Nonclassical Pharmacology of the Dopamine Transporter: Atypical Inhibitors, Allosteric Modulators, and Partial Substrates


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ABSTRACT

The dopamine transporter (DAT) is a sodium-coupled symporter protein responsible for modulating the concentration of extraneuronal dopamine in the brain. The DAT is a principle target of various psychostimulant, nootropic, and antidepressant drugs, as well as certain drugs used recreationally, including the notoriously addictive stimulant cocaine. DAT ligands have traditionally been divided into two categories: cocaine-like inhibitors and amphetamine-like substrates. Whereas inhibitors block monoamine uptake by the DAT but are not translocated across the membrane, substrates are actively translocated and trigger DAT-mediated release of dopamine by reversal of the translocation cycle. Because both inhibitors and substrates increase extraneuronal dopamine levels, it is often assumed that all DAT ligands possess an addictive liability equivalent to that of cocaine. However, certain recently developed ligands, such as atypical benztropine-like DAT inhibitors with reduced or even a complete lack of cocaine-like rewarding effects, suggest that addictiveness is not a constant property of DAT-affecting compounds. These atypical ligands do not conform to the classic preconception that all DAT inhibitors (or substrates) are functionally and mechanistically alike. Instead, they suggest the possibility that the DAT exhibits some of the ligand-specific pleiotropic functional qualities inherent to G-protein-coupled receptors. That is, ligands with different chemical structures induce specific conformational changes in the transporter protein that can be differentially transduced by the cell, ultimately eliciting unique behavioral and psychological effects.

Perspectives in Pharmacology

Introduction

The dopamine transporter (DAT) is a transmembrane protein that regulates dopaminergic signaling in the central nervous system. DATs help to modulate the concentration of extraneuronal dopamine by actively shuttling released transmitter molecules back across the plasma membrane into dopaminergic neurons, where they can be sequestered for later reuse or enzymatic catabolism. Dopaminergic signaling is involved in many aspects of brain function, most notably cognition, motor function, affect, motivation, behavioral reinforcement, and economic analysis (reward prediction and valuation) (Greengard, 2001; Montague and Berns, 2002; Salamone et al., 2009). As such, perturbation of DAT function is implicated in a number of neuropsychiatric disorders: attention deficit/hyperactivity disorder, Parkinson’s disease, depression, anhedonia, and addictive/compulsive disorders (Gainerdin and Caron, 2003; Felten et al., 2011; Kurian et al., 2011). The DAT is also of significant pharmacological interest, because it is a target of several popular medications and a number of recreational drugs. Notable clinically used DAT ligands include psychostimulants (e.g., dextroamphetamine, methylphenidate, and modafinil), antidepressants (e.g., bupropion), and certain anorectics (e.g., phendimetrazine, a prodrug that is converted to the DAT ligand phenmetrazine in vivo). Interaction with the DAT also underlies the powerful

ABBREVIATIONS: β-CIT, 2β-carboxyethyl-3β-(4-iodophenyl)tropane; DA, dopamine; DAT, dopamine transporter; E\(_{\text{max}}\), maximum efficacy; ENAP, (N-ethyl)-1-(2-naphthyl)propan-2-amine; GBR12909, 1-(2-[bis(4-fluorophenyl)ethy]l)-4-(3-phenylpropyl)piperazine; JHW007, (N-butyl)-3-[bis(4-fluoro phenyl)methoxy]tropane; LeuT, leucine transporter; MPP\(^+\), 1-methyl-4-phenylpyridinium; NAP, 1-(2-naphthyl)propan-2-amine; NET, noradrenaline transporter; NSS, neurotransmitter sodium symporter; RTI-371, 3β-(4-methylphenyl)-2β-[3-(4-chlorophenyl)isoxazol-5-yl]tropane; SERT, serotonin transporter; SoRI-9084, (N-benzhydryl)-2-phenylquinazolin-4-amine; SoRI-20040, (N-diphenylethyl)-2-phenylquinazolin-4-amine; TM, transmembrane domain.
reinforcing and locomotor stimulant effects of cocaine, one of the most prominent drugs of addiction (Gainetdinov and Caron, 2003; Schmitt and Reith, 2010).

