2007

Exploring The Ligand Binding Site On The Dopamine Transporter By Photoaffinity Labeling And Site-Directed Mutagenesis

Maria Laura Parnas

Follow this and additional works at: https://commons.und.edu/theses

Part of the Psychology Commons

Recommended Citation

Parnas, Maria Laura, "Exploring The Ligand Binding Site On The Dopamine Transporter By Photoaffinity Labeling And Site-Directed Mutagenesis" (2007). Theses and Dissertations. 737.

https://commons.und.edu/theses/737

This Dissertation is brought to you for free and open access by the Theses, Dissertations, and Senior Projects at UND Scholarly Commons. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of UND Scholarly Commons. For more information, please contact zeineb.yousif@library.und.edu.
EXPLORING THE LIGAND BINDING SITE ON THE DOPAMINE TRANSPORTER BY PHOTOAFFINITY LABELING AND SITE-DIRECTED MUTAGENESIS

by

Maria Laura Parnás
Bachelor of Science, Universidad Católica de Córdoba, 2001

A Dissertation
Submitted to the Graduate Faculty
of the
University of North Dakota
in partial fulfillment of the requirements

for the degree of
Doctor of Philosophy

Grand Forks, North Dakota
August
2007
This dissertation, submitted by Maria Laura Parnas in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

Chairperson

This dissertation meets the standards for appearance, conforms to the style and format requirements of the Graduate School of the University of North Dakota, and is hereby approved.

Dean of the Graduate School

Date

June 31, 2007
Title 
Exploring The Ligand Binding Site on the Dopamine Transporter By Photoaffinity Labeling and Site-Directed Mutagenesis

Department: Biochemistry and Molecular Biology

Degree: Doctor of Philosophy

In presenting this dissertation in partial fulfillment of the requirements for a graduate degree from the University of North Dakota, I agree that the library of this University shall make it freely available for inspection. I further agree that permission for extensive copying for scholarly purposes may be granted by the professor who supervised my dissertation work or, in her absence, by the chairperson of the department or the dean of the Graduate School. It is understood that any copying or publication or other use of this dissertation or part thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of North Dakota in any scholarly use which may be made of any material in my dissertation.

Signature

Date 6-19-07
TABLE OF CONTENTS

LIST OF FIGURES ...................................................................................... viii
LIST OF TABLES ...................................................................................... x
ABBREVIATIONS ...................................................................................... xi
ACKNOWLEDGEMENTS ........................................................................... xiii
ABSTRACT .................................................................................................. xvi

CHAPTER

I. INTRODUCTION ....................................................................... 1

The Discovery of Chemical Neurotransmission .......... 1
The Characterization of Dopamine as a Central Neurotransmitter ......................... 2
Dopaminergic Pathways in the Mammalian Central Nervous System ......................... 7
Dopamine Uptake, Cloning and Localization of the Dopamine Transporter .................. 10
Structural Properties of the Dopamine Transporter ..... 13
Post-translational Modifications ................................. 16
Oligomerization ............................................................... 17
Protein-Protein Interactions ......................................... 18
Functional Properties of DAT ......................................... 18
Prokaryotic Leucine Transporter and Relationship to DAT .................................... 21
Pharmacological Aspects of the Dopamine Transporter . 23
III. RESULTS

Pharmacology of $[^{125}\text{I}]$MFZ 2-24 Incorporation to DAT.... 60

Peptide Mapping of the DAT $[^{125}\text{I}]$MFZ 2-24 Incorporation Site by Trypsin Proteolysis 65

Peptide Mapping of the DAT $[^{125}\text{I}]$MFZ 2-24 Attachment Site by CNBr Cleavage 74

Site-Directed Mutagenesis, CNBr and Antibody 16 Analysis of $[^{125}\text{I}]$MFZ 2-24 Labeled hDAT 80

Localization of TM1 as the $[^{125}\text{I}]$MFZ 2-24 Attachment Site in DAT 85

Photoaffinity Labeling of SERT with $[^{125}\text{I}]$MFZ 2-24 and $[^{125}\text{I}]$RTI 82 91

Pharmacology of $[^{125}\text{I}]$JHC 2-48 Incorporation to DAT and SERT 94

CNBr Analysis of $[^{125}\text{I}]$JHC 2-48 Labeling in DAT 97

Functional Properties of D-147 100

Pharmacology of $[^{125}\text{I}]$D-147 Incorporation to DAT 105

Peptide Mapping of the DAT $[^{125}\text{I}]$D-147 Incorporation Site by Trypsin Proteolysis 105

IV. DISCUSSION 114

TM1 is a Determinant for Cocaine Binding in DAT 115

Relationship with Bacterial Leucine Transporter (LeuT$_{\text{Aa}}$) 117
Photolabels with Identical Pharmacophore Exhibit Differential Incorporation ..................................................... 124

Analysis of the Incorporation of a Novel Cocaine Analogue to DAT ................................................................. 124

[$^{125}$I]D-147 Incorporation Provides Additional Evidence for Proximity of Domains........................................ 127

APPENDICES ............................................................................................................................... 130

APPENDIX A .............................................................................................................................. 131

APPENDIX B .............................................................................................................................. 133

APPENDIX C .............................................................................................................................. 134

REFERENCES ............................................................................................................................. 136
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Schematic Diagram of a Dopaminergic Synapse</td>
<td>3</td>
</tr>
<tr>
<td>2.</td>
<td>Dopamine Biosynthesis and Degradation Pathway</td>
<td>5</td>
</tr>
<tr>
<td>3.</td>
<td>Dopaminergic Pathways in the Human Brain</td>
<td>8</td>
</tr>
<tr>
<td>4.</td>
<td>Schematic Diagram of the Human DAT (hDAT) Primary Sequence</td>
<td>14</td>
</tr>
<tr>
<td>5.</td>
<td>Alternating Access Mechanism for Substrate Translocation</td>
<td>19</td>
</tr>
<tr>
<td>6.</td>
<td>The Dopamine Hypothesis of Reinforcement</td>
<td>25</td>
</tr>
<tr>
<td>7.</td>
<td>Dopamine Transporter Substrates and Inhibitors</td>
<td>28</td>
</tr>
<tr>
<td>8.</td>
<td>DAT Incorporation Domains for Photoaffinity Labels</td>
<td>36</td>
</tr>
<tr>
<td>9.</td>
<td>Chemical Structures of Photoaffinity Labels</td>
<td>41</td>
</tr>
<tr>
<td>10.</td>
<td>Schematic Diagram of the 6xHis-hDAT pcDNA 3.1/His®B Plasmid</td>
<td>54</td>
</tr>
<tr>
<td>11.</td>
<td>Chemical Structure of Cocaine-Based Photoaffinity Labels</td>
<td>61</td>
</tr>
<tr>
<td>12.</td>
<td>[^{125}\text{I}]\text{MFZ 2-24 Photoaffinity Labeling Displays DAT Pharmacological Specificity}</td>
<td>63</td>
</tr>
<tr>
<td>13.</td>
<td>Potential Trypsin Cleavage Sites and Antibody Epitopes in rDAT</td>
<td>66</td>
</tr>
<tr>
<td>14.</td>
<td>[^{125}\text{I}]\text{MFZ 2-24 and [}^{125}\text{I}]\text{RTI 82 Attach to Distinct Domains in DAT}</td>
<td>69</td>
</tr>
<tr>
<td>15.</td>
<td>The Cocaine Analogue [^{125}\text{I}]\text{MFZ 2-24 Attaches to TMs 1-2 Domain in DAT}</td>
<td>71</td>
</tr>
<tr>
<td>16.</td>
<td>Methionine Residues in DAT</td>
<td>75</td>
</tr>
<tr>
<td>17.</td>
<td>CNBr Hydrolysis of [^{125}\text{I}]\text{MFZ 2-24 Labeled rDAT and hDAT}</td>
<td>78</td>
</tr>
</tbody>
</table>
18. Expression and Photolabeling of TM1/TM2 Methionine Mutants ... 81
19. $[^{125}\text{I}]{\text{MFZ}}$ 2-24 Attaches to TMs 1-2 N-terminal to M106 .......... 86
20. $[^{125}\text{I}]{\text{MFZ}}$ 2-24 Attaches to TM1 Between Residues 67 and 80 .... 89
21. $[^{125}\text{I}]{\text{MFZ}}$ 2-24 and $[^{125}\text{I}]{\text{RTI}}$ 82 Label SERT ......................... 92
22. $[^{125}\text{I}]{\text{JHC}}$ 2-48 Photoaffinity Labeling of hDAT and hSERT Displays Pharmacological Specificity .............................................................. 95
23. $[^{125}\text{I}]{\text{JHC}}$ 2-48 Incorporates to DAT Distinctively from $[^{125}\text{I}]{\text{MFZ}}$ 2-24 and $[^{125}\text{I}]{\text{RTI}}$ 82 ........................................................ 98
24. Chemical Structures of GBR-Based Photoaffinity Labels ................. 101
25. D-147 Binds to DAT with High Affinity ........................................... 103
26. $[^{125}\text{I}]{\text{D-147}}$ Photoaffinity Labeling Displays DAT Pharmacological Specificity .............................................................. 106
27. The GBR Analogue $[^{125}\text{I}]{\text{D-147}}$ Binds to Distinct Domains in DAT .. 108
28. Specificity of Immunoprecipitation of $[^{125}\text{I}]{\text{D-147}}$ Labeled Fragments .............................................................. 111
29. Amino Acid Sequence Alignment of hDAT and LeuT$	ext{Aa}$ .................. 119
30. Structures of Cocaine and $[^{125}\text{I}]{\text{MFZ}}$ 2-24 .............................. 122
31. Photolabels with Identical Pharmacophore Bind to Distinct Sites in DAT .............................................................. 125
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. QuikChange® Reaction Mixture</td>
<td>52</td>
</tr>
<tr>
<td>2. QuikChange® Thermal Cycling Parameters</td>
<td>53</td>
</tr>
<tr>
<td>3. FuGene 6 Transfection Reaction Volumes</td>
<td>56</td>
</tr>
<tr>
<td>4. Kinetic Properties of wild-type and TM1/TM2 Methionine Mutant hDATs</td>
<td>83</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine/serotonin</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>AADC</td>
<td>amino acid decarboxylase</td>
</tr>
<tr>
<td>AD-96</td>
<td>4-[2-(diphenylmethoxy)ethyl]-1-[(4-azido-3-iodophenyl)-methyl]-piperidine</td>
</tr>
<tr>
<td>ADHD</td>
<td>attention deficit hyperactivity disorder</td>
</tr>
<tr>
<td>AMPH</td>
<td>amphetamine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>bDAT</td>
<td>bovine dopamine transporter</td>
</tr>
<tr>
<td>BCA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAMKII</td>
<td>calcium-calmodulin dependent kinase II</td>
</tr>
<tr>
<td>CFT</td>
<td>2β-carbomethoxy-3β-(4-fluorophenyl)tropane</td>
</tr>
<tr>
<td>CNBr</td>
<td>cyanogen bromide</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>D-147</td>
<td>1-{2-[(4-Azido-3-iodo-phenyl)-phenyl-methoxy]-ethyl}-4-phenethyl-piperazine oxalate</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>dopamine transporter</td>
</tr>
<tr>
<td>DEEP</td>
<td>1-[2-(diphenylmethoxy)ethyl]-4-2-(4-azido-3-iodophenyl) ethyl piperazine</td>
</tr>
<tr>
<td>EL</td>
<td>extracellular loop</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GA 2-34</td>
<td>N-[n-butyl-4-(4''''-azido-3''''-iodophenyl)]-4',4''''-difluoro-3 alpha-(diphenylmethoxy)tropane</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GAT</td>
<td>GABA transporter</td>
</tr>
<tr>
<td>GBR 12909</td>
<td>1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-3-phenylpropyl)piperazine</td>
</tr>
<tr>
<td>hDAT</td>
<td>human dopamine transporter</td>
</tr>
<tr>
<td>HEK 293 cell</td>
<td>human embryonic kidney 293 cell</td>
</tr>
<tr>
<td>IL</td>
<td>intracellular loop</td>
</tr>
<tr>
<td>JHC 2-48</td>
<td>3-(4'-azido-3'-iodo-phenyl)-8-methyl-8-aza-bicyclo-[3.2.1]octane-2-carboxylic acid methyl ester</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>LeuT&lt;sub&gt;AA&lt;/sub&gt;</td>
<td>leucine transporter from <em>Aquifex aeolicus</em></td>
</tr>
<tr>
<td>LLCPK&lt;sub&gt;1&lt;/sub&gt; cell</td>
<td>lewis lung carcinoma porcine kidney cell</td>
</tr>
<tr>
<td>MAB</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>METH</td>
<td>methamphetamine</td>
</tr>
<tr>
<td>MFZ 2-24</td>
<td>N-[4-(4-azido-3-iodophenyl)butyl]-2-carbomethoxy-3β-(4-clorophenyl)tropane</td>
</tr>
<tr>
<td>MPP+</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>NE</td>
<td>norepinephrine</td>
</tr>
<tr>
<td>NET</td>
<td>norepinephrine transporter</td>
</tr>
<tr>
<td>NSS</td>
<td>neurotransmitter:sodium symporter</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PEE</td>
<td>polyethylemine</td>
</tr>
<tr>
<td>PICK1</td>
<td>protein interacting with C-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2A</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>RACK1</td>
<td>receptor associated with C-kinase</td>
</tr>
<tr>
<td>rDAT</td>
<td>rat dopamine transporter</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RTI 82</td>
<td>3β-(p-chlorophenyl) tropane-2-β-carboxylic acid, 4'-azido-3'-iodophenylethyl ester</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SERT</td>
<td>serotonin transporter</td>
</tr>
<tr>
<td>SN</td>
<td>substantia nigra</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

"When you want something, all the Universe conspires in helping you achieve it"

Paulo Coehlo, The Alchemist

To start I would like to acknowledge my advisor, Dr. Roxanne Vaughan. She has trusted me from the beginning with a fascinating project and has given me the tools to become passionate for science. She has been patient, understanding, encouraging, generous, and an outstanding scientific role model. She has constantly encouraged me and has always found a positive attitude when things seemed slow. I will forever be grateful for the opportunity to learn from her, not only with respect to science but also when it is about life, ethics and family. She has been amazingly understanding of me being a foreign student and has always supported my trips home. I am also thankful for the opportunities Dr. Vaughan has given me to attend scientific meetings, which have allowed me to place my research in perspective and understand its implications, which were essential to promote my scientific career.

I would not have been able to have one of the best experiences in my life here without the wonderful people in the Department of Biochemistry. I would like to thank Dr. Karl Wald for opening my eyes and getting me through the application process, introducing me to many people, encouraging and supporting
me through hard times, and just being a great friend. Mary and Marlys were from the beginning welcoming and have since been a joy to chat with and extremely helpful with administrative duties. They have made me feel home and cared about me as moms. I will miss them very much. I would also like to thank Pete Leary, Dr. Rania Elsabrouty, Dr. Mohamad Hamad, Dr. Gwendolyn Barcelo-Coblijn, Dr. Tom Hill and Dr. Ann Flower for making my time at UND so enjoyable and for being examples of true friendship.

I am very grateful for having had the opportunity to meet and work with such an amazing group of people. Dr. Jon Gaffaney has been my mentor since the early days and has been a pleasure to interact and become friends with. Dr. James Foster is an essential part of the Vaughan lab and has always been available scientifically and personally, with the best advice and the best attitude. He is an amazing scientist and a great friend. Most importantly, Jamie has introduced me to the world of ice hockey and I have developed into a proud Sioux Fan. I will miss the hockey games (and his company) very much. Dr. Mark Cervinski has been my moral, spiritual and emotional support in the lab since the beginning, and has always guided and encouraged me through this process. Thank you Jon, Jamie and Mark for your friendship.

I want to thank my husband Pablo. He is my rock, my backbone, and is the one who coped with me after hours, understood every step of this process, was willing to stay so far away and always looked for alternative ways to be close. He made me feel as if we were always home and most importantly had infinite patience. Thank you Pablo for your unconditional love.

xiv
Finally, I want to thank my family. My parents have been incredibly supportive of this whole process, especially being 6,000 miles away. Thank you for believing in me, for giving me the foundations to be strong, for always stimulating my curiosity for life, culture and education, and for giving me the freedom to do whatever makes me happy. My brothers and sister have been the best throughout my absence and have always kept me connected and helped my parents deal with me being so far away.

My friends from Argentina Florencia, Belen, Maia and Paola have showed me that the long distance becomes short when true friendship exists. They have always been curious about my life and my work in the US, and have always kept in touch and made me feel as if I was still there sharing many important moments with them. I miss you very much and I promise I will stay only two more years and I will be back.

And to the rest of the people that I don't want to leave out, I have learned something from every single person I have ever interacted with, in the Medical School and outside. So thank you very much. Thank you for being part of the Universe that conspired for my dreams to become true. I am indebted to you all.
A mis padres, Nora y Mario, por enseñarme que el cielo es el límite

A mi amor Pablo, por ser siempre incondicional
ABSTRACT

The dopamine transporter (DAT) is a neuronal presynaptic transmembrane protein that clears released dopamine (DA) from the synaptic space, regulating the neurotransmitter concentration and availability. DAT and the related serotonin (SERT) and norepinephrine (NET) transporters belong to the SLC6 family of Na⁺ and Cl⁻ dependent symporters, and are major targets for the action of several drugs, including the psychostimulant cocaine. DAT is predicted to possess 12 transmembrane spanning domains (TMs), with both N- and C-termini located intracellularly. Extensive research efforts to gain insight on the molecular aspects of DAT have been attempted, but the three-dimensional arrangement of the protein as well as the molecular mechanisms involved in substrate translocation remain undiscovered.

Cocaine and other structurally diverse compounds bind to DAT with high affinity and inhibit its transport activity, but their binding site within the protein and the mechanism by which they block DA transport remain unclear. Two distinct ligand interaction sites at transmembrane domains (TMs) 1-2 and 4-6 have been identified using irreversible uptake-blockers. The current studies explore the incorporation site of the novel cocaine analogue [¹²⁵I]MFZ-2-24, which is structurally similar to the previously characterized [¹²⁵I]RTI 82, but the reactive azido (N₃) group is differentially positioned within the cocaine pharmacophore.

Trypsin and cyanogen bromide (CNBr) proteolytic maps of [¹²⁵I]MFZ-2-24
labeled rat and human DATs, followed by epitope-specific immunoprecipitation were used to localize the incorporation site of the ligand to a 13 amino acid stretch in Tl , between residues I67 and L80. This highly conserved region harbors the functionally essential D79 and residues involved in substrate and inhibitor binding. In marked contrast, incorporation of $[^{125}\text{I}]$RTI 82 occurs in TM6, demonstrating that differential positioning of the N$_3$ group on the cocaine pharmacophore leads to distinct incorporation patterns. This further indicates that TMs 1 and 6 are in close proximity three-dimensionally and participate in the reversible binding of cocaine to DAT.
CHAPTER I

INTRODUCTION

The Discovery of Chemical Neurotransmission

Until the end of the 19th century the nerve impulse was believed to propagate in an electrical manner, but this concept was not proven to be responsible for the transference of information between nerves and their target cells. The idea of chemical transmission was initially proposed through studies involving the physiological effects of adrenal extracts on a variety of tissues. In 1904 Elliott reported the actions of adrenaline in sympathetic axons and hypothesized that sympathetic nerves communicate chemically with their target cells, which contain specific recognition sites for these substances [1, reviewed in 2]. Between 1920 and 1940 the laboratory of Otto Loewi in Austria studied frog hearts and proposed that vagal stimulation is due to the actions of acetylcholine and sympathetic stimulation is mediated by adrenaline [reviewed in 2]. However, there were many excitatory effects that could not be explained through the adrenaline hypothesis, especially in tissue of mammalian origin, and in 1946 Ulf von Euler in Sweden was able to demonstrate that noradrenaline was indeed a neurotransmitter, and further that it was the main transmitter mediating mammalian sympathetic nerve communication [reviewed in 2].

