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De novo mutation in the dopamine transporter gene associates dopamine dysfunction with autism spectrum disorder

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Abstract

De novo genetic variation is an important class of risk factors for autism spectrum disorder (ASD). Recently, whole exome sequencing of ASD families has identified a novel *de novo* missense mutation in the human dopamine (DA) transporter (hDAT) gene, which results in a Thr to Met substitution at site 356 (hDAT T356M). The dopamine transporter (DAT) is a presynaptic membrane protein that regulates dopaminergic tone in the central nervous system by mediating the high-affinity re-uptake of synaptically released DA, making it a crucial regulator of DA homeostasis. Here, we report the first functional, structural, and behavioral characterization of an

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ASD-associated *de novo* mutation in the hDAT. We demonstrate that the hDAT T356M displays anomalous function, characterized as a persistent reverse transport of DA (substrate efflux). Importantly, in the bacterial homolog leucine transporter, substitution of A289 (the homologous site to T356) with a Met promotes an outward-facing conformation upon substrate binding. In the substrate-bound state, an outward-facing transporter conformation is a required for substrate efflux. In *Drosophila melanogaster*, expression of hDAT T356M in DA neurons lacking *Drosophila* DAT leads to hyperlocomotion, a trait associated with DA dysfunction and ASD. Taken together, our findings demonstrate that alterations in DA homeostasis, mediated by aberrant DAT function, may confer risk for ASD and related neuropsychiatric conditions.

Keywords

dopamine; transporter; autism spectrum disorder; anomalous dopamine efflux; *de novo* mutation; *Drosophila melanogaster*

INTRODUCTION

Genetic factors have been implicated as important components in the etiology of autism spectrum disorder (ASD). It is now accepted that rare genetic variation affecting single nucleotides of protein-coding DNA, as well as rare genomic copy number variants (CNVs), are significant ASD risk factors[1–4]. In particular, increasing evidence suggests that *de novo* genetic variation is a risk factor in ASD and other neuropsychiatric diseases[1, 3, 5–7]. Several groups have conducted whole exome sequencing (WES) on ASD families, and collectively, these studies indicate that discrete *de novo* mutation (single nucleotide variation (SNV) or small indels) contribute to the overall genetic risk of ASD[2, 8–10]. Among these variations is the first ASD-associated, *de novo* mutation found in the human dopamine (DA) transporter (hDAT) gene (*SLC6A3*)[8]. This mutation results in a Thr to Met substitution at position 356 (hDAT T356M). The consequences of this *de novo* mutation and its impact on DA neurotransmission have yet to be elucidated.

The neurotransmitter DA plays an important role in the central nervous system by regulating a variety of functions, including motor activity, motivation, attention, and reward[11–14]. Disrupted DA function is implicated in a number of neuropsychiatric disorders, including bipolar disorder, schizophrenia, attention-deficit hyperactivity disorder (ADHD)[15–17] and, more recently, ASD[18–27]. The dopamine transporter (DAT) is a presynaptic membrane protein that regulates DA neurotransmission via the high-affinity reuptake of synaptically released DA[28]. It is the major molecular target of cocaine, amphetamine (AMPH; Adderall™), and methylphenidate (Ritalin™)[29–33]. Due to DAT's role in DA neurotransmission, *SLC6A3* variants have been a focus of genetic association studies linking the etiology of brain disorders to dysregulated DA neurotransmission[34, 35]. Recent studies have identified a rare, inherited, functional missense *SLC6A3* variant, hDAT A559V (rs28364997)[36, 37], that has been associated with ADHD, which is commonly comorbid in ASD subjects[38–41]. These studies point to a contribution of DAT genetic variants in complex brain disorders.

While the role of DA in ADHD has been established[42], DA's role in ASD is poorly understood[43]. Many individuals with ASD exhibit co-occurrence of ADHD symptoms (~20–45%)[44–47]. The comorbid nature of ADHD with ASD points to dysregulation of common signaling pathways (e.g. DA) as a mechanism underlying these neuropsychiatric disorders[48].

Here, we characterized the first ASD-identified, *de novo* mutation in hDAT by presenting structural, functional, and behavioral analysis of this *de novo* variant. These results implicate altered DA homeostasis as a potential liability in ASD risk.

METHODS

Subjects and clinical assessment

Subjects from this family included the proband, both parents and unaffected sibling, who were recruited by the Boston Autism Consortium as described previously[8,49]. Clinical assessment followed standard research criteria for ASD diagnosis. The proband was classified as having a comparatively "narrow" diagnosis (as opposed to a "broader ASD") based on diagnostic algorithms from the Autism Diagnostic Interview Revised (ADI-R)[50] using criteria described by Risi et. al[51], and classification resulting from the diagnostic algorithm of the Autism Diagnostic Observational Session (ADOS)[52]. Proband IQ was assessed at age 5 years, 9 months using the Wechsler Preschool and Primary Scale of Intelligence (WPPSI; Wechsler, D. (1967)). The Social Responsiveness Scale (SRS; Western Psychological Services) was performed on both parents to index the presence and severity of broader autism phenotype traits, followed by medical and family history provided by the biological mother.

SLC6A3 T356M de novo discovery

Methodological details and validation of the *de novo* mutation are published[8]. Briefly, DNA derived from whole blood of both parents and the probands was subjected to whole exome sequence analysis. The T356M variant, identified as a heterozygote in the proband and absent in both parents, was experimentally validated and confirmed to be a *de novo* mutation that does not appear in the unaffected sibling.

