Cocaine Binding Site from the Structure Function Analysis of the Neurotransmitter Reuptake Transporters

Dissertation

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Abstract

Cocaine abuse is a worldwide health problem. Cocaine inhibits monoamine reuptake proteins including: dopamine transporter (DAT), norepinephrine transporter (NET), and serotonin transporter (SERT). The location of a cocaine binding site on these proteins has not yet been identified. Locating the residues that form a cocaine binding site could be highly useful in novel cocaine therapeutic designs.

The three monoamine transporters are integral membrane proteins that have multiple conformations; therefore, atomic resolution of a crystallized cocaine bound transporter protein is a very difficult task. To determine the cocaine binding site, alternative protein structure function analysis methods must be used to identify how cocaine inhibition occurs. Since cocaine inhibits these transporters at similar concentrations and these proteins share homology, it is presumed that they would share a similar cocaine binding site. This dissertation uses protein mutagenesis in three methods to locate the cocaine binding site: disulfide crosslinks, cocaine analog screening, and computer modeled inhibition.

Cysteine is a unique amino acid; within a protein, two neighboring cysteine residues can form a disulfide linkage identifying residue proximity. Cross-linking these residues can reveal the DAT protein tertiary structure and possible cocaine binding sites. A fully functional D. melanogaster DAT mutant with all non-critical cysteines removed (dDATx7) was mutated to include factor Xa protease recognition sequences (fXa-dDATx7). Using fXa-dDATx7, 54 single cysteine positional mutants were generated in six transmembrane domains (TMs) (1, 2, 3, 5, 7, and 12). Of these, 18 transported DA more than 50% of wild type dDAT. Functional mutants could be paired together in a single protein to determine if cross-linked residues affect cocaine inhibition or if cocaine inhibits cross-linking. However, crystal structures of a monoamine transporter homolog were solved revealing the overall tertiary structure of the transporter superfamily.
An alternative approach utilized cocaine analog compounds that contain different chemical modifications to the cocaine structure. Cocaine methiodide (CM), a charged cocaine analog, cannot pass the blood brain barrier. Therefore, the effects of systemic CM doses represent cocaine actions in peripheral tissues. However, the half maximal inhibition values (IC$_{50}$) of CM have not been clearly determined for major cocaine targets: DAT, NET, SERT, and sodium channels. In cells transfected with individual monoamine transporters, mouse dorsal root ganglion neurons, and synaptosomes from adult male mice, the IC$_{50}$ of CM were at least 31-fold to 184-fold higher than cocaine. These results indicate that an equal dose of this cocaine analog will not produce the equivalent inhibition effect that cocaine produces.

A more potent analog of cocaine is the DAT-selective uptake inhibitor, RTI-113. Using site-directed mutagenesis, putative aqueous-facing residues were switched between the mouse DAT and NET at the same position. Changing a specific tyrosine residue in NET to phenylalanine increased RTI-113 sensitivity. Conversely, switching a tyrosine into the phenylalanine expressing DAT decreased RTI-113 sensitivity. In contrast, neither mutation significantly altered the sensitivities to non-transporter-selective inhibitors or transporter function. Random mutagenesis at this residue of DAT or NET also does not significantly affect inhibitor sensitivity. This indicates a critical residue contributing to the potent uptake inhibitions of a cocaine analog and a possible residue at the cocaine binding site.

Lastly, using a molecular-dynamic determined cocaine-bound DAT model as a guide, five positions within 3Å of the predicted cocaine binding site on DAT were randomly mutated. Some mutations increased cocaine potency, other mutations, such as a proline to glycine mutation in extracellular loop 4, decreased cocaine potency 23-fold. Effects of these positional mutations on cocaine inhibition give support to the computationally determined cocaine bound DAT model.

In conclusion, data presented here show that specific amino acid residues in DAT or NET can affect cocaine or cocaine analog binding. Some of these residues are presumed to form the cocaine binding site. The experimental models generated from these DAT and NET mutants can guide future studies to fully understand how cocaine
inhibits monoamine transporters. Complete understanding of the mechanism of cocaine inhibition can assist drug designs for cocaine addiction treatments.
Dedication

To K.

I guess I didn’t want to die in Ohio either.
Acknowledgements

I would like to thank my advisor, Dr. Howard Gu, for everything. Your guidance, drive, and patience molded me into the scientist I am today. The latitude and freedom you provided me, allowed me to either succeed or to fail on my terms. You were always there to ask me what I learned from both. I can’t possibly thank you enough for everything you have provided me.

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Lastly, thanks again to Kelly because apparently I can’t thank you enough for this. You win.
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Fields of Study

Major Field: Biochemistry
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Chapter 1: A cysteine removed dopamine transporter to assist with identification of the cocaine binding site


1.1 Abstract
Cysteine is a unique amino acid; within a protein, two neighboring cysteine residues can form a disulfide linkage. Disulfide producing reagents can induce individual pairs of cysteines to crosslink. Knowing the original positions of these crosslinked residues can determine the orientation of a protein’s transmembrane domains (TMs). A fully functional *Drosophila melanogaster* dopamine transporter (dDAT) mutant with all non-essential cysteines removed (dDATx7) was mutated to include individual factor Xa (fXa) protease recognition sequences in extracellular loops (fXa-dDATx7). Using fXa-dDATx7 as a template, 54 single cysteine mutants were generated inside six TMs (1, 2, 3, 5, 6, and 12). Of these single cysteine mutants, 18 transported dopamine at least 50% as well as wild type, indicating that dDAT is tolerant of multiple mutations throughout TM helix regions. Functional single mutant transporters with cysteines existing in different TMs could be paired together into dual mutant transporters. Dual mutant transporters would be used to determine if chemically cross-linked cysteine residues in different TMs, could prevent cocaine inhibition or if cocaine could prevent reagent induced cysteine cross-linking. However, the crystal structure of a homolog to the monoamine transporters was identified during single cysteine mutant screening. This discovery limits the value of TM cross-linking experiments to reveal the DAT tertiary structure that would assist in identifying the cocaine binding site.

1.2 Background
Cocaine abuse is a worldwide social and health problem and this abuse is
prevalent in the United States. The major neurochemical mechanisms behind cocaine’s CNS effects are caused by a blockade of the major neurotransmitter reuptake proteins: the dopamine transport protein (DAT), the norepinephrine transport protein (NET) and the serotonin transport protein (SERT) (Ritz et al., 1990).

In neurons, many solute carrier proteins such as DAT, NET and SERT are membrane bound (Amara and Kuhar, 1993). Their highly hydrophobic nature hampers modern structural analysis techniques, such as x-ray crystallography, to analyze integral membrane transporters under native conditions, (Kaback et al., 2001). The discovery of any cocaine binding sites that may exist within the monoamine transporter proteins, and residues that play a critical role in transporter inhibition, is therefore impeded.

Cysteine is a unique amino acid; within a protein two neighboring residues can form disulfide linkage. This disulfide linkage can occur spontaneously or can be assisted via application of an exogenous chemical cross-linker (Kaback et al., 2001). Commercially available reagents have previously been utilized to identify the proximity of cysteine residues in situ, such as Lac permease (Zhang et al., 2002). This method approximately deduces the 3-dimentional tertiary structure of a membrane bound protein. Positional mutation of key TM residues to a cysteine, followed by reagent induced disulfide linkage could conclusively determine the local orientation of nearby transmembrane domains in native conditions. Finally, cross-linked residues may impact cocaine inhibition of the mutant transporter, reducing the number of possible locations of where a cocaine binding site might exist.

This experimental process would follow several steps. First, all cysteines need to be removed from the transporter, generating a cysteine-less transporter. Cysteines then need to be systematically inserted into the cysteine-less transporter at different aqueous interacting residues in different TM domains. In order to measure the effectiveness of the dual cysteine-crosslinked model, the single cysteine mutant transporters need to retain successful transport function. A plasmid library of different functional single cysteine mutant transporters is produced. These transporter plasmids would then be sub-cloned together to produce a second plasmid library of dual mutant cysteine transporters,
assembled from across different TMs. From this dual mutant library, transporter function would be assayed in the presence of cocaine and in the presence of chemical cross-linking reagents. This assay would determine if one compound could prevent the function of the other on the dual cysteine mutants.

However, the transmembrane domains of the Na⁺/Cl⁻-dependent neurotransmitter transporter family, which includes DAT, are presumed to contain many endogenous cysteine residues (Ferrer and Javitch, 1998). Both the number and distribution of these residues would therefore make it difficult to obtain conclusive cross-linking results with a commercial reagent. Table 1.1 highlights several species DATs and the numbers of cysteine residues contained within each transporter homolog.

<table>
<thead>
<tr>
<th>Selected Species DAT</th>
<th>Cysteine Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo sapiens</td>
<td>13</td>
</tr>
<tr>
<td>Bos taurus</td>
<td>18</td>
</tr>
<tr>
<td>Rattus norvegicus</td>
<td>13</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>13</td>
</tr>
<tr>
<td>Danio rerio</td>
<td>13</td>
</tr>
<tr>
<td>Caenorhabditis elegans</td>
<td>15</td>
</tr>
<tr>
<td>Eloria noyesi</td>
<td>12</td>
</tr>
<tr>
<td>Bombyx mori</td>
<td>12</td>
</tr>
<tr>
<td>Trichoplusia ni</td>
<td>12</td>
</tr>
<tr>
<td>Drosophila melanogaster</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 1.1: Cysteine residues in DAT homologs
Of these eukaryotic transporter homologs, *Drosophila melanogaster* DAT (dDAT) contains the least number of endogenous cysteines. There are 13 cysteine residues in human, rat, and mouse DAT (hDAT, rDAT, and mDAT, respectively) (Hastrup et al., 2001), (Park et al., 2002), 15 cysteines in *C. elegans* DAT (ceDAT) (Chen et al., 2006), and 9 cysteines in dDAT (Porzgen et al., 2001). Figure 1.1 is a diagram showing the predicted topological distribution of the transmembrane domains and endogenous cysteine residues in the wild type dDAT protein, based on hydrophobicity analysis. An additional advantage of the dDAT model system is that dDAT has similar transport rate and cocaine inhibition as hDAT. The dDAT is therefore a desirable candidate protein for determining the structural features of the DAT protein via cysteine removal, positional reinsertion, and reagent induced cross-linking. Therefore, dDAT was chosen to generate a cysteine-less DAT.

![Figure 1.1: Predicted locations of dDAT transmembrane domains and endogenous cysteine residues.](image)

The diagram indicates the putative locations of 12 transmembrane domains of the dDAT protein and terminals. The approximate locations of the endogenous cysteine residues are indicated with an asterisk.

The putative locations of cysteine residues in dDAT are based on a hypothesized model of dDAT derived from hydrophobicity plot and homology alignment (Porzgen et al., 2001). This model places two cysteines in the second extracellular loop (C148 and
C157), one cysteine (C98) in the first intracellular loop, and the remaining six cysteines within TMs. Most of these cysteine residues would need to be mutated so the reagent-induced disulfide linkage would only occur between the specific reintroduced cysteine residues. Yet, these mutations cannot compromise normal transporter reuptake functionality to measure the cocaine inhibition effects.

