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Purification and Reconstitution into Planar Bilayers of the Human Dopamine Transporter

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PURIFICATION AND RECONSTITUTION INTO PLANAR BILAYERS OF THE HUMAN DOPAMINE TRANSPORTER

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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ACKNOWLEDGEMENTS

For their patience and belief in me, I thank my parents, Dr. Edgar Leal-Pinto, and my mentor, Dr. Louis De Felice. I have had the opportunity to learn from some very gifted, inspiring individuals along this journey. Thank you all as this would not have been possible without your care, your guidance, and support.
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LIST OF ABBREVIATIONS

AMPH = (+) Amphetamine

COC = Cocaine

DA = Dopamine

DA neurons = Dopaminergic neurons

H6-hDAT = Hexahistidine tagged- Human Dopamine Transporter

HBA = Homogenization Buffer A

hDAT = Human Dopamine Transporter

LeuT = Leucine Transporter

MBSS = MES-Buffered Saline Solution

MDMA = (+) 3,4-methylenedioxy-N-methylamphetamine, Ecstasy

METH = (+) Methamphetamine

SLC6 = Solute Carrier 6

VMAT2 = Vesicular Monoamine Transporter 2

VTA = Ventral Tegmental Area
ABSTRACT

The human dopamine transporter (hDAT) provides the primary mechanism for dopamine clearance in synapses and thus facilitates the regulation of dopaminergic functions in cognition and reward. It is the molecular target of many centrally-active agents including amphetamines and cocaine. Therefore, an understanding of hDAT function and its modulation by these therapeutic drugs and drugs of abuse can provide insight into the mechanisms of abuse and addiction. In the presented studies, hDAT is tagged with a hexahistidine construct and heterologously expressed in *Xenopus laevis* oocytes. The plasma membranes are isolated, solubilized, and applied to a Nickel affinity column to obtain purified hDAT with preserved functionality. Purified hDAT reconstituted in planar lipid bilayers exhibited channel behaviors at physiological membrane potentials. We observed that the current mediated by single hDAT molecules is (1) induced by dopamine, (2) dependent on the sodium electrochemical gradient, and (3) blocked by cocaine. Our data support hDAT channel activity that is associated with dopamine uptake and presents a novel electrophysiological approach to studying monoamine transporter function and modulation by drugs.
INTRODUCTION

Dopamine and the brain reward circuitry

Dopamine (DA) is a monoamine neurotransmitter with important roles in behavior, cognition, locomotor activity, motivation, punishment and reward, attention, and memory (Fig. 1). Its synthesis occurs in dopaminergic neurons, which are primarily located in the substantia nigra and adjacent ventral tegmental area (VTA). DA neurons in the VTA project to the nucleus accumbens to form the mesolimbic pathway, which serves as the major neurochemical pathway of the reward system in the animal brain. Nearly all addictive drugs affect the brain reward circuit by, directly or indirectly, elevating its extracellular DA levels. Numerous in vivo microdialysis studies have demonstrated a vast increase in DA concentration in the nucleus accumbens and other DA terminal fields upon administration of psychostimulants, opiates, nicotine, phencyclidine, and cannabis (Wise, 1996). Additionally, in vivo and in vitro electrophysiological recordings of mesocorticolimbic DA neurons show that amphetamine and cocaine administration begets increases in the number of active VTA DA neurons and in their basal firing rates (White, 1996).

Figure 1. Chemical structures of dopamine and other select compounds that act on hDAT. Drugs that target the dopaminergic system are generally categorized as dopamine releasing agents (DRAs, blue) or dopamine reuptake inhibitors (DRIs, red). *Tyramine is a naturally-occurring trace amine.
hDAT regulation of dopaminergic signals

Once DA is released, DA neurotransmission occurs through specific postsynaptic receptors. To prevent over activation of the postsynaptic cell, extracellular dopamine is removed by diffusion to the perisynaptic cleft, reuptake by the presynaptic neuron and surrounding glia, and enzymatic degradation by monoamine oxidase and catechol-O-methyl transferase. The human dopamine transporter (hDAT, SLC6A3) provides the major mechanism for DA synaptic clearance in all brain regions except the prefrontal cortex, where DA uptake depends primarily on the closely-related norepinephrine transporter (Morón et al., 2002). At the cellular level, functional hDAT are localized in the plasma membrane of axons, dendrites, and pre-synaptic axon terminals of DA neurons. More specifically, high-resolution immunoelectron microscopy studies in the rat nucleus accumbens have revealed that the transporters are absent at synaptic densities but are found highly-expressed on the perisynaptic axonal membranes (Nirenberg et al., 1997). These findings indicate that released DA diffuses out of the synaptic cleft to be transported into axon terminals and that hDAT potentially regulates the diffusion of DA to extrasynaptically-located receptors (Fig.2).

Figure 2. hDAT provides the primary mechanism for dopamine clearance from synapses. hDAT cotransports extracellular dopamine with sodium and chloride ions (not shown) into the presynaptic neuron.
Given its critical role in regulating the dopaminergic reward circuitry, hDAT is the primary target of numerous drugs of abuse. Figure 1 includes the chemical structures of a few well-studied compounds that act on hDAT. With the exception of tyramine, each compound is synthetic and is utilized as an abused or therapeutic drug. All of these drugs enhance DA neurotransmission; However, depending on its principal mechanism of action, each drug is classified as a dopamine releasing agent (DRA) or dopamine reuptake inhibitor (DRI). Figure 3 illustrates the different processes by which two highly-abused psychostimulants, amphetamine (AMPH) and cocaine (COC), raise extracellular DA levels. Like many DRAs, AMPH enters the presynaptic cell mainly through hDAT. Once inside the cell, AMPH interacts with the vesicular monoamine transporter 2 (VMAT2) to inhibit the vesicular repackaging of DA, and reverses the action of hDAT to produce an efflux of DA into the synaptic cleft (Fig. 3a). In contrast, DRIs such as COC (Fig. 3b) bind to hDAT and block the transporter’s ability to translocate DA back into the presynaptic cell. Table 1 lists select DRAs alongside their potencies for releasing DA, norepinephrine (NE), and serotonin (5-HT). Table 2 lists select DRIs with their approximate potencies for inhibiting DA, norepinephrine (NE), and serotonin (5-HT) reuptake through their respective transporters. Due to the high similarity between these monoamine neurotransmitters and their respective transporters, there are no known compounds that act exclusively on one monoaminergic system.
Figure 3. Drug mechanisms of action at hDAT. Amphetamine (AMPH) and cocaine (COC) are widely-abused psychostimulants that primarily target the dopamine transporter (DAT). (a) AMPH acts as a substrate and is transported by DAT. Once inside the presynaptic cell, AMPH interacts with vesicular monoamine transporter 2 (VMAT2) to inhibit dopamine sequestration into vesicles. Additionally, it induces conformational changes within DAT that cause reverse transport of dopamine through the transporter. (b) COC acts as an inhibitor by binding to DAT and blocking the reuptake of dopamine from the synaptic cleft. Although through different mechanisms, both drugs produce significant increases in the extracellular dopamine level. (Image source: Katzung, B.G.)
### Table 1. DAT Releasers: EC$_{50}$ values for DA release. Adapted from Rothman et. al., 2006.