Cell culture and transfection

The GFP-hDAT-pCIHygro expression vectors containing either hDAT or hDAT T356M sequence were generated, confirmed and transiently transfected into Chinese hamster ovary cells using FuGENE-6 (Promega, Fitchburg, WI, USA). Assays were conducted 24–48 hours post transfection.

Amperometry and patch clamp electrophysiology

hDAT and hDAT T356M cells were plated at a density of ~30,000 per 35-mm culture dish and experiments conducted as previously described[53, 54].

[^{3}H]DA uptake

hDAT and hDAT T356M cells were seeded (50,000 cells/well) into polyornithine coated, 24-well plates, 48 hours before assaying. Uptake kinetic assays were performed as described in the supplementary information of Rickhag *et al.*[55] and in Rasmussen *et al.*[56].

AMPH uptake

Plated hDAT and hDAT T356M cells were washed with KRH buffer and incubated for 5 min at 37°C with 10 nM AMPH. Cells were washed three times with ice-cold KRH. AMPH was quantified using a HPLC system previously described[57].

Cell surface biotinylation and western blot

For cell surface biotinylation assays and Western blots, hDAT and hDAT T356M cells were cultured in 6-well plates and experiments conducted as in Mazei-Robinson *et al.*[54].

Homology Modeling of hDAT and construction of the T356M simulation system

As the template, the homology model uses the known crystal structure for the cognate and homologous structure of the recent outward-open crystal structure of LeuT[58]. The substrate DA, two Na^+ ions and a Cl^- ion were positioned as described in Shan *et al.*[59]. To model the mutant hDAT construct with T356M, the mutation was introduced using the free energy perturbation (FEP) method[60].

Double Electron Electron Resonance

Cysteine residues were introduced using site directed mutagenesis into LeuT and LeuT A289M constructs[61]. Experiments were conducted as in Claxton *et al.*[61]. In Figure 4, Apo refers to ion Na^+ and leucine-free transporter while the +NaL state was obtained by addition of 200 mM NaCl and 4-fold molar excess of Leu relative to LeuT. Double Electron Electron Resonance (DEER)[62] was performed at 83K on a Bruker 580 pulsed EPR spectrometer operating at Q-band frequency using a standard 4-pulse sequence[63]. DEER echo decays were analyzed to obtain distance distributions[64].

Drosophila Genetics

Drosophila homozygotes for the DAT null allele DAT^{fmn} (dDAT KO)[65] and flies harboring TH-Gal4[66] were outcrossed to a control line (Bloomington Indiana (BI) 6326) and selected by PCR or eye color. TH-GAL4 (Bl 8848) and M{vas-int.Dm}ZH-2A, M{3xP3-RFP.attP'} ZH-22A (Bl 24481) were obtained from the BI stock center and outcrossed to dDAT KO flies carrying the *white* (w^{1118}) mutation (BI stock number 6236) for 5–10 generations. Transgenes (hDAT or hDAT T356M) were cloned into pBI-UASC[67] and constructs were injected into embryos from M{vas-int.Dm}ZH-2A, M{3xP3-RFP.attP'}ZH-22A (Bl 24481). Initial potential transformants were isolated and selected.

Behavioral Analysis

Three days post eclosion male *Drosophila* were collected and placed into tubes with food for 72 hours. Locomotion was recorded by beam breaks and analyzed using equipment/ software from Trikinetics (www.trikinetics.com). For the AMPH-induced locomotion, males

were starved for six hours and then fed sucrose (5 mM) containing either AMPH (10 mM) or vehicle. Data were analyzed by One-way ANOVA followed by a Newman-Keuls Multiple Comparison Post-test.

RESULTS

T356M *de novo* DAT variant has impaired function

A recent study assessed the role of *de novo* variation in ASD by using WES in 175 ASD parent-child trios[8]. In this study, a *de novo SLC6A3* variant was identified, resulting in a Thr to Met substitution at site 356[8]. Given the rarity of non-synonymous *de novo* events, it is not surprising that this mutation was absent in the ~1,000 unrelated ASD cases and controls[8] and has not been deposited in 1,000 genomes[68], dbSNP (build 137)[69], or the NHLBI Exome Sequencing Project. The subject carried no other coding *de novo* mutations. The T356 is completely conserved across several species (Fig. 1A). Importantly, the T356 residue is located in the seventh transmembrane domain of hDAT and resides in a highly conserved region implicated in ion binding[70]. The molecular modeling of hDAT and *in silico* mutagenesis of T356 is shown in Figure 1B.

The subject harboring this *de novo* mutation is the elder male child of healthy nonconsanguineous Caucasian parents (proband has a healthy younger sibling). There is no immediate family history of ASD or related psychiatric conditions. The subject has a normal IQ (94) and has no history of seizures or other co-morbidites. By the age of 6 years-old, the proband was diagnosed with ASD (see supplemental material for full clinical reports).

