Localization of Cocaine Analog [\textsuperscript{125}I]RTI 82 Irreversible Binding to Transmembrane Domain 6 of the Dopamine Transporter*

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The site of cocaine binding on the dopamine transporter (DAT) was investigated using the photoactivatable irreversible cocaine analog $[^{125}\text{I}]\text{3\beta-(p-chlorophenyl)tropane-2\beta-carboxylic acid, 4'-azido-3'-iodophenylethyl ester}$. The incorporation site of this compound was mapped to transmembrane domains (TMs) 4–6 using epitope-specific immunoprecipitation of trypsin fragments and further localized using cyanogen bromide (CNBr), which hydrolyzes proteins on the C-terminal side of methionine residues. CNBr hydrolysis of $[^{125}\text{I}]\text{RTI} 82$-labeled rat striatal and expressed human DATs produced fragments of $~5–10$ kDa consistent with labeling between Met\textsuperscript{271/272} or Met\textsuperscript{290} in TM5 to Met\textsuperscript{279/271} in TM7. To further define the incorporation site, substitution mutations were made that removed endogenous methionines and inserted exogenous methionines in combinations that would generate labeled CNBr fragments of distinct masses depending on the labeling site. The results obtained were consistent with the presence of TM6 but not TMs 4, 5, or 7 in the labeled fragments, with additional support for these conclusions obtained by epitope-specific immunoprecipitation and secondary digestion of CNBr fragments with endoproteinase Lys-C. The final localization of $[^{125}\text{I}]\text{RTI} 82$ incorporation to rat DAT Met\textsuperscript{290}–Lys\textsuperscript{346} and human DAT I291M to R344M provides positive evidence for the proximity of cocaine binding to TM6. Residues in and near DAT TM6 regulate transport and transport-dependent conformational states, and TM6 forms part of the substrate permeation pathway in the homologous \textit{Aquifex aeolicus} leucine transporter. Cocaine binding near TM6 may thus overlap the dopamine translocation pathway and function to inhibit TM6 structural rearrangements necessary for transport.

The dopamine transporter (DAT)\textsuperscript{2} is a neuronal protein that clears dopamine (DA) from the synapse via $\text{Na}^+ / \text{Cl}^-$-dependent active transport. DA reuptake is necessary for appropriate control of dopaminergic function and may be dysregulated in disorders such as Parkinson disease and drug abuse (1). Cocaine and many other compounds bind to DAT and inhibit DA reuptake, resulting in increased transmitter levels believed to mediate drug reinforcement (2). Despite extensive pharmacological investigation of transport inhibitors, the molecular mechanisms by which they are recognized by DAT and exert their effects remain largely unknown.

DAT and the related norepinephrine and serotonin transporters (NET and SERT) are integral membrane proteins that consist of 12 transmembrane domains (TMs), connecting intracellular (IL) and extracellular (EL) loops, and intracellular N and C termini (3). Substrate translocation is thought to occur by an alternating access mechanism that sequentially exposes extracellular and intracellular portions of the substrate permeation pathway to opposite sides of the membrane (4). The three-dimensional structures of these proteins are unknown, although they are recently proposed to be similar to that of the homologous \textit{Aquifex aeolicus} leucine transporter, LeuT\textsubscript{AA} (5). The substrate binding site on LeuT\textsubscript{AA} is composed of residues from TMs 1, 3, 6, and 8, and some analogous sites have been identified in DAT, NET, and SERT (6, 7).

LeuT\textsubscript{AA} however, is not cocaine-sensitive, and its structure does not indicate how cocaine or other uptake blockers might interact at monoamine transporters. Cocaine binding to DAT is reduced by mutagenesis of many amino acids throughout the primary sequence (6, 7), but in most cases it is not clear if these effects are due to disruption of specific ligand interaction sites or to conformational alterations that indirectly impact binding. Thus the structure of the inhibitor binding site on DAT and the identities of the residues that dock cocaine and other blockers

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\textsuperscript{2} The abbreviations used are: DAT, dopamine transporter; NET, norepinephrine transporter; SERT, serotoninn transporter; DA, dopamine; TM, transmembrane domain; IL, intracellular loop; EL, extracellular loop; HEK 293 cells, human embryonic kidney cells; [\textsuperscript{125}I]RTIB2, [\textsuperscript{125}I]3\beta-(p-chlorophenyl)tropane-2\beta-carboxylic acid, 4'-azido-3'-iodophenylethyl ester; [\textsuperscript{125}I]MFZ 2-24, N-[4-(4-azido-3-[\textsuperscript{125}I]iodophenyl)butyl]-2-carboxamethoxy-3\beta-(4-chlorophenyl)tropane; CFT, 2\beta-carboxamethoxy-3\beta-(4-fluorophenyl)tropane; CNBr, cyanogen bromide; Lys-C, endoproteinase Lys-C; LeuT\textsubscript{AA} of \textit{A. aeolicus} leucine transporter; h, human; r, rat; WT, wild type.
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A.  

B.  

C.  

distance in Angstroms  

N - N\textsubscript{3} 10.3  

C - N\textsubscript{3} 8.6  

Ph - N\textsubscript{3} 11.2

FIGURE 1. Structures of cocaine and [125I]RTI 82. A, structure of [125I]RTI 82. The phenyl azido (N3) moiety that covalently attaches to the protein is appended to the tropine ring C2 carbon. B, structure of cocaine. C, two-dimensional depiction of intramolecular distances in [125I]RTI 82, obtained using the molecular modeling program SYBYL 6.7 (Tripos Inc.) and the crystal structure of WIN 35,428 as a template. The energy minimization was carried out by conjugate gradient method until a convergence gradient of 0.001 kcal/mol/Å was achieved.

remain largely unknown. Using photoaffinity labeling as a positive function approach to investigate inhibitor binding domains, our laboratory has found two distinct regions of DAT containing TMs 1–2 and TMs 4–7 that are incorporation sites for photoactive cocaine, benztprine, and GBR analogs (8–10). One of the GBR compounds labels both domains, suggesting that the sites are adjacent and form a binding pocket for multiplets classes of transport inhibitors.