Similar to its fellow monoaminergic siblings, the neuronal serotonin transporter (SERT) and noradrenaline transporter (NET), the DAT is a member of the neurotransmitter/sodium symporter (NSS) protein superfamily. NSS proteins use the electrochemical potential energy inherent to the inwardly directed transmembrane Na⁺ gradient to facilitate the thermodynamically unfavorable process of moving substrate molecules against their concentration gradient (Gether et al., 2006; Forrest et al., 2011). Ligands acting at the DAT and other NSS proteins have historically been divided into two categories: inhibitors and substrates. Inhibitors are compounds that bind to the symporter and impede substrate translocation but are themselves not transported inside the cell (cocaine, for example, is a prototypical monoamine transporter inhibitor). Substrates, in contrast, are actively translocated across the plasma membrane into the cytosolic compartment. Substrates (particularly exogenous substrates, such as amphetamine and phenmetrazine) are also referred to as releasers, because the uptake of substrates can provoke efflux of cytosolic transmitter molecules via reversal of the symport cycle (Robertson et al., 2009). Reverse transport by the DAT depends on the concentration of intracellular Na⁺ (Khoshbouei et al., 2003), which is increased by the sodium influx accompanying uptake of amphetamine substrates, thereby promoting dopamine efflux (Sitte et al., 1998). In addition to releasing dopamine by reverse transport, exogenous substrates also inhibit dopamine uptake by competing with dopamine for access to unoccupied DATs. Therefore, despite having virtually orthogonal mechanisms of action, both DAT inhibitors and substrates act to increase extracellular dopamine levels.

Because of their effects on extraneuronal dopamine, it was originally assumed that all DAT-affecting drugs would elicit behavioral effects identical to those of cocaine—that is, they would be readily self-administered, strong psychomotor stimulants with extremely high addictive liability, differing solely in potency (Ritz et al., 1987; Bergman et al., 1989; Cline et al., 1992; Katz et al., 2000). However, a multitude of studies conducted over the past 10–15 years indicate that this notion is incorrect: although certain DAT inhibitors do produce the anticipated cocaine-like behavioral reactions, various atypical DAT inhibitors, such as benztpmine, modafinil, and vanoxerine (GBR12909; 1-(2-[bis(4-fluorophenyl)methoxy]ethyl)-4-(3-phenylpropyl)-piperazin), have far milder reinforcing and locomotor stimulant properties, particularly in humans (Søgaard et al., 1990; Carroll et al., 2009; Vosburg et al., 2010). Moreover, exceptionally potent dopamine uptake inhibitors that exhibit no reinforcement efficacy in animal models have also been reported, indicating that addictiveness is not a constant property of DAT inhibitors (Desai et al., 2005; Li et al., 2011). Figure 1 shows a number of examples of both cocaine-like (Fig. 1A) and atypical DAT inhibitors (Fig. 1B).

Similar to the demonstration of atypical DAT inhibitors, recent investigation of substrate-like ligands revealed compounds with unique partial substrate properties (Rothman et al., 2012). Similar to traditional full substrates (e.g., amphetamine), partial substrates are translocated by the DAT, but are significantly less effective at inducing reverse transport (DAT-mediated substrate efflux). Examples of compounds that act as either full or partial substrates are given in Fig. 2, A and B, respectively.

Although the categorization of novel ligands, such as atypical inhibitors and partial substrates, has challenged the notion that DAT ligands are functionally homogeneous, the molecular mechanism underlying this newly discovered heterogeneity is still poorly understood. Recent studies comparing cocaine-like and atypical DAT inhibitors suggest that the behavioral and phenomenological effects of a particular ligand are contingent on how the compound interacts with the transporter. Inhibitors with differing chemical structures exert unique conformational effects on the transporter, stabilizing the protein in different structural states after binding (Reith et al., 2001; Loland et al., 2008; Schmitt and Reith, 2011), and the nature of these conformational effects can, in turn, influence an inhibitor's rewarding effects (Loland et al., 2008; Li et al., 2011). The finding that different DAT ligands induce specific conformational changes, which are somehow differentially transduced by the cell, ultimately eliciting distinct downstream effects, suggests the possibility that NSS proteins exhibit some of the ligand-specific pleiotropic functional qualities inherent to G-protein–coupled receptors (Urban et al., 2007). In parallel with this insight has come evidence that NSS protein membrane trafficking is dynamically and rapidly regulated by interaction with substrates and other ligands (Schmitt and Reith, 2010). These findings have led to tacit speculation that NSS proteins can function as both transporters and as receptor-like sensors (transceptors), with respective intra- and extracellular substrate-binding domains serving as real-time detectors of substrate concentration on both sides of the plasma membrane (Taylor, 2009; Kriel et al., 2011). In this review, we discuss the conformation-specific activity of certain DAT ligands, with an emphasis on potential protein/ligand interaction mechanisms. Because there is no direct DAT crystallographic structure available, much of our discussion is based on the wealth of structural data provided by crystals of prokaryotic NSS family members in various conformations, such as leucine transporter (LeuT), a bacterial leucine transporter from Aquifex aeolicus (Yamashita et al., 2005), and subsequent homology models developed from these prokaryotic structures by our laboratory and others.

