

The rare DAT coding variant Val559 perturbs DA neuron function, changes behavior, and alters in vivo responses to psychostimulants

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Despite the critical role of the presynaptic dopamine (DA) transporter (DAT, *SLC6A3*) in DA clearance and psychostimulant responses, evidence that DAT dysfunction supports risk for mental illness is indirect. Recently, we identified a rare, nonsynonymous *Slc6a3* variant that produces the DAT substitution Ala559Val in two male siblings who share a diagnosis of attention-deficit hyperactivity disorder (ADHD), with other studies identifying the variant in subjects with bipolar disorder (BPD) and autism spectrum disorder (ASD). Previously, using transfected cell studies, we observed that although DAT Val559 displays normal total and surface DAT protein levels, and normal DA recognition and uptake, the variant transporter exhibits anomalous DA efflux (ADE) and lacks capacity for amphetamine (AMPH)-stimulated DA release. To pursue the significance of these findings in vivo, we engineered DAT Val559 knock-in mice, and here we demonstrate in this model the presence of elevated extracellular DA levels, altered somatodendritic and presynaptic D2 DA receptor (D2R) function, a blunted ability of DA terminals to support depolarization and AMPH-evoked DA release, and disruptions in basal and psychostimulant-evoked locomotor behavior. Together, our studies demonstrate an in vivo functional impact of the DAT Val559 variant, providing support for the ability of DAT dysfunction to impact risk for mental illness.

dopamine | transporter | transgenic | mouse | polymorphism

The neurotransmitter dopamine (DA) plays a key role in regulating brain circuits that control reward, attention, and locomotor activity (1–3). Accordingly, dopaminergic dysfunction is believed to contribute to several neuropsychiatric disorders including Parkinson's disease (4), bipolar disorder (BPD) (5), drug abuse and addiction (6), and attention-deficit hyperactivity disorder (ADHD) (7, 8). The presynaptic DA transporter (DAT) is the primary mechanism for terminating DA signaling at the synapse (9) and is the primary target for several psychostimulant drugs including cocaine (COC), methylphenidate (MPH), and amphetamine (AMPH). COC and MPH are DAT antagonists, elevating extracellular DA levels by preventing DAT-mediated DA reuptake (10). AMPH actions are more complex (11). AMPH is structurally similar to DA and, as a result, is transported by DAT, competing with DA during the reuptake process. AMPH also induces DAT-mediated nonvesicular release, also termed "DA efflux," a process that involves the actions of intracellular signaling proteins such as CamKII α (12–16) and PKC β (17–19), alterations in interactions with DAT-associated proteins and phospholipids (12, 14, 20), and changes in DAT phosphorylation (12, 16, 21–23) that biases the transporter toward an efflux-competent mechanism. Despite their mechanistic differences, MPH and AMPH both rapidly elevate DA in the CNS and are components of the most frequently prescribed medications for ADHD, Ritalin and Adderall, respectively. The DA modulatory actions of MPH and AMPH reinforce hypotheses derived from

brain imaging studies (24) and the analysis of common genetic variation (25–30) that DA signaling perturbations contribute to risk for ADHD. Noncoding variation in the *SLC6A3* gene that encodes DAT has also been associated with ADHD medication response (26–28).

Attention to the impact of DAT genetic variation on behavioral changes underlying mental illness was raised with the studies of Giros et al. in their pioneering analysis of the DAT knockout (DAT KO) mouse (29). These investigators found that elimination of DAT expression leads to reduced presynaptic DA levels, increased extracellular DA, and profound locomotor hyperactivity in a novel environment (29), suggesting the utility of these mice as an ADHD model. Others have adopted DAT heterozygous or DAT knock-down mice as ADHD or BPD disease models, with reference to relevance for ADHD and/or bipolar disorder (31–34), although as with the DAT KO, these models do not replicate known genetic alterations in DAT and, thus, do not meet the important criterion for disease models of construct validity (35). Reliance on model phenotypic similarities to disease features, or face validity, can lead to pursuit of mechanisms that may be of little relevance to the human condition. For example, the hyperactivity of DAT KO mice can be suppressed by antidepressant drugs (30), whereas the core

Significance

Dopamine (DA) signaling provides important, modulatory control of movement, attention, and reward. Disorders linked to changes in DA signaling include Parkinson's disease, attention-deficit hyperactivity disorder, schizophrenia, autism spectrum disorder, and addiction. We identified multiple, functional polymorphisms in the human DA transporter (DAT) gene and showed that one of these variants, which produces the amino acid substitution Val559 (wild-type DATs express Ala559), exhibits normal DA uptake accompanied by a spontaneous outward efflux of the neurotransmitter, reminiscent of the actions of the psychostimulant amphetamine. Here, we identify multiple biochemical, physiological, and behavioral perturbations that arise from DAT Val559 expression in vivo, supporting spontaneous DA efflux as a heretofore-unrecognized mechanism that may underlie multiple DA-linked neurobehavioral disorders.

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features of ADHD are not responsive to these medications (36–39). Further to this point, humans bearing homozygous, loss-of-function DAT mutations as seen in the DAT KO model, present with a complex disorder that, in juvenile stages, resembles Parkinson's disease (40, 41). These findings suggest that DAT contributions to disorders such as ADHD and BPD may derive from less severe or qualitatively distinct changes in DAT activity than simple loss of function.

Increasingly, risk for neuropsychiatric disorders is suspected to derive from a complex interplay of common and rare gene variation that intersect with environmental factors (42, 43). Rare variation can be of larger effect than that attributed to common variants and, when localized to functionally annotated regions of the genome, such as protein coding sequences, they afford the generation of animal models whose study may be informative as to disease mechanisms (44–46). Following this logic, we pursued both a functional analysis of rare DAT coding variants identified in various disorders such as BPD and schizophrenia (8) and screened ADHD genomic DNA for evidence of rare variation in DAT coding sequences (47). These efforts converged on a polymorphism that produces the substitution DAT Ala559Val. Grunhage et al. (48) first identified the variant in a female subject with BPD. Transmission status could not be assessed because of the possibly transmitting parent being deceased and a lack of information as to the status of other carriers in the pedigree. We identified the same variant in two male siblings with ADHD (47), establishing a pedigree that demonstrated heritable transmission through two generations on the maternal side. The mother transmitting the variant lacked a clinical diagnosis, although she described mild learning disabilities as a child. The transmitting grandmother was found in standardized assessments of adult ADHD traits to be above the 95th percentile for impulsivity traits. Recently, Bowton et al. described the transmission of the DAT Val559 variant to two, unrelated male subjects with ASD (49) that was not seen in matched unaffected subjects, extending the possible disease associations to a third disorder with evidence of DA signaling perturbations (50–57).

