PLASMA MEMBRANE MONOAMINE TRANSPORTERS: STRUCTURE, REGULATION AND FUNCTION

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The classical biogenic amine neurotransmitters — dopamine, noradrenaline, and 5-hydroxytryptamine — control a variety of functions including locomotion, autonomic function, hormone secretion, and the complex behaviours that are associated with affect, emotion and reward. A key step that determines the intensity and duration of monoamine signalling at synapses is the reuptake of the released transmitter into nerve terminals through high-affinity plasma membrane transporters. In recent years, molecular, pharmacological and genetic approaches have established the importance of monoamine transporters in the control of monoamine homeostasis and have provided insights into their regulation.

About four decades ago, Julius Axelrod introduced the concept of reuptake to explain how noradrenaline was taken up by sympathetic nerve terminals[1]. He proposed that the reuptake process was an important mechanism for inactivating neurotransmitters. Soon afterwards, similar but distinct uptake mechanisms were shown to exist for dopamine and 5-hydroxytryptamine (5-HT, serotonin)[2] (FIG. 1). The main effect of this research was the discovery that certain antidepressants and psychostimulants blocked the reuptake of monoamines in the brain. As a result, many of the therapeutic agents that are now used in the treatment of some mental disorders were developed. Among these agents, fluoxetine (Prozac) and methylphenidate (Ritalin) remain the most commonly prescribed drugs for treating depression and attention-deficit/hyperactivity disorder (ADHD), respectively.

Since Axelrod's initial experiments, thousands of studies have dealt with the pharmacological and functional properties of monoamine reuptake sites in the brain. With advances in molecular cloning techniques, the genes that code for the proteins that are responsible for monoamine reuptake were identified in the early 1990s, facilitating the development of several important areas of research. First, immunocytochemical and *in situ* hybridization approaches were rapidly developed to examine the localization of monoamine transporters (BOX 1). Second, the use of heterologous expression systems and mutagenesis has been crucial for the identification of the structural and functional domains of the transporters. Third, animal models with targeted disruption of monoamine transporter genes are now available, providing a unique opportunity to examine the contribution of these proteins to brain function. In addition, *in vivo* imaging approaches have become increasingly important for examining the changes in monoamine transporters that are associated with psychiatric and movement disorders.

So, the integration of pharmacological, neurochemical and molecular approaches has provided exciting opportunities to examine aspects of the function, cellular and subcellular localizations, and regulation of monoamine transporters under physiological and pathological conditions. Recent findings indicate that monoamine transporters are highly regulated at the cellular and molecular level. Furthermore, these studies highlight the primary role of monoamine transporters, not only in the regulation of the extracellular concentrations of monoamines, but also in the homeostatic maintenance of presynaptic function. Here, we present an overview of the progress

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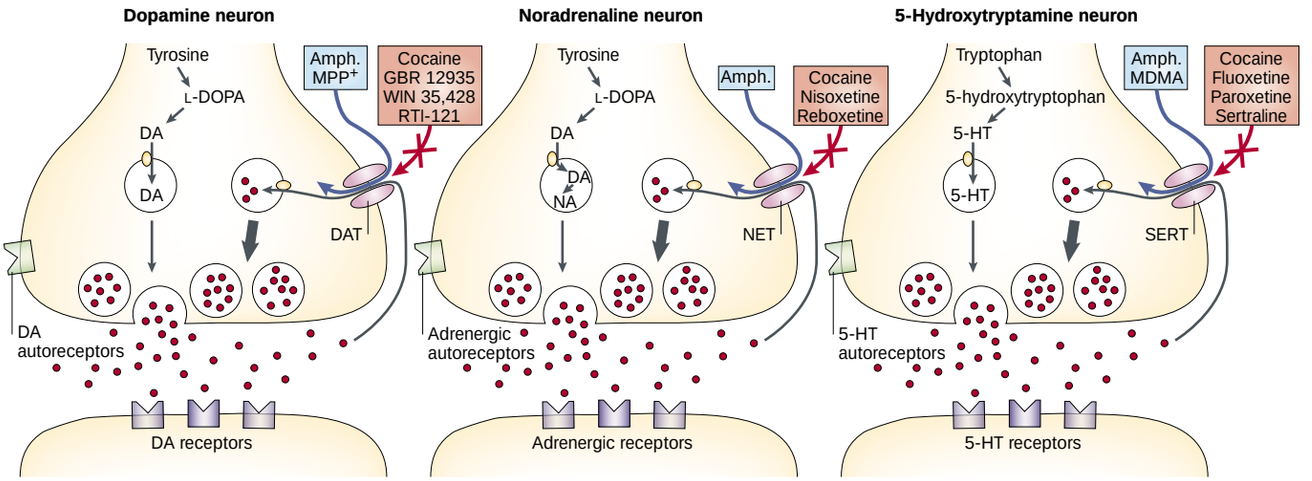


Figure 1 | **Schematic representation of dopamine, noradrenaline and 5-HT synaptic terminals.** Monoamine transporters are localized to perisynaptic sites, where they are crucial for the termination of monoamine transmission and the maintenance of presynaptic monoamine storage. Several selective pharmacological agents acting at each monoamine transporter are shown. Amph., amphetamine; DA, dopamine; DAT, Dopamine transporter; L-DOPA, L-3,4-dihydroxyphenylalanine; 5-HT, 5-hydroxytryptamine; MPP^+, 1-methyl-4-phenylpyridinium; MDMA, (+)-3,4-methylenedioxymethamphetamine; NA, noradrenaline; NET, noradrenaline transporter; SERT, 5-HT transporter.

made in the research on neurotransmitter transporters for dopamine, noradrenaline, and 5-HT in the central nervous system, emphasizing the most recent developments.

Pharmacology of monoamine transporters

Transporters for dopamine, noradrenaline, and 5-HT — DAT, NET and SERT, respectively — represent established targets for many pharmacological agents that affect brain function, including psychostimulants, antidepressants and neurotoxins[3–5] (FIG. 1). The sensitivity of monoamine transporters, for substrates and inhibitors, has been examined in brain preparations and in heterologous systems with recombinant transporters. In general, there is good agreement between these two approaches. Although all three monoamines are substrates of their cognate transporter, it has been shown that either DAT or NET can transport both dopamine and noradrenaline[6]. The heterologous uptake of dopamine by NET has been shown *in vivo* by MICRODIALYSIS[7] and in synaptosomal preparations from mouse cerebral cortex[8].

Box 1 | Localization and distribution of monoamine transporters

In the brain, monoamine transporters are expressed almost exclusively in the neurons that contain their cognate transmitter. *In situ* hybridization studies showed prominent dopamine transporter (DAT) expression in the cell bodies of the substantia nigra and ventral tegmental area (VTA), noradrenaline transporter (NET) expression in the locus coeruleus and other brainstem nuclei, and 5-HT transporter (SERT) expression in the median and dorsal raphe nuclei[161]. In the mouse brain, DAT-like immunoreactivity was detected in the striatum, nucleus accumbens, olfactory tubercle, nigrostriatal bundle and lateral habenula. In addition, cell bodies from neurons of the substantia nigra and VTA, as well as neuronal processes in the substantia nigra, in layers I, II and III of the cingulate cortex and in the medial prefrontal cortex, were DAT positive[54,162,163]. NET immunoreactivity in the brain was confined to noradrenergic somata, dendrites and axons within the hippocampus and cortex[164]. SERT immunoreactive fibers were found widely distributed throughout the brain, with the highest densities in regions that receive a dense serotonergic innervation, such as the cerebral cortex and the CA1 and CA3 regions of the hippocampus. Immunopositive staining for SERT revealed that it was also present in the cell bodies of the raphe nucleus[165,166]. In general, there is good agreement between the distribution of monoamine transporters by immunocytochemical approaches and the use of radioactive uptake inhibitors to label transporter sites.

