J. exp. Biol. ***196****, 263–281 (1994)*
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MOLECULAR PHYSIOLOGY OF NOREPINEPHRINE AND SEROTONIN TRANSPORTERS

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Summary

Cocaine- and antidepressant-sensitive norepinephrine and serotonin transporters (NETs and SERTs) are closely related members of the Na^+/Cl^- transporter gene family, whose other members include transporters for inhibitory amino acid transmitters, neuromodulators, osmolytes and nutrients. Availability of cloned NET and SERT cDNAs has permitted rapid progress in the definition of cellular sites of gene expression, the generation of transporter-specific antibodies suitable for biosynthetic and localization studies, the examination of structure–function relationships in heterologous expression systems and a biophysical analysis of transporter function. *In situ* hybridization and immunocytochemical studies indicate a primary expression of *NET* and *SERT* genes in brain by noradrenergic and serotonergic neurons, respectively. Both NET and SERT are synthesized as glycoproteins, with multiple glycosylation states apparent for SERT proteins in the brain and periphery. N-glycosylation of NET and SERT appears to be essential for transporter assembly and surface expression, but not for antagonist binding affinity. Homology cloning efforts have revealed novel NET and SERT homologs in non-mammalian species that are of potential value in the delineation of the precise sites for substrate and antagonist recognition, including a *Drosophila melanogaster* SERT with NET-like pharmacology. Electrophysiological recording of human NETs and SERTs stably expressed in HEK-293 cells reveals that both transporters move charge across the plasma membrane following the addition of substrates; these currents can be blocked by NET- and SERT-selective antagonists as well as by cocaine.

Introduction

The space surrounding neuronal synapses is a tightly regulated compartment. In this microenvironment, concentrations of extracellular neurotransmitter are actively limited in magnitude and duration by proteins responsible for neurotransmitter clearance, establishing spatial and temporal constraints on chemical signaling. At the vertebrate neuromuscular junction, irreversible enzymatic degradation terminates the actions of acetylcholine (ACh). At non-cholinergic synapses, extracellular neurotransmitter concentrations are regulated by integral membrane Na^+/cotransporter proteins that can rapidly move neurotransmitters (and Na^+) back inside presynaptic terminals or surrounding glia (Iversen, 1975; Rudnick and Clark, 1993). Although these mechanisms

Key words: biogenic amine, antidepressant, cocaine, symporter, glycosylation, *in situ* hybridization, immunocytochemistry, patch-clamp.

of neurotransmitter clearance are distinct, their disruption yields intriguing parallels of tragically mixed consequences. Blockers of ACh breakdown are not only therapeutically beneficial (acetylcholine esterase, AChE, inhibitors provide some relief in myasthenia gravis), but are also potent toxins (irreversible AChE inhibitors can be found in nerve gases and insecticides); antagonists of neurotransmitter transporters rank among our most important tools in biological psychiatry (e.g. antidepressants), but also represent potent addictive substances (cocaine, amphetamines). Experimentally, pharmacological blockade of transporters can be used to deduce the contribution of transporters to postsynaptic responses (e.g. Bruns *et al.* 1993; Mennerick and Zorumski, 1994). Depending upon the kinetics of receptor response, receptor desensitization and the spatial zone of receptor expression relative to release sites, transport may play small or large roles in transmitter clearance relative to diffusion. Even for receptors that are essentially desensitized before transporters can remove transmitter, the recovery from desensitization and the ability to respond appropriately to the next packet of released transmitter are likely to be constrained by rates of transporter-mediated clearance.

Although the presence of transmitter-selective transport systems in presynaptic terminals and glial membranes has been clear for decades, we have had no knowledge of their structure and only a limited ability to manipulate them experimentally. Recently, multiple gene products encoding amino acid and biogenic amine neurotransmitter transporters have been cloned and characterized in heterologous expression systems. These advances herald improved understanding of the intrinsic mechanisms of neurotransmitter transport, of how tightly their activity and cellular distribution are regulated and of whether alterations in transporter activity contribute to neuropathologies and mental illness. In order to reduce redundancy with several more comprehensive biogenic amine transporter reviews (Amara and Kuhar, 1993; Rudnick and Clark, 1993; Barker and Blakely, 1994), we take this opportunity to organize our comments around recent findings derived from the exploitation of cloned transporter cDNAs. Our focus in the present review will be restricted to the cocaine- and antidepressant-sensitive norepinephrine (NE) and serotonin (5-hydroxytryptamine, 5-HT) transporters, molecules that our laboratories have directly investigated.

NE and 5-HT transporters: from sequence to structure and function

Like many transporters, progress in our understanding of NET and SERT structure and regulation was limited by an inability to obtain transport proteins in sufficient purity for direct sequencing. Furthermore, the absence of cloned NET and SERT cDNAs restricted evaluation of gene expression or transporter biophysical properties. To overcome the absence of purified transporters for elucidation of NET primary amino acid sequence, Pacholczyk *et al.* (1991) opted for an expression cloning approach whereby cDNAs were screened for their ability to confer NET function on non-neuronal cells. The intracellular accumulation of the surrogate NET substrate ^{125}I-labelled metaiodobenzylguanedine (MIBG) served as an autoradiographic marker for COS cells expressing the human NET cDNA (derived from the medulloblastoma SK-N-SH). The working assumption in these studies was that the NET exists as a monomer or as a homomonomeric complex, such that

transfection of a single cDNA would transfer function from the SK-N-SH cells to the COS cells. In support of this assumption, other transporters identified by that time, including *Escherichia coli* lac permease, facilitated glucose transporters and the Na^+/glucose transporter, were all known to be encoded by single cDNAs. Potential NET cDNAs identified in this screen were retested for specificity by incubating transfected COS cells with MIBG in the presence or absence of desipramine, a NET-selective antidepressant. In this screen, a single cDNA conferring desipramine-sensitive MIBG transport was isolated. *Vaccinia*-virus-mediated expression (Blakely *et al.* 1991*b*) of the NET cDNA in non-neuronal HeLa cells confirmed the synthesis of a catecholamine-selective transporter bearing high sensitivity to cocaine, antidepressants and amphetamines (Pacholczyk *et al.* 1991).

The cloning of the NET occurred just as Guastella *et al.* (1990) were reporting the cloning of the first isoform of a Na^+- and Cl^--coupled GABA transporter (GAT1). Alignment of the GAT1 and NET sequences demonstrated 46 % amino acid identity and revealed the sequence motifs of a novel neurotransmitter transporter gene family. Both transporters exhibit 12 regions of high hydrophobicity in spans long enough to form transmembrane domains (TMDs; Fig. 1). Since GAT and NET cDNAs encode transporters with markedly different pharmacological properties, we reasoned that transporters with drug sensitivities similar to those of NETs would exhibit at least 50 % amino acid identity with NET and thus could be identified by homology-based cloning techniques. Of particular interest was the antidepressant-sensitive serotonin transporter