The Conformational Cycle of the NSS Protein Family

One of the first proposed mechanisms for secondary active transport was the alternating access model of Jardetzky (1966), published nearly a half-century ago. According to this model, for a membrane protein to be capable of electrochemical-coupled active transport, it needed to fulfill three structural requirements: (1) it must contain a cavity in the core of the protein large enough to accommodate a binding site for associated substrate(s); (2) it must be able to adopt two different conformations, in which the substrate-binding cavity is alternatively open to the extracellular space and the cytosolic space, respectively; and (3) the substrate-binding cavity must exhibit differing affinities for its cognate ligand(s) when the protein exists in the respective outward- and inward-facing conformations (Jardetzky, 1966). The transition between conformations would be accomplished via
a chemical reaction contingent on substrate binding to the outward-open state. This reaction would then trigger the rearrangement of the transporter, simultaneously closing off external access to the binding site while exposing the site to the intracellular milieu. Because the affinity of the central binding site for its transported substrate(s) decreased after allosteric rearrangement of the transporter to the inward-facing state, substrate molecules would naturally dissociate away from the tiny binding cavity into the larger volume of the cytosol by diffusion (because of the binding cavity’s diminutive volume, the apparent local concentration of substrate(s) would be many orders of magnitude greater than that of the entire intracellular volume).

After elucidation of the prototypical 12 transmembrane domain (TM) structure inherent to the NSS superfamily with the crystallization of LeuT, this exceeding subtle model has proven to be astoundingly accurate (Yamashita et al., 2005). Consonant with the alternating access model, the LeuT structure revealed a hydrophobic substrate-binding pocket in the center of the plasma membrane. Residues in partially unwound, flexible regions of TMs 1 and 6 and certain residues of TMs 3 and 8 interact to form this transmembrane cavity, which is large enough to accommodate two Na⁺ ions and a variety of different amino acid substrates (Singh et al., 2008). In the original LeuT/leucine crystal, the central substrate-binding pocket (dubbed the S1 site) is protected from both the periplasmic and the cytoplasmic space by gating networks—proximal residue side chains that are linked to one another via salt-bridging (joint hydrogen and ion-pair bonding), cation–π bonding, and aromatic π-stacking interactions (Yamashita et al., 2005). These gating residues are critical for functional substrate translocation and are highly conserved throughout the entire NSS protein family. The gating residue networks move as a group, functioning as intracellular and extracellular lids that occlude the hydrophobic S1 site from water infiltration after binding of ions and substrate (Nyola et al., 2010; Forrest et al., 2011). Therefore, in addition to the two low-energy conformations predicted by Jardetzky (outward-open and inward-open), LeuT revealed a third low-energy state: a dually occluded, substrate-bound intermediate (Jardetzky, 1966). In the DAT, the extracellular gate is formed by strong hydrogen/ionic interactions (a salt-bridge) between residues Arg85 and Asp476 and a π-cation interaction between Arg85 and the aromatic residue Phe320. In addition, the charged side chain of Asp79 forms a hydrogen bond with the hydroxyl moiety of Tyr156, helping the two aromatic rings of Tyr156 and Phe320 form a lid that obstructs a substrate molecule bound at the S1 site (the LeuT residue corresponding to Asp79 is the neutral Gly24, because the bound substrate leucine provides a necessary charged carboxyl moiety). The composition of the S1 site is exceptionally well conserved among the DAT, NET, and SERT, suggesting

**Fig. 1.** Chemical structures of example cocaine-like and atypical DAT inhibitors. Whereas classic cocaine-like DAT inhibitors (A) stabilize an open-to-out transporter conformation, atypical inhibitors (B) stabilize a more inward-facing (closed-to-out) conformational state. β-CFT, 2β-carbomethoxy-3β-(4-fluorophenyl)tropane; β-CPT, 2β-carbomethoxy-3β-phenyltropane.
that their respective substrates bind in a similar orientation, with substrate selectivity determined by subtle differences in local hydrophobic/hydrophilic character (Koldso et al., 2013). The intracellular gate consists of a salt-bridge interaction between Arg60 and Asp436 and a π-cation interaction between Arg60 and Tyr335, with the side chain of Glu428 helping to stabilize the gate via a hydrogen bond with the hydroxyl group of Tyr335. Figure 3A depicts the relative configuration of the gating residue networks in the occluded and outward- and inward-facing transporter conformations.