The specific localization of monoamine transporters within synaptic terminals has also been examined[164,167–169]. Immunoelectron microscopy revealed that DAT, NET and SERT were localized at the plasma membrane, away from the synaptic area (peri-synaptic area) indicating that transmitter release at the synapse diffuses out of the cleft to be transported back into the terminal. These studies also found DAT, NET and SERT immunoreactivity that was associated with intracellular organelles of tubulo-vesicular structure.

Monoamine transporters have also been found in peripheral locations[170]. *In situ* hybridization and immunohistochemical studies indicate that DAT is expressed in the stomach, pancreas, and kidney[170]. NET is expressed in sympathetic peripheral neurons, the adrenal medulla, the lung and the placenta[170]. The SERT protein has been detected in platelets[171], the intestinal tract[172] and the adrenal gland[173]. In addition, SERT activity has been reported in astrocytes in culture[174].

MICRODIALYSIS
Analytical technique that is used to monitor extracellular levels of neurotransmitters or other molecules *in vivo*. A cannula is inserted into the brain and test solution is perfused through it. Dialysis takes place between the test and the extracellular solutions, making it possible to measure the transmitter levels at the tissue surrounding the tip of the cannula.

Table 1 | Synthetic inhibitors of monoamine transporters

Inhibitors*	DAT	NET	SERT
Cocaine	267	872	392
RTI-55	3.2	2.5	0.49
WIN 35,428	26.1	31.9	127
GBR 12935	21.5	225	6,514
Bupropion	2,784	1,389	45,026
Nisoxetine	477	5.1	383
Desipramine	78,720	4	61
Nortryptyline	13,920	3.4	161
Mazindol	27.6	3.2	153
Imipramine	24,576	67	7.7
Amytriptyline	3,000	100	14.7
Citalopram	10,000	>1,000	5.4
Paroxetine		312	0.25

Table modified from REF. 175 *Figures correspond to K_i values (inhibition of uptake of the substrate appropriate for the given trasporter; in nM).

Cocaine and amphetamines, two important psychostimulants, interact with monoamine transporters. Cocaine and other chemically related drugs are non-selective, competitive inhibitors of monoamine transporters[9]. By contrast, amphetamine-like drugs are substrates for monoamine transporters[10–12]. Once inside the synaptic terminal, these drugs act as weak bases at synaptic vesicles, causing the redistribution of vesicular monoamines into the cytoplasm, and a reversal in the direction of neurotransmitter transport at plasma membrane monoamine transporters[10–12]. As a result, the application of amphetamines induces a massive release of monoamines into the extracellular space. Although cocaine and amphetamines similarly affect at all three monoamine transporters, the behavioural and reinforcing effects of these psychostimulants seem to depend primarily on their interaction with DAT[13,14].

Several synthetic compounds, all with some degree of specificity for a monoamine transporter, have been developed (TABLE 1). For example, cocaine analogues, such as GBR 12935 and WIN 35,428, are selective DAT inhibitors. Fluoxetine (Prozac), citalopram (Celexa), paroxetine (Paxil) and sertraline (Zoloft) are selective inhibitors of SERT, whereas desipramine, nisoxetine and reboxetine are selective inhibitors of NET. Non-selective reuptake inhibitors include methylphenidate (Ritalin), TRICYCLIC ANTIDEPRESSANTS, nomifensine and bupropion. Many of these compounds are currently used in the treatment of neuropsychiatric disorders, including depression, ADHD, and eating disorders. Among the amphetamine-like compounds, only a few agents are known to be selective. They include 3,4-methylene-*N*-dioxymethamphetamine (MDMA, Ecstasy), which is relatively selective for SERT[15]. Another important pharmacological agent is 1-methyl-4-phenyl-1,2,3,6-tetrahydropridine (MPTP), a neurotoxin that causes a parkinsonian syndrome in humans and animals. In the brain, MPTP is converted to its principal metabolite, the 1-methyl-4-phenylpridinium ion (MPP^+), which is taken into dopamine neurons by DAT[16,17].

TRICYCLIC ANTIDEPRESSANTS
Molecules that inhibit monoamine reuptake, therefore prolonging the period during which these neurotransmitters are active at the synaptic cleft.

EXPRESSION CLONING
Cloning strategy that is based on the transfection of cDNAs such that functional proteins are expressed, followed by a screening of the functional activity of the gene of interest.

SPLICE VARIANTS
Further forms of a protein derived from alternative processing of its mRNA.

POLYMORPHIC VARIANTS
Genotypic variants that exist in the same population in frequencies that cannot be explained by recurrent mutations.

POLYTOPIC
A term that refers to a transmembrane protein that traverses the membrane two or more times.

Molecular characterization

A breakthrough in monoamine transporter biology came in 1991, when Susan Amara and her colleagues, using an EXPRESSION CLONING system, isolated a single cDNA clone that encoded the NET protein[18]. Comparison of the NET amino acid sequence with the cloned GABA (γ-aminobutyric acid) transporter that had been previously reported by Kanner and colleagues from the purified neuronal protein[19], revealed significant amino acid identity between these two transporters. These observations led to the identification of a new gene family for neurotransmitter transporter proteins. On the basis of conserved sequences from the GABA transporter and NET, several groups have isolated genes that code for DAT and SERT proteins in different species[20–41], as well as transporters for glycine, betaine, creatine, proline, taurine and several 'orphan' transporters, the substrates of which have yet to be identified[42].

The human *DAT* gene (*SLC6A3*) has been localized[26,43] to chromosome 5p15.3. It spans about 65 kb and is divided into 15 exons, but there is no evidence for RNA SPLICE VARIANTS[44,45]. The human *NET* gene (*SLC6A2*) is located on chromosome 16q12.2, it spans about 45 kb and consists of 14 exons[33,46]. Three splice variants with different carboxyl termini have been identified for the human *NET* gene[47]. Characterization of these *NET* isoforms in heterologous cells revealed that only two isoforms are functional[47,48]. However, a detailed analysis of the tissue distribution of these splice variants is still lacking. The gene for human *SERT* (*SLC6A4*) has been mapped to chromosome 17q11, it spans about 24 kb and contains 13 exons[28]. No RNA splice variants for the *SERT* gene have been identified. The cloning of the promoter region of the human *DAT*, *NET* and *SERT* genes has revealed the presence of several important elements for transcriptional regulation[44,49–52]. However, despite the clinical and physiological significance of monoamine transporter gene regulation, little is known about the transcriptional control mechanisms that govern their expression. POLYMORPHIC VARIANTS of monoamine transporters, in coding and non-coding regions, have recently been identified and are discussed below.

Hydrophobicity analysis of their deduced amino acid sequence indicates that monoamine transporters are POLYTOPIC membrane proteins, containing 12 putative transmembrane domains (TMDs) (FIG. 2). The human DAT, NET and SERT proteins contain 620, 617, and 630 residues, respectively (FIG. 2a). Conservation of amino acid sequences seems to be highest within the putative transmembrane domains, whereas the least conserved regions are at the amino and carboxyl termini (FIG. 2a). One large putative extracellular loop is positioned between TMD3 and TMD4, and has several potential N-glycosylation sites. Owing to the absence of a signal sequence, the amino and carboxyl termini of these proteins are proposed to reside within the cytoplasm (FIG. 2b). So far, all experimental data have confirmed this topological model for all three monoamine transporters[53–56] (FIG. 2b).

