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Antagonist-Induced Conformational Changes in Dopamine Transporter Extracellular Loop Two Involve Residues in a Potential Salt Bridge

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Abstract

Ligand-induced changes in the conformation of extracellular loop (EL) 2 in the rat (r) dopamine transporter (DAT) were examined using limited proteolysis with endoproteinase Asp-N and detection of cleavage products by epitope-specific immunoblotting. The principle N-terminal fragment produced by Asp-N was a 19 kDa peptide likely derived by proteolysis of EL2 residue D174, which is present just past the extracellular end of TM3. Production of this fragment was significantly decreased by binding of cocaine and other uptake blockers, but was not affected by substrates or Zn^{2+} , indicating the presence of a conformational change at D174 that may be related to the mechanism of transport inhibition. DA transport activity and cocaine analog binding were decreased by Asp-N treatment, suggesting a requirement for EL2 integrity in these DAT functions. In a previous study we demonstrated that ligand-induced protease resistance also occurred at R218 on the C-terminal side of rDAT EL2. Here using substituted cysteine accessibility analysis of human (h) DAT we confirm cocaine-induced alterations in reactivity of the homologous R219 and identify conformational sensitivity of V221. Focused molecular modeling of D174 and R218 based on currently available *Aquifex aeolicus* leucine transporter crystal structures places these residues within 2.9 Å of one another, suggesting their proximity as a structural basis for their similar conformational sensitivities and indicating their potential to form a salt bridge. These findings extend our understanding of DAT EL2 and its role in transport and binding functions.

Keywords

cocaine; amphetamine; methamphetamine; SCAM; molecular modeling

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1. INTRODUCTION

1.1 The dopamine transporter

Clearance of dopamine (DA) from the synapse is regulated by the dopamine transporter (DAT), a $\text{Na}^+\text{-Cl}^-$ dependent transporter that couples the energy of downhill ion flow to transmitter translocation. DAT plays a critical role in neuronal homeostasis (Giros et al., 1996; Mager et al., 1994) and is targeted by addictive drugs such as cocaine, amphetamine (AMPH), and methamphetamine (METH) (Amara and Kuhar, 1993; Pramod et al., 2013). These drugs affect transport by distinct mechanisms, as cocaine and other uptake blockers bind to DAT and prevent DA translocation, while AMPH and other substrates compete with DA for uptake and induce transmitter efflux (Sulzer, 2011). Different classes of inhibitors induce varying neurochemical, behavioral, and molecular properties, indicating the potential to develop pharmacological reagents for treatment of drug addiction and other dopamine imbalance disorders (Andersen et al., 2009a; Dutta et al., 2003; Henry and Blakely, 2008; Li et al., 2011; Loland et al., 2008; Schmitt and Reith, 2010; Tanda et al., 2009).

DAT and the related norepinephrine and serotonin transporters (NET and SERT) are members of the SLC6 family of symporters that are composed of 12 transmembrane spanning domains (TMs) connected by extracellular and intracellular loops (ELs and ILs) (Fig. 1A) (Broer and Gether, 2012; Kristensen et al., 2011; Pramod et al., 2013). Substrates are translocated by an alternating access mechanism in which the protein cycles through outwardly and inwardly facing states that allow solutes to enter or exit the permeation pathway from opposite sides of the membrane (Forrest and Rudnick, 2009; Jardetzky, 1966). These forms are generated by the coordinated opening and closing of extracellular and intracellular gates that control substrate access and direction of movement (Kniazeff et al., 2008). The structures of some of these conformations have been captured through crystallization of the *Aquifex aeolicus* leucine transporter (LeuT_{Aa}) in different phases of the cycle, providing templates for computational modeling of DAT and other homologous mammalian transporters (Krishnamurthy and Gouaux, 2012; Singh et al., 2008; Yamashita et al., 2005; Zhou et al., 2009). Recently, *Drosophila* DAT (dDAT) complexed with the antidepressant nortriptyline was crystallized in an 'outward-open' conformation (Penmatsa et al., 2013), although stabilization of the protein for crystal formation required deletion of 43 amino acids from EL2 and inclusion of five thermostable mutations. The modified dDAT was inactive for transport and lacked the functionally relevant zinc binding site present in mammalian DATs formed by residues from EL2 and EL4 (Norgaard-Nielsen and Gether, 2006; Stockner et al., 2013), which may limit the application of its structure to mammalian DAT. Recently, a valid computational model of hDAT EL2 in the outward-facing transporter conformation has been constructed using the molecular constraints provided by the zinc binding site and conserved disulfide bond (Stockner et al., 2013).

1.2 Substrate and antagonist binding sites on DAT

Substrate binding in LeuT_{Aa} occurs in a pocket referred to as S1 that is formed between the extracellular and intracellular gates (Yamashita et al., 2005). This site is formed from residues in TMs 1, 3, 6, and 8, and similar regions of DAT, NET, and SERT have been implicated substrate binding and transport. Some findings also support the presence of and

S2 substrate site on the extracellular side of the extracellular gate in both LeuT_{Aa} and mammalian transporters (Piscitelli et al., 2010; Plenge et al., 2012; Quick et al., 2012; Shi et al., 2008; Singh et al., 2007; Wang et al., 2012; Zhou et al., 2009). Findings obtained from mutagenesis approaches showing interaction of DAT and SERT inhibitors with residues in TM1, TM3, TM6, and TM8 (Andersen et al., 2009b; Beuming et al., 2008; Chen et al., 1997; Field et al., 2010; Henry et al., 2003; Henry et al., 2006; Kitayama et al., 1992; Lin et al., 2000), adduction of irreversible cocaine analogs to DAT near S1 residues in TM1 and TM6 (Akula Bala et al., 2012; Parnas et al., 2008; Vaughan et al., 2005), and molecular modeling of cocaine analog binding (Beuming et al., 2008), strongly support the binding of neurotransmitter transport inhibitors in S1. Further support for high-affinity antagonist binding to S1 comes from recent crystal structures of a LeuT engineered with SERT residues in the central substrate binding pocket (Wang et al., 2013) and from dDAT complexed with nortipityline (Penmatsa et al., 2013). Some computational studies however, suggest that inhibitors can also bind at S2 (Hill et al., 2011; Huang et al., 2009; Kristensen et al., 2011; Plenge et al., 2012; Plenge and Wiborg, 2005; Pramod et al., 2013; Shi et al., 2008).