<table>
<thead>
<tr>
<th></th>
<th>DA Release (nM ± SD)</th>
<th>NE Release (nM ± SD)</th>
<th>5-HT Release (nM ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-Methamphetamine</td>
<td>24.5 ± 2.1</td>
<td>12.3 ± 0.7</td>
<td>736 ± 45</td>
</tr>
<tr>
<td>(+)-Amphetamine</td>
<td>24.8 ± 3.5</td>
<td>7.07 ± 0.95</td>
<td>1765 ± 94</td>
</tr>
<tr>
<td>Dopamine</td>
<td>86.9 ± 9.7</td>
<td>66.2 ± 5.4</td>
<td>8489 ± 200</td>
</tr>
<tr>
<td>Tyramine</td>
<td>119 ± 11</td>
<td>40.6 ± 3.5</td>
<td>2775 ± 234</td>
</tr>
<tr>
<td>MDMA</td>
<td>278 ± 7</td>
<td>110 ± 10</td>
<td>72 ± 3</td>
</tr>
</tbody>
</table>

### Table 2. DAT Inhibitors: $K_i$ values for [H]$^3$DA uptake. Adapted from Rothman et. al., 2006.

<table>
<thead>
<tr>
<th></th>
<th>DA Uptake (nM ± SD)</th>
<th>NE Uptake (nM ± SD)</th>
<th>5-HT Uptake (nM ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocaine</td>
<td>478 ± 25</td>
<td>779 ± 30</td>
<td>304 ± 10</td>
</tr>
<tr>
<td>GBR 12909</td>
<td>4.3 ± 0.3</td>
<td>79.2 ± 4.9</td>
<td>73.2 ± 1.5</td>
</tr>
<tr>
<td>GBR 12935</td>
<td>4.90 ± 0.3</td>
<td>277 ± 23</td>
<td>289 ± 29</td>
</tr>
<tr>
<td>Methylphenidate</td>
<td>90.2 ± 7.9</td>
<td>118 ± 12</td>
<td>inactive</td>
</tr>
<tr>
<td>Desipramine</td>
<td>5946 ± 193</td>
<td>8.32 ± 1.19</td>
<td>350 ± 13</td>
</tr>
</tbody>
</table>
**hDAT structure and activity**

hDAT is a member of the solute carrier 6 (SLC6) gene family, which includes nine sodium- and chloride-dependent transporters for GABA, glycine, and the monoamine neurotransmitters. It consists of 620 amino acids and exhibits high sequence identity with other mammalian dopamine transporters. Photoaffinity labeling studies suggest that functional hDAT is heavily glycosylated with N-linked sugars and sialic acids and that depending on the extent of glycosylation, the molecular weight of hDAT varies from 50 to approximately 80 kDa (Vaughan et al., 1996). hDAT trafficking and activity is also mediated by in vivo phosphorylation of the intracellular domains. Activation of protein kinase C has been shown to result in sequestration of the transporter and down-regulation of transport activity while the inhibition of other protein kinases such as ERK1/2, PI-3-kinase, and tyrosine kinase produced the same effects (Ramamoorthy et al., 2011).

Hydropathy analysis of the hDAT primary sequence predicts a topology with 12 transmembrane domains with both the N- and C- termini located in the cytosol (Fig.4). However, as with most membrane-spanning proteins, a three-dimensional crystal structure of hDAT has yet to be resolved. Consequently, much of the current information on hDAT structure has been obtained from the crystal structure of a bacterial member of the SLC6 family, the leucine transporter (LeuT). Although the overall sequence identity between LeuT and its eukaryotic homologues is only 20-25%, the sequence identity near the substrate and sodium binding sites is especially high, approaching 50% (Singh, 2008). The LeuT tertiary structure has thus served as a template for constructing homology models of hDAT (Fig. 5) and has allowed for unprecedented insights into hDAT structure and function. Co-crystals of LeuT with various substrates and inhibitors have been analyzed in view of the alternating access model for active transport. In this model, a cation-substrate symporter such as the SLC6 members undergoes a cycle of distinct conformations to move solutes across the cell membrane (Fig.6). In the outward-facing (open to out) state, the substrate and ions can enter their respective binding sites from the extracellular
environment. Upon substrate binding, the protein undergoes a conformational change that closes access to the extracellular space; the substrate and ions are sealed off in this *occluded* state. Ultimately, the transporter rearranges to the *inward-facing (open to in)* conformation, in which the binding sites are exposed to the cytosol and the substrate and ions are released. In this proposed mechanism, the substrate binding site essentially alternates between the *outward- and inward-facing* conformations and the relative substrate binding affinities of the two conformations largely determines the net direction of transport. For importers, such as LeuT and hDAT, the *outward-facing* conformation will have higher substrate binding affinities.

