. 5). The role of these transcription factor motifs in the regulation of 5-HTT gene expression has been studied by transfection studies using reporter gene fusion constructs of 5-HTT 5′-flanking sequences [41]. An approx. 1.4-kb fragment, which had been ligated into a luciferase reporter vector and transiently expressed in JAR human placental choriocarcinoma cells, displayed both constitutive and forskolin/cholera toxin induced promoter activity (Fig. 6). Functional promoter mapping revealed a negative attenuating element and several positive elements. Studies with deletional mutants also indicated that core promoter sequences are contained within approx. 100 bp of the transcription start site and that regulation of cAMP- and protein kinase C inducible promoter activity depends on multiple *cis*-acting elements including two AP1 binding sites and a single CRE-like element. The findings suggest that (a) the 5-HTT gene promoter is active in human JAR cells but inactive in 5-HTT deficient human SK-N-SH neuroblastoma and HeLa cells, (b) the information contained within 1.4 kb of 5′-flanking sequence is sufficient to confer its cell-specific expression, (c) the promoter responds to cAMP induction, and (d) the expression of the 5-HTT gene is regulated by a combination of positive and negative *cis*-acting elements operating through a basal promoter unit defined by a TATA-like motif.

The negative attenuating element, a tandemly repeated sequence associated with the transcriptional apparatus of the human 5-HTT gene, displays a complex secondary structure (G4-DNA) [92], represses promoter activity in nonserotonergic neuronal cells, and contains positive regulatory components (Fig. 5). A systematic search for length variations of this 20- to 23-bp repeat element revealed a common polymorphism of this repetitive element and provides evidence for allele-dependent differential 5-HTT promoter activity. This 5-HTT gene linked polymorphic region (5-HTTLPR) is generated by a deletion with an overall length of 44 bp, involves repeat unit 6–8, and is located close to a “hot spot” for deletion mutagenesis [22]. The frequency of the variant short allele in a white population was approx. 39%, with an observed heterozygosity of about 52%. In vitro functional studies of the native 5-HTT promoter containing a long and a short 5-HTTLPR in a human placental choriocarcinoma cell line (JAR) indicated that this polymorphism may contribute to interindividual differences in 5-HTT ex-