1.3 Conformational changes in DAT induced by antagonist binding

The conformational changes that occur in DAT during the transport cycle establish the transport kinetic rate, overall level of DA clearance, and strength of neurotransmission, and are affected by regulatory mechanisms that may become disrupted in dopaminergic disorders and drug abuse (Pramod et al., 2013; Schmitt and Reith, 2010; Vaughan and Foster, 2013). These events are not fully explained by information gleaned from static transporter crystal structures, and their elucidation remains an important area of research. Biochemical and molecular approaches used to probe structural rearrangements of neurotransmitter transporters include protease- and alkylation-protection analyses and the substituted cysteine accessibility method (SCAM), (Chen et al., 2000; Chen et al., 2004; Ferrer and Javitch, 1998; Hastrup et al., 2003; Loland et al., 2004; Norregaard et al., 2003; Reith et al., 2001; Reith et al., 1996; Wenge and Bonisch, 2013; Xu et al., 1997). Our lab previously described a pronounced reduction in the sensitivity of rat (r) DAT EL2 residue R218 to proteolysis by the arginine/lysine specific protease trypsin in response to binding of uptake blockers that we attributed to conformational movements generated during transport inhibition (Gaffaney and Vaughan, 2004).

Here we continue our analysis of uptake blocker-induced changes in EL2 using the aspartic acid specific enzyme endoproteinase Asp-N and identify ligand-induced conformational sensitivity of D174, a residue just C-terminal to the extracellular end of TM3. Comparative modeling of DAT and LeuT_{Aa} places D174 and R218 in close proximity, suggesting a structural basis for their similar uptake inhibitor sensitivities and indicating their potential to form a salt bridge. Using SCAM we examine the regions around rDAT D174 and human (h) DAT R219 and identify conformational activity of hDAT V221. Similar to previous findings obtained with trypsin, we show that DA transport and cocaine analog binding activities are decreased after Asp-N cleavage of EL2, suggesting a role for this domain in these functions. These findings suggest that conformational changes in this region of EL2 following

antagonist binding may represent part of the transport inhibition mechanism and add to our understanding of an under-characterized region of DAT.

2. MATERIALS AND METHODS

2.1 Tissue preparation and proteolysis

Male Sprague Dawley rats (175–300 g) were decapitated and the striatum was quickly removed, weighed, and placed in ice-cold sucrose phosphate buffer (SP) consisting of 0.32 M sucrose, 10 mM sodium phosphate, pH 7.4. The tissue was disrupted with a Polytron homogenizer and centrifuged at $20,000 \times g$ for 10 min at 4 °C. The resulting membranes were washed twice and resuspended to 20 mg/ml original wet weight (o.w.w.) in ice-cold SP buffer. Equal volumes (25 μ l) of membranes and endoprotease Asp-N (1–5 μ g/ml final) prepared in SP buffer were gently mixed and incubated for 45 min at room temperature. Proteolysis was stopped by addition of 500 μ l of ice-cold SP buffer, membranes were centrifuged at $15,000 \times g$ for 8 min at 4 °C and the supernatant was removed. The resulting pellet was solubilized in sample buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris-HCl, pH 6.8) at 20 mg/ml o.w.w. and subjected to electrophoresis and immunoblotting. For experiments testing the effects of ligands on proteolysis, striatal membranes were incubated on ice for 1h in the presence or absence of DAT uptake inhibitors (2 μ M), substrates (30 μ M), or $ZnCl_2$ (10 μ M) followed by addition of Asp-N. For sodium replacement studies SP buffer was prepared with 10 mM monobasic/dibasic potassium phosphate. Proteolysis of DAT was quantified as described below, with statistical evaluation of proteolysis performed using ANOVA with significance set at $p < 0.05$. All experiments were performed three or more times.

2.2 Immunoblot analysis

Solubilized striatal membranes (25 μ l) were electrophoresed on 4–20% Tris/glycine polyacrylamide gels and transferred to 0.2 μ m PVDF membranes. DAT and its proteolytic fragments were detected by immunoblotting as previously described (Gaffaney and Vaughan, 2004) with mouse monoclonal antibody 16 (mAb 16; EMD Millipore; 1:1000 dilution) generated against rDAT N-terminal tail amino acids 42–59 or goat polyclonal antibody raised against rDAT C-terminal tail amino acids 601–619 (Research Diagnostic Inc.; 1:100 dilution). Bound antibodies were detected with anti-mouse or anti-goat IgG 2° antibodies linked to alkaline phosphatase (1:5000 dilution) and membranes were developed with the alkaline phosphatase substrate, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue (BCIP/NBT). Blots were dried, scanned, and quantified using LumiAnalyst software (Roche/Boehringer-Mannheim). Specificity of mAb 16 immunostaining was verified by preabsorbing antibody with 30 μ g/ml peptide 16, using 30 μ g/ml peptide 5 (a.a. 225–236) as a negative control. Tissue linearity experiments verified that mAb 16 signal intensity was linear between 0.1 and 10 mg/ml tissue (not shown).