Single-molecule dynamics studies, molecular simulations, and subsequent crystals of LeuT in outward-open and inward-open conformations have since hinted at a plausible mechanism for substrate translocation. A schematic demonstrating the formational dynamics of LeuT, is presented in Fig. 3B. Starting from an ion/substrate-free (apo) outward-facing state, binding of substrate (Claxton et al., 2010; Krishnamurthy and Gouaux, 2012). Subsequent binding of substrate at the S1 site increases the probability of salt-bridge formation between extracellular gating residues, helping to close the extracellular gate. Substrate binding at the S1 site also induces conformational changes in TM helices, particularly TM1, which are propagated to the intracellular gate via a cascade of allosteric interactions, breaking salt bridge and cation-π interactions among the cytoplasmic gating residues of TMs 1, 6, and 8 and causing the inner portion of TM1 to flex upward and away from TM6 (Zhao et al., 2010, 2011). Finally, because the transporter completes the shift to an inward-facing state, release of Na⁺ ions is promoted by water penetration into the interior vestibule, hydration of the binding sites, and ultimately by release of the substrate into the cytosol (Zhao and Noskov, 2011; Zhao et al., 2011). Putative substrate interaction pockets and permeation pathways for the DAT modeled in the outward-facing, occluded, and inward-facing states are highlighted in Fig. 3A. The complete substrate permeation pathway, rendered as an overlay following superposition of the three respective modeled DAT conformations, is shown in Supplemental Fig. 1. The procedures used in modeling the different DAT conformations are detailed in the Supplemental Methods and References.

Since the proposal of the alternating access model, it has traditionally been assumed that NSS proteins possess a single, centrally localized substrate interaction site. However, Shi and colleagues presented evidence of a novel variation in this mechanism in LeuT (Shi et al., 2008). The authors proposed that binding of a second leucine molecule to a high-affinity allosteric secondary site (termed the S2 site) in the extracellular vestibule of the transporter, located 11 Å above the primary (S1) substrate site (Supplemental Fig. 1), helps to trigger the conformational shift from an occluded to an inward-facing state and is required for cytosolic release of Na⁺ and leucine from the primary site. The S2 site is rather promiscuous and has been shown to bind compounds from a diverse array of structural classes, including tricyclic antidepressants (Zhou et al., 2007), fluoxetine, and other selective SERT inhibitors (Zhou et al., 2009) and alkylglucoside detergents (Quick et al., 2009). Tricyclics bind to the S2 site with relatively low (≥ 10 μM) affinity and act as LeuT inhibitors, stabilizing the protein in an occluded conformation nearly identical to the original LeuT/leucine crystal (Zhou et al., 2007). The nature of the S2 site in LeuT and its
relevance to NSS protein function is the subject of contentious debate (Quick et al., 2012; Wang and Gouaux, 2012). Although molecular simulations and radioligand dissociation assays suggest the presence of a high-affinity secondary site, no crystallographic evidence of LeuT with a substrate-like molecule occupying the S2 site has been found. Our recent investigation of bivalent phenethylamines (novel DAT ligands bearing two substrate-like moieties linked by a flexible polymethylene spacer) show increasing binding potency (up to 82-fold greater than parent monovalent substrates) as the spacer approaches a critical length of roughly 10–12 Å, consonant with the distance separating the S1 and S2 sites in LeuT (Schmitt et al., 2010). Computational modeling of the potent bivalent ligands bound to the DAT indicated

![Image](https://example.com/image.png)

