0026-895X/04/6503-692–701$20.00
MOLECULAR PHARMACOLOGY
Vol. 65, No. 3
2948/1132902
Mol Pharmacol 65:692–701, 2004
Printed in U.S.A.

Uptake Inhibitors but not Substrates Induce Protease Resistance in Extracellular Loop Two of the Dopamine Transporter

Jon D. Gaffaney and Roxanne A. Vaughan

Department of Biochemistry and Molecular Biology, University of North Dakota School of Medicine and Health Sciences, Grand Forks, North Dakota

Received September 5, 2003; accepted December 4, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Changes in protease sensitivity of extracellular loop two (EL2) of the dopamine transporter (DAT) during inhibitor and substrate binding were examined using trypsin proteolysis and epitope-specific immunoblotting. In control rat striatal membranes, proteolysis of DAT in a restricted region of EL2 was produced by 0.001 to 10 μg/ml trypsin. However, in the presence of the dopamine uptake blockers [2-(diphenylmethoxyl) ethyl]-4-(3phenylpropyl) piperazine (GBR 12909), mazindol, 2β-carbomethoxy-3β-(4-flourophenyl)tropane (β-CFT), nomifensine, benztropine, or (−)-cocaine, 100- to 1000-fold higher concentrations of trypsin were required to produce comparable levels of proteolysis. Protease resistance induced by ligands was correlated with their affinity for DAT binding, was not observed with Zn^{2+}, (+)-cocaine, or inhibitors of norepinephrine or serotonin transporters, and was not caused by altered catalytic activity of trypsin. Together, these results support the hypothesis that the interaction of uptake inhibitors with DAT induces a protease-resistant conformation in EL2. In contrast, binding of substrates did not induce protease resistance in EL2, suggesting that substrates and inhibitors interact with DAT differently during binding. To assess the effects of EL2 proteolysis on DAT function, the binding and transport properties of trypsin-digested DAT were assayed with [^{3}H]CFT and [^{3}H]dopamine. Digestion decreased the B_{max} for binding and the V_{max} for uptake in amounts that were proportional to the extent of proteolysis, indicating that the structural integrity of EL2 is required for maintenance of both DAT binding and transport functions. Together this data provides novel information about inhibitor and substrate interactions at EL2, possibly relating the protease resistant DAT conformation to a mechanism of transport inhibition.

The dopamine transporter (DAT) is a Na^+/Cl^--dependent transport protein responsible for clearing synaptic dopamine. DAT is a site of action for psychostimulant and therapeutic drugs (Kuhar et al., 1991) and shares significant structural and functional homology with the norepinephrine (NET), serotonin (SERT), and γ-aminobutyric acid transporters. DAT is predicted to contain 12 transmembrane domains (TMs) connected by internal and external loops (ILs and ELs), the largest of which, EL2, between TMs 3 and 4, contains N-linked carbohydrates and a putative disulfide bond (Fig. 1). Little is known about the three-dimensional structure of DAT, spatial orientation of helices, or functions of connecting loops. It is presumed that closely positioned TM helices provide a pathway for substrates through the lipid bilayer and that transport occurs in response to conformational changes in TMs and loops; how these movements are accomplished and how uptake blockers such as cocaine interfere with transport remain unknown.

Mutagenesis and chimera studies have identified residues or domains in TMs 1 to 3 and TMs 5 to 8 that contribute to inhibitor binding or substrate transport (reviewed in Chen and Reith, 2000). Our group has shown that structurally different irreversible DAT uptake blockers become incorporated into TMs 1 to 2, 4 to 6, or both regions (Vaughan et al., 1999, 2001; Vaughan and Kuhar, 1996), consistent with these domains being in close proximity. Together these studies indicate that residues contributing to active sites on DAT

This work was supported by National Institute on Drug Abuse grants F31-DA14857 (to J.D.G.) and DA13147 (to R.A.V.).

ABBREVIATIONS: DAT, dopamine transporter; NET, norepinephrine transporter; SERT, serotonin transporter; TM, transmembrane domain; EL, extracellular loop; IL, intracellular loop; SP, sucrose/sodium phosphate; o.w.w., original wet weight; PVDF, polyvinylidene difluoride; BSA, bovine serum albumin; PBS, phosphate-buffered saline; IGEPAL-CA-630, (octylphenoxy)polyethoxyethanol; mAb, monoclonal antibody; TAME, *p*-toluenesulphonyl-L-arginine methyl ester; GBR 12909, [2-(diphenylmethoxy)ethyl]-4-(3phenylpropyl) piperazine; β-CFT, 2β-carbomethoxy-3β-(4-flourophenyl)tropane; ^{125}I-RTI82, ^{125}I-3β-(*p*-chlorophenyl)-tropane-2β-carboxylic acid, 4′-azido-3′-iodophenylethyl ester; $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid)trimethine oxonol.

for transport or inhibitor binding are found throughout the primary sequence in regions that flank EL2 but are juxtaposed in the native protein.

EL2 is the largest loop in the cocaine-sensitive neurotransmitter transporters, but its function in these proteins has not been extensively characterized. Chimeras in which EL2 of SERT was replaced with that of NET displayed dramatically reduced serotonin transport activity with no pronounced change in ligand binding pharmacology (Stephan et al., 1997; Smicun et al., 1999), suggesting that it does not directly contribute to inhibitor binding but helps to maintain TM structural relationships necessary for substrate translocation. A requirement for precise EL2 structure in DAT is also suggested by studies showing that mutation of tryptophan and glutamate residues in EL2 led to loss of activity and/or cell-surface expression (Chen et al., 2001).

In previous studies of photolabeled DATs, we found that despite its extensive post-translational modifications, EL2 was the most sensitive site on the protein to proteolysis (Vaughan, 1995; Vaughan and Kuhar, 1996; Vaughan et al., 2001). Tryptic digestion of rat striatal membrane suspensions generate a 45-kDa fragment of DAT that contains the N-terminal tail and N-linked carbohydrates, but not EL2 antibody epitope 5 (Fig. 1), and a 32-kDa fragment that is not glycosylated but contains epitopes for both antibody 5 and the C-terminal tail (Vaughan, 1995; Vaughan and Kuhar, 1996). Thus, the initial site of tryptic proteolysis in DAT occurs in EL2 between the consensus glycosylation sites and the epitope 5, most likely at Arg^{218}, although we cannot rule out the possibility that Arg^{227} within epitope 5 is cleaved. Proteolysis at one or both of these residues separates DAT into essentially N- and C-terminal halves, and further proteolysis at tryptic sites in IL1 and other loops occurs only after EL2 cleavage. The initial inaccessibility of these secondary protease sites suggests that DAT possesses a compact structure that relaxes after EL2 cleavage, leading to increased accessibility of other loops. We also found a pattern of increasing EL2 protease resistance that correlated with the extent of photoaffinity label incorporation in the TM 4 to 6 domain, suggesting that binding of ligands induced alternative conformations in EL2 (Vaughan et al., 2001). However, the lack of ability to assess proteolysis of nonlabeled DAT in these studies and the limited number of available irreversible ligands precluded further characterization of this effect.

We have now extended these observations using an epitope-specific immunoblot assay to detect EL2 proteolysis and examine the effects of reversible binding of a wide range of DAT inhibitors. We have identified a protease resistant state in EL2 that is promoted by uptake inhibitors but not substrates, and we showed that DAT binding and transport are profoundly inhibited by EL2 cleavage. These results provide novel information about DAT function, potential conformations induced by inhibitors and substrates, and indicate a crucial role for EL2 in binding and transport activities.

Materials and Methods

Tissue Preparation and Proteolysis. For membrane preparations, male Sprague-Dawley rats (175–300 g) were decapitated, and the striata were quickly removed, weighed, and placed in ice-cold sucrose phosphate (0.32 M sucrose and 10 mM sodium phosphate, pH 7.4) (SP) buffer. The tissue was homogenized with a Polytron PT1200 homogenizer (Kinematica, Basel, Switzerland) for 10 to 12 s and centrifuged at 20,000*g* for 10 min at 4°C. The resulting pellet was washed twice and resuspended to 20 mg/ml original wet weight (o.w.w.) in ice-cold SP buffer. Proteolysis was started by addition of equal volumes (typically 25 μl each) of membranes and 2× trypsin stock prepared in SP buffer, and samples were gently mixed and incubated for 10 min at 22°C. Proteolysis was quenched by addition of 1 vol of 1 mg/ml trypsin inhibitor prepared in SP buffer, membranes were centrifuged at 15,000*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 dithiothreitol, 60 mM Tris-HCl, pH 6.8) at 20 mg/ml o.w.w. and subjected to electrophoresis and immunoblotting.