2.3 Quantification of DAT proteolysis

Immunoblots were scanned at 600 dpi with an Epson Perfection 12000U scanner and saved as grey-scale images (.tif). Grey scale values were converted to Boehringer light units by LumiAnalyst 3.0 software. Proteolysis of DAT was quantified dividing the

immunoreactivity of the 80 kDa DAT form by the combined immunoreactivity of all DAT bands (full length protein and proteolytic fragments), with results converted to percent and subtracted from 100%. This allowed for correction of low amounts of endogenous proteolysis observed in some experiments, and served as an internal loading and transfer control. PeptideCutter (Expasy) was used to determine the calculated M_r of peptide fragments.

2.4. Deglycosylation analysis

DAT and DAT Asp-N fragments were immunoprecipitated with polyclonal antibody 16 as described previously (Foster et al., 2002). Protein A sepharose beads containing the immune complex were incubated with 1.5 units of glycopeptidase-F (PNGF) for 18h at 22 °C to deglycosylate DAT (Vaughan and Kuhar, 1996). Beads were washed twice with immunoprecipitation buffer (50 mM Tris-HCl, 0.1% Triton X-100) and proteins were eluted with sample buffer followed by immunoblotting with mAb 16.

2.5 Asp-N activity assay

The Asp-N peptide substrate Azocoll[®] which generates a blue product when cleaved was used to determine the activity of Asp-N in the presence or absence of 5 μ M cocaine, GBR 12909, mazindol, benztropine, ZnCl₂, dopamine, or amphetamine. Azocoll[®] was incubated with 1–5 μ g/ml Asp-N at 37 °C for 15 min in the presence of ligands, particulates were removed by filtration through a Whatman No. 1 filter and the absorbance of the filtrate was measured at 520 nm in a Molecular Devices SpectraMax 190 spectrometer. Absorbance was linear with enzyme concentration and activity was not affected by any of the DAT compounds tested (not shown).

2.6 [³H]CFT binding and [³H]DA uptake

For binding assays rat striatal membranes were treated with or without 5 μ g/ml Asp-N for 45 min at 22 °C, followed by addition of ice-cold SP buffer, centrifugation, and removal of supernatant. Membrane pellets were resuspended in SP buffer to a concentration of 6 mg/ml o.w.w. and triplicate samples were incubated on ice with 2 nM [³H]2 β -carbomethoxy-3 β -(4-fluorophenyl)tropane ([³H]CFT) for 2h with non-specific binding determined by addition of 100 μ M (–)-cocaine. Reactions were terminated by rapid vacuum filtration using a Brandel tissue harvester over Whatman GF/B glass fiber filters soaked in 0.1% BSA for 2h. Filters were counted in a Beckman model 1600 scintillation counter. For uptake assays P₂ synaptosomal fractions were prepared in SP buffer from freshly dissected rat striatum (Krueger, 1990), and resuspended at 6 mg/ml o.w.w. in ice-cold SP buffer. Aliquots were treated with or without 1 μ g/ml of Asp-N for 45 min and dispensed into assay tubes. Dopamine uptake assays were performed in triplicate in modified Krebs phosphate buffer (16 mM potassium phosphate, 126 mM NaCl, 4.8 mM KCl, 1.4 mM MgSO₄, 10 mM glucose, 1.1 mM ascorbic acid, and 1.3 mM CaCl₂, pH 7.4) containing 10 nM [³H]dopamine plus 100 nM dopamine (Vaughan et al., 1997) with non-specific uptake determined by addition of 100 μ M (–)-cocaine. Uptake assays were initiated by addition of 100 μ l of synaptosomes to the reaction tube and conducted for 5 min at 30 °C. Uptake was stopped by addition of 5 ml of ice-cold SP buffer and immediate vacuum filtration using a Brandel tissue harvester over a Whatman GF/B filter soaked for 2h in 0.1% BSA. Filters were

counted using a Beckman model 1600 liquid scintillation counter. Aliquots of each sample were subjected to immunoblotting to determine the extent of DAT proteolysis. Results were analyzed by student's t-test with significance set at $p < 0.05$.

2.7 SCAM analysis

The expression plasmid for hDAT E2C with two extracellularly facing Cys residues (C90 and C306) mutated to Ala was the generous gift of Dr. Jonathan Javitch, Columbia University and was used for analysis of R219 and flanking residues. The homologous rDAT E2C (C90A, C305A) was generated in our lab for analysis of D174 and flanking residues. E2C and Cys mutations in the E2C background were made using the Stratagene QuikChange® kit with codon substitution verified by sequencing (Alpha Biolabs; Northwoods DNA). For production of human stable transformants, GripTight cells™ (Invitrogen) were transfected using FuGENE, (Roche Applied Bioscience) and pooled lines were maintained under selection with 250 µg/ml Hygromycin. For production of stable rat transformants, Lewis Lung Carcinoma Porcine Kidney (LLC-PK₁) cells were transfected using X-tremeGENE, (Roche Applied Bioscience) and pooled lines were maintained under selection with 800 µg/ml G418. For western blotting, cells were lysed with solubilization buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 5 mM NEM, and 1% Triton X-100), protein content was determined using the BCA method and 10 µg of total protein was immunoblotted with mAb 16 for rDAT or mAb 369 (Chemicon; Temecula, CA) for hDAT.