Our initial in vitro characterization of the DAT Val559 variant in transfected cells (8) revealed normal total and surface DAT protein expression, and normal DA uptake rates. Subsequent studies, however, that monitored the ability of transfected cells to retain preloaded DA revealed that DAT Val559-transfected cells exhibited a spontaneous, DAT-mediated outward “leak” of cytoplasmic DA (58), which we have termed anomalous DA efflux (ADE). We found ADE to be voltage-dependent, being enhanced by cell depolarization, and to reflect a higher affinity for intracellular Na^+ , a major determinant of the driving force for inwardly directed DA uptake. Remarkably, we also found that AMPH exhibited no ability to induce DAT-mediated DA efflux in DAT Val559-transfected cells. Instead, AMPH actions resembled the actions of MPH and COC, because all three agents block ADE. Finally, Bowton et al. (13) provided evidence that the ADE induced by DAT Val559 derives from basal D2 DA receptor (D2R) and $\text{CamKII}\alpha$ -dependent DAT phosphorylation at N-terminal sites, modifications that normally support AMPH-induced DA efflux (12). Together, these data support a mechanism whereby the ADE of DAT Val559 could short-circuit the highly efficient DA clearance seen with wild-type DAT, with disease trajectory dependent on various genetic and/or environmental modifying factors.

Although fascinating from a mechanistic perspective, our in vitro studies with DAT Val559-transfected cells, including neurons (13), suffer from obvious limitations with respect to their relevance to human mental illness. Additionally, despite having been identified in three different disorders associated with DA dysfunction, the overall rarity of the DAT Val559 variant, and the small, largely uninformative pedigrees that house the

affected subjects, thwart efforts to argue strongly for causation. Here, we significantly advance the case that the DAT Val559 variant can contribute to DA-linked disease states with a description of the biochemical, physiological, pharmacological, and behavioral phenotypes present in mice, engineered to express the variant from the native *Slc6a3* locus. Our findings provide compelling evidence that the DAT Val559 substitution disrupts multiple aspects of DA neuron function in vivo, consistent with the generation of ADE. Moreover, we demonstrate that these alterations establish detectable changes in behavior and in vivo psychostimulant responses, consistent with a dominant, gain-of-function action of the DAT Val559 variant.

Results

Biochemical Consequences of in Vivo Expression of DAT Val559. Recently, we reported the generation and general growth characteristics of DAT Val559 knock-in mice (59). As noted in the prior publication, these mice reproduce normally and display no overt sensorimotor deficits. Our previous heterologous expression studies demonstrated no impact of the DAT Val559 substitution on total or cell surface expression, inward DA transport, or sensitivity to psychostimulants (8, 58). Consistent with these findings, striatal extracts from DAT Val559 animals demonstrate normal transporter protein levels (Fig. 1A). Similarly, striatal synaptosomes from Val559 heterozygous (HET) and homozygous (HOM) animals demonstrated equivalent synaptosomal DA uptake kinetics as compared to WT samples (Fig. 1B). DAT Val559 mice demonstrate no genotype-dependent changes in striatal tyrosine hydroxylase (TH) levels (Fig. 1C) or in total levels of DA, DA metabolites, or DA turnover in striatum (Fig. 1D). Additionally, no changes were observed for DA measures in cortex (Fig. 1E) and midbrain (Fig. 1F). Small but significant differences in striatal (Fig. 1G) and cortical (Fig. 1H), but not midbrain (Fig. 1I) serotonin (5-HT) levels were detected without concomitant changes in 5-hydroxyindoleacetic acid (5-HIAA; a 5-HT metabolite) or 5-HT turnover (Fig. 1G–I). Finally, radioligand-binding studies to assess cortical, midbrain, and striatal D1 DA receptor (D1R) and D2R densities revealed no changes in the levels of these proteins (Fig. 1J and K).

DA Neurons from DAT Val559 Mice Display Alterations in Basal and AMPH-Augmented D2R-Mediated IPSCs. DAT is expressed somatodendritically on DA neurons where it can limit the ability of D2Rs to reduce DA neuron firing, because D2R activation in the midbrain is tightly controlled by reuptake (60). Vesicular somatodendritic release of DA in the substantia nigra (SN) and ventral tegmental area elicits an inhibitory postsynaptic current (IPSC) via D2R activation of a G protein-coupled inwardly rectifying potassium (GIRK) channel, which can inhibit DA neuron firing (61, 62). To examine the impact of DAT Val559 on basic physiological properties of DA neurons, and D2R-mediated currents, we performed whole-cell recordings of SN DA neurons in acute brain slices. We found whole-cell capacitance to be reduced (WT: 35.8 ± 0.67 pF, $n = 139$; HOM: 32.7 ± 0.56 pF, $n = 153$; $P = 0.001$, Mann–Whitney test) and resistance to be increased (WT: 315.7 ± 14.1 M Ω , $n = 138$; HOM: 347.3 ± 12.6 M Ω , $n = 149$, $P = 0.012$, Mann–Whitney test). Resting membrane potential, percent of quiescent cells, and firing rates in response to current injection exhibited no genotype effects.

D2R-mediated IPSCs were evoked by electrical stimulation with glutamatergic, GABAergic, and cholinergic inputs silenced with receptor blockers. A significant difference was observed in the time to peak of the D2R-mediated IPSC evoked by a single stimulus (WT: 254.0 ± 7.3 ms, $n = 32$; HOM: 288.8 ± 8.8 ms, $P = 0.001$, ANOVA), and the duration of the IPSC evoked by a single or five stimuli (one stimulus, WT: 658.5 ± 24.5 ms, $n = 32$, HOM: 791.1 ± 40.5 ms, $P < 0.01$; five stimuli, WT: 702.0 ± 14.2 ms, $n = 64$; HOM: 775.2 ± 26.1 , $n = 67$, $P < 0.05$, ANOVA with