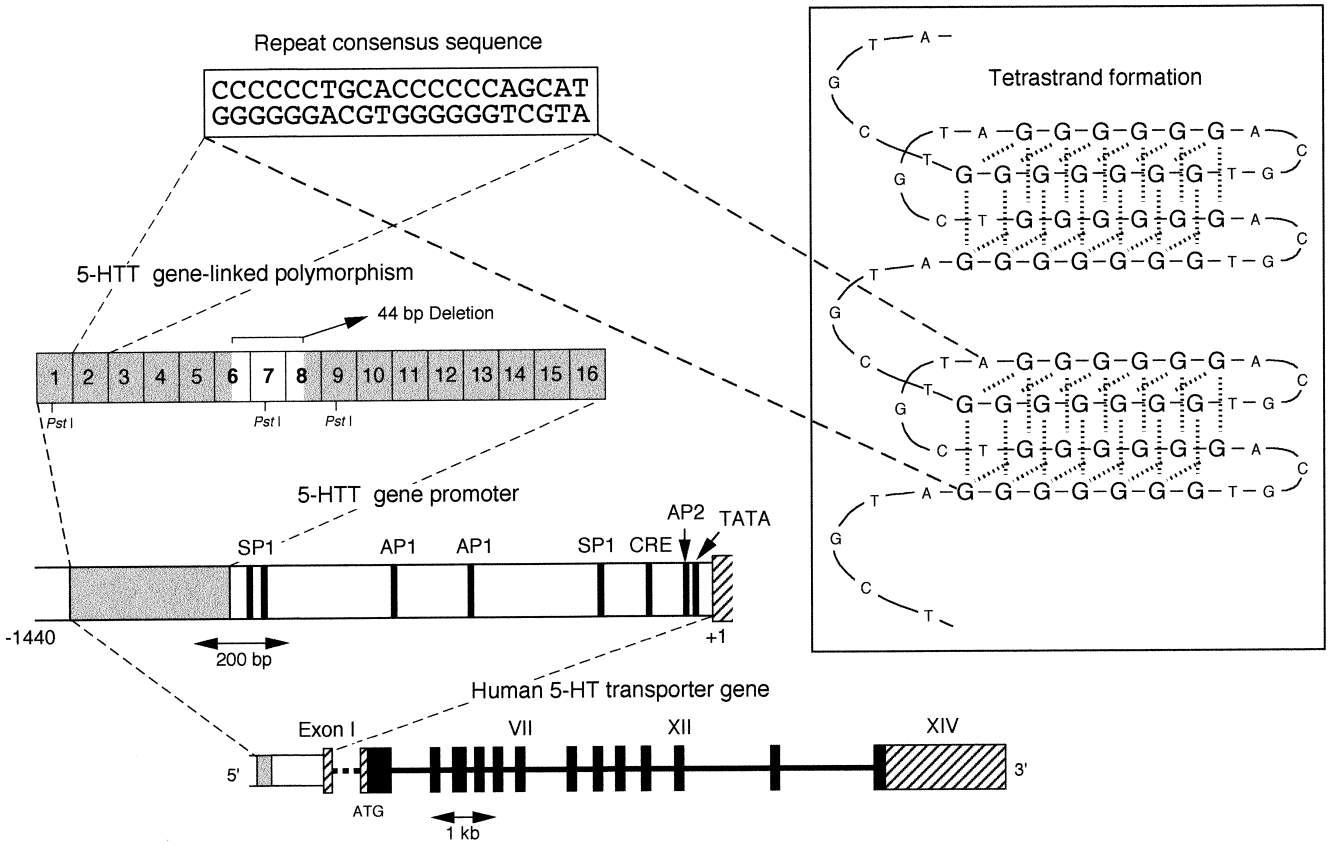


Fig. 5 Structure of the human serotonin transporter (*5-HTT*) gene and its 5′ flanking regulatory region on chromosome 17q11.2. *Black boxes*, coding regions; *hatched boxes*, noncoding regions. The 5-HTT gene promotor is defined by a TATA-like motif, and several potential binding sites for transcription factors including AP1, AP2, SP1, and a CRE-like motif are present in the 5′-flanking region. The position (*shaded box*) and consensus sequence (*open box*) of the 5-HTT gene-linked polymorphic region (*5-HTTLPR*) is indicated. The potential of the 5-HTTLPR to form G4-DNA is also depicted

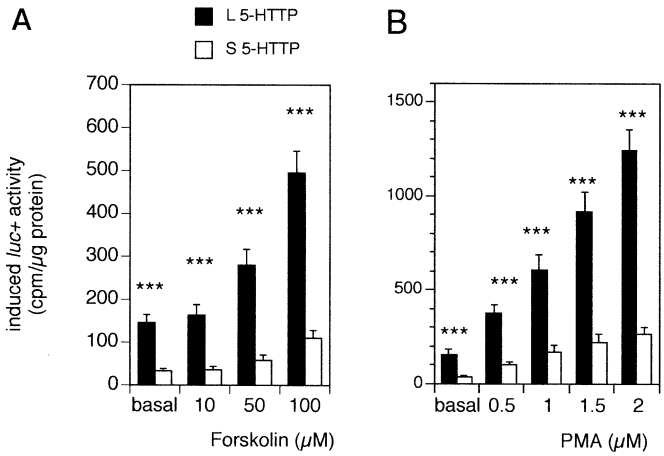


Fig. 6 Cyclic AMP (**A**) and protein kinase C induced (**B**) transcriptional activity of the long (*L*) and short (*S*) 5-HTT gene promoter (*5-HTTP*; L vs. S: ***$P<0.01$, one-way ANOVA followed by *t* tests) [42]. Human JAR placental choriocarcinoma cells were transfected with 5 μg of the long and short h5-HTTP *luc* + construct followed by treatment with forskolin (10–100 μM) or phorbol 13-myristate 12-acetate (*PMA*, 0.5–2 μM). Results represent means ±SD for triplicate measurements

pression and regulation. Comparison of the polymorphic alleles revealed that the basal activity of the long variant is significantly higher than the 5-HTT gene promoter with the deletion [42]. Cyclic AMP and protein kinase C dependent mechanisms induce transcriptional activity in both variants of the 5-HTT promoter, but the dose-dependent increase following induction with forskolin or the phorbol ester phorbol 13-myristate 12-acetate remains proportionally smaller in the allele with the deletion (Fig. 6).

Studies with the 5-HTTLPR containing promoter suggest that basal and induced human 5-HTT gene transcription is differentially modulated by allelic variants of the 5-HTT gene promoter, and that the 5-HTTLPR's differential silencing and/or activating action may be attributable to a complex interaction with other 5-HTT promoter regulatory elements. Considering that the 5-HTTLPR and the previously reported insulin gene linked polymorphic region (ILPR) share several structural and functional characteristics, the findings may have implications for manifold 5-HTT regulated physiological functions such as mood, cognition, food intake, and sleep. Both the 5-HTTLPR and the ILPR contain a tandemly repeated GC-rich sequence unique for primates/humans, form a G string dependent tetrastrand structure (G4-DNA), are located in close proximity to the basal pro-