![Diagram of transporter](image)

**Figure 4.** Without a X-ray crystallographic structure of hDAT, a hydropathy plot of the protein primary sequence predicts a topology of 12 transmembrane segments with both the N- and C- termini located in the cytosol. Image adapted from Torres et al., 2003.
Figure 5. 3D model of hDAT made by homology modeling with the LeuT structure as template. Image is courtesy of Krasnodara Cameron.
Figure 6. Modified alternating access model based on a LeuT crystal structure (black boxes). Substrate and ions bind at their unique sites when the transporter is in the outward-facing (open to out) conformation. Substrate (S) occupying the binding pocket promotes the formation of the occluded state, in which both the extracellular and intracellular gates are closed. When the protein assumes the inward-facing (open to in) conformation, the substrate and ions are released into the cytosol. Image adapted from Singh, 2008.
hDAT transports DA against its concentration gradient by harvesting free energy stored in the Na$^+$ and Cl$^-$ electrochemical gradients across the plasma membrane. Previous biochemical studies on the ion dependence of hDAT suggest that each DA molecule is cotransported with two Na$^+$ ions and one Cl$^-$ ion (McElvain and Schenk, 1992; Gu et al., 1994). This stoichiometry predicts that the translocation of each DA molecule will result in the movement of two net positive charges and thereby generating an inward current (DA is positively-charged at physiological pH). However, hDAT exhibits substrate-dependent currents that exceed those predicted by the 2Na$^+$/1Cl$^-$/1DA$^+$ stoichiometry and the alternation access model. Comparable currents have not been observed in LeuT and accordingly, there is no crystal structure at present to reliably construct a channel-like conformation in hDAT.

Channel properties of hDAT

Electrophysiological studies of both heterologously-expressed and native hDAT show substrate-induced currents that are too large to be accounted for by the fixed stoichiometric coupling, the slow turnover rate of transporters, and transporter density (Sonders and Amara, 1996). The alternating access model estimates an uptake current of less than 1 pA for one million simultaneously operating transporters (DeFelice and Goswami, 2007). Figure 7 displays whole-cell recordings of C. elegans DA neurons with hDAT-mediated currents of at least 5.5 pA in size (a,b) and single channel recordings in outside-out patches with a current amplitude of 0.8 ± 0.1 pA in magnitude (c,d) (Carvelli et al., 2004). These currents display substrate affinity and pharmacological sensitivities comparable to those of DA uptake activity. Furthermore, hDAT has been shown to mediate substrate-independent leak currents, mimicking the electrical activities of ion channels. The leak currents are blocked by reuptake inhibitors such as COC as illustrated in Figure 8 (Sonders et al., 1997). Such experiments that have revealed the existence of channels in hDAT as well as other transporters disagree with the traditional models of cotransport and summon new models with channel-like properties.
Figure 7. Whole-cell (macroscopic) and single-channel (microscopic) recordings in C. elegans DA neurons. (a) DA-induced inward currents observed when the membrane potential was stepped from -40 to -100 mV. These hDAT-mediated currents were blocked by coapplication of imipramine (IMP), a uptake inhibitor. (b) Whole-cell current noise increased with extracellular DA concentration. Membrane potential stepped from -40 to -120 mV. (c) Single-channel events from outside-out patches with holding potential at -120 mV. (d) Amplitude histograms constructed from the channel events. (Carvelli et al., 2004)
Figure 8. Drug applications (solid bars) to *X. laevis* oocytes voltage-clamped at -60 mV. Perfusing 10 uM COC to a hDAT-expressing oocyte generated a small outward current that slowly returned to baseline after 10 min of washout. Applying 20 uM DA to the same cell elicited an inward current that rapidly returned to baseline upon removing the extracellular DA. (Sonders et al., 1997)
Ion channel reconstitution

*Ion channel reconstitution* is a voltage-clamp technique used to characterize channel activities and involves incorporation of the channel protein into artificial phospholipid bilayers. Besides providing an alternative approach to measuring channel function, this technique allows for the investigation of channels that are not accessible to conventional electrophysiological approaches such as those located in intracellular membranes. It also makes possible the study of purified protein molecules (Ashley, 1995). Figure 9 illustrates the standard setup of the planar bilayer system, in which the experimenter has complete control over the components in both the cis (intracellular) and trans (extracellular) compartments. The solution in the trans side of the bilayer is held at ground while the solution in the cis side is clamped at a potential relative to ground. The experimenter can also manipulate the lipid composition surrounding the proteins and in turn determine where the channel function is modulated by specific fatty acids, cholesterol, and phospholipids. Although it confers several advantages over the more conventional electrophysiological techniques of whole-cell voltage clamp and patch clamping, ion channel reconstitution is not as widespread due to technical complexities that must be overcome in order to achieve high signal to noise ratios.
**Figure 9.** Setup of the planar lipid bilayer experiment. Proteoliposomes containing purified hDAT (yellow rings) are fused into the planar bilayer, which is positioned at the interface of the *cis* (intracellular) and *trans* (extracellular) chambers. Once the protein is reconstituted in the artificial bilayer, dopamine and/or other experimental drugs are introduced into the *trans* side and the current flow through hDAT is monitored over time at a constant voltage.
Purification of transmembrane proteins

The primary advantage of the ion channel reconstitution technique is that auxiliary and regulatory proteins that associate with the channel in the endogenous system are not likely present. Therefore, this method is most valuable when purified channel proteins are incorporated into the bilayers for functional characterization. However, the reconstitution of most channels is from native or semi-purified membranes because of the difficulties associated with transmembrane protein isolation. The general workflow for the purification of membrane proteins is depicted in Figure 10. Only few membrane proteins are naturally abundant and most prove difficult to express heterologously. Yet the greatest challenge is defining the most stable conditions during the solubilization and extraction processes in order to preserve fully-functional protein as the purified product.
Figure 10. General workflow for the production and purification of membrane proteins.
**Purpose of this study**

Using techniques that had been used to study classical ion channel proteins, electrophysiologists have accumulated surmounting evidence for the existence of channels in transporters. Despite the numerous studies on hDAT-mediated macroscopic and microscopic currents, there have been no reported attempts at reconstituting purified or partially-purified hDAT into artificial bilayers. By purifying hDAT and subsequently utilizing the ion channel reconstitution approach, we aim to observe single channel events in the lipid bilayer and ultimately, characterize the channel properties of hDAT in a highly-controlled and isolated system.