To evaluate whether the T356M variant may be a risk factor in the proband's ASD, we compared the activities of hDAT and hDAT T356M in a heterologous expression system. We examined radioactive [^{3}H]DA uptake and affinity. In hDAT T356M cells, the maximal velocity of DA influx (V_{max}) was significantly reduced, whereas the apparent DA affinity (K_m) of hDAT T356M was not significantly different from that of hDAT (Fig. 2A, top). A representative plot of DA uptake kinetics for hDAT and hDAT T356M is shown in Fig. 2A (bottom). The reduced [^{3}H]DA transport capacity was not associated with a reduction in either total or DAT surface expression (Fig. 2B, top), as assessed by measuring changes in DAT proteins in the total and biotinylated fraction, respectively. The total fraction for hDAT and hDAT T356M contained both glycosylated and non-glycosylated forms of the DAT. Surface fractions were quantitated, normalized to total DAT (glycosylated), and expressed as a percent of hDAT (Fig. 2B; bottom). Furthermore, normalizing the total DAT fraction (glycosylated) to actin loading control yielded no significant differences between hDAT and hDAT T356M total expression (data are expressed as a percentage of hDAT; hDAT 100 ± 17.6% versus hDAT T356M 96.4 ± 13.7%; $p \geq 0.87$ by Student's t-test; n = 8–11).

hDAT T356M displays ADE

Although hDAT T356M displays similar surface expression to that of hDAT, it demonstrates reduced ability to accumulate intracellular DA. One possibility is that constitutive DA efflux, here referred to as anomalous DA efflux (ADE), contributes to this

reduced DA uptake in the hDAT T356M cells. This efflux would impede the intracellular accumulation of DA.

To determine whether hDAT T356M exhibits ADE, cells were whole cell patch clamped and perfused for 10 minutes with an internal solution containing 2 mM DA[53]. The electrode, in current clamp configuration, allows the cell to control its membrane voltage. In addition, this technique ensures that cells expressing either hDAT or hDAT T356M were equally loaded with DA. DA efflux was quantified through amperometry[53]. We have previously shown that, in the presence of ADE, cocaine decreases the amperometric signal through blockade of DAT[54]. In hDAT cells, amperometric currents were unaffected by application of cocaine (Fig 3A, top, hDAT, COC), indicating no ADE. In contrast, amperometric signals from hDAT T356M cells were significantly reduced by the application of cocaine (Fig. 3A, top, hDAT T356M, COC), revealing ADE. hDAT T356M-expressing cells displayed a significant increase in ADE, compared to hDAT transfected cells (Fig. 3A, bottom).

Cell membrane depolarization has been shown to support DA efflux[71]. Figure 3B reveals that there is not a significant difference in resting membrane potential (measured in current clamp) between cells expressing hDAT or hDAT T356M. This indicates that differences in the function of hDAT and hDAT T356M are not due to resting membrane potential.

Next, we determined possible changes in the ability of AMPH to cause DA efflux by patch delivering DA into hDAT or hDAT T356M cells while recording DA efflux with amperometry. AMPH dose-response assays (measuring the peak of the amperometric current at different AMPH concentrations) revealed that hDAT T356M and hDAT cells have comparable AMPH EC_{50} (EC_{50}; hDAT: 0.15 ± 0.05 μM; hDAT T356M: 0.16 ± 0.07 μM; n = 4; $p \geq 0.95$ by Student's t-test). Then, using a saturating AMPH concentration (10 μM) we determined DA efflux in either hDAT or hDAT T356M cells (Fig. 3C, top). AMPH-induced DA efflux was significantly reduced in hDAT T356M cells in comparison to hDAT cells (Fig. 3C). These results strongly suggest that ADE does not share common mechanisms with AMPH-induced DA efflux.

In LeuT, substitution of Ala289 with a Met promotes an outward-facing conformation

To investigate the structural consequences of the T356M *de novo* mutation in a DAT homolog with a known crystal structure, we analyzed changes in the conformational cycle of the leucine transporter (LeuT). We substituted A289 (the homologous amino acid to T356) with a Met (LeuT A289M). We measured distances between pairs of spin labels (r (Å)) and the distance distribution (P(r), the probability of a given distance between the two labels) monitoring the intraand extracellular gates by double electron electron resonance (DEER)[72]. First, we examined the pair 309/480 (Fig. 4A, left) that monitors the relative movement of the extracellular loop 4 (EL4) in LeuT. This loop obstructs the permeation pathway in the Apo conformation[61], as indicated by the close proximity of the pair 309/480 (Fig. 4A, middle, Apo black line). Upon Na^+ binding, the distance between EL4 and TM12 increases[61], indicating opening of the extracellular vestibule and enabling substrate access (data not shown)[61]. Subsequent Leu binding resets the closed EL4 conformation in the occluded conformation of the transporter (Na^+ and Leu bound in the vestibule) (Fig. 4A, right; +Na/L black line). The extracellular Apo state (Fig. 4A, middle; Apo; compare red

and black lines) as well as Na^+ bound state (data not shown) are similar in LeuT and LeuT A289M. Yet, LeuT A289M with Na^+ and Leu bound in the vestibule has a destabilized bound structure with fluctuations on the extracellular side (Fig. 4A, right; +Na/L; compare red and black lines). The probability distribution in the Na/Leu bound state contains distinct populations of conformations that indicate fluctuations of LeuT A289M to a permeation pathway that has increased probability to be open to the outside, relative to LeuT (Fig. 4A, right; red line, arrows).