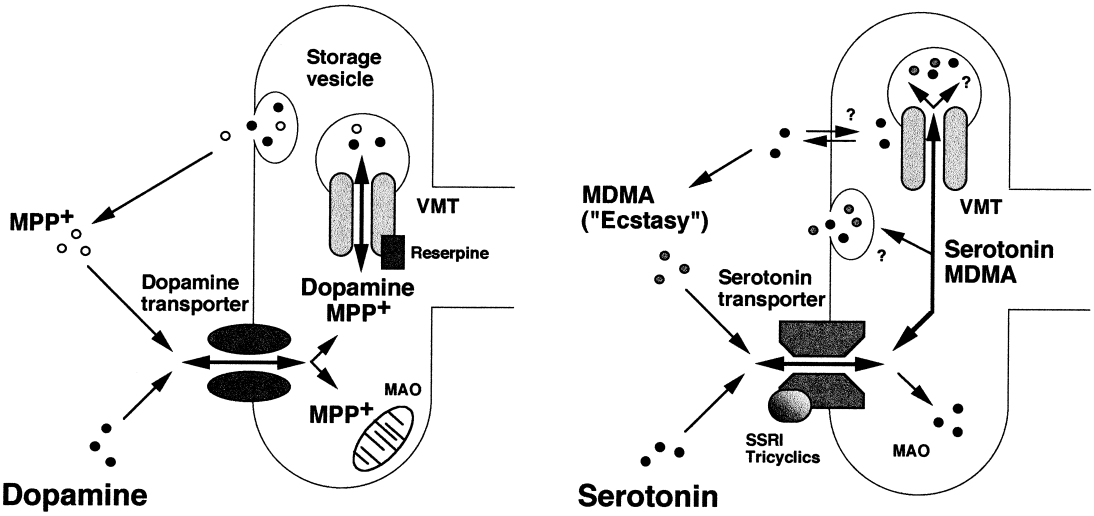


Fig. 7 Neurotransporters and putative mechanisms of 1-methyl-4-phenylpyridinium ion (*MPP+*) and amphetamine neurotoxicity. MPP^+, the neurotoxic metabolite of MPTP, enters neurons through the dopamine transporter. In pathological conditions, dysfunctional transport process may contribute to an increased susceptibility to exogenous MPP^+-like neurotoxins. This vulnerability to neurotoxins may be further aggravated by an impaired capacity of the brain vesicular monoamine transporter (*VMT*), which plays a central role in the sequestration of cytoplasmic toxins and thus in the limitation of mitochondrial damage. Amphetamines and their highly lipophilic, neurotoxic analogs, such as MDMA ("ecstasy"), are either substrates of serotonin and dopamine transporters or enter nerve terminal by diffusion, displace the biogenic amine from its storage vesicle, and ultimately induce 5-HT efflux by a carrier-dependent process. Although the dopamine-releasing action of MDMA is likely to be related to the mechanism of reinforcement, it is also a potent releaser of 5-HT, and long-term administration results in toxic degeneration of serotonergic terminals. MDMA-induced 5-HT release and neurotoxicity can be prevented by tri- and heterocyclic uptake inhibitors

moter, repress transcriptional activity in a tissue-specific mode, and carry activating response elements [8, 9, 50] (K.P.L. et al. manuscript in preparation). In addition, there are several lines of evidence that the ILPR is a susceptibility locus for type 1 diabetes [10]. Since differential transcriptional activity of 5-HTT promoter variants may contribute to quantitative traits, identification of allelic variation in 5-HTT expression and the combined evidence for the 5-HTTLPR's complex transcriptional function provide the first step toward better understanding of the molecular basis for the previously reported genetic control of 5-HTT function and its potential relevance for complex traits and disease. The hypothesis that genetic control of and disease-related alteration in 5-HTT function are more likely to be related to differential regulation of 5-HTT expression than to amino acid substitutions is further supported by preliminary results indicating pronounced allelic variability in glucocorticoid-induced derepression of 5-HTT promoter activity (K.P.L. and A.H., unpublished results) and the fact that mutation screening of 5-HTT gene exons in the general population and samples of patients with affective spectrum and obsessive-compulsive disorders detected only rare coding variants [1, 26, 61]. Taken together, the characterization of the 5-HTT 5′-flanking sequence has identified a gene regulatory unit specific for serotonergic neurons. The fusion of this 5-HTT gene regulatory unit to similar or unrelated genes may aid their expression in a specific neuronal system and open the way to novel therapeutic strategies.

While tri- and heterocyclic antidepressants inhibit monoamine uptake, several agents increase monoaminergic transmission by facilitating nonexocytic transporter-mediated monamine release. Amphetamines and their highly lipophilic analogs, such as MDMA, are either substrates of 5-HT and dopamine transporters or enter nerve terminal by diffusion, displace the biogenic amine from its storage vesicle, and ultimately enhance carrier-mediated release [62, 87, 90] (Fig. 7). Although the dopamine-releasing action of MDMA is likely to be related to the mechanism of reinforcement, it is also a potent releaser of 5-HT, and long-term administration results in toxic degeneration of serotonergic terminals. MDMA-induced 5-HT release and neurotoxicity can be prevented by tri- and heterocyclic uptake inhibitors. In analogy, the 5-HT releasing effect of the anorectic drug fenfluramine is blocked by clomipramine or fluoxetine. Targeted disruption of the 5-HTT gene in mice will eventually clarify the behavioral and biochemical consequences of MDMA and other psychostimulants/neurotoxins as well as its relevance for CNS de/regeneration and neural plasticity.

Dopamine transporter

Following exocytic release into the synaptic cleft, the action of dopamine at its receptors is terminated by neuronal uptake via a Na^+/Cl^--dependent transporter. Various pharmacological, structural, and functional aspects of the dopamine transporter (DAT) have recently been discussed in detail [5, 15, 35]. Although DAT is associated predominantly with dopaminergic neurons, it is also expressed in platelets and several neuroblastoma cell lines (Koutsilieri et al., unpublished results). After its retrieval dopamine is either repackaged into synaptic vesicles or