**Hypothesis**

The hexahistidine tag is small in size (~660 Da) and should not interfere with the functions of the transporter; hDAT and H6-hDAT should mediate equal DA uptake as well as DA-elicited currents. Therefore, H6-hDAT will be used to study the wild type hDAT upon purification. After reconstitution into planar bilayers, individual transporters will elicit microscopic currents in response to extracellular DA application. These currents will be dependent on a Na$^+$ electrochemical gradient across the bilayer and will be diminished by the application of COC.
METHODS AND MATERIALS

cDNA construct and cRNA synthesis

The human dopamine transporter gene (DAT1 or SLC6A3) was subcloned into the KpnI/XbaI cassette of the p3Z vector. A 60bp segment coding for the Kozak sequence, an initiator methionine, a hexahistidine tag, and a TEV recognition sequence was then linked to the 5’ end of DAT1 via insertion into the upstream EcoRI/KpnI sites. The translation product is fused to an additional 17 amino acids at the N-terminus and only 3 of these residues remain upon digestion with TEV protease (Nacalai USA, San Diego, CA). For the heterologous expression of the human dopamine transporter (hDAT) in *Xenopus laevis* oocytes, the entire gene construct was subcloned into the oocyte expression vector pOTV between the unique KpnI and XbaI restriction sites. Transformed DH5α competent cells (Invitrogen) were selectively grown in LB-medium supplemented with 100µg/mL ampicillin. The plasmids were extracted and the presence of the correct recombinant DNA was confirmed via restriction fragment analysis as well as automated DNA sequencing (Virginia Commonwealth University Nucleic Acids Research Facility). Recombinant pOTV vector was digested at the single NotI site to yield a linear fragment from which the corresponding cRNA was transcribed using the Ambion mMessage Machine T7 kit (Ambion Inc., Austin, TX). cRNA preparations were diluted to 1 µg/µl in nuclease-free water (Ambion) and stored at -70°C until use.

Oocyte microinjection and maintenance

Stage V or VI oocytes harvested from adult *Xenopus laevis* females were selected and allowed to rest overnight at 18°C in Ringers solution (in mM: 96 NaCl, 2 KCl, 10 MgCl₂, 10 HEPES, 0.6 CaCl₂ pH = 7.6) supplemented with sodium pyruvate (550 µg/ml), streptomycin (100 µg/ml), tetracycline (50 µg/ml), and dialyzed horse serum (5%). On the following day, each oocyte was injected with 23.0 - 27.6 ng His₆X-hDAT cRNA, untagged hDAT cRNA, or nuclease-free water (Ambion) utilizing the Nanoject
AutoOocyteInjector (Drummond Scientific Co., Broomall, PA). The oocytes were further incubated at 18°C in the supplemented Ringers solution for 7-10 days with fresh solution provided daily.

**Two-electrode voltage clamp**

Transporter currents were measured by two-electrode voltage clamp (TEVC, Figure 11) using AxoPatch 200B Amplifier with a Digidata 1200 series interface. Current and voltage electrodes were filled with 3M KCl and resistances ranged from 0.8 - 6.5 MΩ. Oocytes were voltage-clamped to -60 mV and TEVC recording buffer solution (in mM: 5.4 KGlucolate, 1.2 CaGluconate, 7.2 HEPES, 120 NaCl; pH = 7.4) was continually perfused until a stable holding current was obtained. Experimental drugs – 5 µM dopamine, 5 µM amphetamine, 5 µM methamphetamine, 10 µM cocaine – were individually or simultaneously perfused for indicated periods.

![Figure 11. Two-electrode voltage clamp (TEVC) allows measurement of whole-cell channel currents while holding the membrane potential constant. The oocyte is placed in an experimental chamber perfused with recording solution and is impaled with current (i) and voltage (v) microelectrodes. Drugs solubilized in the recording solution are perfused through the chamber while the membrane current is continuously monitored.](image-url)
Preparation of plasma membrane fractions

40-70 oocytes were rinsed in MES-buffered saline solution (MBSS; 80 mM NaCl, 20 mM MES, pH = 6.0, supplemented with protease inhibitor cocktail) and incubated under mild agitation for 10 min at room temperature in MBSS with 0.005% subtilisin A (Sigma-Aldrich). Polymerization was performed at 4°C by two sequential 60 min incubations under mild agitation in MBSS, first with 1% ludox (Sigma-Aldrich) and then with 0.1% polyacrylic acid (Sigma-Aldrich), with MBSS rinses between each step. The oocytes were homogenized on ice in 5 ml of cold homogenization buffer A (HBA; in mM: 5 MgCl₂, 5 NaH₂PO₄, 1 EDTA, 80 sucrose, 20 Tris pH = 7.4) with 5 strokes of a Dounce homogenizer (Wheaton). Homogenates were centrifuged at 16 g for 30 sec and the supernatants were gently removed and replaced with fresh HBA. With the same procedure, the membrane samples were centrifuged again at 16 g, followed by 25 g and 35 g. Purified plasma membranes were pelleted upon a final centrifugation at 16,000 g for 20 min and were resuspended in HBA. Membranes were stored in -70°C until use.
Figure 12. Plasma membrane preparation

* MBSS = MES-buffered saline solution (80 mM NaCl, 20 mM MES, pH = 6.0)

** Discard supernatant and add fresh Homogenization Buffer A (HBA)
Solubilization

Isolated membrane preparations were resuspended in Buffer 0 (20 mM sodium phosphate buffer, 500 mM NaCl, pH = 7.4) with protease inhibitor (Roche) and either 0.25% DDM, 0.50 DDM, or 1% CHAPS by gently pipetting (~15 mg of membrane). Resuspended membranes were incubated on ice for 45 min in the 4°C room then centrifuged at 16,000xg for 60 min at 4°C. Supernatants were filtered through 0.2 um filters and transferred to clean tubes.

