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Antagonist-induced conformational changes in dopamine transporter extracellular loop two involve residues in a potential salt bridge

Jon D. Gaffaney, Madhur Shetty, Bruce Felts, Akula-Bala Pramod, James D. Foster, L. Keith Henry *, Roxanne A. Vaughan *

Department of Basic Sciences, University of North Dakota School of Medicine and Health Sciences, Grand Forks, ND 58202, United States

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ABSTRACT

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

1. Introduction

1.1. The dopamine transporter

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

DAT and the related norepinephrine and serotonin transporters (NET and SERT) are members of the SLC6 family of symporters that are composed of 12 transmembrane spanning domains (TMs) connected by extracellular and intracellular loops (ELs and ILs) (Fig. 1A) (Broer and Gether, 2012; Kristensen et al., 2011; Pramod et al., 2013). Substrates are translocated by an alternating access mechanism in which the protein cycles through outwardly and inwardly facing states that allow solutes to enter or exit the

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

* Corresponding authors. Address: Department of Basic Sciences, University of North Dakota School of Medicine and Health Sciences, 501 N. Columbia Road, Grand Forks, ND 58201, United States. Tel.: +1 701 777 2295; fax: +1 701 777 4490 (L.K. Henry). Tel.: +1 701 777 3419; fax: +1 701 777 2382 (R.A. Vaughan).

E-mail addresses: keith.henry@med.und.edu (L.K. Henry), roxanne.vaughan@med.und.edu (R.A. Vaughan).

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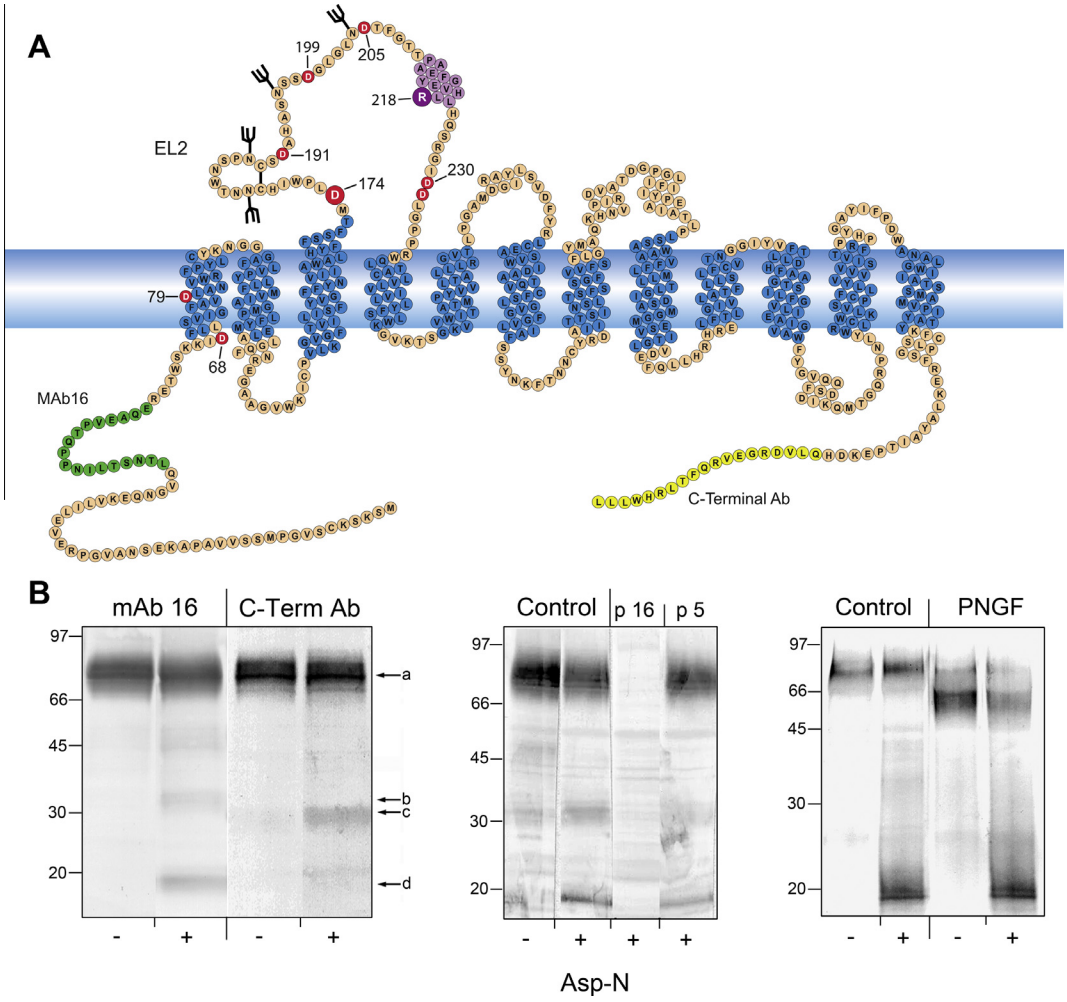