metabolized by mitochondria-associated monoamine oxidase. Vesicular dopamine uptake (via the vesicular monoamine transporter) is unique with regard to its ion and energy requirements, substrate specificity, and kinetic properties (see also section on proton-dependent transporters). cDNAs for DAT have been cloned from both rat and man. Molecular studies have shown that the DAT is an 80-kDa glycoprotein with multiple forms defined by different carbohydrate side chains in different brain areas. The sequence of the human DAT cDNA predicts a hydrophobic 620 amino acid protein, and several features of the deduced amino acid sequence and secondary structure are shared by other gene family members including 5-HTT, norepinephrine, and GABA transporter [35, 101]. The amino and carboxyl termini reside in the cytoplasm, and following transmembrane segment III these transporters contain an extended extracellular loop with multiple glycosylation sites and multiple intracellular sites for serine and threonine phosphorylation (Fig. 1). It has recently been reported that differential glycosylation of the DAT occurs during postnatal development and aging [74]. While the glycosylation status of a transporter protein appears to be relevant to folding stability, transport, and possibly ligand recognition, post-translational modification such as phosphorylation of the amino-terminal tail is linked to acute hormone-dependent regulation of transport activity [21, 23, 33, 46, 71, 81, 100]. Studies utilizing chimeric 5-HTT, norepinephrine transporter, and DAT have revealed that the amino-terminal transmembrane domains including an aspartate residue in the first transmembrane segment appear to be involved in the uptake mechanism and ion dependence, a cluster of serine residues in the transmembrane segments VI–VIII define antagonist and neurotoxin binding, and the remaining carboxyl-terminal region likely determines stereoselectivity and high-affinity sites for the respective substrate [14, 52, 36] (Fig. 7). Moreover, cysteines in DAT's second extracellular loop may provide sulfide residues crucial to full transporter expression, possibly through interference with membrane insertion or formation of quarternary structures [11, 105]. The DAT protein also contains a potential degenerate leucine zipper motif and thus could oligomerize to a tetrameric assembly of identical units (Fig. 3) [72]. Isolation and characterization of the human and murine DAT genes has allowed elucidation of similarities between each and other members of this transporter gene family. Sequences upstream of the transcriptional start sites contain GC-rich, TATA-less, and CAAT-less regions with remarkable conservation between human and murine gene flanking regions. These sequence elements are likely to contribute to DAT's dopaminergic cell-specific expression [27].

Preferential dopamine uptake inhibitory and releasing action at the DAT (in addition to its interaction with norepinephrine and 5-HTTs) by cocaine and its analogs induces multiple neurochemical and behavioral effects that are thought to represent the molecular basis of reinforcement, sensitization, and addictive behavior. In contrast, although amphetamine stereoisomers have considerable affinities for the norepinephrine transporter, their interaction with DAT is stereoselective. This stereoselective property at biogenic amine transporters is likely to be involved in their psychotomimetic actions. Recent work indicates that there is a plasmalemmal component and a vesicular component in the dopamine-releasing action of amphetamines, while the releasing action of cocaine depends on the existence of a vesicular pool of the neurotransmitter and seems to be linked to inhibition of the plasmalemmal DAT [78]. Interestingly, targeted disruption of the DAT gene in mice results in behavioral and biochemical hyperdopaminergic functions and failure to respond to psychostimulants [37].

Abnormalities in DAT function have been postulated for a variety of neurodegenerative disorders. Postmortem studies indicate that [^{3}H]cocaine and [^{3}H]GBR-12935 binding are decreased in Parkinson's disease (PD). Hemisphere-to-hemisphere differences in the binding of [^{11}C]nomifensine in the striatum and accumulation of [^{18}F]fluorodopa in the putamen has been confirmed by positron emission tomography studies and loss of striatal [^{123}I]β-CIT binding by single-photon emission tomography [15, 17, 45]. Neurodegeneration of the nigrostriatal dopaminergic system induced by the 1-methyl-4-phenylpyridine ion (MPP^{+}), the neurotoxic metabolite of MPTP, has implicated DAT in the etiology some forms of PD (Fig. 7). Since MPP^{+} enters neurons through the DAT, a dysfunctional transport process may contribute to an increased susceptibility to exogenous MPP^{+}-like neurotoxins. This vulnerability to neurotoxins may be further aggravated by an impaired capacity of the brain vesicular monoamine transporter (VMT), which plays a central role in the sequestration of cytoplasmic toxins and thus in the limitation of mitochondrial damage. With aging even modest reductions of vesicular uptake would accumulate MPP^{+}-like toxins in the cytoplasmic pool and cause neuronal cell death. In this regard it is of interest to note that in humans there is evidence for progression of MPP^{+}-induced dopaminergic lesions, thus indicating that transient exposure to a toxin may cause a protracted decline in nigrostriatal dopaminergic function more rapid than in normal aging and similar to PD [103]. While the status of VMT expression in PD remains to be investigated, an age-related drastic and sudden onset reduction of DAT mRNA which is contrasted by a more gradual decline in DAT protein has been described postmortem in human substantia nigra [5]. Functional and structural analyses of DAT (and possibly VMT) using in vitro and in vivo strategies, such as expression in cultured cells and neuroimaging with single-photon emission tomography and positron emission tomography, are likely to enhance our understanding of MPP^{+} neurotoxicity and the role of DAT in the progression of PD.

Disorders of the schizophrenic spectrum are thought to be associated with an aberrant dopaminergic (and glutamatergic) neurotransmission. Cocaine and amphetamine act as psychostimulants or psychotomimetics. Both amphetamine-evoked psychotic symtomatology in vulnerable individuals or patients with a preexisting psy-

chotic illness and cocaine-induced kindling and arousal are believed to result from their effects on the mesolimbic dopamine system, which has been implicated in the integrative process of perception, cognition, and emotion. While changes in the kinetics or inhibitor binding of 5-HTT have been reported in disorders of the schizophrenic spectrum [47], studies on DAT have produced conflicting findings [40, 75]. Moreover, no evidence for a linkage of the DAT gene with schizophrenic disorders in multigenerational pedigrees has been found [18]. Finally, an increase in DAT sites has been reported in Gilles de la Tourette's syndrome, and a deficient DAT-related uptake has been proposed to play a role in compulsive self-mutilating behavior, as observed in Lesch-Nyhan syndrome.

