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The top 20 dopamine transporter mutants: structure–function relationships and cocaine actions

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Abstract

Our laboratory and others elucidated the primary amino acid sequences of the dopamine transporter (DAT) by cloning its cDNA and genomic sequences more than 12 years ago. Motivations for this work included the ideas that cocaine's interactions with DAT accounted for its rewarding properties and that selective inhibitors of DAT/cocaine interactions might thus provide good anticocaine medications. Such ideas supported interest in the detailed structure–function relationships of cocaine/DAT interactions, and in the construction and characterization of extensive series of site-directed DAT mutants. We can now select the most interesting 20 cocaine-analog selective mutations of the more than100 single- and multiple amino acid substitution mutations that we have characterized. These mutants selectively reduce the affinities of the mutant DATs for cocaine analogs, but (absolutely or relatively) spare their affinities for dopamine. Several themes relevant to cocaine/DAT interactions emerge from these mutants. First, such mutations are found in a number of different DAT domains. Secondly, many but not all of these mutations lie in groups, near each other and near the same faces of presumably helical DAT transmembrane domains. Third, most are also conserved in the serotonin transporter (SERT), a transporter that is now strongly implicated in cocaine reward based on data from knockout mice. We discuss the results from these "top 20" mutants in light of the strengths and limitations of current DAT models and data from other studies. Taken together, these studies appear to indicate direct or indirect participation of several specific portions of DAT in selective recognition of cocaine analogs. These studies provide a strong basis for redirected studies aimed at producing dopamine- and serotonin-sparing cocaine antagonists that would represent combined DAT/SERT disinhibitors.

Keywords: DAT (dopamine transporter); SERT (serotonin transporter); Transmembrane domain; Mutant; Cocaine selective; Antagonism

1. Introduction

The activity and levels of expression of dopamine transporter (DAT) provide vital determinants for the functions of dopamine, a key neurotransmitter for locomotor control and reward systems. DAT acts to terminate dopaminergic neurotransmission by re-accumulation of dopamine into presynaptic neurons. It plays a central role in the spatial and temporal buffering of released dopamine and key roles in its recycling. DAT's rich physiology includes its ability to concentrate dopamine within the cytoplasm of dopaminergic neurons that depends on its co-transporting two molecules of Na^+- and a molecule of Cl^- down their concentration gradients. DAT can even provide reverse transport of dopamine from intracellular to extracellular compartments under some circumstances.

DAT has a rich pharmacological profile. Much progress in understanding DAT has come from studies that have elucidated and expanded the repertoire of compounds that interact with DAT and with the closely related transporters that accumulate norepinephrine (NET) and serotonin (SERT) into noradrenergic and serotoninergic neurons, respectively. DAT is a principal target for the psychostimulants that enhance mood and locomotor activities. Psychostimulants including cocaine and amphetamine and drugs used for attention deficit-hyperactivity disorder (ADHD) such as methylphenidate interact with DAT, as well as with NET and SERT. Cocaine and related compounds that include GBR-12909, WIN35428 and their analogs block DAT. Amphetamine-like drugs both block dopamine uptake and are

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themselves transported by DAT. DAT also provides the portal for entry and selective concentration for the dopamine-cell-specific neurotoxins 1-methyl-4-phenylpridinium (MPP^+) and 6-hydroxydopamine.

Indirect inferences about the ways in which DAT interacts with stimulants and dopamine have come from structure–activity studies of small molecule substrates and inhibitors of DAT, especially cocaine congeners. These studies suggest roles for both cationic and aromatic interactions between DAT, dopamine and cocaine. Removing the nitrogen from cocaine's tropane ring reduces its affinity for DAT. Cocaine's phenyl ring is also necessary for normal cocaine recognition by DAT. Only limited substitutions on this ring can be tolerated without significant losses of affinity. Cocaine analogs in the series represented by WIN35428 lack the ester linkages between tropane and phenyl moieties that serve as principal sites for cocaine inactivation and degradation. WIN35428 derivatives gain potency with halogenation of their phenyl rings. These structures can tolerate addition of long extended groups at their tropane ring 2 positions. They also tolerate *N*-methylation well with little loss of potency. These features can also be found reflected in the more limited structure–activity work on amphetamines and on other compounds that have high affinities for DAT, such as mazindol, GBR-12909, GBR-12935, benztropine analogs, and DAT substrates related to the dopaminergic toxins MPP^+ and 6-hydroxydopamine.

Thinking about how dopamine might interact with DAT has also been influenced by thinking about how this molecule and other catecholamines are recognized by G-linked, 7-transmembrane domain receptors. These sorts of studies suggest that polar interactions with amine and catechol hydroxyl residues are good candidates to play roles in recognition of the monoamine substrates for these transporters, perhaps through aromatic interactions with catechol rings.

Progress in understanding DAT came from cloning cDNAs and genomic sequences encoding DAT from several species in the early 1990s. The primary amino acid sequence of the dopamine transporter (DAT) was elucidated from successful cloning of its cDNAs. This DAT gene encodes a solute and ligand carrier; the human DAT gene is thus given an alternate designation SLC6A3. We and others originally proposed a 12-transmembrane domain topology for DAT based on hydrophobicity analyses of its amino acids (Shimada et al., 1991; Kilty et al., 1991). Such a structure has been supported by numerous features since this time, including the locations of the intron/exon borders of the DAT gene (Donovan et al., 1995), the intracellular localization of DAT N- and C-terminal epitopes in immunoelectron microscopic studies (Nirenberg et al., 1997a,b), the extracellular localization of asparagine residues involved in N-linked glycosylation (Vandenbergh et al., 1992; Wang and Uhl, unpublished observations), the intracellular location of serine and threonine residues involved in DAT phosphorylation, and comparisons of the DAT sequence with those of other gene family members (Uhl and Johnson, 1994; Povlock and Amara, 1997). Such a topology would fit with the intracellular location of the N- and C-termini of the DAT protein that is consistent with the absence of any signal sequences in the DAT translation product, and with the fact that many of the putative membrane spanning regions are "anchored" by charged residues. DAT displays residues likely to be charged at 9 of its 12-transmembrane domain intracellular faces and at 5–6 of its extracellular borders. The primary sequence of the dopamine transporter is not the end of the story. DAT sequences contain multiple sites where posttranslational changes can alter this structure:

1. We and others have identified direct evidence for dopamine transporter phosphorylation and its regulation by PKC and MAP kinases in several cell expression systems/striatal preparations tested ex vivo (Kitayama et al., 1994; Huff et al., 1997; Vaughan et al., 1997; Carvelli et al., 2002; Lin et al., 2003). While these effects do not substantially alter DAT's affinities for dopamine or cocaine analogs, they do alter uptake rates. These phosphorylation events could allow rapid neuroadaptations to follow cocaine administration and contribute to the differences in the rewarding properties of rapidly vs. slowly administered cocaine, for example.