**Fig. 3.** (A) Computational models of the DAT, demonstrating the configuration of the extra- and intracellular gating networks and the substrate permeation pore in the open-to-out, occluded, and open-to-in conformational states. Formation and disruption of salt bridges and \(\pi\)-cation interactions between residues in the two gating networks (labeled and rendered as highlighted yellow sticks) underlies the alternating access translocation mechanism. As the gates are reciprocally opened and closed, the respective periplasmic and cytoplasmic substrate permeation pores (rendered as a translucent molecular surface, with hydrophobic regions in green, polar regions in purple, and solvent-exposed regions in red) grow significantly, facilitating water infiltration and diffusion of the substrate. (B) An illustration of the putative substrate translocation cycle for the DAT protein. In the absence of bound ions or ligands, the transporter protein exists in dynamic flux between outward- and inward-facing states. Binding of \(\text{Na}^+\) at the S1 site stabilizes a fully outward-facing conformation with an open extracellular gate, primed to bind substrate molecules. Substrate binding at the S1 site induces closure of the extracellular gate, establishing an occluded conformation (closed-to-out). It has been suggested that interaction of a second substrate molecule with the S2 site helps facilitate opening of the intracellular gating network, giving rise to a fully inward-facing (open-to-in) conformation capable of releasing the S1-bound substrate and ions; however, no crystallographic evidence for simultaneous interaction of two substrate molecules with an NSS protein has been found. Apo, an unbound, ligand-free conformational state of a protein.
simultaneous occupancy of both the S1 and putative S2 sites (Supplemental Fig. 2). These data support the idea that multiple substrate-interaction sites exist in a single DAT protein; however, they do not enable any conclusions to be made regarding the affinity of substrates for the S2 site or the purported need for S2 site occupancy to achieve translocation (Shi et al., 2008; Shan et al., 2011).

Partial Substrates and Allosteric Modulators: Differential Effects on Uptake Versus Efflux

Building on the concepts introduced with the alternating access model of transporter function, one of the earliest explanations of DAT-mediated substrate efflux was the facilitated exchange model (Robertson et al., 2009). According to this model, when an extracellular substrate (e.g., amphetamine) is taken up via the DAT, a cytosolic dopamine molecule can bind to the now inward-facing DAT and be transported (in reverse) during reorientation of the transporter to an outward-facing conformation. If the facilitated exchange model is correct, one might expect that any differences among substrates in their ability to induce efflux would be strictly determined by their uptake kinetics: that is, uptake and efflux would be expected to covary directly. However, more recent studies suggest that uptake and efflux of substrates are two mechanistically distinct processes that can be differentially regulated. During a previous chemical library screen for potential NSS ligands, we found several 4-quinazolinamine derivatives (see Fig. 2C for structures) that bind to the DAT with moderate (1–5 μM) affinity and exhibit a novel mechanism of action as DAT ligands: partial allosteric modulation of transporter function (Pariser et al., 2008; Rothman et al., 2009). Each of the 4-quinazolinamine ligands [(N-benzhydryl)-2-phenylquinazolin-4-amine (SoRI-9084), (N-diphenylethyl)-2-phenylquinazolin-4-amine (SoRI-20040), and (N-diphenylpropyl)-2-phenylquinazolin-4-amine (SoRI-20041)] inhibited the binding of the phenyltropane radioligand [(125)I] 2-β-carboxy-3-β-(4-iodophenyl)tropane (β-CIT); however, none of the SoRI ligands exhibited a classic dose-dependent competitive binding profile. At equilibrium, a sufficient concentration of a competitive DAT inhibitor (such as cocaine) will block virtually 100% of the binding of another competitive inhibitor (in this case, [(125)I]β-CIT); however, each of the SoRI compounds showed a ceiling in their ability to inhibit [(125)I]β-CIT binding to the DAT, with maximum efficacy (E_max) of 40–60%. The allosteric modulators increased the K_D value and decreased the B_max value for [(125)I]β-CIT binding, as well as slowed the dissociation rate of prebound [(125)I]β-CIT, further suggesting that these ligands do not compete for the same binding site as β-CIT (Pariser et al., 2008). In addition, each of the compounds inhibited uptake of [3H]DA (dopamine) by the DAT, but with a similar asymptotic ceiling in their effect (unlike competitive inhibitors, which produce complete inhibition of uptake). Perhaps of most importance, although two of the quinazolinamine modulators (SoRI-9084 and SoRI-20040) partially inhibited both uptake of [3H]DA (forward transport) and DAT-mediated release of preloaded [3H]DA (reverse transport), the third compound (SoRI-20041) similarly inhibited substrate uptake, but had no appreciable effect on efflux (Rothman et al., 2009). This compound is the first DAT ligand that has been shown to differentially affect substrate uptake versus release, indicating that the two functional modes of substrate translocation are unique and that it is possible to design compounds that selectively affect a single part of the NSS translocation cycle. In addition, SoRI-20041 and the other 4-quinazolinamine ligands demonstrate that DAT activity can be partially modulated in a noncompetitive, saturable (i.e., allosteric), and functionally selective manner, akin to a G-protein–coupled receptor.