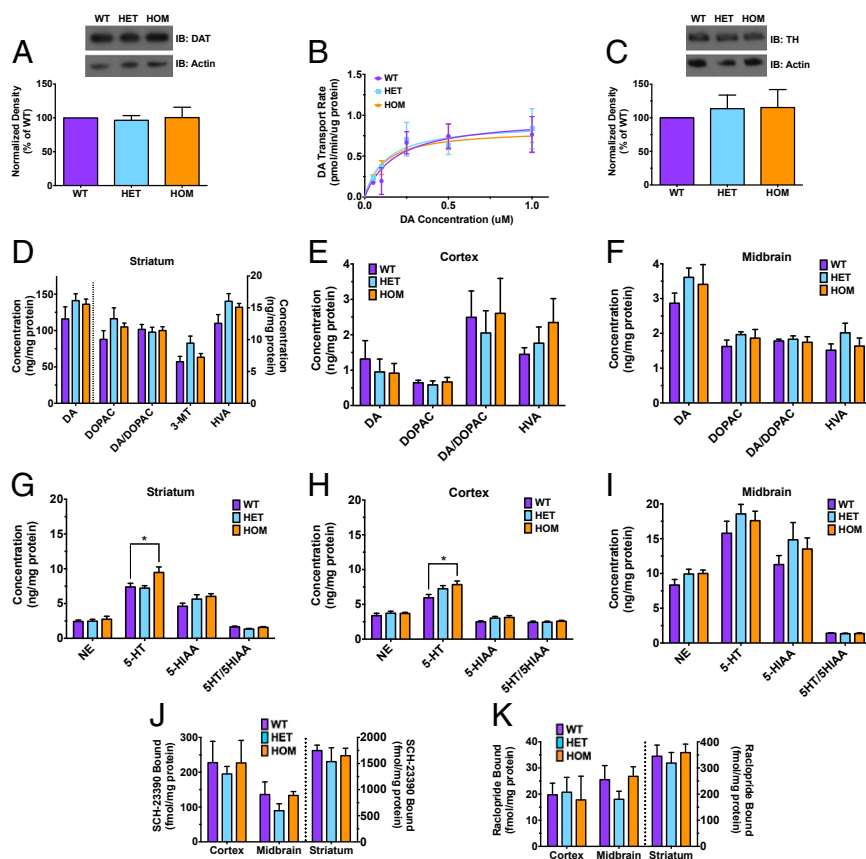


Fig. 1. General lack of gross neurochemical changes associated with in vivo DAT Val559 expression. Striatal DAT expression ($n = 6$ per genotype) (A); DA transport kinetics ($n = 5$ WT, 4 HET, 7 HOM; $V_{\max} \pm \text{SEM}$ (pmol μg of protein $^{-1}$ min^{-1}): WT = 1.01 ± 0.215 , HET = 0.8284 ± 0.1822 , HOM = 0.8847 ± 0.1770 ; $K_m \pm \text{SEM}$ (μM): WT = 0.1774 ± 0.03839 , HET = 0.08762 ± 0.0261 , HOM = 0.1407 ± 0.04754) (B); striatal TH expression ($n = 3$ per genotype) (C); total striatal levels of DA and metabolites DOPAC, HVA, and 3-MT, and DA turnover (DA/DOPAC ratio) ($n = 8$ WT, 8 HET, and 9 HOM) in WT and DAT Val559 mice (D); DA levels in cortex (E) and midbrain (F); (G) Striatal, (H) cortical, and (I) midbrain 5-HT, 5-HIAA, 5-HIAA/5-HT (5-HT turnover), and NE levels ($n = 8$ WT, 8 HET, 9 HOM; cortex and midbrain, $P < 0.05$, one-way ANOVA, $*P < 0.05$, Tukey's multiple comparisons test). (J) D1R density estimated with [^3H]-SCH-23390 binding in cortex and midbrain was plotted on left y axis; striatum values were plotted on right y axis. (K) D2R density was estimated with [^3H]-raclopride binding: Cortex and midbrain was plotted on left y axis, and striatum was plotted on right y axis.

Fisher's LSD post hoc test) (Fig. 2A). In the midbrain, vesicular DA release occurs spontaneously, without electrical stimulation, eliciting spontaneous D2R-mediated inhibitory postsynaptic currents (sIPSCs) (62). sIPSCs were also prolonged in DAT Val559 slices (WT: 380.7 ± 8.7 ms, $n = 135$ sIPSCs; HOM: 427.5 ± 10.9 ms, $n = 150$ sIPSCs, $P < 0.01$, Mann-Whitney) (Fig. 2A), with no difference in amplitude. In contrast to D2R-mediated currents, no differences were evident in the time to peak or duration of evoked GABA $_B$ -mediated IPSCs (Fig. 2B).

To assess AMPH effects on DA neurons, AMPH was bath applied to slices from which D2R-mediated IPSCs were recorded. As expected from the actions of methamphetamine or COC (61, 63), the amplitude, time to peak, and duration of evoked D2R-mediated IPSCs in WT slices treated with AMPH increased significantly relative to no-drug conditions and remained elevated throughout the AMPH application. However, the AMPH-induced increase in D2R-mediated IPSC amplitude was blunted in DAT Val559 slices (Fig. 2C), with some cells showing a brief enhancement followed by a decrease to baseline amplitude. However, there were no differences in the AMPH-induced increase in time to peak (WT: $+89.9 \pm 9.6$ ms; HOM: $+65.4 \pm 11.7$ ms, $P = 0.16$, Student's unpaired t test) and duration (WT: $+330.6 \pm 43.7$ ms; HOM: $+350.6 \pm 122.0$, $P = 0.90$, Student's unpaired t test) of D2R IPSCs between genotype. To determine whether the changes seen in D2R-mediated IPSC amplitude was a consequence of changes in DAT-mediated DA clearance ver-

sus a unique property of AMPH lost in the DAT Val559 mice, we repeated these studies by using MPH, finding no genotype differences in MPH augmentation of IPSC amplitude (Fig. 2D). Finally, maximal D2R-mediated currents were elicited by iontophoretic application of DA onto DA neurons. Consistent with the midbrain D2R binding studies, there was no difference in the maximal DA current between genotypes (WT: 10.0 ± 0.4 pA/pF, $n = 65$; HOM: 9.4 ± 0.4 pA/pF, $n = 76$, $P = 0.46$, Mann-Whitney).

Nerve Terminal DA Release Evaluated in Striatal Slices Displays Blunted AMPH- and 4-AP-Evoked DA Release Accompanied by Tonic Presynaptic D2R Stimulation. Our findings that DA neurons exhibit blunted DAT and D2R-mediated AMPH responses led us to evaluate whether projections of these cells exhibit changes in AMPH-evoked DA release. We first preloaded acute striatal slices from WT and DAT Val559 with [^3H]DA, observing no genotype effects on [^3H]DA accumulation as predicted by synaptosomal uptake studies. Next, we determined a concentration of AMPH (1 μM) that, when applied to preloaded slices, generated an intermediate capacity for DAT-dependent DA release so as to preclude ceiling or floor effects on evoked [^3H]DA release. At this concentration, WT slices demonstrated a significant, $\sim 50\%$ increase from baseline in DAT-dependent [^3H]DA release (Fig. 3A). Notably, DAT Val559 slices showed a significantly reduced capacity for evoked DA efflux compared with