Of the photoaffinity labels analyzed to date, the cocaine analog [125I]RTI 82 (Fig. 1) is the only one that becomes incorporated solely into the TM 4–7 region (10). More precise identification of its incorporation site, however, has been limited by the use of antibody-based procedures. In this study we use chemical cleavage and site-directed mutagenesis to further localize the site of [125I]RTI 82 attachment and identify its incorporation in a sequence containing TM6. This increases our understanding of the structure of the cocaine binding pocket on DAT and the molecular mechanism of cocaine action.

EXPERIMENTAL PROCEDURES

Materials—The amino precursor for [125I]RTI 82 was synthesized by Dr. Jooh W. Cha (Medicinal Chemistry Section, National Institute on Drug Abuse-Intramural Research Program) using modifications (11, 12) of the original procedure (13), and radioiodinated as previously described (14). [3H]CFT was from PerkinElmer Life Sciences (Wellesley, MA) and [3H]DA and Rainbow molecular mass markers were from Amersham Biosciences (Piscataway, NJ). HEK 293 cells were from ATCC (Manassas, VA); electrophoresis reagents were from Bio-Rad (Hercules, CA); cell culture reagents were from Invitrogen; protease inhibitors and FuGENE transfection reagents and trypsin were from Roche Applied Science; endoproteinase Lys-C was from Wako (Richmond, VA); cyanoagen bromide, (−)-cocaine, GBR 12909, nomifensine, mazindol, desipramine, and imipramine were from Sigma-Aldrich (St. Louis, MO); (−)-cocaine was the generous gift of Dr. Maarten Reith (New York University); the QuikChange mutagenesis kit was from Stratagene; and synthetic oligonucleotide primers were purchased from Genscript Corp. (Piscataway, NJ) or Integrated DNA Technologies (Corval, IA). Male Sprague-Dawley rats were obtained from Charles River Laboratory (Wilmington, MA) and were housed and treated in accordance with regulations approved by the University of North Dakota Institutional Animal Care and Use Committee.

In Situ Proteolysis—Labeling of rat striatal tissue with [125I]RTI 82, in situ proteolysis, and DAT immunoprecipitation assays were performed essentially as previously described (15). For pharmacological analysis of photolabeling, (−)-cocaine, (+)-cocaine, GBR 12909, mazindol, nomifensine, desipramine, or imipramine were added to the photolabel binding reaction to final concentrations of 1 or 10 μM. For proteolysis studies photolabeled rat striatal membrane suspensions were treated with or without 20–200 μg/ml trypsin at 37 °C for 5 min, and membranes were centrifuged, washed, and solubilized. Photolabeled DAT and DAT fragments were immunoprecipitated with antisera 16 generated against rDAT N-terminal amino acids 42–59 or antisera 5 directed against rDAT EL2 amino acids 225–242 and analyzed by SDS-PAGE and autoradiography.

Site-directed Mutagenesis—A 3×FLAG-His\textsubscript{6}-human (h) DAT cDNA in pdDNA3.1/His\textsuperscript{8} vector was kindly provided by Dr. J. B. Justice, Jr. (Emory University). This sequence contained a previously described arginine to methionine variation at residue 344 (16). The methionine was reconverted to arginine using the QuikChange mutagenesis kit (Stratagene), and the resulting product is indicated as the wild-type (WT) protein. Several additional point mutations were made singly or in combinations in the hDAT WT or R344M backgrounds to remove or insert methionines to generate custom CNBr cleavage sites. The mutations made were M272L, M272L/I291M, M272L/R344M, and all were confirmed by sequencing the entire hDAT coding region (Northwoods DNA, Solway, MN).

Cell Culture—Initial experiments on hDAT were performed using HEK 293 cells stably expressing the hDAT R344M variant, which has been previously characterized (16, 17), and subsequently modified by the addition of a 3×FLAG-His\textsubscript{6} epitope tag at the N terminus (10). For generation of new cell lines expressing methionine mutants, HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and maintained in 5% CO\textsubscript{2}/95% O\textsubscript{2} at 37 °C. Cells in 12-well plates were transfected with the indicated mutant plasmids using FuGENE transfection reagent and 0.5 μg of DNA/well, and transformed cells were selected after 24 h
by addition of 400 μg/ml G418 to the culture medium. Stably transfected cell pools were grown to 70–80% confluence in 12- or 24-well plates for use. DAT expression was verified by Western blotting cell lysates with hDAT monoclonal antibody 369 (MAB 369, Chemicon International) as previously described (Gaffaney and Vaughan (18)).

**[^3H]DA Transport and[^3H]CFT Binding Assays—**Cells expressing WT and mutant DATs were assayed for[^3H]DA transport and[^3H]CFT binding essentially as previously described (18). Cells were plated in 24-well plates, washed with Krebs-Ringer-HEPES (KRH) buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 10 mM HEPES, 10 mM glucose, pH 7.4), and assayed in triplicate for DA transport using 10 nM[^3H]DA plus 10 μM DA in KRH and using 30 μM (−)-cocaine to define nonspecific uptake. Assays were conducted for 8 min at 37 °C, transport was stopped by washing cells with ice-cold buffer, and cells were solubilized with 1% Triton X-100. For[^3H]CFT binding, washed cells were incubated in triplicate with 5 nM radioligand in KRH for 1 h at 0 °C using 30 μM (−)-cocaine to define nonspecific binding. Binding was stopped by washing cells with ice-cold buffer, and cells were solubilized with 1% Triton X-100. Radioactivity in solubilized uptake and binding lysates was assessed by liquid scintillation counting at 62% efficiency. IC50 values for cocaine inhibition of[^3H]DA uptake and[^3H]CFT binding were determined by conducting assays in the presence of 10−9–10−3 M (−)-cocaine. All uptake and binding assays were performed at least three times with similar results. Non-linear regression analysis and analysis of variance were performed using Prism 3 software.