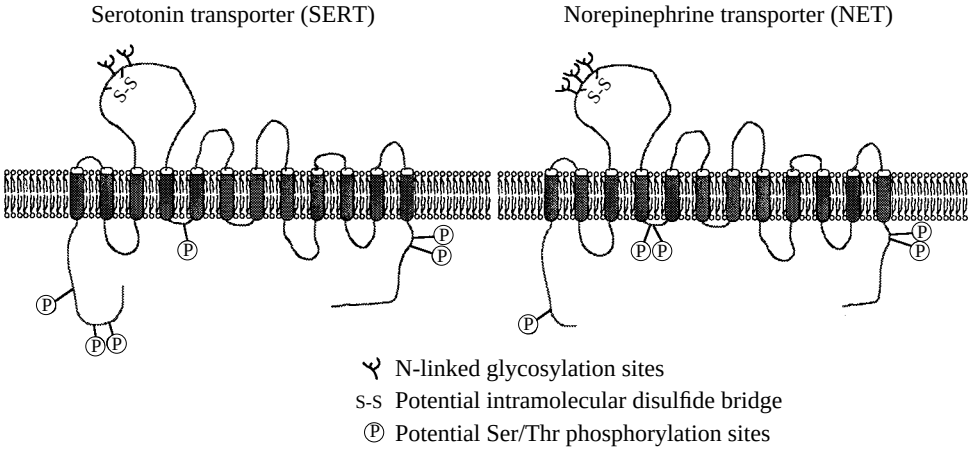

Fig. 1. Predicted topological structures of plasma membrane norepinephrine transporters (NETs) and serotonin transporters (SERTs). Both carriers are predicted to span the membrane with 12 transmembrane domains (TMDs). Both NETs and SERTs bear a large hydrophilic loop between TMD3 and TMD4, within which are located the only canonical sites for N-linked glycosylation. These glycosylation sites have recently been shown to be used by NET in transfected cells (Melikian *et al.* 1994) and by SERT in transfected cells, brain and platelets (Y. Qian, H. E. Melikian, D. B. Rye, A. I. Levey and R. D. Blakely, in preparation; G. C. Tate and R. D. Blakely, in preparation). Potential sites for serine or threonine protein phosphorylation are also indicated.

(SERT), long implicated in a variety of central nervous system (CNS) disorders, including depression (Tuomisto and Tukianen, 1976; Meltzer *et al.* 1981; Paul *et al.* 1981; Stanley *et al.* 1982; Ellis and Salmond, 1994). Using degenerate oligonucleotides derived from conserved NET and GAT sequences, we initially identified eight distinct transporter homologs expressed in rodent and human brain (Peek *et al.* 1991). To determine whether any of these homologs might represent a SERT, we characterized their anatomical expression patterns by Northern blots and *in situ* hybridization. One clone hybridized selectively to a 3.7 kb midbrain and brainstem RNA that exhibited localized synthesis in the serotonergic raphe complex. Isolation of a more complete midbrain cDNA, using the original isolate as a probe followed by examination of its function in transfected HeLa cells, confirmed the expression of a 5-HT-selective transporter sensitive to selective SERT antagonists, including paroxetine, citalopram and fluoxetine (Fuller and Wong, 1990). 5-HT uptake in transfected cells, like NE uptake in NET-transfected cells, could be blocked by low concentrations of addictive amphetamines and cocaine (Blakely *et al*. 1991*a*).

The structure predicted for the rat brain SERT, like that for the NE and GABA carriers, indicates a protein with 12 transmembrane domains (TMDs) bearing greatest sequence identity to other gene family members in TMDs 1–2 and 5–8 (Fig. 1). Concurrent with the publication of the rat brain SERT, Hoffman *et al.* (1991) reported what now appears to be an identical SERT from the mast cell line RBL (see Blakely *et al.* 1993, for a resolution of sequencing differences between the original reports of brain and RBL SERTs), supporting a longstanding contention that peripheral and CNS SERTs are largely identical in functional properties. This hypothesis has been given further validity by the identification of identical human placental (Ramamoorthy *et al.* 1993*a*), brain and platelet SERTs (Lesch *et al.* 1993*b*). Complexity exists in the primary transcripts encoding human and rat NETs (Pacholczyk *et al.* 1991; Lorang *et al.* 1994) or human SERTs (Ramamoorthy *et al.* 1993*a*,*b*; Austin *et al.* 1994); at the time of writing, no definitive investigations of the multiple NET and SERT hybridization products have been reported. However, like human NETs (Brüss *et al.* 1993), human SERTs appear to derive from a single genomic locus (Fig. 2), which for the *SERT* gene lies on the long arm of chromosome 17 (Ramamoorthy *et al.* 1993*a*). It is clear that both *NET* and *SERT* genes, as well as genes encoding homologous transporters (Liu *et al.* 1992), are fragmented by multiple introns (Brüss *et al.* 1993; Lesch *et al.* 1994) and thus could give rise to multiple transcripts by alternative RNA processing.

Several groups are presently engaged in a dissection of structure–function relationships for NETs, SERTs and their specific substrates and antagonists. Blakely *et al.* (1993) and Moore and Blakely (1994) have described the ability of NET and SERT chimeras with interchanged NH_2 and COOH termini (tail chimeras) to maintain appropriate substrate and antagonist recognition. However, a chimera formed within the NET TMD1, just a few amino acids distal to the site utilized by a functional NET chimera bearing a SERT NH_2 terminus, loses NE transport activity (Moore and Blakely, 1994). NET antibodies verify that both the tail and the TMD1 chimera are synthesized at equivalent levels and migrate appropriately by SDS–PAGE. These findings implicate residues of TMD1 in folding, plasma-membrane targeting or NE recognition. Although many chimeras

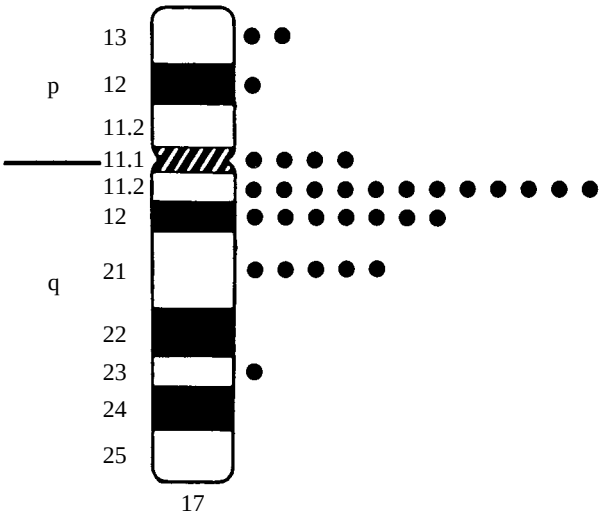

Fig 2. Chromosomal localization of the human *serotonin transporter* gene. An ideogram representing the deposition of silver grains on chromosome 17 after *in situ* hybridization with *rSERT* or *hSERT* cDNA probes. No other human chromosomal locus was labeled above background levels, and localization to chromosome 17 was confirmed by somatic cell hybrid analysis. Figure reproduced with permission from Ramamoorthy *et al.* (1993*a*).

between SERTs and NETs lack function, functional chimeras have been achieved between more closely related NETs and dopamine transporters (DATs) (Buck and Amara, 1993; Giros *et al.* 1993), suggesting that important progress will be forthcoming in the definition of biogenic amine neurotransmitter and antagonist recognition sites.