The fascinating finding that SoRI-20041 partially inhibits [3H]DA uptake primarily via a decrease in the maximal uptake rate but does not alter amphetamine-induced, DAT-mediated efflux of either [3H]DA or [3H]MPP+ (1-methyl-4-phenylpyridinium) suggests that SoRI-20041 influences DAT function via an allosteric binding site distinct from the canonical S1 substrate site. One can speculate that SoRI-20041 subtly alters the conformation of the DAT, such that inward transport of substrate is impaired, but outward efflux of substrate is not. At present, it is not possible to define how SoRI-20041 does this on a molecular level. However, it is likely that SoRI-20041 could prove to be a useful tool for further research into the alternating access model and the impact that auxiliary binding sites (such as the putative S2 site discussed above) have on forward and reverse substrate translocation. The ability of these partial allosteric modulators to decrease the affinity and maximal binding potential of cocaine-like phenyltropane stimulants via interaction with an orthogonal binding site also suggests that such compounds could serve as worthwhile leads for designing cocaine addiction therapeutics.

Another line of evidence for the selective modulation of reverse transport comes from our recent report that different DAT substrates can have variable efficacies for inducing DAT-mediated efflux of the labeled substrate [3H]MPP+. For example, whereas the full substrate naphthylaminopropane (NAP, the (2-naphthyl)-analog of amphetamine, also known as PAL-278; see Fig. 2A for structure) produced complete efflux of preloaded [3H]MPP+ from rat synaptosomes within 30 minutes (E_max ~100%), N-ethyl-naphthylaminopropane (ENAP, also known as PAL-1045; Fig. 2B) was unable to elicit complete [3H]MPP+ release within the experimental period (efflux reached a plateau, with E_max = 78%). Similarly, although the empathogen 3,4-methylenedioxymphetamine is a full DAT substrate (Rothman et al., 2009), the ethyl analog 3,4-methylenedioxy-N-ethylamphetamine behaves as a partial substrate, with an E_max value of roughly 65%. Of importance, the plateau in transporter-mediated [3H]MPP+ efflux was insurmountable; merely increasing the concentration of a partial substrate did not produce complete release. In addition, the attenuated response observed for partial substrates in [3H]MPP+ release assays was also demonstrated in vivo: whereas NAP produced clear dose-dependent increases in locomotor stimulation and extraneuronal DA levels in rats, ENAP showed a flat dose-response curve (Rothman et al., 2012). However, the question of whether the attenuated monoamine-releasing effect of partial substrates, such as ENAP, is genuinely consequential in vivo will require further tests of such compounds in relevant behavioral assays, such as self-administration, drug discrimination, and conditioned place preference.

Although the molecular mechanisms that might explain the observation of partial release remain enigmatic, the
DAT-mediated [3H]MPP⁺ efflux experiments do provide some critical information. In particular, partial DAT substrates, such as ENAP (PAL-1045), stimulate efflux at a slower rate than do full DAT substrates, such as amphetamine. One possible interpretation of these findings is that partial substrates are less effective at promoting the conformational changes in the DAT protein required for the overall process of alternating exchange. This would result in an overall slower turnover rate of the exchange process, a slowed efflux of [3H]MPP⁺, and a lower efficacy in the release assay during the typical 30-minute period. Of note, the existence of partial substrates is not limited to the DAT, because both SERT and NET partial substrates were also identified (Rothman et al., 2012).