Proton-dependent vesicular transporters

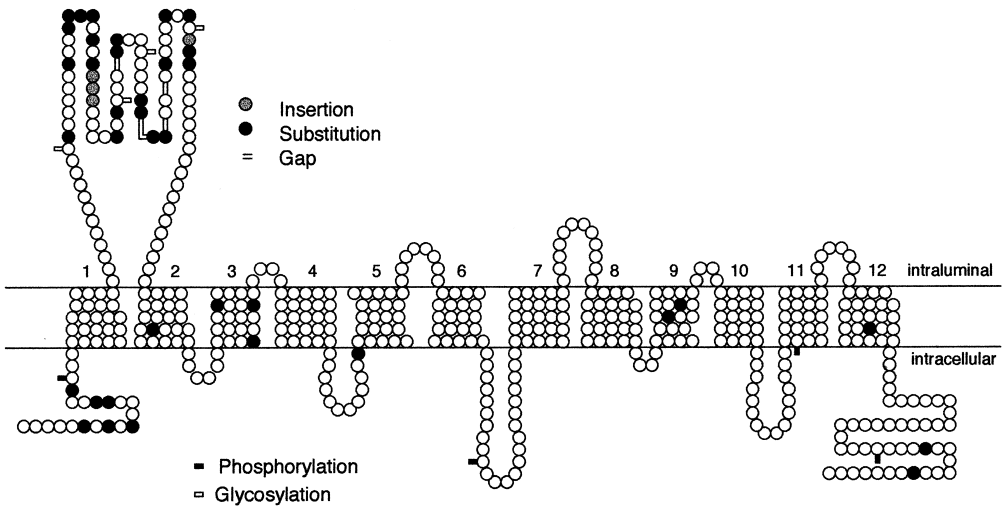
Vesicular transporters constitute a distinct gene family of carriers that accumulate cytoplasmic biogenic amine neurotransmitters or neurotoxins (serotonin, catecholamines, histamine, amphetamines, MPP^+) and acetylcholine by ATPase-dependent and H^+-driven uptake into storage vesicles of presynaptic neurons and platelets [28, 85]. Vesicular transport is inhibited by drugs such as reserpine and tetrabenazine (for the amines) and vesamicol (for acetylcholine) that are distinct from agents that act on plasma membrane transporters (Fig. 1).

Monoamine transporters

The potent antihypertensive drug reserpine prevents the transport of biogenic amines into synaptic vesicles and chromaffin granules, thus depleting amine stores. However, its clinical utility is limited because reserpine also causes side effects that resemble depression. Although the psychobiology of depressive illness is extremely complex, this observation provided the framework for the archetypal amine hypothesis of affective disorder. Reserpine acts by binding irreversibly to VMT which is accelerated by a pH gradient and competed for by amine substrates (Fig. 1). MPP^+ and tetrabenazine, the other prototypical inhibitors of the VMT, compete poorly with reserpine binding, indicating that they interact at different sites [89]. Nevertheless, transport of MPP^+, which depends on a pH gradient, is potently inhibited by both reserpine and tetrabenazine (Fig. 7). Interestingly, tetrabenazine which is being used to treat movement disorders including tics and dystonias depletes central amines more effectively than peripheral amines. [^{11}C]Tetrabenazine has been useful in the in vivo imaging of the VMT in human brain with positron emission tomography [51].

Cloning and sequencing of cDNAs encoding VMTs from rat brain, adrenal medulla, and basophilic leukemia cells have identified two distinct but related proteins (521 and 515 amino acids) with 12 putative transmembrane segments and a large luminal loop located between the first two transmembrane domains which are likely to participate in ion/substrate translocation and antagonist binding [30, 65, 85]. The VMTs show a remote sequence similarity with several bacterial drug resistance genes and have been shown to suppress the neurotoxic effects of MPP^+ by subcellular compartmentalization. Sequence divergence between the central and peripheral VMT may play a central role in the differential neurotoxic effects of MPP^+ in brain and adrenal medulla [77]. Recently a VMT cDNA was isolated from human midbrain and its expression confirmed in human platelets [58]. Although the human brain VMT is 90.7% homologous to the rat

Fig. 8 Topology of the human brain/platelet vesicular monoamine transporter (*hVMT*). *Circles*, individual amino acids; *black/shaded circles, gaps*, amino acids differing from the rat brain VMT; *open boxes*, putative glycosylation sites; *black boxes*, potential phosphorylation sites for cAMP-dependent protein kinase and protein kinase C. An extensive sequence divergence occurs in the large luminal loop located between the first two transmembrane domains. This region differs in 23 of 86 amino acids (26.7%) and contains three additional, two positively and one negatively, charged amino acids. Compared to the rat VMT cDNA, these structural changes result in a reduced overall amino acid identity of 64.0% in the large luminal loop of the human VMT, although four putative glycosylation sites are conserved



protein, extensive sequence divergence occurs in the large luminal loop located between the first two transmembrane domains (Fig. 8). This region of the polypeptide differs in 23 out of 86 amino acids (26.7%) and contains three additional, two positively and one negatively, charged amino acids. Compared to the rat VMT cDNA, these structural changes result in a reduced overall homology of 64.0% in the large luminal loop of the human VMT, although four putative glycosylation sites are conserved. Analysis of the coding sequence flanking the large luminal loop has detected 12 out of 16 conservative amino acid substitutions with an overall homology of 96.3%.

