Biogenic Amine Neurotransmitter Transporters: Just When You Thought You Knew Them

Plasma membrane transporters have long been known to support the reuptake of biogenic amine neurotransmitters following release in the central and peripheral nervous systems. Using high-resolution imaging, patch-clamp and amperometric approaches, as well as molecular manipulations of transporter-regulatory pathways, surprising new details have been uncovered as to how transporters work and are influenced by signaling pathways and psychostimulants.

Beginning in the early 1990s, a new era dawned in studies of neurotransmitter transporter structure, function, and regulation, illuminated by the cloning of transporter cDNAs and genes, the development of transporter-specific gene and protein probes, and the characterization of heterologous expression systems suitable for advanced biophysical analyses. Among the advances in this field over the next decade were the elucidation of critical domains and residues supporting substrate and antagonist recognition, the discovery that transporters exist as multimeric protein complexes, the definition of N-glycosylation and phosphorylation as important posttranslational modifications, the recognition that transporters exhibited ion-channel states, and the elucidation of the first human disorders associated with transporter mutations. Many reviews are available that highlight these and other areas (4–6, 8, 15, 20, 31, 51). Together, they illustrate a field reaching maturity, yet poised for a renaissance, in which long-held ideas are challenged by new techniques and data. In this short review, we describe several new approaches and findings from our labs and from colleagues in the field that have challenged us to reconsider aspects of biogenic amine transporter structure, function, and regulation.

Substrate Binding and Transport: Watching Transporters at Work

The measurements of substrate flux through neurotransmitter transporters has relied extensively on radiolabeled substrates. Although such molecules are sensitive probes for transporter activity from populations of transporter proteins, radiolabeled substrates have limited utility for the real-time monitoring of either binding or transport; they cannot illuminate a substrate’s local environment or how it changes during substrate flux, and they cannot be used to identify cellular subdomains or relate population properties to individual molecules. Amperometric approaches offer some solutions to these limitations, as we will note below, but they are limited to paradigms such as in vitro (26, 45) or in vivo clearance measurements (10, 14), in which the presence or absence of oxidizable amine changes significantly as a function of time. Binding and transfer events that occur before changes in extracellular levels of diffusible neurotransmitter are achieved cannot be resolved. To move beyond these approaches, Schwartz et al. (49) developed a cellular-imaging approach using the fluorescent substrate, 4-(4-(dimethylamino)styryl)-N-methyl-pyridinium (ASP+). ASP+ was appropriated for biogenic amine transporter studies in recognition of its physical similarity to the neurotoxin N-methyl-4-phenylpyridine (MPP+), a well-recognized biogenic amine transporter substrate (24), and its prior utility for monitoring substrate flux through organic cation transporters (37, 40). After ASP+ exposure, transfected cells expressing the human (h) norepinephrine (NE) transporter (NET) immediately (within seconds) acquire ASP+ labeling due to substrate binding and secondarily (within minutes) build up ASP+ fluorescence due to intracellular accumulation, resolved via confocal imaging as two time-resolved phases (49). Both phases of ASP+ emission are sensitive to NET inhibitors and competitive substrates. Morphological markers distinguish plasma membrane-localized ASP+ (bound to the cell surface) from mitochondrial ASP+ (transported into the cell). ASP+ solution fluorescence can be quenched by the collisional quencher Trypan blue. The presence of fluorescence associated with the membrane in the presence of Trypan blue suggests that the substrate binds deep into the membrane in a narrow pore. Such a finding appears to be distinct from the large, open vestibule that describes the entryway to the substrate-binding sites of bacterial L-glutamate transporters, now resolved by X-ray diffraction (54). However, bound substrate in bacterial glutamate transporters appears occluded...
from the vestibule by hairpin loops, suggesting that Trypan blue-insensitive ASP+ fluorescence must be interpreted cautiously. Importantly, ASP+ labeling can be observed associated with NET-expressing, neuronal preparations such as the soma and processes of cultured superior cervical ganglion cells (49); it can also be observed with dopamine (DA) transporter (DAT)-expressing cells and can be monitored in real time in both NET and DAT cell populations using automated, microplate fluorimeters (36).