1.3 Materials and methods

1.3.1 Specific mutagenesis of dDAT

First, cDNA encoding from Drosophila melanogaster was obtained as previously described. (Wu et al., 1996) Using a BLAST search, three cysteine sites were mutated using site directed mutagenesis with oligonucleotide primers designed from homologous mammalian biogenic amine transmitter reuptake proteins (DAT, NET, SERT). These positions contained similar hydrophobic residues contained within similar protein segments. Four remaining cysteine sites on dDAT were removed using site directed random mutagenesis with position specific degenerate oligonucleotide primers (Chen et al., 2007). The resulting seven cysteine removed dDAT transporter (dDATx7) was modified to include a c-myc epitope (EQKLISEEDLN EQKLISEEDLN) at the N-terminus, and a 6-his (GGHHHHHHH) tag at the c-terminus (dDATx7mh).

Factor Xa protease digestion could confirm crosslinks between cystenes on different TMs. Since no digestion sites exist in WT dDAT, factor Xa (fXa) digestion sites (IEGR or IDGR) were systematically introduced singularly into loop sections of the dDAT protein where amino acid homology to WT could be mostly conserved. These single fXa site, dDATx7mh constructs were then examined via transport assay to confirm dopamine reuptake remained similar to WT. A particular fXa-dDATx7mh construct with the fXa site in the second extracellular loop (named 2a3) was most pharmacologically similar to WT and was used for future cysteine mutagenesis.

To generate cysteine mutations at a particular position, PCR primers were designed and purchased with cysteine encoding nucleotides (TGT or TGC) for the codon of the particular position to be mutated to Cys. The oligonucleotide primers included
various silently added restriction enzyme sites to confirm mutation insertion and the sequences for the primers are listed in Appendix A. The mutants were then assayed for uptake activity (see below) and functional mutants were selected for further characterization. The nucleotide sequences of the mutant constructs were confirmed by DNA sequencing (OSU Comprehensive Cancer Center).

1.3.1 \[^{3}H\]dopamine uptake into transiently transfected cells

HeLa cells (American Type Culture Collection, Rockville, MD) were grown in 96-well plates, infected with recombinant vTF-7 vaccinia virus, which carries the T7 polymerase gene, and transiently transfected in triplicate with the plasmids either containing wild type or cysteine mutant dDAT cDNAs using Lipofectin (Invitrogen Corp., Carlsbad, CA) as described before (Wu and Gu, 2003). 20–24 h post transfection, HeLa cells were assayed for dopamine uptake in 96-well plates at room temperature using PBS+MC buffer (phosphate buffered saline solution containing 1 mM MgCl\(_2\), 0.1 mM CaCl\(_2\), and 50 µM L-ascorbic acid). For the determination of Km and Vmax values, cells were incubated in PBS+MC buffer containing 60 nM \[^{3}H\]-dopamine in the presence of increasing concentrations of unlabeled dopamine (0.1–20 µM) for 10 min at room temperature. For determination of IC\(_{50}\) values, cells were incubated in the PBS+MC buffer containing at least 60 nM \[^{3}H\]-dopamine in the presence of increasing concentrations of cocaine. Reactions were terminated by two successive washes with PBS+MC, and cells were dissolved in 0.1 M NaOH. Amounts of \[^{3}H\]-dopamine accumulated in the cells were quantitated by liquid-scintillation counting. All experiments were performed in triplicate. Cells transfected with vehicle alone were used as controls and radioactivity associated with these cells were considered the background and subtracted from the total uptake. The Km, Vmax, and IC\(_{50}\) values were determined by a one-site sigmoidal concentration-response nonlinear regression analyses of experimental data using GraphPad Prism 3.0 (San Diego, CA) and t-test was performed to determine the significance of the differences between a particular mutant and the wild type dDAT. Protein concentrations were determined in triplicate using Bio-Rad dye and bovine serum albumin (gamma V) as the standard. The Km, Vmax, and IC\(_{50}\) values presented are averages and standard error of means calculated from 3 or more
experiments.

1.3.2 6-His purification and protein digestion by factor Xa

For factor Xa (fXa) digestion, HeLa cells in a 6-well tray were transfected as previously described. 20-24hrs post transfection, cells were washed twice with PBS then lysed on ice with 0.1% non-ionic detergents (either NP-40 or digitonin) in PBS. Cysteine mutant dDAT proteins were isolated from the whole cell lysate on Co^{2+}-sepharose beads (TALON metal affinity resin), then digested with fXa overnight at either 4ºC or RT with increasing concentrations of SDS (0.0-0.2%). Digested protein samples were then visualized on film via western blot using mouse anti c-myc Ab (1:1000, 0.05% PBS-Tween 20 in 5% low fat milk, 4ºC o/n, Cell Signaling Technologies), then peroxidase conjugated goat anti-mouse Ab(1:5000, 0.05% PBS-Tween 20 in 5% low fat milk, RT 1hr, Santa Cruz Biotechnology) followed by two 5 min PBS-T washes before addition of chemiluminescent substrate (Millipore).

1.4 Results

Using phylogenetically conserved homology, seven normal DAT cysteines were systematically removed by mutation from *Drosophila melanogaster* DAT protein (dDATx7). One pair of neighboring extracellular cysteines that are understood to form a separate disulfide linkage were retained in this protein (Chen et al., 2007). As individual cysteines were mutated out of the protein and terminal tags were added, the mutant protein remained functional, retaining similar reuptake characteristics to the wild type control (Chen et al., 2007). Normal dopamine transport with the seven cysteine-removed mutant remained functional, showing that this mutant protein retained dopamine transport characteristics.

To facilitate biochemical analysis, marker tags were added to each end of the dDATx7; a double c-Myc tag at the N-terminus and a His tag (6 Histidine residues) at the C-terminus (dDATx7mh). (N.B. Howard Gu assisted with the insertion of terminal tags.) The Km and Vmax data all confirm that mutations removing cysteine residues from the dDAT, and the addition of the two tags, did not affect transport activity (Figure 1.2). The
Km values (µM) for dDAT and dDATx7mh were 2.43 ± 0.61 and 1.42 ± 0.39, respectively. The Vmax (pmole/ mg protein/min) values for dDAT and dDATx7mh were 13.2 ± 1.07 and 9.28 ± 1.31, respectively.

![Graph showing dopamine uptake](image)

**Figure 1.2:** Saturation uptake of tagged mutant dDAT protein.

Dopamine uptake activities by HeLa cells transiently transfected with dDAT (squares) or dDATx7mh (open squares) were measured in the presence of increasing concentrations of unlabeled dopamine at room temperature. The Km values (µM) for dDAT and dDATx7mh were 2.43 ± 0.61 and 1.42 ± 0.39, respectively. The Vmax (pmole/mg protein/min) values for dDAT and dDATx7mh were 13.2 ± 1.07 and 9.28 ± 1.31, respectively. The presented data are averages of triplicates and the error bars represent standard error of means (SEM).

The terminal-tagged seven cysteine removed dDAT proteins also have a slightly shifted Ki value indicating possible insensitivity to cocaine inhibition (Figure 1.3). The IC50 values of cocaine inhibition for WT dDAT and dDATx7mh were 6.0 ± 0.1 and 17.4 ± 1.9 µM (P < 0.05 versus dDAT, t-test). As previously shown (Wu and Gu, 2003), specific introduction of cysteine residues can have dramatic effects on DAT functionality.
in the presence of cocaine.

Figure 1.3: Cocaine inhibition of tagged mutant dDAT protein.

HeLa cells were transiently transfected with dDAT (squares) or dDATx7mh (open squares). Dopamine uptake was measured in the presence of increasing concentrations of cocaine. The uptake activities are presented as fractional activities relative to those in the absence of drugs. The IC$_{50}$ values of cocaine inhibition for dDAT and dDATx7mh were 6.0 ± 0.1 and 17.4 ± 1.9 µM (P < 0.05 versus dDAT, t-test). Each data point is expressed as mean ± SEM (n = 3).

Digestion with fXa protease has previously been used for confirming the location crosslinked transmembrane domains of a integral membrane protein, E. coli membrane protein nucleotide transhydrogenase transpoter (Althage et al., 2003). The fXa protease recognition sequence (IEGR) does not natively exist in dDAT. Therefore, factor Xa (fXa) sites were systematically singularly introduced into predicted extracellular loop sections of dDAT. To confirm that the mutation to insert the fXa sequence into extracellular loops did not affect cocaine inhibition of transporter function, substrate uptake assays with the mutated fXa-dDATx7mh constructs determined which fXa mutant dDAT constructs
retained WT pharmacological profile (Figure 1.4). (N.B. Rong Chen assisted with the insertion and assessment of fXa mutant dDAT constructs)

Figure 1.4: Effect of factor Xa recognition sequence on dDATx7mh transport function.

Dopamine uptake activities by HeLa cells transiently transfected with indicated fXa mutant constructs. The uptake activities are presented as percent uptake relative to the parent dDATx7mh plasmid. The data presented are triplicate measures and the error bars represent ± SEM.

While the introduction of fXa sites did have some effect on transport efficiency when compared to controls, the specific locations selected within the predicted loops had a much greater effect on transport than the introduction of the fXa digestion sequence itself (Figure 1.4). Inserting IEGR sequence residues at one position in the intercellular loop between TM2 and TM3 retained near normal transport (TM2a3). However, mutations at extracellular locations near TMs 4, 5, and 6 did not have normal transport. Therefore, the 2a3 construct was selected.

To confirm protease digestion of the selected 2a3-fXa construct, the mutant protein was isolated using Cobalt-sepharose beads from whole-cell lysate of transiently
transfected HeLa cells. The bound protein was then digested using an fXa protease added
digestion buffer with 0.2% SDS, overnight at either room temperature or 4°C. The
digestion buffer was then removed and new buffer including 100mM imidazole was used
to elute the protein from the sepharose beads. Western blot using anti-myc antibody
confirmed that the cyststine removed fXa-dDATx7mh construct can be isolated and
digested with fXa protease within a certain temperature and detergent range (Figure 1.5).
(N.B. Mike Tilley assisted with Cobalt-sepharose isolation of the proteins.)

Figure 1.5: Western Blot of fXa-dDATx7mh digestion on Cobalt-sepharose beads.

Transiently transfected HeLa cells were lysed on ice with detergents concentration
indicated in PBS. Lysate was added to Co⁺²-sepharose beads then digested with fXa
protease in 0.2% SDS buffer overnight at either 4°C or RT. Supernatant digestion buffer
was then separated and 100mM imidazole was added. Digested protein samples were then visualized on film via western blot using mouse anti c-myc Ab (1:1000), then anti-mouse Ab (1:5000).