2. Analyses of the DAT amino acid sequence and results of mutagenesis and biochemical studies reveal several sites for N-linked glycoslyation in the large second DAT extracellular loop. DAT is a glycoprotein when its glycosylation patterns are studied using radiolabeled photoaffinity ligands or immunopurified protein from a variety of brain and expressed-cell sources. Changes in the apparent molecular masses of canine and rat DAT proteins following digestion with sugar-cleaving enzymes suggest that glycosylation contributes 10–12 kDa to the observed mature mass of the rat striatal protein and up to 40 kDa to the mass of the protein expressed by COS cells. Since 20–25% of the mass of the mature striatal DAT is composed of sugar residues (Grigoriadis et al., 1989; Sallee et al., 1989; Lew et al., 1991a,b; Berger et al., 1991), DAT may thus represent one of the more heavily glycosylated molecules important for nervous system function. Some studies suggest that DAT transport functions can be altered by enzymatic modifications of these sugars (Lew et al., 1992). DAT mutants deleted in one or more of the potential asparagine targets for N-linked glycosylation can reduce DAT function, while deletion of multiple sites can reduce levels of expression. Roles of sugars in trafficking of the transporter to the proper cellular compartment, and even in cell–cell recognition, are suggested by analogy with the roles of sugars in other important membrane biomolecules (Asano et al., 1992).

3. There is substantial evidence for the importance of disulphide bonds in the mature functional DAT structure. Reducing agent effects on DAT functions had been eluci-

dated prior to cloning this transporter's cDNAs. Mutants in the two cysteines located in the second extracellular loop so dramatically reduced DAT expression that they also supported essential roles for disulfide bonds in this large loop in DAT assembly and in the functions of the mature protein.

4. Recent studies have suggested that DAT might function as a multimer. No data available to date suggests that it cannot function as a monomer. Since the effects of mutants can be best interpreted in light of monomeric DAT models, we use these models primarily in this review. However, it is important to keep in mind radiation inactivation data that support both monomeric and multimeric molecular masses, possible DAT dimerization due to cross-linking between cysteine residues (Hastrup et al., 2001) and indirect evidence for DAT oligomerization based on dominant-negative mutants. Poorly functioning mutant transporters often reduce activities of co-expressed wild-type DAT. We have observed this effect using a rare human DAT variant that appears to exert semi-dominant effects in reducing the levels of expression of co-expressed wildype transporter, for example (Lin and Uhl, 2003).

5. No DAT crystallographic data available to date clearly defines how DAT is actually configured in membranes in its functional state. In fact, it is only relatively recently that any structures has become available from a distantly related transporter protein NhaA (Williams, 2000; also see Ravna et al., 2003; Abramson et al., 2003; Huang et al., 2003). The NhaA is a Na+/H+ antiporter of relative molecular mass 42,000, found in the inner membrane of *Escherichia coli*, that was predicted to have 12-transmembrane helices based on primary amino acid sequence and found, by electron cryo-microscopy of two-dimensional crystals at 7–14 Å resolution, to have 12 tilted, bilayer-spanning helices with a linear arrangement of six helices adjacent to a compact bundle of six helices. The ability of the NhaA primary sequence to accurately predict a 12-transmembrane domain topology provides some confidence that major features of the current working models for DAT structures are plausible. Much of current DAT modeling still remains tentative and is supported only indirectly. Nevertheless, studies over the decade that has elapsed since this initial domain assignment have failed to provide any strong evidence against such a structure.

As initial site-directed mutagenesis and other data were being accumulated, we and others began to get results from cocaine reward testing in DAT knockout (KO) mice (Sora et al., 2001). We and others identified full cocaine reward, as assessed by conditioned place preference or self-administration, in DAT KOs. We therefore asked about the possibility that cocaine would be able to exert reward in DAT KO mice by actions on other transporters. We thus studied multi-transporter KO mice, and identified ablated cocaine conditioned place preference (CPP) in DAT/SERT combined KO mice. Since cocaine CPP was nearly intact or enhanced in SERT/NET or DAT/NET combined KO mice (Hall et al., 2002), these sorts of behavioral findings focused our attention on both DAT and SERT. Since we had generated a large dataset on DAT mutants, and since cocaine CPP can only be eliminated if mice have no DAT and wither 0 or 1/2 of wild-type levels of SERT, we focus discussion here on the DAT mutants and note their conservation in SERT as well.

2. Mutagenesis approaches

Valuable sources of indirect information about DAT structure and function have come from studies in which DAT is modified, and the influences of these modifications on its functions have been studied. Cloning DAT cDNAs from several species has allowed the study of DAT structure–function relationships through site-directed mutants in single amino acids that removed or swapped single amino acid side chains, as well as chimeras that created larger changes. The abilities of wild-type DAT and many mutants to express well in non-neuronal cells free of most other cocaine targets provide powerful tools for elucidation of DAT structure–function relationships. Much attention focused on transmembrane regions. Transmembrane regions serve as candidate sites for substrate recognition, substrate translocation and cocaine recognition.

Mutagenesis data support candidate DAT sites for several sorts of interactions that have been thought to be important based on small molecular structure–activity studies. Some DAT sites are candidates for mediating cationic interactions between small molecules and the transporter. Cationic features of dopamine and of cocaine, largely contributed by nitrogens, could interact with acidic DAT amino acids. Alternatively, cationic–π interactions between ligand nitrogens and DAT phenylalanine, tryptophan, or tyrosines are also possible. There are sites for hydrophobic interactions. Cocaine's phenyl is important for its function; many substitutions on this ring produce significant losses of cocaine affinity (Lieske et al., 1998). Aromatic DAT transmembrane residues could play roles in DAT recognition of catecholamine catechol groups, cocaine phenyl groups, and the halogenated phenyl groups of high-affinity cocaine analogs such as CFT [(−)-2-β-carbomethoxy-3-β-(4-fluorophenyl)-tropane]. They could also serve to orient DAT transmembrane regions, helping to direct the correct amino acids toward ligand- and substrate-recognizing pockets.

These general considerations have helped drive the rationales for focusing on specific classes of amino acids for mutagenesis studies aimed at elucidating selective DAT/cocaine interactions. These include the following.

2.1. Polar and charged amino acids

Polar DAT residues are likely to act individually and interactively in recognizing cocaine and dopamine. Polar

portions of dopamine and cocaine important for DAT recognition include amine, hydroxyl and even ester moieties, suggesting that interactions with acidic and basic DAT residues might both be important for interactions of both cocaine and dopamine. Dopamine recognition by other proteins also appears to involve polar interactions. Mutations in 7-transmembrane domain G-protein linked catecholamine receptors support the idea that dopamine's catechol could interact with serine residues, such as those lying in several DAT transmembrane domains including transmembrane domain 7.

At least many of the sites for DAT cocaine and dopamine recognition are likely to lie within DAT transmembrane domains. These domains contain many of the amino acids that maintain the highest homologies between DAT and the SERT and NET monoamine transporters. These domains thus contain many of the amino acids that maintain the highest homologies between transporters that each recognize cocaine congeners with high affinities. It appears likely that transporter roles in translocating polar substrates across hydrophobic neuronal cell membranes would involve amino acids lying in transmembrane domains. Competitive antagonism of this process, such as that displayed by cocaine and related drugs, could also involve transmembrane residues. Initial mutagenesis studies involving charged and polar amino acids lying in transmembrane domains did reveal substantial influences on dopamine transporter functions (see below), and many of the implicated residues are conserved in SERT.

