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Conformational changes in dopamine transporter intracellular regions upon cocaine binding and dopamine translocation

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ABSTRACT

The dopamine transporter (DAT), a member of the neurotransmitter:sodium symporter family, mediates the reuptake of dopamine at the synaptic cleft. DAT is the primary target for psychostimulants such as cocaine and amphetamine. We previously demonstrated that cocaine binding and dopamine transport alter the accessibility of Cys342 in the third intracellular loop (IL3). To study the conformational changes associated with the functional mechanism of the transporter, we made cysteine substitution mutants, one at a time, from Phe332 to Ser351 in IL3 of the background DAT construct, X7C, in which 7 endogenous cysteines were mutated. The accessibility of the 20 engineered cysteines to polar charged sulfhydryl reagents was studied in the absence and presence of cocaine or dopamine. Of the 11 positions that reacted with methanethiosulfonate ethyl ammonium, as evidenced by inhibition of ligand binding, 5 were protected against this inhibition by cocaine and dopamine (S333C, S334C, N336C, M342C and T349C), indicating that reagent accessibility is affected by conformational changes associated with inhibitor and substrate binding. In some of the cysteine mutants, transport activity is disrupted, but can be rescued by the presence of zinc, most likely because the distribution between inward- and outward-facing conformations is restored by zinc binding. The experimental data were interpreted in the context of molecular models of DAT in both the inward- and outward-facing conformations. Differences in the solvent accessible surface area for individual IL3 residues calculated for these states correlate well with the experimental accessibility data, and suggest that protection by ligand binding results from the stabilization of the outward-facing configuration. Changes in the residue interaction networks observed from the molecular dynamics simulations also revealed the critical roles of several positions during the conformational transitions. We conclude that the IL3 region of DAT undergoes significant conformational changes in transitions necessary for both cocaine binding and substrate transport.

Abbreviations: ANOVA, analysis of variance; BCA, bicinchoninic acid; DA, dopamine; DAT, dopamine transporter; FEP, free energy perturbation; GABA, gamma-amino butyric acid; GAT, GABA transporter; HRP, horseradish peroxidase; IL3, intracellular loop 3; MD, molecular dynamics; MTS, methanethiosulfonate; MTSEA, MTS ethyl ammonium; MTSES, MTS ethylsulfonate; MTSET, MTS ethyltrimethylammonium; NET, norepinephrine transporter; NSS, neurotransmitter:Na^+ symporter; PVDF, polyvinylidene fluoride; S1, primary substrate binding site; S2, second substrate binding site; SASA, solvent accessible surface area; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SMD, steered molecular dynamics; SCAM, substituted cysteine accessibility method; SERT, serotonin transporter; TCA, tricyclic antidepressants; TM, transmembrane segment; VMD, Visual Molecular Dynamics.

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1. Introduction

Eukaryotic members of the neurotransmitter:Na^+ symporter (NSS) family, which include transporters for GABA, dopamine (DA), norepinephrine, and serotonin (GAT, DAT, NET, and SERT, respectively), terminate signaling by recapturing released neurotransmitter (Amara and Sonders, 1998; Rudnick, 2002; Sonders et al., 2005). These secondary active transporters enable the thermodynamically uphill transport of their respective substrates across the plasma membrane of the presynaptic neuron in a co-transport (symport) mechanism driven by the Na^+ electrochemical gradient (Gu et al., 1994; Krause and Schwarz, 2005; Torres et al., 2003). Drugs that interfere with reuptake profoundly influence behavior and mood. DAT is the primary target for the psychostimulants cocaine and amphetamine (Koob, 1992) and is a target for treatment of attention deficit hyperactivity disorder and depression (Volkow et al., 2002). Genes encoding more than 200 putative NSS homologs have been identified computationally in prokaryotic genomes, thereby expanding the functional spectrum of this transporter family (Beuming et al., 2006).

A structural context for the information gathered from extensive experimental studies of DAT activity under various conditions has emerged even before the recent elucidation of a Drosophila DAT construct (Penmatsa et al., 2013), from the crystal structure of LeuT, a prokaryotic NSS homolog (Yamashita et al., 2005). In this structure, one Leu and two Na^+ are bound in a pocket formed by the unwound portions of transmembrane segment (TM) 1 and TM6, and residues in TM3 and TM8 that are highly conserved among NSS. The LeuT structure has been invaluable for structure-based interrogation of functional mechanisms and ligand binding (Beuming et al., 2008; Celik et al., 2008; Forrest et al., 2006; Forrest et al., 2008; Huang et al., 2009; Kanner and Zomot, 2008; Kaufmann et al., 2009; Kniazeff et al., 2008; Rudnick, 2006; Shan et al., 2011; Shi et al., 2008; Zhao et al., 2010; Zhao et al., 2011).