For activity assays cells expressing h/rDAT E2C or E2C Cys mutants were grown on 12- or 48-well plates to 90–95% confluency. Cells were washed 2X with Krebs-Ringer HEPES buffer (25 mM HEPES, 1.2 mM KH₂PO₄, 125 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 5.6 mM glucose, and 1.3 mM CaCl₂, pH 7.4). For binding assays cells were incubated with 1 nM (12-well) or 10 nM (48-well) [³H]CFT for two hours on ice, rinsed twice with ice-cold KRH, and solubilized in 1% SDS. For uptake assays, cells were incubated for 10 min at 37 °C with 10 nM [³H]DA plus 3 µM DA. Non-specific binding and transport were determined using 100 µM (–)cocaine. Cells were washed twice with ice-cold KRH, solubilized in 1% SDS, and analyzed for radioactivity by scintillation counting. Uptake and binding values were normalized for protein content and results for mutants are indicated as percent of E2C activity set to 100%. For SCAM analysis MTSET, MTSES, or MTSEA (Biotium; Hayward, CA) were prepared immediately prior to use, and added to the cells (1 mM final) for 10 minutes at room temperature. For cocaine protection assays cells were incubated with 10 µM (–)cocaine for 10 min prior to MTSET addition. Following incubation the cells were washed 3 times with KRH and assayed for [³H]CFT binding or [³H]DA transport.

2.8 Molecular modeling

To determine distance between residues L126 and S150 that are homologous to D174 and R218 in rDAT, the X-ray coordinates for the second extracellular loop (EL2) of LeuT were analyzed from the crystal structures representing the 'apo-out' (PDBID: 3TT1), 'open-to-out' (3F3A), 'outward-occluded' (2A65), 'outward-occluded' (3TU0), 'apo-in' (3TT3), and 'occluded with sertraline-bound' (3GWU). With the exception of the apo-inward structure, residues 132 through 135 were unresolved and were therefore re-built using Prime 3.1 in Schrödinger suite (Schrödinger LLC), with the native crystal structures as templates. The

resulting structures differed only 0.000 to 0.005 RMSD from their respective templates. The mutations L126D and S150R were introduced and EL2 structures modeled using Rosetta Backrub which allows for focused flexible backbone modeling (Smith and Kortemme, 2008). The top scoring Backrub models were analyzed for distances between D126 and R150. Possible backbone-dependent rotamers for D126 and R150 were analyzed in PyMol. Figures were generated using PyMol. (The PyMOL Molecular Graphics System (Schrödinger, LLC.))

2.9 Materials

Endoproteinase Asp-N and Azocoll® were purchased from Calbiochem (San Diego, CA). Bovine serum albumin, Tris base, sodium phosphate and potassium phosphate were from Fischer Scientific (Pittsburgh, PA). GBR 12909, β -CFT, nomifensine, mazindol, benztropine, and (–)-cocaine were from Sigma/RBI (Natick, MA). (+)-cocaine was a generous gift from Maarten E.A. Reith (New York University School of Medicine, New York, NY). [^3H]CFT (specific activity 76 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Boston, MA) and [^3H]DA (specific activity 45 Ci/mmol) was from Amersham Biosciences (Piscataway, NJ). All other chemicals were obtained from Sigma (St. Louis, MO) or as indicated. Rats were purchased from Charles River Laboratories (Wilmington, MA). All animals were housed and treated in accordance with regulations established by the National Institutes of Health and approved by the University of North Dakota Institutional Animal Care and Use Committee.

3. RESULTS

3.1 Endoproteinase Asp-N digestion of rat DAT

In a previous study we found that *in situ* proteolysis of $^{32}\text{PO}_4$ -labeled rat striatal membranes with Asp-N generated a phosphorylated 19 kDa DAT fragment that precipitated with N-terminal polyclonal Ab16 (Foster et al., 2002), suggesting that Asp-N could provide a suitable enzyme for analysis of DAT proteolysis. In the N-terminal region of the protein rDAT contains two Asp residues (D68 and D79) that are embedded in TM1 and inaccessible to protease during *in situ* treatments, and six that are in EL2 (D174, D191, D199, D205, D230 and D231) (Fig. 1A). To develop an immunoblot assay for detection of Asp-N fragments that could be used to test effects of ligands we examined the overall Asp-N digestion pattern using both N-terminal (mAb 16) and C-terminal (a.a. 601–619) antibodies (Fig. 1B, left). In control membranes both antibodies detected full length DAT migrating at ~80 kDa (arrow a). In Asp-N treated membranes the primary N-terminal proteolysis product was a 19 kDa fragment (arrow d) that corresponded to the phosphorylated peptide we identified by immunoprecipitation, and a lower intensity 32 kDa cleavage product (arrow b) was also produced in some but not all experiments. Because there are no Asp residues in EL1, IL1, or the N-terminal tail and those in TM1 are protected from proteolysis, these fragments extend from the transporter N-terminus to the cleavage site. The mass of the 19 kDa band is thus consistent with proteolysis at D174 (calculated M_r 19,243), while that of the 32 kDa band is consistent with proteolysis at D191 or D199, which would generate fragments of calculated protein M_r 21,244 and 22,032 with the remaining mass contributed by N-linked carbohydrates on N181, N188, or N196 (Fig. 1A). The 32 kDa fragment was

not more prominent than the 19 kDa fragment at lower Asp-N doses or treatment times, or seen in its absence, suggesting that D174 is the preferred site of proteolysis with occasional missed cleavages producing the larger peptide. Immunoblotting of Asp-N digests with the C-terminal mAb detected a 30 kDa fragment (arrow c) that is similar in mass to a 32 kDa C-terminal fragment produced by cleavage of R218 (Gaffaney and Vaughan, 2004; Vaughan and Kuhar, 1996), and is thus consistent with proteolysis of D205, D230, or D231.

The specificity of N-terminal fragment immunoreactivity was confirmed by preabsorbing mAb 16 with its antigenic peptide (peptide 16), which blocked staining of full-length DAT and all fragments (Fig. 1B, middle), while inclusion of peptide 5 (rDAT a.a. 225–236) had no effect. To further characterize the fragments we treated control and Asp-N proteolyzed DATs with glycopeptidase-F to remove N-linked carbohydrates (Fig. 1B, right). This reduced the mass of the full-length protein by ~20–25 kDa as previously shown (Li et al., 2004; Vaughan, 1995), but did not affect the mass of the 19 kDa fragment. This result strongly indicates that the 19 kDa fragment is produced by cleavage of D174, which is the only Asp residue in EL2 that is N-terminal to all glycosylation sites. We note that we have been unable to drive *in situ* proteolysis of heterologously expressed DATs and thus have not been able to confirm usage of protease sites by site-directed mutagenesis.