2.2. Phenylalanines

Cocaine contains an important aromatic ring, as do virtually all DAT substrates or inhibitors. These rings could contribute to ligand and substrate recognition through aromatic or π−π interactions with DAT transmembrane domain phenyl rings (Burley and Petsko, 1985; Baldock et al., 1996). DAT transmembrane aromatic residues could also contribute to recognition of the positive charges or polarities that can be found in virtually all DAT substrates and inhibitors, since cation binding to the π faces of aromatic rings can produce surprisingly strong non-covalent cation−π interactions (Dougherty, 1996).

2.3. Prolines

Prolines can play important roles in forming and maintaining DAT structures, breaking or kinking alpha helical motifs commonly found in transmembrane protein secondary structures, since their amide nitrogens cannot form all of the polypeptide backbone hydrogen bonds possible with other amino acids. Prolines bend helices in globular proteins by about 26°, often producing bent or curved helices that can pack into funnel- or cage-like structures that appear to contribute to numerous functions of complex proteins (Barlow and Thornton, 1988). Proline residues can also provide sites for interactions with cationic ligands.

2.4. Tryptophans

Tryptophan residues lying in DAT transmembrane domains could contribute to both aromatic and cationic interactions between DAT, dopamine and cocaine. Cocaine's important aromatic ring could contribute to ligand and substrate recognition through aromatic interactions that include π−π interactions between cocaine phenyl and tryptophan indole (Burley and Petsko, 1985; Baldock et al., 1996). DAT transmembrane tryptophan residues could also contribute cation−π interactions to recognition of the positive charges or polarities that can be found in virtually all DAT substrates and inhibitors. Tryptophans mediate cation binding to the π-like faces of tryptophan indole rings as well as many aromatic residues (Dougherty, 1996).

2.5. Cautions

For mutagenesis studies, it is important to emphasize that mutations can exert both direct and indirect effects on ligand affinities. Alanine substitutions could remove side chains of amino acids that could contribute to DAT structures without ligands or substrates, to DAT structures important for ligand recognition, or to dynamic DAT structural changes such as those likely to accompany DAT-mediated substrate and ion translocation.

3. Results

3.1. Mutations that selectively reduce cocaine affinities

Residues that selectively contribute to the recognition of cocaine can be defined in several ways that try to take into account the strengths and limitations of the site-directed mutagenesis approach.

Mutants that can be characterized appropriately must be expressed at levels sufficient to allow pharmacological characterization. Many DAT variants and chimeras express suboptimally. DAT/NET transporter chimeras at most of the junctional sites selected to date do not express properly (Giros et al., 1994; Buck and Amara, 1995). Removal of even the single amino acid side chain of W162, 4W255, W310, 10W496, 1F86A, 1P87A, 2F98A, 2P112A, P136A, 7F357A, 7F364A, F390A, 8P401A, 8F411A, 11P528A, and P515A (Lin et al., 1999, 2000a,b; Itokawa et al., 2000) and residues in extracellular domains 2, 3, 4, and 6 can disrupt DAT expression, for example (Wang et al., 1995; Lin et al., 1999; Itokawa et al., 2000).

Several mutations non-selectively alter DAT affinities for both dopamine and cocaine analogs. While these may prove to be of interest, the lack of specificity of such mutations makes their interpretation difficult. We thus focus here on

the 20 mutants that provide interesting patterns of specificity: these are the mutations that are much more effective in reducing cocaine-analog affinities than in reducing DAT affinities for dopamine (Table 1).

3.2. Segment by segment localization of the 20 mutants that selectively reduce cocaine-analog affinities

These analyses derive largely from mutagenesis studies that have focused individually on transporter amino acids of several classes that lie in one of the DAT transmembrane, extracellular loop or intracellular loop domains into which we subdivide the transporter for analysis. DAT is the prototype for this discussion; we extend these analyses to SERT (Table 1).

3.2.1. Transmembrane 2

Transmembrane 2 sequences follow very highly conserved amino acids that begin in DAT's first extracellular domain and continue into the first two transmembrane domain 2 amino acids. This sequence is followed by a functionally conserved aromatic residue and by a proline and tyrosine pair 3 to 4 amino acids later. This spacing of highly conserved residues suggests the importance of maintaining specific residues on one "face" of this transmembrane helix.

Cocaine-analog affinities are reduced with substitutions for three transmembrane domain 2 mutants. Alanine substitutions for phenylalanine residues at positions 98 and 105 produce up to 7-fold reductions in cocaine-analog affinity, and alanine substitution for the proline at position 101 reduces cocaine-analog affinities by more than 2-fold. The selective reduction in cocaine-analog affinity with the F98A mutant and effects at nearby 101 and 105 positions suggest that the extracellular half of the transmembrane domain 2 is a good candidate site for cocaine antagonist actions. This is especially true since the F98 and P101 residues are each conserved in SERT, suggesting that they could make similar contributions to cocaine recognition by DAT and SERT. In fact, several transmembrane domain 2 residues including F98, P101, P112, F114 and Y115 are each highly conserved. Even F105 is replaced with methionine in SERTs from only some, but not all, species. Amino acid side chains in which mutations produce such selective effects may well be directly involved in DAT recognition of cocaine analogs. The findings and data from transmembrane domain 3 phenylalanine mutants (see below) could be consistent with contributions of these amino acids contributions to a hydrophobic binding pocket. Alternatively, it is also possible that removing phenyl and proline side chains could each exert functional effects through changes in DAT secondary structures important overall for cocaine-analog recognition. In either case, a drug that exerted similar effects on DAT and SERT could have a significant impact on cocaine recognition.

3.2.2. Transmembrane domain 3

This transmembrane domain shows somewhat less sequence conservation than transmembrane domain 2, with substantial variation in at least 18 of 22 positions. Losses of the side chains of F154 selectively reduce cocaine-analog affinities by >3-fold, while loss of the side chain of F155 also reduces cocaine-analog affinity with less selectivity (Lin et al., 1999; Lin and Uhl, 2002). If transmembrane domains 2 and 3 are adjacent in the mature DAT structure, as predicted by current models, then these phenyl side chains could contribute to an aromatic pocket along with side chains of residues including the extracellularly disposed transmembrane domain 2 residue F98. This pocket could be relatively unobstructed by an extracellular domain, due to the intracellular localization of the loop that connects transmembrane domains 2 and 3. In total, this pocket could be much more involved with cocaine-analog recognition than dopamine recognition. Transmembrane domains 2–3 extracellular border regions thus appear worthy of further investigation. It is also conceivable that these phenylalanine residue side chains are important for proper orientation of a transmembrane domain 3 helical segment.

The conservation of F154 is not complete. The serine that is substituted here in SERT could even contribute to the modest differences in cocaine affinity found between DAT and SERT.

3.2.3. Transmembrane domain 4

Relatively conserved basic residues flank this 22 amino acid hydrophobic transmembrane domain. Its amino acids are somewhat less conserved than those of transmembrane domain 2, although the cytoplasmic side/C-terminal YFSLWKGVK sequence is conserved among DAT and SERT The short length of the third intracellular loop makes it likely that transmembrane domains 4 and 5 are near

Table 1
Mutations that selectively reduce cocaine-analog affinities: locations in current DAT models

TM2	TM3	TM4	TM5	ECL3	TM6	TM7	ECL4	TM8	TM9	TM11
2F98A	3F154A(S)	4Y251A	5Y273A(W)	P287A	6T315A(A)	7F361A	F390A	8W406A	9T455A(C)	11W519A
2P101A					6Q316A			8F410A	9S459A	11W523A
2F105A(M)[a]					6F331A				9T464A	11F530A

TM, transmembrane domain; ECL, extracellular loop.

