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Identification of the benztropine analog [^{125}I]GA II 34 binding site on the human dopamine transporter

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ABSTRACT

The dopamine transporter (DAT) is a neuronal membrane protein that is responsible for reuptake of dopamine (DA) from the synapse and functions as a major determinant in control of DA neurotransmission. Cocaine and many psychostimulant drugs bind to DAT and block reuptake, inducing DA overflow that forms the neurochemical basis for euphoria and addiction. Paradoxically, however, some ligands such as benztropine (BZT) bind to DAT and inhibit reuptake but do not produce these effects, and it has been hypothesized that differential mechanisms of binding may stabilize specific transporter conformations that affect downstream neurochemical or behavioral outcomes. To investigate the binding mechanisms of BZT on DAT we used the photoaffinity BZT analog [^{125}I]*N*-[*n*-butyl-4-(4‴-azido-3‴-iodophenyl)]-4′,4″-difluoro-3α-(diphenylmethoxy)tropane ([^{125}I]GA II 34) to identify the site of cross-linking and predict the binding pose relative to that of previously-examined cocaine photoaffinity analogs. Biochemical findings show that adduction of [^{125}I]GA II 34 occurs at residues Asp79 or Leu80 in TM1, with molecular modeling supporting adduction to Leu80 and a pharmacophore pose in the central S1 site similar to that of cocaine and cocaine analogs. Substituted cysteine accessibility method protection analyses verified these findings, but identified some differences in structural stabilization relative to cocaine that may relate to BZT neurochemical outcomes.

1. Introduction

1.1. Dopaminergic signaling

Reuptake of dopamine (DA) from the synapse following vesicular release is mediated by the dopamine transporter (DAT), a Na^+/Cl^-–dependent symporter that represents one of the major determinants of spatiotemporal regulation of dopaminergic neurotransmission (Bröer and Gether, 2012; Kristensen et al., 2011; Pramod et al., 2013). DA controls numerous processes including movement, emotion, behavior, and memory (Kristensen et al., 2011; Kurian et al., 2011, 2009; Pramod et al., 2013), and dysregulation of reuptake has been implicated in a variety of diseases including attention deficit hyperactivity disorder, Parkinson disease, and bipolar disorder (Grünhage et al., 2000; Hahn and Blakely, 2002; Pramod et al., 2013). DAT and the closely related serotonin and norepinephrine transporters (SERT and NET) comprise the monoamine transporter (MAT) family, and serve as major targets for addictive and therapeutic psychostimulants that elevate extracellular monoamine levels by inhibiting reuptake (Cheng et al., 2015; Gether et al., 2006; Rothman and Baumann, 2003; Sealover et al., 2016; Sulzer et al., 2005). Cocaine and many other DAT inhibitors induce locomotor stimulation and behavioral reinforcement, but some blockers lack these effects and are able to ameliorate cocaine outcomes in animal models (Reith et al., 2015; Rothman et al., 2008; Schmitt et al., 2008; Schmitt and Reith, 2011). This suggests the potential for development of pharmacotherapies for drug abuse and DA

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disorders, but the molecular basis by which differential neurochemical and behavioral outcomes can follow from blockade of reuptake by structurally distinct ligands is unclear.

1.2. Monoamine transporter structure-function

The MATs are composed of 12 transmembrane (TM) spanning domains connected by intracellular (IL) and extracellular (EL) loops with cytoplasmically oriented N- and C-termini (Bröer and Gether, 2012; Kristensen et al., 2011; Pramod et al., 2013; Rudnick et al., 2013). Transport occurs by an alternating access mechanism (Jardetzky, 1966) in which the transporter cycles through multiple conformational states that generate outwardly-facing, occluded, and inwardly-facing forms (Forrest et al., 2008; Krishnamurthy and Gouaux, 2012; Yamashita et al., 2005) that bind and release substrate on opposite sides of the membrane (Forrest et al., 2008; Rudnick et al., 2013). Mechanistic insights into these events have been obtained from high-resolution structures of the *Aquifex aeolicus* leucine transporter (LeuT), *Drosophila melanogaster* DAT (dDAT), and human SERT (hSERT) crystallized in multiple states of the transport cycle that correspond to these forms (Coleman et al., 2016; Wang et al., 2015; Yamashita et al., 2005). The primary substrate binding site (S1) is located near the center of the protein, and in the outwardly-facing form is connected to the external medium by an aqueous vestibule (Fig. 1). Binding of substrate triggers closure of an extracellular gate that occludes the active site, followed by opening of an intracellular gate, release of solutes into the cell interior, and return of the empty protein to the outward form (Cheng and Bahar, 2015; Felts et al., 2014; Forrest et al., 2008; Henry et al., 2011; Koldsø et al., 2011; Shan et al., 2011; Shi et al., 2008; Tavoulari et al., 2016; Zhao et al., 2011). A proposed secondary substrate binding site is located in the outer vestibule above the extracellular gate (Fig. 1).

1.3. Transport inhibition

Uptake blockers arrest the transport cycle in one or more of these phases, with significant evidence from biochemical, molecular, and crystallization studies supporting high-affinity binding of cocaine and many other inhibitors to S1, where they compete with substrates and stabilize outwardly-facing or outwardly-occluded conformations that cannot complete the transport cycle (Henry et al., 2003; Kristensen et al., 2011; Pramod et al., 2013; Rudnick et al., 2013; Yamashita et al., 2005). However, alternative modes of binding are possible, as atypical MAT inhibitors such as ibogaine bind to and stabilize inwardly-facing transporter conformations (Bulling et al., 2012). MATs may also accommodate substrate and inhibitor binding at the low affinity S2 site (Fig. 1) that may allosterically regulate binding and transport functions (Coleman et al., 2016; Penmatsa et al., 2015, 2013; Quick et al., 2009; Singh et al., 2008, 2007a; Wang et al., 2015; Zhou et al., 2009, 2007).