3.2 Uptake blockers reduce Asp-N proteolysis

To determine if uptake blockers or substrates affect the Asp-N sensitivity of DAT, rat striatal membranes were incubated with various DAT ligands during protease treatment (Fig. 2). In control membranes, Asp-N treatment caused robust production of the 19 kDa fragment (Fig. 2A), with DAT proteolysis levels averaging $62 \pm 4\%$ (Fig. 2B). Incubation of membranes with the DA uptake inhibitors (–)-cocaine, GBR 12909, mazindol, nomifensine, or β -CFT strongly inhibited production of the 19 kDa fragment, with proteolysis levels ranging from 12 ± 5 to $33 \pm 6\%$ (all $p < 0.05$ to $p < 0.001$ relative to control). Proteolysis was not affected by the inactive cocaine stereoisomer (+)-cocaine or by the NET inhibitor desipramine (not shown), demonstrating the pharmacological specificity of the effect. In contrast to the effects of transport inhibitors, incubation of membranes with the substrates DA, AMPH, or METH, or with Zn^{2+} , did not affect DAT proteolysis (all $p > 0.05$ relative to control) (Figs. 2A and 2B).

To further verify that the reduced proteolysis of DAT was due to ligand binding, we tested for the effects of Asp-N in the absence of Na^+ (Fig. 3), which significantly reduces the affinity of DAT for cocaine (Wang et al., 2003). In Na^+ -containing buffer, DATs showed robust proteolysis that was strongly inhibited by uptake blockers (Figs. 3A and 3B). However, when K^+ was substituted for Na^+ , uptake blockers did not prevent proteolysis, consistent with reduced ligand affinity resulting in lack of transporter conformational change. No difference was found in the levels of control DAT proteolysis in the presence or absence of Na^+ , indicating that Na^+ binding alone does not induce transporter conformational changes detectable with this assay. Finally, as an additional control, we determined that none of the DAT blockers or substrates tested affected Asp-N cleavage of a synthetic substrate (not shown), indicating that reduced production of DAT fragments by

ligands was not the result of Asp-N inhibition. Together these results demonstrate that binding of uptake blockers but not substrates or Zn^{2+} leads to reduced proteolysis of D174.

3.3 Asp-N treatment disrupts DAT function

To determine if proteolysis of EL2 affects DAT function, rat striatal membranes or synaptosomes were treated with Asp-N followed by assessment of [^3H]CFT binding (Fig. 4A) or [^3H]DA transport (Fig. 4B). Immunoblotting of membranes and synaptosomes confirmed production of Asp-N fragments with proteolysis levels averaging $18 \pm 3\%$. After Asp-N treatment, binding and transport activities of DAT were reduced by $31 \pm 6\%$ and $20 \pm 4\%$, respectively, roughly correlating with the extent of digestion, and suggesting that loss of EL2 integrity leads to reduction of DAT transport and binding functions.

3.4. SCAM analysis of EL2 residues

We then performed SCAM analysis of the regions around rDAT D174 and hDAT R219 to further assess the conformational sensitivity of these residues and to examine flanking sequences. For analysis of the N-terminal side of EL2 we generated an rDAT E2C construct as the background for insertion of Cys mutations at residues 171–177 (F171, T172, M173, D174, L175, P176, and W177) (Fig. 5). The corresponding human sequence (FTTELPW) differs slightly but retains conservation of the negative charge at Glu174. rDAT E2C displayed uptake and binding activities that were similar to those of the WT protein (not shown). Immunoblotting showed that all mutants expressed full-length DAT at ~20–100% of E2C levels except for W177C which showed no mature protein (Fig. 5A). A pronounced band that migrated at ~200 kDa was present in T172C, T173C and D174C forms. As surface projections indicate that these residues are at the transporter surface (not shown), this suggests the possibility for disulfide bond formation to occur between DAT monomers via the inserted cysteines. [^3H]DA uptake and [^3H]CFT binding activities of these mutants roughly paralleled their expression levels, suggesting that kinetic properties were not strongly altered by the mutations. Consistent with its lack of expression, W177C showed 5% of E2C levels of transport and binding activity (Fig. 5B), and could not be further analyzed. The remaining mutants were analyzed by SCAM using [^3H]CFT binding as the functional read-out. Treatment of cells with the positively charged MTS reagent MTSET caused only slight (~5–20%) reductions in binding for mutants at positions 172–176 and larger but more variable reduction ($31 \pm 19\%$) for F171C, none of which were significantly different from E2C (all $p > 0.05$), and inclusion of (–)-cocaine during the treatment did not alter these effects (Fig. 5C). [^3H]DA transport activity of D174C was also not affected by MTSET, and [^3H]CFT binding activity for D174C was not significantly affected by the neutral and negatively charged MTS reagents MTSES and MTSEA (not shown). Pull down experiments showed that MTSEA biotin reacted with D174C (not shown) indicating that the residue was accessible to the reagent. These results thus suggest that binding and transport activities of these DAT forms were not affected by modification of the inserted cysteines.