[a] Equivalent residues in SERT displayed in parentheses, where they differ from DAT residues or identical otherwise.

neighbors; studies of the changes in binding energies from combined mutants support this idea (Itokawa et al., 2000). Some of the areas of highest sequence conservation among transporters in this gene subfamily begin toward the cytoplasmic aspect of transmembrane domain 4, continue through the connecting loop, and extend into the cytoplasmic half of transmembrane domain 5.

Specific patterns of mutation effects can be noted in DAT mutants with substitutions for some of the more conserved residues located in the mid- to cytoplasmic aspects of transmembrane domain 4. Alanine substitution for Y251 and the serine substitution that replaces the polar but not the aromatic features of the wild-type tyrosine side chain each enhance dopamine affinities while decreasing cocaine-analog affinities by 150–600% of wild-type values. Phenylalanine substitutions that replaced the aromatic moiety of the wild-type tyrosine, on the other hand, decrease affinities for both dopamine and CFT.

3.2.4. Transmembrane domain 5

Transmembrane domain 5 displays substantial conservation of at least 13 of its 24 residues among the monoamine transporter family. Interestingly, the highest level of sequence conservation is again found in the transmembrane domain 5's cytoplasmic half.

Y273A mutants reduce cocaine-analog affinities by almost 3-fold, but retain near normal dopamine affinities. Since current DAT models place this residue at approximately the same "depth" in the transmembrane domain 5, domain as the Y251 in transmembrane domain 4, it is conceivable that these two tyrosine side chains could act separately or interactively to contribute to selective features of the cocaine-analog recognition conferred by DAT. SERT's tryptophan residue, located in the homologous transmembrane domain 5 position, could confer similar functions.

Combined 4Y251A and 5Y273A dual alanine substitution DAT mutants appeared to confirm the possible importance of these transmembrane domain regions. These combined mutants actually increase cocaine-analog affinities, with little change in dopamine affinities (Itokawa et al., 2000).

3.2.5. Extracellular loop 3

Third extracellular loop sequences at the borders of transmembrane domain 5 and transmembrane domain 6 are conserved, as is an YLxxxxxxxL sequence in the mid portion of this loop. The heavily conserved aspartic acid that forms the putative extracellular transmembrane domain 6 border is one of three acidic residues at extracellular transmembrane domain junctions. By contrast, there are five acidic and six basic residues at intracellular transmembrane domain borders. Other sequences in this loop are relatively poorly conserved.

Mutations at the proline that forms the extracellular loop 3 N-terminus, P287A, confer 4-fold reductions in cocaine-analog affinity, with only a trend toward lower dopamine affinity. This effect fits with the idea that amino acids closer to transmembrane domains are both more conserved and more involved in cocaine recognition. Conceivably, the effects here could interact with the effects of amino acids lying in the more extracellular faces of transmembrane domain 5 or 6.

3.2.6. Transmembrane domain 6

Transmembrane domain 6, the third intracellular loop, and transmembrane domain 7 display highly conserved residues at a number of positions. Since the third cytoplasmic loop is relatively short, it is also likely that these three parts of the DAT are juxtaposed in the transporter's mature structure.

Alanine substitutions for T315, Q316 and F331 each enhance dopamine affinities and reduce cocaine-analog affinities. Cocaine-analog affinities drop to 1/4, 1/3, and 2 wild-type values for T315A, Q316A, and F331A. The same mutants elevate dopamine affinities 1.6-, 3.6-, and 3-fold, respectively. These data suggest that more careful scrutiny of the transmembrane domain 6 would be of value for targeting dopamine-sparing cocaine antagonists. The conservation of residues 316 and 331 in SERT fits with the idea that SERT could be similarly affected by drugs that targeted these residues. Interestingly, SERT's sequence provides the equivalent of the alanine substitution for T315. It is conceivable that this variation might even contribute to the differential cocaine affinities noted for DAT and SERT.

3.2.7. Extracellular loop 4

The conserved sequences DAAaxQxxFSLGxGxGVLxAFxSS of transmembrane domain 6, YNKxxNNCYxD of the fourth intracellular segment and AxxTxxxNxxTSFxxGFxxFxxL of transmembrane domain 7 represent one of the more conserved transporter domains. The longer stretch of extracellular loop 4 sequence conservation is located in a hydrophobic stretch of more conserved amino acids flanked by prolines and acidic residues.

The hydrophobic side chains of extracellular loop 4 amino acids could interact with membrane lipids or hydrophobic stretches of other transmembrane domain or loop segments. Evidence consistent with such a role comes from studies of F390A. This mutant reduces affinity for cocaine analogs almost 6-fold, with only modest, 2-fold loss of dopamine affinities. It is easy to imagine that these changes could take place through interactions of this segment with a hydrophobic portion of the DAT recognition pocket especially important for cocaine recognition. To evaluate possible contributions of other DAT segments to such a pocket, we have used analyses of Gibbs free energy alterations with combined mutants, and identified evidence for synergistic or complementary interactions, most notable for effects on dopamine affinity.

3.2.8. Transmembrane domain 7

This serine-rich transmembrane domain 7 of the dopamine transporter can be visualized as having elevated hydroxyl density on two sides of a nearly triangular side chain configuration. Transmembrane domain 7 is rich in phenylalanines. Several of their aromatic side chains seem likely to be disposed at skew angles from the hydroxyl serine side chains in current DAT models of transmembrane domain 7.

There are relatively selective effects of the 7F361A mutation on cocaine-analog affinity. Alanine substitutions reduce cocaine-analog affinity more than 8-fold without appreciable alteration in dopamine affinity. However, mutations of nearby transmembrane domain 7 serines can alter DAT interactions with dopamine. Taken together with data from mutated catecholamine receptors which suggest that paired serines could play a significant role in recognizing dopamine's catechol ring, overall transmembrane domain 7 mutagenesis results suggest that nearly but possibly separable aspects of this transmembrane domain play distinct roles in cocaine and dopamine affinities. In contrast to the greater impact of transmembrane domain 6 mutations on dopamine, transmembrane domain 7 might be described as lying at a border between separable dopamine- and cocaine-analog DAT recognition domains.

3.2.9. Transmembrane domain 8

This transmembrane domain displays substantial sequence conservation near its extracellular border and in a segment FFxMLxxLGx(D) that extends from mid- to cytoplasmic aspects of this transmembrane domain.

Mutants in the outer- to mid-aspects of transmembrane domain 8, W406A and F410A reduce cocaine-analog affinity 3.5- and 2-fold. Strikingly, W406A and F410A enhance dopamine affinity 6- and 1.6-fold. Outer to mid-aspects of transmembrane domain 8 thus appear to be a good potential site for targeting cocaine antagonists directed at DAT. Further, although these residues are not absolutely conserved, in at least some species SERT displays residues homologous to T399, P401, S403 and W406. The residue homologous to S404 is a threonine in SERTs from most species. F410 and F411 are conserved in most transporters. Drugs targeted appropriately to this DAT segment might thus exert similar effects toward SERT.