Recently, E. L. Barker, H. L. Kimmel and R. D. Blakely (in preparation) have verified that cloned rat and human SERTs exhibit different sensitivities to tricyclic antidepressants (the human SERT being more sensitive) and amphetamines (the rat SERT being more sensitive). Chimeras formed between these SERT species variants are functional; the site(s) of species selectivity for tricyclic antidepressants appear to reside at or near the last TMD. This region appears to provide species-variant sensitivity for amphetamines as well. Importantly, 5-HT, cocaine and heterocyclic antidepressant antagonist (paroxetine, fluoxetine) recognition appear to be equivalent for rSERT- and hSERT-expressing HeLa cells. These data confirm the hypothesis that tricyclic antidepressants and amphetamines interact with a site on the transporter distinct from sites binding cocaine or heterocyclic antidepressants. This idea is consistent with recent findings of differential ion- and pH-sensitivity for substrate and antagonist recognition (Wall *et al.* 1993). Site-directed mutagenesis paradigms are now being employed to locate candidate residues involved in NET and SERT antagonist selectivity.

The effort to define important structural determinants of transporter function may be greatly accelerated by the discovery of a distantly related SERT expressed in *Drosophila melanogaster* (Corey *et al.* 1994; Demchyshyn *et al.* 1994). *Drosophila* SERT (dSERT) bears approximately 50% identity with rodent and human SERTs and displays

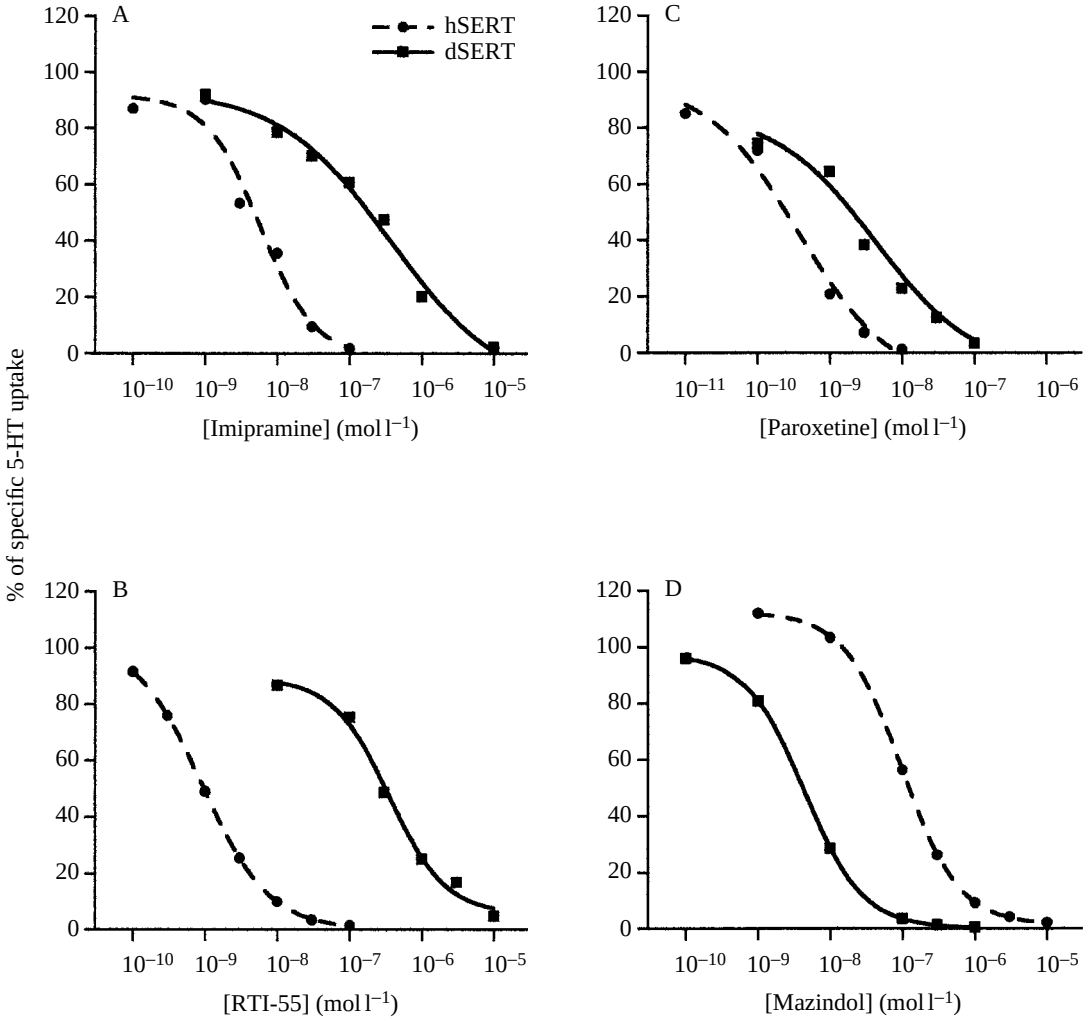

Fig. 3. Differential inhibitor sensitivity of human and *Drosophila* SERTs expressed in HeLa cells. Cloned cDNAs were expressed in HeLa cells using the *Vaccinia*-T7 expression method (Blakely *et al.* 1991*b*). 5-HT uptake assays were performed in the presence or absence of varying concentrations of SERT inhibitors. Note the significantly poorer recognition by dSERT relative to hSERT for imipramine (A), paroxetine (B) and RTI-55 (C), but the much greater sensitivity to the mammalian catecholamine transport inhibitor mazindol (D). Like hSERT, dSERT exhibits higher affinity for 5-HT than for catecholamine substrates and exhibits equivalent potency to hSERT for cocaine recognition. Figure derived, with permission, from data presented in Demchyshyn *et al.* (1994).

substantial selectivity for 5-HT over dopamine (DA), NE, histamine and octopamine. However, as can be seen in Fig. 3, the sensitivity of dSERT to the SERT-specific inhibitors imipramine, RTI-55 and paroxetine is markedly reduced, whereas the NET- and DAT-selective antagonist mazindol is considerably more potent with dSERT than on

its human homolog. The observation that dSERT does not differ from mammalian SERTs for recognition of all ligands (e.g. 5-HT and cocaine) suggests that divergent residues may be associated with the binding pocket for certain substrates and antagonists but not for others. The striking differences in pharmacology for species variants of SERTs recorded in the same heterologous expression system argue against a role of environment-specific post-translational modifications in establishing pharmacological properties, but instead focus attention on the shared and divergent residues of SERTs from fly to man.

Residues shared *among* biogenic amine transporters but *altered in other* transporter homologs of the gene family presumably provide for specific recognition of catecholamine and indoleamine substrates and selective antagonists, such as cocaine, that block NETs, SERTs and DATs with relatively similar potency. Kitayama *et al.* (1992) have presented mutagenesis data supporting a critical role of conserved TMD1 Asp and TMD7 serine residues in DAT for dopamine and/or cocaine recognition. To account for these findings, Kitayama and co-workers propose charge–charge interactions between the TMD1 Asp residue and the protonated NH_2 group of catecholamines, and hydrogen bonding of catechol OH groups with TMD7 serine residues, in a model analogous to that worked out for catecholamine recognition by β-adrenergic receptors (Strader *et al.* 1989). In a preliminary report, Melikian *et al.* (1993) have described similar studies utilizing SERTs and NETs as mutagenesis templates, wherein the conserved TMD1 Asp residue is also found to reduce greatly NE or 5-HT transport, respectively. Antibody experiments verify that *NET* and *SERT* mutations eliminating or reducing function do not do so because of an inability to synthesize transporter protein, suggesting more subtle perturbations of ligand recognition. However, the exact nature of the interaction with ligands at these residues will require more detailed studies. Future issues to resolve include the fact that substrates for other transporters, including GABA, glycine and proline, also bear protonated NH_2 groups at physiological pH, yet lack the TMD1 Asp residue (switched to Gly). Perhaps this site recognizes some other unique attribute of biogenic amine ligands, such as the polar OH groups attached to the catechol and indole rings of NE and 5-HT, respectively. Examination of the comparative effects of mutations on modified substrates, such as NH_2-substituted catecholamines, proved instrumental in supporting the model for G-protein receptor–ligand binding and would probably be a useful test of present models.