The western blot revealed a smaller band (15kDa) following addition of the fXa protease, as well as the expected dDAT protein (60kDa). Multiple higher weight bands have been previously observed in transfected dDATx7 constructs. These bands are presumed to be glycosylated versions of the protein (Chen et al., 2005). The 120kDa+ bands are presumed to be the result of dDAT homo-dimers, but there appeared to be an increase in the 80kDa band following fXa addition. Because the 80kDa increase could be the possibility of spontaneous protein crosslink between the 15kDa fragment to unknown membrane proteins to form the upper weight bands remains, and could cloud the observation of an actual crosslink. To confirm that the multiple higher weight bands observed in the eluted fraction and crude lysate were from glycosylation of the dDAT protein and not from a spontaneous protein crosslink, the eluted fraction was treated with endoglycosidase Hf in 2% SDS to further disrupt any non-specific dimerization resulting in the 120kDa band. Figure 1.6 shows the western blot of EndoHf treated whole cell lysates. The western blots reveal that the 80kDa band from the eluted fraction is the glycosylated protein, and not a spontaneous crosslink.
Figure 1.6: Western Blot of fXa-dDATx7mh with EndoHf.

The imidazole eluted fraction following fXa digestion was incubated with EndoHf in 2%SDS for 1hr at 4°C. Digested protein samples were then visualized on film via western blot using mouse anti c-myc Ab (1:1000), then anti-mouse Ab (1:3000).

Because the cysteine removed fXa-dDATx7mh construct retained near normal transport in pharmacological and functional assays, uniquely positioned cysteine residues could now be reintroduced into the protein. Using site directed mutagenesis, individual cysteine residues were systematically reintroduced into key transmembrane portions of the fXa-dDATx7mh construct with specifically designed oligonucleotide primers (see Appendix A for primer details). Six transmembrane domains near the largest predicted extracellular loops 1, 2, 3, 5, 7, and 12 were selected for mutagenesis. From these domains, 54 single cysteine mutant constructs were generated and protein functionality was confirmed via dopamine transport assay (Figure 1.7).
Figure 1.7: Effect of single cysteine mutants on fXa-dDATx7mh substrate transport.

Dopamine uptake activities by HeLa cells transiently transfected with indicated single cysteine mutant constructs. The uptake activities are presented as percent uptake relative to the parent fXa (2a3) plasmid. The data presented are triplicate measures and the error bars represent ± SEM. The asterisk indicates that dDATx7 was previously mutated to C58A.
Figure 1.7
Single amino acid changes to cysteine did not alter function in any TM segments screened. The A57C mutation was the reverse of one of the original dDATx7 mutations. An interesting observation was that the re-introduction of cysteine at this position severely disabled transporter function.

Of the 54 mutants screened, 18 retained >50% DA transport function of the dDATx7mh-2a3 parent transporter construct. From this library of single cysteine transporters, single mutations in TM1, 2 and 12 retained the highest level of function. This library of cysteine residues can be used to generate double mutants with a high probability of functional transport. Chemical crosslinks can be induced to link domains, which can be confirmed after fXa protease digestion by SDS-PAGE.

1.5 Discussion

Because DAT proteins are integral membrane proteins, modern structural analysis using X-ray crystallization is very difficult. The bulk purification of membrane bound proteins for crystallization attempts would be expensive in both time and money with a low probability for success. Cysteine crosslinks were selected as a more economical and simpler method to localize the amino acid residues in DAT that are involved in cocaine inhibition.

While the library of single dDATx7 cysteine mutants was being generated, Yamashita et al. identified the crystal structure of a bacterial homolog to the 12TM Na+/Cl−-dependent transporters, LeuTaa. The bacterial homolog retained 20% homology to human DAT. (Yamashita et al., 2005) Although the homolog lacked key extracellular loops found on all mammalian biogenic amine transporters, the TM domains are highly conserved at uniquely occurring residues. Additionally, the LeuTaa protein lacks cysteine residues. While the crystal structure doesn’t conclusively show the DAT 3-dimensional structure, it provides an additional guide for the proposed structural and transmembrane alignments. Figure 1.8 is a revised 2-dimensional diagram of dDATx7 based on the 3-dimensional LeuTaa crystal structure model.
Figure 1.8: Two dimensional topological diagram of the Drosophila dopamine transporter.

The diagram is based on sequence alignment with the crystal structure of a Leucine transporter, LeuTaa, which is a bacterial homolog of the Na⁺/Cl⁻ coupled neurotransmitter transports. Transmembrane residues are indicated with gray circles and are drawn in the shaded area representing cell membrane. The seven residues where cysteines were replaced are highlighted in black circles with bold white letters. The two remaining extracellular cysteines are highlighted in enlarged gray circles with bold black letters and they are shown to be linked by a disulfide bond.
The LeuTaa structure also highlighted an inherent flaw in the design of the crosslink experiments. Some of the putative TM domains that had been identified previously by hydrophobicity plots were incorrect. Cysteine mutations occurring in critical ion, salt bridge, or substrate binding locations could have impeded the uptake measurements in many of the single mutants, as was shown in Figure 1.7. A second result of the shifting domain location is that regions selected for cysteine mutation due to extracellular proximity had moved to locations outside the distances that could produce disulfide crosslinks in the native transporters. Recently, this cysteine crosslinking method has been used to confirm the proximity of TM domains in SERT using the LeuTaa crystal structures as a guide (Tao et al., 2009).

In conclusion, the dDAT can accommodate multiple mutations. While it had previously been shown that all but two cysteine residues can be mutated without affecting protein function, the data presented here shows that other specific mutations to dDAT do not negatively impact dDAT function. The addition of terminal tags, fXa protease recognition sites, and some unique single cysteine mutants in different TM domains did not significantly impact protein function. The data presented here solidifies the concept that specific residues in the DAT protein are responsible for either substrate binding and/or inhibitor interactions. Discovering the specific residues that are uniquely responsible for affecting inhibitor binding is the major step towards identifying the cocaine binding site.
1.6 References


2.1 Abstract

Cocaine methiodide (CM), a charged cocaine analog, cannot pass the blood brain barrier. It has been assumed the effects of systemic CM represent cocaine actions in peripheral tissues. However, the IC$_{50}$ values of CM have not been clearly determined for the major cocaine targets: dopamine, norepinephrine, and serotonin transporters, and sodium channels. Using cells transfected with individual transporters from mice and synaptosomes from mouse striatum tissues, the inhibition IC$_{50}$ values for monoamine uptake by CM were 31-fold to 184-fold higher compared to cocaine at each of the transporters. In dorsal root ganglion neurons, cocaine inhibited sodium channels with an apparent IC$_{50}$ of 75 µM, while CM showed no observable effect at concentrations up to 3 mM. These results indicate that an equal dose of CM will not produce an equivalent peripheral effect of cocaine.

2.2 Background

Cocaine produces complex behavioral and physiological effects including: addiction and locomotor stimulation, cardiac arrhythmias, and hormonal changes (Kalivas, 2007) (Koob and Kreek, 2007). The high affinity targets of cocaine include the dopamine (DA) transporters (DAT), norepinephrine (NE) transporters (NET), and serotonin transporters (SERT) (Amara and Kuhar, 1993). Cocaine inhibits these transporters with similar potencies at micromolar or submicromolar levels (Han and Gu, 2006).
Cocaine produces effects in the central nervous system (CNS) primarily by inhibiting three monoamine transporters, DAT, NET and SERT (Amara and Kuhar, 1993). These transporters clear neurotransmitters from neural synapses and surrounding areas through monoamine reuptake (Kalivas, 2007). Cocaine inhibition of these reuptake processes results in prolonged monoamine elevation in brain regions that promote reward and addiction (Kalivas, 2007). Cocaine also blocks sodium channels but with lower potencies (50 \( \mu \text{M} \) or higher) (Ma et al., 2006). These cocaine targets are expressed in both the CNS and the peripheral systems (Billman, 1995) (Brown and Kiyatkin, 2006).

Many chemical analogs of cocaine have previously been synthesized (Strycker and Long, 1968), (Tufariello, 1979). Cocaine methiodide (CM) is a chemical analog of cocaine with a stable positive charge at physiological pH. The positive charge of CM prevents a systemic administered dose from crossing the blood brain barrier (Schindler et al., 1992). Therefore, CM should only inhibit the functions of cocaine target proteins in peripheral tissues.

It has been observed that the toxic effects of systemic CM, measured in vivo by median lethal doses (LD\(_{50}\)), are similar to that of cocaine (Shriver and Long, 1971) (Witkin et al., 1993), leading to the presumption that the potencies of CM and cocaine for peripheral targets might be similar. Accordingly, several investigations examined the effects of systemic CM with the presumption that the results represented cocaine interactions with peripheral cocaine targets at similar doses (Dickerson et al., 1999; Schindler et al., 1992).

However, some studies have shown that CM and cocaine may have different potencies at cocaine targets. CM was shown to be less potent than cocaine at inhibiting NE uptake in aortic tissues dissected from guinea pigs and rats (Tessel et al., 1978). CM was found to be less potent than cocaine at inhibiting the binding of mazindol (Ritz et al., 1990) to rat striatal tissue preparations. In addition, in vivo data showed that CM via intracranial delivery did not produce comparable results to cocaine in rat self-administration tests (Witkin et al., 1991).

While these prior CM studies are relevant to compare the effects of CM to
cocaine, they were performed in tissue preparations that contain multiple cocaine targets with varying expression levels. Accordingly, the concentration-responses for these two drugs have not been clearly determined for each major target of cocaine (DAT, NET, SERT or sodium channel subtypes). Therefore, it is important to determine the potency of CM, as compared to cocaine, for individual major cocaine target proteins expressed in the CNS and in peripheral tissues.

2.3 Materials and Methods

2.3.1 Substrate reuptake into transiently transfected cells

Plasmid DNA containing mDAT, mNET, and mSERT were cloned into bluescript vector with a T7 promoter as described (Chen et al., 2005) (Wei et al., 2009). HeLa cells (American Type Culture Collection, Rockville, MD) were grown in 96-well plates, infected with recombinant vTF-7 vaccinia virus, carrying the T7 polymerase gene, and transiently transfected with the plasmids bearing cDNAs using Lipofectin (Invitrogen Corp., Carlsbad, CA) as described previously (Chen et al., 2005).

About 20–24 h after transfection, HeLa cells were assayed for substrate uptake in 96-well plates at room temperature using PBS/Ca/Mg buffer. For determination of IC₅₀ values, cells were co-incubated in the PBS/Ca/Mg buffer with added 60 nM [³H]-labeled monoamine substrates and increasing concentrations of an inhibitor (e.g., cocaine or CM). Uptakes were terminated by two successive washes with PBS/Ca/Mg. Amounts of [³H]-labeled substrates accumulated in the cells were quantitated by liquid-scintillation counting. All experiments were performed in triplicates. Cells transfected with vehicle were used as controls and radioactivity associated with these cells were considered the background. This background was subtracted from the total scintillation counts of the wells.

2.3.2 Dopamine reuptake into synaptosomes

N.B. Mike Tilley performed, while Erik Hill assisted in synaptosome preparation and assays.