3.2.10. Transmembrane domain 9

The modest length of the fifth and sixth extracellular loops and of the fifth intracellular loop suggests that transmembrane domains 9, 10, 11 and 12 could be near neighbors. Transmembrane domain 9 displays only two highly conserved amino acids, situated near its termini. Transmembrane domain 9 alanine substitutions produce several interesting results. Substitutions for T455, S459, and T464 reduce cocaine-analog affinities by 20-, 2-, 7-, and 7-fold, respectively, with much less influence on dopamine affinities. Conceivably, these amino acids' side chains could lay on the same side of helical transmembrane domain, well disposed to contribute to a pocket that could play important roles in DAT's cocaine-selective features. Interestingly, T455, S459 and T464 are relatively conserved in SERT sequences. Extracellularly disposed transmembrane domain 9 segments thus represent quite reasonable targets for anticocaine therapeutics.

The T455 and S459 residues, likely located in the first and second helical turns from the extracellular border of this segment, could conceivably align with serine and threonine residues at similar positions in transmembrane domains 4, 5, 5, 7, 8, 10 and/or 11 and possibly with the tyrosine hydroxyls from transmembrane domains 1, 2 and 3. Such alignments could provide a more polar, "water-accepting" environment at the center of a pit or pore at which substrate and/or ions might be recognized.

3.2.11. Transmembrane domain 11

Transmembrane domain 11 contains a number of amino acids that are conserved among monoamine transporters but not among amino acid or orphan transporter subfamilies. DAT and SERTs from some species display xxWRxCWxxxSPxFLL sequences followed by amino acids with smaller aliphatic or polar side chains. Mutations in mid- to cytoplasmic residues, W519A, W523A, and F530A reduce cocaine-analog affinities by 3–4-fold. However, none reduces dopamine affinities. These mutants can be modeled as lying on the same side of a transmembrane helix; current DAT models describe them as lying on cytoplasmic- to mid-domains of the presumed transmembrane domain 11 helix. While this transmembrane domain displays only moderate DAT/SERT homology overall, these three amino acids are conserved.

4. Discussion

Cocaine remains a major public health problem without a broadly effective pharmacotherapeutic. A major strategy for anticocaine pharmacotherapeutics was proposed based on the idea that cocaine reward was solely mediated at DAT. To avoid producing just a super cocaine, we and other postulated that an effective blocker would need to inhibit DAT recognition of cocaine while still allowing DAT to recognize and transport dopamine. No clear-cut current pharmacologic term unambiguously describes such a pharmacologic property. Cocaine itself blocks dopamine uptake by DAT with generally competitive kinetic properties, features that have been used to suggest that dopamine and cocaine share overlapping recognition sites on DAT. Such overlap, however, does not exclude the possibility that some DAT regions could be selectively involved with cocaine recognition but not as heavily implicated in dopamine transport. A compound that could act at such regions could provide selective cocaine antagonism by blocking only the DAT sites important for cocaine recognition and sparing those essential for

dopamine transport. Such a compound could be termed a cocaine antagonist. Alternatively, it seems preferable to term such an activity "disinhibition". A disinhibitor could antagonize DAT (and, possibly, SERT) blockade by cocaine but not block transport itself. A complete antagonist disinhibitor would thus allow unimpeded movement of dopamine (and, possibly, serotonin) in the joint presence of dopamine or serotonin, cocaine and the disinhibitor. A partial antagonist or disinhibitor would possibly exert mild dopaminergic blockade at concentrations that could block cocaine's actions.

Assessment of the feasibility to this approach, and even identification of small molecule DAT disinhibitor candidates that could serve as selective cocaine antagonists, would be facilitated by identification of transporter regions necessary for cocaine recognition but not for dopamine transport (and visa versa). Work on DAT and/or SERT could parallel efforts to elucidate selective compounds that could provide cocaine antagonism with less potency in dopamine transport blockade. We and others thus began to approach the structure–function relationships of the DAT with focus on implications for anticocaine medication development. Work on DAT structure–function relationships progressed toward the goals of both improving understanding of DAT and identifying possible cocaine recognition site features that might include areas not involved in dopamine uptake.

4.1. Limitations of the focus on DAT and development of selective DAT disinhibitors

The therapeutic rationale for development of selective DAT disinhibitor cocaine blockers was altered precipitously by data from DAT knockout mice developed in our laboratory and in the laboratory of M. Caron. Giros et al. (1996) initially administered cocaine to DAT knockout mice, found that cocaine could not elevate their high baseline levels of locomotion, and suggested that the mice were "indifferent" to cocaine in the absence of DAT. These observations produced guarded optimism that, since DAT was a primary mechanism for cocaine locomotion, it was the likely locus for cocaine reward as well.

We directly studied cocaine's rewarding properties in our DAT knockout mice by measuring their cocaine-conditioned place preferences (Sora et al., 2001). To our surprise, mice with absent DAT displayed perfectly intact cocaine-conditioned place preferences. Parallel studies of the DAT knockout mice from Caron et al. also found that they avidly self-administered cocaine. We thus reached the inescapable conclusion that DAT was not necessary for cocaine reward. These observations led to the conclusion that a DAT-selective cocaine blocker might also be ineffective in antagonizing cocaine reward.

We next asked if the serotonin transporter could be the site for cocaine reward. SERT knockout mice displayed robust cocaine-conditioned place preference, again indicating that SERT was not necessary for cocaine reward. Xu et al. (2000) found that NET knockout mice also displayed robust cocaine-conditioned place preferences; this transporter was not necessary for cocaine reward.

Observations that elimination of no single monoamine transporter eliminated cocaine reward raised several possible roles for DAT, SERT and NET in cocaine reward in wild-type mice. These included the possibility that none of cocaine's actions at transporters plays a role in cocaine reward in wild-type mice, but that actions at other cocaine targets mediate cocaine reward. Developmental adaptations noted in the knockout mice could also supervene and allow occupancy of another site to substitute for occupancy of the deleted transporter.

We were attracted to another possibility, that cocaine might normally work as a "dirty drug" and provide reward by simultaneous actions at more than one primary site. If cocaine normally altered activities in several different parallel, interactive neurochemical systems with substantial redundancy, sites remaining in single-transporter knockouts could possibly compensate for loss of the single transporters and maintaining cocaine reward.

We initially focused on the possibility that DAT and SERT could form such a relationship, due to the evidence for participation of each of these transmitter systems in reward. We tested whether DAT and SERT could each provide such redundancy in the absence of the other transporter by constructing double knockout mice deleted in one or two copies of both the DAT and SERT genes. Cocaine place preference was maintained in DAT −/− and in SERT −/− single-gene knockout mice and even in SERT −/−/DAT+/− mice that express no SERT and only half of wild-type levels of DAT. However, neither DAT −/− SERT+/− nor DAT −/− SERT −/− mice exhibited any significant preference for the environments paired with cocaine. Eliminating all of one cocaine target, DAT, and at either 50% or all of its SERT target thus eliminated cocaine reward. These data contrasted with data from SERT −/− NET −/− and DAT −/− NET −/− mice. Although the combined SERT/NET knockout mice display substantially enhanced cocaine reward, neither SERT/NET nor DAT/NET combined knockout mice display any large reductions in cocaine reward (Sora et al., 2001; Hall et al., 2002; Dykstra and Caron, personal communication).