For analysis of the C-terminal side of EL2 we used the hDAT E2C construct (Loland et al., 2004) as the background for insertion of Cys mutations at residues 210–226 (Fig. 6). This sequence is identical in hDAT and rDAT. The mutants were analyzed by immunoblotting and [^3H]CFT binding, and in some cases for [^3H]DA transport (Figs. 6A and 6B). E215C,

Y216C and F217C forms showed little to no expression, [^3H]DA uptake, or [^3H]CFT binding, and could not be analyzed by SCAM. The remaining mutants showed ~15–100% of E2C levels of [^3H]CFT binding (Fig. 6B), which roughly corresponded to the protein expression levels, suggesting that the mutations did not strongly impact binding characteristics. The proportionally lower uptake levels for E218C and R219C compared to binding could be consistent with impairments in transport or surface expression, although further work will be necessary to confirm the mechanism. Using [^3H]CFT binding as the functional read-out, we found that MTSET caused ~60% reduction in binding for A213C; ~20–30% reductions in binding for R219C, G220C, V221C, L222C, and H223C; ~10–15% increases in binding for T211C, P212C, L224C, and Q226C; and lesser changes for the remaining residues (Fig. 6C). Statistically different changes relative to that of E2C were seen for A213C ($p < 0.001$), R219C ($p < 0.01$), and V221C, L223C, and H223C ($p < 0.05$), indicative of modifications that altered binding. Inclusion of (–)-cocaine during the MTSET treatment did not alter the binding changes at most of these residues, but significantly reduced the inhibition of binding at R219C ($p < 0.001$) and V221C ($p < 0.05$) (Fig. 6C), confirming the conformational sensitivity of R218/219 to cocaine and identifying cocaine-induced conformational changes at V221.

3.5 Molecular modeling

To further understand the significance of these findings we performed molecular modeling of D174 and R218 using homology to LeuT_{Aa} structures solved in the ‘apo-out’, ‘open-to-out’, ‘outward-occluded’, ‘inward-occluded’, ‘apo-in’, and ‘occluded with sertraline-bound’ forms that represent different states of the transport cycle (Krishnamurthy and Gouaux, 2012; Singh et al., 2008; Yamashita et al., 2005; Zhou et al., 2009) (Fig. 7). The LeuT_{Aa} residues homologous to D174 and R218 are L126 and S150, and their positions have been solved in all LeuT_{Aa} crystal structures, providing a reasonable template for low level modeling of D174 and R218. The results revealed that L126 and S150 were present within ~11 Å of each other (Fig. 7A), but showed no significant differences in proximity or orientation in any of the transporter forms (Fig. 7B), consistent with a lack of movement during transporter cycling or ligand binding in LeuT_{Aa}. We then substituted LeuT_{Aa} residues L126 and S150 *in silico* with D and R to model D174 and R218 positions. The structures were subjected to refinement using Rosetta Backrub which is designed to sample and energy-minimize local backbone and side chain conformations following residue substitution (Smith and Kortemme, 2008). A manual backbone-dependent rotamer search revealed that the longer R and D side chains can come within 2.9 Å of one another (Fig. 7C), suggesting the possibility of an ionic interaction between these two residues in DAT. Similar to the endogenous LeuT_{Aa} residues, however, the relative positions of D174 and R218 side chains showed no significant variations across the *in silico* mutated models (Fig. 7D).

4. DISCUSSION

4.1 Conditions associated with EL2 proteolysis and SCAM protection

Our findings from protease and SCAM analyses suggest that rDAT/hDAT EL2 residues D174, R218/219, and V221 undergo conformational changes in response to binding of uptake blockers or achieve protection indirectly through conformational changes of nearby

residues. Because the effects were not obtained with substrates, it is possible that the changes may contribute to the mechanism of transport inhibition, for instance by promoting outward conformations thought to bind to or be stabilized by cocaine (Loland et al., 2004), or by otherwise impacting structural rearrangements needed for transport. Comparative modeling places these residues in close three-dimensional proximity, which could provide a structural basis for their similar sensitivities to uptake blockers, and indicates the potential for D174 and R218 to form a salt bridge. We found no ability of substrates to induce protease resistant forms, but because substrates were analyzed in binding rather than transport conditions, it cannot be excluded that these states might exist during post-binding phases of the transport cycle. Similar to our previous results with tryptic proteolysis of R218, we found no induction of D174 resistance to Asp-N by Zn^{2+} , which slows DA transport by suppressing conformational movements needed for substrate translocation (Norregaard et al., 1998; Stockner et al., 2013). This suggests that inhibition of transport induced by Zn^{2+} and by pharmacological blockers occurs via distinct mechanisms that might be therapeutically exploited if more fully understood.

4.2 Potential mechanisms underlying EL2 protease and SCAM protection

It is not known how the conformational information from ligand binding is transmitted to these residues or if the D174 and R218 changes occur independently or via charge interactions. TM3 directly interacts with uptake blockers (Chen et al., 1997; Henry et al., 2003; Penmatsa et al., 2013), suggesting the likelihood for direct transmission of ligand-induced conformational changes to D174, although the specific nature of these changes is unknown. R218 and V221 are predicted by PsiPred and JUFO to be present in an α -helical conformation (Fig. 1A). In LeuT_{Aa} crystal structures, the homologous helical domain undergoes significant compression and rotation during the 'apo-out' to 'apo-in' transitions (Krishnamurthy and Gouaux, 2012) that could alter accessibilities of constituent residues. Although modeling in LeuT_{Aa} templates supports D174-R218 salt bridge interactions in all transporter states, leak conductance (Mager et al., 1994) and SCAM studies of SERT (Chen et al., 1997) indicate the occurrence of dynamic conformational changes in the absence of substrate. Similar reorientations in resting DATs could intermittently alter D174 and R218 interactions to allow protease access, while inhibitors that block transport may stabilize a conformation that locks the D174-R218 interaction. Because Asp-N and trypsin require binding of the charged side chains to position the scissile bond in the catalytic site, formation of the salt bridge could suppress enzyme recognition. Such alterations in trypsin resistance and sensitivity in response to salt bridge formation and disruption have been reported (Rajabi et al., 2008).