(a) Induced by DA

(b) Dependent on Na

Figure 13. Artificial bilayer studies. Major steps involved in the reconstitution of H6-hDAT into planar bilayers to test for (a) DA-induced transporter currents and (b) their dependency on a transmembrane Na gradient.
[^3]H DA uptake

Proteoliposomes formed from solubilized hDAT, H6-hDAT, or control membranes enclosed intracellular solution (in mM: 5 CsCl, 8 NaAc, 130 CsAc, 10 HEPES; pH = 7.4). For uptake studies, the vesicles were incubated in extracellular solution (in mM: 105 NaCl, 5 CsCl, 33 NaAc, 10 HEPES; pH = 7.4) and 3%[^3]H DA (5 uM total DA) for 0, 10, 20, 30, 60, and 600 sec before being transferred to 0.20 um filter paper and immediately washed three times with rinse solution (extracellular solution + 10 uM COC). For COC inhibition, 10 uM COC was included during the incubation periods. For the Na+ dependency assays, the proteoliposomes were incubated in extracellular solution #2 (in mM: 130 LiCl, 5 CsCl, 8 NaAc, 10 HEPES; pH = 7.4) and the rinse solution consisted of extracellular solution #2 with 10 uM COC. Each filter paper was dissolved in 10 mL of scintillation fluid before counting. Experiments were conducted at room temperature.

Affinity chromatography

H6-hDAT membrane fractions solubilized in 1% CHAPS was applied to a His-Trap HP column (GE Healthcare) for protein purification at 4°C. Proteins were solubilized in Buffer 0 and allowed to bind to the column. Buffers with constant concentrations of sodium phosphate (20 mM, pH = 7.4) and NaCl (500 mM) but with increasing imidazole (up to 500 mM) was passed through the column at a constant rate. Washes were collected in 1 mL fractions and elutions were collected in 500 uL fractions. Eluted protein was detected by UV absorbance at 280 nm.

Protein detection

Detergent-solubilized proteins were incubated with Laemmli sample buffer (Bio-Rad) with 5% beta-mercaptoethanol at 42°C for 5 min. Each samples was run as duplicates on two separate 10% polyacrylamide gels. One gel was stained with Coomassie for total protein detection while the other was
used for protein transfer to PVDF membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) and probed with an HRP-linked antibody specific for DAT (Santa Cruz Biotechnology). Lastly, membranes were incubated with horseradish peroxidase-linked secondary antibodies for enhanced chemiluminescence by FluorChem E (Cell Biosciences, Santa Clara, CA).

**Planar bilayer experiments**

Partially-purified H6-hDAT was incorporated into liposomes of 1:1 phosphatidylserine (PS, 10 mg/mL) and phosphatidylethanolamine (PE, 10 mg/mL) via sonication at 80,000 kHz. The resulting proteoliposomes were fused with a planar bilayer of the same lipid composition. The bilayer spanned an approximately 100 um diameter hole separating two 1-mL compartments; the cis side (intracellular) was voltage clamped in reference to the trans (extracellular) ground chamber. Proteoliposomes were placed into the trans compartment and stirred until channel activity was observed; hDAT was inserted into the bilayer with the intracellular surface orientated toward the cis side. Records were collected at 10 kHz, and channel events with open times > 1 ms and noise level twice the background were filtered at 1 kHz during analysis (pCLAMP Clampfit 10.2).
RESULTS

Comparison of hDAT and H6-hDAT function

(a) Radiolabeled DA uptake

\[^{3}\text{H}]\) DA uptake experiments were conducted on proteoliposomes formed with plasma membrane fractions from uninjected, hDAT-injected, and H6-hDAT-injected oocytes. Both hDAT and H6-hDAT vesicles accumulated more tritiated DA than the control vesicles at five various time points up to 600 seconds (Fig. 14, n = 2 or 3 for each time point). The presence of 10 uM COC reduced uptake by 52 and 48 % in hDAT and H6-hDAT proteoliposomes, respectively, after 60 sec of incubation in DA and COC (Fig. 15, top). The decreases were 32 and 27 %, respectively, after 600 sec (Fig. 15, bottom). COC had no significant effect on DA uptake in control vesicles. DA accumulation in hDAT and H6-hDAT proteoliposomes were also diminished in the absence of a transmembrane sodium gradient. Figure 16 shows that \[^{3}\text{H}]\) DA uptake in these vesicles was reduced to the level of the control (n = 2 or 3 for each time point).
**Figure 14.** $[^3]H$ DA uptake by proteoliposomes containing hDAT, H6-hDAT, or neither (control). Both hDAT and H6-hDAT vesicles accumulated more tritiated DA than the control vesicles at five various time points up to 600 seconds ($n = 2$ or 3 for each time point).
Figure 15. Cocaine inhibits $[^3]$H DA uptake. DA accumulation was reduced by 52 and 48 % in hDAT and H6-hDAT proteoliposomes, respectively, after 60 sec of incubation in 5 uM DA and 10 uM COC (top). The decreases were 32 and 27 %, respectively, after 600 sec incubation (bottom). COC had no significant effect on DA uptake in the control vesicles (n = 2 or 3 for all conditions).
Figure 16. Lack of Na gradient inhibits [$^3$H] uptake. DA accumulation in proteoliposomes containing hDAT or H6-hDAT was reduced to the level of the control in the absence of a transmembrane sodium gradient (n = 2 or 3 for each time point).
(b) Whole cell hDAT currents

Figure 17a displays the changes in membrane current that occur upon 30 second superfusion of various drugs over hDAT-expressing oocytes voltage-clamped at – 60 mV. The application of 5uM DA induced inward currents that stabilized in between 10 to 40 nA depending on the expression level. These steady-state currents endured for the duration of the DA perfusion, returning to baseline upon removal of extracellular DA. The introduction of either 5 uM AMPH or METH elicited steady-state currents that were comparable to those achieved by DA perfusion in both magnitude and kinetics. When DA was subsequently reapplied to oocytes, only a proportion of the initial current amplitude was achieved. Co-perfusion of 5 uM DA and 10 uM COC eliminated the DA-induced current. Figure 17b shows the electrophysiological responses of H6-hDAT-expressing oocytes to DA, AMPH, METH, and COC, which were generally indistinguishable from those of hDAT-expressing oocytes. Application of the same drugs to uninjected or water-injected oocytes did not elicit any detectable currents (not shown).