1.4. Atypical DAT inhibitors

Major efforts have been directed toward development of a pharmacotherapeutic drug for cocaine abuse that would induce little to no psychomotor stimulation or reinforcement (Cao et al., 2016; Desai et al., 2005; Kohut et al., 2014; Newman and Kulkarni, 2002; Reith et al., 2015; Rothman, 1990; Tunstall et al., 2018; Zhang et al., 2017; Zou et al., 2017). One candidate that has emerged is the DAT antagonist benztropine (BZT), which possesses a tropane-based pharmacophore similar to that of cocaine, but does not induce cocaine-like behavioral profiles and can ameliorate some cocaine-induced behaviors (Agoston et al., 1997b; Hiranita et al., 2009; Katz et al., 2004, 1997; Newman et al., 1995, 1994). The mechanisms by which these distinct outcomes are mediated are unknown, as computational and mutational analyses support both BZT and cocaine coordination at the S1 binding site where they function as competitive uptake inhibitors (Beuming et al., 2008;

Fig. 1. Surface representation of monoamine transporters (side view) with the S1 (magenta) and S2 (yellow) binding sites. The spheres represent the first shell (within 4 Å) of ligand coordinating residues from the co-crystallization of two s-citalopram molecules in the human serotonin transporter by Coleman et al., 2016), PDBID 5i75. The conserved residue D79 is labeled for reference. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Bisgaard et al., 2011). The apparent similarities of cocaine and BZT binding mechanisms has led to the speculation that their distinct endpoints may follow from the stabilization of different conformations of the transporter that impact transporter regulatory properties that lead to different outcomes (Abramyan et al., 2017; Chen et al., 2004; Kohut et al., 2014; Reith et al., 2001; Rothman et al., 2008; Ukairo et al., 2005; Vaughan et al., 1999).

To examine the unique properties of BZT and uncover its molecular interactions with DAT, we developed the irreversible BZT analog [^{125}I]GA II 34 ([^{125}I]N-[*n*-butyl-4-(4′-azido-3′-iodophenylethyl-ester)]-4′,4″-difluoro-3α-(diphenylmethoxy)tropane) (Agoston et al., 1997a), which contains a benztropine pharmacophore with a photoactivatable 4′-azido-3′-iodophenylethyl ester (AIP) moiety appended to the tropane N via a four carbon linking chain (Fig. 2). This moiety can form a covalent bond with proximal N, H or C atoms in the bound protein upon photoactivation (Geurink et al., 2012; Kotzyba-Hibert et al., 1995), allowing for identification of residues proximal to the binding site. This information can be used in conjunction with molecular modeling to predict the binding pose and identify key binding elements, an approach we used to identify binding sites of the cocaine photoaffinity analogs RTI 82 and MFZ 2–24 (shown in Fig. 2) (Dahal et al., 2014a; Krout et al., 2017). We previously demonstrated that [^{125}I]GA II 34 cross-links to rat (r) DAT in TMs 1–2 (Vaughan et al., 1999), and here we extend these studies in human (h) DAT using Met substitution mutagenesis and cyanogen bromide digestion, identifying Asp79 or Leu80 in TM1 as the [^{125}I]GA II 34 attachment site. Computational modeling of GA II 34 docking to DAT homology models narrowed the adduction site to Leu80 and identified binding in S1, where it shared significant overlap with the binding site of cocaine, RTI 82, and MFZ 2–24. Substituted cysteine accessibility (SCAM) protection analyses verified the accuracy of this pose, but identified a more outwardly open conformation for GA II 34 than that induced by cocaine (Dahal et al., 2014a).

2. Materials and methods

2.1. Reagents

DAT polyclonal Ab 16 and monoclonal Ab 16 generated against N-terminal residues 42–59 were used for immunoprecipitation and immunoblotting as previously described (Foster et al., 2002; Gaffaney and Vaughan, 2004). Triton X-100 was from VWR (Batavia, IL), SDS and 4–20% polyacrylamide gels were from Bio-Rad (Hercules, CA), Complete Mini Protease Inhibitor was from Roche Applied Sciences (Indianapolis, IN), HEPES buffer, glucose, trypsin, and trypsin inhibitor were from Sigma-Aldrich (St. Louis, MO), X-ray film was from GE Healthcare Life Sciences (Piscataway NJ), and MTSEA-biotin was from Biotium (Freemont, CA). All other chemicals and reagents were obtained from Fisher Scientific (Waltham, MA). Male Sprague-Dawley rats were obtained from Charles River Laboratory, (Wilmington, MA) and 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.

Fig. 2. Structures of DAT ligands and hDAT labeling with [^{125}I] GA II 34. Structures of (A) cocaine, (B) benztropine, (C) [^{125}I]GA II 34, (D) [^{125}I]MFZ 2–24, and (E) [^{125}I]RTI 82 showing the shared tropane nitrogen pharmacophore and orientation of IAP moiety. (F) Autoradiogram of hDAT photoaffinity labeled with 15 nM [^{125}I]GA II 34 in the absence or presence of 10 μM mazindol followed by immunoprecipitation with Ab16. Molecular mass marker (kDa) is shown on the right.