Since then a plethora of information has become available regarding the understanding of chemical neurotransmission and its regulation, and many
chemically diverse compounds have been identified as neurotransmitters in a variety of nerve terminals across the peripheral and central nervous systems.

Neurotransmitter molecules are synthesized in the body of the neuron and transported through the axon toward the nerve terminal, where they are actively packed into storage vesicles that assemble and dock in the active zone. Upon nerve stimulation neurotransmitters are secreted into the synaptic space via calcium (Ca$^{2+}$)-mediated exocytosis. The released transmitters bind to their post-synaptic receptors and activate them, generating downstream effects that lead to propagation of the action potential. Termination of the signaling events is accomplished by neurotransmitter enzymatic degradation or reuptake through membrane transport proteins. Once inside the cell, transmitters are either repackaged into synaptic vesicles or degraded by intracellular enzymes (Figure 1).

The Characterization of Dopamine as a Central Neurotransmitter

Dopamine (3,4-dihydroxyphenylethylamine; DA) is the most newly discovered catecholaminergic transmitter in the mammalian brain. It is synthesized from the essential amino acid tyrosine through a two-step enzymatic reaction that involves the actions of tyrosine hydroxylase (TH) and L-aromatic amino acid decarboxylase (AADC) (Figure 2).

Until the mid 1950s DA was solely considered a biosynthetic intermediate of norepinephrine and epinephrine, widely known sympathetic neurotransmitters. It was then when Carlsson and coworkers demonstrated the normal occurrence of DA in the brain and suggested that DA was itself a neurotransmitter. These
Figure 1. Schematic Diagram of a Dopaminergic Synapse. Newly synthesized neurotransmitter molecules (DA, blue dots) are actively packed into synaptic vesicles by the vesicular monoamine transporter 2 (VMAT-2, red cylinders). Upon arrival of an action potential DA is released via Ca$^{2+}$-mediated exocytosis and binds to pre-synaptic (magenta cylinder) and post-synaptic (light blue cylinders) receptors, resulting in modulation of the pre-synaptic neuron and propagation of the action potential (green signals), respectively. Termination of the dopaminergic signal is achieved through Na$^+/Cl^-$-coupled DA uptake via the action of the dopamine transporter (DAT) (purple trapezoids). Once inside the cell DA is either repackaged into synaptic vesicles or degraded by the mitochondrial monoamine oxidase (MAO) (orange oval).
Figure 2. Dopamine Biosynthesis and Degradation Pathway. Biosynthetic enzymes are labeled in red and catabolic enzymes are labeled in purple. The conversion of Tyrosine to DOPA via Tyrosine Hydroxylase (TH) is the rate-limiting step in the synthesis of DA.
Catechol-O-Methyl Transferase (COMT)

Tyrosine → Tyrosine Hydroxylase (TH) → 3,4-Dihydroxyphenylalanine (DOPA) → Aromatic Amino acid Decarboxylase (AADC) → Dopamine

Catechol-O-Methyl Transferase (COMT) → Methoxytyramine (MTA)

Monoamine Oxidase (MAO) → 3,4-Dihydroxyphenylacetaldehyde

Aldehyde dehydrogenase → 3,4-Dihydroxyphenylacetic acid (DOPAC)

COMT → Homovanillic acid (HVA)
researchers observed depletion of catecholamine stores in rabbit's adrenal gland, heart and brain upon treatment with the antipsychotic reserpine and hypothesized that replenishment of the catecholamine stores would revert the drug's effects. After administration of the precursor 3,4-dihydroxyphenylalanine (L-DOPA) they found anti-reserpine effects in treated animals and central stimulation in untreated animals [3, 4, reviewed in 5]. These findings corresponded with the accumulation of DA, but not norepinephrine in the brains of these animals, which was, in turn, consistent with DA being a neurotransmitter on its own. It was not long until the unique distribution of DA was mapped to the basal ganglia [6], and the development of fluorescent techniques in combination with pharmacological methods allowed scientists to describe the localization and distribution of the monoaminergic pathways in the central nervous system (CNS) [7-11].

Dopaminergic Pathways in the Mammalian Central Nervous System

Dopamine is estimated to account for about 80% of the total brain catecholamine content. However, the human brain has relatively few dopaminergic neurons, about 150,000-200,000 in each hemisphere, mainly localized in the midbrain and forebrain. Ultrashort DA systems are comprised by retina and olfactory bulb neurons, and intermediate-length systems include the Tubero-infundibular pathway with DA cells that project from the mesobasal hypothalamus to the anterior pituitary gland (Figure 3) [12].

The long DA systems have been the most extensively studied dopaminergic neurons and are equally divided between the substantia nigra (SN)
Figure 3. Dopaminergic Pathways in the Human Brain. DA neurons from the substantia nigra project their axons to the striatum, giving rise to the Nigrostriatal pathway. DA neurons from the ventral tegmental area extend projections to the mesial components of the limbic system, and to the frontal and prefrontal cortices to form the Mesolimbic and Mesocortical pathways. The Tubero-infundibular pathway contains DA neurons that extend from the mesobasal hypothalamus to the anterior pituitary gland. The physiological functions associated with each of these pathways are indicated in bold letters.
Image modified from Pearson Education/Benjamin Cummings Silverthorn:
Human Physiology, 3rd Edition, Figure 9.19c, with permission
and the ventral tegmental area (VTA) in the basal ganglia. Neurons from the SN project their axons into the striatum giving rise to the Nigrostriatal pathway, and neurons from the VTA extend to the mesial components of the limbic system (nucleus accumbens, amygdala, mesial frontal cortex) and to the frontal and prefrontal cortices to originate the Mesolimbic and Mesocortical pathways (Figure 3) [12].

The dopaminergic pathways have a key physiological role in a variety of processes that include voluntary movement and motor control (Nigrostriatal pathway), pleasure and reward (Mesolimbic pathway), motivation and attention (Mesocortical Pathway), and prolactin secretion (Tuberoinfundibular pathway) (Figure 3). Malfunctions or dysregulation in these systems have been implicated in several neuropsychiatric and behavioral disorders, including Parkinson's disease (PD), Attention Deficit Hyperactivity Disorder (ADHD), depression, schizophrenia, and drug abuse.

Dopamine Uptake, Cloning and Localization of the Dopamine Transporter

Termination of acetylcholine signaling was known to be due to enzymatic degradation via acetylcholinesterase, but it soon became evident that the mechanisms for catecholamine inactivation were distinctively different. Axelrod and coworkers discovered that the sympathetic inactivation of norepinephrine (NE) was caused by its uptake and accumulation back into the nerve terminal [13], and further that these processes were inhibited by the psychoactive compounds reserpine, amphetamine (AMPH), imipramine and cocaine [13, 14]. Subsequent characterization revealed that NE uptake followed Michaelis Menten
saturation kinetics, was stereospecific for the L-isomer, required sodium (Na$^+$), and was temperature dependent, suggesting that an active transport membrane carrier system was accountable for this mechanism [15, reviewed in 16 and 17].

In addition, distinctive uptake and pharmacological properties for NE and DA were observed in brain regions where these transmitters are commonly found. Although cocaine and AMPH exhibited similar inhibition patterns for both transmitters, desipramine differentially affected the NE uptake mechanism in the cortex in contrast with its minor effects on the striatal dopaminergic uptake system [18]. These observations were extended to synaptosomal preparations of different brain regions [19], which reinforced the hypothesis that the sites for NE and DA uptake are different in noradrenergic- and dopaminergic-specific neurons. The use of a variety of psychostimulants allowed further differentiation of the catecholaminergic uptake systems in the CNS and the identification of the DA-specific uptake site in mouse, rat and human striatal tissue [20-22].

The first indication of an association between DA uptake sites and cocaine was originated by Kennedy and Hanbauer [23]. They observed a Na$^+$-dependent increase in saturable cocaine binding to rat striatal membranes without a change in affinity, and a strong correlation between the capability of a variety of drugs to inhibit DA uptake and prevent cocaine binding [23, reviewed in 24]. Subsequently Ritz and coworkers proposed that the cocaine receptor related to substance abuse was the site linked to inhibition of DA uptake [25]. They reported that cocaine and cocaine analogues triggered self-administration in non-human primates, and that these substances were potent inhibitors of binding at the
transport sites for DA [25, reviewed in 24]. Although the inhibitory effects of cocaine were shown at nerve terminals for DA, serotonin (5-HT) and NE, this group of researchers demonstrated that the inhibition of DA transport was the principal mechanism involved with cocaine reinforcing effects. Furthermore, their study indicated a non-significant association between cocaine reinforcement and either 5-HT or NE inhibition of transport [25].

With these studies it became even clearer that dopamine transporters were an essential component of the functional dopaminergic synapse and further that they were pharmacologically important as they were affected by a variety of substances. In addition, these discoveries sparked deep interest to unveil the identity of these proteins and, using the sequence for the γ-aminobutyric acid (GABA) transporter (GAT) as a probe, four groups of investigators successfully cloned the dopamine transporter (DAT) from rat (rDAT) and bovine (bDAT) origin [26-29]. Soon after and using the rDAT sequence as a template, the human isoform was cloned [30], followed by the mouse and monkey variants [31,32], and non-mammalian DATs were identified in the genome of *Caenorhabditis elegans* [33], *Drosophila melanogaster* [34], *Eloria noyesi* and *Bombix mori* [35].

In the human brain, DAT is exclusively targeted to dopaminergic neurons, with the highest expression levels comprised to midbrain neurons that arise in the SN and VTA. Other regions such as the cortex, amygdala, hypothalamus and habenula express lower protein levels. At the subcellular level, DAT localizes to the plasma membrane of neuronal perikarya, dendrites, axons, synaptic terminals and may also be found in tubulovesicular intracellular structures [36-
A deeper analysis of DAT localization within the synaptic terminals revealed that the protein is distant from the synaptic area of the presynaptic membrane, suggesting that clearance of DA happens away from its release site [37]. Outside the CNS, DAT has been found in several other systems, including stomach, pancreas, kidney and lymphocytes [39,40].

**Structural Properties of the Dopamine Transporter**

DAT belongs to the SLC6 family of Na+/Cl⁻ dependent neurotransmitter transporters, also known as neurotransmitter:sodium symporters (NSSs), which are secondary active transporters that mediate solute translocation via coupling of Na⁺ and Cl⁻ movement down their electrochemical gradients to upward movement of substrate. Other members of this family include the related norepinephrine (NET) and serotonin (SERT) transporters as well as carriers for GABA, glycine, proline, taurine, betaine, and creatine [reviewed in 41 and 42].

The rDAT and hDAT sequences are the most studied and contain 619 and 620 residues, respectively. Hydropathy profiling predicts 12 transmembrane spanning domains (TMs), mainly composed of α-helical structure, connected by extracellular and intracellular loops (ELs and ILs), and the amino- and carboxy-terminal tails located intracellularly (Figure 4). Alignment of the mammalian DATs' sequences revealed significant homology among them, with the TMs displaying the highest degree of conservation, and the loops and tails the lowest. The segment connecting TM3 and TM4, EL2, is the largest loop and represents a unique feature found within the SLC6 family.
Figure 4. Schematic Diagram of the Human DAT (hDAT) Primary Sequence. This shows the extracellular side of the protein at the top and the predicted transmembrane helices as cylinders. Cysteines 180 and 189 are shown forming a disulfide bond, and the N-glycosylated sites are indicated as branched structures.
Post-translational Modifications

The presence of a variety of post-translational modifications in DAT suggests its function is highly regulated. These modifications are located in regions unique to eukaryotic NSSs, particularly EL2 and the N-terminal tail, and may thus imply the importance of these domains in regulation of their properties, perhaps absent in their prokaryotic counterparts.

Sequence analysis revealed the presence of 3 and 4 potential N-glycosylation sites (N-X-S/T) in EL2 of hDAT and rDAT respectively (Figure 4). N-linked glycosylation at N181, N188, and N205 in hDAT has been associated with proper cell-surface expression, and changes in transport kinetics and in the potency of cocaine analogues [43,44]. Two strictly conserved cysteines in DAT are found in EL2 (C180 and C189) and have been demonstrated to be disulfide bonded and to participate in the appropriate folding of the protein [45,46] (Figure 4).

The finding of several consensus sites for phosphorylation by protein kinases in the primary sequence of DAT sparked interest for the role of this modification in transporter function [reviewed in 47,48 and 49]. DAT has been demonstrated to become phosphorylated in vivo both in constitutive and stimulated manners in a cluster of serine residues located in the N-terminal tail [50,51]. This modification has been related to mechanisms involving protein trafficking, substrate efflux, and protein-protein interactions in DAT, but many other mechanistical and regulatory aspects of its function remain unclear [reviewed in 49]. In addition, DAT undergoes constitutive and stimulated
modification by single and short-chain ubiquitin complexes on lysines 19, 27 and 35, located in the N-terminal tail [52]. Constitutive ubiquitylation occurs through the ubiquitin ligase Parkin, whose mutations lead to deficient removal of abnormal DATs and have been associated with PD [53,4]. Stimulated ubiquitylation through the ubiquitin ligase Nedd4-2 triggers movement of DATs from the plasma membrane into early and then late endosomes, suggesting degradation via the lysosomal pathway [55,56].

**Oligomerization**

The presence of oligomeric DAT complexes was initially suggested by *in situ* radiation inactivation studies [57]. Soon after, chemical cross-linking studies provided direct evidence for the existence of DAT homodimers via the TM6 residue C306 located within a conserved oligomerization motif (GVXXGVXX). In addition, the TM4 residue C243 was found to be part of a symmetrical interface that resulted in tetramers composed of dimers of dimers [58,59].

The use of co-immunoprecipitation and dominant-negative mutants revealed the presence of non-covalent high molecular weight DAT complexes, necessary for proper cell surface expression [43]. Additionally, DAT oligomerization between wild-type proteins was demonstrated in living cells using fluorescence resonance energy transfer (FRET) analysis. The loss of FRET signals upon coexpression of wild-type and endoplasmic reticulum export-deficient mutants provided supplementary evidence for the importance of oligomer formation in the proper assembly and targeting of DATs to the plasma membrane [60].
Protein-Protein Interactions

A number of studies using yeast two-hybrid, co-immunoprecipitation, FRET analysis and mass spectroscopy revealed that DATs interact with various proteins, mainly via the N- and C-termini [reviewed in 61]. At the N-terminus, DAT interacts with protein kinase C (PKC) isoforms β1 and βII, the catalytic subunit of protein phosphatase 2A (PP2A_C), RACK1 (receptor associated with C-kinase) and syntaxin 1A. Interaction partners at the C-terminus include the PDZ protein PICK1 (protein interacting with C kinase-1), Hic-5, α-synuclein, and CaMKII (calcium-dependent calmodulin kinase-II). The identification of this wide variety of DAT interacting proteins implies that the transporter is present in the membrane as a complex, rather than as an isolated entity. Furthermore, these protein-protein interactions have been associated with the regulation of several aspects of DAT function, including trafficking and transport. The mechanisms by which these interactions are integrated and regulated remain unclear and its elucidation will provide a better understanding of DAT function in normal and pathological states [61].

Functional Properties of DAT

It is well known that the mechanism by which uptake of DA takes place at the DAT requires concomitant binding and co-transport of Na⁺ and Cl⁻ ions, with stoichiometry of 1DA:2Na⁺:1Cl⁻ and energy from the plasma membrane Na⁺/K⁺ ATPase. During the translocation process, DAT is proposed to go through a series of conformational movements that result in alternating exposure of the central binding site to both the extracellular and intracellular sides of the
Figure 5. Alternating Access Mechanism for Substrate Translocation. Resting DATs are open to the extracellular medium in an “outward” conformation. Upon binding of Na⁺ (red circles), Cl⁻ (yellow circles) and DA (blue circles) the transporter undergoes a series of conformational changes that result in an “inward” facing conformation, where DATs are closed to the extracellular medium and open to the intracellular side of the membrane. After DA and ions are released to the intracellular medium DATs reset to the “outward” conformation and are ready for a new transport cycle.
membrane. In this "molecular gating" mechanism, DAT is maintained in a conformation "open" to the extracellular side and "closed" to the intracellular side of the membrane, where DA and ions bind. Binding triggers a conformational change and DAT is now "closed" to the extracellular side and "open" to the intracellular side of the membrane, where DA and ions are released. The transporter then resets to the "open" outwardly setting and is ready for a new transport cycle (Figure 5) [62,63]. Under the influence of amphetamine (AMPH), DAT promotes release of DA from the cytosol to the extracellular environment via reverse transport [reviewed in 64]. Additionally, DATs exhibit non-coupled ion conductances attributed to non-stoichiometric ion movements across the membrane, analogous to ion channels. These channel-like properties of the substrate translocation process manifest the presence of "leaking" mechanisms, demonstrate that the permeation pathway is not perfectly sealed, and are associated with AMPH-induced efflux [65-68].

Prokaryotic Leucine Transporter and Relationship to DAT

Although many structural and functional characteristics of DAT have been elucidated since it was cloned in the early 1990s, there is currently limited understanding regarding its three dimensional orientation in the plasma membrane, the binding sites for DA, ions and uptake blockers, the way the TMs rearrange during substrate translocation, and the mechanisms that drive blockade of transport by inhibitors. The lack of understanding of the above mentioned structural aspects could be attributed mainly to the difficulty in
isolating and obtaining a crystal structure from eukaryotic members of the SLC6 family.

A significant advance in this field was provided by studies in the laboratory of Eric Gouaux, who reported the high-resolution crystal structure of a bacterial homologue of the NSSs \[69\]. LeuT_Aa is a Na⁺-dependent leucine transporter isolated from *Aquifex aeolicus* that shares 20-24% identity with its eukaryotic counterparts DAT (20%), SERT (21%) and NET (24%). The LeuT_Aa crystal structure reveals a pseudo two-fold axis arrangement that associates TMs 1-5 and TMs 6-10, where the leucine and Na⁺ binding sites are located in a pocket comprised of TMs 1, 3, 6 and 8. Interestingly, TMs 1 and 6 are juxtaposed and oriented antiparallel. These domains possess the highest amount of conserved residues and are not continuous helices. Instead, halfway across the membrane, the helices display an extended, non-helical conformation that links the two halves of each domain and allows the exposure of main chain atoms for coordination of Na⁺ and leucine binding. The long TMs 3 and 8 also arrange in a pseudo two-fold axis, exhibiting a \(-50^\circ\) angle with respect to the membrane, and possess highly conserved residues that are positioned near the unwound portions of TMs 1 and 6. The rest of the TMs surround this central core, and function as support structures for the correct positioning of the helices in the active site and as the main contact structures with the lipid bilayer. The Na⁺ and leucine binding sites are located contiguously toward the middle portion of the protein core. In the LeuT_Aa crystallized conformation, these sites are obstructed from the aqueous environment by both the extracellular and intracellular gates,
providing evidence that the protein undergoes three possible conformations during transport: open to the extracellular medium, closed at both sides, and open to the intracellular environment. In addition, LeuT<sub>As</sub> was crystallized as a dimer whose interface is composed of EL2, and TMs 9 and 12. Despite evidence that the eukaryotic NSSs form high-order oligomers, the natural occurrence of the LeuT<sub>As</sub> dimers or the extent of their functional implications remains unclear.