**Atypical Uptake Inhibitors: Conformation-Specific Binding Mechanisms**

There is ample evidence that different classes of DAT inhibitors preferentially bind to (or induce after binding) distinct transporter conformational states, and such conformation-specific activity has been recently posited to affect the addictive liability of a given ligand (Loland et al., 2008). Conformation-specific DAT interaction was first suggested by the finding that cocaine and benztropine differentially affect the vulnerability of extracellular-facing DAT cysteine residues toward reaction with membrane impermeant sulfhydryl–reducing reagents, indicating that these inhibitors stabilize different transporter conformations (Reith et al., 2001). Similarly, binding of cocaine-like compounds was shown to protect DAT transmembrane arginine residues from covalent reaction with phenylglyoxal, whereas benzotropine-like compounds failed to impact phenylglyoxal reactivity (Volz et al., 2004). Whole-cell binding studies performed in the presence of Zn²⁺ (a DAT modulator that loosely binds the extracellular face of the transporter, stabilizing an outward-facing conformation) or in the absence of extracellular Na⁺ (which increases the relative number of inward-facing DATs) further hint at specific conformational effects that vary depending on the structure of the bound inhibitor (Loland et al., 2002; Schmitt and Reith, 2011). For example, the affinity of cocaine-like inhibitors for displacement of [3H]β-carbomethoxy-3β-(4-fluorophenyl)tropane binding is strongly decreased in the wake of extracellular Na⁺ depletion, but binding of GBR12909, modafinil, and JHW007 ([N-buty1]-3α-[bis4-fluorophenyl]methoxytropane) (Fig. 1B) is only nominally impacted (and binding of 3α-benzoxytropane is actually increased). This suggests that, unlike cocaine-like ligands, atypical ligands do not require an outward-facing transporter to bind (Kopajtic et al., 2010; Schmitt and Reith, 2011).

To more easily screen the conformational binding preference of various ligands, investigators have used mutagenesis to create DAT mutants with altered conformational equilibrium. For instance, we found that mutation of certain residues in the extracellular vestibule, either Trp84 to leucine or Asp313 to asparagine (W84L and D313N, respectively), interferes with the DAT’s transition between conformational states, biasing the transporter toward an outward-facing conformation (Chen et al., 2001, 2004). Because these mutations promote an open-to-out DAT state, a compound’s wild-type to mutant binding ratio can indicate whether the compound preferentially interacts with a more open, outward-facing conformation or a more closed (inward-facing or occluded) conformation. Each of these mutations considerably increased the affinity of cocaine and related 3β-aryltropanes and the classical DAT inhibitor methylphenidate (Fig. 1A). However, the mutations displayed negligible or opposite effects on the binding affinity of benztropine, GBR12909, bupropion, modafinil, and 3α-benzoxytropane (Fig. 1B), as well as DAT substrates, such as dopamine and amphetamine (Schmitt et al., 2008, 2010). Loland and colleagues have also used a conformationally biased mutant DAT, in which the intracellular gating network residue Tyr335 was mutated to alanine (Y335A), to investigate the relationship between inhibitor-binding mechanism and cocaine-like effects (Loland et al., 2008). By disrupting a critical π-cation interaction needed for closure of the cytoplasmic gate, the Y335A mutation leads to a predominantly inward-facing transporter (Kniazeff et al., 2008). In the Y335A mutant, binding of cocaine-like compounds was essentially ablated, but binding of benztropine, JHW007, and modafinil was less impacted, giving further credence to the idea that benztropine-like atypical inhibitors preferentially interact with the inward-facing conformational state of the DAT (Loland et al., 2008, 2012). Notably, compounds that exhibited a cocaine-like loss of binding at the Y335A mutant more readily substituted for cocaine in rat drug discrimination tests and were more potent locomotor stimulants in mice, demonstrating a correlation between conformational preference and cocaine-like behavioral effects.

It is worth noting, however, that additional factors (other than DAT conformational selectivity) may underlie the reduced cocaine-like effects observed in behavioral tests of certain atypical DAT inhibitors. One factor thought to impact the rewarding efficacy of a given psychostimulant is the rate at which the compound enters the brain and interacts with the DAT; compounds with a rapid onset of action tend to exhibit greater addictive potential than do those with a slower onset rate (Wee et al., 2006). Even cocaine was found to have lower reinforcing efficacy in a primate progressive-ratio self-administration paradigm when injected over a period of 10 minutes, as opposed to a period of 10 seconds (Woolverton and Wang, 2004). It has thus been argued, for example, that the relatively slow DAT association kinetics observed for the benztropine-derived atypical inhibitor JHW007 is responsible for its lack of cocaine-like behavioral effects (Desai et al., 2005). However, a recent study of newer benzotropine analogs that do not induce cocaine-like place preference or locomotor stimulation but also have rapid onset rates suggests that onset rate is not the sole determinant of a ligand’s behavioral profile (Li et al., 2011).