Additionally, the ability of the transporters to generate a persistent current upon prolonged AMPH application was investigated (Fig. 18). In both hDAT- and H6-hDAT-expressing oocytes, a 1 min perfusion of 5 uM AMPH induced a steady inward current that did not fully recover to baseline after removal of extracellular AMPH. The remaining current did not diminish after 1 min of washout and plateaued at a magnitude that was less than half of the initial AMPH-induced current. Exposing the oocytes to 10 uM COC, however, quickly eliminated this persistent inward current; furthermore, the COC application evoked a small outward current that slowly returned to the original baseline after minutes of washout.
Figure 17. Raw TEVC traces representative of drug-elicited responses in (a) hDAT- and (b) H6-hDAT- expressing *Xenopus laevis* oocytes. 5 uM dopamine (DA), 5 uM amphetamine (AMPH), 5 uM methamphetamine (METH), and 10 uM cocaine (COC) were applied at -60 mV. DA, AMPH, and METH each induces an inward current that is eliminated by COC. H6-hDAT-expressing oocytes generate drug-induced currents that are comparable in magnitude and kinetics to those of hDAT-expressing oocytes. Water-injected and uninjected controls produced no response to drug applications (data not shown).
Figure 18. Prolonged exposure to AMPH elicits an inward current that persists after the drug is removed from the extracellular environment. After a 1 min exposure to AMPH, both hDAT- and H6-hDAT-expressing oocytes produce persistent inward currents, which remain even after 1 min of washout but are blocked by COC.
Purification

(a) Chromatography

To determine the imidazole concentration at which H6-hDAT would elute from the nickel resin as a pure molecule, a continuous gradient ranging from 0 to 500 mM imidazole was applied to the column by the ÄKTA FPLC system after binding protein solubilized in 1% CHAPS. As detected by UV absorption at 280 nm, a significant amount of protein eluted after passing 250-300 mM imidazole (Fig. 19). However, this sharp absorption peak did not correspond to purified transporter after examining the content of the respective elution fractions. Inset A shows the Coomassie-stained gel containing fractions 10, 11, and 12. There is a heterogeneous population of proteins but none of them were recognized by the hDAT antibody in the Western blot (not shown). Fractions collected after the peak did not contain any significant amount of H6-hDAT as well (not shown). Accordingly, subsequent purification efforts involved a step gradient of 150, 200, 250, and 500 mM imidazole using a peristaltic pump. Although most of the non-specific proteins were eliminated in the preceding flow through and wash steps (20 mM imidazole), there was still a significant amount of contaminants that eluted with H6-hDAT in 150 mM imidazole. Total protein detection with Coomassie staining (Fig. 20a) revealed their approximate their sizes as 30, 33, 85, 130, and > 250 kDa. These bands were also apparent in fractions collected from control (uninjected) oocyte samples (Fig. 20c). The Western blot (Fig. 20b) shows anti-DAT detection of the transporter at approximately the 68 and 100 kDa marks, corresponding to the non-glycosylated and glycosylated forms, respectively. There was no hDAT-specific immunodetection in fractions collected from the higher imidazole concentrations.
Figure 19. H6-hDAT solubilized in 1% CHAPS was applied to a nickel resin column for FPLC. Imidazole was passed through the column at gradually increasing concentrations (0-500 mM, red line). As determined by absorption at 280 nm, a significant amount of protein eluted in 250-300 mM imidazole. Inset A: Total protein detection of elution fractions 10, 11, and 12 reveals a heterogeneous mix of proteins. None of these bands were detected by anti-DAT in Western blot, indicating that these elution fractions did not contain any significant traces of the transporter.
Figure 20. Proteins eluted with 150 mM imidazole in three 500 mL fractions. A sample of each fraction was run on a SDS-PAGE gel for Coomassie staining and transferred to a PVDF membrane for Western blot analysis. (a) The Coomassie-stained gel shows a heterogeneous population of proteins in the elution fractions. (b) Immunochemiluminescence assay with 1:1000 rat anti-DAT (Santa Cruz Biotechnology) and 1:5000 goat anti-rat (Thermo-Scientific) reveals two populations of hDAT in the fractions. The 68 kDa band represents a non-glycosylated hDAT while the 100 kDa band corresponds to a highly-glycosylated form of the transporter. (c) Uninjected oocyte membranes that were subjected to identical solubilization (1% CHAPS) and purification procedures yielded elution fractions at 150 mM imidazole containing the non-specific bands present in the H6-hDAT elution fractions. None of these bands were detected by anti-hDAT (not shown).
(b) Detergent screening

Plasma membrane isolated from H6-hDAT-expressing oocytes were solubilized with 0.25% DDM, 0.50% DDM, or 1% CHAPS (Table 2). All samples were solubilized and purified with identical protocols. After eluting with 150 mM imidazole from the nickel affinity column, purity was qualitatively assayed by Coomassie stain and Western blot analysis. All samples were partially-purified. The total protein yield was the highest in samples solubilized in 1% CHAPS. Solubilization in 0.25% DDM provided the least stability, generating significant levels of large protein aggregates that were visible during gel electrophoresis. hDAT molecules in 0.25% DDM were also nonfunctional in the lipid bilayer studies. Samples in 0.50% DDM formed considerably less aggregates during the course of purification but did not generate functional hDAT in lipid bilayers. Solubilization with 1% CHAPS yielded the largest amount of partially-purified protein and was the only condition that produced functional transporter molecules as assayed by the bilayer technique. All downstream investigations proceeded using those samples of H6-hDAT in 1% CHAPS.
Table 3. H6-hDAT-expressing oocyte membranes were solubilized with 0.25% DDM, 0.50% DDM, or 1% CHAPS. The type and concentration of detergent affected the outcome of downstream purification and functional assays. Solubilization with 1% CHAPS yielded the largest amount of partially-purified hDAT and was the only condition to generate functional transporter as assayed by electrophysiological studies.

