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Genetic targeting of the amphetamine and methylphenidate-sensitive dopamine transporter: On the path to an animal model of attention-deficit hyperactivity disorder

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Abstract

Alterations in dopamine (DA) signaling underlie the most widely held theories of molecular and circuit level perturbations that lead to risk for attention-deficit hyperactivity disorder (ADHD). The DA transporter (DAT), a presynaptic reuptake protein whose activity provides critical support for DA signaling by limiting DA action at pre- and postsynaptic receptors, has been consistently associated with ADHD through pharmacological, behavioral, brain imaging and genetic studies. Currently, the animal models of ADHD exhibit significant limitations, stemming in large part from their lack of construct validity. To remedy this situation, we have pursued the creation of a mouse model derived from a functional nonsynonymous variant in the DAT gene (*SLC6A3*) of ADHD probands. We trace our path from the identification of these variants to in vitro biochemical and physiological studies to the production of the DAT Val559 mouse model. We discuss our initial findings with these animals and their promise in the context of existing rodent models of ADHD.

Keywords

ADHD; Dopamine; Transporter; Transgenic; Mouse; Animal model

1. Introduction

Attention-deficit/hyperactivity disorder (ADHD) is the most commonly diagnosed neuropsychiatric disorder of childhood, affecting an estimated 4–12% of school-age children (Biederman and Faraone, 2005; Polanczyk et al., 2007; Willcutt, 2012). Adult ADHD is also fairly common, estimated at 4–5% of adults (Fayyad et al., 2007; de Graaf et al., 2008; Kessler et al., 2006). More recent studies suggest that the rates of adult ADHD may actually be greater than 10% (Cahill et al., 2012; Garnier-Dykstra et al., 2010). Indeed, though

ADHD is often considered a disorder of childhood and adolescence, studies suggest that ADHD symptoms persist into adulthood in 60–70% of cases (Biederman et al., 2000; Kessler et al., 2005). Like all neuropsychiatric disorders, ADHD presents with a spectrum of behavioral alterations, with features of motor hyperactivity, impulsivity, and/or inattention providing the diagnostic criteria used for diagnosis (American Psychiatric Association, 1994). There are no biomarkers for ADHD, and therefore diagnoses are based on clinical observation, as well as parent and teacher reports (Visser et al., 2013; Wolraich et al., 2003, 2013). ADHD diagnoses exhibit an ~3:1 male:female bias (Gaub and Carlson, 1997; Getahun et al., 2013). Whether this sex bias arises from cultural or biological factors (or both) that impact ADHD risk is unknown. Interestingly, rates of ADHD diagnosis are consistent among different cultural groups, as studies of populations in Africa (Bakare, 2012), Asia (Chien et al., 2012), and Europe (Bianchini et al., 2013; Ezpeleta et al., 2013) report similar prevalence and sex bias. These findings suggest that although environmental factors may be shared across communities, strong biological risk factors likely drive features and risk of the disorder and ultimately, diagnosis.

1.1. Support for a DA Connection to ADHD

A large body of research demonstrates that the dopamine (DA) system underlies the hallmark symptoms of ADHD. For example, locomotor hyperactivity, a main feature of ADHD as well as ADHD animal models, can be induced by treatment with a DA D1 receptor agonist (Brent, 1991; Dreher and Jackson, 1989; Tirelli and Terry, 1993) or psychostimulants that block DAT and increase synaptic DA concentrations (van Rossum and Hurkmans, 1964; Smith, 1964; Zubrycki et al., 1990). Conversely, depletion of DA with reserpine (Johnels, 1982; Sugita et al., 1989) or lesion of DA neurons with 6-hydroxydopamine (Erinoff et al., 1979; Joyce and Koob, 1981) or MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Colotla et al., 1990; Gnanalingham et al., 1995; Sahgal et al., 1984; Tsai et al., 1991) leads to a hypokinetic state. Similar hypokinetic characteristics are observed in Parkinson's disease (Morris et al., 1994; Sian et al., 1999), a brain disorder characterized by death of DA neurons in the substantia nigra.