2.2. GA II 34 synthesis and radioiodination

GA II 34 and the amino precursor of [^{123}I]GA II 34 were synthesized using modifications of the original procedure (Agoston et al., 1997b; Vaughan et al., 1999) and radioiodinated as previously described (Agoston et al., 1997a).

2.3. Cell culture and site-directed mutagenesis

Photoaffinity labeling experiments utilized wild-type (WT) and previously-generated hDAT mutant constructs stably expressed in Lilly Laboratory Cell-Porcine Kidney (LLC-PK_1) cells as described (Dahal et al., 2014a). For SCAM analysis, mutants D79C, W84C, R85C, V152C, I159C, F319C, S421C, D475C, and A479C were generated in the E2C background (C90A and C305A) of pcDNA3-rDAT and expressed transiently in HEK-GripTite cells as previously described (Dahal et al., 2014a; Krout et al., 2017).

2.4. Photoaffinity labeling and tryptic proteolysis of rDAT

Rat striatal membranes prepared as described (Parnas et al., 2008a; Vaughan et al., 2007) were resuspended at a concentration of 20 mg/ml original wet weight (O.W.W.) in Krebs Ringers HEPES (KRH) buffer (25 mM HEPES, 125 mM NaCl, 4.8 mM KCl (Sigma-Aldrich, St. Louis, MO), 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mM glucose (Sigma-Aldrich); pH 7.4). Aliquots were incubated on ice for 2 h with 10 nM [^{125}I]GA II 34, and ligand was covalently cross-linked to DAT by irradiation with UV light (254 nm) for 5 min. Membranes were washed 3X with 50 mM Tris-HCl pH 8.0 and resuspended to a final concentration of 40 mg/ml O.W.W. Aliquots (45 µl) were treated with 10 µg/ml trypsin in 50 mM Tris-HCl, pH 8.0, for 20 min at 30 °C, and digestion halted by adding soybean trypsin inhibitor and centrifugation at 20,000 x *g* for 20 min. Membranes were solubilized with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS; pH 7.5) containing Complete Mini protease inhibitors, centrifuged at 20,000 x *g* for 12 min at 4 °C, immunoprecipitated with Ab16 and electrophoresed on 4–20% polyacrylamide gels (Bio-Rad, Hercules, CA) followed by autoradiography (GE Healthcare Life Sciences, Piscataway, NJ).

2.5. Photoaffinity labeling of hDAT

WT and mutant cells were grown on 6 well plates to 80–90% confluency, washed with KRH, and incubated on ice for 2 h with 15 nM [^{125}I]GA II 34. Ligand was covalently cross-linked to DAT by irradiation with UV light (254 nm) for 5 min and unbound ligand removed by washing twice with ice cold KRH. Samples were lysed with RIPA, insoluble material removed by centrifugation, and the supernatant subjected to SDS-PAGE and autoradiography on 8% Tris-glycine polyacrylamide gels. Photolabeled DAT bands were excised, extracted by electroelution and dialyzed against deionized water (Vaughan, 1995).

2.6. CNBr peptide mapping

Aliquots of gel purified DATs were counted in a scintillation counter and within each experiment, equal amounts of radioactivity were lyophilized to dryness for analysis. For CNBr digestion samples were resuspended in 70 µL of 70% formic acid with or without addition of 1M CNBr and incubated for 24 h at 22 °C in the dark. Reactions were quenched with MilliQ water and CNBr was removed by 3 cycles of lyophilization and resuspension in water. Final samples were resuspended in immunoprecipitation buffer (50 mM Tris-HCl, 0.1% Triton X-100, pH 8.0) and aliquots were analyzed directly by SDS-PAGE/autoradiography to visualize the total fragment pattern or were subjected to immunoprecipitation with Ab16. For each experiment, control and mutant DATs were photoaffinity labeled and analyzed exactly in parallel and all experiments were replicated twice with similar results. Peptide masses were calculated using PeptideCutter via ExPASy (Gasteiger et al., 2005).

2.7. Computational docking and molecular dynamic simulations

An ensemble of comparative hDAT homology models ('open to out' transporter conformation) were built using Rosetta.3.1 as previously described (Dahal et al., 2014a; Krout et al., 2017) and was based on the nortriptyline-bound dDAT co-crystal (PDB ID: 4M48). dDAT rather than LeuT was chosen as the input structure to create the hDAT homology model because dDAT shares greater homology with hDAT. The top-scoring models from the Rosetta *Etotal* energy function were visually inspected in Pymol (Schrodinger, 2015) for structural integrity. The top 10 models were forwarded for *in silico* ensemble ligand docking using RosettaLigand (Combs et al., 2013). The top 10% of docked complexes based on *interface_delta (IF_delta)* scores were analyzed by ligand-based r.m.s.d clustering (Combs et al., 2013). *IF_delta* is calculated as the difference between the energy with and without the ligand. This approximates the energetic contribution to the complex afforded by the bound ligand. Clusters were ranked by the number of complexes per cluster and filtered to include models where the azido moiety was within 7.5 Å to Asp79 or Leu80 as previously described (Dahal et al., 2014a; Krout et al., 2017). The top scoring model from the distance constraint analysis underwent molecular dynamic simulations for 150 ns in a 1-palmitoyl-2-oleoyl- phosphatidylcholine (POPC) lipid bilayer buffered with water and Na^+ and Cl^- ions using GROMACS (Dahal et al., 2014a; Krout et al., 2017). The dimensions of the simulation box were 20 × 20 × 14 Å containing one protein, one ligand, 50,000 TIP3P water molecules, and 225 POPC molecules. Electroneutrality for the system was obtained by addition of 186 Na^+ and 188 Cl^- counter-ions yielding a salt concentration of 150 mM. All simulations were carried out with GROMACS version 4.5.4 (Hess et al., 2008) using CHARMM27 force field under periodic boundary conditions. The remaining parameters can be found in our previous publication (Dahal et al., 2014a). Interatomic distances were obtained using *g_dist* in GROMACS.