All animal work was conducted in adherence to OSU IACUC approved protocols and guidelines for animal welfare. C57B6 mice (aged 6-8 weeks) were decapitated and
striatum were dissected from both sides of the brain and stored on ice. The tissues were placed in ice-cold Krebs’-Ringer’s solution buffer (KRB) (in mM: 125 NaCl, 1.2 KCl, 1.2 MgSO4, 1.2 CaCl2, 22 NaHCO3, 1 NaH2PO4, and 10 glucose, adjusted to pH 7.4) with an additional 0.32 M sucrose. Tissue samples were homogenized by using a glass homogenizing tube and with a Teflon-coated pestle. The samples were centrifuged for 10 min at 1,000xg. Supernatant was collected and the debris pellet was discarded. Supernatant was centrifuged for 15 min at 16,000x g. The resulting pellet contained synaptosomes, and was resuspended in KRB supplemented with pargyline (50µM) and ascorbic acid (100µM) then applied to a 96-well microfilter plate (Millipore, Irvine, CA). Synaptosomes were assayed for substrate uptake at room temperature using a PBS/Ca/Mg buffer (phosphate buffered saline solution containing 1 mM MgCl2, 0.1 mM CaCl2, and 50 µM L-ascorbic acid). For determination of IC50 values, synaptosomes were co-incubated in the PBS/Ca/Mg buffer with added 60 nM [3H]-labeled dopamine, 100 µM desipramine (NET-selective inhibitor) and increasing concentrations of an inhibitor (e.g., cocaine or CM). Cocaine and cocaine methiodide were provided by the Drug Supply Program, National Institutes on Drug Abuse (NIH, Bethesda, MD). Uptakes were terminated by two successive washes with PBS/Ca/Mg and vacuum removal through the filter plate. Amounts of [3H]-labeled dopamine accumulated were quantitated by liquid-scintillation counting. All experiments were performed in triplicates. Synaptosomes with the highest dose of inhibitor were used as background controls and radioactivity associated with these wells were subtracted from the total scintillation counts of all wells.

2.3.3 Whole-cell voltage clamp recording of sodium channel currents in dorsal root ganglion neurons

N.B. Jinbin Tian performed, while Erik Hill assisted in making voltage clamp recordings.

Dorsal root ganglion (DRG) neurons (T1-T10) from adult male C57B6 mice (aged 6-8 weeks) were isolated as per Malin et al. (Malin et al., 2007) Briefly, ganglia were dissected under stereo microscope and washed in Ca2+/Mg2+-free Hank’s Buffered Salt Solution (HBSS). DRGs were digested enzymatically, first with papain and then collagenase II and dispase II, each for 10 min at 37°C. Digested DRGs were then
triturated in culture media (F-12 supplemented with 10% FBS and 5 mg/ml penicillin/streptomycin) by a fire-polished Pasteur pipette until solution becomes cloudy. Isolated DRG neurons were plated on poly-ornithine coated glass coverslips and maintained in a 37°C, 5% CO₂ incubator. Individual DRG neurons were recorded after overnight culture.

Plated coverslips were centered in a perfusion chamber filled with extracellular solution (ECS) containing (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, pH 7.4. Cocaine and CM were dissolved in ECS and delivered via a pressure-driven perfusion system (SmartSquirt 8, AutoMate Scientific) with the tip positioned so the DRG neuron being recorded was fully within the direct stream of perfusate. Recording pipettes were pulled from micropipette glass (World Precision Instruments, Sarasota FL) to 2-4 MΩ when filled with an intracellular solution containing (in mM): 140 CsCl₂, 1 CaCl₂, 2 MgCl₂, 11 EGTA, 10 HEPES, 2 Mg₂ATP, pH 7.2). Whole-cell recordings were made using an EPC10 amplifier and the PatchMaster 2.2.0 software (both from HEKA Electronik, Germany). As soon as the whole-cell configuration was established, fast and slow capacitances were cancelled and the holding potential (Vh) was set to -70 mV. A step protocol (16 steps from -70 mV to 10 mV with 5 mV increment for each step) was applied to determine the testing voltage (Vt) that generated the maximal inward current in the following experiment. Sodium channels were activated by a 10 consecutive pulse stimulation (10 Hz, 50 ms duration, depolarized from Vh to Vt). Data were filtered at 3 kHz and digitized at 20 kHz. All recordings were made at room temperature (22-24°C). Only one cell per coverslip was recorded to avoid possible drug contamination of other cells. Representative current traces were redrawn in Origin 8.0 SR1 (Northampton, MA).

A range of drug concentrations, starting from low to high, were tested for each DRG neuron. The sodium channel currents were recorded 30 s before the drug application, during the 30 s drug application, and 30-60 s after drug washout by perfusion with ECS. For each set of tests, current amplitude was measured by subtracting the baseline value from the peak current. The current amplitude during the drug was normalized to that before the drug application, and the normalized values were used to
plot the dose-response curve. The currents measured after the drug washout were used to confirm the complete recovery before testing the next drug concentration.

2.3.4 Data analysis

The IC\textsubscript{50} values were determined by a one-site sigmoidal concentration-response nonlinear regression analyses of experimental data using GraphPad Prism 3.0 (San Diego, CA). IC\textsubscript{50} values presented are averages ± standard error of means (SEM) calculated from 3 independent uptake experiments or recordings of 4 different DRG neurons. Statistical analyses for the difference between the IC\textsubscript{50} values of the two drugs were performed by Student’s paired t-test using SPSS 17.0 (Chicago, IL).

2.4 Results

Previous pharmacological studies of CM on CNS proteins utilized dissected tissues or tissue homogenates. Depending on the source, these homogenized tissue samples have variable expressions of multiple cocaine target proteins. In addition, monoamine transporters share substrates and high affinity inhibitor compounds (such as mazindol) commonly used to study drug binding to the transporters. To study the effect of CM on individual transporter cocaine targets, cells transiently transfected with individual transporter cDNAs were used. Because mouse models were used in recent publications on the study of CM (Brown and Kiyatkin, 2006), (Espana et al., 2008), (Wise et al., 2008) the three mouse monoamine transporter cDNAs were selected for transfection. Figures 2.1A–2.1C show the concentration-response curves for CM and cocaine inhibition of monoamine uptake by each of the three transporters. Each experiment was run in triplicate and the experiments were repeated 3 times with similar results. The average IC\textsubscript{50} values for cocaine and CM respectively were: for mDAT, 0.45±0.11 µM and 83.2±2.1 µM, a 184 fold increase; for mNET, 0.67±0.09 µM and 20.9±3.1 µM, a 31 fold difference; and for mSERT, 0.68±0.39 µM and 84.3±4.8 µM, a 123 fold difference. Student’s paired t-tests showed that the IC\textsubscript{50} values of CM and cocaine were significantly different for each of the 3 transporters (p<0.001 for all three comparisons). These results are summarized in Table 2.1.
Figure 2.1: Concentration-response curves for cocaine and cocaine methiodide.

Dopamine uptake by mouse DAT (A), norepinephrine uptake by mouse NET (B), and serotonin uptake by mouse SERT (C) into transfected cells were measured in the presence of increasing concentrations of cocaine or cocaine methiodide. The Na+ channel currents in isolated mouse DRG neurons were recorded by whole-cell patch clamping (D). The data are presented as the percent of the pre-drug activity. Dopamine uptake by striatal synaptosomes were measured in the presence of increasing concentrations of cocaine or cocaine methiodide (E). For A, B, C, and E, each data point represents the average of triplicate measurements ± standard error of means and the experiments were repeated three times with similar results. For D, each data point was obtained from four cells.
Table 2.1: Cocaine and cocaine methiodide IC50 values for the inhibition of monoamine transporters and DRG neuron Na+ channels.

<table>
<thead>
<tr>
<th></th>
<th>Cocaine</th>
<th>Cocaine Methiodide</th>
<th>ratio</th>
<th>p value^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Striatal synaptosomes</td>
<td>0.35±0.11</td>
<td>11.5±5.1</td>
<td>33</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mDAT</td>
<td>0.45±0.11</td>
<td>83.2±2.1</td>
<td>184</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mNET</td>
<td>0.67±0.09</td>
<td>20.9±3.1</td>
<td>31</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mSERT</td>
<td>0.68±0.39</td>
<td>84.3±4.8</td>
<td>123</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DRG Na+ Channels</td>
<td>84.8±5.9</td>
<td>not measured</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

^a IC50 values are mean ± standard error of means calculated from 3 independent experiments.
^bSignificance was determined by Student’s paired t-test.

In addition to the experiments with cultured cells expressing the transporters, CM and cocaine inhibition of dopamine uptake were examined in mouse brain tissues. Synaptosomes were prepared from the striatum of healthy adult C57B6 mice. (N.B. Mike Tilley performed, while Erik Hill assisted in synaptosome preparation and assays.) Standard uptake assays were performed to measure CM and cocaine inhibition. The results are shown in Figure 2.1E (n = 3, p<0.001). The average IC50 values for cocaine and CM were 0.35±0.11 μM and 11.7±5.1 μM respectively, a difference of 33 fold.

Cocaine is well known to block the function of sodium channels (Scholz, 2002). However, the effect of CM on sodium channels has not been reported. Since CM is primarily used to identify cocaine effects in the periphery, where sodium channels are potential targets, it is important to determine whether CM and cocaine had similar potencies on sodium channels in peripheral nerves. DRG neurons express several different fast and slow responding sodium channel subtypes, including NaV1.1, NaV1.6, NaV1.7, NaV1.8, and NaV1.9 (Rush et al., 2007). DRG neurons provide convenient samples to examine the inhibitory effect of cocaine and CM on multiple sodium channels.

Whole-cell recordings were performed using mouse DRG neurons. (N.B. Jinbin
Tian performed, while Erik Hill assisted in making voltage clamp recordings.) Sodium currents were elicited by depolarization to desired test potentials from the holding of $-70 \text{ mV}$ in the absence and presence of cocaine or CM as described in Materials and Methods. Figure 2.2 shows representative inward current traces recorded from DRG neurons, before and during cocaine application, as well as after cocaine washout. As shown in Figure 2.2A–2.2D, cocaine dose dependently inhibited the rising phase of the inward current. Peak currents obtained from 10 voltage pulses were averaged for each drug concentration and normalized to the control value before the drug application. The concentration-response curve is shown in Figure 2.1D for comparison with the monoamine transporters. IC$_{50}$ values were determined for cocaine inhibition of the sodium channels in mouse DRG neurons with an average value of 84.8±5.9 µM (n = 4) (Table 2.1). This data is consistent with previous work that the potency of cocaine in inhibiting sodium channels is roughly 100 fold lower than those for the monoamine transporters (Ma et al., 2006).
Figure 2.2: Effects of cocaine and cocaine methiodide on sodium channels in DRG neurons.