**Photoaffinity Labeling—**For irreversible labeling of hDAT with[^125I]RTI 82, cells expressing WT or mutant proteins were washed once with KRH and incubated with 5 nM radioligand in KRH buffer for 1 h on ice with shaking to allow reversible binding to occur. Cells were then irradiated with 254 nm ultraviolet light for 45 s to covalently attach the ligand to DAT, washed once with ice-cold KRH buffer, and lysed with 1% Triton X-100 containing protease inhibitors on ice for 1 h with shaking. Lysates were centrifuged at 15,000 × g for 15 min at 4 °C, and the supernatant was collected for analysis.

**Electrophoresis and Autoradiography—**Photoaffinity-labeled samples were subjected to SDS-PAGE and autoradiography on 10% acrylamide gels for intact DAT or 17–20% or 18% acrylamide gels for peptide fragments using high and low range Rainbow markers as molecular mass standards (15). For gel purification of DAT the 80-kDa region of the gel containing labeled protein was excised, and the protein was extracted by electroelution as previously described (19). Electrophoresed samples were dialyzed against MilliQ purified water in 10-kDa cutoff Slide-A-Lyzer dialysis cassettes (Pierce) and evaporated to dryness in a SpeedVac concentrator.

**CNBr Hydrolysis—**Dried electroeluted samples were incubated for 24 h at 22 °C in the dark with 0.1 ml of 1 M CNBr prepared in 70% formic acid or with 70% formic acid only (20). Reactions were stopped by addition of 1 ml of water followed by drying in a SpeedVac concentrator, and the resulting pellets were subjected to three additional rounds of suspension in water and drying. Dried samples were either prepared directly for electrophoresis by solubilization in gel sample buffer or were resuspended in 50 mM Tris–HCl, pH 8.0, for immunoprecipitation with antiserum 5. All CNBr digestion experiments were replicated two to six times with similar results. Molecular masses of CNBr and trypsin fragments were calculated using the ExPASy peptide mass program (us.expasy.org/tools/peptide-mass.html). For ease of discussion, the fragments obtained by CNBr or trypsin digestion are referred to by their flanking methionine or arginine and lysine residues, although at the N termini the fragments begin at the residues following the cleavage sites. The residue numbers used to designate fragments or mutagenesis sites in various experiments are specific for rDAT or hDAT isoforms, which differ by one position at a number of sites discussed.

**Lys-C Digestion of CNBr Fragment—**CNBr digestion and electrophoresis of[^125I]RTI 82-labeled rDAT were performed as described above. The gel region containing the labeled fragment was excised, and the peptide was eluted by rotating gel pieces in 1 ml of 10 mM ammonium bicarbonate, pH 8.0, overnight at 21 °C. The eluate was dried in a SpeedVac concentrator, resuspended in 0.1 ml of 0.1 M Tris HCl, pH 9.0, and treated with 10 μg/ml endoproteinase Lys-C for 1 h at 37 °C. Digested samples were analyzed by electrophoresis and autoradiography on 18% SDS-polyacrylamide gels.

**RESULTS**

**Pharmacology of[^125I]RTI 82 Labeling of DAT—**To examine the pharmacological specificity of[^125I]RTI 82 binding to DAT, labeling was performed in the presence of several DA transport blockers (Fig. 2). Labeling obtained in the control sample was completely displaced by the DA uptake blockers (−)-cocaine, mazindol, GBR 12909, and nomifensine, but was not affected by (+)-cocaine or the NET and SERT blockers desipramine and imipramine. These findings reflect the ability of these compounds to inhibit the reversible binding of[^125I]RTI 82 to DAT and support the hypothesis that irreversible ligand attachment occurs in or near a pharmacologically relevant region of the protein.

**Tryptic Mapping of[^125I]RTI 82 Labeling Site—**In situ proteolysis of[^125I]RTI 82-labeled rat striatal membranes with trypsin, which cleaves polypeptides at the C-terminal side of lysine and arginine residues, yielded several fragments of DAT that were immunoprecipitated by EL2 antibody 5 (Fig. 3A). Promi-
nent fragments of ~32, 25, and 16 kDa produced by 20 μg/ml trypsin have been previously characterized (15). These fragments are not glycosylated (15), indicating that their N termini are generated by cleavage at Arg218 in EL2 (Fig. 3B), which is the only trypsin site between epitope 5 and the N-glycosylation residues. The 32-kDa fragment also precipitates with a C-terminal tail antibody and thus contains all of the TMs between Arg218 and the C terminus, while the other fragments lose the C-terminal epitope, indicating cleavage between Arg218 and the C-terminal tail. Although the 16-kDa fragment is the smallest trypsin product previously identified, in more recent studies using higher trypsin concentrations (200 μg/ml) we isolated a serum 5-precipitable-labeled fragment that migrated at ~10 kDa (Fig. 3A, arrow).

Because in situ proteolysis is performed using membrane suspensions, residues within the TMs are protected from protease and only amino acids in exposed loops are accessible to enzyme. Based on the LeuTa structure, lysine and arginine residues in rat (r) DAT between Arg218 and the C-terminal tail that are exposed and could produce fragments of ~25, 16, and 10 kDa are present in IL5 (Lys314 and Arg320), EL4 (Lys373 and Arg379), and IL3 (Lys336 and Arg343), respectively (Fig. 3B). Retention of the photoaffinity label in the 16-kDa fragment thus indicates that ligand attachment occurs N-terminal to EL4, and the mass difference between the labeled 16- and 10-kDa fragments is consistent with excision of TM7 and retention of label in TMs 4–6 (Fig. 3B). The 10-kDa fragment is the smallest [125I]RTI 82-labeled peptide we have been able to precipitate with antibody 5, because more extensive proteolysis with trypsin separates the labeled domain from the antibody epitope (10).