We then examined the pair 7/86 (Fig. 4B, left), to determine the distance between the N-terminus and intracellular loop 1 (IL1) and to monitor fluctuation dynamics on the intracellular side[72]. This is necessary to describe changes in the population of transporters with an inward facing conformation[72]. In the LeuT background, distance distributions between spin[73] or fluorescent probes[74] in the Apo state are bimodal, reflecting the equilibrium of this intracellular gate between closed and open conformations (Fig. 4B, middle; Apo; black lines). Introduction of the A289M leads to a shift in the equilibrium to favor the closed conformation side (Fig. 4B, middle; Apo; compare red and black lines, arrow). Na^+ binding does not alter the equilibrium between the two conformations (data not shown), while Na^+ and Leu binding resets this shift to LeuT-like conformations (Fig. 4B, middle; +Na/L; compare red and black lines).

Our results demonstrate that, because of the A289M, the presence of substrate and Na^+ fails to completely close the extracellular pathway as in LeuT, inducing fluctuations on the extracellular side. These fluctuations to an open-to-the-outside permeation pathway persist, possibly enabling substrate release. This is in contrast to LeuT, where substrate binding closes the extracellular permeation pathway.

Drosophila expressing hDAT T356M in DA neurons are hyperactive

Locomotion is an elemental behavior regulated across species, including *Drosophila melanogaster*, by DA[75–77]. Thus, locomotion in flies offers a powerful model for elucidating the behavioral impact of ADE associated with hDAT T356M.

We expressed hDAT or hDAT T356M in flies homozygous for the *Drosophila* DAT null allele, DAT^{fmn} (dDAT KO)[65], by using the Gal4/UAS system to express a single copy of hDAT or hDAT T356M in a $dDAT^{fmn}$ mutant background, selectively in DA neurons[78]. To generate the transgenic flies, we used phiC31 based integration, which leads to the expression of comparable levels of mRNA for the relevant transgenes (hDAT or hDAT T356M). Locomotion was quantitated by beam crossing detection over a >24 hour period (data binned in 15 minute intervals) during both the light (horizontal white bar) and dark (horizontal black bar) cycle. While dDAT KO are hyperactive[65], flies expressing hDAT in DA neurons have reduced locomotion as compared to dDAT KO, demonstrating the behavioral significance of our approach (Fig. 5A, compare hDAT to dDAT KO).

We hypothesized that flies harboring the hDAT T356M would be hyperactive with respect to hDAT expressing flies due to an increase in extracellular DA promoted by ADE. This is shown in Figure 5A. Figure 5B shows total (24 hour) locomotor activity in the different fly

lines. Total activity (24 hours) of hDAT T356M and dDAT KO flies is significantly higher than hDAT flies (Fig. 5B).

hDAT T356M cells display compromised AMPH-induced DA efflux (Fig. 3C). This suggests a reduced ability of AMPH to increase locomotion in flies expressing hDAT T356M. Changes in locomotion were determined upon AMPH or vehicle exposure (15 minutes) and calculated as beam crosses. We observed no significant increase in locomotion in hDAT T356M flies when exposed to AMPH as compared to vehicle control (hDAT T356M (vehicle) 9.7 ± 0.7 beam breaks versus hDAT T356M (AMPH) 12.7 ± 1.6 beam breaks; $n = 24$; $p \geq 0.05$). This is in contrast to flies expressing hDAT, where AMPH induced a significant increase in locomotion (hDAT (vehicle) 6.2 ± 0.9 beam breaks versus hDAT (AMPH) 18.2 ± 1.0 beam breaks; $n = 24$; $p \leq 0.001$). Moreover, in the dDAT KO flies, similar to the hDAT T356M flies, AMPH failed to induce a significant increase in locomotion (data not shown).

DISCUSSION

Alterations in DA tone underlie multiple neuropsychiatric disorders, including bipolar disorder, schizophrenia, and ADHD[15–17]. With respect to ADHD, altered DA signaling, including changes in DAT function, may contribute to the cognitive and hyperactive traits of the disorder[53, 54]. ASD, like ADHD, is phenotypically and etiologically complex. However, there is mounting evidence that risk for ASD resides, at least in part, in dopaminergic factors. Variants in DA receptor (DRD) sub-type genes, including *DRD1*, *DRD3*, and *DRD4*, have been associated with increased risk for ASD[21] as well as with specific phenotypic behavior within ASD. These include repetitive or stereotyped behaviors[22–24], oppositional defiant disorder, and separation anxiety disorder[24]. Male children carrying four tandem repeats in the promoter region of the *MAOA* gene (the gene product responsible for degrading amine neurotransmitters, including DA) showed elevated risk for developing ASD[27]. It must be noted that none of these genes are significant in genome-wide association studies of ASD. However, in positron emission tomography (PET) studies in adults with ASD, DAT binding was significantly elevated in the orbitofrontal cortex[18].

Genes harboring *de novo* events are highly significant for understanding the etiology of ASD[8]. This is not surprising since rate of reproduction is typically low in individuals with autism. Consequently, genetic variants would be subject to negative selection[1]. This suggests that the biological networks identified from these *de novo* events, and the broader pathways they function within, are candidate risk factors for ASD. In this study, we examine the functional, structural, and behavioral consequences of the first identified *de novo* hDAT missense variant associated with ASD.