2.8. SCAM protection analysis of S1- and S2-binding sites

GripTite cells were plated at a density of 150,000 cells/cm^2 in 24-well culture plates, incubated for 24 h, and transfected with rDAT constructs as described above. Following transfection (48 h), cells were processed as described previously (Dahal et al., 2014a; Henry et al., 2003). Briefly, cells were incubated with 10 or 50 µM GA II 34 or vehicle for 5 min in PBS/CM (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.1 mM $CaCl_2$, 1.0 mM $MgCl_2$, pH 7.4) followed by addition of 0.1 mM MTSEA-biotin for 10 min (Biotium, Freemont, CA). Cell lysates were obtained with RIPA solubilization buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) containing Halt™ Protease Inhibitor Single-Use Cocktail and incubated with NeutrAvidin-agarose resin to extract surface proteins labeled by MTSEA-Biotin. Protein concentration was determined by BCA assay, and equal amounts of protein from total samples and equivalent volumes of surface protein pools were processed by SDS-PAGE and immunoblotting using anti-DAT monoclonal antibody, MAb 16 (Foster et al., 2002; Gaffaney and Vaughan, 2004). DAT levels were quantified by densitometry using Image Studio (LI-COR). Surface values were normalized to total DAT levels and are expressed as a percent of untreated samples. Data were analyzed using a paired *t*-test (Prism 5, Graphpad), with significance set at $p < 0.05$.

3. Results

3.1. Tryptic mapping of [^{125}I]GA II 34 in native DAT

Our previous [^{125}I]GA II 34 labeling and mapping studies were

Fig. 3. Localization of [^{125}I] GA II 34 adduction to TMs 1–2. A. Rat striatal membranes photoaffinity labeled with [^{125}I]GA II 34 were treated with vehicle or 10 μg/ml trypsin, subjected to IP with Ab16, and analyzed by SDS PAGE/autoradiography. Arrows a and b indicate major tryptic fragments recovered. Schematic diagram a shows TM domains (cylinders), trypsin digestion sites consistent with fragment masses (black circles), and Ab16 epitope. Shading indicates primary sequence domains corresponding to fragments a and b. B. WT or M272L hDATs photoaffinity labeled with [^{125}I]GA II 34 were gel-purified, treated with vehicle or CNBr and analyzed by SDS-PAGE/autoradiography. Arrows a and b indicate major fragments generated. Schematic diagram b indicates positions of all Met residues in WT DAT (open and filled circles), Met residues present in M272L DAT (filled circles), and shading represents primary sequence consistent with fragment a. Molecular mass markers (kDa) for all gels are shown on left.

performed using native DATs from rat striatum (Vaughan et al., 1999), and here we extend the analyses to heterologously expressed hDAT. Irreversible labeling of hDAT with [^{125}I]GA II 34 is shown in Fig. 2F, with displacement by mazindol supporting pharmacological specificity of binding. Preliminary experiments that support a similar mode of N-terminal adduction of [^{125}I]GA II 34 to hDAT in comparison to that of rDAT are shown in Fig. 3. N-terminal adduction of [^{125}I]GA II 34 to rat striatal DAT was demonstrated by tryptic proteolysis and isolation of peptide fragments by IP with Ab 16 (Fig. 3A). This procedure generates a 45 kDa peptide that contains TMs 1–3 via proteolysis of Arg218 and a 10 kDa peptide that contains TM1 generated by proteolysis of Arg85 or Lys92 (Parnas et al., 2008a; Vaughan and Kuhar, 1996), as indicated in schematic diagram a.

3.2. CNBr mapping of [^{125}I]GA II 34 attachment

To investigate the [^{125}I]GA II 34 adduction site in hDAT we used cyanogen bromide (CNBr), which cleaves proteins on the C-terminal side of methionine (Met) residues (Gross, 1967), in conjunction with Met substitution mutagenesis. In this approach, endogenous Mets are removed and exogenous Mets are inserted near the ligand adduction site to generate novel CNBr fragments that reveal the site of ligand adduction relative to inserted cleavage sites. Met constructs used in these analyses were active for cocaine-displaceable [^{3}H]CFT binding and [^{3}H]DA uptake except for M272L and D79M which were inactive for transport (Krout et al., 2017; Parnas et al., 2008b) (data not shown). For preliminary analyses (Fig. 3B) we performed parallel digestions of WT hDAT and construct M111L/M116L/M272L (denoted M272L), previously used for mapping the adduction site of the cocaine analogs analogs [^{125}I]MFZ 2–24 and [^{125}I]RTI 82 (Parnas et al., 2008a). This construct eliminates two of three closely spaced Mets in TM2 and one Met in TM5, generating a protein with only one Met (Met106) present between the N-terminal tail and Met371 in TM7 (Fig. 3B, schematic diagram b). Proteolysis of this construct would thus generate fragments extending from Met1/11-Met106 (calculated mass ~10.5 kDa), or from Met106-Met371 (estimated mass ~55 kDa from peptide sequence plus EL2 glycosylation). In Fig. 3B, WT and M272L hDATs were labeled with [^{125}I]GA II 34, gel purified, and subjected to CNBr digestion in parallel. For both constructs the major product was a fragment of ~10 kDa (arrow a), consistent with the majority of ligand incorporation occurring between the N-terminal Mets and Met106 (shaded region schematic diagram b). Minor bands in both samples at ~65 kDa (arrow b) likely represent fragments extending from the N-terminus to Met371 with missed proteolysis of TM2 and TM5 Mets, and strongly indicate that the majority of [^{125}I]GA II 34 adduction occurred at sites N-terminal to TM2.