Fibroblasts transfected with a cDNA clone encoding the VMT expressed in rat adrenal chromaffin cells confers resistance to MPP^+ [30]. Although the peripheral VMT is highly homologous to the VMT expressed in rat brain, considerable sequence divergence occurs in the large luminal loop flanked by first two transmembrane segments and, to a lesser extent, in the amino-terminal region. Since the large extracellular loop of Na^+-dependent plasma membrane transporters and possibly the large luminal loop of H^+-driven vesicular transporters may play a central role in both intracellular transporter protein trafficking and in ion and/or substrate recognition, sequence divergence in this segment of the VMT expressed in rat brain and adrenal medulla may underlie the tissue-specific differences in MPP^+ toxicity (Fig. 7). The extensive sequence divergence in the large luminal loop of the VMT between the rat and human brain may represent a possible molecular basis for the substantial species differences in the susceptibility to MPP^+ demonstrated among humans, nonhuman primates, and rodents [34]. The extraordinary extent of variation across species reflecting the process of evolution may indicate that this region of the gene is susceptible to mutations and may have implications for the pathogenesis of idiopathic PD. Structural alterations and/or abnormalities in gene expression arising as a consequence of an accumulation of somatic mutations could result in a decreased capacity of the brain VMT to remove neurotoxins or regulate cytoplasmic dopamine selectively taken up into nigrostriatal dopaminergic neurons by DAT [99]. Dysfunctional VMT may not be able to limit the mitochondrial damage of putative exogenous and endogenous toxins, thus leading to degeneration of dopaminergic neurons and clinical PD (see “Dopamine transporter”). Recently the heterogeneous distribution of the VMT, labeled with [^{3}H]dihydrotetrabenazine (TBZOH) and DAT, labeled with [^{3}H]GBR12783, was studied in the rat striatum [54]. The ratio TBZOH/GBR12783 was higher in the anterior part of the striatum than in the caudal part. VMT and DAT were more abundant in the lateral regions than in the regions situated near the midline. In the caudal striatum the ventral part was richer in VMT than the dorsal part. In aged rats (30 months) a significant decrease in the density of both transporters was noticed in the middle part of the striatum. In the anterior part of the striatum the ratio TBZOH/GBR12783 was higher in aged rats than in adult animals. This change may reflect a functional adaptation of the partially diminished population of dopaminergic neurons during aging. Similarly, the pattern of distribution of TBZOH binding also heterogeneous in the human striatum, with higher binding levels in the “matrix” than in the “striosome” compartment [53]. A severe decrease in TBZOH binding was observed in all parts of the striatum in PD brains. The data corroborate the deficiency in striatal dopaminergic transmission and suggest that dopaminergic terminals in PD brains have disappeared and/or no longer contain synaptic vesicles. In Alzheimer’s disease, TBZOH binding is significantly reduced in the ventral striatum and not in the caudate nucleus and putamen. The specific decrease in VMT levels in the ventral striatum confirm that this nucleus is a target area in Alzheimer’s disease.

Finally, VMT abnormalities affecting monoamine transporter system function may contribute to the pathogenesis of affective disorders [60] and other neuropsychiatric disorders in which these systems have been implicated.

Acetylcholine transporter

Cholinergic neurotransmission is fine-tuned by dissociation of acetylcholine (ACh) from nicotinic and muscarinic ACh receptors followed by its enzymatic hydrolysis and uptake of choline into the presynaptic terminal. Choline uptake represents a rate-limiting step in ACh synthesis and is competitively inhibited by the prototypic inhibitor hemicholinium-3. Although a recently reported novel transporter cDNA displayed choline uptake following functional expression, uptake was not blocked by hemicholinium-3, the carrier failed to be associated with cholinergic neurons, and alternate substrates may be transported [68, 104]. Other Na^+-dependent high-affinity choline transport proteins located on the nerve terminal are therefore likely to be cloned and characterized in the near future.

In contrast to the controversy regarding putative choline transporters, identification and structural characterization of the vesicular ACh carrier has progressed remarkably. Vesamicol disrupts cholinergic neurotransmission by inhibiting vesicular accumulation of ACh. Since vesamicol binds to a single population of uptake sites that are present only on synaptic vesicles associated with cholinergic neurons, it has assisted the cloning of the vesicular ACh transporter (VAChT) [7, 31, 82, 102]. The distribution of the VAChT in both the central and peripheral cholinergic neuronal system is associated with choline acetyltransferase (ChAT), the enzyme required for ACh biosynthesis. Intriguingly, the sequence of the human VAChT cDNA is intron-less and contained within the first intron of the ChAT gene. In cultured sympathetic neurons induction of ChAT gene expression with the cholinergic differentiation factor/leukemia inhibitory factor or retinoic acid also increases VAChT mRNA, which is consistent with the existence of a coregulatory mecha-

nism for the embedded ChAT and VAChT genes [12]. Transcription of genes from the same or contiguous promoter/enhancer unit is a novel regulatory mechanism for coordinate expression of two proteins in a specific cell system. This coordinate regulation may play a central role in cholinergic system development and functional loss of the central cholinergic tone in dementias of the Alzheimer type.

