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X-ray structures of *Drosophila* dopamine transporter in complex with nisoxetine and reboxetine

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Abstract

Most antidepressants elicit their therapeutic benefits through selective blockade of Na⁺-Cl⁻ - coupled neurotransmitters transporters. Here we report x-ray structures of the *Drosophila melanogaster* dopamine transporter in complexes with the polycyclic antidepressants nisoxetine or reboxetine. The inhibitors stabilize the transporter in an outward-open conformation by occupying the substrate binding site. These structures explain how interactions between the binding pocket and substituents on the aromatic rings of antidepressants modulate drug – transporter selectivity.

The release of biogenic amines -- dopamine (DA), norepinephrine (NE) and serotonin (5-HT) -- underlies signaling of neural pathways in the central and peripheral nervous systems, regulating mood, alertness, motor function, and reward-seeking behavior¹. Following release, the neurotransmitters are cleared from synaptic and extrasynaptic spaces by biogenic amine transporters (BATs), integral membrane symporters that couple neurotransmitter uptake to sodium and chloride electrochemical gradients across cell membranes². Due to the central role of BATs in controlling the extracellular concentrations of neurotransmitters available for receptor binding, BATs are logical targets for small molecules that include psychostimulants, such as cocaine and amphetamines, and therapeutic agents, including antidepressants and anti-anxiety medications³.

Many clinically prescribed inhibitors of 5-HT and NE uptake act by elevating the concentrations of the neurotransmitter in extracellular spaces and, in so doing, alleviating conditions that can include depression, anxiety, attention deficit hyperactivity disorder (ADHD), narcolepsy, and neuropathic pain³. Early discoveries in treating depressive disorders correlated the ability of tricyclic antidepressants (TCAs), such as imipramine, to treat depression through inhibition of catecholamine uptake⁴. More recently, drugs with increased specificity in the form of selective serotonin reuptake inhibitors (SSRIs) and

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A.P., K.H.W., and E.G. designed the project. A.P. and K.H.W. performed protein purification, crystallography, and biochemical assays. A.P., K.H.W., and E.G. wrote the manuscript.

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serotonin and norepinephrine reuptake inhibitors (SNRIs) have replaced TCAs as the preferred agents to treat depression⁵. The treatment of affective disorders through increased neurotransmitter levels constitutes the “monoamine-hypothesis,” which describes the relationship between monoamine signaling and mood disorders⁶. In this study, we report the x-ray crystal structures of the *Drosophila melanogaster* dopamine transporter (dDAT) in complex with the specific NE uptake inhibitors nisoxetine and reboxetine, structures which yield insight into the molecular basis for inhibitor specificity.

The dDAT has relatively broad substrate specificity, harboring the capacity to transport DA, NE, and tyramine with varying efficacies, and is sensitive to a multitude of inhibitors that act on human biogenic amine transporters⁷. Indeed, *D. melanogaster* lacks a dedicated norepinephrine transporter but retains a 5-HT transporter⁷. Despite a preference to transport DA over NE, the dDAT shows greater sensitivity towards antidepressants and lower affinity for cocaine and amphetamines than mammalian DATs, and exhibits a pharmacological profile closest to mammalian NETs⁷.

We previously solved the x-ray structure of a nortriptyline-bound dDAT that revealed the ability of TCAs to compete for the substrate binding site and lock the transporter in an outward-open state⁸, rather than through a non-competitive mode of inhibition by binding to the extracellular vestibule⁹. TCAs potently inhibit multiple BATs, a phenomenon which likely underlies their multiple side effects and which, in turn, renders them unattractive as a primary medication for depressive disorders. More recently, selective inhibitors of SERT have been developed, including fluoxetine, escitalopram, sertraline and paroxetine, and are widely prescribed antidepressants. By contrast, NET-specific inhibitors such as nisoxetine and reboxetine exhibit high affinity binding to NET as compared to DAT or SERT^{10,11}, with reboxetine useful for treatment of panic disorder and ADHD. Despite the importance of BAT inhibitors as therapeutic agents and tools of neuroscience, there is little understanding of how TCAs, SSRIs and SNRIs bind to BATs and the molecular basis of inhibitor selectivity. We set out to determine the structural basis of NET-specific inhibitor selectivity using dDAT as a model for human NET.