Despite the low overall sequence similarity of LeuT<sub>As</sub> with the mammalian transporters of the SLC6 family, the high conservation found within TMs, especially those comprising the substrate binding site (TMs 1, 3, 6 and 8), has already proved that this structure could be used as a framework for molecular modeling and analysis of NSSs active sites [70-72]. The major differences are found within the loops and cytoplasmic tails, more specifically the much smaller EL2 in LeuT<sub>As</sub> and the absence of N- and C-terminal tails, which harbor post-translational modifications and protein-protein interaction domains in the mammalian counterparts. The divergences observed in these regions highlight their importance in the regulation of ion/substrate ratios and transport function, and further the non-conserved residues in the binding pocket are thought to be important determinants of substrate specificity for each of the NSSs [71].

Pharmacological Aspects of the Dopamine Transporter

The synthesis, storage, release, reuptake, recycling and metabolism of DA tightly control the homeostasis of the dopaminergic system. DAT plays a major regulatory role by modulating the amount of DA in the synaptic cleft, thereby controlling the intensity and duration of the nerve impulse. When the activity of
the transporter is disturbed, such as in the presence of cocaine and AMPH, the levels of DA in the extracellular space are increased. As a consequence, postsynaptic neurons become stimulated to supraphysiological levels, resulting in euphoria and psychomotor stimulation, and ultimately in behavioral reinforcement. This gives rise to what is known as the "dopamine hypothesis of reinforcement" (Figure 6) [73].

Inhibitors

Cocaine is a psychostimulant drug with euphorigenic and reinforcing properties, which are believed to be critical determinants for its abuse and dependence. It is the fifth most widely abused stimulant in western countries and possesses characteristics common to other addictive substances, such as tolerance to some of its effects and psychological withdrawal syndrome. Cocaine is a naturally occurring alkaloid in the leaves of *Erythroxylon coca*, a plant endemic in South America, and has been the topic of extensive scientific investigation since the beginning of the 20th century. Its physiological effects include local anesthesia and sympathetic as well as psychomotor stimulation, which provide this agent with an exceptional potential for abuse [reviewed in 74]. Today it is classified as a Schedule II drug, meaning that it has high potential for abuse, but can be administered by a physician for legitimate medical uses, such as local anesthesia for some surgeries [75]. Cocaine has been demonstrated to act as an uptake inhibitor of NE, serotonin (5-HT) and DA via binding to their cognate transporters, and to have effects on cholinergic, muscarinic and sigma
Figure 6. The Dopamine Hypothesis of Reinforcement. The normal functioning dopaminergic system (left panel) is disturbed by the presence of cocaine (☉) (right panel), which binds to DAT (purple trapezoids) and inhibits its transport activity. As a result, there is an increase in DA concentration at the synaptic cleft (blue dots) with stimulation of the pre- and post-synaptic receptors (magenta and light blue cylinders) to supraphysiological levels (green signals).
receptors [reviewed in 74,76]. However, its reinforcing properties have been specifically related to its inhibitory actions at DAT (Figure 6).

Other substances that function as DAT uptake inhibitors and join cocaine in this group include the therapeutic agents GBR 12909 (originally developed to treat cocaine addiction), bupropion (Wellbutrin®, an antidepressant), methylphenidate (Ritalin®, therapy for ADHD), and mazindol (Mazindol®, an appetite suppressant) (Figure 7).

Substrates
Amphetamines (AMPH and methamphetamine-METH) are also powerful CNS psychostimulants. They were first synthesized at the end of the 19th century from the alkaloid ephedrine, the active principle from the leaves of Ephedra species. The physiological effects of AMPH and METH include euphoria, psychomotor stimulation, decrease in appetite, and general sense of well-being. The main difference between these substances resides in the fact that, at comparable doses, METH reaches the CNS much faster and has longer-lasting effects than AMPH, having greater potential for abuse. AMPH (Aderall®) is currently used as a standard therapeutic agent for ADHD and narcolepsy, but METH is classified as a Schedule II drug due to its high abuse potential [77,78]. In contrast to cocaine and because of their chemical resemblance to dopamine (Figure 7), amphetamines act as competitive substrates for DAT and become transported into the presynaptic neuron. The end result is similar to that of cocaine with respect to increased availability of DA at the synapse. In addition, these substances cause redistribution of vesicular DA into the cytoplasm, and
Figure 7. Dopamine Transporter Substrates and Inhibitors. Chemical structures of A. the physiological substrate dopamine, and the psychostimulants amphetamine and methamphetamine that act as substrates at DAT; B. the neurotoxins 6-hydroxydopamine and 1-methyl-4-phenylpyridinium, which are also transported by DAT and cause intracellular oxidative damage; C. the uptake inhibitors cocaine, GBR 12909 and methylphenidate (Ritalin®) that bind to DAT and block its transport activity.
reverse transport of DA by DAT, inducing a tremendous spill of DA into the synaptic space [79, reviewed in 64 and 77].

Neurotoxins

DAT has also been demonstrated to be the gateway for several neurotoxins that act as substrates and become transported into the cytoplasm due to their structural resemblance to DA. 1-methyl-4-phenylpyridinium (MPP\textsuperscript{+}) is the oxidation product of the widely characterized neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Figure 7). MPP\textsuperscript{+} enters the neuron through DAT and triggers harmful intracellular events that include oxidative stress and disruption of mitochondrial respiration, ultimately leading to selective neuronal deterioration and Parkinson-like syndrome in mammals [80,81]. The naturally occurring toxin 6-hydroxydopamine (6-OHDA) is produced by non-enzymatic hydroxylation of DA and is widely used to obtain animal models of PD. Once inside the cell it causes oxidative stress via production of reactive oxygen species (ROS), leading to cellular, mitochondrial and DNA damage [80,81].

The toxic actions of these substances in dopaminergic neurons and the fact that they cause their detrimental effects by entering the cell through DAT suggest a potentially important role of this protein in the selective degeneration of DA neurons characteristic of PD. Despite the presence of evidence suggesting an environmental component in the etiology of idiopathic PD, researchers have yet to find a specific toxin in the brains of these patients and the DAT hypothesis appears to be one of several factors contributing to the cascade of events that leads to the pathogenesis of PD [80].
Lessons from DAT Knockout Mice

The role of the dopaminergic system in association with the molecular events that lead to drug abuse, and specifically the involvement of DAT in maintaining neuronal homeostasis has been strengthened and extended by studies in knockout animals. In 1996, Caron and coworkers reported the production and analysis of a DAT knockout mouse (DAT<sup>-/-</sup>) that exhibits unique neurochemical and behavioral characteristics [82]. The biochemistry of the nigrostriatal DA system is profoundly altered in these animals, with elevated extracellular DA levels, disrupted DA removal, decreased levels of intracellular DA and TH, and down-regulation of pre- and post-synaptic DA receptors. In these animals, DA persists in the extracellular space at least 100 times longer than in the presence of DAT because diffusion is the only remaining mechanism for DA clearance. DA knockout mice display highly elevated spontaneous locomotion (5-6 times more active), reduced size, cognitive deficiencies, and sleep impairment [reviewed in 83, 84]. Injections of high doses of cocaine and AMPH in the striatal tissue produced no significant increases in locomotor effects. In addition, methylphenidate and MPTP also fail to affect DA-related processes in these animals [reviewed in 83]. Interestingly, despite the lack of locomotor effects in the presence of cocaine, DAT knockouts do self-administer cocaine [85], indicating contribution of a different system in the reward mechanism. Identification of sites for cocaine binding and neuronal activation implicated the 5-HT system [85], as further supported by the lack of conditioned
place preference behavior (an indication of reward) in the DAT/SERT double knockout mouse [86].

These knockout studies demonstrate the involvement of DAT in processes related to modulation of DA concentrations and further substantiate its crucial role in maintaining pre- and post-synaptic DA homeostasis. In addition, these models demonstrate the complexity of the monoaminergic systems and the contribution of various mechanisms in mediating psychostimulant self-administration and reward.

Substrate and Ligand Binding Sites

The first step in the translocation process involves the selective binding of substrates and ions to DAT. Inhibitors also bind to DAT but they are unable to be transported across the plasma membrane. A number of distinct structural elements present in substrates and inhibitors are involved in the high affinity recognition of their binding site on DAT. DA most likely appears in cationic or zwitterionic form at physiological pH, where the catechol ring is postulated to be necessary for binding, and the amine side chain is thought to mediate the conformational switches necessary for transport [87-89]. Additionally, the majority of the non-DA substrates are phenethylamine analogues that contain a protonated amine group, apparently involved in their recognition by the transporter [89]. In the case of cocaine the tropane ring, the bridged tropane nitrogen and the phenyl-ester moiety represent essential elements for normal DAT binding [74]. Together the structural features of DA, phenethylamine
derivatives, and cocaine are potentially recognized by DAT through a variety of interactions involving charged, aromatic and polar residues [90].

The DAT interaction domains for substrates and uptake-blockers have been extensively studied through a variety of approaches in an attempt to obtain insight into the structural implications of the translocation pathway. Understanding the relationship between structure and function at DAT is crucial in the development of pharmacotherapy directed against drug abuse and DA-related syndromes. According to these studies the active sites reside within the TM domains, with the loops functioning in a structural/conformational role. Traditional binding and uptake experiments suggested that the recognition site for DA and inhibitors was indistinguishable or mutually exclusive [91]. However, evidence from chimeras and site-directed mutagenesis implicate several distinct and shared domains in substrate and blocker binding.

DAT/NET constructs revealed the involvement of TMs 1-3 in both DA and blocker interaction, and TMs 5-8 in the recognition of blockers only [92,93]. Additionally, human/bovine DAT chimeras demonstrated that the concomitant presence of TM3 and TMs 6-8 was necessary for high affinity cocaine binding, suggesting that several domains participate in the recognition of uptake blockers [94,95].

More precise localization of the specific amino acids present in the active site within these domains was achieved through site-directed mutagenesis. The first DAT amino acid demonstrated to participate in interactions with both DA and cocaine was D79, located in the middle of TM1 [96]. Interestingly, D79 is one of
only two negatively charged residues located within DAT TMs and further it is conserved within the monoamine NSSs. Replacement of D79 with alanine, glycine or glutamate results in profound decreases in DAT affinities for DA and the cocaine analogue CFT (2β-carbomethoxy-3β-(4-fluorophenyl)tropane) [96]. TM1 additionally contains the residues F76 and W84, which were demonstrated to be involved in cocaine binding by site-directed mutagenesis [97,98]. V152 is predicted to lie within DAT TM3 and analysis of its replacement to isoleucine supports participation in cocaine analogue binding [99]. The TMs 6-8 residues D313, F320, G323, Y335, D345, and D436 have also been implicated in mechanisms involved in transport and inhibitor binding [97,98,100,101].

Although these domains are distant in the primary structure of DAT, their involvement in substrate and blocker binding suggests they lie close together in the tertiary structure and are part of a common binding pocket in DAT. Additional evidence for the three-dimensional juxtaposition of domains was provided by the identification of an endogenous extracellular zinc (Zn$^{2+}$) binding site in DAT. Coordination of Zn$^{2+}$ by residues H193 in EL2, and H375 and E396 in EL4 requires an ideal distance of 4 Å among them, positioning these domains in close proximity in the three-dimensional arrangement of the protein [102-104]. Engineering artificial Zn$^{2+}$ binding sites into DAT has been useful to further define secondary and tertiary structure at the extracellular side of TMs 7 and 8, and to identify residues involved in the conformational changes related to the transport mechanism [105-107].
Photoaffinity Labeling Strategy

In addition to the use of reversible competitive analogues and the above described approaches, the functional domains and the active site in DAT have been studied using photoaffinity ligands. Photoaffinity labeling enables direct probing of the target protein through a covalent bond, which is photochemically introduced between a ligand and its specific receptor. This method requires the use of a bioactive ligand analogue with high affinity for the recognition site that contains a light-sensitive moiety. The action of ultraviolet (UV) light produces a highly reactive species that binds irreversibly to the biological receptor at or near the active site [108,109].

A number of structurally diverse photoaffinity probes have been used to study DAT, including structural analogues of cocaine, GBR 12909 and benztropine. These analogues are composed by their cognate pharmacophore core containing the essential structures for reversible binding to DAT, in addition to a photoactivatable iodo-azido moiety, where the azido (N₃) is the reactive group that enables irreversible attachment and the reactive iodine ([¹²⁵I] serves as a tracer (Figure 8). With this technique, the radiolabeled analogues are covalently incorporated into DAT and subjected to peptide mapping in order to localize the attachment site. For the peptide mapping approach, photolabeled DATs are subjected to protease treatment for relatively short periods of time, after which large, well-defined fragments are generated. These fragments are then analyzed by immunoprecipitation using antibodies directed against specific epitopes in DAT, followed by sodium dodecyl sulphate polyacrylamide gel
Figure 8. DAT Incorporation Domains for Photoaffinity Labels. A. Chemical structures of the photolabels analyzed to date. $^{[125I]}$DEEP and $^{[125I]}$AD-96 are GBR analogues, $^{[125I]}$RTI 82 is a cocaine analogue, and $^{[125I]}$GA 2-34 is a benztropine analogue. B. Schematic diagram of hDAT indicating the domains of ligand incorporation (magenta and blue cylinders), the relative positions of the antibody 5 and 16 epitopes (colored bold lines), and the potential trypsin cleavage sites (yellow circles).
electrophoresis (SDS-PAGE), and autoradiography. Two DAT-specific antibodies have been widely used for the immunoprecipitation procedures and have enabled the identification of distinct labeled regions within the protein. Antibody 16 is directed against amino acids 42-59 in the N-terminal tail, and antibody 5 is generated against amino acids 225-238, located in EL2 (Figure 8).

Using photoaffinity labeling in combination with peptide mapping, two distinct regions in DAT, TMs 1-2 and TMs 4-7, become consistently labeled by structurally diverse uptake-blocker analogues. The GBR analogue \[^{125}\text{I}]\text{DEEP}\) and the benztropine analogue \[^{125}\text{I}]\text{GA 2-34}\) become incorporated in the TMs 1-2 region [110-112], and the cocaine analogue \[^{125}\text{I}]\text{RTI 82}\) becomes incorporated in TMs 4-7 [110,111]. Interestingly, the piperidine analogue \[^{125}\text{I}]\text{AD-96}\) becomes incorporated into both of these regions [113] (Figure 8). This represents additional evidence supporting the proximity of these domains in the three-dimensional structure of DAT, and implies that uptake-blockers bind to a common pocket composed of multiple DAT regions.

**Photolabeling, Chemical Cleavage and Site-Directed Mutagenesis**

Specific characterization of the exact amino acid to which these compounds bind in DAT is limited by the enzymatic cleavage and antibody-based approach. This method only allows positive identification of labeled fragments that retain the antibody epitopes. Since DAT contains many potential cleavage sites within or close to these epitopes, labeled fragments could be generated but they would not be immunoprecipitated by the antibody. Recently, our laboratory was able to localize the specific \[^{125}\text{I}]\text{RTI 82}\) incorporation domain in DAT to TM6
through the use of chemical cleavage in combination with site-directed mutagenesis [114]. Proteolysis of $[^{125}\text{I}]$RTI 82 labeled DAT was performed using cyanogen bromide (CNBr), which specifically cleaves at the C-terminal side of methionine residues. hDAT and rDAT contain only 13 and 15 methionines, respectively, scattered throughout the sequence. Additionally, based on the antibody identification of TMs 4-7 as the $[^{125}\text{I}]$RTI 82 attachment region, several methionines were engineered into the hDAT sequence to create mutants in combinations such that the size of the labeled fragments obtained by CNBr treatment would be consistent with incorporation at a particular domain within this region. This combination strategy allowed the positive identification of a region containing residues from EL3, TM6, and IL3 as the $[^{125}\text{I}]$RTI 82 labeled domain. This finding was further confirmed by subjecting the labeled fragment obtained by CNBr cleavage to a second round of digestion using the enzyme Lys-C. This generated a reduced-mass labeled fragment, consistent with TM6 being the $[^{125}\text{I}]$RTI 82 incorporation domain. This combination approach thus proved to be an effective strategy to delineate the specific domains to which the photolabels become incorporated in DAT.

The present studies describe the characterization and localization of the binding site for $[^{125}\text{I}]$MFZ 2-24 ($N$-[4-(4-azido-3-$[^{125}\text{I}]$iodophenyl)butyl]-2-carbomethoxy-3$\beta$-(4-clorophenyl)tropane) in DAT using photoaffinity labeling, peptide mapping, chemical cleavage and site-directed mutagenesis. $[^{125}\text{I}]$MFZ 2-24 is a cocaine analogue whose pharmacophore is identical to that of $[^{125}\text{I}]$RTI 82 but the phenyl-iodo-azido moiety is placed differentially within the cocaine core.
(Figure 9). Since these compounds share the essential structures for reversible cocaine binding, they should bind in the active site with identical orientation, and the differential positions of the reactive groups should provide distinctive irreversible incorporation patterns within the pocket. This would allow the positive identification of the TMs that constitute the binding pocket for cocaine in DAT, and would provide an estimation of the proximity of these domains within the pocket. In addition, a third cocaine analogue in these series of compounds, $[^{125}\text{I}]$JHC 2-48 (3-(4'-azido-3'-iodo-phenyl)-8-methyl-8-aza-bicyclo-[3.2.1]octane-2-carboxylic acid methyl ester) was initially analyzed and characterized through peptide mapping in comparison to $[^{125}\text{I}]$MFZ 2-24 and $[^{125}\text{I}]$RTI 82. $[^{125}\text{I}]$JHC 2-48 contains the reactive group appended to the phenyl ring in the pharmacophore (Figure 9), and the localization of its incorporation site will provide further evidence for the identity of the domains that compose the binding pocket for cocaine in DAT.

These studies additionally describe the binding characteristics and the irreversible incorporation into DAT of the GBR analogue $[^{125}\text{I}]$D-147 (1-{2-[(4-Azido-3-iodo-phenyl)-phenyl-methoxy]-ethyl}-4-phenethyl-piperazine oxalate) using photoaffinity labeling and peptide mapping. $[^{125}\text{I}]$D-147 is identical in structure to the previously characterized GBR-like compound $[^{125}\text{I}]$DEEP, with the iodo-azido reactive group attached in a different position with respect to the piperazine pharmacophore (Figure 9). The comparison of the irreversible attachment sites for these related compounds will provide essential information.
Figure 9. Chemical Structures of Photoaffinity Labels. A. Cocaine and cocaine-based photolabels are shown. \([^{125}\text{I}]\text{RTI 82}\) attachment to DAT has been previously characterized [110,111,114]. Incorporation profiles of \([^{125}\text{I}]\text{MFZ 2-24}\) and \([^{125}\text{I}]\text{JHC 2-48}\) into DAT are described in these studies. B. GBR 12909 and GBR-based photolabels are depicted. Incorporation of \([^{125}\text{I}]\text{DEEP}\) into DAT has been previously described [110,111]. \([^{125}\text{I}]\text{D-147}\) attachment to DAT is characterized in the present studies.
about the components of the binding site for GBR-like compounds and will further our understanding of how the TMs in DAT are three-dimensionally oriented.