3.3. Localizing [^{125}I]GA II 34 adduction to TM1 in DAT

We recently showed that the cocaine photoaffinity analog [^{125}I]MFZ 2–24 binds to DAT in the S1 pocket with the tropane N oriented toward Asp79 and the AIP moiety undergoing adduction to adjacent residue Leu80 (Krout et al., 2017). Based on the similarity of the [^{125}I]GA II 34 tropane and AIP moieties to those of [^{125}I]MFZ 2–24 (Fig. 2), we hypothesized that [^{125}I]GA II 34 would possess a similar binding pose and incorporation site, allowing us to focus on this region of TM1. For these mapping experiments we thus utilized the previously-described TM1 constructs V78M, D79M, and L80M (Krout et al., 2017) to probe the region surrounding these key residues, using construct M111L/M116L (termed M106) as control. Although D79M DAT was used successfully for analysis of [^{125}I]MFZ 2–24 mapping, it showed insufficient labeling with [^{125}I]GA II 34 for use in these studies (not shown), preventing us from specifically assessing this residue as an adduction site.

Because cleavage of the inserted TM1 Mets would only change the mass of the labeled TM1-2 fragments by a small amount that would preclude accurate characterization by SDS-PAGE, we used immunoprecipitation with Ab 16 to determine the relative location of ligand adduction and proteolysis sites (Fig. 4). M106, V78M, and L80M

Fig. 4. Localization of [^{125}I] GA II 34 adduction to residues 79 or 80. M106, L80M, and V78M hDATs photoaffinity labeled with [^{125}I]GA II 34 were gel purified and treated with vehicle or CNBr. Samples were subjected directly to SDS-PAGE for analysis of total fragment production (A), or were immunoprecipitated with Ab16 (B). Arrows a, b, and c indicate migration of major CNBr fragments, and schematic diagrams a, b, and c represent primary sequence regions consistent with masses and IP characteristics of fragments a, b, and c.

DATs were labeled with [^{125}I]GA II 34, gel purified, and digested with CNBr, and aliquots of each sample were analyzed directly by SDS-PAGE to visualize the total fragment pattern (Fig. 4A) or subjected to IP with Ab 16 to determine presence or absence of the antibody epitope in the fragments (Fig. 4B). In M106 DAT, the primary labeled fragment migrated at ~10 kDa (arrow a) (Fig. 4A, lane 2) and this fragment was immunoprecipitated with Ab16 (arrow a) (Fig. 4B, lane 2), verifying [^{125}I]GA II 34 incorporation between Met1/Met11 and Met106 (shaded region in schematic diagram a). In L80M and V78M DATs, CNBr digestion also produced [^{125}I]GA II 34-labeled fragments of ~10 kDa (arrow a) (Fig. 4A, lanes 4 and 6), that likely result from missed proteolysis of the inserted Mets, as well as lower M_r fragments that ran ahead of the 8 kDa marker (arrows b and c) (Fig. 4A, lanes 4 and 6) and likely derive from proteolysis of the inserted Mets. The 10 kDa fragments in both samples immunoprecipitated with Ab16 (arrow a) (Fig. 4B, lanes 4 and 6), suggesting that they extend from Met1/Met11 to Met106, as in M106 DAT. The low M_r peptide from L80M DAT was immunoprecipitated with Ab16 (arrow b) (Fig. 4B, lane 4), indicating that it extends from Met1/11 to Met80, (shaded region in schematic diagram b). In contrast, there was no appreciable IP of the low M_r fragment from V78M DAT (Fig. 4B, lane 6), indicating that this fragment lacks the Ab16 epitope and thus extends from Asp79-Met106 (shaded region in schematic diagram c). As CNBr cleaves proteins on the C-terminal side of Mets, these results indicate that adduction of GA-II-34 to DAT occurs at Asp79 or Leu80.