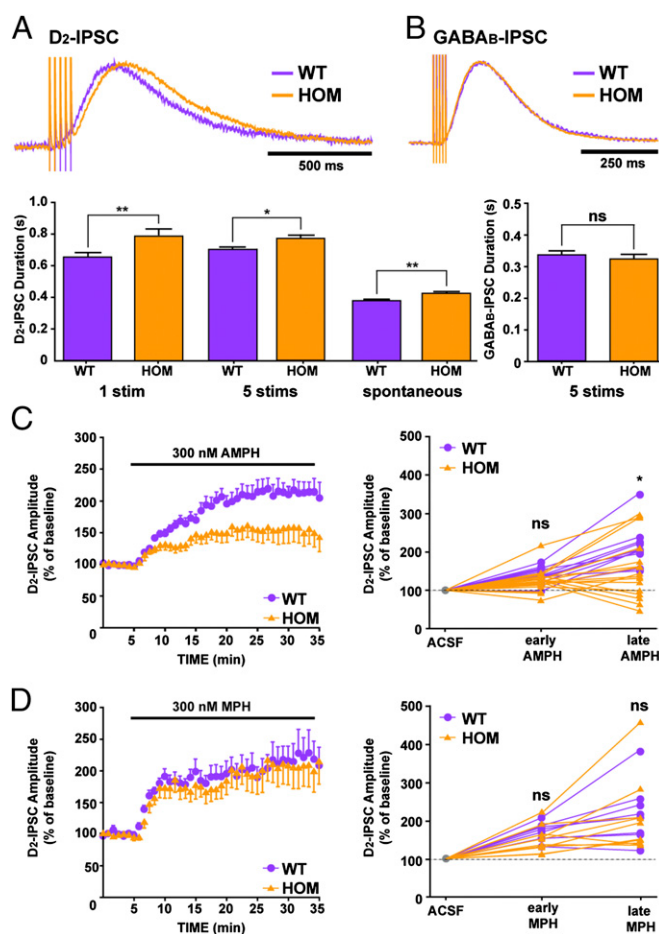


Fig. 2. Dopamine D2R-mediated synaptic currents in midbrain slices from DAT Val559 mice exhibit slower kinetics and blunted enhancement by AMPH, but not MPH. Scaled representative synaptic currents (IPSCs) mediated by D2R (A) or GABA_B receptors (B) were evoked by electrical stimulation in brain slices containing substantia nigra dopamine neurons. D2R-mediated IPSCs recorded in DAT Val559 slices are significantly slower than in WT slices, whether from a single electrical stimulation or using a train of stimuli to evoke DA release (1 stim: $n = 32$ cells WT, 35 cells HOM; $P = 0.003$; 5 stims: $n = 64$ cells WT, 67 cells HOM; $*P < 0.05$, one-way ANOVA), or those occurring spontaneously ($n = 135$ sIPSCs WT, 150 sIPSCs HOM; $**P = 0.01$, Mann-Whitney). No genotype differences were detected in the kinetics of GABA_B-mediated IPSCs ($n = 18$ cells WT, 17 cells HOM). Upon AMPH (C) or MPH (D) application, the amplitude of evoked D2R-mediated IPSCs increases significantly. (C) The AMPH-induced increase in D2R-mediated IPSC amplitude is blunted in DAT Val559 slices [Left, time course of AMPH response; Right, averaged increase during the period 2.5–5 min after AMPH application (early) and averaged increase during 20–30 min (late); normalized to pre-AMPH amplitude; $n = 18$ cells WT, 19 cells HOM, $*P < 0.05$, two-way RMANOVA]. (D) No genotype differences were detected in the MPH-induced increase in D2R-mediated IPSC [Left, time course of MPH response; Right, averaged increase during the period 2.5–5 min following MPH application (early) and averaged increase during 20–30 min (late); normalized to pre-MPH amplitude; $n = 10$ cells WT, 11 cells HOM].

WT controls. To determine whether changes in nonvesicular AMPH-evoked DA release from DAT Val559 animals is accompanied by alterations in vesicular DA release, we challenged preloaded slices with perfusion solution supplemented with 50 μ M 4-aminopyridine (4-AP) to block voltage-gated K⁺ channels, establishing in WT slices a level of evoked [³H]DA release comparable to that observed with AMPH treatment. With 4-AP-treated DAT Val559 slices, we again observed a blunted level of release relative to WT preparations, suggestive

of either reduced DA stores or tonic inhibition of vesicular release (Fig. 3B). Because striatal DA tissue levels are normal (Fig. 1D), we suspected that presynaptic D2Rs in the DAT Val559 slices might be underrepresented stimulation due to ADE. To test this idea, we challenged these receptors with either the D2R agonist quinpirole (250 nM) or the D2R antagonist raclopride (500 nM). Whereas quinpirole reduced 4-AP-induced [³H]DA release in WT slices (Fig. 3C), indicative of presynaptic receptor-mediated inhibition, quinpirole suppression of 4-AP-evoked [³H]DA release was lost in DAT Val559 slices (Fig. 3D). This loss of quinpirole modulation could either arise from an ongoing, maximal stimulation of presynaptic D2Rs by extracellular DA due to ADE, or from D2R desensitization or down-regulation. That ongoing presynaptic D2R stimulation explains the loss of

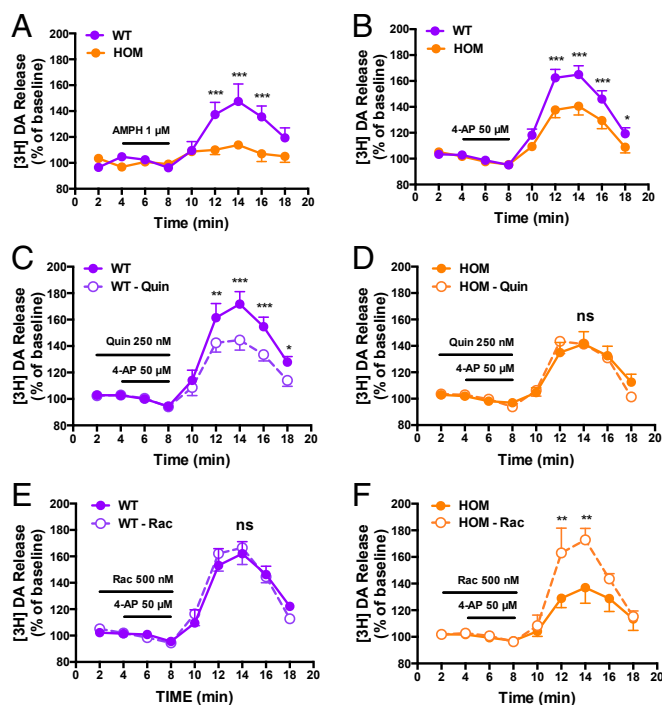


Fig. 3. Blunted AMPH and depolarization-evoked DA release by DAT Val559 striatal DA nerve terminals is accompanied by constitutive presynaptic D2R stimulation. (A) Striatal slices from WT mice exhibit significant elevation in [³H]DA release above baseline upon application of 1 μ M AMPH, whereas DAT Val559 tissue releases significantly less [³H]DA than WT tissue [$n = 5$ WT, 5 HOM; $P(\text{genotype}) < 0.05$, $P(\text{time}) < 0.0001$, $P(\text{interaction}) < 0.0001$, post hoc tests (Sidak's multiple comparisons test) reveal $P < 0.001$ at 12 and 16 min and $P < 0.0001$ at 14 min]. (B) Following application of 50 μ M 4-AP, WT striatal slices exhibit significant [³H]DA release above baseline ($n = 12$; $P < 0.0001$), but DAT Val559 slices show significantly diminished [³H]DA release compared with WT [$n = 12$ WT, 12 HOM; $P(\text{genotype}) < 0.0001$, $P(\text{time}) < 0.0001$, $P(\text{interaction}) < 0.0001$, post hoc tests (Sidak's multiple comparisons test for time-dependent genotype differences) reveal $P < 0.001$ at 12, 14, and 16 min and $P < 0.05$ at 18 min]. (C) Quinpirole (250 nM) significantly decreases 4-AP-evoked [³H]DA release in WT striatal slices [$n = 7$ WT; shows $P(\text{quinpirole effect}) < 0.05$, $P(\text{time}) < 0.0001$, $P(\text{interaction}) < 0.0001$, post hoc tests (Sidak's multiple comparisons test for time-dependent genotype differences) reveal $P < 0.01$ at 12 min, $P < 0.0001$ at 14 min, $P < 0.001$ at 16 min, and $P < 0.05$ at 18 min]. (D) Quinpirole-mediated suppression of 4-AP-evoked, striatal [³H]DA release is absent in DAT Val559 [$n = 7$ HOM; $P(\text{quinpirole effect}) > 0.05$, $P(\text{time}) < 0.0001$, $P(\text{interaction}) > 0.05$]. (E) After 500 nM raclopride, WT striatal tissue showed no changes in 4-AP-evoked [³H]DA release [$n = 5$ WT; $P(\text{raclopride effect}) > 0.05$, $P(\text{time}) < 0.0001$, $P(\text{interaction}) > 0.05$]. (F) Raclopride application enhanced 4-AP-evoked [³H]DA release in DAT Val559 striatal slices [$n = 5$ HOM; $P(\text{raclopride effect}) < 0.05$, $P(\text{time}) < 0.0001$, $P(\text{interaction}) < 0.05$, post hoc tests (Sidak's multiple comparisons test for time-dependent genotype differences) reveal $P < 0.01$ at 12 and 14 min].