Sodium- and potassium-dependent excitatory amino acid transporters

The joint action of neuronal and glial high-affinity exitatory amino acid transporters plays a critical role in the termination of glutamatergic neurotransmission and the maintenance of extracellular glutamate concentrations below a neurotoxic level. Several distinct cDNAs encoding structurally related glutamate/aspartate (EAAC1, GLT1, GLAST) have now been identified and characterized in rat brain, although the precise transmembrane topology remains controversial [30, 48, 49, 79, 98] (Figs. 1, 9). Expression of EAAC1 appears to be confined to the somatodendritic section of neurons in the neocortex, hippocampus, and basal ganglia. GLAST is found on certain neurons and astroglia in cerebellar Purkinje cell layer, and GLT1 is thought to be primarily glial in origin with ubiquitous distribution in the neocortex, hippocampus, and entorhinal cortex [84]. A distinct glutamate transporter (GluT) located on presynaptic nerve terminals has not yet been identified. Recently six human homologs of GluT cDNAs (EAAT1-5) have been cloned from motor cortex [3] (J.L. Arriza, personal communication). Although differences in tissue distributions between species have been demonstrated, the amino acid sequence homologies of EAAT1, EAAT2, and EAAT3 compared to GLAST, GLT1, and EAAC1 are quite remarkable.

The transport activity encoded by the human EAAT4, which is expressed predominantly in the cerebellum, has high apparent affinity for L-aspartate and L-glutamate, and has a pharmacological profile consistent with previously described cerebellar GluT activities. Intriguingly, at transiently expressed EAAT4 L-aspartate and L-glutamate elicit a current predominantly carried by chloride ions. Thus EAAT4 combines the uptake of neurotransmitter with a mechanism for increasing chloride permeability, both of which could regulate excitatory neurotransmission [32].

The neutral amino acid carrier ASCT1 (transports alanine, serine, and cysteine) is structurally closely related to this transporter family, although transmembrane transport of neutral amino acids is expected to differ in its binding site from that of the acidic excitatory amino acids glutamate and aspartate. Three positively charged amino acid residues, Arg-122, Arg-280, Arg-479, and one polar Tyr-405 are conserved in all GluTs but are replaced by apolar amino acid residues in the ASCT1 sequence. Interestingly, recent work suggests a pivotal

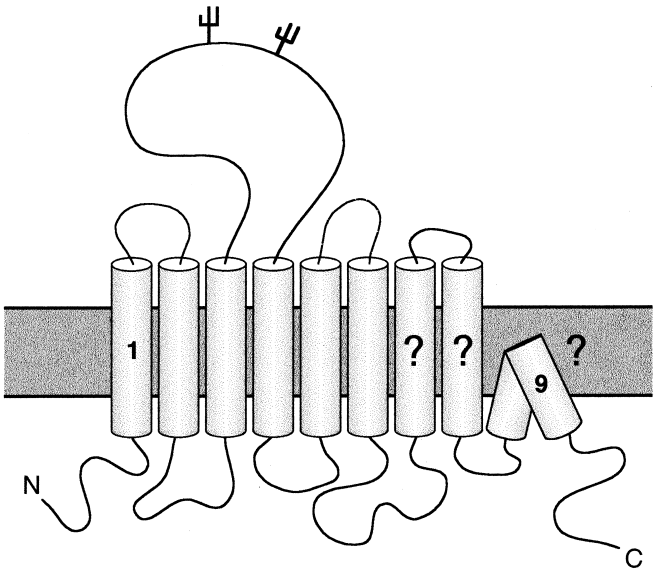


Fig. 9 Hypothetical topology of glutamate transporters. Several distinct cDNAs encoding structurally related glutamate transporter have been identified and characterized, but the precise transmembrane topology of the C-terminus remains controversial

function of the hydroxy group of the highly conserved Tyr-405 and the positively charged Arg-479 in the binding of the negatively charged acidic neurotransmitter glutamate [20].

It has recently been reported that the overall properties of a vesicular GluT in microvesicles of the bovine pineal gland match those of the synaptic vesicle GluT: (a) the major driving force of the glutamate uptake is the membrane potential, (b) Cl^- regulates the glutamate uptake, probably via anion-binding site(s) on the transporter, and (c) the transporter shows strict substrate specificity. It is concluded that the vesicular GluT may be similar if not identical to the neuronal counterpart.

Expression studies with the gene products demonstrate that glutamate transport is an electrogenic process and depends characteristically on both extracellular Na^+ and intracellular K^+ (Fig. 1). During each transport cycle a proton-accepting and thus pH-changing anion (OH^- or HCO_3^-) is also countertransported. This indicates that the uptake of a glutamate molecule is escorted by the cotransport of two Na^+ and by the countertransport of one K^+ and one anion, such as OH^- or HCO_3^-. Hence the glutamate uptake process evokes a depolarizing positive inward current leading to intracellular acidification and extracellular alkalinization. Although there is evidence that this electrogenic uptake is participating in the depolarizing action of glutamate, the functional consequences of the transporter-mediated depolarization and the intra- and extracellular pH changes are not fully understood. Intriguingly, nonvesicular calcium-independent release of glutamate may be induced by reversing the transport process through an extracellular increase in K^+ or a decrease in Na^+ concentrations.