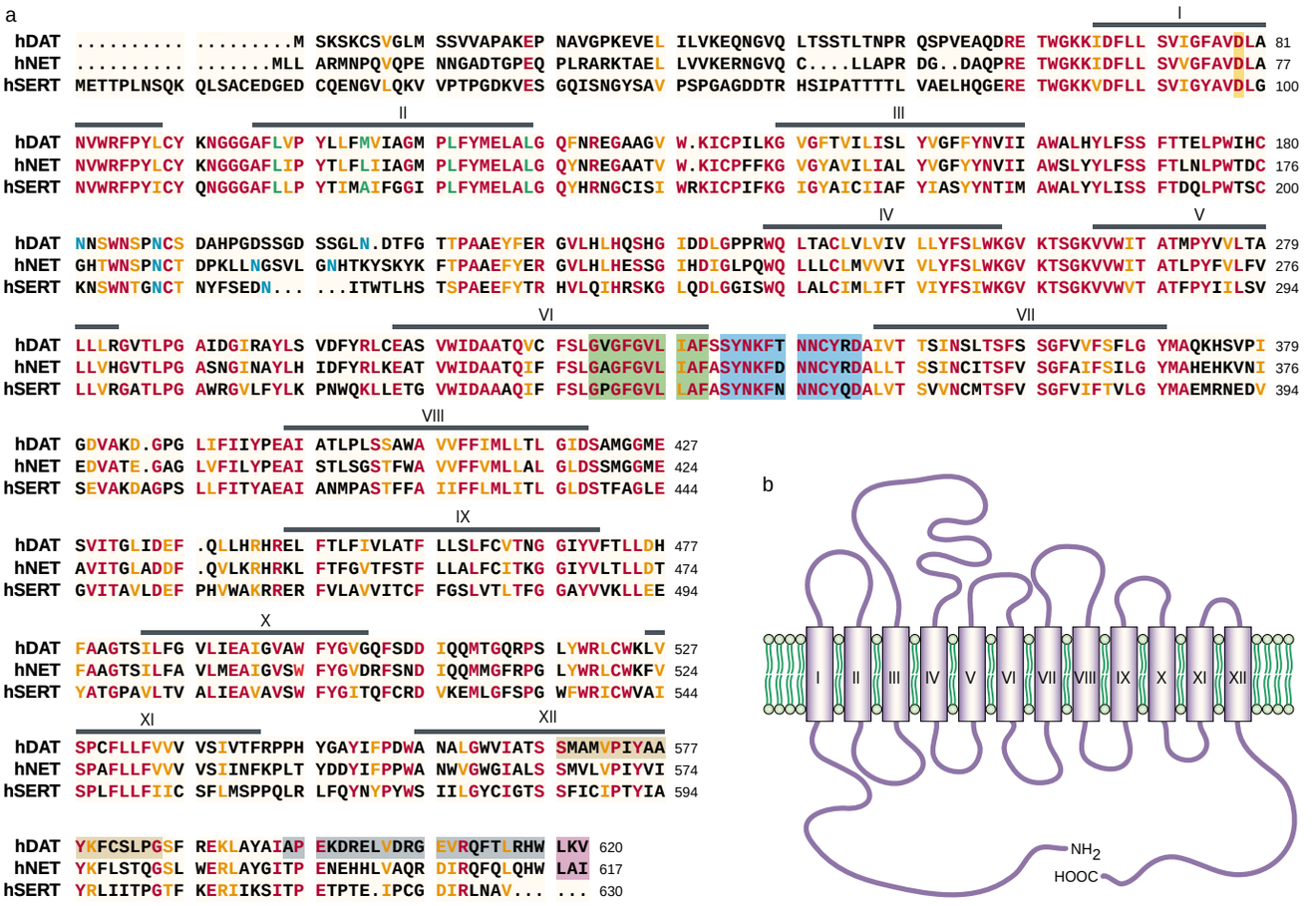


Figure 2 | **Amino acid sequence and topology of monoamine transporter proteins. a** | Amino acid alignment of the human dopamine transporter, noradrenaline transporter and 5-HT transporter. Identical residues are shown in red, whereas similar residues (V, L, and I, K and R, F and Y, or D and E) are shown in orange. Asparagine residues that form part of N-linked glycosylation consensus sequences are shown in blue. A conserved aspartate residue in transmembrane domain 1 that is presumably involved in the interaction with monoamines is shown as a yellow box. A leucine-repeat in transmembrane domain 2, and a glycophorin-like motif in transmembrane domain 6, are shown in green. The intracellular loop between transmembrane domain 6 and 7 contains several residues involved in conformational changes during substrate binding and translocation and is shown in blue. Black bars represent putative transmembrane domains. The colour boxes covering the parts of the intracellular carboxyl termini represent interacting sites with Hic-5 (beige), synuclein (grey), and PICK1 (purple). DAT, dopamine transporter; h, human; NET, noradrenaline transporter; SERT, 5-hydroxytryptamine transporter. **b** | Proposed topology of monoamine transporters depicting 12 transmembrane domains connected by intracellular and extracellular loops.

Structure/function analysis

The identification of residues and domains that participate in substrate binding and translocation has been an area of intense research. Three main approaches have been used to examine this problem: the analysis of CHIMAERIC transporters, SUBSTITUTED CYSTEINE ACCESSIBILITY METHOD (SCAM) and SITE-DIRECTED MUTAGENESIS.

On the basis of the differential pharmacological profiles of DAT and NET, two research groups have developed a series of chimaeric constructs between DAT and NET[6,57,58]. Giros and colleagues identified a discrete region between TMD6 and TMD8 as an important determinant in the affinity of cocaine and tricyclic NET inhibitors, whereas the carboxy-terminal region, encompassing TMD9 through the carboxy-terminal tail, seems to be responsible for the observed stereoselectivity and high affinity for substrates[6]. Using a similar set of chimaeric constructs, Buck and Amara arrived at comparable conclusions[57,58].

The localization of the transmitter translocation pathway proved to be more elusive. According to Giros and colleagues, the region containing the first five TMDs was involved in translocation and ion dependence[6], whereas Buck and Amara proposed that the region from TMD4 to TMD8 was involved in substrate translocation[58]. On the other hand, a series of chimaeras between human and bovine DAT revealed the role of TMD3 in dopamine uptake[59]. Together, these results highlight the complexity of the translocation mechanism and the limitations of this type of approach, as many chimaeras lacked functional activity.

Using SCAM analysis, a residue located in the third intracellular loop of DAT was found to be more reactive to thiol-modifying agents during uptake, implying that

CHIMAERIC
Molecules that are constructed from functional domains that belong to homologue and orthologue proteins. They are commonly used to identify the structural determinants of the functional properties in the parental proteins.

this residue participates in a conformational reorganization of DAT during substrate translocation[60,61]. Consistent with a role for this loop in substrate-mediated conformational changes in DAT, mutation of a tyrosine residue in the same third intracellular loop of DAT abolished uptake activity[62]. Interestingly, the function of the mutant transporter could be rescued by Zn^{++}, which binds to the extracellular face of the transporter[63]. These results not only suggest a role for the third intracellular loop in the translocation pathway, but also indicate that the transporters undergo ligand-mediated conformational changes between active and inactive states.