Just as progress is occurring in a definition of ligand binding sites of biogenic amine transporters, our understanding of the biosynthesis of transporter proteins is rapidly improving. First, however, it was necessary to develop specific transporter antibodies that could identify NET and SERT proteins in transfected and endogenous cells. Melikian *et al.* (1994) have characterized an anti-peptide antibody (N430) that recognizes hNET proteins by immunoprecipitation, immunoblot and immunofluorescence techniques in transfected cells (Fig. 4). In stably transfected LLC-PK1 cells, immunoblots and immunoprecipitation studies reveal that, within 30 min of pulse labelling, NET proteins are synthesized and N-glycosylated. hNET proteins shift to larger M_r forms over 2–4 h in these cells, with the latter forms enriched at the cell surface (H. E. Melikian, C. G. Tate and R. D. Blakely, in preparation). Tunicamycin inhibition of N-glycosylation prevents

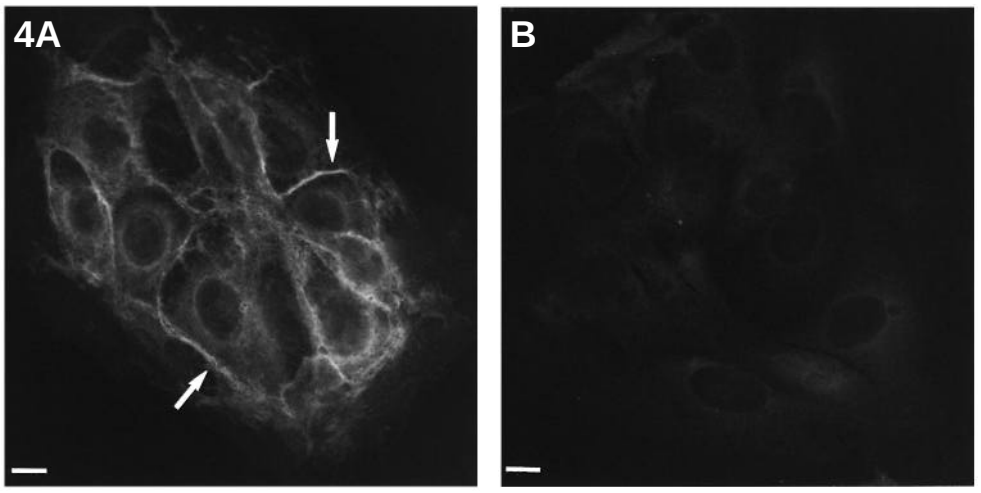

Fig. 4. Immunocytochemical localization of norepinephrine transporters in stably transfected LLC-PK1 cells. An affinity-purified, anti-peptide antibody (N430; 5 μg ml^{-1}) raised against a peptide from an intracellular loop between TMD8 and TMD9 detects hNET expression in transfected cells in the presence of detergent (A) but not in the absence of detergent (B), consistent with an intracellular localization of the N430 epitope (Melikian *et al.* 1994). Arrows in A indicate intense immunoreactivity associated with the plasma membrane. Scale bars, 10 μm. Figure reproduced by permission from Melikian *et al.* (1994).

hNET maturation to the larger (80 kDa) form and reduces whole-cell NE transport activity (Melikian *et al.* 1994). Pulse-chase analysis demonstrates that hNET forms synthesized in the presence of tunicamycin have a greatly reduced half-life compared with controls. Radioligand binding also reveals a loss of antagonist ([^{125}I]RTI-55) binding sites in LLC-PK1 membranes relative to controls following tunicamycin treatment. These data confirm the use of N-glycosylation sites on the hNET protein, consistent with their extracellular location on the proposed model (Fig. 1), and suggest that N-glycosylation is important for the physical maturation and surface targeting of biogenic amine transporters. In support of these studies, Tate and Blakely (1994) have found that removal of glycosylation sites from SERT proteins by site-directed mutagenesis reduces 5-HT uptake activity and [^{125}I]RTI-55 binding, the latter effect derived selectively from a reduction of membrane site density. Together, these data suggest that N-glycosylation itself may play little or no role in direct ligand recognition, but rather sugar addition may increase the probability that *transporter proteins fold properly* and/or are *protected from degradation* so that they can be efficiently trafficked to the plasma membrane. Interestingly, SERT proteins are differentially N-glycosylated in brain and platelets (Y. Qian, H. E. Melikian, D. B. Rye, A. I. Levey and R. D. Blakely, in preparation). Like NET, SERT N-glycosylation may contribute little to the pharmacological characteristics of transporter proteins, which appear to be identical, but reflects the particular biosynthetic constraints imposed by neurons and platelets.

Cellular expression patterns of NE and 5-HT transporters

Although NE and 5-HT uptake activities have long been associated with noradrenergic and serotonergic nerve terminals, respectively, questions have arisen as to whether additional sites of transporter expression exist, for example in glia or target cells (Kimelberg, 1986; Anderson *et al.* 1992). The major problem with previous localization techniques is that they are largely indirect or of limited cellular resolution. Thus, lesion studies support the localization of NET and SERT proteins in neuronal terminals (Kuhar *et al.* 1972), but these studies cannot control for the possibility of alterations in potential target or glial expression once the terminal is destroyed. Autoradiographic studies using radiolabeled NET or SERT antagonists (D'Amato *et al.* 1987; De Souza and Kuyatt, 1987; Hrdina *et al.* 1990; Chen *et al.* 1992; Duncan *et al.* 1992) or the neurotransmitters themselves (e.g. Azmitia and Marovitz, 1980) can generally provide only inferential cell localization patterns.

The availability of *NET* and *SERT* gene probes as well as specific transporter antibodies now permits a direct evaluation of endogenous sites of transporter expression. Blakely *et al.* (1991*b*) and Hoffman *et al.* (1991) demonstrated by *in situ* hybridization a restriction of *rSERT* gene expression to midbrain and brainstem raphe nuclei (Fig. 5), the principal sites of serotonergic neurons in the rat brain (Steinbusch, 1984). Similar studies revealed essentially identical findings for mouse brain SERT localization (A. S. Chang, S. M. Chang, D. M. Staines, S. Schroeter, A. L. Bauman and R. D. Blakely, in preparation). Antisense oligonucleotide probes derived from the cloned human placental SERT cDNA (Ramamoorthy *et al.* 1993*a*) reveal dense SERT hybridization signals overlying neurons, but not glia, of the dorsal and median raphe nuclei and the serotonergic caudal linear nucleus in *post mortem* human brain sections (Austin *et al.* 1994).