<table>
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Electron microscopy

Partially-purified H6-hDAT samples were incorporated into unilaminar liposomes formed from a 1:1 ratio of phosphatidylserine (PS) and phosphatidylethanolamine (PE). The resulting proteoliposomes were negatively-stained with 0.75% uranyl formate and resolved with an electron microscope at 50,000 X magnification (Fig. 21a). Most of the unilaminar vesicles had diameters between 50 to 200 nm. Figure 21b displays multimeric protein complexes that could be discerned at the surface of the liposomes.

Figure 21. Negative staining images of H6-hDAT-containing proteoliposomes prepared with 0.75% uranyl formate. (a) At 50,000X magnification, unilaminar proteoliposomes of various diameters are resolved. (b) A closer look at the individual vesicles reveals the presence of multimeric protein structures embedded in the phospholipids (black box).
Lipid bilayer studies

(a) DA-induced microscopic currents

Planar bilayers were synthesized with a 1:1 PE:PS mix and stabilized at –50mV with extracellular solution (high Na\(^\pm\)/high Cl\(^-\)) on the trans side and intracellular solution (low Na\(^\pm\)/low Cl\(^-\)) on the cis side of the membrane. The current trace in Figure 22a depicts the absence of any channel activity in the empty bilayer. Unilaminar proteoliposomes containing partially-purified H6-hDAT were subsequently fused into the bilayer. Without further treatments, no significant channel activity was observed (Fig. 22b). The all-point amplitude histograms of the bilayer and the H6-hDAT-incorporated bilayer were indistinguishable (Fig. 22d). The bilayer histogram fit under a normal Gaussian distribution with \(\mu = -0.00236 \pm 0.000137\), \(r = 0.992 \pm 0.00503\).

Addition of 5 uM DA to the trans side elicited changes in current in accordance with the stochastic opening of channel proteins in response to extracellular dopamine. Figure 22c illustrates the presence of at least two conductance states, \(O_1\) and \(O_2\). The all-points histogram of DA-induced current trace in Figure 22e displays an uneven bell curve that tails off towards the more negative amplitudes, and represents a mixture of more than one Gaussian distributions. An automated comparison of models using \(n = 1, 2, 3, 4, 5,\) or 6 (Clampfit 10.2) predicts the best fit with \(n = 5\); \(\mu_1 = -0.948 \pm 0.617\), \(\mu_2 = -0.463 \pm 0.0839\), \(\mu_3 = -0.125 \pm 0.0372\), \(\mu_4 = 0.0366 \pm 0.00228\), \(\mu_5 = 0.313 \pm 0.0200\); \(r = 0.995 \pm 0.00279\). Single channel search analysis yields an amplitude histogram with at least three distinct Gaussian distributions (Fig. 22f): \(n = 3\); \(\mu_1 = -1.0162 \pm 0.00342\), \(\mu_2 = -0.468 \pm 0.00100\), \(\mu_3 = -0.00535 \pm 0.000938\); \(r = 0.898 \pm 0.0634\).
Figure 22. Electrophysiological recordings of partially-purified H6-hDAT in a planar lipid bilayer at -50 mV. (a) Empty bilayer and (b) reconstituted H6-hDAT do not generate currents. (c) The application of 5 uM DA to the trans side induces channel activity in the reconstituted H6-hDAT. At least two conductance states appear to exist during the displayed time frame of the recording. C, closed state; O1, open state 1; O2, open state 2. (d) All-point amplitude histogram from the bilayer and reconstituted H6-hDAT recordings were nearly identical, forming a normal distribution centered at zero. (e) On the other hand, the all-point amplitude histogram from the recording of reconstituted H6-hDAT in the presence of 5 uM DA yields an uneven distribution. (f) Results from a single channel search generates an amplitude histogram with at least three distinct Gaussian distributions: \( n = 3; \mu_1 = -1.0162 \pm 0.00342, \mu_2 = -0.468 \pm 0.00100, \mu_3 = -0.00535 \pm 0.000938; r = 0.898 \pm 0.0634. \)
(b) Dependence on Na\textsuperscript{+} gradient

The dependence of the DA-induced hDAT microscopic currents on a transmembrane Na gradient was investigated by eliminating the Na chemical gradient across the bilayer; The high Na extracellular solution in the trans compartment was exchanged for the same low Na intracellular solution used on the cis side. Addition of 5 uM DA to the trans side in the absence of the physiologically-equivalent Na gradient produced no significant channel activity in the reconstituted H6-hDAT (Fig. 23b). Reintroducing the initial Na gradient (trans: 138 mM, cis: 8mM) in the absence of DA did not elicit any channel activity (Fig. 23c). Channel activity was recovered in the bilayer after reapplying DA to the trans side in the presence of the Na gradient (Fig. 23d).

Figure 23. Planar bilayer recordings of reconstituted H6-hDAT at -100 mV. (a,d) DA-induced hDAT microscopic currents require both a transmembrane Na gradient and extracellular (trans) DA. (b) In the absence of a Na gradient across the lipid bilayer, 5 uM DA does not elicit channel activity and (c) vice versa. C, closed; O, opened.
(c) Inhibition by cocaine

The ability of a DA-uptake inhibitor, COC, to block hDAT-mediated currents in the bilayer was investigated. The application of 5 uM DA to the trans side of a H6-hDAT-containing planar bilayer induced channel openings (Fig. 24a). The corresponding all-point amplitude histogram in Figure 24b suggests the existence of at least three conducting states. When 10 uM COC was introduced into the trans solution, channel activity was significantly, although not completely, diminished (Fig. 24c). The resulting all-point amplitude histogram in Figure 24d displays a shift of the current amplitudes towards the smallest conducting state and the closed state (0 pA). An analysis of open probabilities followed (Fig. 25). In the presence of 5 uM DA, the mean open probability of reconstituted H6-hDAT was 0.220 (n = 3; S.E. = 0.0770). Upon addition of 10 uM COC, the mean open probability became 0.0505 (n = 3; S.E. = 0.0270).