Immunoblot Analysis. Solubilized striatal membrane samples (25 μl) were loaded onto 10% Tris/glycine polyacrylamide minigels, and proteins were separated by electrophoresis at 125 V for 1.5 h. After electrophoresis, proteins were transferred to 0.2-μm PVDF membranes for 2 h at 100 V in 4°C transfer buffer (10% methanol, 0.01% SDS, 100 mM glycine, and 10 mM Tris base) rinsed with Milli-Q water, and allowed to air dry. The membranes were hydrated

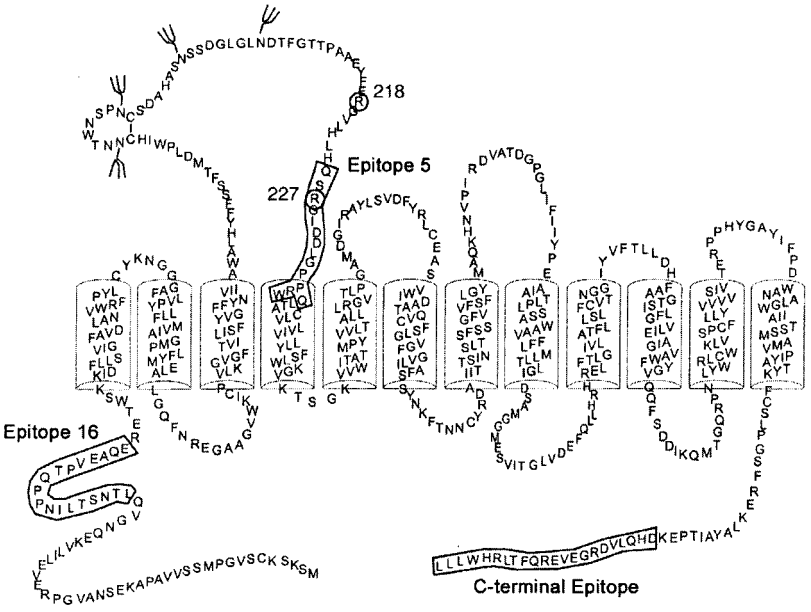

Fig. 1. Schematic diagram of the rat dopamine transporter showing antibody epitopes and potential EL2 trypsin proteolysis sites. The amino acid sequence and predicted transmembrane topology of rDAT are shown with the extracellular face of the protein at the top and cylinders indicating putative positions of transmembrane-spanning helices. The epitopes for monoclonal Ab 16, antibody 5, and C-terminal antibody are outlined and labeled. The potential trypsin proteolysis sites in EL2, arginines 218 and 227, are circled and numbered. Cysteines 180 and 189 are indicated as disulfide bonded, and consensus *N*-glycosylation sites are designated with branched structures.

with methanol and blocked with 3% bovine serum albumin (BSA) in 10 mM phosphate-buffered saline (PBS) for 1.5 h followed by 5% nonfat dry milk in PBS for 0.5 h. Membranes were rinsed twice with wash buffer (PBS with 0.1% IGEPAL-CA-630) and once with PBS. A mouse monoclonal antibody (mAb) was generated by Cell Essentials (Boston, MA) against synthetic peptide 16 containing amino acids 42 to 59 of the rat DAT N-terminal tail (mAb 16). A polyclonal goat antibody raised against amino acids 601 to 620 in the C-terminal tail of human DAT that cross-reacts with rDAT was purchased from Research Diagnostic Inc. (Flanders, NJ). Antibodies were diluted 1:1000 (mAb 16) or 1:100 (anti-hDAT) in blocking buffer (3% BSA in PBS) and incubated with the membrane for 1 h at 22°C, followed by four rinses with wash buffer and one rinse with PBS. Anti-mouse or anti-goat IgG antibodies linked to alkaline phosphatase were diluted 1:5000 in blocking buffer and incubated with the membrane for 1 h at 22°C, followed by four rinses with wash buffer and one rinse with PBS. The membranes were developed 5 to 10 min using the alkaline phosphatase substrate, 5-bromo-4-choloro-3-indolyl phosphate/nitro blue tetrazolium until a strong signal was obtained, then rinsed twice with water, air dried, scanned, and quantitated.

Specificity of fragment immunostaining was verified by presorbing mAb 16 with 30 μg/ml peptide 16, using peptide 5 (amino acids 225–236) as a negative control. For each experiment, control and treated samples were prepared, electrophoresed, transferred, and immunoblotted exactly in parallel so that signal intensities would be quantitatively comparable. Tissue linearity experiments performed by diluting solubilized membrane preparations in 60% increments verified that the signal intensity was linear between 0.1 and 10 mg/ml tissue (not shown).

Deglycosylation Analysis. Rat striatal membranes treated with or without trypsin followed by trypsin inhibitor, were washed by centrifugation, resuspended in SP buffer, and incubated with 1.5 units of glycopeptidase-F for 18 h at 22°C. The membranes were washed twice with SP, solubilized at 20 mg/ml o.w.w. in sample buffer, and immunoblotted.

Inhibitor Binding. For uptake inhibitor single point assays, striatal membranes suspended in SP buffer were incubated with saturating concentrations (2 μM) of DAT uptake inhibitors for 1 h on ice, followed by addition of 3 ng/ml-3 mg/ml trypsin for 10 min at 22°C. For saturation analysis of dopamine uptake inhibitors, membranes were incubated with 3 pM-30 μM of the indicated dopamine uptake inhibitors for 1 h on ice, followed by addition of 3 μg/ml trypsin for 10 min at 22°C. For both procedures, proteolysis was then quenched by addition of 1 mg/ml trypsin inhibitor, centrifugation at 20,000*g*, removal of supernatants, and solubilization of membranes at 20 mg/ml o.w.w. in sample buffer.

Trypsin Activity Assay. The trypsin substrate *p*-toluenesulphonyl-L-arginine methyl ester (TAME) was used to assay the activity of 1 ng of trypsin in the presence or absence of 5 μM cocaine, GBR 12909, mazindol, β-CFT, or dopamine. Hydrolysis of TAME to *p*-toluenesulphonyl-L-arginine was measured by monitoring absorbance at 247 nm for 10 min. The increase in absorbance was plotted as a function of time and the rate of enzymatic activity was calculated from the slope between t = 1 and t = 8.

Quantitation of Fragments. Immunoblots were scanned at 600 dpi with an Epson Perfection 12000U scanner (Epson America, Long Beach, CA) and saved as grayscale TIF images. Grayscale values were converted to Boehringer light units by LumiAnalyst 3.0 software. Digestion was quantitated by dividing the immunoreactivity of the 80-kDa DAT form by the total immunoreactivity (both 45- and 80-kDa fragments) in each lane and expressed as percentage of control (no trypsin). This method allowed correction for the low amount of endogenous proteolysis obtained in some experiments, and served as an internal loading and transfer control.

[^{3}H]CFT Binding. Rat striatal membranes were treated with or without 1 μg/ml-1 mg/ml trypsin for 10 min at 22°C, followed by addition of 1 mg/ml trypsin inhibitor, centrifugation, and removal of supernatant. Pellets were resuspended in ice-cold binding buffer (50 mM Tris-HCl and 50 mM NaCl, pH 7.4) to a concentration of 6 mg/ml o.w.w. Triplicate samples of membranes were incubated on ice with 2 nM [^{3}H]CFT for 2 h followed by rapid vacuum filtration using a Brandel tissue harvester over Whatman GF/B glass fiber filters soaked for 2 h in 0.1% BSA. Nonspecific binding was determined by addition of 100 μM cocaine to the binding buffer. For saturation analysis of [^{3}H]CFT binding, membranes treated with or without 10 μg/ml trypsin were prepared as above, followed by incubation with 2 nM [^{3}H]CFT in the presence of 1 to 1000 nM unlabeled CFT. Filtered radioactivity was counted in a scintillation counter (model 1600; Beckman Coulter, Fullerton, CA) at 45% efficiency. Aliquots of each sample were subjected to immunoblotting to determine extent of digestion.

[^{3}H]Dopamine Uptake. P_2 synaptosomal fractions were prepared in SP buffer from freshly dissected rat striatum using standard procedures (Krueger, 1990), and were resuspended to 6 mg/ml o.w.w. in ice-cold SP buffer. Aliquots were dispensed into assay tubes and dopamine uptake assays were performed in triplicate in modified-Krebs phosphate buffer (126 mM NaCl, 4.8 mM KCl, 16 mM potassium phosphate, 1.4 mM $MgSO_4$, 10 mM glucose, 1.1 mM ascorbic acid, and 1.3 mM $CaCl_2$, pH 7.4) as described previously (Vaughan et al., 1997). [^{3}H]Dopamine was 10 nM and unlabeled dopamine concentrations varied from 1 to 800 nM. Nonspecific binding was determined by addition of 100 μM cocaine to the uptake buffer. Uptake assays were started by addition of 100 μl of synaptosomes to the reaction tube and incubation for 5 min at 30°C. Uptake was stopped by addition of 5 ml of ice-cold SP buffer and samples were immediately vacuum-filtered using a Brandel tissue harvester over a Whatman GF/B filter soaked for 2 h in 0.1% BSA. Filters were counted using a Beckman model 1600 liquid scintillation counter with 45% efficiency. Data were analyzed by nonlinear regression using Prism 3.0 software (GraphPad Software, San Diego, CA). Aliquots of each sample were subjected to immunoblotting to determine extent digestion.