Isolated mouse DRG neurons were voltage-clamped in the whole-cell mode, held at −70 mV and sodium channels were activated by 10 consecutive pulse stimulations (10 Hz, 50 ms duration, depolarized from −70 mV to −25 mV). Representative currents recorded before drug addition (before drug), during the drug (during drug) and after drug washout (after drug) are overlaid for 30 (A), 100 (B), 300 (C) and 1,000 µM (D) cocaine as well as 3 mM cocaine methiodide (E).
However, when CM was applied to isolated DRG neurons, little effect was observed on the sodium channel currents. Even at 3 mM of CM, the highest concentration tested, very little inhibition was observed in peak inward current (Figure 2.2E, n = 4 cells, p>0.05) and the data did not allow the calculation of IC$_{50}$ value. While concentrations higher than 3 mM may inhibit sodium channels, doses equivalent to such concentrations would be much higher than the LD$_{50}$ and thus not likely to be used in animals. Therefore, experiments were limited to 3 mM. Figure 2.1D shows the effect of CM on sodium channel currents from 3 µM to 3 mM. To rule out that the ECS used in the DRG experiments may interfere with CM actions, transport assays with HeLa cells using the ECS as for the DRG experiments were performed. The results were not different from the experiments using the PBS/Ca/Mg buffer (data not shown).

2.5 Discussion
Since CM cannot pass across the blood brain barrier, it had been used in studies attempting to separate the CNS effects from the peripheral effects of cocaine. The effective concentrations used in these studies were based on the assumption that because the LD$_{50}$ values for CM and cocaine are similar, the IC$_{50}$ values for CM and cocaine are similar for major target proteins. The data presented shows very substantial differences between potencies of cocaine and CM in inhibition of major cocaine target proteins.

The individual IC$_{50}$ values determined for CM are 184-fold, 31-fold, and 123-fold higher than those for cocaine at DAT, NET, and SERT respectively. Cocaine inhibits the sodium channels in DRG neurons with an apparent IC$_{50}$ of 85 µM, about 100-fold higher than those for the monoamine transporters. The sodium channel inhibition data shown in Figure 2.1D fit well with a single IC$_{50}$ value, suggesting that the individual CM IC$_{50}$ values for each sodium channel subtypes are similar. In contrast, doses of CM up to 3 mM have little observable effect on sodium channels expressed in DRG neurons. It remains to be determined whether the sodium channel subtypes that are not expressed in DRG neurons can be inhibited by CM and whether those channels have similar sensitivities to CM and cocaine.

Cocaine has been referred as a “dirty drug” due to its multiple sites of action within the CNS and peripheral tissues (Uhl et al., 1996). Separating the cocaine actions
in the CNS from peripheral tissue contributions can be beneficial in understanding complex cocaine effects. The data show that CM and cocaine have very different potencies on the major cocaine targets, and therefore, equimolar systemic doses of CM do not produce equivalent inhibition of major cocaine targets.

The data from DRG neurons raises interesting questions about how systemic CM produces its effects which was hypothesized to result primarily from CM inhibition of peripheral Na\(^+\) channels (Brown and Kiyatkin, 2006). The CM electrophysiology data excludes the five sodium channel subtypes commonly expressed in DRG neurons from mediating this peripheral effect. The effects of CM at other sodium channel subtypes, not expressed in DRG neurons, remain unknown. Indeed, recent reports show that the peripheral glutamate system is involved in rapid CNS effects observed with intravenous administration of cocaine and CM (Wise et al., 2008). However, it is clear from the data that equivalent doses of CM should not be used to examine the effects of cocaine interaction with its peripheral targets.

The use of CM to measure the effects of peripheral cocaine stems from previous reports of similar LD\(_{50}\) values for cocaine and CM (Shriver and Long, 1971) (Witkin et al., 1993), which suggests the two drugs have similar potencies at the targets that mediate the lethal effects. In stark contrast, remarkable differences were observed between the potencies of CM and cocaine. These results suggest that the lethal toxic effects of cocaine and CM are not likely through the inhibition of the monoamine transporters or subtypes of sodium channels expressed in DRG neurons. Other target proteins might be involved. One study indicates that CM produces weaker effect than cocaine in a conditioned taste aversion test (Freeman et al., 2005). Another study shows that cocaine is a 50-fold lower affinity antagonist at α7-nicotinic acetylcholine receptor (nAChR) than α4b4-nAChR, while CM is a high affinity agonist of the α7-receptor, 6-fold higher than ACh (Francis et al., 2001). It has also been proposed that the toxic effects of cocaine emanate from an unknown site in the peripheral tissue (Billman, 1995). Future experiments are needed to identify other CM target proteins and to understand why CM and cocaine have similar LD\(_{50}\) in animals.
The data presented here show that CM is much less potent than cocaine at inhibiting monoamine transporters and thus similar doses of CM will not inhibit the transporters to the same extent. It was also observed that a very high dose of CM does not inhibit sodium channels expressed in DRG neurons. Therefore, systemic CM effects are not good measurements of cocaine actions through its peripheral targets.
2.6 References


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Chapter 3: A critical hydroxyl group affects the potency of RTI-113, a transporter selective cocaine analog compound

3.1 Abstract

Cocaine inhibits the transporters of dopamine, norepinephrine and serotonin. The amino acid residues involved in cocaine inhibition are unknown. A more potent analog of cocaine is the dopamine-selective uptake inhibitor, RTI-113. Site-directed mutagenesis was performed to switch putative aqueous facing residues between the mouse dopamine transporter and norepinephrine transporter at the same position. A residue was identified in transmembrane domain 3 that differs between dopamine transporter (DAT) and norepinephrine transporter (NET). Switching the transporter residues at this position causes a 15-fold increase in RTI-113 sensitivity for NET and 20-fold decrease in DAT sensitivity without altering transporter function. In contrast, the sensitivities to non-selective inhibitors cocaine and RTI-31 are only slightly changed. Performing random mutagenesis at these positions does not affect inhibitor sensitivity. The data reveal a critical residue contributing to the potent uptake inhibitions of the dopamine-selective analog, RTI-113. Furthermore, this drug-sensitive mutant can be used to generate a computer model to study the role of specific residues in cocaine inhibition of uptake transporters.

3.2 Background

Cocaine inhibits the dopamine transporter (DAT) and norepinephrine transporter (NET) at similar concentrations (Han and Gu, 2006). Since cocaine inhibits the monoamine transporters at similar concentrations and these transporters share homology, it is presumed that they have a common similar cocaine binding site (Wu and Gu, 2003), (Gu et al., 2006), (Beuming et al., 2006).

In contrast to cocaine, some cocaine analogs have very different affinities to DAT
and NET (Carroll et al., 1995). Since DAT and NET are highly homologous and believed to have similar structures and conformations, unique residues that are different between DAT and NET could be responsible for the affinity differences. Because the generated cocaine analogs have similar structure to cocaine (Carroll et al., 1995), these residues are likely to be at the cocaine binding site and have direct interactions with specific functional groups on cocaine or cocaine analogs.

Most of these cocaine analog compounds are more potent inhibitors of the neurotransmitter reuptake transporters than cocaine, presumably binding to the same site as cocaine (Singh, 2000), (Dutta et al., 2003). However, despite the transporter homology, some cocaine analogs have transporter-specific affinities, whereby these cocaine analogs inhibit DAT more potently than NET (Carroll et al., 1995).

These cocaine analog compounds contain distinct chemical modifications to the cocaine structure (Carroll, 2003). Figure 3.1a-c shows the structure of cocaine and two cocaine analogs (RTI-31 and RTI-113). RTI-31 has removed the 3β ester from cocaine, resulting in a compound that is 100x more potent in inhibiting DAT and NET compared with cocaine (Carroll et al., 1995). When the methyl group on the 2β ester in RTI-31 is replaced with a phenyl group, the resulting compound (RTI-113) (Figure 3.1c) retains the same potency to DAT but is about 100 fold lower to NET compared to RTI-31 (Carroll et al., 1995). Therefore, these cocaine analogs are a unique tool to identify the critical residues that confer transporter specific affinity and help identify the elusive cocaine binding site.

Figure 3.1 Chemical structures of reuptake inhibitors.

The structure of cocaine (a), the 3β ester removed non-specific inhibitor RTI-31 (b), and dopamine-specific inhibitor RTI-113, (c); the highlighted area indicates the different 2β ester substitutions on the compounds.
3.3 Materials and Methods

3.3.1 Specific mutagenesis of mDAT and mNET

Plasmid DNA containing mouse DAT (mDAT) and mouse NET (mNET) were cloned into bluescript vector with a T7 promoter as described (Chen et al., 2005), (Wei et al., 2009). Using a CLUSTALX alignment screen, putative aqueous facing TM regions of mDAT and mNET were selected. Different multiple residues in TM3, TM6, and TM8 were mutated using site directed mutagenesis with oligonucleotide primers designed using nucleotides encoding homologous residues contained at similar protein segments. The primers used are listed in Appendix A. The mutants were then assayed for uptake activity (see below) and the sequences of the mutant constructs were determined by sequencing.

3.3.2 Substrate reuptake into transiently transfected cells

HeLa cells (American Type Culture Collection, Rockville, MD) were grown in 96-well plates, infected with recombinant vTF-7 vaccinia virus, carrying the T7 polymerase gene, and transiently transfected with the plasmids bearing cDNAs using Lipofectin (Invitrogen Corp., Carlsbad, CA) as described previously (Chen et al., 2005).

About 20–24 h after transfection, HeLa cells were assayed for substrate uptake in 96-well plates at room temperature using the PBS/Ca/Mg buffer. For the determination of Km and Vmax values, cells were incubated in PBS/Ca/Mg buffer containing 60 nM [3H]-labeled monoamine substrates in the presence of increasing concentrations of unlabeled monoamine substrates (0.1–20 µM) for 10 min at room temperature. For determination of IC50 values, cells were co-incubated in the PBS/Ca/Mg buffer with added 60 nM [3H]-labeled monoamine substrates and increasing concentrations of an inhibitor (e.g., cocaine or CM). Uptakes were terminated by two successive washes with PBS/Ca/Mg. Amounts of [3H]-labeled substrates accumulated in the cells were quantitated by liquid-scintillation counting. Protein concentrations were determined in triplicate using Bio-Rad dye and bovine serum albumin (gamma V) as the standard. The Km, Vmax, and IC50 values presented are averages and standard error of means calculated from 3 or more experiments. Cells transfected with vehicle were used as
controls and radioactivity associated with these cells were considered the background. This background was subtracted from the total scintillation counts of the wells.

3.3.3 Random mutagenesis of mDAT and mNET

To generate random mutations at mNET Tyr151/mDAT Phe155 position, PCR primers were used with nucleotides NNS (N being A, T, G, or C; and S being G or C) as the desired mutation codon. Nucleotides NNS encode for all amino acids but reduce the number of stop codons and increase the relative abundance of rare codons for Met and Trp. When necessary, additional primers were designed with specific nucleotides codon at the desired mutation site to encode for generating a specific mutant. The degenerate primers used are listed in Supplementary Table 1. The random mutants were then assayed for uptake activity and functional mutants were selected for further characterization. The sequences of the mutant constructs were determined by sequencing.