Given the structure of monoamines (an aromatic ring containing an amine group), researchers have focused on hydrophobic and charged residues of the TMDs that might interact with the aromatic ring or the amine group. Only a few conserved, charged residues are predicted to lie within TMDs. Mutation of a conserved aspartic acid in TMD1 (Asp79 in human DAT) markedly reduces dopamine uptake activity and the ability of dopamine to inhibit the binding of a cocaine analogue[64], but does not alter the cell surface levels of the mutant protein. In NET and SERT, replacement of the corresponding aspartic acid with alanine, glycine or asparagine residues abolished transporter activity and, in the case of SERT, decreased the affinity for cocaine-related compounds[65]. Together, these results indicate that the aspartic acid in TMD1 is crucial for substrate recognition, presumably through an interaction between its carboxyl group and the positive charge of the amine group of monoamines. Interestingly, this residue is only conserved in monoamine transporters. In other transporter proteins from the same family, a glycine occupies this position. Chen and collaborators have also provided evidence for a potential role for the DAT residues Asp313, Asp435, and Asp476 in substrate recognition[66].

In the search for interacting residues with the aromatic ring of dopamine, most phenylalanine and tryptophan residues that are predicted to lie within the putative TMDs of DAT have been mutated[66–68]. The most profound effect was observed when a phenylalanine, predicted to lie within TMD3, was replaced by an alanine. The Phe155Ala transporter mutant showed a marked decrease in the affinity for dopamine and an analogue of cocaine, compared to the wild-type transporter[66]. In addition, the affinity of the neurotoxin MPP^+ for DAT seems to be influenced by residues within TMD7, TMD11 and TMD12[57,69].

In the case of SERT, the analysis of cross-species chimaeric constructs identified a residue in TMD12 as an important determinant in the affinity of the tricyclic uptake inhibitor imipramine[70], whereas residues in TMD1 were found to determine selectivity for the SERT inhibitor citalopram[71]. A different set of chimaeric constructs, in which part of the second loop in the SERT sequence was replaced by the corresponding sequence in NET, revealed a marked reduction in uptake activity, implying that the second extracellular domain might be involved directly, or indirectly, in substrate translocation[72]. Recently, chimaeras between the human SERT and the SERT from the moth *Manduca sexta* have revealed the importance of the amino terminal domain, which includes TMD1 and TMD2, in cocaine sensitivity[41]. Also, chimaeric constructs between human and bovine SERTs identified Met180, Tyr495, and Phe513 as important molecular determinants of inhibitor interactions[73].

Using SCAM analysis, Chen and colleagues investigated the role of 20 residues, from TMD3 of SERT, in transporter activity[74,75]. Mutant transporters in which Ile172 and Ile179 were replaced one at a time by cysteine residues were functional, but were readily inactivated by the membrane impermeable cysteine-modifying reagent (2-(trimethylammonium)ethyl)methanethiosulphonate (MTSET). The Ile172Cys mutant transporter, but not the Ile179Cys mutant, was protected from MTSET inactivation by 5-HT or cocaine, indicating that Ile172 participates directly in the binding to 5-HT and cocaine. Although Ile179 is not directly involved in substrate binding, the accessibility of this residue was found to be sensitive to conformational changes that result from substrate binding and translocation[74,75]. Together, these results indicate that the TMD3 of SERT is crucial for 5-HT and cocaine binding, and is located in the translocation pathway. Consistent with a role for the third intracellular loop of these transporters in substrate translocation, a reactive cysteine in the third intracellular loop of SERT (Cys357) was found to be sensitive to conformational changes that result from ion and ligand binding[76]. In addition, several other functional residues in SERT have been identified[77–79].

Despite the various approaches that have been used to map the substrate interaction site of monoamine transporters and the translocation pathway, it is fair to say that a clear picture of how the initial docking of the substrate is translated into the movement of the molecule across the cell membrane is still lacking.

Transport mechanisms

The observation that extracellular Na^+ ions were a necessary requirement for substrate uptake in all three monoamine transporters provided one of the first insights into the transport mechanism[80,81]. It is now well established that the mechanism by which transporter proteins mediate monoamine uptake involves sequential binding and co-transport of Na^+ and Cl^- ions. The driving force for transporter-mediated monoamine uptake is the ion concentration gradient that is generated by the plasma membrane Na^+/K^+ ATPase. In the case of DAT, two Na^+ ions and one Cl^- ion are transported with the substrate, whereas NET and SERT co-transport their respective substrates with one Na^+ ion and one Cl^- ion. The outward transport of K^+ ions is involved in the translocation mechanism of SERT. Rudnick and colleagues have proposed a model in which the binding of 5-HT, Na^+ and Cl^- to SERT induces a conformational change in the transporter, allowing the exposure of the binding site on the opposite site of the membrane, with the concomitant translocation of the substrate and ions.

SUBSTITUTED CYSTEINE ACCESSIBILITY METHOD (SCAM). A method used to identify the residues that are probably exposed to a solvent, such as those that line the pore of a channel. It is based on the systematic replacement of native amino acids by cysteines to then test the ability of hydrophilic molecules to react with the added cysteines. If a cysteine is accessible to the hydrophilic reagent, then channel permeability will be affected.

SITE-DIRECTED MUTAGENESIS The generation of a mutation at a predetermined position in a DNA sequence. The most common method involves the use of a chemically synthesized mutant DNA strand that can hybridize with the target molecule.

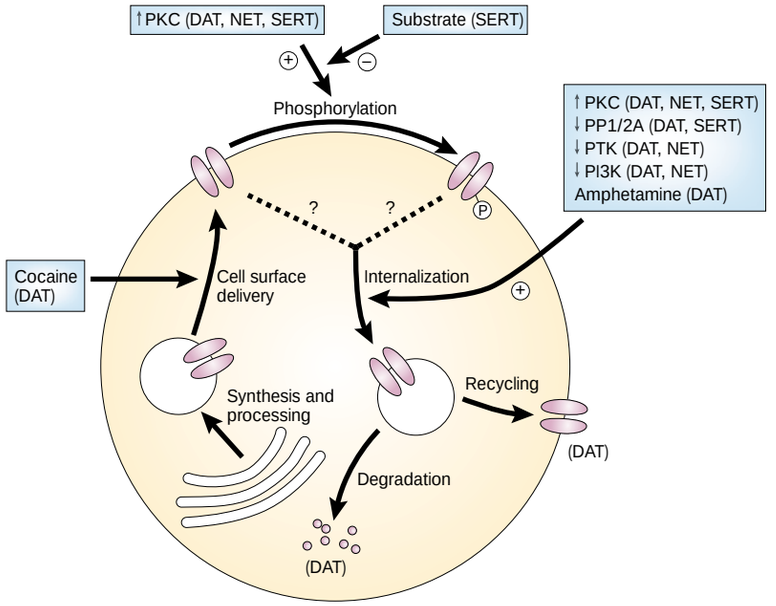


Figure 3 | **Schematic representation of the trafficking mechanisms associated with plasma membrane monoamine transporters.** After being synthesized, monoamine transporters are delivered to the cell surface. Activation of protein kinase C induces the phosphorylation and internalization of all three transporters. However, it is not clear whether phosphorylation of transporters is required for the internalization mechanism. Some pharmacological manipulations have been shown to regulate different steps of the trafficking of monoamine transporters and are shown in boxes. DAT, dopamine transporter; NET, noradrenaline transporter; PI3K, phosphoinositol-3 kinase; PKC, protein kinase C; PP1/2A, protein phosphatase 1/2A; PTK, protein tyrosine kinase; SERT, 5-hydroxytryptamine transporter.