**CNBr Mapping of [125I]RTI 82-Labeling Site**—To more precisely define the [125I]RTI 82 incorporation site on DAT we undertook a study to map photolabeled sites using cyanogen bromide (CNBr), which specifically cleaves peptide bonds on the C-terminal side of methionines. Both rat and human (h) DAT have a limited number of methionines in their sequence (Fig. 4). Within the first seven TMs of hDAT endogenous methionines are present at positions 1 and 11 in the N-terminal tail; 106, 111, and 116 in TM2; 272 in TM5; and 371 at the extracellular end of TM7 (Fig. 4, black circles). The analogous methionines plus another two at positions 173 in EL2 and 290 at the extracellular end of TM5 are present in rDAT (Fig. 4, open circles). The remaining six methionines in TMs 8–12 are present in both hDAT and rDAT, but are C-terminal to the [125I]RTI 82-labeling site and do not impact the results presented here. Our initial CNBr mapping studies were done with the hDAT R344M variant isolated by Eshleman et al. (16); this residue is indicated by the gray circle in Fig. 4.

To determine the cleavage pattern of [125I]RTI 82-labeled DAT with CNBr we used gel-purified, electroeluted DAT as the starting sample. Because the pharmacological displacement of [125I]RTI 82 photolabeling demonstrates that DAT is the only radiolabeled protein that electrophoreses at ~80 kDa (Fig. 2) (15), gel purification produces a starting sample in which all radioactivity originates from DAT, and thus all radioactive fragments generated are DAT cleavage products. In contrast to in situ proteolysis of native protein where TM residues are pro-

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**Figure 3.** [125I]RTI 82-labeled trypsin fragments and their origin in the primary sequence. A, [125I]RTI 82 labeled rat striatal membranes were treated with 0, 20, or 200 μg/ml trypsin, and DAT and DAT fragments were immunoprecipitated with antiserum 5 and analyzed by SDS-PAGE and autoradiography. The arrow indicates the 10-kDa fragment. B, schematic diagram of rDAT showing origin of 10- and 16-kDa fragments in primary sequence. TM domains are shown as cylinders, branched structures indicate rDAT glycosylation sites, antibody 5 epitope in EL2 is shown as a bold line, the black circle in EL2 shows the position of Arg218, black circles in IL3, EL4, and IL5 indicate trypsin sites consistent with production of 10-, 16-, and 25-kDa fragments, respectively. Dark gray shading indicates the region of the 10-kDa labeled fragment, and light gray shading indicates the region excised from the 16-kDa fragment.
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FIGURE 4. Methionine residues in rDAT and hDAT. Schematic diagram showing positions of endogenous methionines in DAT and their relationship to EL2, hDAT glycosylation sites (branched structures), and antibody epitope 5 (bold line in EL2). TM domains are shown as cylinders, black circles show positions of all endogenous hDAT methionines and analogous rDAT methionines with selected residues numbered (rDAT/hDAT), open circles show rDAT methionines 173 and 290 not present in hDAT, and the gray circle shows the position of the R344M variant in hDAT. Positions of methionines are modeled with selected residues numbered (rDAT/hDAT), thus our discussions are oriented toward description of complete cleavage products.

Proteolysis of gel-purified [125I]RTI 82-labeled rDAT with CNBr is shown in Fig. 5A. The untreated protein migrated at ~80 kDa (lane 1), consistent with its original mass, whereas after CNBr treatment most of the intact sample disappeared and the radioactivity appeared as a discrete band of ~5–8 kDa (lane 3, arrow). This fragment was produced with good stoichiometry (30–60% recovery) and thus represents a major photolabeling site. The same cleavage pattern was obtained with shorter and longer CNBr treatment times, indicating that the fragment is a limit digestion product, and in most experiments only minor levels of higher Mₖ incomplete cleavage products were present, indicating close to complete hydrolysis. In this gel and other experiments (see Fig. 7) the fragment migrated as a broad but evenly labeled band and on other gels (see Figs. 6 and 8) displayed a narrower appearance, consistent with the presence of a single peptide.

To verify that the CNBr fragment originated from a part of DAT within or near to the cocaine binding site, a parallel sample was prepared in which [125I]RTI 82 labeling was performed in the presence of 10 µM (−)-cocaine (Fig. 5A). Cocaine displacement of labeling for the sample in lane 2 was obscured by overexposure from lane 1, but aliquots of the same preparations in lanes 5 and 6 show the blockade of [125I]RTI 82 labeling. Importantly, the labeled fragment seen in lane 3 was not obtained from the cocaine displaced sample (lane 4), consistent with the [125I]RTI 82-labeled fragment originating from the cocaine binding site. Finally, to verify that the fragment was generated by CNBr and not by nonspecific acid hydrolysis, labeled DAT was treated with the 70% formic acid vehicle lacking CNBr. In this case only the full-length cocaine-displaceable DAT was observed, and there was no production of any lower Mₖ species (lanes 5 and 6).

Fig. 5B shows the same analysis performed for R344M hDAT labeled with [125I]RTI 82. The results show full-length DAT (lanes 1 and 5) and displacement of label by cocaine (lanes 3 and 6). CNBr treatment produced a fragment of ~5–8 kDa (lane 2) that was displaced by cocaine (lane 4) and was not produced by formic acid (lane 5), indicating the similarity of [125I]RTI 82 incorporation into rDAT and hDAT.

The samples prepared in these studies also frequently contained very low Mₖ radioactivity that migrated ahead of the 3.5-kDa marker and just behind the gel dye front. In most cases (e.g. Fig. 5A), this radioactivity was present in both non-treated and formic acid treated samples and was not displaced by cocaine, indicating that even though it is carried with DAT through gel purification it is not a DAT CNBr peptide. Free [125I]RTI 82 migrates to a similar position on these gels (Fig. 5B, lanes 7), and the high mobility radioactivity may represent free ligand or ligand complexed non-specifically with low molecular weight molecules such as lipids and passively carried through these procedures.

Based on the localization of [125I]RTI 82 incorporation to TMs 4–6 by antibody studies, the masses of the labeled CNBr fragments are most consistent with TM 5–7 containing sequences extending from rDAT Met²⁷¹ or Met²⁹⁰ to Met³⁷⁰ (calculated masses 10.7 or 8.8 kDa) and hDAT Met²⁷² to R344M or Met³⁷¹ (calculated masses 7.8 or 10.7 kDa). The shaded region in Fig. 4 schematically depicts the labeled rDAT and hDAT CNBr domains, taking into account the possibility of missed cleavages at Met²⁹⁰ in rDAT and R344M in hDAT.