To interrogate ASP+ molecules specifically bound to plasma membrane-localized NET, Schwartz et al. (50) used subcellular organelle stains to demarcate cellular features of hNET-293 cells. Cells were stained with 500 nM 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) for 20 min before ASP+ exposure. ASP+ is optically isolated from DiO, a plasma membrane-specific marker. By imaging DiO-stained cells for 10 s before addition of ASP+ and DiO emission was shown to be negligible. Initially, ASP+ staining colocalizes with the DiO fluorescence (first 3 s of ASP+ exposure). After 3 s, a punctate, intracellular ASP+ distribution is evident, labeling that is not colocalized with DiO. Instead, the ASP+ labeling colocalizes with mitochondrial markers, whereas plasma membrane ASP+ still colocalizes with DiO. Subsequent exposure to NE (30 μM) displaces the plasma membrane ASP+ without altering the mitochondrial concentration. Human embryonic kidney 293 cells measured in parallel demonstrate <0.5% membrane binding and <10% mitochondrial staining. However, circumference fluorescence may not report purely plasma membrane labeling. Nonspecific surface labeling is expected due to the relatively low spatial resolution of confocal microscopy and cell movement during the 2-min incubation. To improve resolution, total internal reflectance microscopy was implemented, identifying membrane subdomains enriched for ASP+ labeling (50). These studies are the first to resolve binding and transport of a biogenic amine transporter substrate at the single-cell level and reveal nonuniformities in sites of expression whose presence may relate to membrane subdomains that support transporter regulation (25, 35).

To gather insights into stoichiometric relationships between substrate and protein targets, ASP+ labeling in combination with visualization of green fluorescent protein-tagged hNETs was used to measure substrate-transporter kinetics and stoichiometry (50). Calibrated confocal microscopy and fluorescence correlation spectroscopy revealed that hNETs, which exist as homomultimers, bind one substrate molecule per transporter subunit with an average dwell time (average substrate residence time on the transporter) of 526 ± 25 μs (FIGURE 1). Calibrated images and mass spectroscopy give a transport rate of 0.055 ± 0.0064 ASP+–hNET protein−1·s−1, translating to 36,000 binding events for every transport event. Concurrent imaging and patch-clamp experiments indicate that transporter stoichiometry changes with time, settling to 700 elementary charges/substrate, 50 ms after the appearance of substrate-transporter complexes.
induced currents. Multimeric transporters bind one substrate per gene product, and thousands of binding and unbinding events appear to occur before transport ensues. On the basis of such data, Schwartz and colleagues (50) suggest that ASP+ acts as a ligand that gates a substrate-permeant channel and evokes a substantial depolarizing current but is itself inefficiently transported. They propose that the rate-limiting step for ASP+ translocation is not a large conformational change of the protein occurring continually throughout the transport cycle but rather reflects the low probability of transport following binding and may be more consonant with a gated-pore model of permeation. Because ASP+ is not the natural substrate, important steps now need to be taken to link the phenomena reported by ASP+ fluorescence to those supporting translocation of the biogenic amine itself.

A Transporter “Turn-On” Accompanies Focal Membrane Insertion

Biogenic amine transporters are known to translocate from the plasma membrane in response to activation of cell-signaling pathways, with signaling linked to protein kinase C (PKC) activation the best studied (1, 21, 33, 41, 43). Less well-understood processes presumably also support insertion of transporters and link insertion to demand. Hints of a localized, activity-dependent process were recently covered by Savchenko and colleagues (48), who utilized a new NET-specific ectodomain antibody to visualize NET protein expression on the cell surface of transfected cells and cultured noradrenergic neurons. Labeling of live cells under detergent-free conditions was sensitive to prior treatments with PKC-activating phorbol esters, which, as expected, diminish surface expression and antibody labeling. Of greater interest, labeling on superior cervical ganglion neuronal membranes was found to be noticeably punctate, in register with synaptic vesicle markers stained following subsequent permeabilization. Brain stem cultures enriched for noradrenergic neurons also stained under detergent-free conditions with the ectodomain antibody but not with an intracellular epitope-directed antibody. Strikingly, ectodomain labeling was dramatically increased following brief depolarization of cultures using elevated K+. A similar elevation of NET epitopes was evident after treatments of brain stem cultures with angiotensin II, a neuropeptide previously reported to augment NE uptake rapidly (within minutes) after in vitro treatments. A rapid augmentation of serotonin (5-HT) transporter (SERT) surface density is also evident following adenosine receptor stimulation of RBL-2H3 cells or of receptor/transporter-cotransfected Chinese hamster ovary cells, a process dependent on cGMP elevation and protein kinase G (PKG) activation (56, 57). In preliminary studies using NET ectodomain antibodies from different species (V. Savchenko and R. D. Blakely, unpublished observations), data were gathered to support rapid delivery of transporters to the neuronal membrane from intracellular stores, changes that augment both NE uptake and NET-associated currents. The localization of surface NETs overlying synaptic vesicle pools and the exposition of NETs triggered by chemical and depolarizing stimuli suggests that changes in presynaptic membrane transporter

![Diagram](https://example.com/diagram.png)
density accompany state changes in NE signaling, although this remains to be documented in vivo.