Although *SERT* gene expression appears to be exclusively neuronal in the CNS, *SERTs* are known to be expressed in several peripheral tissues, including lung, placenta (Balkovetz *et al.* 1989) and platelets (Rudnick, 1977). Interestingly, Blakely *et al.* (1991*a*) demonstrated a high level of SERT mRNA expression in the rat adrenal gland, a tissue that is not thought to synthesize 5-HT. The presence of SERT mRNA in the adrenal gland has been confirmed by *in situ* hybridization, where we have detected high-level gene expression by medullary chromaffin cells (S. Schroeter and R. D. Blakely, unpublished results; Fig. 5). At present, a role for SERTs in the adrenal gland is unclear, although it seems unlikely, given the small size of the adrenal medulla, that these transporters are involved in plasma 5-HT clearance. Rather, a hypothesis that we prefer is that SERTs are involved in loading chromaffin granules with 5-HT for later release. It has been proposed that 5-HT has direct effects within the adrenal gland (Idres *et al.* 1989; Vijayaraghavan *et al.* 1993), although the concentrations required for responses may be higher than those normally found free in plasma. Thus, 5-HT concentration within intracellular chromaffin granules after plasma membrane transport by SERTs may allow for extracellular 5-HT to reach significant levels following granule exocytosis. Such a process would be analogous to the uptake, storage and secretion of 5-HT by platelets, which obtain the amine from

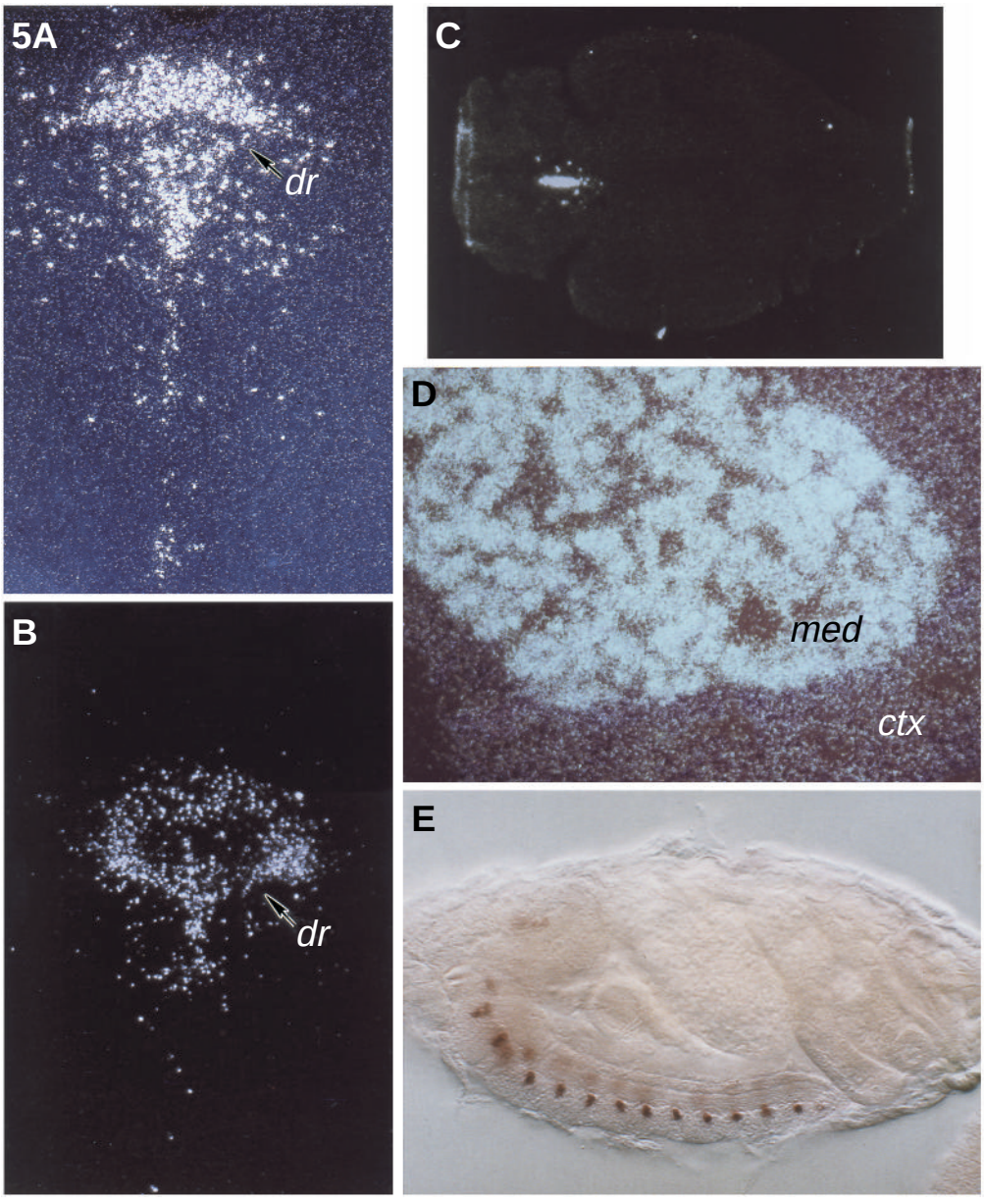

Fig. 5. Localization of serotonin transporter gene expression in rodent, human and fly. *In situ* hybridization images were obtained with *SERT* gene probes specific for each species. Sections were prepared from adult rat and mouse brain, adult mouse adrenal gland, *post mortem* human brain and a late-stage fly embryo. (A) Coronal section of mouse midbrain hybridized to an mSERT antisense cRNA probe, courtesy of S. Schroeter. *dr*, dorsal raphe. (B) Localization of hSERT expression in *post-mortem* human midbrain using an hSERT oligonucleotide probe (Austin *et al.* 1994). (C) Localization of SERT expression in a horizontal section of rat brain (Blakely *et al.* 1991*a*) using an rSERT cRNA probe. (D) Cross section of mouse adrenal gland hybridized with mSERT antisense cRNA probe, courtesy of S. Schroeter. *ctx*, cortex; *med*, medulla. (E) Expression of the *dSERT* gene in a late-stage fly embryo (lateral view). Dense hybridization product is present in cell bodies of each abdominal, thoracic and subesophageal ganglion in a pattern identical to that reported for 5-HT-containing neurons (Demchyshyn *et al.* 1994). Reproduced with permission from cited publications.