Membrane Potential Measurements. Rat striatal synaptosomes were isolated according to (Dodd et al., 1981) and membrane potential was detected by fluorescence as described by Blaustein and Goldring (1975). Briefly, rats were decapitated, the striatum dissected, and dispersed with a Teflon-glass homogenizer in ice-cold 0.32 M sucrose. Cellular debris was removed by centrifugation at 5100 rpm in a 70.1 titanium rotor (w^2t setting 1.57×10^8 rad^2/s). The resulting supernatant was layered onto 4 ml of 1.2 M sucrose, centrifuged with a 70.1 titanium rotor (50,000 rpm; w^2t setting 1.60×10^{10} rad^2/s), and the interface was collected and diluted to 7 ml with 0.32 M sucrose. This suspension was layered onto 4 ml of 0.8 M sucrose and centrifuged under the identical conditions and the pellet (P_2) was collected. The centrifugation steps remove approximately 96% of the mitochondria and 97% of the myelin and microsomes from the final P_2 pellet, which contains approximately 50% synaptosomes (Dodd et al., 1981). The P_2 pellet was resuspended with 1 ml of 0.32 M sucrose and gradually transferred to a physiological buffer (Na + 5K), consisting of 132 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, and 20 mM Tris/succinate, pH 7.4, by adding 5 to 10 volumes over 5 min. The resulting synaptosome suspension was centrifuged at 9000*g* for 5 min and the pellet resuspended into 1 ml of Na + 5K buffer. Protein content was determined with a Bradford assay, and the protein concentration was adjusted to 4 mg/ml.

Fluorescence of bis-(1,3-dibutylbarbituric acid)trimethine oxonol ($DiBAC_4(3)$) was measured using a Shimadzu RF-540 fluorospectrophotometer with a DR-3 data recorder. $DiBAC_4(3)$ was excited at 493 nm and its emission at 512 nm was measured as a function of time. Each cuvette contained 3 ml of Na + 5K buffer (37°C), 6 μl of 2 mM $DiBAC_4(3)$, 100 μl of synaptosomes, and 30 μl of buffer or trypsin. The fluorescence was measured for 8 min followed by depolarization of synaptosomes with 30 mM KCl. Complete depolarization of synaptosomes was confirmed by adding Gramicidin D (9.3 μg/ml) to each cuvette 2 min after addition of KCl.

Materials. *N*-Tosyl phenylalanine chloromethyl ketone-treated trypsin and trypsin assay kit were purchased from Worthington Biochemicals (Freehold, NJ). Bovine serum albumin, Tris base, and sodium phosphate were from Fisher Scientific (Pittsburgh, PA). GBR 12909, β-CFT, nomifensine, mazindol, benztropine, and (−)-cocaine were from Sigma/RBI (Natick, MA). $DiBAC_4(3)$ was purchased from Molecular Probes (Eugene, OR). (+)-Cocaine was a generous gift from Maarten E. A. Reith (New York University School of Medicine, New York, NY). [^{3}H]CFT was from PerkinElmer Life and Analytical Sciences (Boston, MA) and [^{3}H]dopamine was from Amersham Biosciences (Piscataway, NJ). All other chemicals were from Sigma (St. Louis, MO).

Results

Immunoblot Detection of Trypsin Fragments. We have produced a monoclonal antibody (mAb 16) that recognizes N-terminal epitope 16 of DAT (Fig. 1) and have used this to detect N-terminal fragments produced by tryptic digestion. To validate the similarity of the fragments visualized with immunoblotting to those observed with photoaffinity labeling, samples were subjected to N- and C-terminal immunoblotting, peptide blocking, and enzymatic deglycosylation (Fig. 2).

Striatal membranes were treated with or without trypsin and analyzed by immunoblotting with N- or C- terminal antibodies (Fig. 2A). In untreated membranes, a single protein of approximately 80 kDa (Fig. 2A, arrow a) is detected with both antibodies. This band is not detected with these antibodies in cerebellar membranes or in cultured cells that do not express DAT (not shown), verifying that the immunoreactivity represents DAT. Treatment of membranes with 1 and 10 μg/ml trypsin results in production of a 45-kDa fragment (Fig. 2A, arrow b) that is similar to the N-terminal fragment observed previously from DAT photoaffinity-labeled with the GBR analog, [^{125}I]1-[2-(diphenylmethoxy)ethyl]-4-[2-(4-azido-3-iodophenyl)ethyl] piperazine (Vaughan, 1995; Vaughan and Kuhar, 1996). A minor amount of a 14-kDa fragment that occurs because of proteolysis of arginines and lysines in IL1 is produced at higher trypsin concentrations (Fig. 2A, arrow d). The same preparations immunoblotted with the C-terminal antibody and shows production of a single 32-kDa fragment (Fig. 2A, arrow c), similar to that generated from DATs labeled with the irreversible cocaine analog [^{125}I]RTI82 (Vaughan, 1995; Vaughan and Kuhar, 1996). Antiserum 5, generated against residues 225 to 237 in EL2 was not useful for immunoblotting DAT fragments in the present study but does immunoprecipitate the [^{125}I]RTI82–labeled 32-kDa fragment, indicating that the epitope was present on the immunoblotted fragment. The immunological specificity of the 45-kDa fragment was verified by preabsorption of the primary antibody with the immunogenic peptide (Fig. 2B). Inclusion of peptide 16 with the primary antibody eliminated the staining of the full-length DAT and the 45-kDa fragment, while incubation with nonantigenic peptide 5 had no effect. In C, trypsin-digested striatal membranes were treated with or without glycopeptidase-F. The untreated sample shows the full-length protein and the 45-kDa fragment (arrows on left). The masses of the full-length DAT protein and the 45-kDa fragment were reduced by 20 to 25 kDa after glycopeptidase-F treatment (arrows on right), indicating that they had been deglycosylated. This is consistent with our demonstration of *N*-linked glycosylation on the [^{125}I]1-[2-(diphenylmethoxy)ethyl]-4-[2-(4-azido-3-iodophenyl)ethyl] piperazine-labeled 45-kDa fragment (Vaughan and Kuhar, 1996) and shows that proteolysis of EL2 is occurring C-terminal to the consensus glycosylation sites. The mass of the deglycosylated tryptic fragment was approximately 25 kDa, also consistent with proteolysis probably occurring at Arg^{218} or Arg^{227}, which are the only tryptic sites in EL2. This proteolytic pattern is the same as that of the photoaffinity-labeled protein and indicates that the 45- and 32-kDa fragments represent most or all of the N- and C-terminal halves of the protein, respectively. Thus, for most experiments, low concentrations of trypsin were used to restrict the proteolysis of DAT to this region of EL2.

Uptake Inhibitors Protect DAT from Proteolysis. To examine the effects of uptake inhibitors on proteolysis of EL2, rat striatal membranes were incubated with various transport blockers followed by digestion of DAT with multiple low concentrations of trypsin and analysis of fragments by immunoblotting with mAb 16. The experiments shown in Fig. 3 demonstrate that cocaine exerts a pronounced stereospecific effect on the extent of EL2 proteolysis. A shows an immunoblot of DAT digested with trypsin in the presence and absence of (−)- or (+)-cocaine. In the absence of cocaine, production of the 45-kDa fragment was apparent at 0.01 μg/ml trypsin and increased steadily up to the highest protease concentration used (10 μg/ml). In the presence of 2 μM (−)-cocaine, however, DAT proteolysis was strongly reduced, with less than control levels of the fragment obtained at 10 μg/ml trypsin and only minor amounts of the fragment de-