The reorientation of the transporter would require an additional step, involving binding and outward transport of intracellular K^+ ions. Under certain pharmacological conditions, monoamine transporters can function in reverse — that is, substrates are transported from the intracellular to the extracellular compartment. This mechanism is important when considering the actions of amphetamines, which induce a massive release of monoamines by reversing the actions of monoamine transporters[10–12,82].

In addition to the classic substrate transport mechanism, transporters also exhibit channel-like activity. Electrophysiological techniques, using recombinant transporters expressed in heterologous cells, have revealed conductances that cannot be accounted for by the fixed stoichiometry of substrate translocation[83–85]. For all three monoamine transporters, uptake inhibitors block these uncoupled conductances. Whether these conductances are sufficient to alter membrane potential, and whether they participate in the effect of antidepressants and psychostimulants, are questions that are being addressed. Recently, Ingram and co-workers have identified a chloride conductance elicited by DAT substrates in midbrain dopamine neurons in culture[86]. Activation of this chloride conductance increased cell excitability in dopamine neurons and has been proposed as an alternative mechanism to regulate dopamine release.

CONCATAMER
A linear array of identical molecules that are covalently linked in tandem. They can be useful to determine the stoichiometry of homomeric macromolecular complexes.

TAGGED PROTEIN
The lack of specific antibodies against many proteins makes it necessary to develop alternative methods for their visualization. Tagging the amino or carboxyl termini of proteins with short peptides that can then be used as epitopes is one of the most commonly used approaches.

FLUORESCENCE RESONANCE ENERGY TRANSFER
(FRET). A spectroscopic technique that is based on the transfer of energy from the excited state of a donor moiety to an acceptor. The transfer efficiency depends on the distance between the donor and the acceptor.

Regulation of transporter function

Transporter oligomerization. Although most models of transporter function have assumed that transporters function as single subunits, early studies using radiation inactivation and crosslinking experiments indicated that monoamine transporters might exist as oligomers[87,88]. Biochemical, immunological and functional approaches have recently been used to investigate the oligomeric nature of monoamine transporters. Heterologous expression of SERT CONCATAMERS revealed uptake activity for both transporter dimers and tetramers[89]. In HEK 293 cells, flag-TAGGED and myc-tagged SERT proteins can co-immunoprecipitate, providing evidence for the physical association of SERT monomers[90]. In addition, cells co-expressing wild-type SERT in combination with a mutant SERT that conferred resistance to a cysteine modifying reagent, yielded a functional phenotype that was consistent with the idea that an oligomeric transporter complex was formed between wild-type and mutant transporter proteins[90]. Oligomers of SERT were also detected at the plasma membrane of living HEK 293 cells by FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) microscopy[91].

The oligomerization of DAT has also been examined. Hastrup and colleagues observed DAT dimers as a result of a symmetrical crosslinking between cysteine residues located at the extracellular face of TMD6[92]. Mutations of two conserved glycines that form a GLYCOPHORIN-like motif in TMD6 support a role for this domain in transporter oligomerization. We have also obtained evidence for DAT oligomerization using DOMINANT-NEGATIVE mutants of the transporter[93]. Non-functional mutant transporters inhibited wild-type DAT activity, either by interacting with the wild-type transporter at the plasma membrane or by interfering with the normal processing of wild-type DAT proteins. Mutations in the LEUCINE REPEAT of TMD2 abolish transporter delivery to the plasma membrane and interaction with wild-type DAT. We have proposed that TMD2 might be important for transporter assembly, and that the oligomerization process is essential for the trafficking of the transporter to the cell surface[93].

Post-translational regulation. Analysis of the amino acid sequence of monoamine transporters reveals the presence of several consensus sites for protein kinase phosphorylation by cAMP-dependent protein kinase, protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase. It is therefore not surprising that many research groups have turned their attention towards the role of phosphorylation in transporter function. The best-documented effect is the downregulation of transporter activity by acute PKC activation in native preparations and expression systems[94–102]. In heterologous cells, this modulation occurs largely owing to the redistribution of the transporter proteins from the cell surface to intracellular compartments, rather than to changes in the intrinsic transport activity (FIG. 3). A recent study by Daniels and Amara indicates that the PKC-induced internalization of DAT is CLATHRIN-mediated and DYNAMIN-dependent[101], a mechanism that

GLYCOPHORIN
Protein with a single transmembrane domain that has been shown to mediate the non-covalent dimerization of this molecule.

DOMINANT NEGATIVE
A mutant molecule that can form a heteromeric complex with the normal molecule, knocking out the activity of the entire complex.

LEUCINE REPEAT
A leucine-rich domain within a protein that binds to other proteins with a similar domain.

CLATHRIN
A major structural component of coated vesicles that are implicated in protein transport. Clathrin heavy and light chains form a triskelion, the main building element of clathrin coats.

DYNAMIN
A GTPase that takes part in endocytosis. It seems to be involved in severing the connection between the nascent vesicle and the donor membrane.

PDZ DOMAIN
A peptide-binding domain that is important for the organization of membrane proteins, particularly at cell–cell junctions, including synapses. It can bind to the carboxyl termini of proteins or can form dimers with other PDZ domains.

YEAST TWO-HYBRID SYSTEM
A system used to determine the existence of direct interactions between proteins. It involves the use of plasmids that encode two hybrid proteins; one of them is fused to the GAL4 DNA-binding domain and the other one is fused to the GAL4 activation domain. The two proteins are expressed together in yeast; if they interact, then the resulting complex will drive the expression of a reporter gene, commonly β-galactosidase.

LIM HOMEODOMAIN
A domain that is found in several proteins, many of which function as transcription factors. Many LIM-homeodomain-containing proteins are involved in developmental processes or cell differentiation.

closely resembles the internalization process of G protein-coupled receptors. Whether internalized transporters recycle back to the plasma membrane, or are degraded inside the cell, is still not clear[101,102]. In an elegant series of experiments, Ramamoorthy and colleagues showed that SERT phosphorylation and internalization were blunted by transporter occupancy[103] (FIG. 3). These findings point to a mechanism by which substrates can rapidly increase monoamine clearance by preventing transporter internalization. Consistent with this idea, recent studies in heterologous cells have shown that cocaine and amphetamines can also influence the trafficking of DAT[104–106].

An unresolved issue in the trafficking of monoamine transporters is whether phosphorylation of the transporter proteins is required for internalization. Removal of PKC consensus sites from DAT and SERT do not prevent downregulation of transporter activity by PKC activation[107,108]. Foster and colleagues recently reported that the amino terminus of DAT, which contains a cluster of serine residues, is the primary site of phosphorylation by PKC[109]. The role of such residues in transporter function awaits further experimentation. Monoamine transporter function is also regulated by tyrosine kinases, phospatidylinositol 3-kinase (PI3K), arachidonic acid, nitric oxide, MAP (mitogen-activated protein) kinases and ethanol[110] (FIG. 3). However, the exact mechanism by which these pathways affect transporter function remains largely unknown. On the other hand, chronic treatment with antidepressants is known to induce downregulation of transporter function. For example, chronic exposure to NET inhibitors results in reduction in NET binding and protein levels in cells expressing NET[111,112]. Similarly, chronic antidepressant treatment results in a decrease in SERT binding, function and protein levels without affecting mRNA levels[113,114]. These findings indicate that chronic antidepressant treatment might induce an increase in the rate of degradation of transporter proteins.