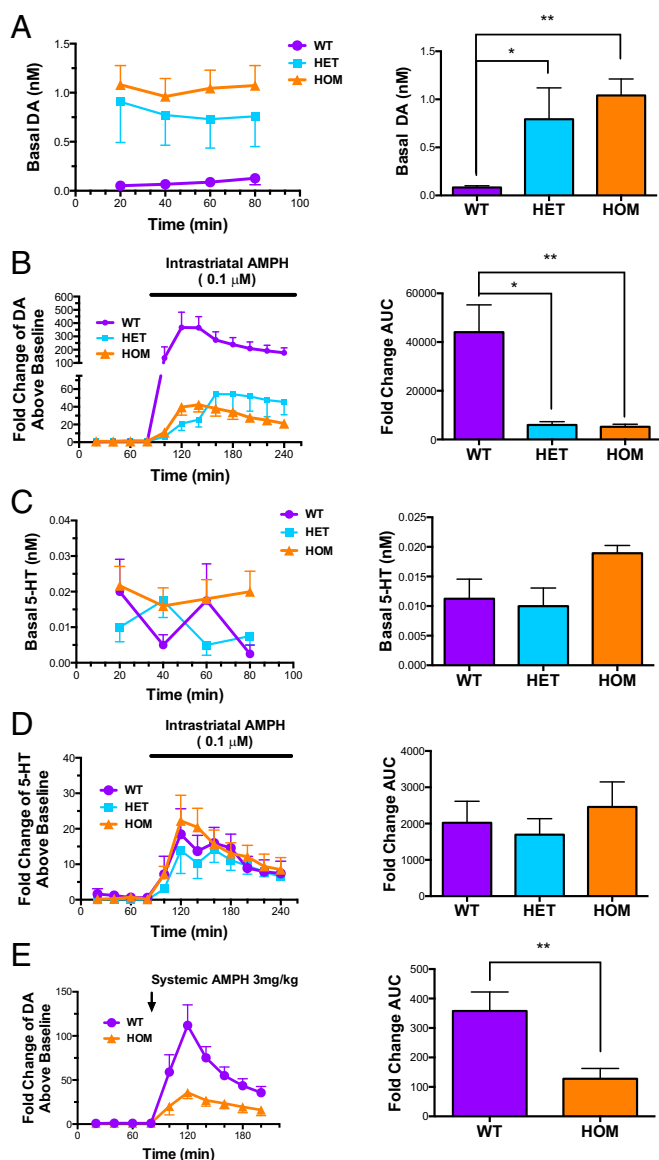


Fig. 4. In vivo microdialysis DAT Val559 mice exhibit elevated basal extracellular DA and significantly blunted AMPH-evoked DA release, but 5-HT levels and release are unaffected. (A) Basal extracellular DA levels are elevated in DAT Val559 mice [$n = 7$ WT, 4 HET, 8 HOM; *Left*, time course of basal DA release, $P(\text{genotype}) < 0.01$, post hoc tests reveal $P < 0.05$ at 20 and 40 min for WT vs. HET, and $P < 0.001$ at 20, 60, and 80 min and $P < 0.01$ at 40 min for WT vs. HOM; *Right*, mean $\pm$ SEM extracellular DA concentration before AMPH stimulation, $P < 0.05$ one-way ANOVA, post hoc testing reveals $P < 0.05$ (*) for WT vs. HET and $P < 0.01$ (**) WT vs. HOM]. With intrastratial AMPH (B), AMPH-evoked DA is reduced ~10-fold in DAT Val559 mice [$n = 7$ WT, 4 HET, 8 HOM; *Left*, time course of evoked DA release, fold change above baseline $\pm$ SEM, $P(\text{genotype}) < 0.01$, post hoc tests reveal $P < 0.05$ at 200 and 220 min, $P < 0.01$ at 180 min, $P < 0.001$ at 160 min, $P < 0.0001$ at 120 and 140 min for WT vs. HET, $P < 0.05$ at 100 min, $P < 0.01$ at 220 and 240 min, $P < 0.001$ at 180 and 200 min, and $P < 0.0001$ at 120, 140, and 160 min for WT vs. HOM; *Right*, mean $\pm$ SEM, integrated fold change of DA relative to baseline, $P < 0.01$ one-way ANOVA, post hoc testing reveals $P < 0.05$ (*) for WT vs. HET and $P < 0.01$ WT vs. HOM (**)]. (C) Basal extracellular 5-HT levels do not differ between genotypes [$n = 4$ WT, 4 HET, 6 HOM; *Left*, time course of basal 5-HT release; *Right*, mean $\pm$ SEM extracellular 5-HT concentration before AMPH stimulation]. (D) Equivalent AMPH-induced increases in extracellular 5-HT ($n = 4$ per genotype; *Left*, time course of evoked 5-HT release, fold change above baseline $\pm$ SEM; *Right*, mean $\pm$ SEM integrated fold change of 5-HT relative to baseline). (E) Systemic AMPH-evoked DA is reduced ~3-fold in DAT Val559 mice [$n = 7$ each genotype; *Left*, time course of evoked DA release, fold change above baseline $\pm$ SEM, $P(\text{genotype}) < 0.05$,

quinpirole effects in the DAT Val559 slices was revealed by subsequent raclopride studies. We found that whereas the D2R antagonist was without effect on WT slices (Fig. 3E), indicative of little if any tonic stimulation of D2Rs under our perfusion conditions, raclopride significantly augmented [^3H]DA release in DAT Val559 slices (Fig. 3F).

In Vivo Striatal Microdialysis of DAT Val559 Mice Reveals Elevated Basal Extracellular DA and a Blunted DA Elevation in Response to Local and Systemic AMPH. To extend our release studies to an in vivo context and assess whether DAT Val559 animals exhibit changes in basal and psychostimulant-induced DA release, we performed striatal microdialysis studies in freely moving animals. When quantifying basal levels of extracellular DA (Fig. 4A), we obtained values for DAT Val559 animals that were significantly elevated compared with WT animals, with DAT Val559 heterozygous animals demonstrating an intermediate elevation. To complement our brain slice efforts, we locally infused AMPH (0.1 μM) and monitored changes in extracellular DA. Here, we detected a significantly blunted ability of AMPH to induce DA release in DAT Val559 animals relative to WT littermates (Fig. 4B). Interestingly, a comparably blunted response to that seen with DAT Val559 homozygous mice was seen with DAT Val559 heterozygotes. In contrast to these findings, no significant differences were evident in basal or AMPH-evoked 5-HT levels (Fig. 4C and D). To determine whether the loss of AMPH responsiveness was also evident with systemic AMPH injections, we first determined a dose of AMPH (3 mg/kg i.p.) that induced an intermediate level of locomotor activation in WT animals (see below). Compared with the striatal DA elevations achieved with WT animals, the changes induced in DAT Val559 animals were significantly reduced (Fig. 4E).