4.2. A jointly selective DAT and SERT disinhibitor

Current evidence thus suggests that cocaine normally works as a "dirty drug", providing reward by actions at both DAT and SERT. Since both DAT and SERT appear to provide such redundancy that cocaine reward can be expressed in the absence of the other transporter's actions, our approach to thinking about DAT structure–function relationships relevant to cocaine therapeutics changed. Results from double knockout mice motivated continued focus on transporter-based mechanisms. They fit with the explanation that normal redundancy of transporter-related

brain reward systems and/or adaptations to chronic deletions of single transporters might render actions at both DAT and SERT necessary to block cocaine reward. They thus support an updated version of the cocaine blocker/transporter disinhibitor strategy: a desired cocaine blocker/disinhibitor should disinhibit both DAT and SERT. Since deletion of two DAT copies and one SERT gene copy ablates cocaine preference just as effectively as deletion of both copies of both genes, pharmacologic interventions for human anticocaine therapy might require near-complete actions at DAT but allow either subtotal or complete accompanying SERT "disinhibition".

Could a "dirty drug" medication active in blocking cocaine recognition by both DAT and SERT but allowing normal uptake of dopamine and serotonin be found? Both the sequence homologies between DAT and SERT and work from structure–function studies that identify DAT domains selectively involved in cocaine recognition support the idea that a single compound could conceivably serve as a monoamine-uptake-sparing cocaine blocker active at both transporters. It is possible that blockade of cocaine actions at NET would also be important. However, data from knockout mice is most consistent with the DAT/SERT focus of this review. Elucidating features of DAT structure–function relationships, and seeking their relationships with SERT structure–function relationships could provide the basis of a strategy for identifying a substrate-sparing combined DAT/SERT cocaine antagonist. With such a compound in hand, behavioral efficacies could be tested. Only such tests could prove or disprove the ability of a pharmacologic bi-transporter disinhibitor to produce reductions in cocaine reward similar to those that we have identified in combined knockout studies.

4.3. Caveats for mutagenesis structure–function analyses: limitations of current mutagenesis approaches

Data from DAT mutagenesis studies fall into several groups. A number of amino acids side chains can be eliminated without substantial effects on DAT expression, cocaine-analog recognition or dopamine transport. Changes in many other amino acids reduce DAT expression. Changes in still other amino acids alter both dopamine transport and cocaine-analog binding, in some cases to such low levels that they effectively eliminate the functional expression of the transporter. However, the DAT mutations on which we focus in this review cause selective effects on DAT affinities for cocaine analogs, and spare affinities for dopamine. Results from these 20 mutants, and others, appear to demonstrate the feasibility of a strategy that could allow selective pharmacological disinhibition of DAT in the presence of cocaine. In particular, the mutagenesis data reviewed here appears to demonstrate the feasibility of allowing continued dopamine—and perhaps even serotonin—uptake in the joint presence of cocaine and a dopamine- and serotonin-sparing cocaine antagonist.

While the mutagenesis data highlighted here appears to provide further support for such ideas, it is also important to remember that mutations can alter DAT functions even when the mutated residues do not directly participate in the functions. Mutations disrupting amino acid side chains that are important for stabilizing interactions between adjacent DAT hydrophobic domains or between DAT domains and membrane lipids could influence DAT functions even though the mutated residues did not directly participate directly in the functions. Alternative interpretations thus need to be carefully considered in interpreting the effects of transporter mutagenesis on the affinities for small molecules. Nevertheless, removing side chains of single amino acids represents one of the smaller changes in DAT that can be readily produced. Other indirect means of examining DAT structure–function relationships also provide substantial cautions. The molecular weights of the sulfhydryl reagents commonly used to infer functional features of transmembrane proteins, for example, are more than three times the size of the alterations produced here.

4.4. Limitations of current DAT models

The models of DAT that we use to discuss these results also need to be used with some caution. These were built based on analyses of primary DAT sequence information (Kilty et al., 1991; Shimada et al., 1991), and have stood the tests of time remarkably well. They can fit reasonably with a variety of data, including the two-dimensional crystal electron cryo-microscopic structure of the 42-kDa *E. coli* inner membrane Na^+/H^+ antiporter NhaA. Based on indirect data similar to that available for DAT, this prokaryotic transporter was also predicted to have a 12-transmembrane helical structure. When studied in 2-D crystals, 12-transmembrane helices are revealed. This observation provides support for current DAT models by analogy. Further, the rough arrangements of NhaA transmembrane helices and subdomains can also fit readily with predictions for DAT. Tilting of several NhaA transmembrane helices with respect to the plane of the membrane again fits with suggestions for DAT transmembrane domains. Nevertheless, the transmembrane domains and other structural assignments such as those employed in this review represent our best current understanding and interim, though not final, DAT models. Conversely, it is also important to note that none of the large body of indirect evidence accumulated since these transporters' cloning invalidates current DAT models.

4.5. Positions of "cocaine-analog selective" vs. "dopamine selective" mutants

It is possible to position mutants that influence cocaine-analog recognition selectively, and place them into the contexts of the sizes of ligands and substrates, the sizes of the cavities into which portions of them might fit in the current DAT models, and the nature of mutagenesis influ-

ences that could influence uptake vs. cocaine-analog recognition. The small size of dopamine (ca. 3×8 Å) relative to that of cocaine (ca. 6×12 Å) makes it plausible that DAT components specifically involved in initial dopamine recognition could be smaller than those involved in cocaine recognition. On the other hand, dopamine uptake might be altered by mutations that directly or indirectly influenced a number of DAT features that are involved in steps into which transport can be divided: (1) sodium, and then (2) dopamine and chloride recognition by an ''outwardly facing'' transporter state, (3) dopamine and ion translocation, (4) intracellular unloading of dopamine and ions, and (5) return of the unloaded carrier to its extracellularly facing state (Povlock and Schenk, 1997).

Despite the broad portions of DAT likely to be involved in dopamine uptake processes, there are still a variety of sites at which mutations attenuate cocaine-analog affinities with selectivity. Cocaine analog affinities are selectively altered by removal of the side chains of three residues located in transmembrane domains 2, 6, 9 and 11, two residues in transmembrane domain 9, and single residues in transmembrane domains 3, 4, 5 and 7. More such residues are identified in DAT transmembrane domains than in loop regions. Although there has been a relative focus of mutagenesis studies on transmembrane domains, as opposed to loop domains, it is nevertheless striking that only two extracellular loop mutants exert such selective effects. None of the intracellular domain mutants characterized to date exerts such striking effects.

It is remarkable that the positions of several of the cocaine-selective transmembrane domain mutants in the DAT primary structure suggest that they lie quite close to each other in primary sequence (e.g. residues 315 and 316 in transmembrane domain 6). Other residues could well lie near each other on similar faces of presumably helical transmembrane segments (see positions of residues in transmembrane domains 2, 8, 9 and 11). Many more of these residues are located on similar transmembrane faces than would be expected by chance.

Cocaine-selective residues could lie next to each other in the mature DAT structure even if they are not nearby in primary sequence. Possible adjacencies could be created by side-by-side fits between transmembrane domains. Further, even the ''extracellular'' variations that alter cocaine-analog affinities with some selectivity may not be independent of transmembrane domain/membrane effects. While current models place F390 in the extracellular space, its location in hydrophobic sequences flanked by prolines could allow it to interact readily with plasma membrane and/or with DAT hydrophobic transmembrane residues. Since mutation of F390 produces some of the most remarkably selective decreases in affinity for cocaine analogs, it is conceivable that this residue could make contributions to small molecule interactions with DAT. It could also interact with residues in transmembrane domain 7, which forms one of the anchoring segments to which this loop is connected. F390 could thus even contribute to formation of the walls of a putative cocaine recognition pocket segment.