Nisoxetine and reboxetine differ in chemical structure from classic TCAs in that they have discontinuous aromatic groups that branch from a central chiral carbon (Fig. 1a) whereas TCAs have a fused tricyclic ring framework. The SSRI fluoxetine shares a similar aromatic ring constellation with nisoxetine, with the difference being the position and identity of the pharmacophore on the phenoxy ring. Furthermore, fluoxetine has a *para*-trifluoromethyl substitution, whereas nisoxetine possesses an *ortho*-methoxy group. Similar to nisoxetine, reboxetine has an *ortho*-ethoxy group, and a unique morpholine ring that hosts the amine group, with an amine moiety a conserved feature of both substrates and inhibitors in BATs (Fig. 1a). Here we provide insight into how these differences in pharmacophores modulate BAT selectivity.

The structures of nisoxetine and reboxetine bound to dDAT were solved at a resolution of 3.0 Å, yielding unambiguous views of the inhibitor position in the central binding site (Fig. 1b, Supplementary Table 1). The dDAT construct used to crystallize nisoxetine was a thermostabilized variant of wild-type dDAT, dDAT_{cryst}, which was originally used to

crystallize the nortriptyline-bound complex and lacks neurotransmitter transport activity⁸. By contrast, the dDAT_{mfc} construct used in the reboxetine-bound structure has fewer thermostabilizing mutations and possesses dopamine transport activity, albeit with lower efficiency as compared to the wild-type dDAT¹². Despite differences in the constructs used in the crystallographic studies, the structures of nisoxetine-dDAT_{cryst} and reboxetine-DAT_{mfc} only differ by a root mean square deviation (r.m.s.d.) of 0.5 Å, indicating that the thermostabilizing mutations do not grossly alter the conformation of dDAT found in these inhibitor-bound structures.

The antidepressant-bound structures of dDAT exhibit an outward-open conformation (Fig. 1c) with the inhibitors lodged in the central, or S1 binding site¹³. The inhibitors of dDAT occupy subsites A, B and C, subsites derived from studies on a LeuT variant engineered to mimic eukaryotic BATs (LeuBAT) and to bind the chemical groups of SSRIs and SNRIs^{14,15}. A comparison of dDAT structures bound to the hNET inhibitors nortriptyline⁸, nisoxetine, and reboxetine reveals a similar compartmentalization of pharmacophores that enables high-affinity binding⁸ (Fig. 1d, e, 2a). The amino groups interact at distances of 2.7-3.0 Å with the carbonyl oxygen of Phe 43, although the carboxylate of Asp46 is within 3.6 Å. The secondary amine group of nisoxetine and the morpholine nitrogen of reboxetine extend into the cavity occupied by Phe43, Ala44, Phe319, and Ser320 that form subsite A (Fig. 2b)¹⁵. The presence of the bulky morpholine ring is akin to the tropane ring of drugs like cocaine and the methylated amine groups of nisoxetine and nortriptyline and sterically prevent inward movements of transmembrane helices (TMs) 1a and 6b that could close or gate the extracellular vestibule (Fig. 2b).

All three inhibitors possess two aromatic rings that insert into a hydrophobic cleft bordered by Val120, Tyr123, and Tyr124 in TM3 and Ser422 in TM8 at subsite B, and Phe319 and Phe325 (TM6-linker) at subsite C¹⁵. Residues constituting subsite B differ among insect and mammalian BATs in that dDAT has polar residues Asp121 and Ser426 at subsite B, whereas both human DAT (hDAT) and hNET have glycine and methionine at positions equivalent to 121 and 426, respectively. Substituting the polar residues in subsite B of dDAT with hNET residues D121G and S426M resulted in a threefold enhancement in nisoxetine binding affinity¹². Interestingly, the trifluoromethyl group of (*R*)-fluoxetine and dichlorophenyl group of sertraline also insert into this cavity in cocrystal structures with LeuBAT¹⁵. Upon generating homology models and analysis of residues making contacts with the inhibitors in dDAT, we observed the binding pocket to be more closely related to the binding site of hNET homology models as compared to hDAT (Fig. 2c; Supplementary Table 2), supporting the similarity between the pharmacological profiles of hNET and dDAT.