DAT Val559 Mice Demonstrate Conditional Hyperactivity and Blunted Motor Activation by AMPH. DAT KO mice exhibit elevated basal extracellular DA and are hyperactive in open field testing (29, 64), with the behavioral disturbance suggestive of an animal model of ADHD (65, 66). In our open field assessments of locomotor behavior, we observed no such hyperactivity with the DAT Val559 mice (Fig. 5A). Additionally, no genotype differences were detected when horizontal activity was divided into center versus surround time (Fig. 5B). However, we detected a significant reduction in vertical activity (rearing) in the DAT Val559 animals when assessed as either number of rearing events or time spent rearing (Fig. 5C and D). Notably, these changes were evident with heterozygous animals, consistent with a dominant effect of the Val559 allele. Seeing no obvious spontaneous hyperactivity, we considered the possibility that the DAT Val559 variant might confer an increased sensitivity to stimuli that normally provoke a locomotor response, particularly because hyperactivity in ADHD subjects is not seen at all times or in all environments (67, 68). Indeed, early on in our handling of these mice, we noticed that when researchers reached to transfer animals between cages, the presence of a DAT Val559 genotype could be fairly reliably assigned based on the presence of a more robust escape response, a behavior we hereafter term “darting.” To quantify darting, we recorded escape responses after successive, stereotyped hand approaches, blind to genotype, and measured the speed for each escape. Analysis of darting responses revealed a significant genotype effect when assessed in terms of distribution (Fig. 6A) or group means (Fig. 6B), such that DAT Val559 mice demonstrated more rapid escape responses than their WT counterparts, with a gene dosage effect evident. The

post hoc tests reveal $P < 0.05$ at 100 min, $P < 0.001$ at 140 min, and $P < 0.0001$ at 120 min for WT vs. HOM; *Right*, mean $\pm$ SEM integrated fold change of DA relative to baseline, $P < 0.01$ (**), WT vs. HOM].

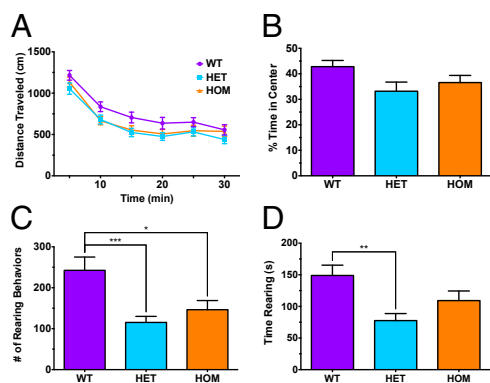


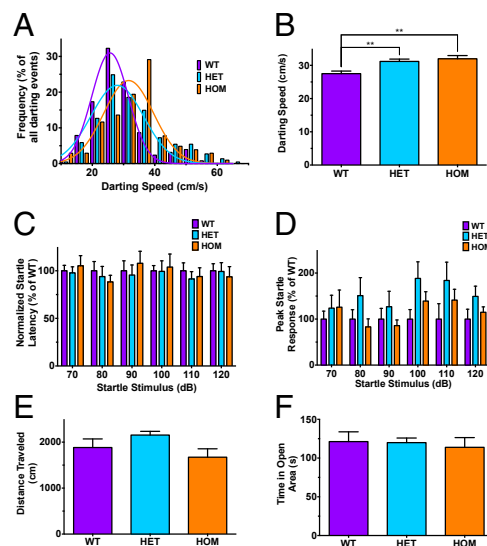
Fig. 5. Spontaneous locomotor response to a novel environment reveals a lack of overt hyperactivity but a significant reduction in vertical activity (rearing). (A) DAT Val559 exhibit reduced horizontal locomotor activity upon initial exposure to open field chamber ($n = 28$ WT, 30 HET, 25 HOM; $P(\text{genotype}) > 0.05$, two-way RMANOVA). (B) Lack of genotype differences in relative time spent in the center versus the edge of the activity chambers. DAT Val559 exhibit reduced number of rearing behaviors ($P < 0.001$, one-way ANOVA, $*P < 0.05$, $***P < 0.001$, Tukey's multiple comparisons test) (C) and reduced time spent rearing $P < 0.01$, one-way ANOVA, $*P < 0.01$, Tukey's multiple comparisons test) (D).

blunted response for rearing counts in relation to WT values. As seen with AMPH, expression of DAT Val559 did not impact stereotypical behaviors (Fig. 7 I and J).

The blunted AMPH and MPH effects on locomotor behavior could arise from altered presynaptic mechanisms or changes in postsynaptic DA receptor responses. D1Rs have been implicated in the hyperactivity triggered by *in vivo* AMPH injections (70, 71). Thus, we injected mice with the D1R agonist SKF83822 at a dose (2 mg/kg *i.p.*) that generates equivalent locomotor effects in our WT mice as 3 mg/kg AMPH. These experiments revealed no genotype differences in horizontal locomotion (Fig. 8A), rearing (Fig. 8B), or stereotypes (Fig. 8C) in response to SKF83822, suggesting that the differences in locomotor response to AMPH and MPH displayed by DAT Val559 mice most likely arises from intrinsic changes in DA neuron function, including those demonstrated here.

Discussion

Rare genetic variation is increasingly recognized as a source for insights into the etiology of complex disorders (72–76). Often, such variation is confined to a single, and sometimes small, pedigree, compelling the demonstration of functional perturbations *in vitro* and *in vivo* to make conclusions as to possible disease associations. Our efforts to date have uncovered multiple rare SLC6A3 coding variants in screens of ADHD subjects, with two of these, A559V and R615C, demonstrating altered function after heterologous expression (58, 77). Here, we present the first opportunity, to our knowledge, to explore the impact of disease-associated, rare DAT variation *in vivo*. Our efforts originated from identification of the Val559 variant in two male siblings with an ADHD diagnosis (47). Although our starting point concerned efforts to detect rare, functional gene variation impacting DAT expression or function that could contribute to risk for ADHD, the variant has also been detected in a female subject with BPD (48), and in two unrelated males with an ASD diagnosis (49). ADHD and BPD are found at higher incidence in