3.4. Computational docking and molecular modeling

RosettaLigand docking analysis of GA II 34 to hDAT homology models derived from the nortriptyline-bound dDAT (Wang et al., 2015) identified a top scoring hDAT-GA II 34 complex in which the ligand is positioned in the S1 binding pocket with the tropane nitrogen of GA II 34 within 4.1 Å of the O of Asp79 (Fig. 5A). In addition, the photoactivated, ring-proximal N of the azido moiety was 7.5 Å from the Leu80 side chain (Fig. 5A). Subsequently, molecular dynamic simulations were performed with the ligand/transporter complex sampled in a solvated lipid bilayer system. The results showed that the phenylazido moiety repositioned such that the azido-reactive N was within 4.5 Å of the C1 of Leu 80 and therefore capable of adduction upon photoactivation (Figs. 5A and 6A). The tropane N rotates slightly but maintains the ~4 Å distance to both O atoms of Asp79 (Figs. 5A and 6B), which would allow for salt bridge formation. These findings are consistent with GA II 34 binding to the S1 site and interacting with many of the same residues that coordinate binding of cocaine (Fig. 5C), DA amphetamine, MFZ 2–24 and RTI 82 (Dahal et al., 2014b; Krout et al., 2017; Wang et al., 2015) (Fig. 5B and D). Whereas this binding pose positions GA II 34 and MFZ 2–24 AIP moieties for cross-linking to Leu 80 (Krout et al., 2017), the differential orientation of RTI 82 AIP arm results in adduction to Phe320 (Dahal et al., 2014b). Interestingly, Asp79 displays bidentate coordination of both Os with the GA II 34 tropane N (Fig. 6B). This is distinct from the monodentate coordination observed with RTI 82 and MFZ 2–24 where the O atoms oscillated such that one interacted with the tropane N and the other O coordinated Na^+ at the Na1 site. Nevertheless, the tropane core of GA II 34 is virtually superimposable with that of cocaine in the dDAT co-crystal (Wang et al., 2015), with the position of the tropane Ns differing by only 1.4 Å (Fig. 5D). One of the fluorophenyl arms of GA II 34 overlays on the phenyl-ester of cocaine and coordinates with Asn157 as previously modeled for BZT (Bisgaard et al., 2011), whereas the other fluorophenyl arm, which increases the bulk of GA II 34 compared to cocaine, is coordinated by residues Val145 and Ile148 in TM3, Val328 in TM6, and Gly426 and Ser429 in TM8 (Fig. 5B). Analysis of side chain packing within 5 Å of the additional fluorophenyl arm of GA II 34 and comparison to packing around MFZ 2–24 from Krout et al. (2017) suggests the side chains of Ile148, Ser149, Phe325, Val328, and Ile483 rotate to accommodate the added bulk in the BZT, though our methods lack the resolution to determine how these changes may impact DAT structure (Fig. 5B). The proximity of the phenylazido arm of GA II 34 to the outer gate residue Arg85, a boundary residue between S1 and S2, suggests formation of a cation-π interaction. In addition, in the hDAT model a salt bridge forms between Asp385 (EL4) and Arg544 (EL6), and as a result the GA II 34-bound DAT possesses a partially 'open-to out' conformation (Fig. 5E). In contrast, the cocaine-bound dDAT crystal structure revealed a fully open-to out structure, which could follow from the presence of negative Glu residues incapable of forming ionic or salt bridges at the homologous positions.

Fig. 5. Computational Docking and Molecular Dynamics Simulations. (A) RosettaLigand docking of GA II 34 to hDAT model with pre-MD pose in (yellow) and post-MD (150 ns simulation) pose in (light blue). Notable side chains are shown as lines in the respective color. Na (purple) and Cl (green) in the post-MD model are shown as spheres. Distances between the tropane N and D79 side chain, the reactive azido N and the L80 side chain and the outergate residues Arg85 and Asp476 are shown as magenta dashed lines (Pymol, Schrodinger). The TM6 helix is not shown for clarity. (B) Represents a 140° rotation of pose in (A) around the y-axis and shows DAT side chains within 5 Å of the additional phenyl-fluoro arm of GA II 34 (yellow, pre-MD) and overlaid with pre-MD MFZ 2-24-DAT complex (green) from Krout et al., 2017) and pre-MD RTI 82-DAT compex (gray) from Dahal et al. 2014a,b. The Asp79 side chain is shown for orientation. Pre-MD complex of GA II 34 was used for this panel for appropriate comparison the pre-MD MFZ 2 24 and RTI 82 poses. V/L328 represents the divergence of rat and human DAT at that position. (C) 2D plot of post-MD GA II 34 from A (left panel) and cocaine (right panel) where polar (blue), aliphatic (green), and charged residues (red) point toward the part of the molecule they coordinate. (D) Stereo image overlay of pre-MD GA II 34 (yellow) and post-MD GA II 34 (blue) with cocaine (magenta) from Wang et al., 2015). All structures were aligned in Pymol and the helical domains shown are from the post-MD structure of hDAT-GA II 34 complex. The image is rotated ~90° around y-axis and ~30° around the x-axis compared to the pose in (A) to better visualize alignment with cocaine. Residue Asp79 is given for reference. (E) Salt bridge between Asp385 (EL4, yellow) and R544 (EL6, green) formed during MD simulation contributing to occluded structure. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. GA II 34 protection of S1- and S2-binding pockets from Cys-directed biotinylation

The cross-linking and computational support for GA II 34 adduction at Leu80 suggests that pharmacophore binding occurs in S1 and positions the tropane N towards Asp79, similar to cocaine and cocaine analogs (Dahal et al., 2014a; Krout et al., 2017; Wang et al., 2015). To further validate this model, we performed SCAM protection analyses of several residues in S1, S2, or the transition region between S1 and S2 (Fig. 7). Cys mutants were constructed in a methanethiosulfonate (MTS)-insensitive rDAT E2C background in which endogenous extracellularly accessible Cys90 and Cys305 were mutated to Ala and assessed for reactivity with MTSEA-biotin (Fig. 7). MTSEA-biotin readily reacts with WT rDAT, but not with E2C rDAT (Fig. 7), confirming the suitability of E2C DAT as a background for analysis of inserted Cys residues (Dahal et al., 2014a). For these experiments we generated Cys mutations in E2C rDAT at S1 residues Asp79, Ala81, Asn82, Val152, and Ser421; at intermediate residues Phe319, Ala479, and Ile483; and at S2 residues Trp84, Arg85, Ile159, and Asp475. Protection assays were performed using 10 μM GA II 34 (denoted as +), except for D79C and S421C DATs, which required 50 μM GA II 34 (denoted as + +), due to reduced GA II 34 potency to inhibit CFT binding in these specific mutants (data not shown). Experiments were performed in whole cells to allow for analysis of plasma membrane (surface) DAT.