Reboxetine and nisoxetine have 130-400 fold higher affinities to hNET over hSERT or hDAT despite the high sequence identity among the three monoamine transporters^{11,16}. In contrast with the *para*-trifluoromethyl group of the SSRI fluoxetine, the *ortho*-positioned substituents of these hNET-specific inhibitors access a hydrophobic cavity below the plane of the drug that is lined by residues Ala117, Gly425, Val327 and Ala428 in dDAT and identical in hNET (Fig. 2c). However, our hDAT homology model predicts that this pocket is polar with serine residues in place of Ala117 and Ala428; the equivalent residues Ser149 and Ser429 in hDAT are likely to lead to less favorable interactions with nisoxetine and

reboxetine (Fig. 2b). In the hNET homology model, reboxetine complements this pocket more effectively than nisooxetine due to the ethoxy group, which could explain its improved selectivity for hNET over hDAT compared to nisooxetine (Fig. 2c)¹⁷.

We next investigated whether the nisooxetine- and reboxetine-bound structures could explain stereoisomer-specific effects on the inhibition of hNET. *R*-nisooxetine and (*S,S*)-reboxetine have greater specificity toward hNET over their stereoisomers¹⁷, although the difference in apparent affinity between enantiomers of nisooxetine and reboxetine are only three-fold and two-fold respectively^{18,19}. The affinity of dDAT_{mfc} for (*R,R*)-reboxetine, the enantiomer used in this study is 20 nM and comparable to the affinity observed for human NET (Fig. 2a)²⁰. Because (*R,R*)-reboxetine and a racemic mixture *R,S*- nisooxetine were available for structural studies, we employed these stereoisomers in our crystallographic experiments. Enantiomers for each antidepressant could be placed into the electron densities in the central substrate binding site (Supplementary Fig. 1)^{12,15}. Specific molecular interactions, upon modeling the stereoisomers within the densities, were not significantly different for either reboxetine or nisooxetine, indicating the ability of stereoisomers to inhibit the transporters with subtle variations in efficacies. Although these antidepressant-bound structures cannot identify specific interactions used in defining the enantiomeric selectivity of hNET, they nonetheless will be useful for modeling studies to understand how the more potent enantiomers are recognized.

The cocrystal structures of dDAT in complex with the NE-specific uptake inhibitors nisooxetine and reboxetine yield insights into the pharmacophores that dictate selectivity toward hNET. Together with the dDAT-nortriptyline complex, these structures support a model where the bilobed aromatic moieties and extended amine groups of antidepressants are important for generating high-affinity inhibition. Chemical modifications on the aromatic rings of antidepressants encode selectivity between biogenic amine transporters, namely away from recognition by hDAT and toward hNET or hSERT. In particular, the identity and position of substituents on the phenoxy ring distinguish certain hNET and hSERT inhibitors based on the environment of subsite B, as demonstrated by the comparison of *ortho*- and *para*-substitutions on nisooxetine and fluoxetine, respectively. This study reveals the contacts between residues in the binding pocket of a eukaryotic neurotransmitter transporter and hNET-specific antidepressants, providing an avenue for the structure-directed design of more selective biogenic amine transport inhibitors.

Structure Deposition

The coordinates for the structure have been deposited in the Protein Data Bank under the accession codes 4XNU (nisooxetine-DAT_{cryst}), 4XNX (reboxetine-DAT_{mfc}) (see Supplementary Table 1 for details).

Methods

Constructs

dDAT constructs employed in this study include:

ts⁵ dDAT_{cryst}. Contains thermostabilizing mutations (V74A, V275A, V311A, L415A, G538L), 1-20, a deletion in EL2 from 164-206, and a thrombin cleavage site (LVPR|GS) replacing residues 602-607.

dDAT_{mfc}. Contains thermostabilizing mutations (V74A, L415A), 1-20, a modified deletion in EL2 162-202, and a thrombin site replacing residues 602-607.

Expression and purification

The dDAT constructs were expressed as C-terminal GFP-His₈ fusions using baculovirus-mediated transduction of mammalian HEK-293s GnTI⁻ cells^{21, 22}. Membranes harvested from cells post-infection were homogenized with 1x TBS (20 mM Tris pH 8.0, 150 mM NaCl) and solubilized with a final concentration of 20 mM n-dodecyl β-D-maltoside (DDM) in 1x TBS. Detergent-solubilized material was incubated with cobalt-charged metal affinity resin and eluted with 1x TBS containing 1 mM DDM and 0.2 mM CHS and 100 mM imidazole (pH 8.0). The GFP-His₈ tag was removed by incubation with thrombin overnight at 4°C. Thrombin-digested protein was subjected to size exclusion chromatography through a Superdex 200 10-300 column pre-equilibrated with buffer containing 20 mM Tris pH 8.0, 300 mM NaCl, 4 mM decyl β-D-maltoside, 0.2 mM CHS and 0.001% (weight as a percentage of volume) 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE). Peak fractions were collected and pooled together. All procedures were carried out at 4 °C.