Antiserum 5 Analysis of [125I]RTI 82-Labeled CNBr Fragment—Although TMs 4 and 5 are present in the trypsin fragments seen in Fig. 3, the CNBr results are not consistent with the presence of TM4 or the region of TM5 N-terminal to Met²⁷¹/²⁷² in the labeled CNBr fragments. If labeling occurred in these regions, the rDAT CNBr fragment would extend at a
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Minimum from Met^{173}–Met^{271} and would include antibody epitope 5 (Fig. 4). Although an Met^{173}–Met^{271} fragment would have 10.8 kDa of mass from protein, it would also contain the N-linked carbohydrate present on EL2, which imparts ~25 kDa of mass to DAT and EL2-containing DAT fragments (15), for a total mass of ~35 kDa. Although the masses of the labeled CNBr fragment are inconsistent with this interpretation, we formally examined this possibility by subjecting the rDAT CNBr fragment to immunoprecipitation with antibody 5 to ascertain the presence or absence of its epitope (Fig. 6). The starting [^{125}I]RTI 82-labeled rDAT and CNBr fragments are shown in lanes 1 and 2. After immunoprecipitation the intact DAT but not the CNBr fragment was recovered in the immune pellet (lanes 3 and 4), whereas the CNBr fragment but not the untreated DAT was recovered in the immune supernatant (lanes 5 and 6). The lack of antibody 5 immunoreactivity in the labeled CNBr fragment indicates that it does not contain the antibody epitope and thus does not contain the Met^{173}–Met^{271} sequence. This result confirms the exclusion of TM4 and the region of TM5 N-terminal to Met^{271} as the site of [^{125}I]RTI 82 incorporation.

Identification of TM6 as the [^{125}I]RTI 82-Labeling Domain—The elimination of regions N-terminal to Met^{271/272} as the site of [^{125}I]RTI 82 incorporation in conjunction with localization of incorporation to within 10 kDa of epitope 5 in trypsin studies strongly indicates that the CNBr fragments in Fig. 5 consist of the sequence between hDAT Met^{272} to R344M or Met^{271} and rDAT Met^{271} or Met^{290} to Met^{370}, because these are the only regions of overlap between the 10-kDa trypsin and 5- to 8-kDa CNBr fragments. To determine which TM in this sequence is the ligand attachment site and verify the residues undergoing CNBr hydrolysis, we used a strategy of deleting endogenous methionines and inserting exogenous methionines in combinations that would produce labeled CNBr fragments of widely differing masses depending on which TM was labeled. We used hDAT for this analysis to minimize the number of potential CNBr cleavage sites and the number of multiple mutations required.

The hDAT proteins used in this study were WT, R344M, M272L/R344M, and M272L/I291M/R344M. All of the mutants expressed full-length DAT protein at 100–150% of WT levels assessed by Western blotting (not shown), and all exhibited easily detectable cocaine-blockable [^{3}H]DA transport and whole cell [^{3}H]CFT binding (Table 1). Although transport and binding values for the R344M and M272L/I291M/R344M mutants were statistically lower than for the WT, this could be due to lower DAT surface expression levels, which was not analyzed, and the increased [^{3}H]CFT binding at M272L/R344M was not pursued. Importantly, the [IC_{50}] values for cocaine inhibition of [^{3}H]DA transport and [^{3}H]CFT binding at the WT and mutant proteins showed no statistical differences (Table 1), and all mutants also displayed robust cocaine-displaceable [^{125}I]RTI 82 labeling (data not shown) that paralleled their [^{3}H]CFT binding levels (Fig. 7). The functional similarities of the WT and mutant proteins indicate that the mutations did not significantly affect the overall structure of the protein or the cocaine binding site.

To directly compare the CNBr cleavage products in this study the WT, R344M, M272L/R344M, and M272L/I291M/ R344M proteins were labeled with [^{125}I]RTI 82, treated with CNBr, and electrophoresed on the same gel exactly in parallel. The untreated samples (odd-numbered lanes) and CNBr products (even-numbered lanes) produced are shown in Fig. 7A. Although the digestion in this experiment was less complete than in other figures, as indicated by the retention of higher M_{r} material, sufficient levels of low M_{r} peptides were produced to assess digestion patterns. The CNBr fragment from the R344M mutant migrated at ~5–8 kDa as previously described (lane 4), whereas that from the WT protein migrated slightly more slowly at ~6–10 kDa (lane 2). Although precise fragment masses cannot be determined from these gels, the relative electrophoretic mobilities of the fragments were clearly distinct, indicating differences in mass. The increased mobility of the fragment from the R344M hDAT relative to the fragment from the WT protein indicates that R344M was hydrolyzed, because otherwise the same fragments (Met^{272}–Met^{371}) would have been produced from both proteins. The mass of the fragment from WT hDAT is consistent with labeling between Met^{272} and Met^{371} (10.7 kDa, shaded region in diagram), whereas the mass of the R344M fragment is consistent with labeling between Met^{272} and R344M (7.8 kDa, shaded region in diagram). The fragment from R344M is unlikely to represent an R344M-Met^{371} cleavage product containing TM7 (unshaded region in diagram), which would contain only 28 residues for a mass of 2.8 kDa. The interpretation that TM7 is not labeled is also sup-