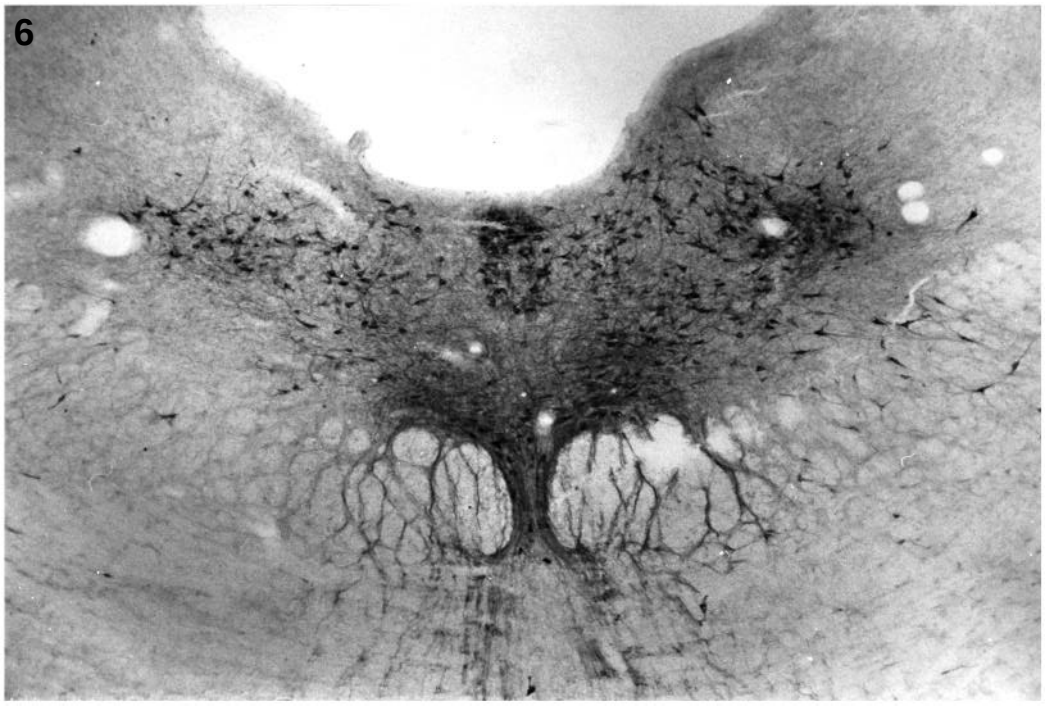

Fig. 6. Immunocytochemical localization of SERT-expressing neurons in the rat midbrain. Anti-fusion protein antibodies developed against the rSERT COOH terminus label serotonergic neurons in a coronal section of the rat midbrain at the level of the dorsal raphe nucleus (Y. Qian, H. E. Melikian, D. B. Rye, A. I. Levey and R. D. Blakely, in preparation). The distribution of SERT-immunoreactive neurons closely matches the pattern observed with anti-5-HT antibodies in adjacent sections and does not appear to indicate glial expression. SERT-immunoreactive fibers are visible throughout the neuropil when examined at higher magnification (Y. Qian, H. E. Melikian, D. B. Rye, A. I. Levey and R. D. Blakely, in preparation).

plasma (Rudnick, 1977), concentrate it in intracellular storage granules and release it at sites of vascular injury.

SERT protein expression in rodent and human brain has recently been directly visualized using SERT-specific fusion protein antibodies (Y. Qian, H. E. Melikian, D. B. Rye, A. I. Levey and R. D. Blakely, in preparation). SERT-immunoreactive neurons are evident throughout the raphe complex and are particularly numerous in the dorsal raphe complex (Fig. 6). The distribution of SERT-immunoreactive neurons closely matches that obtained with anti-5-HT antisera (Steinbusch, 1984; Y. Qian, H. E. Melikian, D. B. Rye, A. I. Levey and R. D. Blakely, in preparation). In addition, SERT-positive fibers course widely throughout the rat forebrain, terminating most densely in regions previously described (1) to receive rich serotonergic projections and (2) to contain a high density of radiolabeled antagonist binding sites (De Souza and Kuyatt, 1987; Hrdina *et al.* 1990; Chen *et al.* 1992). Thus, the cerebellum receives only a sparse serotonergic

innervation and has negligible [^{3}H]paroxetine binding sites relative to other forebrain structures and likewise exhibits few SERT-positive fibers. In contrast, the substantia nigra and the dorsal raphe itself are densely populated with SERT-positive fibers and processes. No glial SERT staining is apparent. Thus, either SERTs expressed by glial cells (Anderson *et al.* 1992) are distinct from neuronal SERT or SERT expression measured *in vitro* is elicited inappropriately by culture conditions. Alternatively, glial SERT expression may occur during embryonic and early postnatal development but later become restricted to serotonergic neurons. Interestingly, polymerase chain reaction (PCR) techniques reveal SERT mRNA in forebrain regions lacking 5-HT neurons, suggesting low but detectable levels of SERT mRNA in axons, targets or glia (Lesch *et al.* 1993*a*). Higher-resolution techniques, such as electron microscopy/immunocytochemistry, are required to resolve whether small glial processes surrounding 5-HT synapses might express SERTs, as the overall abundance may be low enough to escape detection by *in situ* hybridization and light microscope immunocytochemistry.

The distribution of NET-mRNA-expressing neurons in the rat CNS has recently been reported by Lorang *et al.* (1994). In these studies, NET mRNA was localized by *in situ* hybridization performed in parallel with immunocytochemistry of tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH), two enzymes required for NE biosynthesis. Previously defined noradrenergic soma, including the locus ceruleus (Fig. 7), express NET mRNA and exhibit TH and DBH immunostaining. As in the SERT studies, no glial staining is evident, although cross-hybridizing mRNAs are evident with Northern blots using regions lacking NET cell bodies (Lorang *et al.* 1994). Unfortunately, antipeptide NET antibodies that work well for transporter visualization in transfected cells (Melikian *et al.* 1994) appear to be of limited value for studies of NET expression in the brain *in situ*. Higher-affinity fusion protein antibodies, like those capable of revealing SERT localization (Y. Qian, H. E. Melikian, D. B. Rye, A. I. Levey and R. D. Blakely, in preparation), will be important tools to develop for studies of endogenous NET expression and distribution.

Certain brainstem neurons that stain with TH and DBH and also express phenylethanolamine *N*-methyltransferase (PNMT), and thus presumably synthesize and release epinephrine (Epi), exhibit no NET mRNA. Furthermore, the NET recognizes Epi but with a greatly reduced affinity relative to NE (Iversen, 1975; Pacholczyk *et al.* 1991). Possibly, PNMT-positive brainstem neurons express a distinct catecholamine transporter selective for Epi. Epi is released at sympathetic synapses in lower vertebrates, including amphibians, where evidence exists for an antidepressant-sensitive Epi transporter (Pimoule *et al.* 1987). Recently, we (R. D. Blakely and H. C. Hartzell, unpublished results) have identified a partial cDNA from frog sympathetic neurons with high homology to the human NET. Future studies will determine whether this new gene product represents a fragment of an Epi-selective transporter and, if so, which molecular alterations lead to its different substrate selectivity (Epi>NE>DA).