In the transport cycle, substrate binding is thought to initiate a transition from an outward to an inward facing conformation, but the mechanism for this transition and how it is driven by the sodium gradient had been unclear. The first LeuT crystal structure, closed to both the outside and the inside (occluded), appeared to represent an intermediate conformation, and subsequently solved crystal structures of LeuT mutants have captured conformations thought to represent outward-open as well as inward-open configurations (Krishnamurthy and Gouaux, 2012; Singh et al., 2008; Yamashita et al., 2005).

Using steered molecular dynamics (SMD) simulations starting from the initial occluded structure of LeuT, the substrate translocation pathway in LeuT was explored computationally and a second substrate binding site (S2) was identified in the extracellular vestibule (Shi et al., 2008). In LeuT this site is also the binding site for tricyclic antidepressants (TCAs) (Singh et al., 2007; Zhou et al., 2007; Zhou et al., 2009). Substrate binding in the S2 site is thought to constitute an allosteric trigger for the mechanism of intracellular release of Na^+ and substrate from the primary S1 site, whereas TCAs in the S2 site inhibit transport by binding in a way that prevents substrate release (Cheng and Bahar, 2013; Shi et al., 2008; Singh et al., 2007; Zhao et al., 2011). A similar role of the bound S2 substrate was also observed in the SMD simulations of a LeuT-based DAT homology model, where the stably bound dopamine molecule in the S2 site triggers the opening of the intracellular gate in the absence of sodium in the Na2 site, thereby enabling penetration of waters from the intracellular milieu and the gradual rearrangement of intracellular segments towards an inward-open conformation (Shan et al., 2011).

Binding of cocaine to DAT was shown to occur in the S1 site (Beuming et al., 2008). Interestingly, cocaine has been proposed to stabilize DAT in an outward-facing configuration (Chen et al., 2000; Chen and Justice, 1998). Extensive work in the homologous serotonin transporter (SERT) using the substituted-cysteine-accessibility method (SCAM) (Karlin and Akabas, 1998) has shown that cocaine modulates the conformation of this transporter, and has led to the proposal of a rocking-bundle model (Chen et al., 1997; Forrest et al., 2008; Henry et al., 2003; Zhang and Rudnick, 2006). Our combined computational and experimental studies of LeuT and DAT have brought to light detailed elements of the transport mechanism propagated through flexible rearrangements. The global rearrangements in DAT were found in computational simulations of substrate translocation in DAT (Shan et al., 2011) to be quite similar to those observed from similar simulations in LeuT (Shi et al., 2008), and to correspond substantially to inferences from the comparison of LeuT crystal structures. Notably, however, in LeuT some intracellular portions of TM segments exhibited either smaller-scale movements (TMs 4 and 8) or did not appear to move at all (TMs 3, 9 and 10). The structural rearrangements propagated in DAT by substrate translocation were shown (Shan et al., 2011) to take advantage of specific hinge regions and the rearrangement of local interaction networks that result in the conformational transitions observed both computationally and from smFRET measurements of LeuT (Shan et al., 2011; Shi et al., 2008; Zhao et al., 2010; Zhao et al., 2011). In DAT, one such region is the third intracellular loop (IL3), for which we found significant rearrangements associated with the TM1 and TM6 movements in the transition towards inward-facing conformations in mechanisms explored computationally and shown to reach beyond the inferences of rigid motions inherent in comparisons of crystal structures or whole bundle repositioning (Shan et al., 2011).

As described here, we used the substituted-cysteine accessibility method (SCAM) and computational modeling to identify the structural signature of ligand-dependent conformational rearrangements of IL3 in DAT, by evaluating the impact of cocaine binding and DA transport on changes in accessibility of cysteine-targeting reagents. The results are presented in the structural context of an established homology model for DAT based on the LeuT structure in which we simulated the outward and inward facing conformations (Shan et al., 2011), which was shown to be in excellent agreement with the recently determined structure of a construct from the drosophila DAT (Penmatsa et al., 2013). Our results suggest that the TM6–IL3–TM7 region plays an important role in functionally relevant conformational changes and thus shed light on conformational transitions during the transport cycle.

2. Material and methods

2.1. Mutagenesis and subcloning of cysteine mutants

We have shown previously that the accessibility of Cys342 in IL3 of DAT is transport-dependent (Chen et al., 2000) and sensitive to cocaine binding (Ferrer and Javitch, 1998), which is most consistent with this residue being buried in the TM bundle in the outward facing state and becoming intermittently accessible during the transport cycle. To investigate the properties and putative roles of residues in the TM6–IL3–TM7 region, we made a background DAT construct in which seven intra- and extracellular cysteines were mutated to other residues (X7C). Twenty cysteine substitution mutants were made in IL3 and the contiguous intracellular ends of TM6 and TM7 in the X7C background by replacing, one at a time, each residue from Phe332 to Ser351.

We used EM4 cells, which are human embryonic kidney 293 cells stably transfected with macrophage scavenger receptor to increase their adherence to tissue culture plastic (Robbins and

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