The electrochemical environment of glutamate transport may have important implications for a variety of

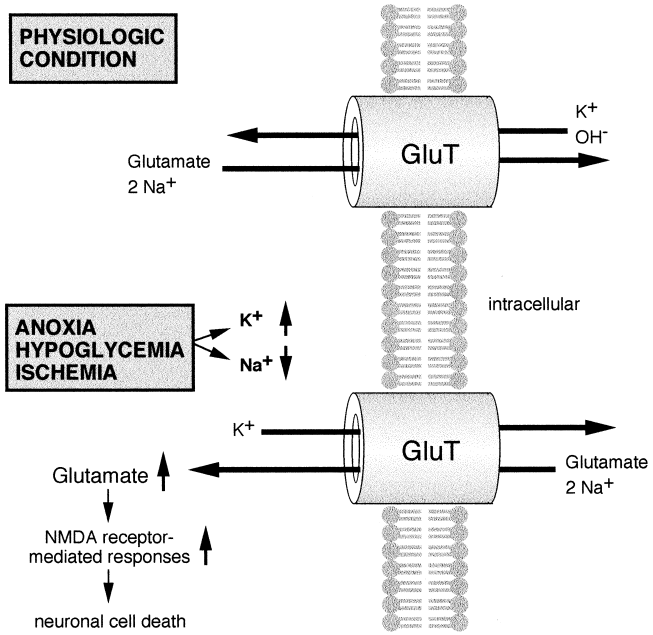


Fig. 10 Reversal of neuronal and glial glutamate transport in pathological conditions. Glutamate, a potent excitotoxin, is increased to neurotoxic levels in anoxic, hypoglycemic, and ischemic states. Following epileptic seizures or stroke, an extracellular increase of K^+ and reduction of Na^+ may favor transport reversal and glutamate release, which in turn would prolong NMDA and non-NMDA receptor-mediated responses and cause acute and chronic neuronal cell death

neuropathological processes (Fig. 10). Glutamate is a potent excitotoxin, and in vitro and in vivo observations indicate that extracellular glutamate is increased to neurotoxic levels in anoxic, hypoglycemic, and ischemic conditions. An extracellular increase of K^+ and reduction of Na^+ following epileptic seizures or stroke may favor transport reversal and glutamate release [62]. Glutamate released in vivo is likely to derive from both neurons and astrocytes. During ischemia and concurrent anoxia/hypoglycemia the K^+-evoked release of aspartate is increased only in cultured neurons, while the spontaneous release (=leakage) of glutamate and aspartate is enhanced in astrocytes but not in neurons. In line with these findings microdialysis studies have demonstrated that inhibition of glutamate uptake prolongs NMDA receptor-mediated responses and causes acute and chronic neuronal cell death. In addition to the blockade of NMDA receptors, the stimulation and/or the protection of the uptake systems for glutamate could be of help in preventing neurotoxic damage [106].

The interrelationship of defective excitatory amino acid transporter function and excitotoxic glutamate concentrations in the synaptic cleft is also indicative of transporter dysfunction in neurodegenerative disorders that are associated with a disrupted glutamate turnover. There is evidence that GluT function and/or concentrations are altered in distinct brain areas of patients with Alzheimer's disease, PD, Huntington's disease, amyotrophic lateral sclerosis, and psychiatric disorders of the schizophrenic spectrum [83, 86, 91, 93]. Aberrant GluT function in these conditions would increase synaptic glutamate which would subsequently initiate a cascade of events leading to neuronal death. Whether abnormalities in glutamate uptake are related to the pathogenesis of these disorders or result from as yet unknown neurodegenerative processes is controversial.

An inborn error of glutamate transport may cause dicarboxylic aminoaciduria, which is characterized by increased renal excretion of glutamate/aspartate and may also be associated with neurological abnormalities and mental retardation [95]. Finally, glutamate transport inhibiting properties of agents such as (*S*)-β-oxalyl-α,β-diaminopropionic acid (ODAP) and kainic acid may significantly contribute to their neurotoxicity. The former is the excitotoxin implicated in lathyrism, a degenerative disorder associated with spastic paraplegia.

Conclusions

There has been considerable progress in the molecular characterization of multiple neurotransporters. New insights into transporter diversity provide the means for more targeted approaches of studying uptake processes at the molecular level. Future research strategies will focus on molecular mechanisms of substrate translocation and antagonist binding and on posttranslational modification and functional regulation of the neurotransporter protein. Exciting information will also be derived from the analysis of genomic regulatory elements and from modeling neurotransporter-related disorders in transgenic animals by targeted gene knock out. Technologies that allow the addition, alteration, or elimination of individual genes from the genome to create transgenic animals are getting increasingly refined. There are clear advantages of having a transgenic animal model of a human disease. These animals often serve as the first unequivocal proof that a particular gene participates in the pathological changes that occur with disease. They can also provide a system suitable for dissection of the successive events that lead to the disease state and can provide a custom-designed whole animal system to test potential therapies and to treat and eventually cure the disease. In this line of thinking the fusion of transporter promoter/enhancer units to similar or unrelated genes could aid their expression in a specific neuronal system and open the way to various approaches of gene therapy. Transgenic strategies are therefore expected to be instrumental in providing new insights into mechanisms of neurotransporter dysfunction at the gene regulatory, neurochemical, and anatomical level during various stages of development. In addition, they may offer exciting possibilities for generating animal models for distinct neurodegenerative disorders. An improved understanding of the modulation of neurotransporters function in the brain may make it possible to identify the molecular factors underlying both the predisposition to and the pathogenesis of neurodegenerative disorders. Due to their specifici-

ty for distinct neuronal systems neurotransporters and their genes are potential targets for novel therapeutic strategies.

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