The involvement of N-linked glycosylation in transporter expression and function has also been investigated. Several potential consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr) are found in the large extracellular loop, between TMD3 and TMD4, of all monoamine transporters. Kuhar and colleagues found differences in the glycosylation pattern of DAT in different brain regions (nucleus accumbens versus striatum)[115], in different species[116] and during development[117]. More recently, the contribution of N-linked glycosylation to monoamine transporter function has been examined using site-directed mutagenesis. N-linked glycosylation seems to be important for the normal expression of monoamine transporters at the cell surface, but not for ligand binding or translocation of substrate[93,118–120].

Protein–protein interactions. Two observations were the starting point that initiated the search for proteins that interact with monoamine transporters. Compelling evidence has shown that monoamine transporters undergo regulated trafficking in cells, indicating the possible involvement of transporter-interacting proteins. In addition, the specific localization of monoamine transporters to perisynaptic sites of nerve terminals (BOX 1) presumably involves interactions with intracellular proteins that target transporters to these sites. Monoamine transporters contain sequence motifs that are known to direct protein–protein interactions, including a leucine repeat in TMD2, a PDZ binding site at the extreme carboxyl termini, and putative tyrosine-based and di-leucine internalization motifs.

Using approaches such as co-immunoprecipitation and the YEAST TWO-HYBRID SYSTEM, an increasing number of interactions are emerging, although the physiological significance of these interactions is not completely understood. Bauman and Blakely found that the protein phosphatase 2A (PP2A) could be co-immunoprecipitated from brain tissue with DAT, NET, or SERT proteins[121]. In the case of SERT, the interaction with PP2A was dynamically regulated by phosphatase inhibition, PKC activation and transporter substrates. Although it is not known whether PP2A binds directly to monoamine transporters, this association might help to explain the molecular events that are responsible for transporter phosphorylation and internalization. An *in vitro* interaction between the carboxyl terminus of SERT and a homologue of MARCKS (myristoylated alanine-rich C-kinase substrate) — MacMARCKS — was identified using the yeast two-hybrid system[122]. It is not clear whether DAT and NET also bind to MacMARCKS, and whether the interaction between SERT and MacMARCKS occurs *in vivo*.

Lee and colleagues, using the yeast two-hybrid system, have identified a direct interaction between DAT and α-synuclein[123], a presynaptic protein that has been implicated in Parkinson's disease[124]. α-Synuclein increases DAT activity when it is co-expressed with DAT in heterologous cells. Consequently, the MPP^+-mediated cellular toxicity through DAT was increased in the presence of α-synuclein[125]. On the basis of these findings, a role for DAT in mediating selective degeneration of dopamine neurons, through its interaction with α-synuclein, has been proposed.

We have recently shown an interaction between the PDZ domain-containing protein PICK1 (protein that interacts with C kinase) and the carboxyl termini of DAT and NET[126] (FIG. 4). In culture, PICK1 co-localizes with DAT and NET in dopaminergic and noradrenergic neurons, respectively. In addition, PICK1 induces DAT and NET clustering in transfected cells and increases the activity of these transporters, therefore implying that PICK1 might participate in the targeting of DAT and NET to nerve terminals (FIG. 4). In cultured neurons, a DAT mutant lacking the PICK1 binding site fails to localize to neuronal processes and remains localized mainly in cell bodies. As PICK1 has been shown to interact with PKC, this raises the possibility that PICK1 might also be involved in the PKC-mediated trafficking of monoamine transporters. More recently, we showed an interaction between DAT and the LIM HOMEODOMAIN-containing protein Hic-5 (REF. 127). As Hic-5 interacts with several signalling molecules, including the glucocorticoid receptor and the

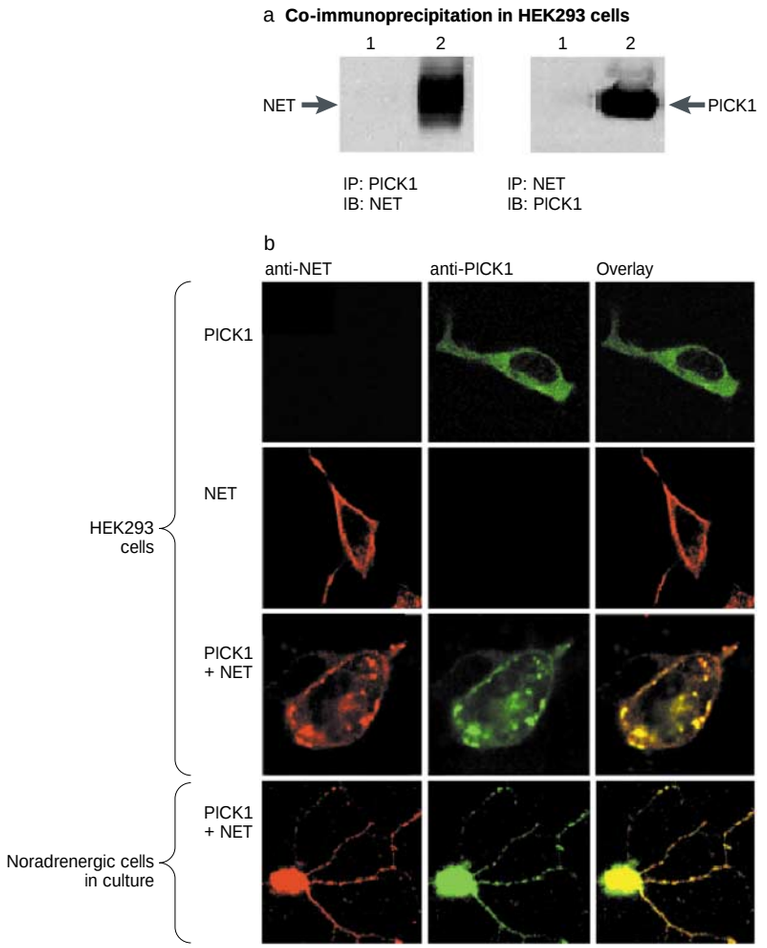


Figure 4 | **Interaction between monoamine transporters and the PDZ domain-containing synaptic protein PICK1. a** | The carboxyl termini of monoamine transporters contain a class II PDZ binding site responsible for the interaction with PICK1. NET and PICK1 co-immunoprecipitate from transfected HEK 293 cells. IB, immunoblotting; IP, immunoprecipitation. **b** | NET and PICK1 co-localize and form clusters in transfected HEK 293 cells and in neurons in culture from mouse locus coeruleus. Reproduced, with permission, from REF. 126 © 2001 Elsevier Science. NET, noradrenaline transporter; PICK, protein that interacts with C kinase.

non-receptor protein tyrosine kinases — focal adhesion kinase (FAK) and Fyn — this observation raises the possibility that this adaptor protein might link DAT to intracellular signalling pathways.

Genetic approaches to transporter function

The disruption of monoamine transporter genes in mice, by knockout technologies, has provided an opportunity to investigate the role of these proteins *in vivo*. Recently, our laboratory and others have developed and characterized mice lacking DAT (DAT-KO)[128], NET (NET-KO)[129] or SERT (SERT-KO)[130]. The elimination of the *DAT* gene in mice resulted in a 300-fold increase in the persistence of dopamine in the extracellular space of striatal tissue, indicating that diffusion is the only relevant mechanism that can clear released dopamine in these mice. The striatal dopamine content in DAT-KO mice was reduced by 95%, but the extracellular dopamine concentration was increased 5-fold[128,131] (FIG. 5). These findings show that DAT is essential for controlling the levels of extracellular dopamine in the brain as, in the absence of dopamine re-uptake, no other mechanism can compensate to maintain the homeostatic control of presynaptic function. In addition, these mice represent an excellent genetic model of persistent functional hyperdopaminergia[132,133].