![FIGURE 6. Antibody 5 analysis of [^{125}I]RTI 82-labeled CNBr fragment. Gel-purified [^{125}I]RTI 82-labeled rDATs were treated with or without CNBr, and samples were subjected to immunoprecipitation with antibody 5 followed by SDS-PAGE and autoradiography on 17–20% gels. The input untreated DAT and CNBr fragment are in lanes 1 and 2, samples recovered in immune pellets in lanes 3 and 4, and samples recovered in immune supernatants are shown in lanes 5 and 6.](image-url)
were photoaffinity-labeled with [125I]RTI 82 in the presence or absence of the extracellular half of TM5 was labeled by [125I]RTI 82, the CNBr methionine, which would prevent separation of TM5 but not TM7. As seen in lane 6, however, a fragment of ~4–6 kDa was produced from this mutant, whereas a ~45-kDa fragment was not apparent, which excludes the extracellular half of TM5 between Met272 and Ile291 as the labeling site. The mass of the low Mᵣ fragment produced is consistent with labeling occurring between I291M/R344M (5.8 kDa, shaded region in diagram), and the greater mobility of the fragment relative to the WT and R344M CNBr fragments is consistent with the loss of the 19 residues between Met272–I291M (2 kDa). This result also supports the exclusion of TM7 as the labeling site, because if TM7 were labeled, the same CNBr fragments (R344M-Met371) would be produced from the R344M and M272L/I291M/R344M proteins. The same pattern of progressively smaller [125I]RTI 82-labeled CNBr fragments from these proteins and the clearly detectable differences in their electrophoretic mobilities were obtained in a separate identically conducted experiment in which samples were labeled, hydrolyzed, and electrophoresed on the same gel exactly in parallel. These results thus identify the TM6-containing sequence between hDAT Met291 and R344M as the [125I]RTI 82 labeling site.

To further substantiate this conclusion and confirm that the N termini of the fragments obtained in these studies were generated by hydrolysis of the residues indicated, we replaced Met272 in the R344M hDAT protein with leucine to remove the TM5 CNBr cleavage site. If the labeling site is in TM6, removal of Met272 would prevent its separation from TMs 2–5, which would prevent formation of the 5- to 8-kDa fragment seen in lane 4 and result in production of a labeled CNBr fragment that would extend from Met116 to R344M (Fig. 7B, lower panel). This fragment would contain 228 amino acids (25-kDa protein) plus ~25 kDa of carbohydrate for a total mass of ~50 kDa. This prediction was supported because the 5- to 8-kDa fragment was not produced from this mutant (Fig. 7A, lane 8), and the only lower Mᵣ radioactivity seen migrated at the gel dye front. A higher Mᵣ peptide that could be the predicted 50-kDa fragment appears to be present near the top of lane 8, but its mass on this high percent gel is not clear. To better characterize this sample, a separate experiment was performed in which the M272L/I291M/R344M protein was labeled in the presence or absence of cocaine, treated with CNBr, and electrophoresed on a 10% gel (Fig. 7B). A single [125I]RTI 82-labeled cocaine-displaceable CNBr fragment of ~50–65 kDa was generated (lanes 2 and 4), confirming the production of the higher Mᵣ labeled fragment, and the radioactivity migrating at the gel dye front was not displaced by cocaine (lane 4), substantiating that this is not a binding site fragment. This result verifies that the N-terminal

| hDAT form         | DA uptake pmol/min/μg | [3H]CFT binding pmol/μg | Cocaine IC₅₀ μM|
|-------------------|-----------------------|-------------------------|----------------|
| WT                | 796 ± 47              | 3.73 ± 0.27             | 1060 ± 57      |
| R344M             | 215 ± 14              | 0.93 ± 0.09             | 507 ± 133      |
| M272L/R344M       | 834 ± 31              | 6.20 ± 0.75             | 1172 ± 438     |
| M272L/I291M/R344M | 341 ± 51             | 1.50 ± 0.17             | 759 ± 202      |
|                  |                       |                         |                |
| *p < 0.05, relative to WT values (analysis of variance). |
| *p < 0.001, relative to WT values (analysis of variance). |


![FIGURE 7. CNBr analysis of [125I]RTI 82-labeled hDAT methionine mutants. A, WT, R344M, M272L/I290M/R344M, and M272L/R344M hDATs were labeled with [125I]RTI 82 and gel-purified. Equal amounts of radioactivity were subjected to treatment with or without CNBr as indicated followed by SDS-PAGE and autoradiography on a 17–20% gel. Schematic diagrams of the first three proteins below the autoradiograph indicate the positions of endogenous and engineered methionines cleaved to produce the fragments obtained (black circles). Met477 that remains in the sequence but does not affect the masses of labeled fragments (open circle), and protein regions consistent with masses of labeled fragments (shading). B, M272L/R344M hDATs were photoaffinity-labeled with [125I]RTI 82 in the presence or absence of cocaine, treated with or without CNBr as indicated, followed by SDS-PAGE and autoradiography on a 10% gel. The schematic diagram of this mutant below the autoradiograph shows the position of methionines cleaved to produce the fragments obtained (black circles), the open circle indicates the position of Met371, and shading indicates the region present in the labeled fragment. df, gel dye front. |

portated by the trypsin studies and by additional results presented below.

In the next construct (lanes 5 and 6) we mutated Met272 in the R344M background to leucine and replaced I291 with methionine, which would prevent separation of TM5 but not TM6 from the TM2–TM4 sequence that contains EL2. If the extracellular half of TM5 was labeled by [125I]RTI 82, the CNBr fragment produced from the M272L/I291M/R344M mutant would extend from Met116–I291M (calculated mass of 19.1 kDa) plus the ~25 kDa of carbohydrate. This fragment would have a total mass of ~45 kDa, and importantly, a low Mᵣ labeled fragment would not be produced. As seen in lane 6, however, a

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TABLE 1
Kinetic characteristics of wild-type and methionine mutant hDATs
Data are presented as mean ± S.E. (n = 3).
cleavage sites of the low Mr fragments produced by CNBr in lanes 2, 4, and 6 of Fig. 7A occurred by hydrolysis of Met<sup>272</sup> in the WT and R344M proteins and I291M in the M272L/I291M mutant and not at nonspecific sites or methionines elsewhere in the protein. Because R344M is accessible to cleavage by CNBr (Fig. 7A, lane 4), this result also further supports exclusion of TM7 as the labeling site, because if TM7 were labeled a fragment of low Mr (2.8 kDa) but not high Mr would be produced from the M272L/R344M protein. Taken together, the results shown in Figs. 4–7 identify a CNBr sequence that contains TM6 but not TMs 4, 5, or 7 as the incorporation site of [<sup>125</sup>I]RTI 82.