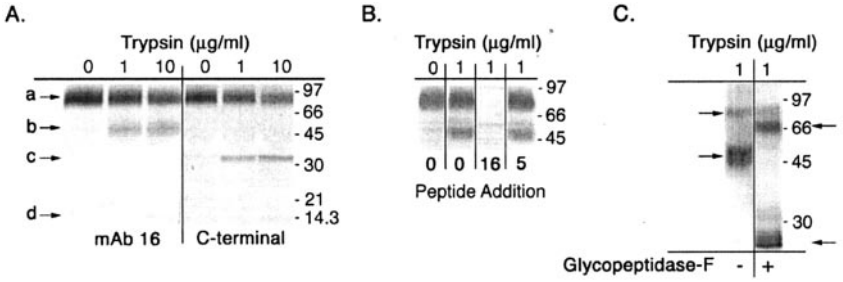

Fig. 2. Detection of N- and C-terminal tryptic fragments by immunoblotting. A, rat striatal membranes were treated with or without 1 or 10 μg/ml trypsin, separated on a 10% Tris/glycine gel and transferred to PVDF membranes. Membranes were immunoblotted with N-terminal mAb 16 or a C-terminal antibody as indicated. Full-length DAT migrates at 80 kDa (arrow a), N-terminal proteolytic fragments migrate at approximately 45 and 14 kDa (arrows b and d), and a single C-terminal fragment migrates at 32 kDa (arrow c). B, rat striatal membranes were treated with or without 1 μg/ml trypsin, followed by immunoblotting with mAb 16 in the presence of no addition, 30 μg/ml peptide 16, or 30 μg/ml peptide 5. C, rat striatal membranes were digested with 1 mg/ml trypsin followed by treatment with glycopeptidase-F and immunoblotting with mAb 16. Arrows on the left indicate full-length DAT and the 45 kDa fragment; arrows on the right indicate the deglycosylated forms of full-length DAT and the proteolytic fragment.

tectable at 0.1 to 1 μg/ml trypsin. Incubation of membranes with the inactive stereoisomer (+)-cocaine had no effect, in that the fragment was produced at the lower trypsin doses effective in the control membranes and at levels comparable with the control samples. Quantitation of these results in B shows that 100-fold more trypsin was required to produce comparable amounts of DAT proteolysis in the presence of (−)-cocaine, indicating that cocaine binding to DAT results in protease resistance in EL2.

To verify the specificity of these results for DAT ligands, we examined DAT proteolysis in the presence of uptake blockers for NET and SERT (Fig. 4). In the absence of exogenous ligand, 0.1 μg/ml trypsin produced a 22% greater level of DAT digestion compared with the untreated sample with endogenous proteolysis. In the presence of 2 μM imipramine or desipramine, the same levels of DAT proteolysis EL2 occurred, whereas addition of the cocaine analog β-CFT induced significant protease resistance, consistent with the effect being induced by ligand binding to DAT.

To determine whether this effect was specific for cocaine or extended to other uptake blockers, we examined a wide range of structurally diverse dopamine uptake blockers, including mazindol, GBR 12909, β-CFT, benztropine, and nomifensine, for their effects on DAT proteolysis (Fig. 5). Representative immunoblots of each ligand are shown at the left, and quantitation of three or more experiments with each ligand is shown at the right. Each inhibitor tested induced protease resistance that was 100- to 1000-fold more resistant to proteolysis than control. The uptake blockers themselves had no effect on the ability of trypsin to proteolyze the synthetic substrate TAME (not shown), demonstrating that the reduced proteolysis induced by ligands was not caused by decreased trypsin catalytic activity. Interestingly, Zn^{2+}, which also inhibits dopamine uptake (Norregaard et al., 1998), did not induce protease resistance (Fig. 5), suggesting that Zn^{2+} and the classic DAT uptake inhibitors prevent transport by different molecular mechanisms.

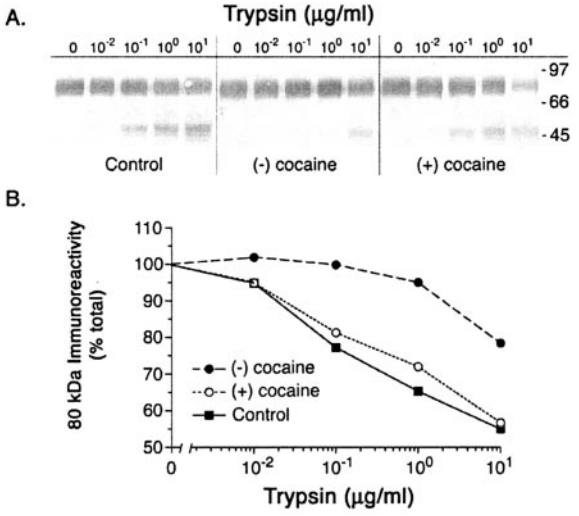

Fig. 3. Cocaine induces EL2 protease resistance in a stereospecific manner. Rat striatal membranes were digested with the indicated concentrations of trypsin in the presence or absence of 2 μM (−)- or (+)-cocaine. Full-length DAT and its proteolytic fragments were separated on 10% tris/glycine gels, transferred to PVDF, and immunoblotted with mAb 16. A, representative immunoblot of samples with mAb16. B, densitometric quantitation of the immunoblot shown in A. This experiment has been replicated three times with similar results.

To further verify that the change in proteolysis was caused by ligand binding, we examined the ligand concentration at which protease resistance was induced at a fixed concentration of trypsin (1 μg/ml). This concentration was chosen because it generated the greatest difference in extent of digestion between control and inhibitor-treated preparations. Membrane suspensions were incubated with 10^{-12} to 10^{-3} M concentrations of each uptake inhibitor followed by digestion with trypsin (Fig. 6). Fig. 6A shows a representative immunoblot obtained using (−)-cocaine as the ligand. In the absence of ligand, there is robust production of the 45-kDa fragment, which is defined as 100% digestion. Production of the fragment is strongly reduced in the presence of 1 mM (−)-cocaine but increases as the (−)-cocaine concentration is decreased until control fragment levels are obtained at 1 to 10 nM ligand. Similar results, shown quantitatively in Fig. 6B, were obtained with GBR 12909, mazindol, and β-CFT. All of the tested compounds induced protease resistance between 0.01 and 1 μM, roughly correlating with their affinities for binding to DAT (Heikkila and Manzino, 1984; Reith and Selmeci, 1992; Wiener and Reith, 1992). A low reproducible level of protease protection was produced by extreme subnanomolar concentrations of GBR 12909. Because these levels are below the binding affinity for DAT, they may reflect nonspecific effects of the compound on the membrane or other uncharacterized properties.

Substrates Do Not Protect DAT against Proteolysis. We then used the protease assay to examine the effects of substrate binding to DAT, using experimental conditions that do not support substrate transport. Membrane potential necessary for transport was disrupted by shearing the tissue with a Polytron homogenizer in low-ionic-strength buffer containing no Cl^- (0.32 M sucrose, 18 mM Na^+, and 10 mM PO_4, pH 7.4). Membranes were digested with trypsin in the presence or absence of 10 μM dopamine, amphetamine, or methamphetamine, followed by immunoblotting with mAb 16 (Fig. 7). Parallel samples were treated with 2 μM GBR 12909 as a positive control for protease protection. Representative immunoblots are shown in Fig. 7A, and quantitated averages are shown in Fig. 7B. There was no significant difference in the extent of EL2 proteolysis in the presence of any of the substrates tested, whereas aliquots of the same membranes incubated with GBR 12909 showed substantial protease resistance.

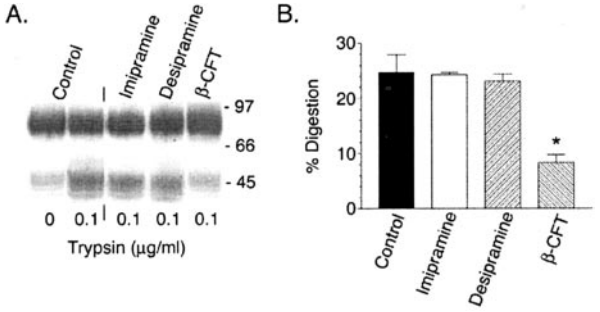

Fig. 4. NET and SERT specific uptake inhibitors do not induce EL2 protease resistance. A, rat striatal membranes were incubated in the presence or absence of 2 μM imipramine, desipramine, or β-CFT, then digested with 0.1 μg/ml trypsin. Full-length DAT and its proteolytic fragments were separated on 10% tris/glycine gels, transferred to PVDF, and immunoblotted with mAb 16. B, quantitation of immunoblot data, shown as mean ± S.E. of two independent experiments. *, $p < 0.001$, statistically different from control value.

Binding and Transport Activities of DAT Are Inhibited by EL2 Proteolysis. Because these experiments suggested the presence of a conformational link between EL2 and the binding site for uptake blockers, we then examined the ability of DAT to function after EL2 proteolysis. The concentrations of trypsin used in these experiments produced primarily N- and C-terminal halves of the protein, and it is possible that the resulting domains would remain associated and continue to function, as has been found for some transmembrane proteins (Bibi and Kaback, 1990; Loo and Clarke, 1994; Juul et al., 1995; Adamo et al., 2000). To address this question, the binding and uptake properties of the digested transporter were studied in membranes and synaptosomes, respectively.