Fig. 1. Characterization of DAT Asp-N fragments. (A) Schematic diagram of rDAT illustrating 12 transmembrane spanning domains, epitopes for N- and C-terminal tail antibodies (green and yellow), and EL2 components including N-linked glycosylation (branched structures), disulfide bond (solid line), Asp residues (red), Asp174 (large red circle) and Arg218 (large purple circle). The full rDAT sequence was analyzed by PsiPred and JUFO secondary structure prediction algorithms (Leman et al., 2013; McGuffin et al., 2000) which predicted the region surrounding and including R218 is likely a helical structure (purple). (B) Rat striatal membranes were treated with (+) or without (−) 1 μg/ml Asp-N and analyzed as indicated. Left panel, immunoblotting of samples with N- and C-terminal specific DAT antisera. Arrow a, full-length DAT; arrows b and d, 32 and 19 kDa fragments detected by mAb 16; arrow c, 30 kDa fragment detected with C-terminal antibody. Middle panel, immunoblotting of Asp-N treated samples with mAb16 containing no addition (control), 30 μg/ml peptide 16 (p16), or 30 μg/ml peptide 5 (p5). Right panel, DAT and DAT Asp-N fragments were immunoprecipitated with polyclonal antibody 16 and treated with or without 1.5 units PNGF, followed by immunoblotting with mAb 16.

permeation pathway from opposite sides of the membrane (Forrest and Rudnick, 2009; Jardetzky, 1966). These forms are generated by the coordinated opening and closing of extracellular and intracellular gates that control substrate access and direction of movement (Kniazeff et al., 2008). The structures of some of these conformations have been captured through crystallization of the *Aquifex aeolicus* leucine transporter ($LeuT_{Aa}$) in different phases of the cycle, providing templates for computational modeling of DAT and other homologous mammalian transporters (Krishnamurthy and Gouaux, 2012; Singh et al., 2008; Yamashita et al., 2005; Zhou et al., 2009). Recently, *Drosophila* DAT (dDAT) complexed with the antidepressant nortriptyline was crystallized in an 'outward-open' conformation (Penmatsa et al., 2013), although stabilization of the protein for crystal formation required deletion of 43 amino acids from EL2 and inclusion of five thermostable mutations. The modified dDAT was inactive for transport and lacked the functionally relevant zinc binding site present in mammalian DATs formed by residues from EL2 and EL4 (Norgaard-Nielsen and Gether, 2006; Stockner et al., 2013), which may limit the application of its structure to mammalian DAT. Recently, a valid computational model of hDAT EL2 in the outward-facing transporter conformation has been constructed using the molecular constraints provided by the zinc binding site and conserved disulfide bond (Stockner et al., 2013).

1.2. Substrate and antagonist binding sites on DAT

Substrate binding in $LeuT_{Aa}$ occurs in a pocket referred to as S1 that is formed between the extracellular and intracellular gates (Yamashita et al., 2005). This site is formed from residues in TMs 1, 3, 6, and 8, and similar regions of DAT, NET, and SERT have been implicated in substrate binding and transport. Some findings also support the presence of an S2 substrate site on the extracellular side of the extracellular gate in both $LeuT_{Aa}$ and mammalian transporters (Piscitelli et al., 2010; Plenge et al., 2012; Quick et al., 2012; Shi et al., 2008; Singh et al., 2007; Wang et al., 2012; Zhou et al., 2009). Findings obtained from mutagenesis approaches showing interaction of DAT and SERT inhibitors with residues in TM1, TM3, TM6, and TM8 (Andersen et al., 2009b; Beuming et al., 2008; Chen et al., 1997; Field et al., 2010; Henry et al., 2003, 2006; Kitayama et al., 1992; Lin et al., 2000), adduction of irreversible cocaine analogs to DAT near S1 residues in TM1 and TM6

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