Previous studies using both GBR and cocaine classes of compounds have suggested that they share a common binding pocket composed of multiple TM domains. The present studies represent the first time that photoaffinity labels carrying identical pharmacophores will be used to directly compare their incorporation patterns. The obtained results will allow for correlation of the information obtained from identifying the attachment sites for both classes of inhibitors, and to further analyze the degree of similarity or difference among their binding sites.
CHAPTER II
MATERIALS AND METHODS

Materials

Animals

Male Sprague-Dawley rats (175-300 g) were obtained from Charles River Laboratories (Wilmington, MA) and were housed and treated in accordance with regulations approved by the University of North Dakota Institutional Animal Care and Use Committee and the National Institutes of Health.

Reagents

$[^{125}\text{I}]$MFZ 2-24, $[^{125}\text{I}]$RTI 82, $[^{125}\text{I}]$D-147, and $[^{125}\text{I}]$JHC 2-48 were synthesized by Dr. Amy Newman (NIDA), Dr. Mu-Fa Zou (NIDA), Dr. Joo Whan Cha (NIDA) and Dr. Aloke Dutta (Wayne State University), and radioiodinated by Dr. John Lever (University of Missouri) as previously described [115-118]. Trypsin, trypsin inhibitor, dopamine, (-)-cocaine, GBR 12909, nomifensine, mazindol, desipramine, imipramine, citalopram, nisoxetine, fluoxetine and CNBr were from Sigma-Aldrich (St Louis, MO). (+)-Cocaine was the generous gift of Dr. Maarten E.A. Reith (New York University School of Medicine, New York, NY). $[^{3}\text{H}]$2β-carbomethoxy-3β-(4-fluorophenyl)tropane (CFT) was from Perkin Elmer Life and Analytical Sciences (Boston, MA). Protein A Sepharose CL4B beads, $[^{3}\text{H}]$dopamine, and high- and low-range Rainbow Molecular Weight Markers
Markers were from Amersham Biosciences/GE Healthcare (Piscataway, NJ). Electrophoresis reagents were from Bio-Rad (Hercules, CA). Human Embryonic Kidney (HEK) 293 cells were from ATCC (Manassas, VA), HEK 293 cells expressing hSERT were kindly provided by Dr. Randy Balely (Vanderbilt University, Nashville, TN), and Lewis Lung Carcinoma Porcine Kidney (LLCPK₁) cells expressing rDAT were the generous gift of Dr. Gary Rudnick (Yale University, New Haven, CT). Complete Mini protease inhibitor and FuGENE 6 transfection reagent were from Roche Applied Sciences (Indianapolis, IN). Cell culture reagents were from Mediatech (Herndon, VA); the QuikChange Mutagenesis kit was from Stratagene (La Jolla, CA). Synthetic oligonucleotide primers were purchased from Genscript Corporation (Piscataway, NJ), or MWG Biotech Inc (High Point, NC). All other chemicals and reagents were from Sigma-Aldrich (St Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Equipment

Centrifuges

A Beckman Avanti J-25 centrifuge with a JA 25.50 or a JA 16.250 rotor was used to prepare rat striatal membranes and for purification of plasmids. Beckman Microfuge R and Microfuge 18 centrifuges were used for general microcentrifugation under refrigerated and non-refrigerated conditions, respectively. A Beckman J6-MI swinging bucket centrifuge with a JS 5.2 rotor was used for Protein A Sepharose beads preparation and cell pelleting.
Electrophoresis, electroelution and dialysis

Sodium dodecyl sulphate (SDS) poly-acrylamide gel electrophoresis (PAGE) was performed using the Gibco/BRL Life Technologies Model V16 gel system or the Bio-Rad Mini-Protean III electrophoresis device. Transfer of electrophoresed proteins was performed using a Bio-Rad Mini trans blot electrophoresis transfer cell. Electrical voltage and current for both processes was monitored by the Gibco/BRL Life Technologies 250 EX power supply. Gels were dried using a Bio-Rad Model 583 gel dryer.

Electroelution of proteins was performed using the Bio-Rad Model 422 Electroeluter with 3,500 MW cutoff membrane caps. Electroeluted proteins were dialyzed using Pierce Slide-A-Lyzer® cassettes with 10,000 MW cutoff.

Spectroscopy

A Molecular Devices SpectraMax 190 plate reader was used to quantify Bicinchoninic acid (BCA) protein assays and a Beckman DU640 spectrophotometer was used to quantify plasmid DNA. A Rackard 1900CA or a Beckman LS6500 liquid scintillation counter was used to analyze incorporation of radioactivity during binding and uptake experiments.

Cell culture, molecular biology and miscellaneous equipment

Mammalian cells were grown and maintained in a Nuaire 2700-30 water-jacketed CO₂ incubator, and steriley managed in a Nuaire Class II type A/B3 laminar flow hood. An Eppendorf Mastercycler Personal thermocycler was utilized to perform procedures involving Polymerase Chain Reaction (PCR). A Polytron PT2100 homogenizer was used to homogenize rat striatum for
membrane preparations. A Thermo Savant SpeedVac® evaporator was used to dry dialyzed and CNBr digested samples. A Fotodyne ultraviolet (UV) lamp model 3-6000 was used in photolabeling studies.

Methods

Photoaffinity Labeling

Rat Striatal Membranes

Male Sprague-Dawley rats (175-300 g) were decapitated and the striatal tissue was immediately removed and weighed, after which it was placed in ice-cold sucrose-phosphate (SP) buffer (10 mM sodium phosphate plus 0.32 M sucrose, pH 7.4) and homogenized with a Polytron Homogenizer in setting 11 for 12-15 sec. Homogenate tissue was subjected to centrifugation at 20,000 xg for 12 min at 4 °C, and the resulting membranes were washed twice by centrifugation in ice-cold SP buffer and resuspended to 20 mg/mL original wet weight (o.w.w.). \([^{125}}\)MFZ 2-24, \([^{125}}\)RTI 82, \([^{125}}\)D-147 and \([^{125}}\)JHC 2-48 reaction mixtures were prepared in SP buffer, added to the membranes at a final concentration of 5 nM, and incubated for 60 min at 0 °C to allow reversible binding. For pharmacological competition studies, saturating concentrations (1-10 μM) of non-radioactive transporter inhibitors and substrates were added to the binding mixture. Irreversible attachment of the photoaffinity ligands to DAT was carried out by directly irradiating the sample with UV light (254 nm) for 45 sec at a distance of 1 cm. The resulting photolabeled membranes were washed three times by centrifugation with SP buffer and subsequently solubilized at 20 mg/mL o.w.w. in SDS-PAGE sample buffer (60 mM Tris-HCl pH 6.8, 100 mM
dithiothreitol, 10% glycerol, 2% SDS and 0.001% bromophenol blue) or SP buffer for \textit{in situ} proteolysis or 0.5% SDS sample buffer (60 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 10% glycerol, and 0.5% SDS) for immunoprecipitation.

\textbf{hDAT/rDAT/hSERT Expressing Cells}

Wild type hSERT, and wild type and mutant hDAT and rDAT cells were plated onto 6-well plates and grown to 90-95% confluence. Growth medium was removed and the cells were incubated with 5 nM radioligand reaction mixtures prepared in Krebs Ringers HEPES (KRH) buffer (25 mM HEPES, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.3 mM CaCl$_2$, 1.2 mM MgSO$_4$, 5.6 mM glucose, pH 7.4) for 60 min at 22 °C to allow reversible binding. For cocaine displacement studies, a 50 μM solution of cocaine was prepared in KRH buffer and added to the binding mixture. The ligands were covalently incorporated into DAT by direct irradiation with UV (254 nm) light for 45 sec at a distance of 1 cm. Photolabeled cells were washed twice with 1 mL/well KRH and solubilized with 500 μL/well Triton buffer (1% Triton X-100, 25 mM Tris base, 150 mM NaCl, 1mM EDTA) supplemented with Complete Mini protease inhibitor at 0 °C for 30-45 minutes. Lysates were centrifuged at 20,000 xg for 15 min at 4 °C and supernatants were collected and used for further analysis.

\textbf{Gel Purification, Electroelution and Dialysis}

Solubilized radiolabeled DATs from rat striatal membranes or cells were subjected to purification by SDS-PAGE on 10% tris-glycine gel systems. Gels were dried and exposed to autoradiographic film for 6-16 h. The ~80 kDa labeled bands that correspond to DAT were excised, removed and rehydrated in 1X...
SDS-PAGE running buffer (25 mM Tris, 192 mM Glycine and 0.1% SDS). Rehydrated gel pieces were subjected to electrophoresis at 10 mA/tube for 5.5 h, and electrophoresates were dialyzed against 1 L of MilliQ purified water for 20-24 h followed by evaporation to dryness in a SpeedVac\textsuperscript{©} concentrator.

**In situ** Trypsin Proteolysis

Photolabeled rat striatal membrane suspensions prepared at 20 mg/mL in SP buffer were subjected to treatment for 10 min at 22 °C with equal volumes of trypsin prepared in SP buffer at final concentrations of 10-200 μg/mL. At the end of the incubation one volume of 1 mg/mL trypsin inhibitor was added to halt the digestion process, followed by centrifugation at 20,000 xg for 12 min at 4 °C. The resulting pellets were solubilized with 0.5% SDS sample buffer.

**In solution** Cyanogen Bromide Digestion

Dried electroeluted and dialyzed extracts were incubated for 24 h at 22 °C in the dark with 0.1 mL of 1M CNBr prepared in a 70% formic acid solution or with 70% formic acid solution alone. Quenching of the reaction was achieved by addition of 0.9 mL MilliQ water followed by evaporation to dryness in a SpeedVac\textsuperscript{©} concentrator. The samples were subjected to three additional rounds of suspension in water and drying to remove residual volatile acidity. The final dried samples were resuspended in either sample buffer for analysis by electrophoresis and autoradiography, or immunoprecipitation buffer (50 mM Tris-HCl, 0.1% Triton X-100, pH 8.0) (IPB) for analysis by immunoprecipitation, electrophoresis and autoradiography.
Immunoprecipitation, Electrophoresis and Autoradiography

Solubilized radiolabeled DATs or DAT fragments were subjected to epitope-specific immunoprecipitation as described previously [112,113] with minor modifications, using antiserum 16 generated against amino acids 42-59, or antiserum 5 generated against amino acids 225-238. Briefly, protein A sepharose beads were hydrated and washed three times with IPB, and resuspended in IPB plus 0.05% sodium azide (NaN₃). Polyclonal antibody (20 μg) was bound to the hydrated beads for 3-6 h at 4 °C with rotation followed by three washes with IPB. Alternatively, protein A sepharose beads were hydrated in triethanolamine (TEA) buffer (0.2 M TEA, pH 8.0), incubated with polyclonal antibody for 45 min at 22 °C and washed twice with TEA buffer; the antibody was cross-linked to the beads by incubation with DMP (Dimethyl pimelimidate) for 45 min at 22 °C, after which the beads were washed twice with 100 mM Tris pH 8.0, rinsed with IPB and resuspended in IPB plus 0.05% NaN₃. Samples were incubated with either bead slurry for 3 h at 4 °C with rotation, subsequently washed four times with IPB and eluted with sample buffer. For peptide competition experiments, diluted antibodies were preabsorbed with 50 μg/mL of peptide 16 or peptide 5 before sample addition. Total and immunoprecipitated samples were electrophoresed on SDS-PAGE 9-16%, 16%, 18% or 20% gels at 6-8 mA for 16 h, followed by autoradiography using Hyperfilm™ MP or Kodak BioMax MS film for 1 to 4 days at -80 °C. Low- and high-range Rainbow Markers were used as molecular mass standards.
**Immunoblot Analysis**

Solubilized rat striatal membranes or cell lysates were subjected to separation by SDS-PAGE on 10%, 4-20%, or 10-20% tris-glycine polyacrylamide gels at 125 V for 2 h. Separated proteins were transferred to 0.45 or 0.2 μm polyvinylidene fluoride (PVDF) membranes at 100 V for 2 h in transfer buffer (10 mM Tris, 100 mM glycine, 0.01% SDS and 10% methanol) at 4 °C. Subsequently, PVDF membranes were blocked for >3 h with 3% bovine serum albumin (BSA) in phosphate buffer saline (PBS) (BSA/PBS buffer) at 4 °C. DAT protein was detected with the hDAT specific rat monoclonal antibody MAB 369 (Chemicon/Millipore, Temecula, CA) generated against DAT N-terminal tail. A 1:1000 dilution of MAB 369 in BSA/PBS buffer was incubated with the blocked PVDF membranes for 1 h at 22 °C. Removal of unbound antibodies was achieved by washing the membranes four times with PBS/Tween buffer (PBS plus 0.1% Tween-20). Detection of the primary antibody was achieved by incubating the membranes with a 1:20,000 dilution of alkaline-phosphatase linked anti-rat IgG (Sigma-Aldrich) for 1 h at 22 °C, followed by removal of unbound antibodies by washing the membrane four times with PBS/Tween buffer. The membranes were developed using the alkaline phosphatase substrates Immun-Star™ AP (Bio-Rad) for 5 min or 5-bromo-4-chloro-3-indolyl phosphate/nitro blue (BCIP/NBT) (Sigma-Aldrich) for 5-10 min. Immun-Star™ treated membranes were visualized with a Boehringer-Manheim Lumi Imager and quantified using LumiAnalyst 3.0 software. BCIP/NBT treated membranes
were dried, scanned on an Epson 1200 scanner as tagged image format files (TIFF) images, and quantified using LumiAnalyst 3.0 software.

**QuikChange® Site-Directed Mutagenesis**

An N-terminal wild-type (WT) 6xHis-human (h) DAT in a pcDNA 3.1/His®B vector was used as the starting template for mutagenesis. Selected residues were mutated using the QuikChange® method in order to generate or eliminate CNBr cleavage points. The oligonucleotide primers utilized for codon substitution were designed through the PrimerX website (http://bioinformatics.org/primerx) and synthesized by GenScript Corporation or MWG Biotech (Appendix B). The reaction mixture was prepared in 200 µL PCR tubes following the parameters in Table 1. The reaction was initiated by the addition of 1 µL of *Pfu Turbo* DNA Polymerase (2.5 U/µL) and cycled according to the cycling parameters described in Table 2.

**Table 1. QuikChange® Reaction Mixture**

<table>
<thead>
<tr>
<th>Volume (µL)</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>10X Reaction buffer (100 mM KCl, 100 mM(NH₄)₂SO₄, 200 mM Tris-HCl pH 8.8, 20 mM MgSO₄, 1% Triton® X-100, 1 mg/mL nuclease-free BSA)</td>
</tr>
<tr>
<td>20</td>
<td>Template DNA (5 ng/µL)</td>
</tr>
<tr>
<td>1.25</td>
<td>Oligonucleotide primer #1 (125 ng)</td>
</tr>
<tr>
<td>1.25</td>
<td>Oligonucleotide primer #2 (125 ng)</td>
</tr>
<tr>
<td>1</td>
<td>dNTP mix</td>
</tr>
<tr>
<td>21.5</td>
<td>Double distilled DNAse free H₂O</td>
</tr>
<tr>
<td>50</td>
<td>Final Volume</td>
</tr>
</tbody>
</table>

52
Table 2. QuikChange® Thermal Cycling Parameters

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95 °C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>95 °C</td>
<td>30 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55 °C</td>
<td>1 minute</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68 °C</td>
<td>8 minutes</td>
</tr>
</tbody>
</table>

Following the thermal cycling process, 1 µL of the *DpnI* restriction enzyme (10 U/µL) was added to the reaction mixture and incubated at 37 °C for 1 h to degrade the template supercoiled double-stranded DNA. The resulting 6xHis-hDAT pcDNA 3.1/His®B plasmid (Figure 10) was transformed into either NovaBlue competent cells (Novagen) or XL1-Blue supercompetent cells (Stratagene) according to the manufacturers' guidelines.

**Plasmid Amplification and Isolation**

Transformed cells were plated on 1.5% agar plates containing 100 µg/mL carbenicillin, followed by incubation at 37 °C for >16 h to obtain colonies. Colony development was achieved by collection of one colony of cells from the selective agar followed by growth in Glucose M9Y medium complemented with 100 µg/mL carbenicillin. The plasmid DNA was isolated using the PureYield® Plasmid Midiprep System (Promega), visualized through separation by electrophoresis on 1% agarose gel (0.5 µg/mL ethidium bromide) with the GrabiT Annotating Grabber 2.04.6 (UVP Inc, Upland, CA), and quantified using the BioRad Molecular Analyst™ software version 1.5 (1992-1996). Additionally, the DNA concentration of the sample was measured by spectrophotometry at 260 nm using the following formula: [DNA (µg/mL)] = A_{260} (50) (dilution factor). The final
Figure 10. Schematic Diagram of the 6xHis-hDAT pcDNA 3.1/His\textsuperscript{B} Plasmid. The major regions of this fusion vector are depicted here. Shown are the CMV immediate-early promoter (yellow), the 6xHistidine sequence, the hDAT sequence (blue), the SV40 early promoter (green), the Neomycin resistance gene (purple), the Ampicillin resistance gene (magenta), and the polyadenylation signal (black and white). Additionally, the restriction sites used to insert the hDAT coding sequence are indicated.
hDAT
pcDNA 3.1/His®B
concentration of the plasmid DNA was obtained as an average of the two methods used for quantification. To verify the presence of the desired mutation(s) oligonucleotide primers were generated (Appendix C), the DAT insert was sequenced by Northwoods DNA (Solway, MN) or Alpha Biolabs (Sunnyvale, CA) and analyzed using Vector NTI software (Invitrogen).

Transfection of HEK 293 Cells

Stable transfection of Human Embryonic Kidney (HEK) 293 cells was achieved by growth of parental HEK 293 cells to 40-60% confluency in T25 flasks and transfection through the FuGENE 6 reagent in a 3:1 FuGENE 6 to DNA ratio mixture (Table 3). The FuGENE 6/DNA mixture was prepared in serum-free complete medium (Dulbecco's modified Eagle's medium-DMEM, 2 mM L-glutamine), gently mixed and incubated at 22 °C for 15-30 min.

Table 3. FuGENE 6 Transfection Reaction Volumes

<table>
<thead>
<tr>
<th>Mutants</th>
<th>M116A</th>
<th>M111L/M116L</th>
<th>L80M/</th>
<th>I67M/</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>M111L/M116L</td>
<td></td>
</tr>
<tr>
<td>FuGENE 6 (µL)</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>DNA (4 µg) (Concentration)</td>
<td>21 (193 ng/µL)</td>
<td>14 (300 ng/µL)</td>
<td>11 (356 ng/µL)</td>
<td>19 (212 ng/µL)</td>
</tr>
<tr>
<td>Media</td>
<td>217</td>
<td>234</td>
<td>227</td>
<td>219</td>
</tr>
<tr>
<td>Final Volume</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
</tr>
</tbody>
</table>

Following incubation, the transfection reaction mixture was added in a drop-wise manner to the cell medium in the T-25 flask containing the parent cells and placed in the incubator at 37 °C until 90-95% confluency is reached. For
selection of effectively transfected cells, the original medium was removed, the
cells were split 1:2, and selection medium was added (DMEM, 10% fetal bovine
serum-FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin,
and 600 µg/mL G418). Cells were kept under selection conditions for 2-3 days,
dead cells were removed, flasks were rinsed with Hanks Balanced Salt Solution
(HBSS), and selection medium was added. Cells were grown in selection
medium for ~2 weeks, after which growth medium (DMEM, 10% FBS, 2 mM L-
glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 250 µg/mL G418)
was added.