3.3.4 Data analysis

The Km, Vmax, and IC50 values were determined by a one-site sigmoidal concentration-response nonlinear regression analyses of experimental data using GraphPad Prism 3.0 (San Diego, CA). The Km, Vmax, and IC50 values presented are averages ± standard error of means (SEM) calculated from 3 independent uptake experiments. Statistical analyses for the differences between the IC50 values were performed by Student’s paired t-test using SPSS 17.0 (Chicago, IL).

3.4 Results

The process of identifying a cocaine analog binding site involved three steps. First, selected aqueous-facing residues that differ between mDAT and mNET needed to be switched. Next, the various switched residue mutants were compared to WT in inhibition and kinetic assays. Individual residues that altered drug specificities were randomly mutated to every amino acid to determine the basis of the interaction. Lastly, the interaction identified can be confirmed via computer docking model to identify residues that form the cocaine binding site.
3.4.1 TM screening

Identifying transmembrane (TM) regions to switch residues utilized the neurotransmitter/Na+ symporter bacterial homolog, a 12 TM *Aquifex aeolicus* leucine transporter (LeuTaa) (Yamashita et al., 2005). LeuTaa is proposed as a bacterial homolog to various mammalian neurotransmitter transporters. LeuTaa shares ~25% homology to mDAT and mNET (Yamashita et al., 2005). The LeuTaa crystal structure identifies that TM1, TM3, TM6 and TM8 have contact with the leucine binding site (Yamashita et al., 2005). Aqueous facing residues at near-extracellular positions inside these four transmembrane domains may be directly responsible for the transporter specificity that some cocaine analogs exhibit. By extension these residues might also be critical for cocaine binding. Figure 3.2 is a graphical protein alignment of mDAT and mNET indicating the location of residues that differ between mouse DAT (mDAT) and NET (mNET), highlighting these four critical TMs. Figure 3.3 is an alignment map, featuring the various mNET and mDAT mutants generated for the four TMs, aligned to WT mDAT residues.
Figure 3.2 Protein differences between mDAT and mNET.

A graphical representation of an alignment of protein sequences of mDAT and mNET with dark circles highlighting different residues at the same position, boxed regions identify the four substrate facing domains (TM1, 3, 6, 8) as determined from LeuTaa crystal structure.
Figure 3.3 mNET and mDAT residues differ in substrate facing domains.

The protein alignment of the four critical TMs, (1, 3, 6, 8) of mDAT and mNET as determined by LeuTaa crystal structure, the same residues occurring at the same position are identified with a period, extra and intra refer to extracellular and intracellular, respectively.
3.4.2 mNET 151/mDAT155 positional screening

For the second part of identifying the cocaine analog binding site, inhibition assays were performed with the generated mutants using pairs of cocaine analogs, with and without chemical substitutions at the 2β position. Uptake inhibition of the WT transporters produced IC$_{50}$ values similar to published results (Carroll et al., 1995), Figure 3.4a-b. The average IC$_{50}$ values for cocaine, RTI-31, and RTI-113 respectively were: for mNET, 398±66.8 nM, 2.04±0.96 nM, and 170±25.3 nM, an 83-fold increase; for mDAT, 318±126 nM, 0.91±0.52 nM, and 0.65±0.28 nM. Student’s paired t-tests showed that the IC$_{50}$ values were significantly different for each of the 3 drugs (n=3, p<0.001 for all three comparisons). These results are summarized in Table 3.1.

![Figure 3.4](image_url)

Figure 3.4 Inhibition of substrate uptake in wildtype transporters.

Dopamine and norepinephrine uptake of transiently transfected HeLa cells. A 2β phenyl-substitution increases the half maximal inhibition constant 83-fold for mNET (A). The same effect is not observed with mDAT (B).

From switching residues between mDAT and mNET TM domains, no effect was observed in RTI-113 inhibition by switching the residue positions between TM6 or TM8. There were significant changes to transporter function, (data not shown). However, switching residues in TM3 generated an RTI-113 sensitive mNET mutant, shown in Figure 3.5. The average IC$_{50}$ values with RTI-113 for the switched mutants respectively were: for WT mNET, 170±25.3 nM; for mNET TM3, 1.89±1.29 nM, a 90-fold decrease; for mNET TM6, 341±107 nM; for mNET TM8, 269±76.8 nM.
Figure 3.5 Removing a hydroxyl group on mNET increases RTI-113 potency.

The half maximal inhibition constant for phenyl substituted RTI-113 is 90-fold lower for a triple mutant mNET. The same effect is not observed with the constructs switched between TM6 and TM8.

Further mutagenesis revealed the mNET Y151/mDAT F155 position as having a unique role in 2β-phenyl ester inhibition specificity. These results are also shown in Figure 3.6(a-f) (n=3, p<0.001). Replacing Y with F in mNET (mNET-Y151F), resulted in 15-fold increase in the potency for RTI-113 (the 2β phenyl substituted cocaine analog). The average IC₅₀ value with RTI-113 for the Y151F mutant is 12.1±0.8 nM, a 15-fold decrease from WT. For the non-specific inhibitor, RTI-31, there is minimal effect in potency compared to WT (1.44±0.4 nM for Y151F, 2.02±0.5 nM for WT; n=3) (Figure 3.6b). The mNET-Y151F mutation has minimal effect on transporter function as compared to WT (figure 3.6c). The Km and Vmax NE values respectively were, for WT mNET, 1.01±0.2 uM and 11.5±2.5 pmol/mg/min; for mNET Y151F, 1.73±0.6 uM and 8.14±1.8 pmol/mg/min.

To determine if the hydroxyl of the tyrosine is responsible for the change in potency for RTI-113, the reverse mutation was performed in mDAT. As expected, adding the hydroxyl group to the residue in mDAT by F to Y substitution at position 155 decreases the potency for RTI-113 20-fold (Figure 3.6d, 13.4±1.9 nM for mDAT F155Y,
0.65±0.28 nM for WT; p<0.001, n=3) but similar to Y151F, the F155Y also has little effect on the binding of RTI-31 (Figure 3.6e, 1.56±0.5 nM for mDAT F155Y, 0.91±0.25 nM for WT; p<0.001, n=3). This data suggests that the presence of a tyrosine hydroxyl group at the mNET Y151/mDAT F155 position in is affecting phenyl ester analog binding either through specific interactions or causing a conflict that affects binding. The tested RTI inhibitor results are summarized in Figure 3.7a-b. The various mNET and mDAT mutants generated for the four TMs, aligned to WT mDAT residues.

To determine if the observed mNET-Y151F effect occurs with other residues, random mutagenesis was utilized at this residue position. Mutant mNET Y151X plasmids were created with degenerate primers at the 151 position to assist in determining if other residues at this position could be affected by this 2β-specific structural inhibition. Table 3.1 also lists RTI-113 IC50 values for 17 of the 20 possible NET Y151X mutations. None of the random mutations tested produced IC50 values for RTI-113 or isopropyl-substituted RTI-121 that were observed with the RTI-113 and the Y151F mutation.
Figure 3.6 Removing a hydroxyl group on mNET increases RTI-113 potency.

The half maximal inhibition constant for RTI-113 is 15-fold lower for mNET-Y151F (a). The same effect is not observed with the 2β methyl-substituted non-specific inhibitor RTI-31 (b). The mNET-Y151F mutation did not affect transporter function as compared to WT (c). The reverse mutation in mDAT shows a corresponding insensitivity to RTI-113(d), without affecting non-specific inhibition (e) or substrate uptake (f).
<table>
<thead>
<tr>
<th>Transporter</th>
<th>RTI-113 IC50 [nM]</th>
<th>Km</th>
<th>Vmax</th>
</tr>
</thead>
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<tr>
<td>mNET</td>
<td>170±25.3</td>
<td>1.01±0.2</td>
<td>11.5±2.5</td>
</tr>
<tr>
<td>mNET TM8</td>
<td>269±35.8</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>mNET TM6</td>
<td>341±77.2</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>mNET TM3</td>
<td>1.89±1.29</td>
<td>1.98±0.5</td>
<td>3.76±1.3</td>
</tr>
<tr>
<td>mNET Y151F</td>
<td>12.1±0.8</td>
<td>1.73±0.6</td>
<td>8.14±1.8</td>
</tr>
<tr>
<td>mDAT</td>
<td>0.65±0.28</td>
<td>1.60±0.19</td>
<td>17.2±2.6</td>
</tr>
<tr>
<td>mDAT F155Y</td>
<td>13.4±1.9</td>
<td>1.66±0.34</td>
<td>13.9±2.8</td>
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<tr>
<td>mNET Y151A</td>
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<tr>
<td>mNET Y151C</td>
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<tr>
<td>mNET Y151D</td>
<td>306±105</td>
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<tr>
<td>mNET Y151E</td>
<td>770±55.3</td>
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<tr>
<td>mNET Y151G</td>
<td>165±28.4</td>
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<td>mNET Y151H</td>
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<tr>
<td>mNET Y151I</td>
<td>199±65.1</td>
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<tr>
<td>mNET Y151K</td>
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<td>mNET Y151L</td>
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<tr>
<td>mNET Y151M</td>
<td>679±174</td>
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<tr>
<td>mNET Y151N</td>
<td>291±51.5</td>
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<td>mNET Y151P</td>
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<tr>
<td>mNET Y151Q</td>
<td>831±69.0</td>
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<td>mNET Y151R</td>
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<tr>
<td>mNET Y151S</td>
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<tr>
<td>mNET Y151T</td>
<td>115±33.2</td>
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<td>mNET Y151V</td>
<td>181±81.1</td>
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</tr>
<tr>
<td>mNET Y151W</td>
<td>256±66.9</td>
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</tbody>
</table>

Table 3.1 RTI-113 IC50 values for selected transporters.
Figure 3.7 Adding a hydroxyl group on mDAT decreases RTI-113 potency.

The half maximal inhibition constant for 2β phenyl-substituted RTI-113 is 15-fold lower for mNET Y151F and 20-fold higher for mDAT-F155Y (a). The same effect is not observed with the 2β methyl-substituted non-specific inhibitor RTI-31 (b).

3.5 Discussion

According to a published computer model of cocaine bound DAT, the 2β position of cocaine is within 3Å of residue F155 of cocaine-bound DAT-DA (Huang et al., 2009). The corresponding residue in NET is Y151. It is important to test whether the difference of a hydroxyl group between Phe and Tyr contributes to the large difference in cocaine analog affinities. By replacing Tyr with Phe in NET (NET-Y151F), it was found that removing the hydroxyl group at this position resulted in large increase in the potency for RTI-113 (the 2β phenyl substituted cocaine analog), suggesting specific interactions or removal of conflict. Introducing a hydroxyl group, replacing F with Y in DAT, has the opposite effect (Figure 3.7a).

Interestingly, this change has no effect on the potency of RTI-31 (Figure 3.7b) and minimal impact on transporter function (Figures 3.6c, 3.6f). This result is not likely due to an overall conformational change of the cocaine binding site because the affinity for the RTI compound with a 2β methyl group is not affected. Switching the selected positions between mDAT and mNET did not affect the binding of cocaine and other nonspecific monoamine uptake inhibitors (data not shown).