MTSEA-biotinylation of WT rDAT was not affected by incubation with GA II 34 (Fig. 7, Control) indicating lack of protection of residues Cys90 and/or Cys305 (Loland et al., 2004), which are relatively distant from the S1 ligand binding site. The S1 residues D79C, V152C, and S421C exhibited altered sensitivity to MTSEA-biotin upon co-incubation with GA II 34, with biotinylation reduced at D79C and S421C and increased at V152C (Fig. 7, S1). Changes in sensitivity were not observed for S1 residues A81C and N82C. For S2 residues and those intermediate between S1 and S2, bound GA II 34 did not change reactivity of F319C, I483C, R85C, I159C, and D475C, but did decrease reactivity of intermediate residue A479C and S2 residue W84C (Fig. 7).

Fig. 6. Interatomic distances between GA II 34 and key transporter residue side chains during molecular dynamics simulations. Panel A, the nitrene N formed during the activation of the phenylazido moiety and distance to the δ1 (black) and δ2 (red) carbons of Leu80. Panel B, the distance between the tropane nitrogen and the δ1 (black) and δ2 (red) oxygens of Asp79. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

4.1. Benztropines - atypical DAT antagonists

DAT is the biological target responsible for the abuse liability of cocaine (Kuhar et al., 1991), and several high-affinity DAT ligands have been developed and tested for the purpose of treating cocaine addiction (Schmitt and Reith, 2011). Whereas most DAT antagonists induce neurochemical or behavioral outcomes similar to cocaine, the BZT class of DAT antagonists do not elicit cocaine-like behavioral profiles in animal models, despite their similar affinities (Katz et al., 2001; Vaughan et al., 1999) and potencies for inhibiting DA uptake (Agoston et al., 1997b; Katz et al., 2004). Furthermore, BZT treatment in rats has also been implicated in reducing relapse to methamphetamine seeking (Dassanayake and Canales, 2018). For example, GA 103, the non-photoactivatable precursor to [^{125}I]GA II 34, demonstrates high-affinity and selectivity for DAT, but does not significantly increase locomotor activity in mice (Agoston et al., 1997b) or substitute for cocaine in drug discrimination assays in rats (Katz et al., 1999, 1997). Further, extensive behavioral evaluation of JHW007 (Desai et al., 2005), a now classic atypical DAT inhibitor that is structurally related to GA II 34 (Agoston et al., 1997b), and a recent novel analog JJC7-043 (Zou et al., 2017), have supported the development of atypical DAT inhibitors for the treatment of psychostimulant use disorders. These findings underscore the importance of determining BZT interaction with DAT and determining how those interactions may participate in the divergent pharmacological profile of these BZT analogs from cocaine.

4.2. GA II 34 binding site

Our findings reveal that GA II 34 binds in S1, with the AIP arm oriented toward the outer vestibule but curving back toward the S1 site, in a U-shaped configuration. The BZT pharmacophore virtually overlaps the binding poses reported for cocaine (Beuming et al., 2008; Wang et al., 2015) RTI 82 (Dahal et al., 2014a), and MFZ 2–24 (Krout et al., 2017) such that the critical interaction is maintained between the tropane N of GA II 34 and the O atoms on the Asp79 side chain (Amara and Kuhar, 1993; Penmatsa et al., 2015).

SCAM analysis showed that co-incubation with GA II 34 reduced reactivity of S1 residues D79C and S421C, intermediate residue A497C, and S2 residue W84C, an outcome that is virtually identical to the SCAM analysis of MFZ 2–24, which like GA II 34 has the photoaffinity moiety attached to the tropane N (Krout et al., 2017). This suggests that the added bulk from the AIP moiety alters the density of residue packing around Trp84 and/or Ala479 making them less accessible to MTS-mediated biotinylation (Krout et al., 2017). In contrast, RTI 82, which has the AIP moiety appended from the 2β methyl ester, undergoes adduction to TM6 residue Phe320 and exhibits a protection profile more similar to cocaine (Dahal et al., 2014a). However, the SCAM profile of cocaine differed from the three photoaffinity analogs with respect to enhanced protection of intermediate residues, suggesting that the cocaine-bound structure is more outwardly-occluded than the photoaffinity bound structures which are more open-to-out, consistent with the smaller size of the cocaine molecule. This SCAM outcome for cocaine contrasts with the outwardly open conformation observed in the dDAT-cocaine co-crystal (Wang et al., 2015). This discrepancy may result from a minimally functional dDAT stabilized by a Fab fragment and crystallized in a single conformation compared to SCAM queries with a functional mammalian DAT that can undergo normal conformational dynamics.