Our amperometric recordings demonstrate that the *de novo* hDAT T356M mutation confers cocaine sensitive ADE. We also show that ADE does not share common mechanisms with AMPH-induced DA efflux since hDAT T356M has an impaired AMPH response. It is intriguing to speculate that anomalous transporter-mediated neurotransmitter efflux may be an unappreciated source of risk for mental illness, especially in disorders associated with altered DA signaling. It is possible that ADE, driven by DAT variants or variants in other

genes in the DAT regulatory network (such as DRD subtypes), could impact risk for ASD. A similar point for ADE has been argued previously, in the context of ADHD, for the hDAT variant A559V, in its functional identification[54] and original characterization[53].

The question remains as to how the hDAT T356M *de novo* mutation perturbs transporter structure to trigger ADE. Since the crystal structure of hDAT is unavailable, we analyzed changes in the conformational cycle of the hDAT bacterial homolog, LeuT. In LeuT A289M, we measured the distances between pairs of spin labels monitoring the intra- and extracellular gates by DEER. The spin labels monitoring the extracellular gate clearly show that in LeuT A289M, in contrast to LeuT, the presence of Na^+ and leucine promotes a permeation pathway unoccluded to the outside. In terms of transporter function, it is difficult to draw parallels between hDAT T356M and LeuT A289M. Nevertheless, it is compelling to speculate that the mechanism by which the substrate promotes an outward open conformation in LeuT A289M, could also support the ability of hDAT T356M to promote ADE when cytoplasmic DA is available. This would suggest that the mechanism of ADE for hDAT T356M is distinct from that of hDAT A559V, which is a result of a tonic activation of DRD2 and the downstream kinase CaMKII[53]. Thus, there may be multiple mechanistic routes to promote hDAT-mediated ADE, and yet ADE might support the comorbid nature of ASD with ADHD.

In an *in vivo* context, hDAT T356M may alter extracellular DA levels and, as a consequence, increase locomotion[65]. We selectively expressed hDAT T356M specifically in DA neurons of dDAT KO flies. *Drosophila* expressing hDAT T356M exhibited prominent hyperactivity as compared to *Drosophila* expressing hDAT. In addition, AMPH has an impaired ability to increase locomotion in hDAT T356M and dDAT KO flies. This might stem from the decreased ability of AMPH to cause DA efflux in hDAT T356M cells.

Here, we report novel and profound functional abnormalities associated with the hDAT *de novo* mutation T356M, resulting in enhancement of non-vesicular, DAT-dependent DA release, referred to as ADE. Our data raise the possibility that ADE could impact the risk for ASD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ASD	Autism spectrum disorder

5-hydroxytryptamine, DA	dopamine
hDAT	human dopamine transporter
AMPH	amphetamine

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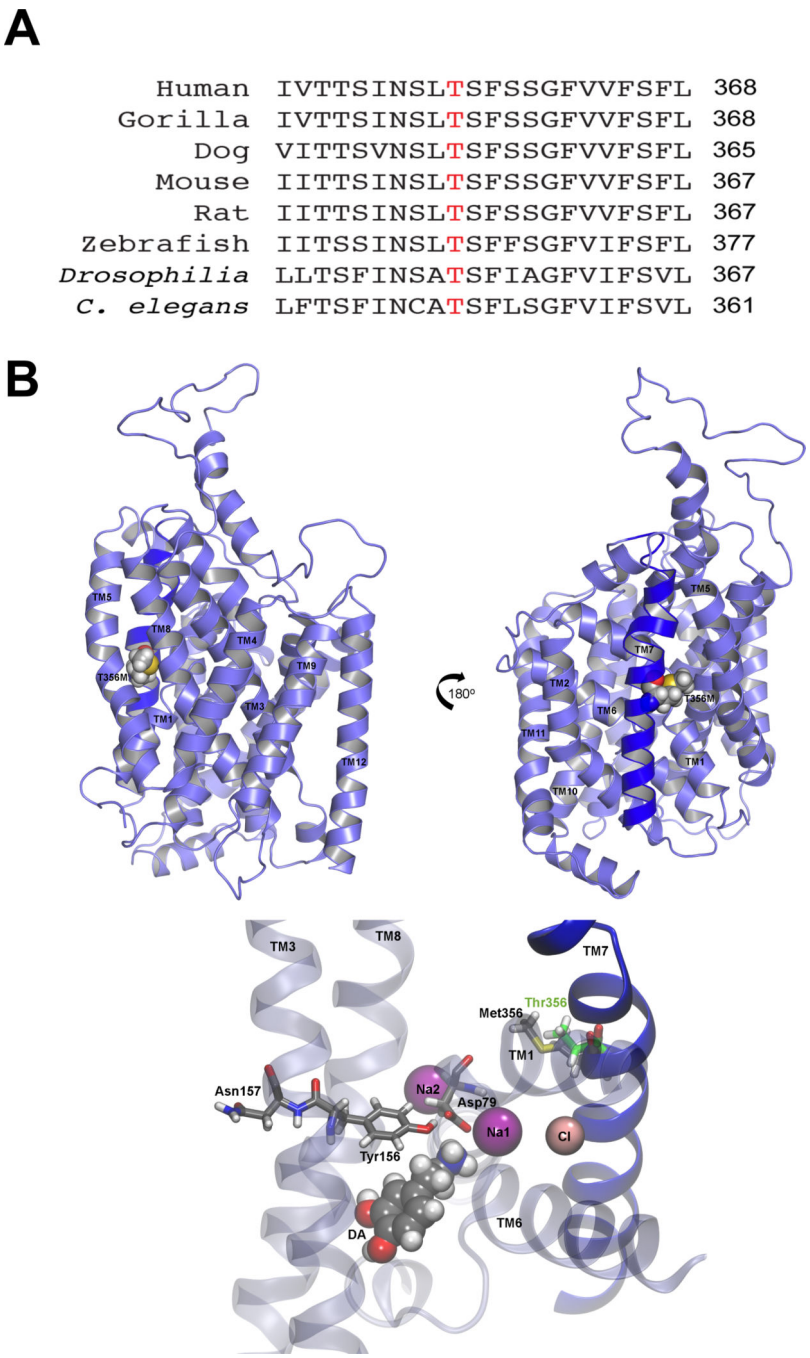
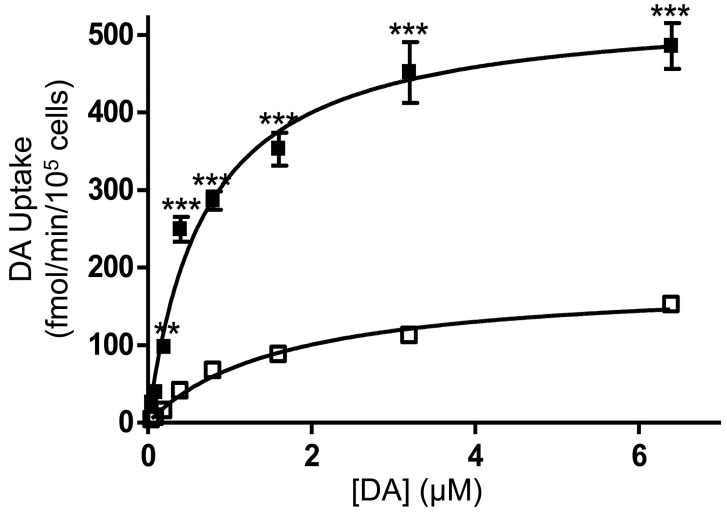