Regulation of NE and 5-HT transporters

Most aspects of chemical signaling at synapses are under tight control, including

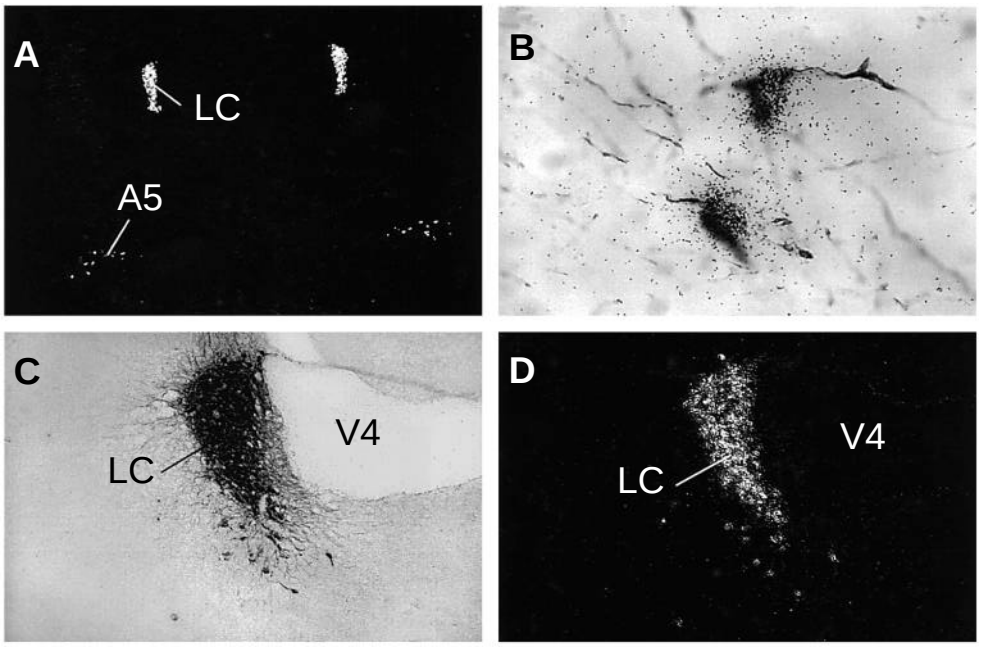

Fig. 7. *In situ* localization of *NET* gene expression in the rodent brainstem. (A) Dark-field photomicrograph of NET-mRNA-containing neurons in the locus ceruleus. The A5 group of cells is also marked. (B) Doubly labeled neurons in the A7 group illustrate the density of silver grains overlying dopamine β-hydroxylase (DBH)-immunoreactive neurons. (C,D) Bright-field (C) and dark-field (D) photomicrographs of the same field showing the appearance and distribution of DBH-immunoreactive (C) and NET-mRNA-containing (D) neurons in the locus ceruleus. LC, locus ceruleus. Figure reproduced with permission from Lorang *et al.* (1994).

neurotransmitter biosynthesis and target responsiveness. Viewed in this context, it would be expected that neurotransmitter uptake would also be subject to both acute and chronic levels of regulation. Multiple observations lend credence to the possibility that second messengers, whose concentrations are elevated by receptor activation and/or membrane depolarization, may rapidly alter the activities of NETs and SERTs *in situ* (reviewed in Barker and Blakely, 1994). Both NETs and SERTs contain canonical protein phosphorylation sites (Fig. 1); these sites may be utilized to transmit a demand for increased or decreased clearance from second messenger to transporter. Casado *et al.* (1994), working with endogenous and cloned glutamate transporters, have recently described direct protein phosphorylation as the mechanism by which phorbol esters increase glutamate uptake in C6 glioma cells and transfected HeLa cells. Although it is not known at present how phosphorylation increases glutamate transport, several groups are actively investigating whether analogous mechanisms might regulate biogenic amine transport. For example, Launay *et al.* (1994) have recently described the effects of histamine in elevating rates of 5-HT transport in platelets, effects that may be mediated through increases in the level of cyclic GMP and perhaps the activity of cyclic-GMP-dependent protein kinases. Activation of protein kinase C has also been implicated in

platelet SERT down-regulation (Anderson and Horne, 1992). Since SERT proteins are expressed at high levels in platelet membranes (Y. Qian, H. E. Melikian, D. B. Rye, A. I. Levey and R. D. Blakely, in preparation), this peripheral model for 5-HT neurons should continue to receive attention as an experimental tool to unravel acute regulation of biogenic amine transporters.

Although molecular mechanisms for acute regulation of NETs and SERTs are only now beginning to be documented, chronic hormonal regulation of transporter gene expression has become evident both *in vivo* and in model cell lines. Figlewicz *et al.* (1993*b*) have shown that chronic intraventricular insulin administration in rats significantly reduces steady-state NET mRNA levels. *In vitro*, chronic insulin treatment reduces NE uptake and levels of desipramine-labeled NETs in PC12 cells (Figlewicz *et al.* 1993*a*). Pertussis toxin treatment of chromaffin cells also appears to regulate NET expression in a delayed fashion consistent with reduced NET gene expression (Bunn *et al.* 1992). Although mechanistic studies have yet to be conducted to evaluate points of control in NET expression, it is clear that NET expression can be chronically modulated. Improper control of NET gene expression might lead to an inability to express the large number of NETs required for transmitter re-uptake in the far-removed terminal fields of noradrenergic neurons, leading to chronically elevated NET levels and desensitized adrenergic receptors. CNS studies of NET mRNA expression in human disease have yet to be reported; however, NET activity has been reported to be up-regulated in heart in human diabetic cardiomyopathy (Ganguly *et al.* 1986).

Alterations in SERT expression have been associated with a number of disorders, particularly major depression (Tuomisto and Tukianen, 1976; Meltzer *et al.* 1981; Paul *et al.* 1981; Stanley *et al.* 1982; Ellis and Salmond, 1994). Although it may be tempting to implicate changes in SERT activity directly in molecular hypotheses of mental illness, few data are available to describe endogenous mechanisms of *SERT* gene regulation in normal physiology. The availability of cloned SERTs as gene probes has permitted several investigators recently to explore drug- and hormone-dependent changes in *SERT* expression in animal models and cultured cell lines. Lesch *et al.* (1993*a*) has described reduced SERT mRNA levels in rat brain following chronic tricyclic antidepressant treatment. Seemingly opposite results have been obtained by Lopez *et al.* (1994), suggesting a sensitivity of *SERT* gene expression to drug and administration variables in these different paradigms. Cool *et al.* (1990) have demonstrated that SERT activity in human placental JAR cells can be modulated by chronic exposure to cyclic-AMP-elevating agents, including cholera toxin (CTX), forskolin and isobutylmethylxanthine. Although elevations in intracellular cyclic AMP concentrations are observable within minutes of stimulation, many hours are required to achieve maximal increases in SERT activity. The effects of CTX are blocked by translation and transcription inhibitors and derive from an increase in the V_{max} for 5-HT transport, suggesting a transcription-mediated increase in SERT abundance. Ramamoorthy *et al.* (1993*b*) have recently extended these findings to demonstrate that SERT density, assessed by [^{125}I]RTI-55 binding, increases in JAR membranes after CTX treatment and that this density increase is paralleled by a significant increase in SERT mRNA levels. Further studies are required to demonstrate directly that elevations in cyclic AMP concentration increase transcription