Fab complexation and crystallization

Antibody fragment (Fab) 9D5 was used to complex with the protein at a molar ratio of 1.2 (Fab):1 (protein)⁸. The Fab-DAT complex solution was incubated with racemic nisoxetine or (*R,R*)-reboxetine at 1 mM and concentrated using a 100 kDa cutoff concentrator to 3-4 mg·ml⁻¹. The concentrated protein was spun down to remove excess drug and insoluble aggregates and plates were set up by hanging drop vapour diffusion. Crystals of Fab-DAT complexes grew in 0.1 M glycine pH 9 and 38% PEG 350 monomethyl ether. Crystals of dDAT_{mfc} were obtained by streak seeding with a cat whisker, 7 days after drops were set up. All crystals were grown at 4 °C.

Data collection and structure refinement

Crystals were directly flash cooled in liquid nitrogen. Data were collected at APS (24-IDC). Data were processed using either HKL2000²³ or XDS²⁴. Molecular replacement was carried out for all datasets using coordinates 4M48 with Fab 9D5 and dDAT_{cryst} used as independent search models, using PHASER in the PHENIX software suite^{25, 26}. Iterative cycles of refinement and manual model building were carried out using PHENIX and COOT²⁷, respectively till the models converged to acceptable levels of R-factors and stereochemistry. Homology modeling of hNET and hDAT was done using SWISS-MODEL²⁸ using structure based sequence alignments generated using deposited coordinates (PDB id 4M48) for dDAT, using PROMALS3D²⁹.

Radiolabel binding

All binding assays were carried out by scintillation proximity assay (SPA)³⁰. Reactions contained 5-20 nM protein, 0.5 mg·ml⁻¹ Cu-YSi beads, SEC buffer, and [³H-nisoxetine] from 0.1-300 nM for saturation binding assays. Competition binding assays were done with 30 nM ³H-nisoxetine and increasing concentrations of unlabeled competitor. Each data point was performed in triplicate, and error bars denote standard deviations. K_i values were estimated from IC₅₀ values using the Cheng-Prusoff equation. Fits were plotted using Graphpad Prism v4.0.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Critical interactions for antidepressant recognition in the binding pocket of dDAT. **a**, Chemical structures of NET-specific reuptake inhibitors (NRIs), the TCA nortriptyline, and the SSRI fluoxetine. **b, c**, Overall views of the dDAT_{mfc}-reboxetine structure in cartoon and surface representations. In panel **b**, the central sections of TMs 1 (blue), 3 (orange), 6 (cyan), and 8 (light orange) interact with reboxetine, depicted in magenta sticks. The $F_o - F_c$ omit density (2.0σ) for reboxetine is shown in pink mesh. **d, e**, Close-up views of the binding pocket for nisoxetine- and reboxetine-bound dDAT, respectively. Residues Tyr124 and Phe319 are cyan and sodium and chloride ions are purple and green spheres, respectively.

Figure 2.

Comparison of ligand orientations in antidepressant-bound dDAT structures and predicted interactions in hNET and hDAT. **a**, Inhibition of ³H-nisoxetine binding to dDAT_{mfc} by (*R,R*)-reboxetine with an inhibition constant (K_i) value of 20 nM. One representative plot of two independent trials is shown, and error bars denote s.d. of technical replicates ($n=3$). **b**, Superposition of nortriptyline (orange, PDB id 4M48), nisoxetine (blue), and reboxetine (magenta) using the dDAT_{mfc}-reboxetine structure as a template. Reboxetine is also depicted in sphere format, and hydrogen bond acceptors are labeled with red spheres. Van der Waals' radius of the morpholine nitrogen atom in reboxetine represented as blue dots. **c**, Homology model of hNET using dDAT_{cryst}-nortriptyline (PDB id 4M48) as a template, with pocket residues depicted as gray sticks and spheres, and TMs depicted as cylinders. Residues that differ between an hDAT homology model and hNET are shown in purple sticks, and residue numbering follows that of hNET with equivalent positions in hDAT in parentheses. Dashed line (purple) highlights residues that dictate specificity towards hNET over other BATs. Nisoxetine and reboxetine are shown as blue and magenta sticks and spheres, respectively.