The data suggest an interaction or close proximity of NET-151Y / DAT-155F with the 2β ester group on cocaine based uptake inhibitors. It is likely that when cocaine
based analogs bind to norepinephrine transporter cocaine binding site, there is a conflict that occurs between the hydroxyl group of the tyrosine residue and the 2β phenyl group on the cocaine analog compound, RTI-113, that prevents tighter binding observed when the hydroxyl group is not present. Support for this theory is that RTI-113 is not selective for the serotonin transporter (Carroll, 2003), which also has a tyrosine residue at the equivalent Y151/F155 position.

Due to the strong structural similarity between RTI-113 and cocaine, the data strongly support the model of an alternative cocaine binding site as separate from the dopamine binding site (Huang et al., 2009). Future computer modeling of this inhibition could explain the biochemical data observed as changes in modeled binding affinity.
3.6 References


Han, D. D., Gu, H. H., 2006. Comparison of the monoamine transporters from human and mouse in their sensitivities to psychostimulant drugs. BMC Pharmacol 6, 6.


Chapter 4: Positional mutagenesis confirms a molecular model of a cocaine and dopamine bound dopamine transporter

4.1 Abstract

Molecular modeling and dynamics simulations produced a cocaine-bound dopamine transporter (DAT) model in which the cocaine binding site is near but not overlapping the dopamine binding site. In this model the residues forming the cocaine binding site are part of an outward facing pocket of the DAT structure that naturally accommodates cocaine. The model predicted five residues of mouse DAT to be within 3Å of the bound cocaine molecule. These five residues were randomly mutated to any amino acid and the mutants were analyzed for changes in cocaine inhibition potencies. While most mutations had relatively small impact on cocaine inhibition, several DAT mutants had significant changes in cocaine sensitivity, including a Pro to Gly mutation in extracellular loop 4 that decreased the potency of cocaine by 23-fold. The effects of these mutations on cocaine binding energy were also calculated using molecular modeling and dynamics simulations. The biochemical data and cocaine binding energy analysis together support our cocaine inhibition model, proposing that cocaine binds to an initial binding site that is not overlapping with the dopamine binding site.

4.2 Background

Molecular modeling and dynamics simulations have been useful in determining specific amino acid residues of the dopamine transporter (DAT) that affect how sodium and chlorine binding assist transport of dopamine substrate (DA) (Beuming et al., 2006), (Huang and Zhan, 2007). A previously generated computationally-determined DAT-DA binding model in which the cocaine binding site is near the DA binding site,(Huang et al., 2009) This model is different than a previous overlapping substrate and inhibitor binding site where DAT makes some necessary conformational change to expand substrate
binding site cavity (Beuming et al., 2008). This binding site determined is presumed to be the initial cocaine to DAT binding site, composed of residues from TM helices 1, 3, 6, 8, and 10. In this outward facing conformation, the transporter can naturally accommodate cocaine. (Huang et al., 2009) It is important to understand how cocaine inhibition of dopamine transport results from the external binding of cocaine to DAT.

In DAT, the substrate transport process is similar to other members of the twelve transmembrane neurotransmitter sodium symporter (NSS) family. (Nelson, 1998) The co-binding of two Na\(^+\) ions and one Cl\(^-\) ion, assists in substrate transport. (Chen and Reith), (Yamashita et al., 2005) Therefore, the NSS transporters must utilize different conformational states to bind substrates or inhibitors. (Forrest et al., 2007), (Zhang and Rudnick, 2006) The determination of LeuTaa in complex with substrate and ions was a critical advance in understanding structural and functional relationships of the NSS members. (Gouaux, 2009) Following discovery of this structure, searches for the binding sites of ions and substrates of different NSS members were performed. (Beuming et al., 2008), (Singh et al., 2007), (Zomot et al., 2007), (Zhou et al., 2007) Additionally, cocrystal structures of LeuTaa with antidepressant drugs (PDB 3F3A) determined that noncompetitive ligands bind a vestibule facing the extracellular side, called “extracellularly open” (Gouaux, 2009), (Singh et al., 2007), (Zhou et al., 2007). The binding site for the noncompetitive ligands in this structure is approximately 11Å above the presumed binding sites of both substrate and Na\(^+\) ions (Singh et al., 2007), (Zhou et al., 2007). Binding to this vestibule is presumed to stabilize the “extracellularly open” conformation of the transporter. These structures and models have provided critical information about the ligands that interact with NSS members such as DAT. This work guided our computationally-determined model at atomic resolution of the cocaine bound DAT structure (Huang et al., 2009).

Using this new cocaine and dopamine bound DAT structure, a number of amino acid residues are predicted to have direct interactions with the initial binding site of cocaine. In order to confirm the predicted model in vitro, five residues within 3Å of the modeled cocaine position were chosen for study. The residues selected were from TM helices 1 and 10 and extracellular loop 4 (EL4). The residues are W84, Y88, G386, P387,
and L475. Random mutagenesis and functional analysis of mutants at these five positions will determine if the computationally-determined cocaine bound DAT-DA structure is an accurate model.

4.3 Materials and Methods

4.3.1 Specific random mutagenesis of mDAT

Plasmids containing the cDNA for mouse DAT (mDAT) was cloned into bluescript vector with a T7 promoter as described (Chen et al., 2005), (Wei et al., 2009). Site specific mutagenesis was performed using a PCR method similar to that described (Chen et al., 2005), (Wu and Gu, 2003). Random mutations were also introduced with PCR (Chen et al., 2007). To generate random mutations at a specific residue position, PCR primers were used with nucleotides NNS (N being A, T, G, or C; and S being G or C) as the codon for the desired residue position. NNS nucleotides encode all amino acids, increase the relative abundance of rare amino acid codons such as Trp and Met, and remove two of the three stop codons. When necessary, additional primers were designed with specific nucleotide codons at the desired mutation site to increase the number of mutants at a given position. The primers used are listed in Table 1. The random mutants were then assayed for uptake activity (see below) and functional mutants were selected for further characterization. The sequences of the mutant constructs were confirmed by DNA sequencing (OSU Comprehensive Cancer Center).

4.3.2 Substrate reuptake into transiently transfected cells

HeLa cells (American Type Culture Collection, Rockville, MD) were grown in 96-well plates, infected with recombinant vTF-7 vaccinia virus, carrying the T7 polymerase gene, and transiently transfected with the plasmids bearing mDAT mutant constructs using Lipofectin (Invitrogen Corp., Carlsbad, CA) as described previously (Wei et al., 2009).

About 20–24 h after transfection, HeLa cells were assayed for substrate uptake in 96-well plates at room temperature using the PBS/Ca/Mg buffer. For determination of IC_{50} values, cells were co-incubated in the PBS/Ca/Mg buffer with added 60 nM [^{3}H]-labeled monoamine substrates and increasing concentrations of an inhibitor (e.g., cocaine
or CM). Uptakes were terminated by two successive washes with PBS/Ca/Mg. Amounts of [3H]-labeled substrates accumulated in the cells were quantitated by liquid-scintillation counting. Protein concentrations were determined in triplicate using Bio-Rad dye and bovine serum albumin (gamma V) as the standard. The Km, Vmax, and IC50 values presented are averages and standard error of means calculated from 3 or more experiments. Cells transfected with vehicle were used as controls and radioactivity associated with these cells were considered the background. This background was subtracted from the total scintillation counts of the wells.

4.3.3 Data analysis

The Km, Vmax, and IC50 values were determined by a one-site sigmoidal concentration-response nonlinear regression analyses of experimental data using GraphPad Prism 3.0 (San Diego, CA). The Km, Vmax, and IC50 values presented are averages ± standard error of means (SEM) calculated from 3 independent uptake experiments. Statistical analyses for the differences between the IC50 values were performed by Student’s paired t-test using SPSS 17.0 (Chicago, IL).

4.4 Results

To confirm a computer model of cocaine bound DAT-DA inhibition, mutants of five positions in mDAT were screened for changes to cocaine sensitivity. While a majority of mutations had a relatively small impact on cocaine inhibition, several DAT mutants had significant changes to cocaine sensitivity. The present study then confirmed the pharmacological effects of mutations at the five selected positions using molecular modeling and dynamics simulations on cocaine binding energy.

Because mouse DAT (mDAT) has 99% overall homology to the modeled human DAT, (Donovan et al., 1995) it was selected for the random mutagenesis and functional analysis of the five selected residue positions. The selected positions were 100% homologous between the modeled human and mDAT. Random mutations were introduced at these five positions and functional mutants were identified and sequenced.

The mutants were transfected into HeLa cells and DA uptake was assayed in the presence of increasing concentrations of cocaine (100 pM – 1 mM) to determine IC50
values. Mutant and WT mDAT constructs were analyzed in the same experiments and the ratios of the IC$_{50}$ values (mutants/WT) were determined. Table 1 lists the IC$_{50}$ values and ratios calculated from triplicate measurements of each mutant. Some nonfunctional mutants were also sequenced to determine the mutations that resulted in nonfunctional transporters.
<table>
<thead>
<tr>
<th>mDAT</th>
<th>Cocaine IC$_{50}$ [µM]$^a$</th>
<th>ratio to WT</th>
<th>p value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>0.84±0.34</td>
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<td>---</td>
</tr>
<tr>
<td>W84G</td>
<td>0.61±0.24</td>
<td>0.73</td>
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</tr>
<tr>
<td>W84L</td>
<td>0.12±0.07</td>
<td>0.14</td>
<td>&lt;0.001</td>
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<td>W84R</td>
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<td>---</td>
<td>---</td>
</tr>
<tr>
<td>W84S</td>
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<td>W84T</td>
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<td>W84V</td>
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<td>Y88A</td>
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<td>Y88E</td>
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<td>Y88G</td>
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<td>Y88V</td>
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<td>&lt;0.001</td>
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<td>Y88W</td>
<td>0.94±0.09</td>
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<td>G386N</td>
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<td>G386R</td>
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<tr>
<td>G386S</td>
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<td>0.44±0.11</td>
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<td>P387C</td>
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<tr>
<td>P387R</td>
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<tr>
<td>P387S</td>
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<tr>
<td>L475S</td>
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<tr>
<td>L475T</td>
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<td>&lt;0.001</td>
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<tr>
<td>L475V</td>
<td>3.96±0.45</td>
<td>4.72</td>
<td>&lt;0.001</td>
</tr>
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</table>

Table 4.1 Cocaine IC$_{50}$ values and fold difference for the inhibition of mutant mDAT transporters.