4.3 Potential mechanisms underlying differences observed in DAT functional analyses and comparative modeling

Whatever the mechanism responsible for D174 and R218 protease resistance, our findings indicate that molecular modeling of D174 and R218 in LeuT_{Aa} templates, which identifies no significant structural alterations of these residues, does not accurately represent the properties of DAT, in which these residues clearly undergo either direct or indirect ligand-induced alterations. These inconsistencies may result from differences between DAT and LeuT_{Aa} in EL2 structure and/or ligand binding site. EL2 of DAT differs considerably from

that of LeuT_{Aa} by containing 21 additional residues, a disulfide bond, multiple N-linked glycosylation sites, and Zn²⁺ coordinating residues (Chen et al., 2007; Li et al., 2004; Meinild et al., 2004; Norgaard-Nielsen et al., 2002; Stockner et al., 2013). Few of these elements have been solved at high resolution, and their contributions to EL2 structure are incompletely understood. It is conceivable that given these large differences, EL2 of DAT may undergo distinct or greater conformational changes than that of LeuT_{Aa} and that even small rotations in connecting elements could alter D174-R218 interactions. Such conformational changes in EL2 are supported by computational models of this domain based on well-defined molecular constraints which show that the zinc binding site present in the outward-facing form of DAT is destabilized in the inward-facing structure, due in part to movement of EL2 (Stockner et al., 2013). In addition, in the sertraline-bound form of LeuT_{Aa} the ligand is complexed at low affinity in the S2 site, which may not accurately represent structures produced by high affinity and/or S1 binding of cocaine or other inhibitors in DAT. Dose-response studies of antagonist-induced R218 protease resistance showed strong agreement with published ligand affinities, indicative of responses to high affinity binding (Gaffaney and Vaughan, 2004). Previously the only insight into competitive binding at S1 was the Trp-bound LeuT_{Aa} co-crystal (Singh et al., 2008), presenting the possibility that binding of larger molecules such as cocaine at S1 could induce distinct conformational changes in EL2. In fact, in the dDAT-nortriptyline co-crystal (Penmatsa et al., 2013) the residues homologous to D174 and R218 are almost twice as far apart (19.7 Å) as compared to LeuT (11.1 Å) (Fig. 7E), suggesting differences between DAT and LeuT_{Aa} in EL2 structure or conformational responsiveness. While the distance between D174-R218 in this inhibitor-bound dDAT structure is too great to allow salt bridge interactions, it is possible that the native EL2 structure is not accurately represented due to the removal of 43 residues needed to crystalize the protein. In addition, the dDAT residue homologous to rDAT D174 is Ser, which may also impact the final loop structure and the potential for this site to interact with R218. Thus many questions remain to be addressed with respect to these issues.

4.4 EL2 and antagonist binding in neurotransmitter transporters

Our current and previous findings that conditions that cleave EL2 but leave the resulting N- and C-terminal segments of the protein largely intact lead to reductions of DA transport and CFT binding suggest that EL2 is important for overall transporter structure and/or contributes a functional property that is lost after proteolysis. A connection between EL2 and transport inhibitor binding was also recently identified in hNET (Wenge and Bonisch, 2013). Alkylation of hNET EL2 residue His222 suppressed binding of the uptake inhibitor [³H]nisoxetine, although mutation of this residue was without effect. This suggested that the residue was not directly involved in binding but that its alkylation sterically blocked access of nisoxetine to its binding pocket, implicating its proximity to the binding pathway or the binding site. This residue is conserved in r/hDAT (H224/225) and is just downstream of R218/219 in the EL2 α -helix, suggesting a potential structural similarity and further supporting a mechanistic connection between this region of EL2 and uptake inhibitor binding.

4.5 Conclusion

High-resolution insight into neurotransmitter transporter molecular mechanisms has been obtained by homology comparison to the LeuT_{AA} and dDAT ligand binding sites, but lack of information on EL and IL structures has delayed progress in understanding the roles of these domains. Though EL2 has typically been thought to serve a supportive structural role rather than being directly involved in transport or ligand binding functions (Koldso et al., 2013), our results now begin to identify functional properties associated with the region. In addition, because our protease resistance findings differentiate responses of these residues to antagonists and substrates, they suggest this region as a possible target for development of reagents to reduce inhibitor binding without directly impacting the substrate site.

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ABBREVIATIONS

DAT	dopamine transporter
NET	norepinephrine transporter
SERT	serotonin transporter
TM	transmembrane domain
EL	extracellular loop
IL	intracellular loop
o.w.w	original wet weight
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue
CFT	2β-carbomethoxy-3β-(4-fluorophenyl)tropane
GBR12909	[2-(diphenylmethoxy) ethyl]-4-(3-phenylpropyl)piperazine
SCAM	substituted cysteine accessibility method
PNGF	glycopeptidase-F
MTSET	[2-(trimethylammonium)ethyl]-methanethiosulfonate
MTSES	(2-sulfonatoethyl) methanethiosulfonate
MTSEA	2-aminoethylmethanethiosulfonate

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Highlights

1. Uptake inhibitor binding induces protease resistance of DAT at residues in EL2.
2. These changes may be associated with the mechanism of transport inhibition.
3. Modeling of DAT with LeuT indicates a potential salt bridge between D174 and R218.
4. These findings suggest a link between EL2 and transport inhibitor action.