**Lys-C Digestion of CNBr Fragment**—Although we did not perform the methionine mutagenesis experiments for rDAT, the mapping results indicate a similar interpretation for the rat protein. The evidence shown in Fig. 7 that all the endogenous and exogenous methionines surrounding the photolabeled site in hDAT (Met<sup>277</sup>, I291M, R344M, and Met<sup>377</sup>) are cleaved strongly suggests that the rDAT CNBr fragment is a complete digestion product extending from Met<sup>290</sup> to Met<sup>370</sup>. As an additional method to confirm this we treated an [<sup>125</sup>I]RTI 82-labeled rDAT CNBr fragment with endoproteinase Lys-C, which specifically cleaves peptides on the C-terminal side of lysine residues, and a single lysine (Lys<sup>336</sup>) that could serve as a site of Lys-C proteolysis is present in rDAT midway through this sequence (Fig. 8). An rDAT CNBr fragment identified by SDS-PAGE and autoradiography was excised, eluted from the gel, and treated with or without Lys-C, followed by electrophoresis on an 18% gel (Fig. 8). The sample treated with vehicle migrated at ~6.5 kDa, consistent with the mass of the starting fragment, whereas the Lys-C-treated sample displayed a reduced mass of ~3.5 kDa. This finding supports Met<sup>290</sup>–Met<sup>370</sup> as the starting fragment, and because photolabeling does not occur C-terminal to Arg<sup>343</sup> (Figs. 3 and 7), the labeled rDAT CNBr/Lys-C fragment thus likely represents the sequence between Met<sup>290</sup>–Lys<sup>336</sup> (Fig. 9, shaded region in bottom panel), which eliminates most of IL3 as the labeling site.

**DISCUSSION**

Using a combination of proteolysis, epitope-specific immunoprecipitation, and site-directed mutagenesis, we identify in this study a sequence of DAT that contains TM6 as the site of cocaine analog [<sup>125</sup>I]RTI 82-irreversible incorporation. The smallest [<sup>125</sup>I]RTI 82-labeled fragment identified extends from rDAT Met<sup>290</sup> to Lys<sup>336</sup>, which includes over half of EL3, all of TM6, and a few residues of IL3. We cannot at present exclude the possibility that incorporation of the ligand occurs in loop structures but will focus on the transmembrane spanning sequence as the likely site of attachment, because most currently available evidence indicates that determinants for uptake blocker binding in DAT and related transporters are present in TMs rather than loops (21, 22).

Covalent attachment of [<sup>125</sup>I]RTI 82 in DAT TM6 provides positive evidence that reversible binding of cocaine occurs in or near this domain. Other results consistent with TM6 participation in cocaine binding include findings from DAT-NET and human-bovine DAT chimeras that implicated TMs 4–8 (23) and TMs 6–8 (22) in maintenance of cocaine or CFT affinity. In addition, at least twenty residues in the DAT 290/291–336/337 sequence have been analyzed by site-directed mutagenesis (6, 7), with several mutants showing altered cocaine-binding properties. Of the proteins that display proper processing and surface expression, mutations that reduced cocaine or cocaine analog binding were found at hDAT residues Trp<sup>311</sup>, Thr<sup>316</sup>, Glu<sup>317</sup>, Ser<sup>321</sup>, and Tyr<sup>335</sup> (6, 24, 25, 26) and rDAT Phe<sup>325</sup>, Phe<sup>329</sup>, and Phe<sup>331</sup> (27), whereas increased cocaine affinity occurred at D313N (26). Most of the inhibitory mutations decreased cocaine affinity by only modest amounts (2- to 3-fold) that may be consistent with indirect effects on binding site structure rather than alteration of direct interaction sites.
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TM6 and IL3 also participate in conformational changes that regulate the transition between extracellularly and intracellularly facing transporter forms during DA translocation. These conformational rearrangements are suppressed by mutation of Asp$^{313}$, Tyr$^{335}$, Tyr$^{343}$, and Asp$^{345}$ (26, 33, 34), which leads to transport inhibition. The participation of TM6 and its adjacent loops in structural rearrangements necessary for DA transport suggests that cocaine interaction with this domain may prevent these changes and/or stabilize a transport deficient state.

In the substrate-bound form of LeuT$_{AA}$ the region of TM6 intracellular to the unwound sequence is closed to substrate passage via contacts with TMs 1, 3, and 8, while the region to the extracellular side is outwardly open and may provide substrate access. Such a form of DAT may be the conformation recognized by cocaine and/or stabilized for prevention of transport, because kinetic studies indicate that cocaine binds to an outwardly facing form of NET (35), and some of the mutations that increase (D313N) or decrease (Y335A and D345A) cocaine affinity act by, respectively, stabilizing extracellularly or intracellularly facing DAT conformations (26, 33, 34). These studies are consistent with cocaine contact points in or near TM6 occurring at residues extracellular to the unwound region. Cocaine can tolerate significant steric bulk at the C2 carbon of the tropane ring with maintenance of binding affinity (36), suggesting that this aspect of the molecule is oriented toward an opened protein region. The C2 tropane carbon is the site to which the phenyl azido arm of $[^{125}I]$RTI 82 is appended (Fig. 1A), compatible with a ligand binding orientation in which the pharmacophore occupies the same site as cocaine near the TM6-unwound region while the phenyl azido arm extends toward the opened region where it forms its covalent attachment.

The cocaine elements crucial for high affinity binding at DAT include the phenyl ring, tropane ring, and tropine nitrogen (Fig. 1B) (36). Because the phenylazido group on $[^{125}I]$RTI 82 is appended to a site that is non-essential for binding, irreversible attachment will occur near to but presumably not at the residues that directly contact the cocaine pharmacophore. The interatomic distances between the azido group of $[^{125}I]$RTI 82 and the tropane ring bridge nitrogen, C2 carbonyl carbon, and phenyl ring were measured on the three-dimensional minimum energy conformation of RTI 82. These distances were measured as N–N$_3$ = 10.35 Å, C–N$_3$ = 8.6 Å, and Ph–N$_3$ = 11.2 Å (Fig. 1C). These measurements are predicted to define maximum distances between the $[^{125}I]$RTI 82 addiction site and other ligand-protein contact points in the cocaine binding pocket, with the possibility that, in the bound form of the ligand, the phenylazido arm, which is likely to be highly flexible, may adopt a conformation different from the energy minima projection from which these distances were measured.