$^a$The IC$_{50}$ values are mean ± standard error of means calculated from 3 independent experiments. $^b$Significance was determined by Student’s paired t-test.
TM1 mutation sites – W84 and Y88

mDAT-W84

The mutation screening generated five functional W84 mutants. W84G and W84V had IC₅₀ values nearly identical to WT. W84L, W84S and W84T were 5-8 times more sensitive to cocaine than WT. W84R was sequenced but non-functional in the substrate uptake assay.

mDAT-Y88

Screening generated ten mutants at the Y88 position. Mutations at this position could accommodate charged residues that other positions selected could not. Most mutants at Y88 transported substrate with Km and Vmax values equivalent to WT. The Y88 position appeared to directly affect cocaine binding. There is a large difference in IC₅₀ measures between two different mutants at this Y88 position, Y88V and Y88R; Y88V shows an increase in cocaine sensitivity while Y88R shows a decrease. Y88L and Y88V were roughly four-fold more sensitive to cocaine than WT mDAT, however, Y88R was roughly four-fold less sensitive to cocaine than WT mDAT. Mutations to charged amino acid residues (Y88E, H, and K) and to Y88S are tolerated and result in mild shifts in cocaine sensitivity, while altering residue size had small impact. Y88A, Y88G and Y88W generated IC₅₀ values similar to WT.

EL4 mutation sites - G386 and P387

G386

Five mutants were generated at G386. 386A, N, R, and S showed no change to transport or cocaine sensitivity. Only G386T showed a mild increase in sensitivity.

P387

Nine mutants at position P387 were studied. The corresponding residue of P387 is an alanine in mouse and human norepinephrine transporters, therefore the proline did not appear to be critical for correct secondary structure or correct protein folding. Randomly mutating this position generated the most insensitive mutant screened during these
experiments. P387G was highly cocaine insensitive with an IC$_{50}$ value 23 fold greater than that for WT mDAT. Charged or polar residues at this position appeared to increase cocaine potency, with P387K, N, and S showing slight increases in cocaine sensitivity compared to WT. The mutants P387A, C, L, M, and R all showed cocaine sensitivities similar to WT.

**TM10 mutation site – L475**

**mDAT-L475**

Six mutants were tested and screened, identifying that L475V and L475T increased cocaine IC$_{50}$. Most of the mutations at this position were slightly cocaine insensitive. Interestingly, this position was observed to accommodate a proline without impacting cocaine inhibition, only transporter function. The L475 S, C and N mutations were not observed to affect cocaine potency.
Measurement of mutant protein function

Following these inhibition assays, kinetic measures were made of several mutants at each of the positions selected. Table 4.2 lists the Km and Vmax values calculated for these selected mutants. Figure 4.1 shows DA uptake and IC$_{50}$ plots generated for select mutants from each of the five residue positions.

<table>
<thead>
<tr>
<th>mDAT</th>
<th>Dopamine Km [µM]$^a$</th>
<th>ratio to WT</th>
<th>Vmax ratio to WT</th>
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<td>9.67±0.24</td>
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<td>11.7±0.64</td>
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<td>Y88R</td>
<td>1.13±0.42</td>
<td>1.52</td>
<td>4.76±0.32</td>
</tr>
<tr>
<td>Y88V</td>
<td>1.28±0.42</td>
<td>1.72</td>
<td>6.31±0.54</td>
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<td>P387A</td>
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<td>7.57±0.90</td>
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<tr>
<td>P387G</td>
<td>6.46±1.46</td>
<td>8.72</td>
<td>3.73±0.53</td>
</tr>
<tr>
<td>L475V</td>
<td>2.86±0.58</td>
<td>3.87</td>
<td>14.7±0.74</td>
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Table 4.2 Cocaine Km and Vmax values and fold difference for the mutant mDAT transporters.

$^a$These values are mean ± standard error of means calculated from 3 independent experiments. $^b$Significance was determined by Student’s paired t-test.
Figure 4.1 Cocaine and DA concentration-response curves for mutant mDAT proteins.

Cocaine inhibition and dopamine uptake by wild-type mouse DAT and W84S(A, B), Y88H (C, D), Y88R (E, F), Y88V (G, H), P387A (I, J), P387G (K, L), and L475V (M, N) into transfected cells were measured in the presence of increasing concentrations of cocaine or dopamine. The IC$_{50}$ data are presented as the percent of the pre-drug activity. Each data point represents the average of triplicate measurements ± standard error of means and the experiments were repeated three times with similar results.
Figure 4.1 (cont)

A. % DA Uptake vs. log [cocaine, M]

B. Log DA Concentration (μM) vs. DA Uptake Vmax (pmole/mg protein/min)

C. % DA Uptake vs. log [cocaine, M]

D. Log DA Concentration (μM) vs. DA Uptake Vmax (pmole/mg protein/min)

E. % DA Uptake vs. log [cocaine, M]

F. Log DA Concentration (μM) vs. DA Uptake Vmax (pmole/mg protein/min)

G. % DA Uptake vs. log [cocaine, M]

H. Log DA Concentration (μM) vs. DA Uptake Vmax (pmole/mg protein/min)
Figure 4.1

Graphs showing the effect of various concentrations of cocaine on DA uptake for different mutant DAT proteins: mDAT, P387A, P387G, and L475V. The graphs compare the percentage DA uptake and the maximum uptake velocity (Vmax) with different log concentrations of cocaine.
Because the basis for these pharmacological studies was computational modeling and simulations of DAT with inhibitor, MD simulations were then performed on each of the 35 mutants listed in Table 4.1. In collaboration with the Zhan group at the University of Kentucky, the modeled cocaine binding energy for each of the mutants tested and was compared to the previously published DAT-DA model. For some mutants the initial modeling interaction data did not correspond to the biochemical IC₅₀ data. Of the mutants, secondary MD simulation analysis was performed with more precise model constraints. The result of the molecular modeling found that the calculated binding energy correlated to the changes observed in IC₅₀. Because MD simulations of energy and binding fit with the biochemical data from random mutagenesis, the mutation screening data supports the DAT-DA computational model as a correct simulation of DAT and inhibitor binding.

4.5 Discussion

Since the genes for NSS transporters were identified nearly two decades ago, DAT structure-function analysis has repeatedly used site-directed mutagenesis. (Wei et al., 2009), (Wu and Gu, 2003), (Kitayama et al., 1992) The present study advances on previously unguided site-directed mutagenesis screening studies with the application of a MD generated model of cocaine inhibition. The present study also combines site-directed mutagenesis pharmacological study with MD simulations of the same mutations. While a majority of mutations assayed had relatively small impact on cocaine inhibition, several DAT mutants, in TM1, EL4 and TM10, had significant shifts in cocaine sensitivity.

In DAT TM1, large functional deficits are observed when the residue size is reduced to alanine or glycine. (Kitayama et al., 1992), (Ukairo et al., 2005) However, the increase in cocaine potency seen with some W84 mutants is probably not due to a loss in physical interaction based on tryptophan size, since the W84G and W84V mutants showed no change to IC₅₀. The influence of residue size was also not observed for the other TM1 helix position selected, the Y88 positional mutants. Y88A, G, and W had no difference in IC₅₀ from wildtype mDAT. While polar mutations Y88E, H, K and S show non-significant increases cocaine potency, Y88R mutation is slightly cocaine insensitive with a 4-fold decrease in cocaine potency. However, the non-polar mutations to leucine
and valine both had a 4-fold increase. A charge interaction occurring between TM1 positions and other DAT residues has also previously been proposed from a mutagenesis study of the TM1 arginine residue. (Ukairo et al., 2005)

In EL4, the data showed that mutations could affect the shape of the -GPGL- hairpin turn and thereby produce a unique interaction with cocaine. Altering the shape of this hairpin turn has already been shown to change NSS member inhibition, such as serotonin transporter (SERT) recognition of tri-cyclic antidepressants. (Zhou et al., 2009) Previous cysteine scanning of EL4 residues in SERT found that the analogous G386C mutant is non-functional, while P387C was nearly 100% functional. (Mitchell et al., 2004) The mutated mDAT P387C was also similar to WT in both IC50 and uptake while a functional G386C mutant was not generated. The comparable P387C mutant was also completely insensitive to MTSET reagent inactivation in the presence of excess native substrate. (Mitchell et al., 2004) The MD determined basis DAT-DA model in this study is also modeled with the substrate binding site occupied by DA.

Because the -GPGL- turn in EL4 may function as a dynamic hinge during substrate or inhibitor binding, the mutation to -GGGL- may have prevented the conformational changes necessary for normal cocaine inhibition, observed in the 23-fold decrease in cocaine IC50. The mutation to -GGGL- should provide a high degree of EL4 flexibility at the hairpin bend of the loop, and prevent potent cocaine binding. It was observed that adding a methyl group can remove the cocaine insensitive effect, because the P387A construct was observed to have cocaine IC50 similar to WT. In addition to being highly homologous to DAT, the norepinephrine transporter (NET) has a species-conserved alanine instead of the normally NSS-conserved proline at this position. However, mNET and mDAT have a similar cocaine IC50. (Hill et al., 2009) Steric interactions generated from the methyl group of P387A may be enough to hold the hairpin loop structure together. This position could influence the binding affinity of potent DAT selective inhibitors such as GBR-12909 or RTI-113. Therefore, the function of P387 in substrate/inhibitor recognition remains unclear.

Also unclear is the effect of L475 mutations. Following the discovery of the LeuTaa structure, the hydrophobicity-based locations of the putative EL5-TM10 domains
were determined to be incorrect. Therefore, the role of actual EL5-TM10 residues in monoamine transporter substrate and inhibitor recognition has only recently been identified in SERT with serotonin selective inhibitors. The LeuTaa-based TM10 helix is now described as a part of the ‘permeation pathway’ for the binding of substrates and inhibitors (Rudnick, 2006). The role of L475 in this pathway was shown in a cysteine scanning search of EL5-TM10 in SERT. That data showed that transporter function of an analogous L475C mutant was rapidly deactivated by the addition of the negatively charged MTSES reagent (Keller et al., 2004). The mDAT L475C mutant had only slight changes to IC50.

According to the DAT-DA model proposed, L475 would be within 3Å with the 2β position of cocaine. The random mutagenesis data revealed that L475V and L475T had slightly decreased cocaine potency. Many random mutations at the L475 position were observed to have reduced transporter function (data not shown), but few of the mutants screened presented a significant difference in cocaine inhibition from WT. Mutations to the L475 and W84 residue positions could affect the neighboring D476-R85 salt-bridge previously shown to be conserved in 12TM Na+/Cl− transporters. (Rudnick, 2006; Yamashita et al., 2005) The effect of extracellular facing TM10 residues on cocaine inhibition requires further experimentation.

In conclusion, 35 site-directed mutants encompassing five different residue positions were analyzed on the presumption based on the DAT-DA model that they would interact with cocaine. While many mutants slightly affected cocaine inhibition, some positions showed significant increases or decreases in cocaine sensitivity. Specifically, residues in EL4 have a unique structural interaction with cocaine, where mutation to the center NSS conserved proline can decrease the potency of cocaine 23-fold. Data from biochemical assays and calculated energy show that mutations at the selected residue positions, predicted by the published DAT-DA model to be 3Å from cocaine, affected cocaine sensitivity to inhibit substrate uptake. The present study supports the published cocaine bound DAT-DA model and highlights the importance of the first binding interactions between DAT-DA and cocaine on causing cocaine inhibition of monoamine transporters.
4.6 References

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Appendix A – Table of oligonucleotide primers used
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