Several of the findings from these mutagenesis studies fit relatively well with data from other laboratories that have approached this problem with different methods. Wu and Gu (2003) tracked down the molecular basis for the lower cocaine affinity of the drosophila DAT to transmembrane domain 2, and localized this effect to the F105 residue that we had previously identified as selectively important for cocaine recognition. Cocaine affinity decreased by mutations of aromatic residue phenylalanine 105 in the transmembrane domain 2 of dopamine transporter. The possibility that cocaine and dopamine recognition by DAT involve different domains is supported by findings that cocaine and dopamine were able to differentially protect the mazindol binding sites from alkylation by *N*-ethylmaleimide in rat striatal membranes.

Selective alterations in affinity for dopamine can also be produced by variations in a number of putative intracellular or extracellular sequences that have effects on cocaine-analog B_{max}, but less effect on their affinities. DAT C-terminal truncation mutants with removal of as few as 22 C-terminal amino acids appeared to display significantly reduced expression, as reflected in reduced cocaine-analog B_{max} values, but 10–100-fold lower K_i values for dopamine and other substrates than those observed for wild-type DAT. Truncation/substitution mutants in the dopamine transporter carboxyl-terminal tail selectively confer high affinity dopamine uptake while attenuating recognition of the ligand-binding domain (Lee et al., 1996). Mutagenesis data support the influences of oxidative alterations in DAT cysteines, especially Cys (342) in the influences of dopamine ortho-quinone (DAQ) oxidation products on cocaine-analog binding B_{max} (Whitehead et al., 2001). The role of conserved tryptophan and acidic residues in the human dopamine transporter has also been characterized by site-directed mutagenesis (Chen et al., 2001). Altering D345 in the DAT third intracellular formed a transporter that still provided considerable dopamine uptake and nearly full potency of cocaine analogs in inhibiting dopamine uptake. While each of these results and the data from our extracellular loop 2 and 3 selective mutants argue against focusing too tightly on interactions with transmembrane residues, none of these results provides the sorts of selectivity for cocaine-analog recognition provided by most of the transmembrane domain mutations.

The body of work here continues to provide evidence that encourages thinking about selective interactions between monoamine transporters and cocaine. The identification of these focal regions in likely DAT structures that harbor amino acids whose side-chain removals so selectively influence cocaine-analog recognition is striking. These lines of evidence should continue to provide motivation for pharmacological approaches to anticocaine medication development based on transporter disinhibition.

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References

Abramson, J., Smirnova, I., Kasho, V., Verner, G., Kaback, H.R., Iwata, S., 2003. Structure and mechanism of the lactose permease of *Escherichia coli*. Science 310, 610–615.

Asano, T., Katagiri, H., Takata, K., Tsukuda, K., Lin, J.L., Ishihara, H., Inukai, K., Hirano, H., Yazaki, Y., Oka, Y., 1992. Characterization of GLUT3 protein expressed in Chinese hamster ovary cells. Biochem. J. 288, 189–193.

Baldock, C., Rafferty, J.B., Sedelnikova, S.E., Baker, P.J., Stuitje, A.R., Slabas, A.R., Hawkes, T.R., Rice, D.W., 1996. A mechanism of drug action revealed by structural studies of enoyl reductase. Science 274, 2107–2110.

Barlow, D.J., Thornton, J.M., 1988. Helix geometry in proteins. J. Mol. Biol. 201, 601–619.

Berger, B., Gaspar, P., Verney, C., 1991. Dopaminergic innervation of the cerebral cortex: unexpected differences between rodents and primates. Trends Neurosci. 14, 21–27.

Buck, K.J., Amara, S.G., 1995. Structural domains of catecholamine transporter chimeras involved in selective inhibition by antidepressants and psychomotor stimulants. Mol. Pharmacol. 48, 1030–1037.

Burley, S.K., Petsko, G.A., 1985. Aromatic–aromatic interaction: a mechanism of protein structure stabilization. Science 229, 23–28.

Carvelli, L., Moron, J.A., Kahlig, K.M., Ferrer, J.V., Sen, N., Lechleiter, J.D., Leeb-Lundberg, L.M., Merrill, G., Lafer, E.M., Ballou, L.M., Shippenberg, T.S., Javitch, J.A., Lin, R.Z., Galli, A., 2002. PI 3-kinase regulation of dopamine uptake. J. Neurochem. 81, 859–869.

Chen, N., Vaughan, R.A., Reith, M.E., 2001. The role of conserved tryptophan and acidic residues in the human dopamine transporter as characterized by site-directed mutagenesis. J. Neurochem. 77, 1116–1127.

Donovan, D.M., Vandenbergh, D.J., Perry, M.P., Bird, G.S., Ingersoll, R., Nanthakumar, E., Uhl, G.R., 1995. Human and mouse dopamine transporter genes: conservation of 5′-flanking sequence elements and gene structures. Brain Res. Mol. Brain Res. 30, 327–335.

Dougherty, D.A., 1996. Cation–pi interactions in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp. Science 271, 163–168.

Giros, B., Wang, Y.-M., Suter, S., McLeskey, S.B., Pifl, C., Caron, M.G., 1994. Delineation of discrete domains for substrate cocaine and tricyclic antidepressant interactions using chimeric dopamine–norepinephrine transporters. J. Biol. Chem. 269, 15985–15988.

Giros, B., Jaber, M., Jones, S.R., Wightman, R.M., Caron, M.G., 1996. Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature 379, 606–612.

Grigoriadis, D.E, Wilson, A.A., Lew, R., Sharkey, J.S., Kuhar, M.J., 1989. Dopamine transport sites selectively labeled by a novel photoaffinity probe: 125I-DEEP. J. Neurosci. 9, 2664–2670.

Hall, F.S., Li, X.F., Sora, I., Xu, F., Caron, M., Lesch, K.P., Murphy, D.L., Uhl, G.R., 2002. Cocaine mechanisms: enhanced cocaine, fluoxetine and nisoxetine place preferences following monoamine transporter deletions. Neuroscience 115, 153–161.

Hastrup, H., Karlin, A., Javitch, J.A., 2001. Symmetrical dimer of the human dopamine transporter revealed by cross-linking Cys-306 at the extracellular end of the sixth transmembrane segment. Proc. Natl. Acad. Sci. U. S. A. 98, 10055–10060.

Huang, Y., Lemieux, M.J., Song, J., Auer, M., Wang, D.N., 2003. Structure and mechanism of the glycerol-3-phosphate transporter from *Escherichia coli*. Science 301, 616–620.

Huff, R.A., Vaughan, R.A., Kuhar, M.J., Uhl, G.R., 1997. Phorbol esters increase dopamine transporter phosphorylation and decrease transport Vmax. J. Neurochem. 68, 225–232.

Itokawa, M., Lin, Z., Cai, N.S., Wu, C., Kitayama, S., Wang, J.B., Uhl, G.R., 2000. Dopamine transporter transmembrane domain polar mutants ΔG and ΔΔG values implicate regions important for transporter functions. Mol. Pharmacol. 57, 1093–1103.