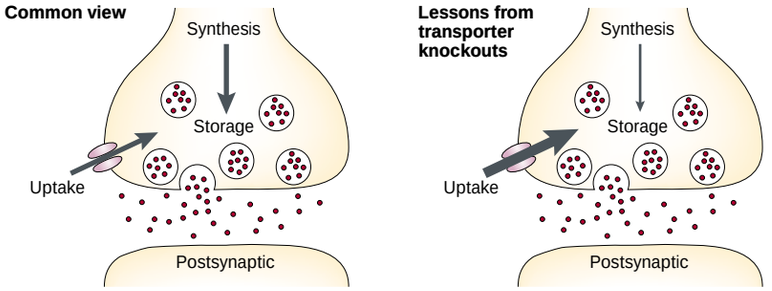
Similar to the findings obtained with the DAT-KO mice, NET-KO mice showed profound alterations in presynaptic homeostasis[129]. In noradrenaline-enriched regions, including the prefrontal cortex, the hippocampus and the cerebellum, the total tissue content of noradrenaline was 55–70% lower than in wild-type animals, and the clearance rate of released noradrenaline was at least 6-fold slower[129] (FIG. 5). These data are also consistent with the idea that diffusion is the main mechanism for noradrenaline clearance in the knockout mice. As a result, the extracellular levels of noradrenaline in the NET-KO mice were increased 2-fold. These observations indicate that NET is also required for noradrenaline presynaptic homeostasis.

The SERT-KO mice showed a 6-fold elevation in the extracellular levels of 5-HT and a marked reduction (60–80%) in 5-HT tissue content in several brain regions[130,134] (FIG. 5). The profound neurochemical alterations that were observed in all three mutant mice show that plasma membrane monoamine transporters are crucial in presynaptic homeostasis at two levels. First, monoamine transporters are the primary mechanism for terminating neurotransmission by removing the released transmitter from the synaptic cleft. Second, the reuptake process by these transporters is the main mechanism for replenishing transmitter stores[133] (FIG. 5).

In addition to changes in monoamine homeostasis, the analysis of transporter knockout animals reveals alterations in pre- and postsynaptic receptor responsiveness. In DAT-KO animals, the protein and mRNA levels for two important postsynaptic dopamine receptors — D1 and D2 — were downregulated[128,133]. In the hippocampus of NET-KO mice, the postsynaptic α1 adrenergic receptor was downregulated[129]. In the case of SERT-KO, postsynaptic 5-HT receptors were also downregulated[133,135]. So, in all three knockout mice, compensatory changes that occur in response to elevated extracellular concentrations of monoamines result in a significant changes in postsynaptic receptor levels and responsiveness[133].

Monoamine transporters in drug addiction

Cocaine and amphetamine are important psychostimulants, the abuse of which has profound medical and social implications worldwide. In the brain, cocaine acts as an inhibitor of monoamine transporters, whereas amphetamines act as substrates of monoamine transporters, displace monoamines from synaptic vesicles and induce the reversal of the transporters. As a result, cocaine and amphetamines increase the extracellular levels of monoamines. The large increase in synaptic dopamine levels that are produced by these drugs result in continuous stimulation of target neurons, an event that is believed to be



Genotype	Extracellular lifetime (fold increase)	Release (% decrease)	Extracellular levels (fold increase)	Storage (% decrease)	Synthesis (fold increase)
DAT$^{-/-}$	300	75–95	5	95	2
NET$^{-/-}$	6	60	2	55–75	1.7
SERT$^{-/-}$	↑	ND	6	65–80	↑

Figure 5 | **Role of monoamine transporters in presynaptic homeostasis — lessons from knockout mice.** In mice that lack DAT, NET or SERT, the extracellular lifetime and levels of monoamine are increased, but intracellular monoamine storage and evoked monoamine release are decreased. Note that the increased monoamine synthesis that is found in these mutants can not replenish the intracellular monoamine stores, owing to the lack of a monoamine recycling mechanism. So, transporter-mediated reuptake of monoamines is critical for the adequate maintenance of intracellular transmitter stores. DAT, dopamine transporter; ND, not determined; NET, noradrenaline transporter; SERT, 5-hydroxytryptamine transporter.

responsible for the rewarding effects of cocaine and amphetamine. The identification of DAT as the 'cocaine receptor'[9] enhanced our understanding of the basic mechanisms of addictive processes[132] and became one the most important findings to support the dopamine theory of addiction[13,14].

Analysis of both the locomotor and the rewarding properties of psychostimulants in monoamine transporter knockout animals provided important insights into the complex mechanisms of action of these drugs (TABLE 2). In DAT-KO mice, cocaine had a robust, albeit altered, rewarding effect, as shown by studies of cocaine self-administration[136] and cocaine-induced PLACE PREFERENCE[137,138]. The analysis of extracellular dopamine by microdialysis in DAT-KO mice indicated that cocaine and amphetamine were not effective in increasing dopamine levels in the striatum[12,136]. However, these drugs could elevate synaptic dopamine levels in the nucleus accumbens, a brain area that is involved in addictive processes[139]. Given the fact that dopamine uptake in brain regions with low levels of DAT might depend partially on NET[8], it has been proposed that the effect of cocaine in the nucleus accumbens of DAT-KO mice could be mediated by the blockade of dopamine uptake through NET[139]. However, this seems unlikely, as NET inhibitors do not affect dopamine clearance when they are applied directly to the nucleus accumbens in the DAT-KO mice, as shown by FAST SCAN CYCLIC VOLTAMETRY[140]. More consistent with these observations would be an indirect modulatory action on dopamine neurons, possibly through serotonergic or noradrenergic inputs at the level of neuronal cell bodies in the ventral tegmental area (VTA), leading to enhanced dopamine release in the nucleus accumbens.

Mice lacking SERT display an increased place preference in response to cocaine[137] but, interestingly, no place preference for cocaine was observed in double knockout mice lacking both DAT and SERT[138] (TABLE 2), indicating that serotonergic and dopaminergic mechanisms are both required for the rewarding effects of cocaine. Conversely, in double knockout mice lacking both SERT and NET, cocaine place conditioning was significantly enhanced[141], implying that the actions of cocaine on the NET system might have aversive effects. So, it seems possible that in DAT-KO mice, the action of cocaine at SERT is sufficient to induce its rewarding effects. However, it should be emphasized that the extracellular levels of dopamine are already elevated in DAT-KO mice[131], and that under these conditions, SERT inhibition might induce rewarding properties.

Paradoxical observations have also been made when locomotor responses to psychostimulants were investigated in DAT-KO mice. It is well established that psychostimulants induce an increase in locomotor activity in response to the elevated dopamine levels caused by these drugs. However, in DAT-KO mice, which are already hyperactive, amphetamine, methylphenidate and cocaine all decreased locomotor activity[142]. Similar effects were observed with the SERT inhibitor fluoxetine, but not with the NET inhibitor nisoxetine, supporting an inhibitory role for 5-HT on dopamine-dependent behavioural activation[142]. Interestingly, psychostimulants have been used as the preferred therapeutic strategy to produce an inhibitory or 'calming' effect on the behavioural manifestations of hyperactivity disorders such as ADHD. Mice that lack DAT recapitulate several features that are typical of this disorder. They display PERSEVERATIVE ERRORS in cognitive tasks, are hyperactive, and their increased locomotor activity is inhibited by the psychostimulants that are commonly used to treat ADHD. Taking into account these observations and the evidence for an association of the polymorphism in the DAT gene with ADHD, we have suggested that similar molecular events might underlie some of the therapeutic effects of psychostimulants in patients with this disorder[133,142].