Expression of mutant DATs was verified through immunoblot analysis of
cell lysates with MAB 369 antibody. Lysates were obtained by solubilizing cells in
Triton buffer for 30-45 min at 4 °C, and immunoblotted as described above. The
protein concentration of the lysates was measured by spectrophotometry using
the BCA method.

Cell Culture and Cell Freezing

Parental HEK 293 cells were grown and maintained in serum-free
complete medium. 6xHis-hDAT HEK 293 cells were selected, grown and
maintained in complete medium with either 250 or 600 µg/mL G418. Growth and
maintenance was performed in T-75 flasks at 37 °C in an incubator gassed with
5% CO2/95% O2.

Once DAT expression was verified by immunoblotting with stably
transfected cells, stock aliquots were prepared for freezing. Cells were cultured in
T-75 flasks until 95-100% confluency was achieved. Once confluent, cells were
collected, pelleted, resuspended in 2-3 mL of freeze medium (10% Dimethyl Sulfoxide-DMSO), and aliquoted in properly labeled cryovials. Aliquots of cells were gently frozen for 24 h in a Nalgene Cryo 1 °C container, and subsequently stored in liquid nitrogen.

Functional Assays

Whole Cell \[^3H\]CFT Binding and \[^3H\]DA Uptake

HEK 293 cells expressing WT and mutant 6xHis-hDAT were grown in 24-well plates until 75-80% confluency was reached, and washed twice with ice-cold KRH buffer. For binding assays cells were incubated in quadruplicates with 5 nM \[^3H\]CFT in KRH for 2 h at 0 °C, subsequently rinsed twice with KRH, and solubilized in 1% Triton X-100. For uptake assays, cells were assayed in triplicate for DA transport using a 3 μM DA mixture containing 10 nM \[^3H\]DA in KRH, and with 30 μM (-)-cocaine to determine non-specific uptake. Assays were carried out for 9 min at 37 °C and stopped by rinsing cells with ice-cold KRH followed by solubilization in 1% Triton X-100. Following solubilization, radioactive binding and uptake lysates were added to 3 mL of scintillation fluid EcoScint A followed by analysis by liquid scintillation counting at 52% efficiency. In order to obtain the IC\(_{50}\) values for cocaine inhibition of \[^3H\]CFT binding and \[^3H\]DA uptake, assays were performed in the presence of 10\(^{-11}\) – 10\(^{-4}\) M (-)-cocaine.

Rat Striatal Membrane \[^3H\]CFT Binding Competition Assay

In order to determine the binding affinity of the novel ligand D-147 for DAT, a \[^3H\]CFT binding competition assay was performed in rat striatal membrane preparations. Rat striatal tissue was homogenized in SP buffer, and
homogenates cc. centrifuged. The resulting membranes were resuspended at 10 mg/mL o.w.w. in the same buffer. To initiate the binding competition assay, triplicate samples of membranes were added to tubes containing $10^{-10} - 10^{-5}$ M concentrations of D-147, and 1 nM $[^{3}\text{H}]$CFT prepared in SP buffer, followed by incubation on ice for 2 h. Membranes were then collected and washed three times with 5 mL of SP buffer using a Brandel tissue harvester over Whatman GF/B glass fiber filters soaked for 1 h in 0.1% BSA/0.05% Polyethylene (PEE) in PBS. Bound radioactivity was quantified by liquid scintillation counting at 52% efficiency.

Statistical Calculations and Graphs

The determination of the cocaine IC$_{50}$ for inhibition of $[^{3}\text{H}]$DA uptake or $[^{3}\text{H}]$CFT binding to DAT in WT and mutant hDAT HEK 293 cells, and the determination of the D-147 IC$_{50}$ for binding to DAT in rat striatal tissue were performed using non linear regression analysis with Prism 3.0 software (Graphpad Software, San Diego, CA). Values were statistically analyzed using Analysis of Variance (ANOVA) with Tukey's Multiple Comparison Test.

Energy Minimization Calculations

Intramolecular distances and energy minimization calculations were carried out by Dr. Amy Newman (NIDA-IRP). The intramolecular distances in $[^{125}\text{I}]$MFZ 2-24 were obtained using the molecular modeling program SYBYL 6.7 (Tripos Inc.) with the CFT crystal structure as a template. Energy minimizations were performed by conjugate gradient method until a convergence gradient of 0.001 kcal/mol/Å was achieved.
CHAPTER III

RESULTS

Pharmacology of $[^{125}\text{I}]$MFZ 2-24 incorporation into DAT

The photoaffinity label $[^{125}\text{I}]$MFZ 2-24 is a cocaine analogue whose structure contains tropane and phenyl rings, known essential elements for cocaine binding to DAT. $[^{125}\text{I}]$MFZ 2-24 is structurally identical to the previously characterized $[^{125}\text{I}]$RTI 82 [114], except for the position of the phenyl-iodoazido reactive group with respect to the cocaine pharmacophore (Figure 11). Synthesis and radiiodination of MFZ 2-24 have been previously described and its $IC_{50}$ value for displacement of $[^{3}\text{H}]$CFT in rat striatum was shown to be $33.0 \pm 4.7$ nM [115,116], indicating high affinity for DAT.

Incorporation of $[^{125}\text{I}]$MFZ 2-24 into rat striatal DATs was evaluated in the presence of a number of uptake blockers and the physiological substrate DA, in order to determine its pharmacological specificity for binding to DAT. Labeling was prevented when binding was carried out in the presence of DA or the DA uptake inhibitors (-)-cocaine, mazindol, GBR 12909 and nomifensine, but was not modified by the inactive enantiomer (+)-cocaine, the NET blocker desipramine or the SERT blocker imipramine (Figure 12). These results demonstrate that $[^{125}\text{I}]$MFZ 2-24 irreversibly attaches to DAT and that the compounds used in these studies act as competitive inhibitors by preventing the reversible
Figure 11. Chemical Structure of Cocaine-Based Photoaffinity Labels. Cocaine and the analogues used in these studies are shown. The phenyl-iodoazido moiety that allows the covalent incorporation into the protein is attached to the tropane ring C2 carbon in $[^{125}\text{I}]$RTI 82, to the tropane bridge nitrogen in $[^{125}\text{I}]$MFZ 2-24, and to the phenyl ring in $[^{125}\text{I}]$JHC 2-48.
Cocaine

\[ \text{[\textsuperscript{125}I]RTI 82} \]

\[ \text{[\textsuperscript{125}I]MFZ 2-24} \]

\[ \text{[\textsuperscript{125}I]JHC 2-48} \]
Figure 12. [\(^{125}\text{I}\)]MFZ 2-24 Photoaffinity Labeling Displays DAT Pharmacological Specificity. Rat striatal membranes were photolabeled with [\(^{125}\text{I}\)]MFZ 2-24 in the presence of the indicated compounds [10 \(\mu\text{M}\) final concentrations, except imipramine (1 \(\mu\text{M}\)) and dopamine (100 \(\mu\text{M}\))]. After labeling, samples were immunoprecipitated with antibody 16 and subjected to analysis by SDS-PAGE and autoradiography.
incorporation of the ligand. This evidence supports the assumption that the
irreversible incorporation site is located in or near a DAT domain of significant
pharmacological importance.

Peptide Mapping of the DAT [\textsuperscript{125}I]MFZ 2-24 Incorporation Site by Trypsin
Proteolysis

Trypsin is a serine protease that cleaves proteins at the carboxyl side of
lysine and arginine residues. The DAT sequence contains several potential
trypsin sites (Figure 13), but for our peptide mapping studies using membrane
preparations, only those located in the interhelical loops are accessible for
enzymatic cleavage. In addition, the presence of both the antibody epitope and
the attached radioactive label is essential for a labeled fragment to be detected
by this approach. Tryptic maps of [\textsuperscript{125}I]RTI 82 and [\textsuperscript{125}I]DEEP labeled rat striatal
membranes showed cleavage at the EL2 residue R218 with subsequent
generation of two distinctive fragments that originate N- and C-terminal to this
amino acid. Visualization of a glycosylated 45 kDa [\textsuperscript{125}I]DEEP fragment that
immunoprecipitates with antibody 16 (against residues 42-59), and a non-
glycosylated 32 kDa [\textsuperscript{125}I]RTI 82 fragment that is precipitated by antibody 5
(against residues 225-238) (Figure 13) demonstrates the differential
incorporation of these ligands into discrete domains in DAT [111]. This strategy
was also utilized to delineate the attachment sites for a variety of structurally
diverse compounds, which became incorporated into one or both of these
domains (Figure 8), supporting the hypothesis that TMs 1-2 and TMs 4-7
contribute at least in part to form a common binding pocket for various classes of
inhibitors [reviewed in 119].
Figure 13. Potential Trypsin Cleavage Sites and Antibody Epitopes in rDAT. Schematic diagram of rDAT showing the arginine (R) and lysine (K) residues as the potential trypsin proteolysis sites (yellow). The N-terminal antibody 16 is directed against residues 42-59 (magenta) and the EL2 antibody 5 reacts against residues 225-238 (blue).
In order to delineate the $[^{125}\text{I}]$MFZ 2-24 irreversible incorporation profile on DAT, a tryptic peptide map was performed in parallel with $[^{125}\text{I}]$RTI 82 for direct comparison of their incorporation sites. Rat striatal membranes were photoaffinity labeled with $[^{125}\text{I}]$MFZ 2 24 and $[^{125}\text{I}]$RTI 82, and subjected to *in situ* trypsin proteolysis followed by immunoprecipitation with N-terminal antibody 16 and EL2 antibody 5, electrophoresis on a 9-16% gel and autoradiography (Figure 14). Cleavage of $[^{125}\text{I}]$MFZ 2-24 labeled DATs generated a prominent 45 kDa fragment that precipitates with antibody 16 and a very faint 32 kDa fragment that precipitates with antibody 5. These results indicate that incorporation of $[^{125}\text{I}]$MFZ 2-24 occurs N-terminal to R218, in a DAT domain containing TMs 1-3. In contrast, proteolysis of $[^{125}\text{I}]$RTI 82 labeled DATs produced the previously characterized 32 kDa fragment that is precipitated by antibody 5, and no detectable antibody 16 precipitable fragments (Figure 14).

$[^{125}\text{I}]$MFZ 2-24 labeled rat striatal membranes were treated with increasing concentrations of trypsin to more precisely localize the incorporation of the ligand into DAT. In addition to the 45 kDa fragment, a 16 kDa fragment that immunoprecipitated with antibody 16 was observed at high trypsin concentrations, consistent with cleavage at IL1 (Figure 15A). To validate the DAT origin of these fragments, photoaffinity labeling was performed in the presence of 10 mM (-)-cocaine, followed by tryptic proteolysis and immunoprecipitation with antibody 16 (Figure 15A). $[^{125}\text{I}]$MFZ 2-24 covalent labeling of the intact DAT, and the 45 kDa and 16 kDa fragments (lanes 1, 3, 5 and 7) was completely displaced.
Figure 14. $[^{125}\text{I}]$MFZ 2-24 and $[^{125}\text{I}]$RTI 82 Attach to Distinct Domains in DAT. Upper panel: Rat striatal membranes labeled with either $[^{125}\text{I}]$MFZ 2-24 or $[^{125}\text{I}]$RTI 82 were treated with or without 50 μg/mL trypsin and immunoprecipitated with antiserum 16 or 5, as indicated. Lower panel: Schematic diagram of rDAT showing predicted structure and membrane topology. Transmembrane (TM) domains are shown as cylinders, the trypsin cleavage site on EL2 (R218) is shown as a yellow circle, and antibody epitopes are indicated by colored bold lines with numbers. Arrows indicate relationship of autoradiograph fragments to domains in schematic DAT. Cleavage at R218 generated an N-terminal 45 kDa fragment labeled with $[^{125}\text{I}]$MFZ 2-24 that originated from TMs 1-3 (magenta), and a C-terminal 32 kDa fragment labeled with $[^{125}\text{I}]$RTI 82 that originated from TMs 4-12 (blue).
Figure 15. The Cocaine Analogue $[^{125}]$MFZ 2-24 Attaches to TMs 1-2 Domain in DAT. A. Rat striatal membranes were labeled with $[^{125}]$MFZ 2-24 in the presence (lanes 2, 4, 6, 8) or absence (lanes 1, 3, 5, 7) of 10 μM (-)-cocaine, treated with (lanes 3-8) or without (lanes 1-2) increasing concentrations of trypsin and subjected to immunoprecipitation with antiserum 16, followed by electrophoresis and autoradiography. Full-length DAT protein migrates at ~80 kDa (arrow a), and together with the 45- and 16-kDa N-terminal fragments (arrows b and c), they display cocaine displacement of labeling. B. $[^{125}]$MFZ 2-24 labeled rat striatal membranes were subjected to trypsin (100 μg/mL) proteolysis and immunoprecipitation with antibody 16 (lane 1), antibody 16 preabsorbed with peptide 16 (lane 2) and antibody 16 preabsorbed with peptide 5 (lane 3), demonstrating the immunological specificity of the precipitation of the 45- and 16-kDa N-terminal fragments. C. Schematic diagram of rDAT highlighting the incorporation domain of $[^{125}]$MFZ 2-24 (magenta). The trypsin sites that originate the labeled fragments, R123, K132 and R218, are shown as yellow circles and a colored bold line indicates antibody 16.
by cocaine (lanes 2, 4, 6 and 8), demonstrating that the origin of the fragments resides within the cocaine binding site in DAT.

Additionally, the immunological specificity of the $[^{125}\text{I}]{\text{MFZ}}$ 2-24 labeled fragments was verified by preabsorbing antibody 16 with the immunizing peptide or the non-immunogenic peptide 5 before subjecting the samples to immunoprecipitation (Figure 15B). Trypsin treated labeled rat striatal membranes were immunoprecipitated with antibody 16 alone (lane 1), antibody 16 preabsorbed with peptide 16 (lane 2), and antibody 16 preabsorbed with peptide 5 (lane 3). Immunoprecipitation of the 45- and 16-kDa fragments with serum 16 (lane 1) was prevented by the presence of the antigenic peptide (lane 2) but not affected by the unrelated peptide 5 (lane 3).

These results indicate that the $[^{125}\text{I}]{\text{MFZ}}$ 2-24 labeled 45 kDa fragment obtained by trypsin proteolysis and immunoprecipitation originates from rDAT N-terminal to the EL2 residue R218 and includes TMs 1-3. More precise localization by tryptic treatment generates an additional 16 kDa fragment, consistent with cleavage at rDAT R123 or K125 and containing TMs 1-2 (Figure 15C). There is a possibility of labeled fragments smaller than 16 kDa being produced by the tryptic treatment, but the proteolytic loss of the antibody epitope did not allow their detection by immunoprecipitation. In contrast to the $[^{125}\text{I}]{\text{RTI}}$ 82 irreversible attachment to TMs 4-7, $[^{125}\text{I}]{\text{MFZ}}$ 2-24 becomes incorporated into the N-terminal TMs 1-2 region in DAT, indicating that differential placement of the phenyl-iodoazido arm in the cocaine pharmacophore results in distinct
incorporation patterns. This represents the first evidence for differential incorporation of photoaffinity labels carrying identical pharmacophore. In addition, these findings strongly support the hypothesis of three-dimensional proximity between the regions encompassing TMs 1-2 and TMs 4-7 in the tertiary structure of DAT, and directly implicate these domains in binding of the psychostimulant cocaine.

Peptide Mapping of the DAT $[^{125}\text{I}]$MFZ 2-24 Attachment Site by CNBr cleavage

In contrast to enzymatic proteolysis, chemical cleavage of proteins is directed to less abundant residues, and consequently results in fewer and larger fragments. One of the most commonly used agents for chemical proteolysis is cyanogen bromide (CNBr), which cleaves at the C-terminal side of unoxidized methionine residues. This digestion occurs with high specificity, few side reactions, and average yields of 90-100% [120,121]. The rat and human isoforms of DAT contain a relatively low number of methionine residues, making them an ideal target for the use of this method. The 13 methionines present in hDAT are located at positions 1 and 11 within the N-terminal tail; 106, 111, and 116 in TM2; 272 in TM5; 371 in EL4; 414, 424, and 427 in TM8; 511 in IL5; and 569 and 571 in TM 12 (Figure 16, black circles). rDAT contains 2 additional methionines at positions 173 in EL2 and 290 in EL3 (Figure 16, grey circles).

Within the TMs 1-2 domain in hDAT and rDAT there are five methionines that represent potential CNBr cleavage sites. In order to characterize the CNBr cleavage profile of $[^{125}\text{I}]$MFZ 2-24-labeled DATs, HEK 293 cells expressing a 6x-Histidine tagged hDAT and LLCPK1 cells expressing rDAT were utilized. This
Figure 16. Methionine Residues in DAT. Schematic diagram of hDAT showing the TM domains (grey cylinders), the N-glycosylation sites in EL2 (branched structures), the position of epitope 16 (highlighted in magenta), the 13 endogenous methionines (black circles), and positions of two additional methionines present in rDAT (grey circles). Residues pertinent to these studies have been selectively numbered (rDAT/hDAT).
chemical fragmentation reaction requires the protein to be isolated, lacking salts and SDS. Thus, a thorough purification procedure was performed in order to have suitable samples for this reaction. For the first step, $[^{125}]$MFZ 2-24 labeled rDAT and hDAT cell lysates were gel purified through SDS-PAGE. The radioactive band located between the 66- and the 97- kDa markers was excised, rehydrated and subjected to a second purification step by electroelution in order to remove DAT from the polyacrylamide gel matrix. Electroeluates were dialyzed against MilliQ-filtered distilled water (MilliQ H$_2$O) to remove salts and SDS, and the final aqueous sample containing isolated, denatured DATs was evaporated to dryness. Labeled rDAT and hDAT samples were treated with or without 1M CNBr in 70% formic acid for 24 h in the dark, washed twice and evaporated to dryness, followed by SDS-PAGE analysis (Figure 17A). In the absence of CNBr, DATs from rat and human origin display their original migration profile at ~80 kDa (lanes 1 and 3), while in the presence of CNBr a labeled fragment at ~12 kDa is stoichiometrically produced from both isoforms (lanes 2 and 4). This fragment is consistent with cleavage at one or more of the TM2 methionines (M106, M111 and M116) and labeling occurring N-terminal to the cleaved residue (Figure 17B). The next potential CNBr site in rDAT is located at position 173 in EL2, and if the ligand were attached C-terminal to M116 in rDAT, the labeled fragment would be ~6 kDa in size; for hDAT the next potential CNBr methionine site is in TM5, at position 272, and the size of the labeled fragment would be ~40 kDa (including ~25 kDa of glycosylation present in EL2). Thus, the presence of the 12 kDa
Figure 17. CNBr Hydrolysis of $^{[125\text{I}]}$MFZ 2-24 Labeled rDAT and hDAT. A. hDAT-HEK 293 and rDAT-LLCPK₁ expressing cells were labeled with $^{[125\text{I}]}$MFZ 2-24 and lysates subjected to purification by SDS-PAGE on a 10% gel. The band located between the 66- and the 97-kDa markers was excised, DATs were extracted by electrophoresis and subsequently dialyzed against water, followed by evaporation to dryness. Dried DATs were solubilized in 70% formic acid and treated with (lanes 2 and 4) and without (lanes 1 and 3) 1M CNBr for 24 h in the dark, followed by SDS-PAGE and autoradiography on an 18% gel. B. Schematic diagram of DAT highlighting the TMs 1-2 domain labeled by $^{[125\text{I}]}$MFZ 2-24 N-terminal to M116 (magenta). Black circles indicate the 13 methionine residues common to hDAT and rDAT, and grey circles indicate the positions of the additional 2 methionines present in rDAT.
fragment in both rDAT and hDAT provides additional evidence for the attachment
of [$^{125}$I]MFZ 2-24 in the TMs 1-2 domain, N-terminal to M116 (Figure 17B).