Figure 1. Characterization of DAT Asp-N Fragments

(A) Schematic diagram of rDAT illustrating 12 transmembrane spanning domains, epitopes for N- and C-terminal tail antibodies (green and yellow), and EL2 components including N-linked glycosylation (branched structures), disulfide bond (solid line), Asp residues (red), Asp174 (large red circle) and Arg218 (large purple circle). The full rDAT sequence was analyzed by PsiPred and JUFO secondary structure prediction algorithms (Leman et al., 2013; McGuffin et al., 2000) which predicted the region surrounding and including R218 is likely a helical structure (purple). (B) Rat striatal membranes were treated with (+) or without (-) 1 μ g/ml Asp-N and analyzed as indicated. Left panel, immunoblotting of samples with N- and C-terminal specific DAT antisera. Arrow *a*, full-length DAT; arrows *b* and *d*, 32 and 19 kDa fragments detected by mAb 16; arrow *c*, 30 kDa fragment detected with C-terminal antibody. Middle panel, immunoblotting of Asp-N treated samples with

mAb16 containing no addition (control), 30 µg/ml peptide 16 (p16), or 30 µg/ml peptide 5 (p5). Right panel, DAT and DAT Asp-N fragments were immunoprecipitated with polyclonal antibody 16 and treated with or without 1.5 units PNGF, followed by immunoblotting with mAb 16.

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Figure 2. Effect of Ligand Binding on Asp-N Proteolysis

Rat striatal membranes incubated in the absence (control) or presence of 2 μ M uptake blockers, 30 μ M substrates, or 10 μ M Zn²⁺, were treated with vehicle or 1 μ g/ml Asp-N and immunoblotted with mAb 16. (A) Representative immunoblot showing full-length DAT and 19 kDa Asp-N fragments produced in indicated conditions. (B) Quantification of DAT proteolysis in the presence of tested compounds. Bars indicate the fraction of DAT digestion in the presence of vehicle (black bar), uptake blockers or Zn²⁺ (hatched bars), or substrates (gray bars). Values shown are means $\pm$ S.E.M. of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to control by ANOVA with a Dunnett's multiple comparison post hoc test.

Figure 3. Uptake Ligand-Induced Protease Resistance Requires Na⁺

Rat striatal membranes suspended in buffer containing either Na⁺ or K⁺ were incubated with uptake blockers (2 μM) or substrates (30 μM) and treated with vehicle or Asp-N followed by immunoblotting with mAb 16. (A) Representative immunoblot. (B) Quantification of DAT proteolysis (means ± S.E.M.) in the presence of Na⁺ (black bars) or K⁺ (hatched bars). **, p<0.01, *** p<0.001 relative to control by ANOVA with a Dunnett's multiple comparison post hoc test.

Figure 4. Asp-N Treatment Disrupts Binding and Transport Activities

(A) Rat striatal membranes or (B) rat striatal synaptosomes were treated with (+) or without (-) 5 μ g/ml Asp-N followed by assessment of (A) [3 H]CFT binding or (B) [3 H]DA uptake. Left panels show verification of proteolysis by immunoblotting. Histograms show quantification of uptake or binding (means $\pm$ S.E.M.). * $p < 0.05$, ** $p < 0.01$ relative to control by Student's t-test.

Figure 5. SCAM analysis of the N-terminal region of EL2

Cells expressing the indicated rDAT mutants were analyzed by (A) immunoblotting or (B) $[^3\text{H}]\text{DA}$ transport (black bars) and $[^3\text{H}]\text{CFT}$ binding (gray bars). (C) Cells were incubated with vehicle (black bars) or 10 μM (-)cocaine (gray bars) followed by addition of 10 mM MTSET prior to binding analysis. The histogram shows % changes in $[^3\text{H}]\text{CFT}$ binding for each form (means $\pm$ S.E.M, n = 3). Treated and untreated samples were not significantly different.

Figure 6. SCAM analysis of the C-terminal region of EL2

Cells expressing the indicated hDAT mutants were analyzed by (A) immunoblotting or (B) $[^3\text{H}]\text{DA}$ transport (black bars) and $[^3\text{H}]\text{CFT}$ binding (gray bars). (C) Cells were incubated with vehicle (black bars) or 10 μM (–)cocaine (gray bars) followed by addition of 10 mM MTSET prior to binding analysis. Responses for R219C and V221C are indicated in blue (vehicle) or red (cocaine) for clarity. Results show % changes in $[^3\text{H}]\text{CFT}$ binding for each form (means $\pm$ S.E.M.). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to E2C by ANOVA with a Dunnett's multiple comparison post hoc test; † $p < 0.05$, ††† $p < 0.001$ for indicated forms relative to the absence of cocaine by Student's t-test. $n = 3$ –5 for all forms except T211C (6), P212C (6), A213C (6), E218C (8), R219C (10), and H223C (6).

Figure 7. Comparative modeling of D174 and D218 from LeuT_{Aa} EL2 crystal structures
 (A) Ribbon diagram of LeuT_{Aa} EL2, highlighting positions and calculated distance between L126 and S150 (dashed line). (B) Superimposed ribbon diagrams of EL2 coordinates from LeuT_{Aa} forms ‘apo-out’, (pink); ‘open-to-out’, (orange); ‘outward-occluded’, (brown); ‘inward-occluded’, (green); ‘apo-in’, (gray); and ‘sertraline-bound’, (cyan), show minimal changes in overall domain structure or L126 and S150 distances. (C) Ribbon diagram of LeuT_{Aa} with *in silico* mutation of L126 and S150 side chains to D and R. Energy minimization using Rosetta Backrub revealed several conformations in which the residues are close enough to form a salt bridge (dashed line). (D) Superimposed ribbon diagrams of EL2 from LeuT_{Aa} forms listed above with DAT substitutions reveal no significant alteration in D174/R218 side chain orientation or distance. (E) Ribbon diagram of dDAT EL2 with *in silico* mutation of S142 to D. Manual rotamer search of D142 and R218 were chosen in PyMol. Measured distance between the side chains is indicated (dashed line).