Kilty, J.E., Lorang, D., Amara, S.G., 1991. Cloning and expression of a cocaine-sensitive rat dopamine transporter. Science 254, 578–579.

Kitayama, S., Dohi, T., Uhl, G.R., 1994. Phorbol esters alter functions of the expressed dopamine transporter. Eur. J. Pharmacol. 268, 115–119.

Lee, F.J., Pristupa, Z.B., Ciliax, B.J., Levey, A.I., Niznik, H.B., 1996. The dopamine transporter carboxyl-terminal tail. Truncation/substitution mutants selectively confer high affinity dopamine uptake while attenuating recognition of the ligand binding domain. J. Biol. Chem. 271, 20885–20894.

Lew, R., Grigoriadis, D., Wilson, A., Boja, J.W., Simantov, R., Kuhar, M.J., 1991a. Dopamine transporter: deglycosylation with exo- and endoglycosidases. Brain Res. 539, 239–246.

Lew, R., Vaughan, R., Simantov, R., Wilson, A., Kuhar, M.J., 1991b. Dopamine transporters in the nucleus accumbens and the striatum have different apparent molecular weights. Synapse 8, 152–153.

Lew, R., Patel, A., Vaughan, R.A., Wilson, A., Kuhar, M.J., 1992. Microheterogeneity of dopamine transporters in rat striatum and nucleus accumbens. Brain Res. 584, 266–271.

Lieske, S.F., Yang, B., Eldefrawi, M.E., MacKerell Jr., A.D., Wright, J., 1998. (−)-3 Beta-substituted ecgonine methyl esters as inhibitors for cocaine binding and dopamine uptake. J. Med. Chem. 41, 864–876.

Lin, Z., Uhl, G.R., 2002. Dopamine transporter mutants with cocaine resistance and normal dopamine uptake provide targets for cocaine antagonism. Mol. Pharmacol. 61, 885–891.

Lin, Z., Uhl, G.R., 2003. Human dopamine transporter gene variation: effects of protein coding variants V55A and V382A on expression and uptake activities. Pharmacogenomics J. 3, 159–168.

Lin, Z., Wang, W., Kopajtic, T., Revay, R.S., Uhl, G.R., 1999. Dopamine transporter: transmembrane phenylalanine mutations can selectively influence dopamine uptake and cocaine analog recognition. Mol. Pharmacol. 56, 434–447.

Lin, Z., Itokawa, M., Uhl, G.R., 2000a. Dopamine transporter proline mutations influence dopamine uptake, cocaine analog recognition, and expression. FASEB J. 14, 715–728.

Lin, Z., Wang, W., Uhl, G.R., 2000b. Dopamine transporter tryptophan mutants highlight candidate dopamine- and cocaine-selective domains. Mol. Pharmacol. 58, 1581–1592.

Lin, Z., Zhang, P.W., Zhu, X., Melgari, J.M., Huff, R., Spieldoch, R.L., Uhl, G.R., 2003. Phosphatidylinositol 3-kinase, protein kinase C, and MEK1/2 kinase regulation of dopamine transporters (DAT) require N-terminal DAT phosphoacceptor sites. J. Biol. Chem. 278, 20162–20170.

Nirenberg, M.J., Chan, J., Vaughan, R.A., Uhl, G.R., Kuhar, M.J., Pickel, V.M., 1997a. Immunogold localization of the dopamine transporter: an ultrastructural study of the rat ventral tegmental area. J. Neurosci. 17, 5255–5262.

Nirenberg, M.J., Chan, J., Vaughan, R.A., Uhl, G.R., Kuhar, M.J., Pickel, V.M., 1997b. Immunogold localization of the dopamine transporter: an ultrastructural study of the rat ventral tegmental area. J. Neurosci. 17, 4037–4044.

Povlock, S.L., Amara, S.G., 1997. The structure and function of norepinephrine, dopamine, and serotonin transporters. In: Reith, M.E.A. (Ed.), Neurotransmitter transporters, Structure, Function, and Regulation. Humana Press, Totowa, NJ, USA, pp. 1–28.

Povlock, S.L., Schenk, J.O., 1997. A multisubstrate kinetic mechanism of dopamine transport in the nucleus accumbens and its inhibition by cocaine. J. Neurochem. 69, 1093–1105.

Ravna, A.W., Sylte, I., Dahl, S.G., 2003. Molecular mechanism of citalopram and cocaine interactions with neurotransmitter transporters. J. Pharmacol. Exp. Ther. [Epub ahead of print].

Sallee, F.R., Fogel, E.L., Schwartz, E., Choi, S.M., Curran, D.P., Niznik,

H.B., 1989. Photoaffinity labeling of the mammalian dopamine transporter. FEBS Lett. 256, 219–224.

Shimada, S., Kitayama, S., Lin, C.L., Patel, A., Nanthakumar, E., Gregor, P., Kuhar, M., Uhl, G., 1991. Cloning and expression of a cocaine-sensitive dopamine transporter complementary DNA. Science 254, 576–578.

Sora, I., Hall, F.S., Andrews, A.M., Itokawa, M., Li, X.F., Wei, H.B., Wichems, C., Lesch, K.P., Murphy, D.L., Uhl, G.R., 2001. Molecular mechanisms of cocaine reward: combined dopamine and serotonin transporter knockouts eliminate cocaine place preference. Proc. Natl. Acad. Sci. U. S. A. 98, 5300–5305.

Uhl, G.R., Johnson, P.S., 1994. Neurotransmitter transporters: three important gene families for neuronal function. J. Exp. Biol. 196, 229–236.

Vandenbergh, D.J., Persico, A.M., Uhl, G.R., 1992. A human dopamine transporter cDNA predicts reduced glycosylation, displays a novel repetitive element and provides racially-dimorphic TaqI RFLPs. Brain Res. Mol. Brain Res. 15, 161–166.

Vaughan, R.A., Huff, R.A., Uhl, G.R., Kuhar, M.J., 1997. Protein kinase C-mediated phosphorylation and functional regulation of dopamine transporters in striatal synaptosomes. J. Biol. Chem. 272, 15541–15546.

Wang, J.B., Moriwaki, A., Uhl, G.R., 1995. Dopamine transporter cysteine mutants: second extracellular loop cysteines are required for transporter expression. J. Neurochem. 64, 1416–1419.

Whitehead, R.E., Ferrer, J.V., Javitch, J.A., Justice, J.B., 2001. Reaction of oxidized dopamine with endogenous cysteine residues in the human dopamine transporter. J. Neurochem. 76, 1242–1251.

Williams, K.A., 2000. Three-dimensional structure of the ion-coupled transport protein NhaA. Nature 403, 112–115.

Wu, X., Gu, H.H., 2003. Cocaine affinity decreased by mutations of aromatic residue phenylalanine 105 in the transmembrane domain 2 of dopamine transporter. Mol. Pharmacol. 63, 653–658.

Xu, F., Gainetdinov, R.R., Wetsel, W.C., Jones, S.R., Bohn, L.M., Miller, G.W., Wang, Y.M., Caron, M.G., 2000. Mice lacking the norepinephrine transporter are supersensitive to psychostimulants. Nat. Neurosci. 3, 465–471.