Monoamine transporters in disease

Monoamine transporter genes have received considerable attention as candidate genes for psychiatric and neurological disorders. Three lines of evidence support this idea. First, DAT, NET and SERT are important target sites for therapeutic agents that are used in the treatment of mood disorders. Second, the levels of monoamine transporters, measured by brain imaging techniques *in vivo* or binding techniques in post mortem brain samples, have been shown to be altered in several psychiatric and neurological conditions, such as Parkinson's disease, ADHD and depression. Third, polymorphic variations in monoamine transporter genes have been proposed to be associated with susceptibility to conditions such as ADHD and depression.

PLACE PREFERENCE
In this experimental model, a drug with a rewarding effect is injected to an animal, which is then placed in a chamber with specific environmental cues. Over time, the animal develops an association between the rewarding effect of the drug and the environmental cues. In this way, if the animal is given the choice between a chamber containing drug-associated cues and a chamber with neutral cues, it spends more time in the chamber with the drug-associated cues.

FAST SCAN CYCLIC VOLTAMETRY
An analytical technique for the real-time measurement of evoked monoamine release and clearance in extracellular brain fluid.

PERSEVERATIVE ERRORS
Cases in which a subject sticks to a specific strategy when solving a problem despite the fact that the strategy is wrong or the rule of the task has changed.

Table 2 | Cocaine CPP in monoamine transporter knockouts

Genotype	Effect	References
$DAT^{-/-}$	Present	156
$NET^{-/-}$	Increased	146
$SERT^{-/-}$	Increased	156
$DAT^{-/-}$, $NET^{-/-}$	Not determined	
$DAT^{-/-}$, $SERT^{-/-}$	Abolished	157
$NET^{-/-}$, $SERT^{-/-}$	Greatly increased	160

CPP, conditioned place preference.

In vivo brain imaging techniques, such as positron emission tomography and SINGLE-PHOTON EMISSION COMPUTED TOMOGRAPHY using selective DAT ligands, have been used to monitor monoamine transporter levels in neurodegenerative and psychiatric conditions[143]. In healthy people, recent studies have shown age-related decreases in the levels of DAT[144]. Studies have also shown that DAT densities are affected in several brain disorders, including Parkinson's disease[145], WILSON'S DISEASE[146], LESCH–NYHAN DISEASE[147], TOURETTE'S SYNDROME[148], major depression[149] and ADHD[150]. To a lesser extent, SERT proteins have also been examined by imaging approaches. Midbrain SERT levels were reduced in patients with impulsive aggressive behaviour[151], alcoholism[152] or major depression[153]. For NET proteins, there are no satisfactory ligands for brain imaging studies. Using brain tissue collected post mortem, Klimek and colleagues reported that the NET levels are reduced in the locus coeruleus in people with major depression[154]. However, when interpreting these observations, it is important to consider one limitation of these approaches; namely, that it is not clear whether the observed changes in transporter densities are part of the cause of the disease, or whether they result from compensatory mechanisms. Also, studies using patient populations cannot exclude the possibility that therapeutic agents given to these patients might directly or indirectly affect the levels of monoamine transporters.

Family and twin studies have provided evidence for a strong genetic component in the aetiology of some psychiatric disorders, prompting the search for candidate genes that are involved in brain disorders. The identification of polymorphic variants in the coding and non-coding regions of monoamine transporters has opened up the possibility that transporter polymorphisms might be associated with human disorders. Several coding and non-coding polymorphic variants for the *DAT* gene have been found. Among these variants, ten different alleles, containing from 3 to 13 copies of a 40-base pair (bp) tandem repeat that is located in the 3′ untranslated region of the transporter, have been detected in humans[44]. Recent genetic studies indicate that the 10-repeat allele is associated with ADHD[155].

Several polymorphisms in the coding and non-coding region of *NET* have also been reported, but on the basis of the available genetic association studies, it is unlikely that any of these *NET* variants are related to brain disorders. In the case of SERT, polymorphisms in the 5′-flanking promoter region of the *SERT* gene have been identified. The reported polymorphisms consist of a multi-allelic 17-bp tandem repeat and an insertion/deletion of a 44-bp sequence, resulting in long (L) or short (S) alleles. The S variant has been shown to reduce the transcriptional efficiency of the *SERT* gene promoter, leading to decreased SERT expression and 5-HT uptake[156]. These polymorphisms might be associated with susceptibility to depression, obsessive–compulsive disorder and ADHD[156–159]. Although several single-nucleotide polymorphisms that result in amino acid changes have been reported for all three monoamine transporters, so far none of these naturally occurring mutations have been linked to a psychiatric or neurological condition. A single mutation in the coding sequence of *NET* has been linked to ORTHOSTATIC INTOLERANCE[160].

Concluding remarks

In recent years, findings using molecular, pharmacological, and genetic techniques have not only established the importance of monoamine transporters in brain function, but have also shown that these proteins are tightly regulated. Pharmacological and genetic studies have shown us that monoamine transporters are indispensable for the control of extracellular monoamine concentrations, the maintenance of presynaptic homeostasis and the actions of psychostimulants, including cocaine and amphetamines. Electron microscopy studies have revealed the strategic targeting of monoamine transporters to perisynaptic sites away from neurotransmitter release sites. At the molecular level, we now believe that these transporters function as oligomeric complexes at the cell membrane, and that they are regulated primarily through phosphorylation, protein–protein interactions and trafficking events. As we continue to decipher the fascinating mysteries of monoamine transporter biology, several important issues remain unsolved.

The increasing number of proteins being identified as interacting with monoamine transporters provides evidence for a more complicated degree of organization for these transporters than was previously anticipated. Proteomic and genetic approaches might be necessary to determine the network of proteins that are associated with monoamine transporters. A clear understanding of all the factors that contribute to monoamine transporter regulation is also needed. As we have learned about the importance of transporters in the control of neuronal homeostasis, it will be important to establish whether alterations in expression levels or post-translational regulatory mechanisms that are associated with monoamine transporters might contribute to brain disorders. We have also learned that transporters can function in reverse and that they possess channel-like activity. However, the physiological significance of these mechanisms is not known. Research aimed at answering these questions will produce a better understanding of monoamine transporter function, and will hopefully generate novel therapeutic strategies for the management of psychiatric and neurological illnesses.

SINGLE-PHOTON EMISSION COMPUTED TOMOGRAPHY (SPECT). A method in which images are generated by using radionuclides that emit single photons of a given energy. Images are captured at multiple positions by rotating the sensor around the subject; the three-dimensional distribution of radionuclides is then used to reconstruct the images. SPECT can be used to observe biochemical and physiological processes, as well as the size and volume of structures.

WILSON'S DISEASE
Genetic disorder that causes excessive copper accumulation in the liver and brain, resulting in hepatitis, as well as in psychiatric and neurological symptoms.

LESCH–NYHAN SYNDROME
X-linked recessive disorder that is caused by alterations in the activity of the enzyme hypoxanthine-guanine phosphoribosyltransferase. It is characterized by self-mutilating behaviours such as lip and finger biting, and head banging.

TOURETTE'S SYNDROME
A disorder that is thought to be caused by abnormalities of the basal ganglia. It is characterized by facial and vocal tics and less frequently by verbal profanities.

ORTHOSTATIC INTOLERANCE
Condition that is characterized by light-headedness and fainting when the upright position is assumed. Its causes are unknown, but it might be related to low blood pressure and an inadequate supply of blood to the brain.

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