For analysis of binding, striatal membrane suspensions were treated with or without varying concentrations of trypsin for 10 min, and proteolysis was quenched with trypsin inhibitor and the trypsin removed by centrifugation. Membranes were resuspended in SP buffer, and aliquots were subjected to immunoblotting to determine the extent of DAT proteolysis (Fig. 8A) or were assayed for their ability to bind [^{3}H]CFT (Fig. 8B). The results show increasing levels of DAT proteolysis with increasing trypsin, with no obvious production of smaller fragments at higher trypsin concentrations. The same samples displayed [^{3}H]CFT binding activity that decreased proportionally with the extent of DAT digestion (Fig. 8B). Saturation analysis of [^{3}H]CFT binding after DAT digestion at 1 mg/ml trypsin showed a 71% decrease in B_{max} (392 fmol/mg o.w.w. tissue for control, 114 fmol/mg o.w.w. tissue for treated) with no change in the K_d for CFT binding. These results are consistent with a loss of binding activity in the proteolyzed proteins and indicate that the integrity of EL2 is required for binding to occur.

To assay the effects of EL2 proteolysis on uptake, intact synaptosomes prepared from rat striatum were digested with 1 to 100 μg/ml trypsin for 10 min and assayed for uptake using [^{3}H]DA (Fig. 9A). As with binding, there was a strong dose-dependent reduction in transport activity even at low trypsin concentrations. To determine the basis for this reduction in uptake, a dopamine uptake saturation analysis was performed for synaptosomes treated with or without 1 mg/ml trypsin (Fig. 9B). The V_{max} and K_m from the control synaptosomes were 0.76 ± 0.04 pmol/mg tissue (o.w.w.) and 51.8 ± 10.1 nM, and the respective values from trypsin-treated synaptosomes were 0.3 ± 0.02 pmol/mg tissue (o.w.w.) and 161.4 ± 32.9 nM. Aliquots of the samples removed for immunoblotting showed 64% digestion of DAT, with little to no proteolysis occurring in regions outside EL2 (Fig. 9C), comparable with the 61% reduction in dopamine transport V_{max}. These results are therefore consistent with a loss of transport activity after cleavage of EL2. Whether this occurs at the step of DA binding to DAT or at a later step in translocation cannot be determined because of the inability of the digested transporter to bind [^{3}H]CFT.

Trypsin-Treated Synaptosomes Retain Their Membrane Potential. To verify that the reduced DA transport activity in these samples was caused by cleavage of DAT and not by a loss of membrane potential as a result of proteolysis of ion pumps or channels, we assayed the membrane potential of control and trypsin-treated synaptosomes using the anionic lipophilic dye, $DiBAC_4(3)$. Under depolarizing conditions, this and related fluorophores display increased fluorescence because of association with plasma membrane and intracellular proteins (Brauner et al., 1984; Ohkuma et al., 2001; Zhang et al., 2001) and have been used to monitor the membrane potential of whole brain synaptosomes (Blaustein and Goldring, 1975).

Figure 10 shows fluorescence traces of control and trypsin-treated rat striatal synaptosomes. The $DiBAC_4(3)$ alone (arrowhead) fluoresces slightly and addition of synaptosomes to the cuvette produces baseline "synaptosomal fluorescence" (Blaustein and Goldring, 1975), which is most probably caused by light diffraction from particulates in the solution. Control assays showed that this baseline fluorescence was achieved within 10 s and was stable over the time course of these experiments. To determine whether trypsin affected the synaptosomal membrane integrity, identical aliquots of tissue were incubated with the dye until the baseline was established, then treated with either buffer (control) or 100 μg/ml trypsin for 8 min. The samples were stirred after 5 min to ensure that no synaptosome settling had occurred. In two independent experiments, there was no difference between the baseline fluorescence of control and trypsin-treated synaptosomes, indicating that trypsin alone did not lead to a loss of membrane electrochemical potential. Eight min after the addition of either buffer or trypsin, 30 mM KCl was added to

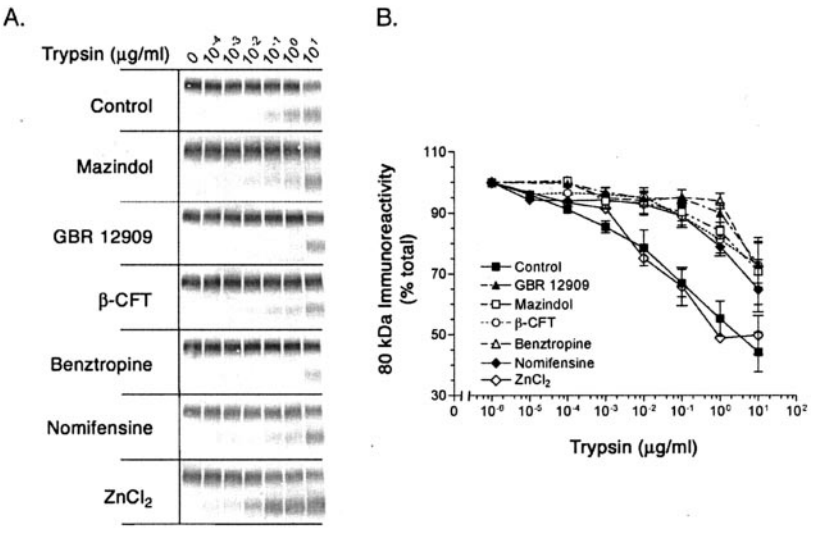

Fig. 5. Multiple dopamine uptake inhibitors induce EL2 protease resistance. A, rat striatal membranes were incubated in the presence or absence of 2 μM dopamine uptake inhibitors or 10 μM $ZnCl_2$, and then were digested with increasing concentrations of trypsin. Full-length DAT and its proteolytic fragments were separated on 10% tris/glycine gels, transferred to PVDF membranes, and immunoblotted with mAb 16. B, quantitation of immunoblot data, shown as mean ± S.E. of three independent experiments.

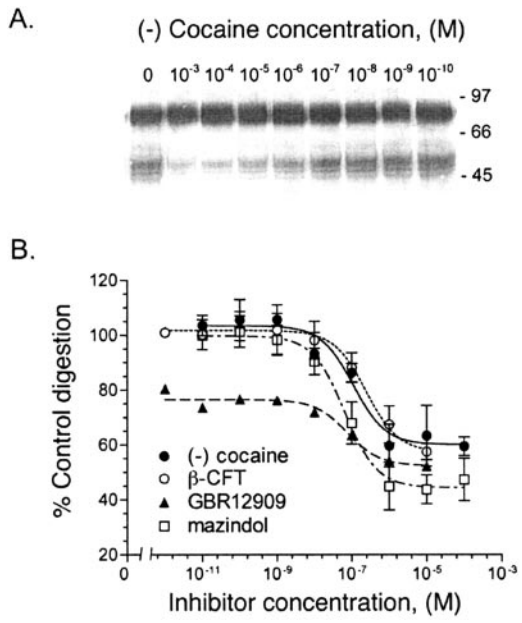

Fig. 6. Dose response of uptake inhibitors for induction of EL2 protease resistance. Rat striatal membranes were incubated for 1 h in the presence or absence of 10^{-10} to 10^{-3} M final concentrations of (−)-cocaine, CFT, mazindol, or GBR 12909, followed by digestion with 1 μg/ml trypsin, electrophoresis, and immunoblotting with mAb 16. A, representative immunoblot result obtained using (−)-cocaine. B, quantitation of immunoblot data shown as mean ± S.E. of three independent experiments performed for (−)-cocaine (●), β-CFT (○), and mazindol (□), or a single experiment replicated with similar results for GBR 12909 (▲).

the cuvette to depolarize the synaptosomes. Both control and trypsin-treated samples displayed immediate and equal increases in fluorescence, indicating the same degree of depolarization occurred in both samples, and providing further evidence that synaptosomal membrane potential was not affected by trypsin. The cationic ionophore, Gramicidin D produced the same effect as KCl in treated and untreated synaptosomes (data not shown), and adding Gramicidin D after KCl produced no further fluorescence changes (Fig. 10A) verifying that the synaptosomes were fully depolarized. The synaptosomal samples analyzed in A were subjected to immunoblot analysis (Fig. 10B) to verify the trypsin treatment resulted in DAT proteolysis. These data strongly support the hypothesis that the loss of DA transport activity in these synaptosomes is caused by proteolysis of DAT EL2.