Site-directed Mutagenesis, CNBr and Antibody 16
Analysis of [$^{125}$I]MFZ 2-24 Labeled hDATs

The CNBr approach proved to be useful in order to determine that
[$^{125}$I]MFZ 2-24 attaches to DAT in a domain located N-terminal to M116, which
includes TM1 and almost all of TM2 (Figure 17B). If the incorporation site lies
anywhere in the short stretch of amino acids that separates the three
methionines in TM2, the resolution of the peptide mapping strategy does not
allow visualization of such a small fragment (<1 kDa). Thus a strategy involving
site-directed mutagenesis in conjunction with photoaffinity labeling and CNBr
peptide mapping, was implemented in order to more precisely determine the
specific [$^{125}$I]MFZ 2-24 incorporation domain in DAT. Using this method two
hDAT mutants were generated, one containing a methionine to alanine mutation
at position 116 (M116A) and another containing simultaneous methionine to
leucine mutations at positions 111 and 116 (M111L/M116L). hDAT expression
levels in these mutants were analyzed by western blotting (Figure 18-upper
panel) and their [$^{125}$I]MFZ 2-24 photoaffinity labeling pattern (Figure 18-lower
panel) was proportional to their expression levels. In addition, their functional
properties were assessed and they exhibited robust cocaine-blockable [$^3$H]DA
transport and whole cell [$^3$H]CFT binding (Table 4).

For these studies hDAT WT and TM2 mutants were labeled with [$^{125}$I]MFZ
2-24 in the presence and absence of cocaine, subsequently gel purified and
subjected to CNBr digestion, followed by immunoprecipitation with N-terminal
Figure 18. Expression and Photolabeling of TM1/TM2 Methionine Mutants. HEK 293 cells stably transfected with hDAT plasmids carrying the indicated mutations were photoaffinity labeled with $^{125}$I-MFZ 2-24, solubilized in 1% Triton buffer and assayed for total protein content. Equal amounts of solubilized protein were separated by SDS-PAGE in a 4-20% gel, transferred to a 0.2 μm PVDF membrane and blotted with monoclonal anti-hDAT MAB 369 (upper panel). The blotted membrane was air-dried and subjected to autoradiography in order to visualize the extent of photoaffinity labeling (lower panel).
Table 4. Kinetic Properties of wild-type and TM1/TM2 Methionine Mutant hDATs.
Data are presented as mean ± S.E. (n=3). Values were statistically analyzed using one-way ANOVA with Tukey's Multiple Comparison Test.
<table>
<thead>
<tr>
<th>hDAT form</th>
<th>DA uptake</th>
<th>[3H]CFT binding</th>
<th>Cocaine DA uptake</th>
<th>IC_{50} [3H]CFT binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol min^{-1} mg^{-1}</td>
<td>pmol min^{-1}</td>
<td>nM</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>236.9 ± 13.2</td>
<td>2.54 ± 0.63</td>
<td>866.6 ± 102.7</td>
<td>169.5 ± 38.8</td>
</tr>
<tr>
<td>M116A</td>
<td>146.2 ± 14.1</td>
<td>0.95 ± 0.19</td>
<td>644.5 ± 95.1</td>
<td>361.6 ± 15.4^{b}</td>
</tr>
<tr>
<td>M111L/M116L</td>
<td>404.7 ± 41.4^{a}</td>
<td>3.71 ± 0.56</td>
<td>607.4 ± 76.1</td>
<td>122.6 ± 11.4</td>
</tr>
<tr>
<td>L80M/M111L/M116L</td>
<td>47.5 ± 2.9^{b}</td>
<td>2.38 ± 0.69</td>
<td>144.6 ± 21.3^{a}</td>
<td>104.4 ± 10.7</td>
</tr>
<tr>
<td>I67M/M111L/M116L</td>
<td>173.6 ± 12.9</td>
<td>2.53 ± 0.15</td>
<td>705.2 ± 147.4</td>
<td>129.1 ± 15.2</td>
</tr>
</tbody>
</table>

^{a} p < 0.01, relative to WT values
^{b} p < 0.001, relative to WT values
antibody 16, SDS-PAGE and autoradiography. Figure 19 shows the untreated (70% formic acid only) samples (odd-numbered lanes) and the CNBr digestion (even-numbered lanes) products. Cleavage of the wild type (WT) protein generates a cocaine-displaceable labeled fragment that migrates at ~12 kDa as previously demonstrated (lanes 2 and 4). Digestion of M116A and M111L/M116L hDATs originates cocaine-displaceable labeled fragments with migration patterns essentially identical to the ones obtained for WT hDAT (Figure 19, lanes 6, 8, 10, 12). In addition, all the fragments are immunoprecipitated by antibody 16, indicating the presence of the N-terminal epitope. These results verify that $[^{125}\text{I}]$MFZ 2-24 attachment occurs N-terminal to M106 (shaded domains in lower panels) in hDAT. If the attachment would occur C-terminal to either M111 or M106, the labeled CNBr fragment obtained from the M116A and the M111L/M116L mutants would contain residues between M111-M272 (~42 kDa) and M106-M272 (~43 kDa) respectively, and would be separated from the N-terminal epitope. These findings provided additional detailed information regarding the attachment site of $[^{125}\text{I}]$MFZ 2-24 in DAT and further validated the CNBr mapping strategy, strengthened by the concomitant use with site-directed mutagenesis and immunoprecipitation. The elimination of potential CNBr cleavage sites allowed for the identification of TM1 and the extracellular-facing portion of TM2 as the DAT $[^{125}\text{I}]$MFZ 2-24 labeled region.

Localization of TM1 as the $[^{125}\text{I}]$MFZ 2-24 binding site in DAT

The use of site-directed mutagenesis combined with CNBr digestion and immunoprecipitation is a powerful approach to increase the existing knowledge of
Figure 19. \([^{125}\text{I}]\text{MFZ 2-24}\) Attaches to TMs 1-2 N-terminal to M106. hDAT HEK 293 cells expressing WT, M116A and M111L/M116L protein were labeled with \([^{125}\text{I}]\text{MFZ 2-24}\) in the presence or absence of cocaine and subjected to purification by SDS-PAGE on a 10% gel. The band located between the 66- and the 97 kDa markers was excised, and DATs were extracted by electroelution and dialyzed against water, followed by evaporation to dryness. Dried DATs were solubilized in 70% formic acid and treated with (lanes 2, 4, 6, 8, 10 and 12) and without (lanes 1, 3, 5, 7, 9 and 11) 1M CNBr for 24 h in the dark at room temperature. Following digestion, samples were immunoprecipitated with antibody 16 and subjected to SDS-PAGE on an 18% gel followed by autoradiography. Schematic diagrams of each protein indicate the positions of the methionine residues present in them as black circles, with the region of the protein corresponding to the labeled fragments shown as magenta shading.
the incorporation domain in DAT for the cocaine analogue [\(^{125}\text{I}\)]MFZ 2-24. To more precisely determine the exact domain to which this compound becomes attached in the TMs 1-2 region in DAT, a series of methionine residues were engineered at positions that would allow the production of distinctive fragments upon CNBr cleavage. Using site-directed mutagenesis the leucine at position 80 and the isoleucine at position 67 were individually replaced by methionine in the hDAT M111L/M116L mutant such that there would be only one methionine (M106) remaining in TM2 and the exogenous methionines would be located in TM1 (L80M/M111L/M116L) and the N-terminal tail respectively (I67M/M111L/M116L). These mutants expressed hDAT protein and incorporated [\(^{125}\text{I}\)]MFZ 2-24 at levels comparable to the WT protein, as demonstrated by western blot analysis and photolabeling (Figure 18). Additionally, their functional properties were assessed by determining the IC\(_{50}\) for cocaine inhibition of [\(^3\text{H}\)]DA transport and whole cell [\(^3\text{H}\)]CFT binding (Table 4).

For these studies WT and TM1/TM2 hDAT mutants (M111L/M116L, L80M/M111L/M116L and I67M/M111L/M116L) were photoaffinity labeled with [\(^{125}\text{I}\)]MFZ 2-24, gel purified and digested with CNBr, followed by immunoprecipitation with N-terminal antibody 16, SDS-PAGE and autoradiography. Results in Figure 20 show the untreated (70% formic acid only) samples (odd-numbered lanes) and the CNBr digestion (even-numbered lanes) products. Cleavage of WT and M111L/M116L hDATs generates the previously characterized ~12 kDa fragment that contains epitope 16 (lanes 2 and 4). Importantly, the CNBr digestion product of L80M/M111L/M116L displays faster
Figure 20. $^{[125\text{I}]}$MFZ 2-24 Attaches to TM 1 Between Residues 67 and 80. hDAT-HEK 293 cells expressing WT, M111L/M116L, L80M/M111L/M116L and I67M/M111L/M116L protein were labeled with $^{[125\text{I}]}$MFZ 2-24 and subjected to purification by SDS-PAGE on a 10% gel. The band located between the 66- and the 97-kDa markers was excised, DATs were extracted by electroelution and dialyzed against water, followed by evaporation to dryness. Dried DATs were solubilized in 70% formic acid and treated with (lanes 2, 4, 6 and 8) and without (lanes 1, 3, 5 and 7) 1M CNBr for 24 h in the dark at room temperature. Following digestion, samples were immunoprecipitated with antibody 16 and subjected to SDS-PAGE on a 20% gel followed by autoradiography. Schematic diagrams of each protein denote the positions of the endogenous and engineered methionines as black circles, with the region of the protein corresponding to the labeled fragments shown as magenta shading.
electrophoretic mobility than the fragments from WT or M111L/M116L proteins and migrates at ~9 kDa (lane 6). In addition, this fragment is precipitated with antibody 16. This indicates that the labeling occurs N-terminal to L80M (shaded region, lower panel) because if the ligand attachment occurred C-terminal to this residue the CNBr fragment would extend from L80M to M106 (~3 kDa), would not contain the N-terminal epitope, and thus would not immunoprecipitate. The next mutant analyzed, I67M/M111L/M116L, produced no precipitable CNBr fragment (lane 8). This represents evidence of labeling occurring C-terminal to I67M and separation of the N-terminal epitope from the labeled TM1 region (unshaded region, lower panel). If the label were incorporated in the cytoplasmic N-terminal tail, the fragment would contain the entire tail (~7 kDa) including epitope 16. These results indicate that a 13 amino acid stretch of TM1, between residues 67 and 80, is the site of covalent incorporation in DAT for the cocaine analogue [125I]MFZ 2-24.

Photoaffinity Labeling of SERT with [125I]MFZ 2-24 and [125]RTI 82

The transporter for the neurotransmitter serotonin (5-HT), SERT, belongs to the NSSs family and shares high homology with DAT and NET. SERT is a well-known target for treatment of mood disorders, such as anxiety, depression, and post-traumatic stress. Several classes of compounds such as cocaine, the tricyclic antidepressants and the serotonin-specific reuptake inhibitors (SSRIs) block SERT and inhibit its uptake activity [reviewed in 41]. The cocaine analogues [125I]MFZ 2-24 and [125]RTI 82 were used to photolabel hSERT expressing HEK 293 cells in parallel with rDAT expressing LLCPK1 cells (Figure
Figure 21. $[^{125}\text{I}]{\text{MFZ}}$ 2-24 and $[^{125}\text{I}]{\text{RTI}}$ 82 Label SERT. A. rDAT LLCPK$_{1}$ cells and hSERT HEK 293 cells were subjected to photolabeling with $[^{125}\text{I}]{\text{MFZ}}$ 2-24 and $[^{125}\text{I}]{\text{RTI}}$ 82, followed by immunoprecipitation with antibody 16 (against amino acids 42-59 in rDAT) or antibody 48 (against amino acids 605-630 in hSERT). B. Photoaffinity labeling of hSERT with $[^{125}\text{I}]{\text{MFZ}}$ 2-24 displays appropriate pharmacological specificity. hSERT HEK 293 cells were photolabeled with $[^{125}\text{I}]{\text{MFZ}}$ 2-24 in the presence of the indicated compounds. Photolabeled lysates were immunoprecipitated with antibody 48 and subjected to analysis by SDS-PAGE and autoradiography [122].
Labeled bands at 70–80 kDa were visible in lysates from both cell lines. Immunoprecipitation of lysates with the corresponding anti-DAT (antibody 16) or anti-SERT (antibody 48) antibodies specifically pulled down radiolabeled proteins (Figure 21A). The pharmacological specificity of $[^{125}\text{I}]\text{MFZ 2-24}$ incorporation into SERT was evaluated in the presence of 5-HT reuptake inhibitors followed by epitope-specific immunoprecipitation, SDS-PAGE and autoradiography. Covalent labeling of SERT was prevented by the SERT blockers (−)-cocaine, citalopram, imipramine, and fluoxetine, but not by (+)-cocaine, the DAT inhibitor GBR 12909 or the NET blocker nisoxetine (Figure 21B) [122]. These results represent the first time a photoaffinity approach has been applied for SERT and further demonstrate that SERT becomes effectively labeled by $[^{125}\text{I}]\text{MFZ 2-24}$ and $[^{125}\text{I}]\text{RTI 82}$. This strongly suggests that the irreversible incorporation sites on DAT and SERT for cocaine analogues are likely to be similar.

Pharmacology of $[^{125}\text{I}]\text{JHC 2-48}$ Incorporation to DAT and SERT

The cocaine analogue $[^{125}\text{I}]\text{JHC 2-48}$ is a novel photoaffinity ligand that contains the cocaine pharmacophore structures present in both $[^{125}\text{I}]\text{RTI 82}$ and $[^{125}\text{I}]\text{MFZ 2-24}$, with the phenyl-iodoazido reactive group appended to the cocaine phenyl ring (Figure 11). Synthesis and radioiodination of JHC 2-48 were recently described, and evaluation of its kinetic properties revealed high affinity for both DAT and SERT [118].

Irreversible incorporation of $[^{125}\text{I}]\text{JHC 2-48}$ into hDAT and hSERT was analyzed in the presence of cocaine in order to determine the pharmacological specificity of the photolabel for these transporters. hDAT or hSERT expressing
Figure 22. $[^{125}\mathrm{I}]$JHC 2-48 Photoaffinity Labeling of hDAT and hSERT Displays Pharmacological Specificity. HEK 293 cells expressing hDAT or hSERT were photolabeled with $[^{125}\mathrm{I}]$JHC 2-48 in the presence or absence of (−)-cocaine and cells were solubilized. Lysates were subjected to immunoprecipitation with hDAT and hSERT specific antibodies, followed by SDS-PAGE on a 10% gel and autoradiography [118].
cells were photolabeled with $[^{125}\text{I}]$JHC 2-48 in the presence or absence of (-)-cocaine followed by immunoprecipitation, SDS-PAGE and autoradiography (Figure 22). Labeling was prevented when binding was carried out in the presence of (-)-cocaine, demonstrating $[^{125}\text{I}]$JHC 2-48 incorporation at a pharmacologically significant site in both DAT and SERT.

**CNBr Analysis of $[^{125}\text{I}]$JHC 2-48 Labeling in DAT**

The use of CNBr cleavage for the analysis of DAT proteins photoaffinity labeled with cocaine analogues has been demonstrated to be a powerful strategy to delineate the attachment domains of these compounds [114]. In order to initially analyze the incorporation pattern of $[^{125}\text{I}]$JHC 2-48 in DAT, HEK 293 cells expressing hDAT were photolabeled with $[^{125}\text{I}]$JHC 2-48, $[^{125}\text{I}]$MFZ 2-24, and $[^{125}\text{I}]$RTI 82 for comparison purposes. Labeled proteins were gel purified, digested with CNBr, and analyzed by SDS-PAGE followed by autoradiography (Figure 23). Cleavage of $[^{125}\text{I}]$RTI 82 labeled DATs produces the previously characterized 6 kDa fragment containing TM6 (lane 2). Similarly, digestion of $[^{125}\text{I}]$MFZ 2-24 labeled protein generates the 12 kDa fragment containing TM1 (lane 4). Interestingly, the digestion product of $[^{125}\text{I}]$JHC 2-48 labeled DATs exhibits a pattern distinct from that of the related cocaine analogues (lane 6), with absence of any distinguishable labeled fragment, suggesting that this ligand becomes incorporated into a region in DAT that has not been previously characterized. If TM3 was the attachment site of $[^{125}\text{I}]$JHC 2-48 a ~45 kDa labeled fragment originating from cleavage between M116 and M272 should be present in the SDS-PAGE analysis of the CNBr fragments, but the absence of
Figure 23. $[^{125}\text{I}]$JHC 2-48 Incorporates to DAT Distinctively from $[^{125}\text{I}]$RTI 82 and $[^{125}\text{I}]$MFZ 2-24. hDAT expressing HEK 293 cells labeled with $[^{125}\text{I}]$JHC 2-48, $[^{125}\text{I}]$RTI 82, and $[^{125}\text{I}]$MFZ 2-24 were subjected to purification by SDS-PAGE on a 10% gel. The band located between the 66- and the 97-kDa markers was excised, DATs were extracted by electroelution and dialyzed against water, followed by evaporation to dryness. Dried DATs were solubilized in 70% formic acid and treated with (lanes 2, 4 and 6) and without (lanes 1, 3 and 5) 1M CNBr for 24 h in the dark at room temperature, followed by SDS-PAGE on an 18% gel, and autoradiography.
any discernable labeled fragment in the CNBr map eliminates this possibility. On the other hand, there are several methionine residues in the C-terminal half of DAT at positions 414, 424 and 427 in TM8, and at positions 569 and 571 in TM12 (Figure 16). \[^{125}\text{I}]\text{JHC 2-48}\) attachment to a residue within the methionines in any of these domains would generate CNBr fragments of 1 kDa or less that would not be resolved by the SDS-PAGE method and are consistent with the absence of labeled fragments in the initial mapping (Figure 23).