The specific amino acid to which $[^{125}I]$RTI 82 becomes attached is not yet known. We mutated several candidate hDAT residues in the extracellular half of the TM6 sequence, including Phe$^{302}$, Leu$^{305}$, Ala$^{314}$, Cys$^{319}$, and Leu$^{322}$, to alanine or leucine, (Fig. 9, circles with bold lines), but these mutations did not prevent $[^{125}I]$RTI 82 labeling (not shown). It is not clear, however, if these results exclude the mutated residues as the incorporation sites, because adduction of

although mutation of multiple contact points may be required to reduce inhibitor affinity more significantly (28). Mutations in the TM6 region that did not affect cocaine binding were found at hDAT residues Leu$^{305}$, Cys$^{306}$, Glu$^{307}$, Ala$^{308}$, Ser$^{309}$, and Ile$^{312}$ (in an H193K background) (29), hDAT Cys$^{319}$ (30), and rDAT Cys$^{305}$ (6). Participation of TM6 in cocaine binding is also supported by findings consistent with formation of TM2–TM6 interhelical contacts that support the optimal configuration of transport and cocaine binding sites (31).

In LeuT$_{AA}$ TMs 1 and 6 are adjacent and form one side of the substrate binding site (5). If DAT conforms to this structure, our result showing $[^{125}I]$RTI 82 attachment to TM6 indicates that cocaine binding occurs close to the DA binding site. TM6 of LeuT$_{AA}$ is a discontinuous α-helix separated by five amino acids of non-helical structure, and leucine binding occurs in and just extracellular to the unwound region at residues corresponding to DAT Cys$^{319}$, Phe$^{320}$, Gly$^{323}$, and Phe$^{326}$ (Fig. 9, asterisks), while sodium interacts with a threonine corresponding to DAT Ser$^{321}$ (Fig. 9, circle with dashed line). Mutation of Gly$^{323}$ in DAT leads to loss of DA transport (32), consistent with substrate involvement. Many of the mutations that decrease cocaine binding to DAT are clustered near this region (Fig. 9, blue circles), consistent with proximity of cocaine and presumed substrate binding sites, whereas most of the mutations that do not affect cocaine binding are located in the more extracellular part of the helix (Fig. 9, tan circles).

FIGURE 9. TM6 and adjacent regions present in $[^{125}I]$RTI 82-labeled CNBr fragment. Schematic diagram of hDAT TM6 region between Ile$^{312}$ and Lys$^{337}$, modeled after LeuT$_{AA}$. Cylinders indicate presumed outer and inner helical regions connected by random coil structure, and the E3 region between Asp$^{292}$ and Tyr$^{297}$ is shown as an α-helix. Asterisks indicate sites homologous to LeuT$_{AA}$ leucine contact points, and the circle with dashed outline indicates residue homologous to LeuT$_{AA}$ sodium contact site. Blue circles indicate DAT residues that reduce cocaine affinity when mutated, and tan circles show residues that do not affect cocaine binding when mutated. Tyr$^{335}$ and Asp$^{313}$, which reduce or increase cocaine affinity when mutated through stabilization of inward or outward facing transporter conformations, respectively, are indicated in green and red, and Gly$^{323}$ which loses DA transport activity when mutated is shown in yellow. Circles with bold outlines indicate DAT residues that retain $[^{125}I]$RTI 82 labeling when mutated to leucine or alanine.
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photoaffinity ligands has been found at all standard amino acids (37), suggesting that ligand-amino acid proximity may be of greater importance in bond formation than the nature of the residue side chain. If \([^{125}I]\)RTI 82 photoincorporation does not require a specific amino acid side chain or occurs in peptide backbone structure, irreversible attachment may be retained at a mutated site, and a positive function approach such as mass spectrometry will be required to identify the added residue.

We have also mapped the attachment site of N-[4-(4-azido-3-\([^{125}I]\)iodophenyl)butyl]-2-carbomethoxy-3β-(4-chlorophenyl)tropane (\([^{125}I]\)MFZ 2-24), a tropane ligand that is identical to \([^{125}I]\)RTI 82 except for the placement of the phenylazido moiety (38, 39). Because the tropane pharmacophores of these ligand are identical, the compounds should bind in the same orientation and differ only in the addition site. In marked contrast to the \([^{125}I]\)RTI 82 findings, attachment of \([^{125}I]\)MFZ 2-24 to DAT has been localized to TM1 or the top of TM2 (40). This directly demonstrates the physical proximity of cocaine analogs to multiple TM domains of DAT and supports the homology of DAT to the Leu\(_{T2}\) aspA structure with respect to arrangement of TM1 and 6. If TM3 and 8 of DAT are involved in substrate transport as found for Leu\(_{T2}\), and cocaine binding occurs near the substrate sites, then these TMs could also contribute to the cocaine binding pocket. This idea is particularly supported by mutagenesis results for TMs 1 and 3 (6, 7). Our finding that SERT can be photoaffinity labeled with both \([^{125}I]\)RTI 82 and \([^{125}I]\)MFZ 2-24 (28) suggests that the cocaine binding pocket in other transporters may be composed of similar interaction sites from multiple TM domains.

GBR 12909- and benztpine-based photoaffinity ligands also become incorporated into TMs 1–2 and/or TMs 4–7 of DAT (10). Although it is not known if these ligands interact with the same TMs or residues as the 3-aryltropane ligands, these findings suggest that multiple classes of uptake blocking drugs share a binding site that is at least partially overlapping with that of cocaine and thus inhibit transport by similar mechanisms. Identification of the residues adducted to \([^{125}I]\)RTI 82 and other irreversible ligands will provide unique information about the three-dimensional structure of uptake blocker binding domains in DAT, the orientation of ligands in the binding pocket, and the relationship of uptake blocker and substrate active sites. This will extend our understanding of transport mechanisms and accelerate the development of therapeutics for pharmacological manipulation of DA transport in drug abuse and disease.

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