Figure 24. Planar bilayer recordings of reconstituted H6-hDAT at -50 mV. (a,b) 5 uM DA induces channel activity. (c) Addition of 10 uM COC significantly inhibits the DA-induced microscopic currents. (d) The all-point amplitude histogram depicts a shift of current amplitudes towards the smallest conducting state and the closed state (0 pA). C, closed; O, opened.
Figure 25. Open probabilities of reconstituted H6-hDAT in varying external conditions. No significant conductance was detected in the empty bilayer; No conductance was observed after incorporation of partially purified H6-hDAT and after establishing a physiologically-equivalent Na gradient. Upon application of 5 uM DA, the open probability was $0.220 \pm 0.0770$ and subsequent addition of 10 uM COC diminished the mean open probability to $0.0505 \pm 0.0270$ (means $\pm$ S.E., n=3).
DISCUSSION

To validate the use of hexahistidine-tagged hDAT (H6-hDAT) for the study of the wild type hDAT, DA uptake and electrophysiological assays were conducted prior to purification. Both hDAT- and H6-hDAT-containing liposomes accumulated significantly more \[^{3}H\] DA than the control proteoliposomes after 10, 20, 30, 60, and 600 sec of incubation in 5 uM DA. According to the data (Figure 14), it also appeared that the unmodified transporter took up more DA than the tagged variant. However, it is unclear whether the histidine tag affected the transport ability of the protein based on this experiment alone because the sample size for each condition was limited to two or three. Furthermore, there was no method for determining the number of transporters incorporated into each liposome during the formation of the proteoliposomes; therefore, the discrepancy in \[^{3}H\] DA uptake between hDAT and H6-hDAT may also be a reflection of varying numbers of transporter proteins in action. To address this limitation, future studies must include larger sample sizes for each uptake experiment. Nonetheless, the coapplication of extracellular DA and COC diminished DA translocation to the level of control vesicles in both the hDAT and H6-hDAT samples. The control proteoliposomes, which were formed from solubilized membranes of uninjected oocytes, exhibited equal \[^{3}H\] DA uptake in the presence or absence of COC. The same phenomenon was observed after removing the Na\(^{+}\) gradient across the vesicle membranes: \[^{3}H\] DA uptake by hDAT and H6-hDAT proteoliposomes was decreased to the level of the controls and uptake by the controls was unaffected. These results suggest that any \[^{3}H\] DA accumulation by control vesicles over time was not mediated by dopamine transporters as hDAT-mediated DA uptake is dependent on a Na\(^{+}\) gradient and is inhibited by COC.

Transporter currents measured by two electrode voltage clamp were consistent between hDAT- and H6-hDAT-expressing oocytes. DA, AMPH, and METH elicited inward currents in both tagged and untagged hDAT that were identical in amplitude and kinetics. COC blocked the DA-induced currents as well as the constitutive hDAT leak currents. Rodriguez-Menchaca et al. reported persistent leak currents
in hDAT after long (>30 min) exposures to AMPH. In both hDAT- and H6-hDAT-expressing oocytes, a 1 min application of AMPH induced a current that only partially recovered to baseline after removal of the drug. This persistent leak current did not vanish even after 1 min of washout but was quickly blocked by COC. Therefore, in the whole cell electrophysiological experiments, H6-hDAT exhibited pharmacological responses to multiple drugs that were indistinguishable from that of the untagged transporter.

H6-hDAT membranes were solubilized in three different detergent conditions: 0.25% DDM, 0.50% DDM, and 1% CHAPS. Total protein detection revealed that all three samples, after completion of the purification procedures, were not completely extracted from all the endogenous proteins in the oocyte membrane. Probing with a DAT-specific antibody revealed the presence of the transporter in all three solubilized samples. hDAT was detected at the approximately 68 and 100 kDa mark, which corresponds to the nonglycosylated and glycosylated forms. However, only the glycosylated transporter has been found to be functional. In addition, significant aggregation was observed in 0.25% DDM-solubilized hDAT. As determined by the channel reconstitution method, only the 1% CHAPS-solubilized hDAT remained functional after purification. No DA uptake experiments were performed with the partially-purified samples because of the lack of available protein. Therefore, it is uncertain whether the 0.25% DDM- and 0.50% DDM-solubilized samples would have been functional in the biochemical [3H] DA uptake assays. Previous studies have indicated the presence of at least three ionic conductances through hDAT, including one that is uncoupled to substrate transport.

Negative staining and electron microscopy revealed the successful formation of unilaminar proteoliposomes. At 50,000 X magnification, multimeric protein complexes could be discerned at the surface of multiple vesicles. Because the proteoliposomes were formed from partially-purified samples, the observed protein structures could not be identified as the proposed tetrameric complexes of hDAT. However, these images propose the possibility to detect functional hDAT complexes with confidence.
once proteoliposomes of purified protein are acquired. Consequently, unprecedented structural studies could follow.

H6-hDAT solubilized in 1% CHAPS and purified via nickel affinity chromatography was incorporated into proteoliposomes and ultimately reconstituted into planar lipid bilayers. Single channel events were elicited after application of DA to the extracellular side of the membrane. These same ionic conductances were significantly eliminated when COC was also added to the extracellular solution or when there was no Na\(^+\) gradient across the bilayer. Single channel analysis using the pCLAMP Clampfit software determined the amplitude of single transporters in response to DA to be approximately 0.468 pA at -50 mV. In accordance to:

\[ I = gV \]

the calculated single hDAT conductance is 9.36 pS. Cocaine neither completely removed the DA-elicited activity nor altered the single channel conductance. However, it significantly decreased the open probability of the hDAT channel to 0.0505 ± 0.0270. Together, these results reveal hDAT-associated channels in the artificial bilayer system.

Future directions will involve maximizing protein yield and purity. This may be achieved by incorporating longer polyhistidines tags or different ones such as myc and haemaglutinin HA. If the longer histidine tags are utilized, the transporter will be expressed in systems other than *X. laevis* oocytes to avoid the significant contamination from those endogenous membrane proteins that interact just as strongly with the nickel column during affinity chromatography. Additional purification techniques may be employed as well, including immunoaffinity chromatography using immobilized antibodies and ligands that bind to hDAT with high specificity and affinity. With more protein, more uptake experiments will be possible to compliment the electrophysiological data, allowing for more accurate investigations between hDAT-mediated substrate flux and the substrate-induced hDAT-mediated currents.
LITERATURE CITED