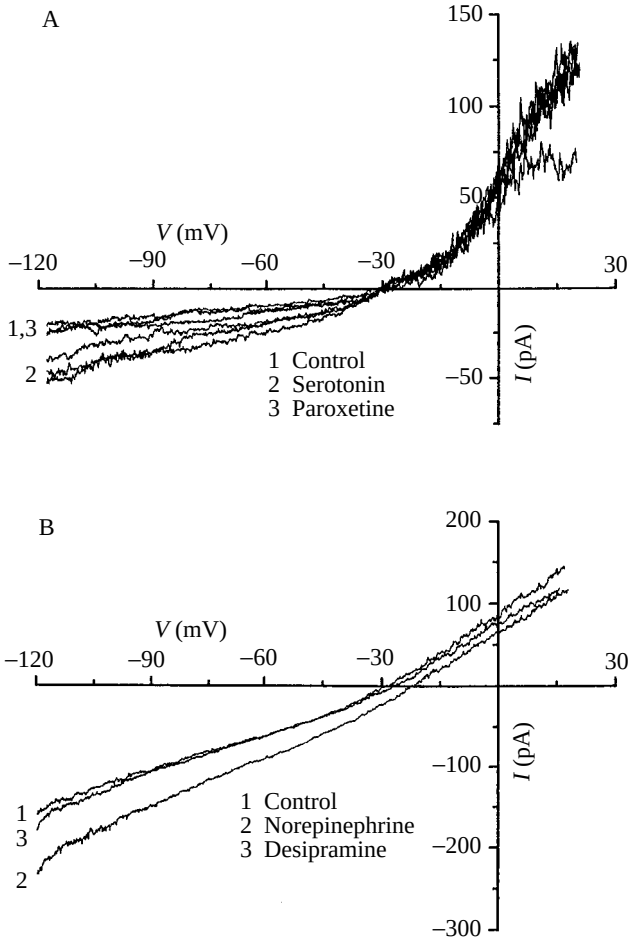

Fig. 8. Electrophysiological measurements of norepinephrine (NE) and serotonin (5-HT) symporter currents. (A,B) Current response profiles of stably transfected HEK-293 cells carrying either a NET or a SERT cDNA, with expression driven by a cytomegalovirus promoter. Recordings were performed on single cells subjected to whole-cell patch-clamp techniques. NET currents are elicited by application of guanethidine, a more stable substrate for NET than NE (NE currents can also be detected, although they are generally of smaller magnitude). SERT currents are observed after application of 5-HT. A slow (7 s) voltage ramp is applied across the ranges indicated in the presence or absence of substrates. Application of symporter antagonists reduces whole-cell currents to baseline levels. The alterations observed in the current traces, indicative of the inward movement of net positive charge during transport, are not observed in the untransfected parental cell line. Unpublished figures courtesy of S. Risso (A) and F. Laezza (B).

rate of the *hSERT* gene. In this regard, potential *cis*-acting cyclic AMP response elements have been described in cloned genomic sequences (Lesch *et al.* 1994), suggesting that *SERT* gene expression may be modulated by receptor activation. Perhaps clinically relevant changes in SERT activity derive less from structural changes in transporter

proteins, but rather arise from alterations in basal transcription rates or improperly orchestrated responses to stimuli integrated by 5-HT neurons.

Biophysical properties of cloned NE and 5-HT transporters

Mechanistic and regulatory studies of biogenic amine transporter function have have traditionally relied upon whole cell and membrane vesicle measurements of radiolabeled neurotransmitter uptake and efflux (Kanner and Schuldiner, 1987; Humphreys *et al.* 1991). Although powerful for transmitter flux measurements, these techniques generally suffer from limited temporal resolution, an inability to control and modulate membrane potential, lack of control over intracellular concentrations of ions and second messengers, and an inability to perform repeated measurements at the single-cell level. Since many transporters move net charge across the plasma membrane with every transport cycle, an opportunity exists with direct electrophysiological recording to follow the movement of these currents as a sensitive index of transporter behavior. In some circumstances, the level of transporter expression and the stoichiometry of ion movements are sufficient to obtain transporter recordings from endogenous preparations, for example for glutamate transporters in retinal glial cells (Barbour *et al.* 1991) or serotonin transporters in leech Retzius cells (Bruns *et al.* 1993). Overexpression of electrogenic transporters in *Xenopus laevis* oocytes (Mager *et al.* 1993) or mammalian cells (S. Risso, L. J. DeFelice and R. D. Blakely, in preparation) offers an opportunity to circumvent low expression levels of endogenous sources and to utilize cloned and characterized transporter cDNAs. Studies seeking to define the 'pore' regions of transporters or the involvement of key protein domains rely more heavily on the latter mode of transporter analysis.

To initiate biophysical studies, one is naturally led by available evidence for net charge transfer, which can usually be obtained in two ways. First, one can determine the dependence of neurotransmitter uptake on concentrations of intracellular and extracellular ions to arrive at an estimate of 'stoichiometry'. Along with the charge on the substrate itself, these data provide an initial indication of whether net charge transfer is to be expected. Second, investigators often attempt to shift the membrane potential of membrane vesicles using ionophores, such as valinomycin, which can generate a K^+ diffusion potential because of its selective K^+ permeability. If transport is unaffected by this manipulation, the carrier is said to be electroneutral, as charge transfer is expected, under most models, to respond to changes in the membrane electric field. Using such data, investigators have described NETs as electrogenic and SERTs as electroneutral (reviewed in Barker and Blakely, 1994).

Using cloned NET and SERT cDNAs, it is now possible to produce stable cell lines manifesting high levels of NET and SERT expression (Gu *et al.* 1994; Melikian *et al.* 1994), suitable for direct electrophysiological analysis of transporter currents. In our initial studies, we demonstrated that bidirectional charge movements accompany the influx and efflux of GAT1 GABA transporters expressed transiently in HeLa cells (S. Risso, L. J. DeFelice and R. D. Blakely, in preparation), consistent with the predicted electrogenicity of GABA transporters. When the same methods are applied to NET- and SERT-transfected cells, substrate-mediated currents are also observed. In the case of

NET, currents can be blocked by desipramine, a NET-selective tricyclic antidepressant, as well as by cocaine (Fig. 8). For SERT, currents are blocked by citalopram and paroxetine, but not by desipramine. These findings support previous indirect indications of NET electrogenicity but differ from previous models ascribing an electroneutral transport of 5-HT by SERTs. Similar observations have been reported for *Drosophila* (Corey *et al.* 1994) and rat SERTs expressed in *Xenopus laevis* oocytes (Mager *et al.* 1993). These observations suggest that additional charge not accounted for by ion-dependence studies can pass through the SERT carrier during 5-HT transport. Alteration of membrane potential in oocytes appears not to affect 5-HT transport itself, suggesting that the critical steps involved in 5-HT transfer are relatively insensitive to voltage and are gated by a smaller number of charged ions (presumably Na^+) than can actually pass through the carrier. As membrane hyperpolarization appears to increase the size of transporter-mediated inward currents (see Fig. 8), the transporter may exist at some point in its transport cycle as an 'ion-pore', in which an increased electrostatic driving force can increase net charge flow. Studies are under way to exploit the power of patch-clamp techniques to develop new models for NET and SERT transport mechanisms and to gain insights into second-messenger control of transporter activity. These techniques also offer the opportunity to understand the behavior of transporters on the time scale of milliseconds involved in synaptic transmission (Bruns *et al.* 1993) as well as to decipher transporter contributions to nonvesicular transmitter release (Attwell *et al.* 1993).

Completion of this review was supported in part by a grant from the National Institutes of Health (DA07390) and a Mallinckrodt Award to R.D.B. The authors thank S. Schroeter, Y. Qian, A. Galli, F. Laezza, M. Austin, M. Forte and D. Lorang for contribution of figure parts and K. Moore and A. Bauman for assistance in completion of the manuscript.

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