Figure 1. Cross-species conservation and *in silico* mutagenesis of T356

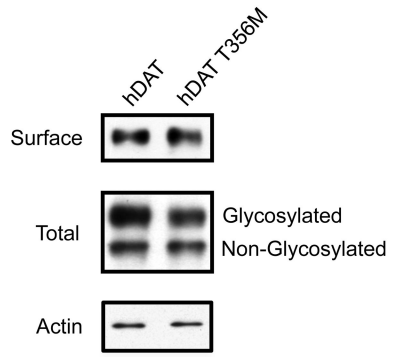
A) Alignment of the DAT amino acid sequence across multiple species. Threonine 356 is represented in red. B) In an equilibrated three-dimensional homology model of hDAT, the T356M mutation is located on transmembrane domain 7 (TM7). Top: Schematic views representing a 180° rotation show T356M with respect to the TM helices. TM7 is shown in dark blue. Bottom: Critical residues that interact with DA[79, 80] are shown, as well as the bound Na^+ and Cl^- ions. The methionine is rendered together at position 356 with the wild type threonine (green).

A

	hDAT	hDAT T356M
V_{max} (fmol/min/10^5 cells)	453 ± 42	154 ± 31
K_m (μM)	0.8 ± 0.1	1.5 ± 0.4



B



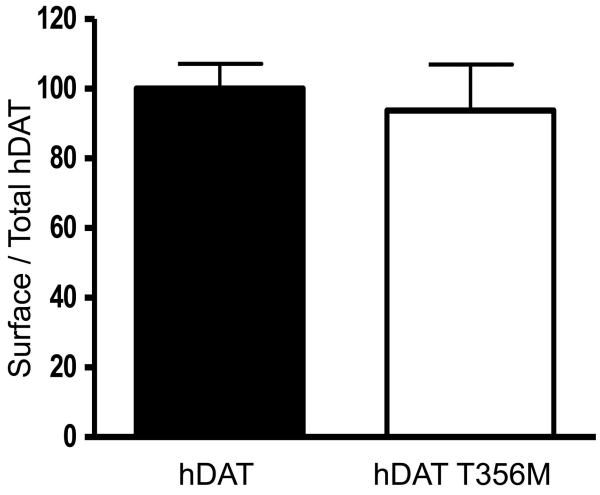


Figure 2. Human dopamine transporter (hDAT) T356M has impaired function

A) Top: Kinetic parameters (V_{max} and K_m) for hDAT and hDAT T356M (V_{max}: $p \leq 0.005$; by Student's t-test; n = 3, in triplicate; K_m: $p \geq 0.20$; by Student's t-test; n = 3, in triplicate). Bottom: Representative plot of [^{3}H]DA uptake kinetics in hDAT (filled squares) or hDAT T356M (empty squares) cells (** = $p \leq 0.01$, *** = $p \leq 0.001$; by two-way ANOVA followed by Bonferroni posttest; n = 3, in triplicate). B) Representative immunoblots for biotinylated (surface) and total protein fractions from hDAT and hDAT T356M cells. Surface fractions were quantitated, normalized to total DAT (Glycosylated), and expressed as a percent of hDAT ($p \geq 0.05$; by Student's t-test; n = 8–11).