In addition to kinetic differences, another theory proposed for the differential effects of cocaine-like and atypical DAT inhibitors is that atypical inhibitors engender few cocaine-like behavioral responses, not because of their unique DAT binding profile, but instead because of interaction with additional targets other than the DAT. For example, antagonism of neuronal σ receptors is thought to contribute to the activity of the atypical DAT ligand rimcazole in reducing cocaine-induced locomotion and self-administration, with sigmaergic inhibition proposed to blunt potential locomotor stimulant effects mediated by DAT inhibition (Hiranita et al., 2011). In another case, the DAT-selective phenyltropane RTI-371
proteins possess a symmetrical intracellular secondary substrate site, that is, an S3 site (Supplemental Fig. 1). Demonstration of an intracellular S3 site might help unravel the mechanism underlying the differences between substrates in their efficacy as releasers. For example, partial substrate ligands, such as ENAP and 3,4-methylenedioxyn-N-ethylamphetamine, may dissociate from the cytosolic S3 site at a slower rate than do full substrates, such as amphetamine and NAP. Ibogaine could also be hypothesized to act as a SERT/DAT partial substrate, albeit one that is translocated so slowly that it effectively functions as an uptake inhibitor after interaction with the transporter protein.

It is possible that altering the conformation of the DAT in different manners can trigger different downstream cellular signaling events. In this case, binding of particular ligand increases the probability that the transporter will adopt a given conformation and that ligand-specific conformation would then be transduced via DAT interaction partners and associated scaffolding proteins. The second-messaging cascades downstream of the DAT have not been completely elaborated at this time, but a vast array of DAT-interacting proteins has been detected (Eriksen et al., 2010; Hadlock et al., 2011). DAT interaction partners include membrane scaffolding and trafficking proteins, cytosolic kinases, phosphatases, and other signaling proteins, G-protein–coupled receptors, and receptor tyrosine kinases. These interaction partners are capable of altering surface DAT levels in real time and can even selectively modulate particular DAT functions, such as reverse transport (Eriksen et al., 2010). Further study of ligands with conformation-specific activity, including effects on putative signaling proteins downstream of the DAT, will help reveal the nature of the transceptor function of NSS proteins and could also lead to improved medications for depression, attention deficit/hyperactivity disorder, cocaine addiction, and other monoamine-linked disorders.

Conclusions: Molecular Mechanisms of Action for Atypical DAT Ligands

The structural basis of the differential interaction of cocaine-like and atypical DAT inhibitors is currently unknown, as is the molecular mechanism of more exotic ligands, such as the newly discovered 4-quinazolinaminelike–based allosteric modulators and partial substrate monoamine releasers. The response that a given ligand has toward certain conformationally biasing mutations and ionic conditions offers some insight but not specific structural information. Molecular modeling studies suggest that cocaine, 2β-carbomethoxy-3β-(4-fluorophenyl)tropane, and methylphenidate promote an outward-facing conformation by breaking a critical hydrogen bond between the side chains of DAT residues Asp79 and Tyr156 (Supplemental Fig. 3A), impeding closure of the extracellular gating network and preventing the transporter from transitioning from an open-to-out state to an occluded state (Beuming et al., 2008; Schmitt and Reith, 2011). By contrast, binding models for the atypical inhibitors benztropine, modafinil and bupropion, as well as the substrates dopamine, amphetamine, and 3,4-methylenedioxy-N-methylamphetamine reveal a preserved Asp79-Tyr156 bond (Supplemental Fig. 3B), indicating that these ligands do not prevent the DAT from transitioning to an occluded conformation (Bisgaard et al., 2011; Schmitt and Reith, 2011). The fact that atypical inhibitors facilitate a closed-to-out state implies that they affect the transporter conformation in a manner more akin to substrates than to cocaine-like ligands but, unlike true substrates, are not translocated into the intracellular milieu.

Although our DAT modeling studies indicate that atypical ligands, such as modafinil and GBR12909, stabilize an occluded transporter state, the biochemical studies do not allow us to discriminate between an occluded or more inward-facing state. It is conceivable that certain DAT ligands stabilize a true inward-facing state by interacting with a cytosol-accessible binding pocket (Supplemental Fig. 1). There is evidence that the psychedelic-like poly cyclic tryptamine ibogaine acts in this manner at both the SERT and DAT, stabilizing an open-to-in, cytoplasmic-facing state of the transporter (Jacobs et al., 2007; Bulling et al., 2012). Of interest, the recent crystallization of another 12TM prokaryotic membrane transporter protein, the L-carnitine/- β-butyrobetaine exchanger CaiT, indicated substrate interaction with three distinct binding domains: one in the center of the protein, one in the extracellular vestibule (akin to the S1 and S2 sites, respectively), and another located at the base of the intracellular vestibule, approximately 6 Å below the S1 site, directed toward the cytosol (Tang et al., 2010). The presence of such a site has led to speculation that NSS

References


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