Discussion

These results show that proteolysis of DAT by trypsin probably occurs at Arg^{218} and/or Arg^{227} in EL2 and is significantly reduced when dopamine uptake blockers are bound to the protein. Multiple potential mechanisms or combinations thereof would explain these results. One possibility is that inhibitor binding to DAT induces a conformational change in EL2 that results in loss of Arg^{218} and/or Arg^{227} accessibility to trypsin. Cocaine binding to hDAT has been shown to induce changes in the accessibility of Cys^{90} in EL1, Cys^{135} in IL1, and Cys^{342} in IL3 (Reith et al., 1992; Ferrer and Javitch, 1998); to our knowledge, however, the present results are the first to implicate ligand-induced conformational changes occurring in EL2. In addition, cocaine binding increased the accessibility of the extracellular facing cysteines in ELs 1 and 3, whereas our results demonstrate that uptake blockers reduce EL2 accessibility. A related possibility is that ligand binding induces a conformational change in a different region of DAT that reduces trypsin access to Arg^{218} and/or Arg^{227}.

Alternatively, it is possible that bound uptake inhibitors prevent proteolysis by direct or indirect steric hindrance of the protease site. Indirect effects could occur if inhibitors bind at a site other than EL2 in such a way that they limit trypsin access to the EL2 site. A direct effect would occur if Arg^{218} and/or Arg^{227} participated in binding so that the ligand itself covered the cleavage site. Although the binding site for DAT ligands is currently unknown, several lines of evidence are compatible with inhibitor binding occurring in TM spanning domains (Giros et al., 1994; Buck and Amara, 1995; Vaughan, 1995; Vaughan and Kuhar, 1996; Chen et al., 1997; Barker et al., 1998; Lee et al., 1998, 2000, 2002; Adkins et al., 2001). Thus, if EL2 also participates in binding, it may be close to the TM domains, although proteolysis clearly shows that in the absence of inhibitors it is also accessible to the aqueous medium. There have been few studies investigating the involvement of DAT EL2 in inhibitor binding. Mutation of Glu^{215} in hDAT (Glu^{214} in rDAT), which is very close to the tryptic proteolysis site, resulted in almost total loss of dopamine uptake and [^{3}H]CFT binding (Chen et al., 2001). However, whether this is caused by a protein structural change or by direct involvement with the binding site is unknown, and in our studies on photoaffinity label binding, we have never observed incorporation of tropane or other ligands into EL2 (Vaughan, 1995, 1999, 2001; Vaughan and

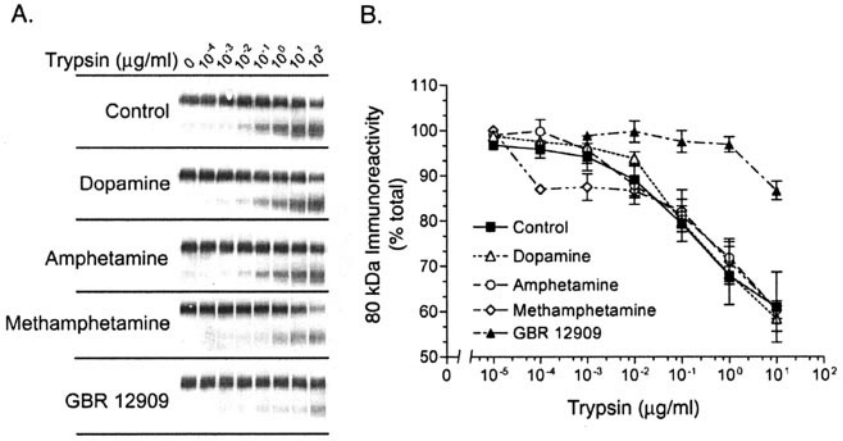

Fig. 7. Substrates do not induce EL2 protease resistance. A, rat striatal membranes were incubated with or without 10 μM substrate or 2 μM GBR 12909 for 1 h followed by digestion with increasing concentrations of trypsin. Digested membranes were separated by SDS-PAGE, transferred to PVDF, and immunoblotted with mAb 16. B, quantitation of immunoblot data, shown as mean ± S.E. of three independent experiments.

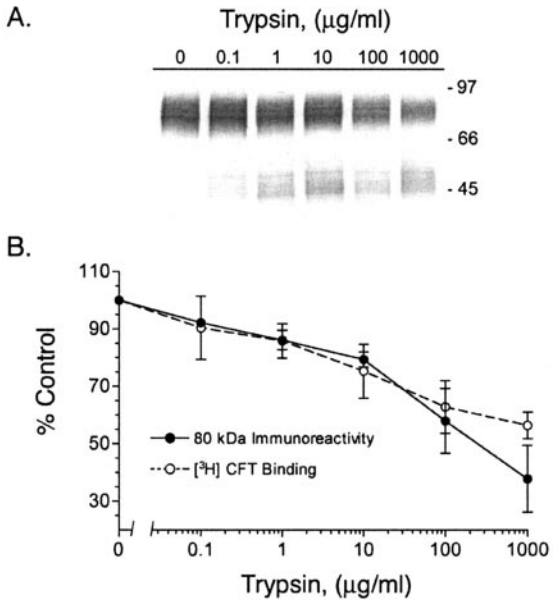

Fig. 8. Proteolysis of EL2 reduces [^{3}H]CFT binding. Rat striatal membranes were treated with or without the indicated concentrations of trypsin for 10 min at 22°C. Trypsin inhibitor was added and the membranes were washed and trypsin removed by centrifugation. The membranes were resuspended and aliquots were assayed for extent of proteolysis or for [^{3}H]CFT binding. A, representative immunoblot with MAb 16. B, quantitation of DAT proteolysis and [^{3}H]CFT binding, means ± S.E. of three separate experiments. Control values for [^{3}H]CFT binding were approximately 6 fmol/mg (o.w.w.) of tissue.

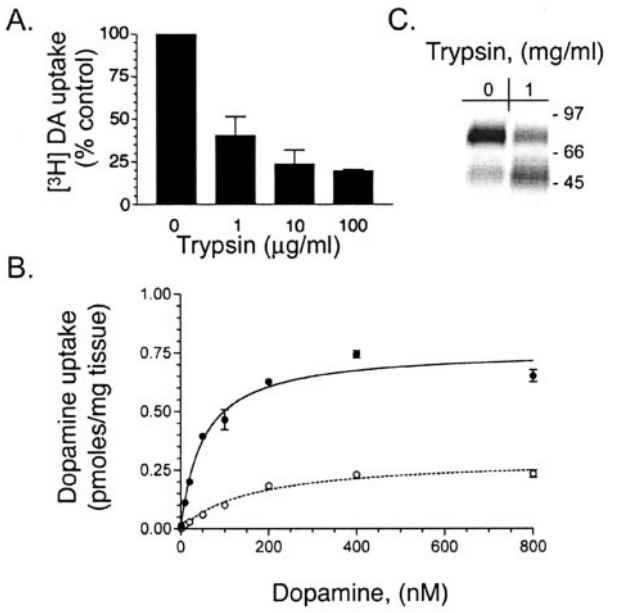

Fig. 9. Proteolysis of EL2 reduces [^{3}H]dopamine transport. Rat striatal synaptosomes were treated with or without the indicated concentration of trypsin for 10 min at 22°C. Trypsin inhibitor was added, samples were washed twice, and aliquots were assayed for the extent of proteolysis or for dopamine uptake. A, histogram showing decreased uptake of [^{3}H]DA after trypsin proteolysis of synaptosomes, means of two experiments performed in triplicate. B, saturation analysis of dopamine transport activity from synaptosomes treated with (○) or without (■) trypsin, shown as means ± S.E. of three independent experiments. C, immunoblot of synaptosomes used for saturation analysis showing the extent of EL2 proteolysis.

Kuhar, 1996). Results from SERT EL2 studies (Stephan et al., 1997) are also not consistent with direct participation of EL2 in inhibitor binding, lending more support to the hypothesis that ligand-induced protease resistance in EL2 is caused by protein conformational changes.

EL2 is by far the largest of the TM domain connecting loops in DAT and the other monoamine transporters. Its size and extensive glycosylation present the possibility that it may extend farther from the lipid bilayer than the other loops, giving it the potential to be the first region of the protein in contact with extracellular ions or substrates. If EL2 of DAT is essential for transport, as suggested for SERT (Stephan et al., 1997; Smicun et al., 1999), then inhibitor-induced reduction in its accessibility may prevent an early step in substrate recognition. It is also possible that stabilization of the C-terminal side of EL2 is propagated into the adjacent TM4 and/or other nearby TM helices, resulting in inhibition of TM conformational movements necessary for transport. Interestingly Zn^{2+}, which reduces dopamine uptake by coordinating residues on both sides of Arg^{218} (His^{193} in EL2 with His^{375} and Glu^{396} in TMs 7 and 8) (Loland et al., 1999), does not induce EL2 protease resistance, indicating that the mechanism of Zn^{2+} transport inhibition is distinct from that produced by ligand inhibitors. Zn^{2+} may inhibit transport by coordinating into a pre-existing binding site in the resting transporter, leading to prevention of movements required for transport by stabilization of the resting structure. Conversely, our results indicate that ligands may actively induce a conformation of EL2 that is substantially different from that of the resting state.