Transporter movements to and from the plasma membrane provide an opportunity to elevate the transport capacity of the neuronal membrane in response to demand (FIGURE 2). New findings regarding how SERT capacity is elevated following adenosine receptor/PKG activation reveal a trafficking-independent process supporting changes in transport activity. Prior work on NET regulation by insulin revealed that the hormone rapidly augmented NE uptake not through a trafficking process but through a change in intrinsic transport activity, supported by p38 MAPK (2). As with NET regulation by insulin, SERT regulation by adenosine receptor/PKG activation in both native and transfected cells was found to be sensitive to the p38 MAPK inhibitor SB-203580 (56). Other p38 MAPK inhibitors afforded similar blockade of SERT upregulation, and inactive structural analogs of SB-203580 lacked activity. What was most remarkable about the effects of SB-203580 was that the antagonist failed to block the adenosine receptor/PKG activation-dependent increase in cell-surface SERT protein. These findings suggested that, just as with NET regulation by insulin, SERT regulation by PKG-linked pathways appears to engage a step of catalytic modulation involving p38 MAPK. This surmise predicts that direct activation of p38 MAPK might well trigger a change in SERT activity with no change in SERT surface density. Such a finding was recently realized using the p38 MAPK activator anisomycin (55). Anisomycin was found to augment SERT activity within minutes of application, an effect blocked by SB-203580, and to significantly reduce the SERT 5-HT $K_i$ (as well as $K_i$ for RTI-55 blockade), suggesting a stabilization of structural conformations necessary for high-affinity substrate binding. These studies raise exciting possibilities for novel paths for the development of SERT-targeted therapeutics that intercept or stimulate either transporter trafficking or catalytic activation (or both).

Interestingly, transfection studies indicate that the two antidepressant-sensitive transporters, NET and SERT, share p38 MAPK-dependent catalytic activation, whereas the DAT protein is, if anything, inhibited by this pathway (55). Ramamoorthy’s group (47) has also shown that in addition to catalytic modulation, basal p38 MAPK activity may also play a role in SERT trafficking. Changes in SERT trafficking in synaptosomes following exposure to p38 MAPK inhibitors or RNA interference coincide with diminished SERT phosphorylation and a loss of associations with the SNARE protein syntaxin 1A and the SERT-associated phosphatase PP2Ac. Whether changes in protein associations support changes in SERT trafficking and catalytic modulation or are consequences of other changes, such as SERT phosphorylation, remain to be established. Regardless, these studies identify p38 MAPK as an important new player in the presynaptic plasticity of noradrenergic and serotonergic neurons and will need to be considered as possible sites of dysregulation underlying mental disorders treated by antidepressants.

Channels and Psychostimulants in a State of Flux

Whole cell currents and transporter-associated channel states first became evident in biogenic amine transporters through Xenopus oocyte expression studies (19, 34, 52, 58) as well as with whole cell and detached-patch recordings of transfected mammalian cells (16, 18, 32, 46). The latter studies were extended to combined patch-clamp/amperometric recordings (17), documenting highly correlated channel and substrate permeation states. A reasonable concern is that such activities could reflect anomalous states of transporter proteins elicited in heterologous models in which natural partners might be underrepresented or absent. Ramsey and DeFelice (44) detected evidence in oocytes that relative transporter/channel properties change as a function of mRNA injection level, possibly indicative of saturable partnerships that, when exceeded, change transporter properties. The elucidation of a number of transporter-associated proteins has added more objective reasons to consider this position, particularly with the realization that at least one of these proteins, syntaxin 1A, could inactivate NE transport and currents through NET (53), a phenomenon seen earlier with the GABA transporter GAT1 (11). Recently, Quick (42) showed that SERT currents in oocytes, but not 5-HT transport, were eliminated by syntaxin 1A coexpression, revealing differences in the consequences of associated proteins on transporter properties. Importantly, SERT currents could be observed in cultured serotonergic neurons and could be enhanced by intracellular perfusion of syntaxin 1A-interacting SERT peptides. DAT transporter currents and channels have also recently been defined in DA neurons of rodents (22) and nematodes (7), respectively, further moving these observations to a more physiological context. These findings suggest that biogenic amine transporter channel states are not artifacts but rather are likely reporters of transporter conformations impacted by interactions with regulatory proteins. Because syntaxin 1A/SERT and NET interactions can be modulated by cell-signaling pathways, these channel states, rather than being artifacts of heterologous expression, more likely coincide with states stabilized by (poorly understood) physiological demands.
How do transporter channel states link to biogenic amine pathophysiology such as that thought to support mental illness and drug abuse? Certainly, this will be a focal point of investigation in coming years, although some indication of possible directions is now evident (FIGURE 3). First, although it has become clear that transporter channels are evident in neuronal preparations, their exact contribution to cell physiology is unclear. Possibly, as suggested by the studies of Ingram (22), transporter channels may depolarize neurons sufficiently to modulate neuronal firing characteristics, influencing the neuronal excitability that underlies transitions from tonic to phasic or bursting modes (12). Recently, altered firing of GABAergic neurons subsequent to opiate withdrawal has been linked to a protein kinase A-linked modulation of GAT1 currents (3). Do similar state changes link DAT, NET, and SERT channels to changes in monoaminergic excitability? If so, do the actions of transporter antagonists have as much to do with dampening these currents as they do reuptake blockade?