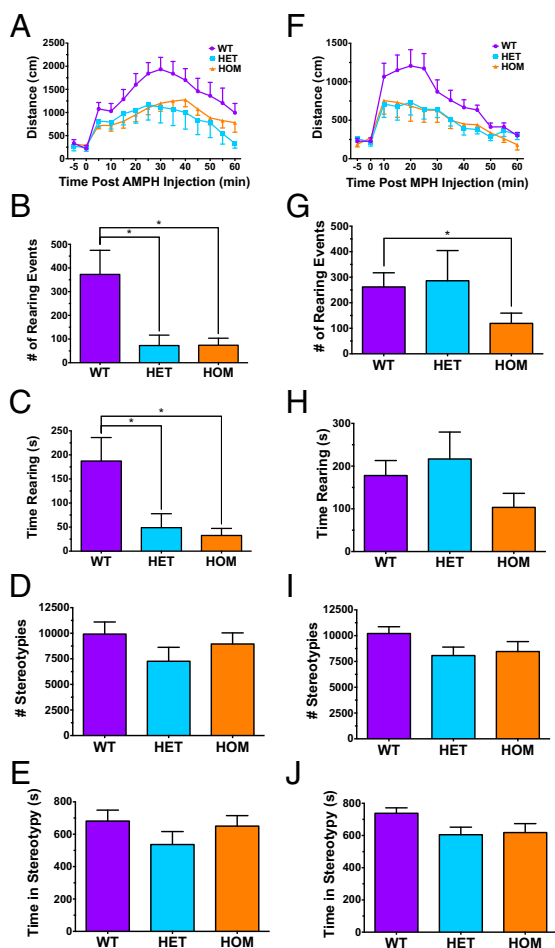


Fig. 7. DAT Val559 mice display blunted horizontal and vertical activity responses to AMPH and MPH. (A) Reduced horizontal locomotor-stimulating effects of AMPH (3 mg/kg i.p.) treatment in DAT Val559 mice ($n = 9$ WT, 9 HET, 6 HOM), $P(\text{genotype}) < 0.05$, post hoc tests reveals $P < 0.05$ at 25, 45, 45, and 55 min and $P < 0.01$ at 30 and 35 min after AMPH injection for WT vs. HET and $P < 0.05$ at 25 and 30 min after AMPH injection for WT vs. HOM, two-way RMANOVA; DAT Val559 mice demonstrate reduced numbers of rearing events $\pm$ SEM in 60 min ($n = 9$ WT, 9 HET, 6 HOM, $P < 0.05$, one-way ANOVA, $*P < 0.05$, Tukey's multiple comparisons test) (B) and time spent rearing $\pm$ SEM ($n = 9$ WT, 9 HET, 6 HOM, $P < 0.05$, one-way ANOVA, $*P < 0.05$, Tukey's multiple comparisons test) (C). DAT Val559 mice demonstrate lack of genotype effect on a number of stereotyped behaviors ($n = 9$ WT, 9 HET, 6 HOM, number $\pm$ SEM in 60 min) (D) and on time in stereotypy ($n = 9$ WT, 9 HET, 6 HOM, time $\pm$ SEM in 60 min) (E). (F) Reduced horizontal locomotor-stimulating effects of MPH (10 mg/kg i.p.) treatment in DAT Val559 mice [$n = 14$ WT, 15 HET, 15 HOM, $P(\text{interaction}) < 0.05$, post hoc tests reveals $P < 0.01$ at 15, 20, and 25 min after MPH injection for WT vs. HET and $P < 0.05$ at 10 and 15 min and $P < 0.01$ at 20 and 25 min after MPH injection]. Impact of genotype on number $\pm$ SEM in 60 min ($n = 16$ WT, 14 HET, 15 HOM, $P > 0.05$, one-way ANOVA, unpaired Student t test, $*P < 0.05$, Tukey's multiple comparisons test) (G) and time spent rearing $\pm$ SEM, $P > 0.05$, one-way ANOVA following MPH injections (H). (I) Lack of genotype effects on number of stereotyped behaviors ($n = 16$ WT, 16 HET, 16 HOM; left axis, number $\pm$ SEM in 60 min) or time in stereotypy time $\pm$ SEM in 60 min (J).

families of probands with one or the other diagnosis (7) and, although not a part of the diagnostic criteria for ASD, many of the latter subjects meet clinical criteria for ADHD (78). These epidemiological findings suggest that the detection of the DAT Val559 variant in ADHD, BPD, and ASD subjects may relate to the differing trajectories of complex neuropsychiatric disorders that emerge from common biological substrates, supportive of

a move away from categorical definitions in the development of animal models of psychiatric disorders (79). The availability of the Val559 mouse model also allows us to explore other, sometimes unexpected, phenotypic consequences that upon further analysis may connect to the existing biological underpinnings of neuropsychiatric disorders. For example, we observed altered cortical and striatal 5-HT levels, which upon further consideration links to evidence of a contribution of prenatal 5-HT signaling in ADHD risk (80).

Our studies argue strongly that the DAT Val559 allele produces functional alterations in vivo and, as such, the animal represents the first construct-valid model of ADHD to our knowledge, although perhaps a better characterization is as a model of DA dysfunction underlying shared physiological substrates of ADHD, BPD, and ASD. With respect to ADHD, other mouse models derived from heritable mutations have been produced, but these models are based on mutations that produce other disorders where ADHD is comorbid (81). The spontaneously hypertensive rat (SHR) is often used as an ADHD model (82–84), although no clear genetic link to alterations found in subjects with ADHD has been presented. Other ADHD animal models, although displaying some of the behavioral features characteristic of the disorder, have not been based on heritable deficits identified in ADHD subjects (85). The DAT KO mouse is one such example (29). Although profound hyperactivity in a novel environment is a highly visible feature of this model (86), human subjects with two loss-of-function DAT alleles display a complex motor phenotype characterized by infantile hyperkinesia and dystonia and, when older, Parkinson's disease-like symptoms (40). The DAT Val559 model shares with the DAT KO an elevation in basal extracellular DA (29), yet lacks overt hyperactivity. These findings suggest that the hyperactive locomotor response to a novel environment observed in the DAT KO model is likely not a consequence of an elevation in extracellular DA per se.

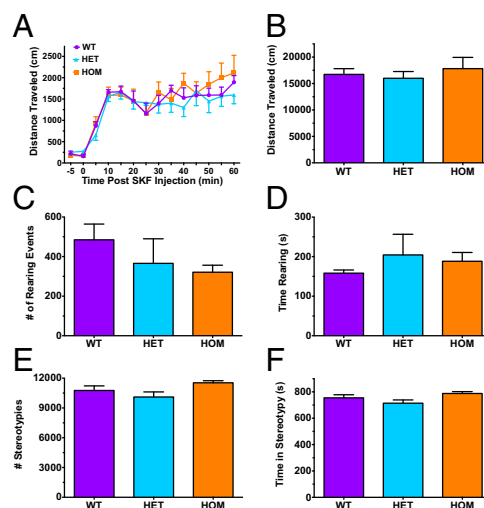


Fig. 8. DAT Val559 mice display normal motor behaviors following D1R agonist injections. (A and B) Horizontal locomotor activity following treatment with D1R agonist SKF83822 does not differ between DAT Val559 mice and WT controls [$n = 8$ per genotype; A, time course of SKF83822 (2 mg/kg i.p.) response; B, total $\pm$ SEM distance traveled in 60 min after SKF83822 administration]. (C and D) Lack of genotype effect on SKF83822-induced changes in rearing behavior ($n = 8$ per genotype; C, number of rearing behaviors $\pm$ SEM in 60 min; D, time spent rearing $\pm$ SEM). (E and F) Lack of genotype effect on SKF83822-induced changes in stereotyped behavior ($n = 8$ per genotype; E, number of stereotyped behaviors $\pm$ SEM in 60 min; F, time in stereotypy $\pm$ SEM in 60 min after 2 mg/kg SKF83822 treatment).

DAT Val559 nerve terminals (and transfected cells) exhibit WT rates of inward transport of DA, whereas DAT KO terminals fully lack such capacity. Other differences include a significant loss of tissue DA levels and reduced levels of TH, D1R, and D2R expression in the DAT KO that are not present in the DAT Val559 model. Together these findings point to important aspects of behavioral changes present in the KO model as derived from one or more compensations arising in the context of a full loss of the transporter.