DAT transport activity is inhibited by a wide variety of structurally diverse abused and therapeutic drugs, and there is great interest in identifying their binding sites and elucidating their relationships to each other and the active sites

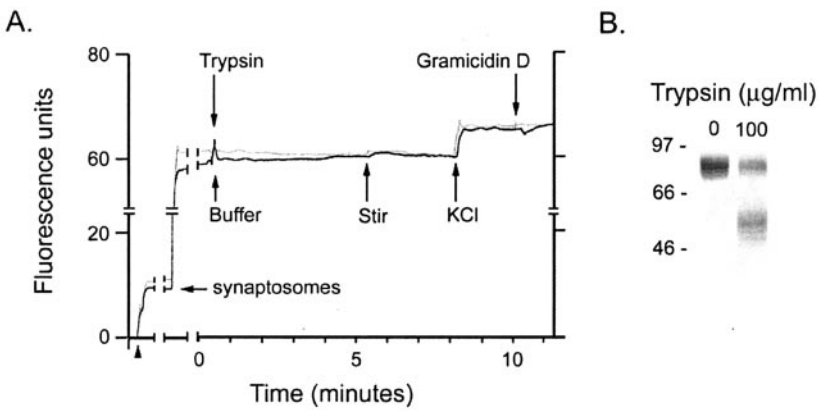

Fig. 10. Trypsin treatment does not affect synaptosomal polarity. Rat striatal synaptosomes were prepared by differential sucrose density centrifugation and assayed for changes in $DiBAC_4(3)$ fluorescence. A, fluorescence traces of synaptosomes incubated with buffer (dark line) or trypsin (light line). Arrows indicate the addition of $DiBAC_4(3)$, synaptosomes, buffer or 100 μg/ml trypsin, 30 mM KCl, and 9.3 μg/ml Gramicidin D. Identical results were obtained in two independent experiments. B, immunoblot of the synaptosomes used in the fluorescence assay showing the extent of EL2 proteolysis.

for transport. Current evidence suggests that the binding properties of many blockers are similar but not identical (Reith et al., 1992, 2001), compatible with the ligands occupying overlapping binding domains. Our results showing that reversible binding of several different classes of DAT uptake blockers induces similar effects in EL2 protease resistance further supports this idea. Many studies have shown that various residues and sites within the first six TM helices in DAT and SERT contribute to binding of cocaine, mazindol, and GBR analogs (Giros et al., 1994; Buck and Amara, 1995; Vaughan and Kuhar, 1996; Chen et al., 1997; Lee et al., 1998, 2000; Vaughan et al., 1999, 2001; Adkins et al., 2001; Lee et al., 2002; Pham et al., 2002), and we have shown that a single GBR-like ligand (Vaughan et al., 2001) and a tropane-based analog of [^{125}I]RTI82 (Zou et al., 2001) can become irreversibly incorporated into domains on both sides of EL2 (J. Gaffaney, R. Vaughan, M. Zou, A. Newman, unpublished results). Thus, the TM domains that flank EL2 may contribute to a shared binding pocket for multiple uptake blockers that is conformationally linked to the C-terminal side of EL2.

In contrast to the effects of uptake blockers, DAT protease sensitivity was not changed by dopamine, amphetamine, or methamphetamine, demonstrating that binding of substrates under conditions in which transport cannot occur does not induce the same changes in EL2 as inhibitors. Different effects of substrates and inhibitors at DAT have also been found in cysteine accessibility and mutagenesis studies (Ferrer and Javitch, 1998; Chen et al., 2000, 2002; Norregaard et al., 2003), although considerable evidence also indicates that substrate binding and translocation sites on monoamine transporters overlap or are similar to the sites occupied by uptake blockers (Buck and Amara, 1994; Barker et al., 1998, 1999; Lin et al., 2000a,b; Adkins et al., 2001; Vaughan et al., 2001; Chen et al., 2002). Thus, substrates may also interact with EL2-flanking TM domains without inducing EL2 stabilization, again compatible with the hypothesis that EL2 functions to maintain flexible domain relationships necessary for transport (Stephan et al., 1997; Smicun et al., 1999). Although we did not detect a change in protease sensitivity associated with substrate binding, changes in cysteine accessibility have been found in IL3 of DAT during transport (Ferrer and Javitch, 1998) and in the GABA and neuronal glutamate transporters using proteolysis (Mabjeesh and Kanner, 1993; Grunewald and Kanner, 1995). These studies suggested that the changes in accessibility and protease sensitivity were a result of conformational changes occurring during the transport process.

Some transport proteins, including the lactose permease and P-glycoprotein, are able to maintain proper structure and function via noncovalent interactions between TM domains after proteolysis or when independent segments are coexpressed (Bibi and Kaback, 1990; Loo and Clarke, 1994; Juul et al., 1995; Adamo et al., 2000), but this does not seem to be the case for DAT proteolyzed at Arg^{218} or Arg^{227}. When DAT was proteolyzed specifically in EL2, the B_{max} for [^{3}H]CFT binding and V_{max} for [^{3}H]DA uptake showed decreases that correlated strongly with the extent of EL2 digestion. Together with our previous results showing substantial loss of photoaffinity label binding after EL2 proteolysis (Vaughan and Kuhar, 1996), these results indicate that EL2 must be intact for DAT to function in both binding and transport. If EL2 is directly involved with the binding of ligands or substrates, then proteolysis may inhibit binding by destroying a necessary point of ligand-protein interaction. If EL2 functions indirectly to maintain proper TM domain spatial relationships, then proteolysis may affect binding and transport active sites by leading to disruption of TM orientations or interactions. Our results showing the irreversible incorporation of ligands on both sides of EL2 and the peptide mapping profiles that show cleavage of secondary connecting loops only after proteolysis of EL2 are compatible with the latter scenario. A more remote possibility that we cannot exclude is that DAT activity in these experiments is lost because of proteolysis of an unidentified accessory protein required for maintenance of function.

This study provides some of the first evidence pertaining to the involvement of EL2 in DAT binding and transport activity, and highlights the importance of this loop domain for proper transporter function. The results further implicate the contribution of TM helices near EL2 in the generation of a binding site for multiple uptake inhibitors and advance our understanding of molecular events associated with transport and transport inhibition by demonstrating the differential effects of substrates and uptake blockers on EL2.

Acknowledgments

We thank Dr. James Foster for valuable discussion and critical review of the manuscript during preparation.

References

Adamo HP, Grimaldi ME, and Bredeston LM (2000) The N-terminal region of the plasma membrane Ca^{2+} pump does not separate from the main catalytic fragments after proteolysis. *Biochim Biophys Acta* **1464:**127–134.

Adkins EM, Barker EL, and Blakely RD (2001) Interactions of tryptamine derivatives with serotonin transporter species variants implicate transmembrane domain I in substrate recognition. *Mol Pharmacol* **59:**514–523.

Barker EL, Moore KR, Rakhshan F, and Blakely RD (1999) Transmembrane domain I contributes to the permeation pathway for serotonin and ions in the serotonin transporter. *J Neurosci* **19:**4705–4717.

Barker EL, Perlman MA, Adkins EM, Houlihan WJ, Pristupa ZB, Niznik HB, and Blakely RD (1998) High affinity recognition of serotonin transporter antagonists defined by species-scanning mutagenesis. An aromatic residue in transmembrane domain I dictates species-selective recognition of citalopram and mazindol. *J Biol Chem* **273:**19459–19468.

Bibi E and Kaback HR (1990) In vivo expression of the lacY gene in two segments leads to functional lac permease. *Proc Natl Acad Sci USA* **87:**4325–4329.

Blaustein MP and Goldring JM (1975) Membrane potentials in pinched-off presynaptic nerve terminals monitored with a fluorescent probe: evidence that synaptosomes have potassium diffusion potentials. *J Physiol* **247:**589–615.

Brauner T, Hulser DF, and Strasser RJ (1984) Comparative measurements of membrane potentials with microelectrodes and voltage-sensitive dyes. *Biochim Biophys Acta* **771:**208–216.

Buck KJ and Amara SG (1994) Chimeric dopamine-norepinephrine transporters delineate structural domains influencing selectivity for catecholamines and 1-methyl-4- phenylpyridinium. *Proc Natl Acad Sci USA* **91:**12584–12588.

Buck KJ and Amara SG (1995) Structural domains of catecholamine transporter chimeras involved in selective inhibition by antidepressants and psychomotor stimulants. *Mol Pharmacol* **48:**1030–1037.