An alternative and perhaps not mutually exclusive view of transporter channels is that transporter channels may be of insufficient magnitude to influence excitability, given other inputs to the neuron, except under extreme circumstances, but that, in terminals, they represent a substrate flux pathway that can clear neurotransmitter rapidly and efficiently in the milliseconds that follow a release event. Because NE triggers both currents and pulsatile NE spikes resolved by amperometry, might channel states not also provide a clearance mechanism for more rapid rates of neurotransmitter clearance than can be afforded through a stoichiometric, alternating-access mechanism? The latter concept suggests that transmitter could flow in an open-channel mode bidirectionally, and indeed, Kahlig and co-workers (27) recently identified outwardly directed DA channels derived from DAT proteins exposed to 1-d-amphetamine. However, they also indicate that with DA on both sides of the membrane, outward channels are not evident, unlike what occurs with amphetamine. Importantly, flux channels were identified both in transfected cells and in DA neurons. Even more interesting, both the outward movement of DA and the outwardly oriented ion flow monitored under voltage-clamp conditions appear to require discrete signaling events linked to phosphorylation of transporter-modulatory domains. PKC activation leads to NH2-terminal phosphorylation of DAT in rat striatum (13). Consistent with this observation, deletion of the first 22 amino acids from DAT (hDAT-del22) essentially eliminates 32P incorporation into DAT in response to PKC activation (21). Surprisingly, this truncation does not alter PKC-induced internalization, thereby demonstrating that NH2-terminal phosphorylation of DAT is not essential for internalization. Instead, Khoshbouei et al. (30) determined that NH2-terminal phosphorylation may be associated with the ability of amphetamine to cause DAT-mediated DA efflux, because amphetamine-induced DA efflux in hDAT-del22 was greatly impaired. Interestingly, the

FIGURE 3. Amphetamine triggers dopamine efflux with assistance from intracellular signaling pathways
Amphetamine triggers the influx of Na+ through channel state (A), leading to a rise in intracellular Ca2+ and kinase activation (B) that may target the DAT NH2-terminus, placing the transporter into a DA-efflux-competent state. Mutation of NH2-terminal phosphorylation sites impacts efflux potential of DAT with little or no effect on DA uptake ability. DA efflux triggered by amphetamine occurs, in part, through a novel channel state.

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uptake, inhibitor binding, and oligomerization of hDAT-delta2 are not significantly different from full-length DAT (21). Whether PKC is the only trigger for moving DAT channels into an efflux-competent state is certainly a point for further investigation.

Amphetamine treatment also results in an increase in Ca2+/calmodulin-dependent protein kinase II (CaMKII) activity in striatal synaptosomes. This amphetamine effect was blocked by the absence of extracellular Ca2+ as well as by the presence of the DAT blocker nomifensine, suggesting that DAT function is required for amphetamine to increase CaMKII activity (23). Additionally, acute intrastriatal administration of amphetamine increases phosphorylated CaMKII immunoreactivity (9). Remarkably, inhibitors of CAMKII blocked a Ca2+-dependent component of amphetamine-induced DA release in the nucleus accumbens of rats chronically treated with cocaine (38) and also blocked the sensitized locomotor response (39). Similarly, Kontar and co-workers (28) found that chronic amphetamine elicits sensitized amphetamine-evoked DA release that can be blocked by CaMKII inhibitors. In PC-12 cells, amphetamine-elicted Ca2+ increases are mediated by L- and N-type Ca2+ channels (29), and thus it is possible that amphetamine activates CaMKII by changing levels of intracellular Ca2+.

How these changes result in efflux competency are as yet unclear, although requirements for intracellular Na+ in the efflux process, independent of changes in voltage, suggest that altered intracellular ion sensing may be important. Additionally, changes in voltage, suggest that altered intracellular Ca2+.

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References


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