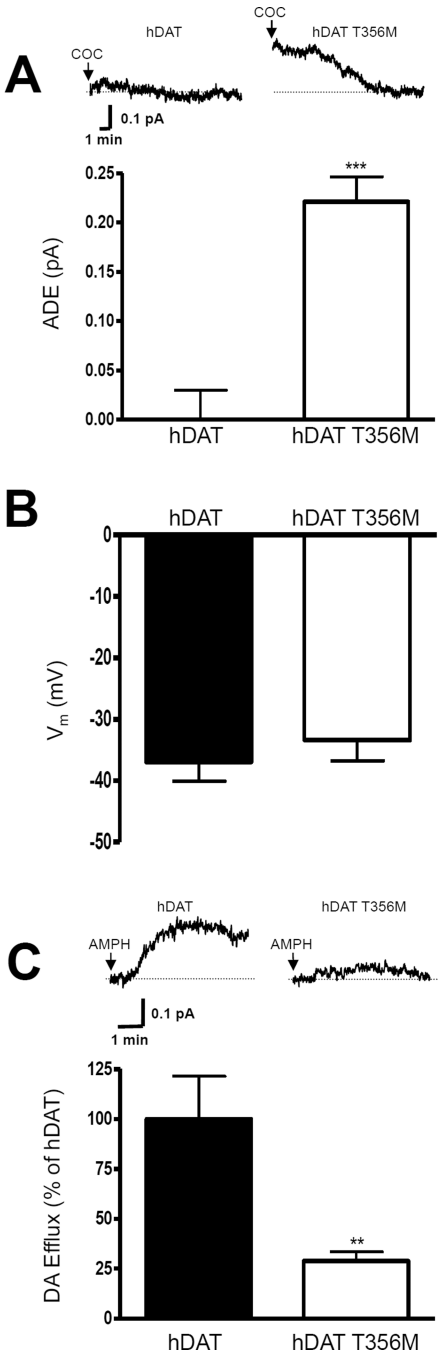


Figure 3. hDAT T356M exhibits robust ADE

A) Top: representative amperometric currents recorded from hDAT and hDAT T356M cells. Arrows indicate application of 10 μM cocaine (COC). Bottom: quantitation of the cocaine-induced reduction in the amperometric current (ADE). Data are reported as maximal current (*** = $p \leq 0.001$ by Student's t-test; n = 4–5). B) De novo mutation in the hDAT gene causes DA dysfunction 25 hDAT T356M cells do not display altered resting membrane potential with respect to hDAT cells ($p \geq 0.05$ by Student's t-test, n = 9–25). C) Representative AMPH-induced amperometric currents recorded from hDAT and hDAT

T356M cells. Arrows indicate application of 10 μM AMPH. Bottom: quantitation of AMPH-induced DA efflux. Data are represented as maximal current expressed as percent of the current recorded in hDAT cells (** = $p \leq 0.01$ by Student's t-test; n = 5–7).