The comparative DAT models in this study were based on the dDAT crystal structure (Wang et al., 2015), and during the modeling process adopted an occluded structure with the S1 binding site separated from the extracellular and intracellular environments by protein density. The relevance of this modeling-derived occluded structure is yet unclear, as homology modeling subjects the structure to multiple relaxation and energy minimization steps (Davis and Baker, 2009) followed by MD simulations. However, it is intriguing to consider that the more occluded structure we observe appears to be stabilized by formation of a salt bridge between Asp385 and Arg544 from EL4 and EL6, respectively (Fig. 5E). This interaction is not possible in dDAT which lacks the requisite acid-base amino acid pairing. Additional work will be needed to investigate this putative interaction and any associated functional impact. Notably, our analyses of RTI 82 and MFZ-2-24 docking found the ROSETTALIGAND-docked pose showed greater agreement with our biochemical constraints than the post-MD complexes whereas for GA II 34 the MD-refined pose better fit the biochemical constraints indicating more refining and/or rearrangement was needed to accommodate the atypical and larger ligand.

Nevertheless, our studies with MFZ 2–24 (Krout et al., 2017), RTI-82 (Dahal et al., 2014a) and GA II 34, those of Wang and colleagues with the dDAT-cocaine crystal structure (Wang et al., 2015), and computational and mutagenesis findings (Bisgaard et al., 2011), strongly support a similar S1 binding pose for BZT and cocaine ligands, with virtual overlap of the bound tropane pharmacophore. This argues against the presence of gross conformational differences between cocaine and BZT-bound DAT complexes, and suggests that subtle

Fig. 7. SCAM protection analysis of S1 and S2 binding pockets. (A) Immunoblots of the surface pool of DAT Cys mutants in the rDAT E2C background isolated with MTSEA-biotin in the absence (−) or presence of GA II 34 (+, 10 μM; ++, 50 μM). (B Quantification of DAT bands in A and total DAT expression (data not shown) were determined using Image Studio (LI-COR). Surface samples were normalized to total DAT then expressed as a percent of GA II 34-treated samples (black bars) to the respective untreated samples (white bars) and shows means ± SE. These data represent three independent experiments. Significant differences between treated and untreated samples were determined with a paired *t*-test *, $p < 0.05$; **, $p < 0.01$. Indicated Cys mutants constructed in E2C background were subjected to whole-cell surface biotinylation with MTSEA biotin in the presence of vehicle or GA II 34, followed by neutravidin extraction and immunoblotting for DAT. (C) Residues analyzed in SCAM analysis mapped on the post-MD GA II 34-DAT complex. Residues protected, unprotected or unchanged from cysteine adduction are represented by the green, magenta and gray shading respectively. The biotinylation (A) was performed in rDAT whereas the modeling was performed on hDAT. To simplify the presentation of panel (C) hDAT numbering for the residues is used. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

conformational changes such as altered Asp79 coordination identified with GA II 34 in this study or those demonstrated for JHW007 may be sufficient to affect pharmacological actions of BZT-based DAT inhibitors (Abramyan et al., 2017).

4.3. Benztropine effects and the DAT interactome

Ligand effects at MATs are increasingly understood to occur by complex mechanisms that may involve secondary and even tertiary binding sites (Chen et al., 2005; Larsen et al., 2016; Nandi et al., 2004; Nightingale et al., 2005; Plenge et al., 2012; Rothman et al., 2015; Schmitt and Reith, 2011; Shi et al., 2008; Singh et al., 2007b; Tomlinson et al., 2018), but the low affinity of many of these interactions complicate the ability to investigate their contributions to drug effects. Like others, our studies assess high-affinity binding of BZT, but may not detect possible interactions at additional site(s) that could be related to the unique behavioral outcomes. Specific conformations stabilized by different ligands could impact structures of cytoplasmic loops or tails to affect DAT interactome or post-translational modifications that have profound effects on multiple DAT functions and signalizing outcomes. For example, recent studies have suggested a connection between DAT and the σ^1 receptor and the presentation of cocaine-like effects (Hiranita et al., 2017; Katz et al., 2017, 2004). In fact, despite the lower affinity of cocaine for σ^1 receptors compared to the DAT, behaviorally relevant doses of cocaine simultaneously occupy both σ^1 receptors and DAT in the brain (Lever et al., 2016), and drugs such as BZTs which can antagonize both DAT and the σ^1 receptor block cocaine self-administration, suggesting that action at both of these protein targets may be requisite for DAT antagonists to exhibit non-cocaine-like effects (Hiranita et al., 2017). While the precise mode of action for BZTs remains unclear, there is evidence that the endoplasmic reticulum-localized σ^1 receptor may physically complex with DAT at the plasma membrane via ER projections (Hong et al., 2017; Sambo et al., 2017). In addition, DAT undergoes extensive post-translational modifications such as phosphorylation, ubiquitylation, and palmitoylation on cytoplasmic N- and C-termini (Foster and Vaughan, 2017; Rastedt et al., 2017; Vaughan and Foster, 2013). These modifications are subject to complex regulation by psychostimulant inhibitors and substrates (Cervinski et al., 2005; Challasivakanaka et al., 2017; Gorentla and Vaughan, 2005), and not only function in regulation of uptake activity (Vaughan et al., 1997), but impact cocaine analog affinity (Moritz et al., 2013), and substrate efflux parameters (Foster et al., 2012) strongly supporting responsiveness to, and outcomes of, DAT ligands. Thus, whereas we do not observe gross conformational differences in the core of DAT when bound to cocaine versus GA II 34, it is possible that subtle conformational changes in loops or other flexible

domains induced by BZT could be transmitted to these regulatory regions to influence DAT/σ^1 interaction and/or lead to altered cell signaling.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuint.2018.08.008.

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