Functional properties of D-147

The GBR series of compounds were originally developed in the early 1980s and shown to possess high selectivity and affinity for DAT. GBR 12909 selectively inhibits DA uptake with an IC\textsubscript{50} of ~1 nM, and is less potent at NET and SERT. This compound exhibits unique neuropharmacological properties, including slow dissociation from DAT and the ability to block cocaine self-administration in non-human primates [123,124]. One of the first photoaffinity labels developed and used to visualize and characterize DA uptake sites was the GBR derivative \[^{125}\text{I}]\text{DEEP}\) (Figure 24) [125-127]. Soon after DAT was cloned, \[^{125}\text{I}]\text{DEEP}\) was used to map GBR binding sites and its attachment was localized to the TMs 1-2 domain [110,111].

The photoaffinity label \[^{125}\text{I}]\text{D-147}\) is an aryl-dialkylpiperazine compound whose structure derives from GBR 12909. \[^{125}\text{I}]\text{D-147}\) is structurally identical to \[^{125}\text{I}]\text{DEEP}, except for the position of the phenyl-iodoazido group with respect to the piperazine pharmacophore (Figure 24). Synthesis of D-147 was performed by
Figure 24. Chemical Structures of GBR-Based Photoaffinity Labels. The structure of GBR 12909, an aryl-dialkylpiperazine dopamine uptake blocker, is shown with its derivative photoaffinity labels. $^{[125]}\text{I}$DEEP is a piperazine-based photoaffinity ligand whose site of incorporation has been identified in TMs 1-2, $^{[125]}\text{I}$D-147 is the piperazine analogue being characterized in these studies, with a structure identical to $^{[125]}\text{I}$DEEP except that the reactive iodo-azido group is placed on the biphenyl moiety at the opposite end of the molecule. $^{[125]}\text{I}$AD-96 is a piperidine analogue, derived from GBR structures and previously characterized.
Figure 25. D-147 Binds to DAT with High Affinity. Rat striatal membranes were prepared at 10 mg/mL in SP buffer. To initiate the binding competition assay, triplicate membrane samples were added to tubes containing $10^{-10}$-$10^{-5}$ M concentrations of D-147 and 1 nM $[^3]$H]CFT, followed by a 2 h incubation on ice. Membranes were collected and washed three times using a tissue harvester over glass fiber filters. Bound radioactivity was quantified by liquid scintillation. The IC$_{50}$ value was determined and graphed using non-linear regression analysis with Prism 3.0 software.
$[^3]H$CFT binding,
% Control

D-147, M

IC$_{50}$ = 25.9 ± 2.5 nM
Dr. Aloke Dutta (Wayne State University, MI) and the IC₅₀ value for displacement of [³H]CFT binding in rat striatum was found to be 26 nM (Figure 25).

Pharmacology of [¹²⁵I]D-147 incorporation to DAT

Irreversible incorporation of [¹²⁵I]D-147 to rat striatal DATs was assessed in the presence of several structurally diverse compounds and the physiological substrate DA, in order to determine its pharmacological specificity for DAT. Photolabeling was prevented when binding was carried out in the presence of DA or the DA uptake inhibitors (-)-cocaine, mazindol, GBR 12909 and nomifensine, but was not modified by the inactive enantiomer (+)-cocaine, the NET blocker desipramine or the SERT blocker imipramine (Figure 26). These results demonstrate that [¹²⁵I]D-147 covalently attaches to DAT and that the compounds used in these studies are competitive inhibitors and prevent the reversible incorporation of the ligand to the transporter. These results support the assumption that the irreversible incorporation site for uptake blockers is located in or near a pharmacologically significant domain in DAT.

Peptide Mapping of the DAT [¹²⁵I]D-147 Incorporation Site by Trypsin Proteolysis

In order to localize the irreversible incorporation site of [¹²⁵I]D-147 on DAT, a tryptic peptide map was performed. [¹²⁵I]D-147 was used to photoaffinity label rat striatal membranes, which were subjected to in situ trypsin proteolysis followed by immunoprecipitation with N-terminal antibody 16 and EL2 antibody 5, SDS-PAGE separation on a 9-16% gel, and autoradiography (Figure 27). Trypsin digestion of [¹²⁵I]D-147 labeled DATs resulted in a 45 kDa fragment and an
Figure 26. $[^{125}\text{I}]$D-147 Photoaffinity Labeling Displays DAT Pharmacological Specificity. Rat striatal membranes were photolabeled with $[^{125}\text{I}]$D-147 in the presence of the indicated compounds [10 μM final concentrations, except imipramine (1 μM) and dopamine (100 μM)]. After labeling, samples were immunoprecipitated with antibody 16 and subjected to analysis by SDS-PAGE and autoradiography.
Figure 27. The GBR Analogue $^{[125]}$I-D-147 Attaches to Distinct Domains in DAT.
A. Rat striatal membranes were labeled with $^{[125]}$I-D-147, treated with (lanes 2-4 and 6-8) or without (lanes 1 and 5) increasing concentrations of trypsin and subjected to immunoprecipitation with antiserum 16 or antiserum 5, followed by electrophoresis on a 9-16% gradient gel and autoradiography. Arrows a, b, c and d indicate the positions of the $^{[125]}$I-D-147 labeled fragments.
additional 16 kDa fragment that immunoprecipitate with antibody 16 (lanes 2-4, arrows a and b), consistent with incorporation at the TM 1-2 region. Interestingly, precipitation of the same trypsin-digested $[^{125}\text{I}]D$-147 labeled sample with antibody 5 isolated a 32 kDa fragment with an additional ~16 kDa fragment (lanes 6-8, arrows c and d) consistent with attachment to the TM 4-6 domain.

Additionally, the immunological specificity of the $[^{125}\text{I}]D$-147 labeled fragments was verified by preabsorbing the antibodies with their respective immunizing peptides or non-immunogenic peptides before subjecting the samples to immunoprecipitation (Figure 28). Trypsin treated labeled rat striatal membranes were immunoprecipitated with antibody 16 alone or antibody 5 alone, antibody 16 preabsorbed with peptide 16 or antibody 5 preabsorbed with peptide 5, and antibody 16 preabsorbed with peptide 5 or antibody 5 preabsorbed by antibody 16, as indicated. Immunoprecipitation of the 45 kDa fragment with serum 16 was prevented by the presence of the antigenic peptide but not affected by an unrelated peptide. Precipitation of the 32 kDa fragment by antibody 5 was prevented in the presence of the immunizing peptide but remained unmodified by an irrelevant peptide.

The relative amount of $[^{125}\text{I}]D$-147 incorporation into DAT appeared to be larger for the TM 4-6 region than for the TM 1-2 region, as estimated from the intensities of the respective 32- and 45-kDa fragments, compared to the full-length protein. This suggests an apparently increased incorporation of $[^{125}\text{I}]D$-147 into the C-terminal half of DAT, similar to $[^{125}\text{I}]RTI$ 82.
Figure 28. Specificity of Immunoprecipitation of $^{125}$ID-147 Labeled Fragments. $^{125}$ID-147 labeled rat striatal membranes were subjected to trypsin proteolysis and immunoprecipitation with antibody 16 or antibody 5. During the immunoprecipitation procedure, samples were supplemented with no peptide, 50 μg/mL peptide 16 or 50 μg/mL peptide 5, as indicated (upper panel). Schematic diagram of rDAT showing predicted structure and membrane topology (lower panel). Transmembrane (TM) domains are shown as cylinders, the trypsin cleavage site on EL2 (R218) is shown as a yellow circle, and antibody epitopes are indicated by colored bold lines with numbers. Arrows indicate relationship of autoradiograph fragments to domains in schematic DAT. Cleavage of $^{125}$ID-147 labeled DATs at R218 generated a N-terminal 45 kDa fragment that originated from TMs 1-3 (magenta), and a C-terminal 32 kDa fragment that generated at TMs 4-12 (blue).
The present results indicate that the $^{[125]}$D-147 labeled 45 kDa fragment obtained by peptide mapping originates from rDAT N-terminal to the EL2 residue R218 and includes TMs 1-3, and the 32 kDa precipitated tryptic fragment is derived from rDAT C-terminal to R218 and contains TMs 4-12 (Figure 28). The presence of smaller ~16 kDa labeled fragments that precipitate with both antibody 16 and antibody 5 indicate that $^{[125]}$D-147 becomes incorporated into both the TMs 1-2 region and the TMs 4-7 region. Thus, in contrast to the closely related GBR-like ligand $^{[125]}$DEEP that incorporates almost exclusively into TMs 1-2, the differential positioning of the reactive iodo-azido group in $^{[125]}$D-147 generates a distinct incorporation pattern.
CHAPTER IV

DISCUSSION

The plasma membrane is an essential component of the cell and functions to surround it, determining its boundaries and separating it from the outside environment [128]. The membrane consists of protein molecules embedded in a thin double layer of lipids. The protein composition varies in different cell types according to their specific functional properties and provides them with exclusive characteristics. Transmembrane proteins, including DAT, traverse the lipid bilayer and contain portions of their structure on both sides of the membrane. They possess amphipathic characteristics, with their hydrophobic regions interacting with the lipids and their hydrophilic domains in contact with water at both sides of the membrane. Due to their amphipathic structure, common methods to study protein tertiary structure such as x-ray crystallography and nuclear magnetic resonance (NMR), are extremely complex to execute and analyze for these transmembrane proteins. Thus, more indirect biochemical techniques have been applied in order to describe DAT’s structural conformation, molecular mechanisms, and active sites.

Photoaffinity labeling was initially utilized to visualize dopamine transport sites in mammalian tissue before DAT became cloned and characterized [125,126]. Additionally, the GBR analogue $[^{125}]$DEEP and the cocaine analogue $[^{125}]$RTI 82 were demonstrated to label the same protein in rat striatal
preparations, with identical pharmacological profiles [127]. After the cloning of DATs in the early 1990s, photoaffinity labeling in combination with peptide mapping and epitope-specific immunoprecipitation was used in order to localize the binding sites for a variety of structurally diverse DAT inhibitors. 

$[^{125}\text{I}]\text{DEEP}$ and $[^{125}\text{I}]\text{RTI 82}$ were found to attach to TMs 1-2 and TMs 4-7 respectively, suggesting the possibility of distinct incorporation sites for different classes of uptake blockers [110,111]. Soon after, the incorporation site of the benztropine analogue $[^{125}\text{I}]\text{GA 2-34}$ was mapped to the TMs 1-2 region, and the GBR analogue $[^{125}\text{I}]\text{AD-96}$ was demonstrated to attach to both the TMs 1-2 and the TMs 4-7 domains [112,113]. The fact that incorporation of numerous structurally diverse photoaffinity labels occurs in the same domains suggests that these uptake blockers bind to a common pocket on DAT, composed at least partially of TMs 1-2 and 4-7, and further that they inhibit transport by a similar mechanism, supporting the hypothesis of three-dimensional proximity between the regions encompassing TMs 1-2 and TMs 4-7 in the tertiary structure of DAT.

**TM1 is a Determinant for Cocaine Binding in DAT**

In this study, using photoaffinity labeling in combination with enzymatic and chemical proteolysis, epitope-specific immunoprecipitation, and site-directed mutagenesis, the incorporation site on DAT for the novel cocaine analogue $[^{125}\text{I}]\text{MFZ 2-24}$ was localized to TM1, more specifically to a domain containing residues 67 to 80. This represents the first time that the use of photolabeling has allowed the positive identification of such a small number of residues as the binding site for a cocaine analogue in DAT. The finding that irreversible
incorporation of $^{[125]}$I-MFZ 2-24 occurs in TM1 strongly suggests that the reversible binding site for cocaine is located within this domain, somewhere between residues I67 and L80.

The DAT region encompassing TM1 has been extensively studied through a variety of approaches due to its high degree of conservation among related transporters, and has been implicated in substrate and inhibitor binding through several lines of evidence. Studies using DAT-NET chimeras suggested interaction of TM1 with cocaine as demonstrated by lower cocaine affinity in constructs whose junction points lie in or near this domain [93].

The chemical structure of DA and other DAT substrates, with the phenethylamine group positively charged at physiological pH, have led the search for charged, aromatic and polar residues as potential targets for mutagenesis. TM1 in DAT harbors D79, the first amino acid shown to be essential for DAT function. Substitution of this residue to alanine, glycine or glutamic acid results in profound losses in DA and cocaine analogue affinities [96]. It has been proposed that the carboxyl group of D79 is essential for interaction with the positively charged amine from DA, and possibly with the tropane nitrogen in cocaine. Importantly, this residue is conserved within the related monoamine transporters NET (D75) and SERT (D98), and similar effects on substrate uptake have been observed upon amino acid substitution at these positions [129]. Mutation of rDAT F76, located one helical turn below D79, to alanine results in extensive loss of affinity for CFT binding to DAT [97]. The analogous Y95 in SERT and F72 in NET exhibit reduced substrate and inhibitor
binding affinities, highlighting the functional importance of this position throughout the monoamine transporter family [130,131]. Conversely, mutation of W84 to alanine in rDAT and leucine in hDAT leads to increased affinity for the cocaine analogue CFT [98,132]. Additionally, ionic dependence studies of the W84L mutant in hDAT revealed that this mutation contributes to stabilizing the transporter on an outward facing conformation, with increased affinity for cocaine [98]. Substitution of D68 for asparagine in hDAT led to a significant loss in cocaine analogue affinity, but an even greater loss in affinity for a GBR analogue, implicating this residue in binding of different classes of inhibitors [133].

The findings from this study using photoaffinity labeling extend and complement previous studies that implicate TM1 in cocaine binding, and further provide a separate line of biochemical evidence that supports the involvement of this domain in the active site of DAT.

Relationship with Bacterial Leucine Transporter (LeuT\textsubscript{Aa})

The leucine transporter from \textit{Aquifex aeolicus} (LeuT\textsubscript{Aa}) is the first member of the Na\textsuperscript{+}/Cl\textsuperscript{−}-dependent family of transporters to be crystallized with exceptionally high resolution [69]. Although distantly related to DAT (only 20% homology) the TMs show high degree of similarity, and a large amount of biochemical evidence, including data from photoaffinity labeling studies, supports the assumption that DAT adopts a similar three-dimensional conformation. In LeuT\textsubscript{Aa} the binding site for leucine and Na\textsuperscript{+} is composed mainly of TMs 1, 6, 3 and 8. Interestingly, TMs 1 and 6 are not continuous helices but contain non-helical unwound regions that are in direct contact with both substrate and ions. In
TM1 leucine binding occurs to both sides of the unwound regions at residues corresponding to DAT A77, L80 and A81 whereas the Na⁺ ions are coordinated by the carboxyl group of leucine, and LeuTₐₐ A22, N27, G20 and V23, corresponding to DAT residues A77, N82, G75, and V78 (Figure 29). All the previously described DAT mutations involved in substrate and inhibitor binding (D79, F76, W84 and D68) are located within the boundaries of this essential region in TM1 and are highly conserved, highlighting their importance in the functional aspects of DAT active mechanism. The presence of a glycine in the unwound region of TM1 in LeuTₐₐ (G24) and the analogous position of its mammalian counterparts, except for the biogenic amine transporters that possess an aspartate, may be explained by the structure of the cognate substrates. An aspartate at this position may contribute a carboxyl group that replaces the one present in amino acid substrates, and could function as a Na⁺ coordination site in addition to directly interact with the amine from the substrates.

The findings from these studies indicate that the cocaine interaction with DAT occurs at residues located within this extremely important and highly conserved region, more specifically at a 13-residue stretch located N-terminal to the substrate contact point at L80. Although the site for irreversible incorporation of the cocaine analogue [¹²⁵I]MFZ 2-24 to DAT will not likely be identical to the residues that directly interact with the cocaine pharmacophore, measurements of the interatomic distances between the reactive phenyl-iodo azido group and the 118
Figure 29. Amino Acid Sequence Alignment of hDAT and LeuT<sub>Aa</sub>. The FASTA formatted sequences from LeuT<sub>Aa</sub> (NP_214423) and hDAT (Q01959) were aligned using the T-Coffee Multiple Sequence Alignment Tool [134], and manually adjusted according to Beuming et al [71]. Colored bars indicate TM domains, with amino acids from LeuT<sub>Aa</sub> highlighted in bold letters.
hDAT 1 MSKSKCSVGLMSSSVAPAKEPNAVGPKEVELILVRKQNVQLTSLTLTNRFSQPSVEAQedTW
LeuTAa -----------------------------------------------MEVKREHW

TM 1

hDAT 64 GKKIDFLLSVIGFAVDLANWRFPPYYLCYNGGGAGFLPVYLDLVMMVIAQMLFLYEMELALGQFNRE
LeuTAa 9 ATRLGLILAMAGNAVGGLGNFLRFPVQAENGGGAPMYIEIAFLVIGIPIMQWENAMGRYGGA

TM 2

hDAT 127 GAAGVWK-ICP------ILKVGGFTVVLILSYVGGFYVNIIAWALHLYFLFSTTEDELPHICCN
LeuTAa 72 QGHTTPAIFYLLWWRNFALIGVImGFLMFPYMEALGQFNRE

TM 3

hDAT 183 SWNSPNCSDAHPGDSGDDSSGGLNDFGTTPAEYFERGVHLHQSQGIDDGPPRQWQTLACLV
LeuTAa 155 T---------------DPDIILRFKEFLYSIYVPGKDEPLKPSFLAFIVFVL

TM 4

hDAT 246 LVIVLLYFLWKGVKT-SQKVVWVITATNPVYVLTALLLRGVTLP-----GAIDGIRAYLSVDFY
LeuTAa 218 ITMFINYSILIGSKGERFAKIAMPTFLILAVFLVIRVEFFILPNTQAAADQLNIFLWTPDFE

TM 5

hDAT 304 RLCEASVWRIATAQVCSLGVGFGVLIAFSSYNKFTNTNGYVRKQDIVENGTAATLNEKAEVILGGSISI
LeuTAa 267 KLDGFWIAAVQQIFPFTSLGFIAGAITYASYVRYKQDIVENGTAATLNEKAEVILGGSISI

TM 6

hDAT 367 FLGYMA--QKSHVIPGAVDGPGLIFIIYPEAIAATLPLLASSAWAVFVIMLLTGLDSAMGM
LeuTAa 329 PAAVAFGGVANAVAIAAKAG--AFNLGFIITLPAIFSGTAGGTFIYMLHFFLFFAGLTSIAIM

TM 7

hDAT 428 ESVITGLIDEFQLLLHRELFLFIATFLVLSSLFCVINGGIYVFELLHDFAAGTSILFGVLI
LeuTAa 391 QPMIAFLEDEKL--SRHKAVLWTAIAVFSAHLVIFL--NKSLDOMCFWACTIGVVFGLT

TM 8

hDAT 491 EAIGVAVFYVGQFSDDIQMTGQRPSLYWRLCWKLVSFCFLFVERYSVVSIIFTRPFHPHYGAYIF
LeuTAa 453 EIIFFWIFGADKAKVEINRGGIIKVPRIYYVQYITPAPWAVAVWHIREYIPKIMEETH-

TM 9

hDAT 554 PDWANALGWITASSAMVPIAYAKCFSLPQFREKLAYAIAPKDRRELVDGVRQFTLIRH
LeuTAa 516 --MIVNITFRFYIIGLFLFIFLFLVFAERRRRHEA----------------------------------

TM 10

hDAT 617 WLKV
LeuTAa ----
essential determinants of cocaine binding will allow more precise identification of potential crucial contact points in DAT (Figure 30).