Chen JG, Sachpatzidis A, and Rudnick G (1997) The third transmembrane domain of the serotonin transporter contains residues associated with substrate and cocaine binding. *J Biol Chem* **272:**28321–28327.

Chen N, Ferrer JV, Javitch JA, and Justice JB Jr (2000) Transport-dependent accessibility of a cytoplasmic loop cysteine in the human dopamine transporter. *J Biol Chem* **275:**1608–1614.

Chen N and Reith ME (2000) Structure and function of the dopamine transporter. *Eur J Pharmacol* **405:**329–339.

Chen N, Sun L, and Reith ME (2002) Cationic interactions at the human dopamine transporter reveal binding conformations for dopamine distinguishable from those for the cocaine analog 2 alpha-carbomethoxy-3 alpha-(4-fluorophenyl)tropane. *J Neurochem* **81:**1383–1393.

Chen N, Vaughan RA, and Reith ME (2001) The role of conserved tryptophan and acidic residues in the human dopamine transporter as characterized by site-directed mutagenesis. *J Neurochem* **77:**1116–1127.

Dodd PR, Hardy JA, Oakley AE, Edwardson JA, Perry EK, and Delaunoy JP (1981) A rapid method for preparing synaptosomes: comparison, with alternative procedures. *Brain Res* **226:**107–118.

Ferrer JV and Javitch JA (1998) Cocaine alters the accessibility of endogenous cysteines in putative extracellular and intracellular loops of the human dopamine transporter. *Proc Natl Acad Sci USA* **95:**9238–9243.

Giros B, Wang YM, Suter S, McLeskey SB, Pifl C, and Caron MG (1994) Delineation

of discrete domains for substrate, cocaine and tricyclic antidepressant interactions using chimeric dopamine-norepinephrine transporters. *J Biol Chem* **269:**15985–15988.
Grunewald M and Kanner B (1995) Conformational changes monitored on the glutamate transporter GLT-1 indicate the existence of two neurotransmitter-bound states. *J Biol Chem* **270:**17017–17024.
Heikkila RE and Manzino L (1984) Behavioral properties of GBR 12909, GBR 13069 and GBR 13098: specific inhibitors of dopamine uptake. *Eur J Pharmacol* **103:** 241–248.
Juul B, Turc H, Durand ML, Gomez de Gracia A, Denoroy L, Moller JV, Champeil P, and le Maire M (1995) Do transmembrane segments in proteolyzed sarcoplasmic reticulum Ca^{2+}-ATPase retain their functional Ca^{2+} binding properties after removal of cytoplasmic fragments by proteinase K? *J Biol Chem* **270:**20123–20134.
Krueger BK (1990) Kinetics and block of dopamine uptake in synaptosomes from rat caudate nucleus. *J Neurochem* **55:**260–267.
Kuhar MJ, Ritz MC, and Boja JW (1991) The dopamine hypothesis of the reinforcing properties of cocaine. *Trends Neurosci* **14:**299–302.
Lee SH, Chang MY, Jeon DJ, Oh DY, Son H, Lee CH, and Lee YS (2002) The functional domains of dopamine transporter for cocaine analog, CFT binding. *Exp Mol Med* **34:**90–94.
Lee SH, Chang MY, Lee KH, Park BS, Lee YS, and Chin HR (2000) Importance of valine at position 152 for the substrate transport and 2β-carbomethoxy-3β-(4-fluorophenyl)tropane binding of dopamine transporter. *Mol Pharmacol* **57:**883–889.
Lee SH, Kang SS, Son H, and Lee YS (1998) The region of dopamine transporter encompassing the 3rd transmembrane domain is crucial for function. *Biochem Biophys Res Commun* **246:**347–352.
Lin Z, Itokawa M, and Uhl GR (2000a) Dopamine transporter proline mutations influence dopamine uptake, cocaine analog recognition and expression. *FASEB J* **14:**715–728.
Lin Z, Wang W, and Uhl GR (2000b) Dopamine transporter tryptophan mutants highlight candidate dopamine- and cocaine-selective domains. *Mol Pharmacol* **58:**1581–1592.
Loland CJ, Norregaard L, and Gether U (1999) Defining proximity relationships in the tertiary structure of the dopamine transporter. Identification of a conserved glutamic acid as a third coordinate in the endogenous Zn^{2+}-binding site. *J Biol Chem* **274:**36928–36934.
Loo TW and Clarke DM (1994) Reconstitution of drug-stimulated ATPase activity following co-expression of each half of human P-glycoprotein as separate polypeptides. *J Biol Chem* **269:**7750–7755.
Mabjeesh NJ and Kanner BI (1993) The substrates of a sodium- and chloride-coupled gamma-aminobutyric acid transporter protect multiple sites throughout the protein against proteolytic cleavage. *Biochemistry* **32:**8540–8546.
Norregaard L, Frederiksen D, Nielsen EO, and Gether U (1998) Delineation of an endogenous zinc-binding site in the human dopamine transporter. *EMBO (Eur Mol Biol Organ) J* **17:**4266–4273.
Norregaard L, Loland CJ, and Gether U (2003) Evidence for distinct sodium, dopamine and cocaine dependent conformational changes in transmembrane segments 7 and 8 of the dopamine transporter. *J Biol Chem.*
Ohkuma S, Katsura M, Higo A, Shirotani K, Hara A, Tarumi C, and Ohgi T (2001) Peroxynitrite affects Ca^{2+} influx through voltage-dependent calcium channels. *J Neurochem* **76:**341–350.
Pham AT, Gaffaney JD, Wirtz SE, Granstrem O, Vaughan RA, Lever J, and Justice JB (2002) Enzymatic and chemical cleavage of [^{125}I]RTI-82 labeled 3XFLAG6XHIShDAT. *Soc Neurosci Abstr* 898.17.
Reith ME, Berfield JL, Wang LC, Ferrer JV, and Javitch JA (2001) The uptake inhibitors cocaine and benztropine differentially alter the conformation of the human dopamine transporter. *J Biol Chem* **276:**29012–29018.
Reith ME, de Costa B, Rice KC, and Jacobson AE (1992) Evidence for mutually exclusive binding of cocaine, BTCP, GBR 12935 and dopamine to the dopamine transporter. *Eur J Pharmacol* **227:**417–425.
Reith ME and Selmeci G (1992) Radiolabeling of dopamine uptake sites in mouse striatum: comparison of binding sites for cocaine, mazindol and GBR 12935. *Naunyn-Schmiedeberg's Arch Pharmacol* **345:**309–318.
Smicun Y, Campbell SD, Chen MA, Gu H, and Rudnick G (1999) The role of external loop regions in serotonin transport. Loop scanning mutagenesis of the serotonin transporter external domain. *J Biol Chem* **274:**36058–36064.
Stephan MM, Chen MA, Penado KM, and Rudnick G (1997) An extracellular loop region of the serotonin transporter may be involved in the translocation mechanism. *Biochemistry* **36:**1322–1328.
Vaughan RA (1995) Photoaffinity-labeled ligand binding domains on dopamine transporters identified by peptide mapping. *Mol Pharmacol* **47:**956–964.
Vaughan RA, Agoston GE, Lever JR, and Newman AH (1999) Differential binding of tropane-based photoaffinity ligands on the dopamine transporter. *J Neurosci* **19:** 630–636.
Vaughan RA, Gaffaney JD, Lever JR, Reith ME, and Dutta AK (2001) Dual incorporation of photoaffinity ligands on dopamine transporters implicates proximity of labeled domains. *Mol Pharmacol* **59:**1157–1164.
Vaughan RA, Huff RA, Uhl GR, and Kuhar MJ (1997) Protein kinase C-mediated phosphorylation and functional regulation of dopamine transporters in striatal synaptosomes. *J Biol Chem* **272:**15541–15546.
Vaughan RA and Kuhar MJ (1996) Dopamine transporter ligand binding domains. Structural and functional properties revealed by limited proteolysis. *J Biol Chem* **271:**21672–21680.
Wiener HL and Reith ME (1992) Determination of radioligand specific activity using competition binding assays. *Anal Biochem* **207:**58–62.
Zhang Z, Huang JM, Turner MR, Rhinehart KL and Pallone TL (2001) Role of chloride in constriction of descending vasa recta by angiotensin II. *Am J Physiol* **280:**R1878–R1886.
Zou MF, Kopajtic T, Katz JL, Wirtz S, Justice JB Jr, and Newman AH (2001) Novel tropane-based irreversible ligands for the dopamine transporter. *J Med Chem* **44:**4453–4461.

Address correspondence to: Dr. Roxanne A. Vaughan, Department of Biochemistry and Molecular Biology, University of North Dakota School of Medicine and Health Sciences, 501 N. Columbia Rd., Grand Forks, ND 58203. E-mail: rvaughan@medicine.nodak.edu