Our findings that DAT Val559 demonstrate an absence of hyperactivity in the open field also raises the question as to whether the validation of rodent models of ADHD should be based primarily on motor hyperactivity and its reversal by psychostimulants, particularly when models are derived from insults absent from ADHD subjects. We were able to detect a context-dependent motor hyperreactivity, a behavior that we termed darting. The more robust escape response of DAT Val559 mice to imminent handling was sufficiently apparent to allow for blinded identification of genotype. Interestingly, darting behavior does not appear to represent a motor manifestation of general anxiety or an elevated startle reaction. Although using a different analytical platform, Kafkali et al. used the term darting to describe stable differences between strains in locomotor acceleration (87). Our findings with the DAT Val559 mice suggest that a contributor to the strain differences observed in darting behavior may arise from heritable differences in DA signaling. With respect to mechanism, the darting phenotype may derive from a deficit of cortical inhibition of DA release (88). Studies in humans with ADHD have demonstrated deficits in response inhibition (reviewed in refs. 89 and 90) that have been explained by using the “activation-suppression model” (91). According to the model, the motor response to a salient but irrelevant stimulus must be suppressed to respond to another relevant stimulus. In this context, the darting behavior may reflect an inability to suppress an escape response to imminent handling that the animals experience regularly during cage transfers, which therefore should be treated as a relatively, nonthreatening manipulation. Whether darting responses derive from the persistent elevation of striatal extracellular DA versus long-term changes in descending control of DA signaling, or both, requires further study. Additional analyses using cognitive tasks, including those that model response inhibition (92, 93), are also needed to explore these issues. DAT Val559 mice also demonstrated significantly reduced rearing compared with WT animals in the open field, when assessed under either basal conditions or following AMPH/MPH injections. Young et al. reported that D1R and D2R KO mice display reduced rearing behavior under basal conditions, similar to that seen after treatment with the stimulant modafinil (94), suggesting that the effects on rearing we observe could reflect a functional perturbation of DA receptor signaling independent of receptor density. Such a perspective should be further pursued, particularly given the evidence we present that both presynaptic and somatodendritic D2Rs demonstrate altered modulation of DA neuron function.

The key phenotypes found for DAT Val559 in transfected cells are ADE and a loss of AMPH-induced DA release (13, 58). Evidence of ADE *in vivo* arises from both our striatal microdialysis studies that demonstrate a significant elevation of basal extracellular DA levels and our findings of elevated presynaptic D2 modulation of vesicular DA release that can be normalized with D2R antagonist (raclopride) treatments. We also observed alterations in somatodendritic D2R responses that may reflect ADE occurring at the cell body level, with a prolonged duration of D2R-mediated IPSCs that may arise from a greater demand on DA clearance. Such an explanation seems reasonable in the context of a lack of genotype effects on midbrain DAT and D2R levels, maximal D2R-mediated current evoked by iontophoretic DA

application, and in the time course of GABA_B-mediated IPSCs. Although compensations related to other aspects of cell structure and excitability cannot be ruled out, the fact that both D2R and GABA_B couple to the same GIRK channels to induce DA neuron inhibition suggests that the intrinsic machinery for these responses is intact. In terms of AMPH response, our microdialysis studies revealed a significantly reduced capacity of DAT Val559 to support DA efflux *in vivo*. Retention of a small response to AMPH, in contrast to the complete loss seen with heterologous expression studies, is not unexpected given the ability of AMPH to continue to act as a competitive DAT antagonist. Consistent with this idea, we observed a more complete loss of AMPH effects in striatal slice studies. Under perfusion conditions, DAT has little access to released DA, and as such, the competitive antagonism of DAT by AMPH to diminish DA clearance would not be expected. We also observed a blunted AMPH response at the cell body level with the loss of sustained effects of AMPH on D2R IPSCs. We speculate that the appearance of this blunted response at late versus early time points may also derive from retention of the more rapid process of AMPH reuptake inhibition, which may predominate at earlier time points, versus loss of the slower, DAT-mediated DA release process (95), that may predominate at later times. The evidence we provide of tonic presynaptic D2R stimulation, and changes in somatodendritic D2R signaling, predict a secondary influence on vesicular DA release *in vivo* and its behavioral manifestations, whether triggered by agents such as MPH or evoked by environmental stimuli.

The presence of similarly altered phenotypes in our microdialysis and behavior studies for DAT Val559 heterozygous and homozygous animals reminds us that the affected subjects so far identified with the DAT Val559 variant are single copy carriers. Our *in vivo* functional studies in the context of the available human genetic data are consistent with a dominant effect of the variant. Possibly this dominant effect relates to the ability of ADE generated from one gene copy to restrict the efficiency of DA clearance from a WT copy sufficiently to trigger functional effects. Inappropriately inactivated or tonic leak conductances lead to dominant effects for a number of disorders, including epilepsy (96) and paralytic disorders (97). An alternative mechanism for dominant effects could involve DAT multimers of mixed Ala559 and Val559 partners, although to date these interactions have not been documented *in vivo* (98, 99).

In summary, the biochemical, pharmacological, and behavioral features of the DAT Val559 mouse corroborate our findings from heterologous expression studies and warrant further consideration of spontaneous DAT reversal as a pathway to risk for ADHD (and BPD and ASD). Prior interpretations of the utility of AMPH and MPH in ADHD treatment have oriented around their ability to elevate extracellular DA in basal ganglia and cortical circuits that support motor, reward, and cognitive function. Our model suggests that the clinical utility of these agents could arise in some individuals from a selective reduction of inappropriate, nonvesicular DA release rather than from simply an elevation of extracellular DA. PET imaging studies of unmedicated ADHD subjects have provided evidence of reduced DAT protein levels (100), findings that predict elevated basal extracellular DA levels either because of competition of elevated DA with *in vivo* antagonist binding to DAT or because of reduced DA clearance. Why then should DAT blockade or nonvesicular DA efflux, which should produce even greater extracellular DA levels, be therapeutic? One explanation is that the elevation of DA produced by these agents overcomes reduced sensitivity of postsynaptic DA receptors or enhances DA neuromodulation of other dysfunctional targets. Our findings raise the possibility that ADE “short circuits” excitation-secretion coupling, with ADHD medications returning control of DA signaling to

vesicular release mechanisms in exchange for reduced psychostimulant activation.

Methods

All biochemical and behavioral experiments were pursued under a protocol approved by the Vanderbilt University Animal Care and Use Committee and used male mice maintained on a hybrid background (~75% 129SvEvTac and ~25% C57BL/6J). Animals were placed on a reverse light cycle with a 12 h light:12 h dark schedule such that behavior and biochemical studies could be performed with animals during their normally active periods. For behavior and microdialysis studies where heterozygous animals were analyzed, animals were derived from matings of DAT Ala559/Val559 heterozygous parents. All other studies used homozygous Ala559

and DAT Val559 animals generated by homozygous parents of the respective genotypes established from initial crosses of heterozygous animals. All studies used male animals except where specified. For electrophysiological studies, animals were studied by following approved protocols at Oregon Health and Science University. Male and female mice (>26 d old) were used. All other methods used in this report are provided in *SI Methods*.

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