The novel structure provided by the crystallization of this bacterial homologue represents a breakthrough in the structure/function studies of this family of transporters, and despite being distantly related, the high homology found within the TMs demonstrate its importance in serving as a template for modeling the mammalian homologues, and complementing the large amount of biochemical data that is consistent with this model. However, there are several structural aspects that are not shared between LeuT_{Aa} and its eukaryotic counterparts such as the size of the second extracellular loop that contains the glycosylation and disulfide bonds, and the length of the N- and C-terminal tails that participate in protein-protein interactions. Perhaps these modifications are part of the evolutionary changes that led to their specialized functional properties, ionic dependences, and substrate specificity. Additionally, LeuT_{Aa} was crystallized in a closed conformation, with leucine and ion sites occluded from the aqueous environment at both sides of the membrane. The extracellular access gate is formed by a small number of amino acids, but the cytoplasmic gate consists of highly packed protein structure, with participating residues from TMs 1,3,6,8 and 10. This stationary structure does not provide information about the mechanistic implications of substrate and ion movement across the membrane or the inhibition of its function by cocaine and other compounds, leaving this area open for future exploration.
Figure 30. Structures of Cocaine and $^{[\text{125}}\text{I}]$MFZ 2-24. A and B. Chemical structures of $^{[\text{125}}\text{I}]$MFZ 2-24 and cocaine. C. The interatomic distances in $^{[\text{125}}\text{I}]$MFZ 2-24 between the phenyl-odoazido arm and the tropane nitrogen or the phenyl ring are indicated as arrows and obtained from energy minimization calculations (see Materials and Methods).
distance in Angstroms

N - N₃  10.5
Ph - N₃  15.5
Photolabels with Identical Pharmacophore Exhibit Differential Incorporation

The irreversible attachment site for $[^{125}\text{I}]\text{RTI 82}$ in DAT has recently been mapped to TM6 [114]. $[^{125}\text{I}]\text{RTI 82}$ and $[^{125}\text{I}]\text{MFZ 2-24}$ are identical cocaine analogues that differ only in the position of the phenyl-iodoazido reactive group (Figure 31). Since these compounds share the cocaine pharmacophore with the crucial elements for cocaine binding, their reversible binding should be identical, with a distinct irreversible incorporation site. The studies presented here demonstrate that, in contrast to $[^{125}\text{I}]\text{RTI 82}$, $[^{125}\text{I}]\text{MFZ 2-24}$ attaches to TM1 in hDAT. This is the first time that photolabels harboring identical pharmacophores have been shown to exhibit distinct incorporation patterns. In addition, these results demonstrate physical proximity of TMs 1 and 6 in the three-dimensional arrangement of DAT and are consistent with DAT adopting a conformation similar to that of LeuT$_{Aa}$ (Figure 31B).

Furthermore, this study strongly supports the idea that multiple domains of the DAT primary structure form a binding pocket and are involved in direct interaction with cocaine. Although the exact amino acid to which $[^{125}\text{I}]\text{MFZ 2-24}$ becomes attached is not yet identified, this study indicates that incorporation occurs within only 13 amino acids in TM1, representing the shortest sequence ever identified by photoaffinity labeling techniques.

Analysis of the Incorporation of a Novel Cocaine Analogue to DAT

The incorporation pattern of the novel cocaine analogue $[^{125}\text{I}]\text{JHC 2-48}$, the third member of this family of tropane-based DAT inhibitors (Figure 11), was found to be different from that of $[^{125}\text{I}]\text{MFZ 2-24}$ and $[^{125}\text{I}]\text{RTI 82}$ in these
Figure 31. Photolabels with identical Pharmacophore Attach to Distinct Sites in DAT. A. Chemical structures of the cocaine analogues $[^{125}\text{I}]$MFZ 2-24 and $[^{125}\text{I}]$RTI 82. They possess identical cocaine pharmacophore but the phenyl-iodoazido reactive group is placed in a different position. Schematic diagram of DAT indicating the incorporation site for $[^{125}\text{I}]$MFZ 2-24 in TM1 between residues I67 and L80 (magenta) and the attachment site for $[^{125}\text{I}]$RTI 82 in TM6 between residues I291 and K336 [108] (blue). B. Schematic diagram of the proposed binding pocket for DAT according to LeuT$\alpha$ [69], showing TMs 1, 6, 3 and 8. Cocaine is placed within this pocket, and the attachment sites for $[^{125}\text{I}]$MFZ 2-24 (magenta) and $[^{125}\text{I}]$RTI 82 (blue) are indicated.
studies. More in-depth characterization, including site-directed mutagenesis, will be necessary in order to localize its attachment domain. However, it is tempting to speculate about the potential candidate incorporation domains for $[^{125}\text{I}]$JHC 2-48. Based on the positions of the 13 methionine residues in hDAT and the preliminary CNBr map of $[^{125}\text{I}]$JHC 2-48 labeled hDAT, the potential domains for incorporation of this compound are the regions encompassing TM8 and TM12. Three methionines are located at positions 414, 424 and 427 in TM8, and two other methionines are situated in TM12 at positions 569 and 572. If incorporation of the ligand occurred in a residue located in between the methionines in these domains, the resulting CNBr fragments would have masses of 1 kDa or less that could not be resolved by the SDS-PAGE method, and are consistent with the initial mapping results. In addition, TM8 residues S355 and I359 in LeuT$_{\text{Aa}}$ are in direct contact with the substrate leucine, while the homologous residues in hDAT correspond to S422 and G426 in TM8 and are located between M414 and M427. This implicates TM8 as a potential target for further investigation regarding the attachment site of $[^{125}\text{I}]$JHC 2-48. Furthermore, mutagenesis and chimeras studies have demonstrated the participation of TM8 in important DAT functions involving substrate and inhibitor binding [92, 93, 135]

$[^{125}\text{I}]$D-147 Incorporation Provides Additional Evidence for Proximity of Domains

The findings from the present studies localize the irreversible incorporation site on DAT for the novel GBR analogue $[^{125}\text{I}]$D-147. This compound is structurally identical to the previously characterized $[^{125}\text{I}]$DEEP but the phenyl-iodoazido reactive group is placed in a different position with respect to the
piperazine pharmacophore (Figure 24), and becomes incorporated into both previously identified labeled regions TMs 1-2 and TMs 4-7. These results complement previous findings where the related GBR-based compounds \[^{125}\text{I}]\text{AD-96}\) and \[^{125}\text{I}]\text{GA 2-34}\), attach to one or both of these regions \(\[112,113\]. However, the exact domain to which any of these compounds becomes incorporated remains to be explored.

The results of these studies suggest that the irreversible binding domains in DAT are shared between structurally diverse uptake blocker analogues, and further indicate that these compounds bind to a common binding pocket in the transporter composed of various domains. The exact epitopes to which each class of inhibitor attaches are not necessarily identical, but are contained within the same domains, and further, their differential orientations within the binding pocket lead to distinct incorporation patterns. These studies also demonstrate that the DAT active site consists of multiple TM domains located distantly in the primary structure but proximally in the three-dimensional conformation of the protein. The identification of TM1 as a cocaine binding domain complements the finding that indicates TM6 is another component of the cocaine binding site. The identical structures of \[^{125}\text{I}]\text{MFZ 2-24}\) and \[^{125}\text{I}]\text{RTI 82}\) allow their binding to DAT with the same spatial orientation in the pocket, and the differential position of the reactive group results in irreversible incorporation into discrete domains. This strongly indicates that, although distant in the primary structure, TMs 1 and 6 are in close three-dimensional proximity and are components, at least partially, of the cocaine binding site in DAT. In addition, these studies provide biochemical
evidence that supports the similarity of the three-dimensional arrangement between DAT and LeuT$_{Aa}$, with TMs 1 and 6 as partial constituents of the active site. Together these results indicate that the substrate and the uptake-blocker binding sites may overlap and that these compounds may inhibit dopamine transport by competing with the substrate for binding, or by locking DAT in an open outwardly conformational state that prevents the necessary movements for dopamine to be transported.
APPENDIX A

Below is the nucleotide sequence of the 6xHis-hDAT insert. The 6xHistidine tag is highlighted in cyan, the EcoRI restriction site is highlighted in grey, the coding sequence is underlined, and TM1 is highlighted in bold letters.

5'---TGCTTACTGG CCTATCGAAA TTAATACGAC TCACATAGG GAGACCAAG
    CTGGCTAGCG TTTAAACTTA AGCTTACCAT GGGGGTTCCT CAATCTATAGGG

101
    GAGACTATGG TATGCTAACG ATGACTGGTG GACAGCAAT ATGGTCGGGAT
    EcoRI

101
    CTGTACGACG ATGACGATAA GGTACCTAAG GATCCAGTGT GGTGAACTTC

201
    CTCAACTCCC AGTGTGCCCA TGAGTAAGAG CAATTGTCTCC TGGGACTCTA
    TGCTTCCGGT GTTGGCCCTCC GCTAAGGAGC CCAATGCCGT GGGGCCCGAAG

301
    AGGGTGGAGC TCATCCTTGGT CAAGGAGCAG AACGGAGTGC AGCTCACCAG
    CTCCACCCCT ACCAAACCGC GCCAGAGCCC GTGAGAAGCC CAGGATCGG

401
    AGACCTGGGG TAAAGAGATC GATTTCTCC AGTGCATCATCG GAACGTTCTTG

501
    TGCCGGCTCC TCTTACTGCTC TTTCTACGTTA ATGAGGAT TTGGGGTCGG
    TGGCTTTGCT GTGGACCTGG CCAACGTCTG GCCGTTCCCC TACCTGTGCT

601
    CTCAACTCCC GCATGGCATCT GACAGGAGC TACCAAGGGG TGGGTGGGTC

701
    TGGCGGTGCC TTTG G TCCCTACCTG CTTCATGGTC ATTGCTGGGA
    GCATCAATCG TACCTCTGCT ATGCGGGAGC AGCATCCCGA TCTGGGCGCT

801
    TGGTGACTCC AGTTGAGACA GCCGCCCTCG TGGACGACACT TTTGGGACCA
    GTGCTGATCG CCTTCTCCAG CTACAACAAG TTCACCAAGA CCAATGCGAG

901
    CACCTTGCGC CGACTTATAT GACAGTCCTCC TGGCAAGCAGG GACAGCGAG

1001
    CTCAGGGAA AGTGGTATGG ATACGAGACA CATGGAGGTT CTCCGCGCTCC
    GCTTCGTCGT CTTCTCCCTT CTTCTCCTCT CTTCCAGGGA GCTCCTCGGG

1101
    ATTCATCGGA AACACTCTAT TCCAGGATCT GGTCTGTGAT GCATGCCGTG

1201
    AGTGGCCTGG TTTCTGCTGG TGGGGTGGCA TGGCAGCTTG ACTGCTGCTG

1301
    CCTCAGGGAA AGTGGTATGG ATACGAGACA CATGGAGGTT CTCCGCGCTCC

1401
    GCCATCCACG CGTCCGCTGG TGGACGACACT TTTGGGACCA GCTTCGTCGT

1501
    TGTTCTGCGT CACCAACGGT GGCATCTACG TCTTCAAGGT CCTGGACCAT

1601
    AGTGGCCTGG TTTCTGCTGG TGGGGTGGCA TGGCAGCTTG ACTGCTGCTG

1701
    TGGTGACTCC AGTTGAGACA GCCGCCCTCG TGGACGACACT TTTGGGACCA

TGACCCGAGA CCGCCCGAGG CTGGTCTCGC TGGGTCGTCG TGGCTGACTG

GAGACCAAGG AGGGAGGAGC AGGGGATGGG CCAAGAGCCC TGGGGTTCGG

GCATCGAGTG CTGGCTGATC TGGGCTGATG AAGCCATCGG

GCCCTCAGGG TTTGGAAGCC GACGACCGAG GCCGCCGAGG CTGGTCTCGC TGGGTCGTCG TGGCTGACTG

131
1801  AGCCCCGTCT TTCTCCTGTT CGTGGTCGTG GTCAGCATTTG TGACCTTCAG
       ACCCCCCAC TACGGAGCCT ACATCTTCCC CGACTGGGCC AACGCCGCTGG
1901  GCTGGGTCTAT CGCCACATCC TCCATGGCCA TGTTGCCCAT CTATGCGGGCC
       TACAAGTTCT GCAGCCTGCC TGGGTCTTTT CGAGAGAAAC TGGCCTACGC
2001  CATTGCACCC GAGAAGGACC GTGAGCTGGT GGACAGAGG GAGGTCGACC
       ACACGCTCCG CCACCTGGCTC AAGGTGTAGA GGGAGCAGAG ACGAAGACCC
2101  CAGGAAGTCA TCTGCAATG GGAGAGACAC GAACAAACCA AGGAAATCTA
       AGTTT --- 3'
APPENDIX B

Site-directed Mutagenesis Primers

The primers used for site-directed mutagenesis by the QuikChange® method are shown below. The substituted codons are highlighted in magenta.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M116A</strong></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>5'-GGGATGCCACTTTTCTACGGGCTGGCC-3'</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'-CCGAGGGCCAGCTCCTGGCATCCC-3'</td>
</tr>
<tr>
<td><strong>M111L/M116L</strong></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>5'-GGTCATTGCTGGGCACCTTTTCTAGCTGGCCC-3'</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'-GGGCCAGCTCGGATGAAAGTGCCCGCAGCAATGACC-3'</td>
</tr>
<tr>
<td><strong>L80M</strong></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>5'-GCTTTGCTGCTGGACGAACTTTGTCCTGGC-3'</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'-GCCAGACGTTGGCTCCACAGCAAAGC-3'</td>
</tr>
<tr>
<td><strong>I67M</strong></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>5'-CTGGGGCAAGAAGACTTTTCTCTGTCC-3'</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'-GGACAGGAGAAAGTCCCTTTGGCCCCAG-3'</td>
</tr>
</tbody>
</table>
APPENDIX C

Shown is the nucleotide sequence of the 6xHis-hDAT insert with the primers used for sequencing purposes in bold letters.

T7 promoter - 1 (F) →

5′--- TGCTTACTGG CTTATCGAAA TTAATACGAC TCACTATAGG GAGACCCAAG
CTGGCTAGCG TTTAACTTAA AGCTTACCAG GGGGGTTCT CATCATCATC
ATCATCATGG TATGGCTAGC ATGACTGGTG GACAGCAAAT GGGTGGGAT
CTGTACGAGC ATGACGATAA GGTACCTAG ATGCCAGTGT GGTGGAATTC
CTCAAACCTCC AGTGTGGCCA TGAGTAAAG CAAATGCTCC GGGGAACTCA
TGTCTTCCGT GGTGGCCCG CDTAAGGAGC CCAATGCCGT GGGCCCAAG
← hDAT 4 (R)
GAGGTGGAGGC TCACTTTTCTGT CAAGGAGCAG AACGGAGTGC GAGTCACCAAG
CTCCACCCCTC ACCAACCAGG GGGGAGACC GGTTGAGGCC CAGGATCGGG
AGACCTGG AACGAAGATC GACTTTTCTCC TGTCGGTCAT TGCGTTTGGCT
GGTGACCTGG CCAACGTCTG GCGTTCCCC TACCTGTGCT ACAAAATAGG
hDAT 2 (F) ←
TGGCCGTTGCC TTCTCTGTCC CCTACCTGCT TTTGCAGTCTG ATGCTGGGAG
TGCCCATTCTT CTACATGGAG CTGGCCTCGG GCCAGTTCAA CAGGGAAGGG
GCCGCTGGTG TCTGAGATC GATGCCCATC TGAAGGTTG TGGGCTTCAC
GGTCATCCTC ATCTCAGTGT ATGTGGGCTT TTTCTACAAAC GTCATCATCG
CCTGGCCGCT GCACATATCTT TTTCTCCTCT TCACACAGGA GTCCTCCTGG
ATCCACTGGA ACAAATCTGT GAACAGCCCC AACTGCTGGG ATGCCCATCC
TGTTGAACCTG AGTGGAGACA GCTGGGCTCTT CAACGACACT TTTGGGACCA
CACCTGCTGC CGAGTACTTTT GAACGTGGCG TGCTGCACCT CCACCAGAGC
← hDAT 3 (R)
CATGGCACTCG ACAGACTTGG GGCCTCAGG GGGCAGCTCA CAGGCTGACTT
GGTGCTGGTG ATCGTGCTGC TCTACTTCAG CCTCTGGGAG GGCGTGGAAGA

134
CCTCAGGGAA GGTGGTATGG ATCACAGCCA CCATGCCATA CGTGGTCCCTC
ACTGCCCTGC TCTTGCCTTG GGTCAACCCTC CCTGGAGCCA TAGACGGCAT
hDAT 3 (F) → CAGAGCATAC CTGAGGGTTG ACTTCTACCG GCTCTGCGAG GCGTCTGTTTT
GGATGGACGC GGCCACCCAG GTGTGCTTCT CCCCAGGGGT GGAGTTCGGG
GTGCTGATCG CCTTCTCCAG CTACAACAAG TTCACAACAAG ACTGCTACAG
GGACGGATT GTCACCACCT CCATCAACTC CCTGAGGAGC TTCTCCTCCG
GCTTGTCTGT CTTCTCCTTC CTGGGTACAA TGGCACAGAA GCACAGTGTG
CCCATCGGGG ACAGTGGCCAA GGACGGGCCA GGGCTGATCT TCACTCATCTA
CCCAGGAGCC ATCGCCAGGC TCCCTCTGTC CTCAGCCTGG GCGGTGTTCT
TCTTACATCAT GCTGCTCACC CTGGGTATCG ACAGCGCATG GGTGGTATAG
← hDAT 2 (R) GAGTCAGTGA TCACCGGGCT CATCGATGAG TTCCAGCTGC TGCAACAGACA
CGTGAGCTC TTCAGCTCTC TCATCGTCTT GGCGACTTCC TCTCCTGCCC
TGTTCTGCGT CACCAAGGCT GCCATCTACG TCTTACAGGT CCTGCCACCAT
TTTGCAAGCG GCACGGCCAT CCTCTTTGGA GTGCTCATCG AAGCCATCGG
hDAT 4 (F) → AGTGCCCCTG TTCTATGGTG TTGGGCAGTT CAGCGACGAC ATCCAGCAGA
TGACCGGCA GCCCGCCAGC CTGTAAGTCG GGCTGTGCTG GAAAGTTGCT
AGCCCTCTGC TTCTCCTGTT CGTGTCGCTG GTCACTATGG TGACCTTCAG
ACCACCCACC TACGGAGCCT ACATCTCCCT CGACTGGGCC AACCAGCTGG
GCTGGGTCAT CGCCACATCC TCCATGGCCA TGGGTCCCCAT CTATCGGGCC
TACAAGTTCT GCAGCCTGCC TGGGTCTTCTT CGAGAGAAAC TGGCCTACGC
CATTGCACCC GAGAAGGACC GTGAGCTGCTG GGACAGAGG GAGGTGCGCC
ACAGGCCTCC CCACCTGCTG CAGGTGTAGA GGGAGCGAG ACGAGAGACC
← hDAT 1 (R) CAGGAAGTCA TCTGCAATG GGAGAGCAC GAAACAACCA AGGAAATCTA
AGTTT --- 3'
REFERENCES


[98] Chen N, Zhen J, Reith ME. Mutation of Trp84 and Asp313 of the dopamine transporter reveals similar mode of binding interaction for GBR12909 and benztropine as opposed to cocaine. J Neurochem 2004;89 (4):853-64.


End
File