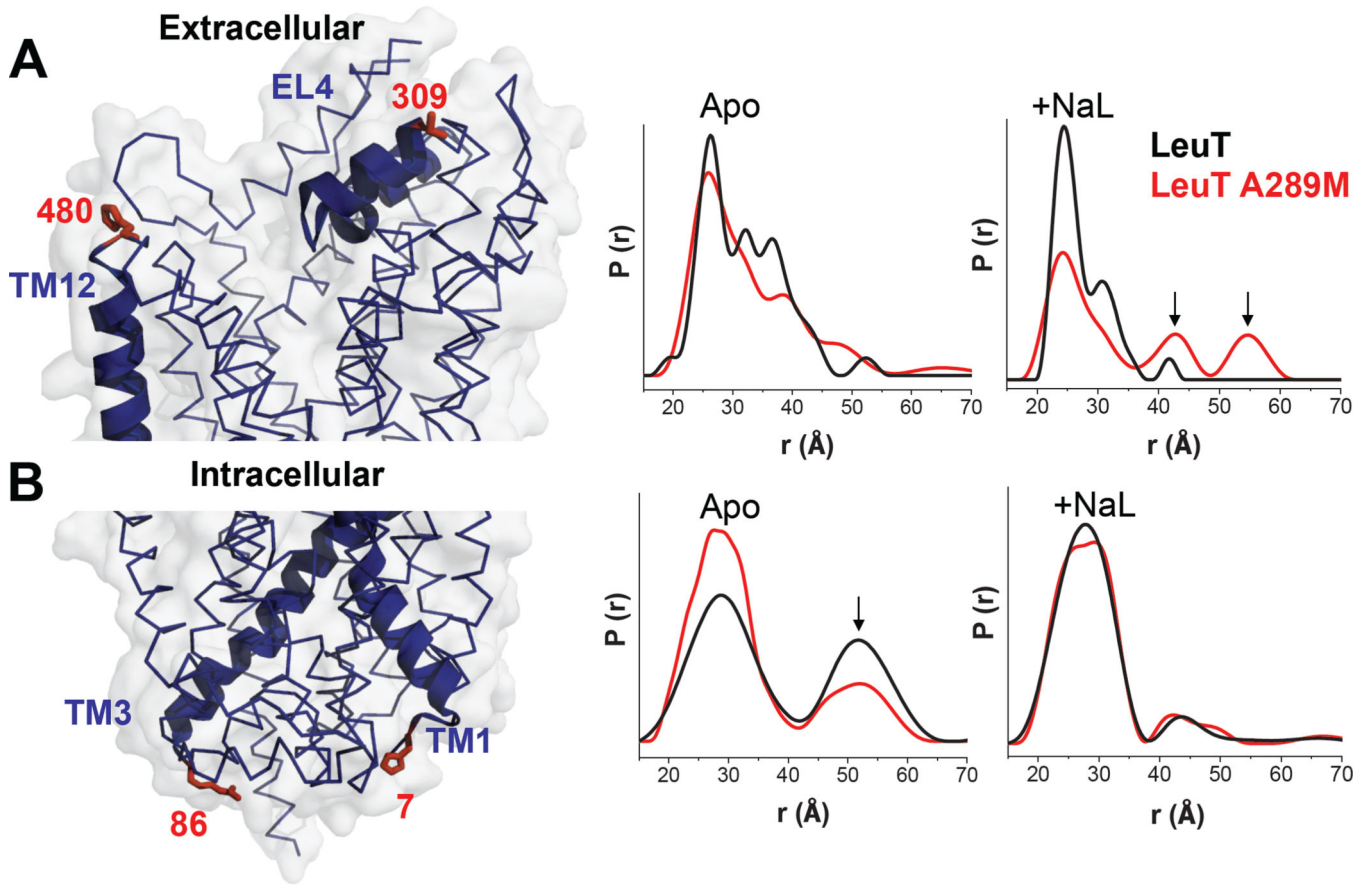


Figure 4. In LeuT, substitution of Ala289 with a Met supports an outward-open facing conformation

Distance distributions of extracellular and intracellular spin labeled Cys pairs in LeuT reveal changes in the conformational equilibrium caused by mutating Ala289 to a Met. A) Left: extracellular reporter pairs (309–480) tagged on three-dimensional structure of LeuT. Right: distance of the extracellular reporter pair for LeuT (black) and A289M (red), in the Apo conformation (Apo) and in the presence of Na^+ and Leu (+NaL). B) Left: intracellular reporter pairs (7–86) tagged on the three-dimensional structure of LeuT. Right: distance of the intracellular reporter pair for LeuT (black) and A289M (red), in the Apo conformation (Apo) and in the presence of Na^+ and Leu (+NaL). The LeuT structure was obtained from PDB 2A65. The structures were generated using PyMOL.

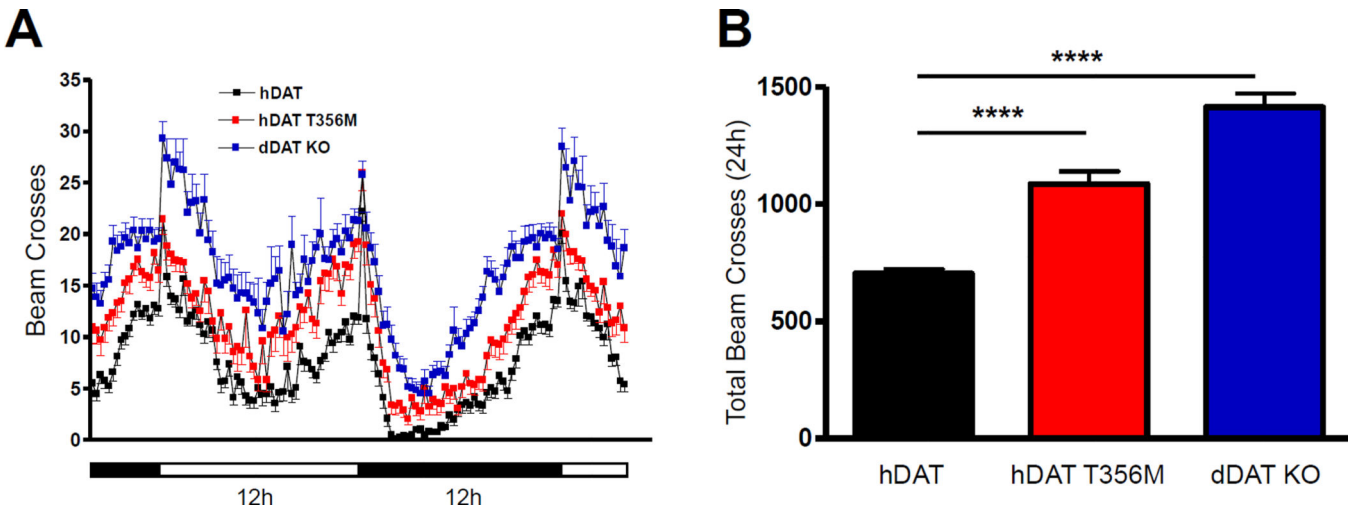


Figure 5. Expression of hDAT T356M in *Drosophila* leads to hyperactivity

hDAT or hDAT T356M was expressed in DA neurons of dDAT KO flies. A) Locomotor activity was assayed over 32 hours during the light (horizontal white bars) or dark (horizontal black bars) cycle. Flies expressing hDAT T356M (red squares), as well as dDAT KO flies (blue squares), were hyperactive throughout the 32 hour period with respect to flies expressing wild type hDAT (black squares) (n = 32; beam breaks binned in 15 minute intervals). B) Quantitation of total beam crosses over 24 hours for hDAT, hDAT T356M, and dDAT KO flies (**** = $p \leq 0.0001$; n = 32).