In addition to locomotor hyperactivity, differences in the DA system have been reported to underlie impulsivity. Work in rodents (Puumala and Sirviö, 1998; Winstanley et al., 2005) and humans (Buckholtz et al., 2010) have demonstrated that differences in DA and DA receptor levels correlate with impulsive traits. Consistent with these observations, animal studies indicate that DA receptor antagonists can reduce impulsivity, whereas AMPH can increase impulsivity (Burton and Fletcher, 2012; Wade et al., 2000). With respect to human studies, striatal DAT (Costa et al., 2013a) and D2-like receptor (Ghahremani et al., 2012; Trifilieff and Martinez, 2014) availability correlate with impulsive traits in healthy human subjects, whereas treatment with DA receptor agonists increases impulsivity (Barake et al., 2013; Ondo and Lai, 2008; Voon et al., 2010). Consistent with these findings, genetic variation in a number of components of DA signaling have been associated with impulsivity (Dalley and Roiser, 2012; Forbes et al., 2009).

1.2. Support for a DAT connection to ADHD: pharmacological considerations

A major connection between DAT and ADHD is the utility of DAT-targeted agents for treatment of the disorder (Bitter et al., 2012; Vaughan and Kratochvil, 2012). Thus, both methylphenidate (MPH) (e.g. Ritalin[®]) and amphetamine (AMPH) formulations (e.g. Adderall[®]) are effective in the treatment of subjects with ADHD (Faraone et al., 2002; Janols et al., 2009; Minzenberg, 2012; Treuer et al., 2013). Psychostimulant treatment in ADHD is often labeled as “paradoxical”, since, as indicated by their categorical definition, ADHD medications, particularly *D*-amphetamine, can produce motor activation (Glick and Milloy, 1973; Ralph et al., 2001) and disruption of cognitive performance (Ornstein et al., 2000; Sanday et al., 2013; Stefani and Moghaddam, 2002), even psychosis (Bramness et al., 2012; Grant et al., 2012; Segal and Kuczenski, 1997; Wallis et al., 1949). Some researchers discount differences in response between ADHD and normal adolescents to psychostimulants, reporting that pre-pubertal adolescents respond to psychostimulants oppositely to that seen in adults (Rapoport et al., 1978; Zahn et al., 1980), and thus the “paradoxical” effect of psychostimulants in ADHD may be more a bias based on expectations from adult actions of the drugs. However, this position seems to be at odds with continued medication benefits seen by adults with the disorder. With respect to animal models, psychostimulant drugs increase locomotor activity in both adult and adolescent rodents (Cirulli and Laviola, 2000; Gainetdinov et al., 1999; Good and Radcliffe, 2011; Kameda et al., 2011). Whether a “normal animal” is an appropriate model for the actions of these drugs in ADHD is certainly debatable. As we discuss later in this report, psychostimulants decrease locomotor hyperactivity in a number of current animal models of ADHD (Gainetdinov, 2010; Russell, 2011). But such observations may be misleading if the underlying causes of ADHD are not mirrored in the model, leading to convergent behavioral characteristics that appear related to the human disorder but that may diverge when it with respect to utility in understanding ADHD mechanisms.

Support for a role of altered DA signaling also comes from the actions of ADHD medications. MPH is a competitive DAT antagonist like cocaine, though more slowly acting, whereas AMPH perturbs DA signaling through multiple mechanisms (Sulzer et al., 2005). First, AMPH is a competitive substrate for DAT, precluding normal DA clearance. Second, the drug acts to trigger depletion of vesicular DA stores by a weak-base action on the intravesicular pH gradient that is required to concentrate DA. Recently, this action has been reported to differentially impact readily releasable versus reserve pool vesicles (Covey et al., 2013), where depletion was observed to occur with a loss of DA from reserve pool vesicles and an enhancement of vesicular release involving the readily releasable vesicle pool. The latter observation may involve the ability of AMPH to elevate intracellular Ca^{2+} , known also to support the phosphorylation of DAT on the transporter’s N-terminus (Fog et al., 2006; Gnegy et al., 2004; Khoshbouei et al., 2004; Wei et al., 2007). Phosphorylation (evidence implicates CaMKII, and possibly PKC β) then leads to an increased probability for DAT-dependent efflux of cytoplasmic DA. Fourth, AMPH is an MAO antagonist, and through a lack of DA catabolism leads to further elevation of DA cytoplasmic levels.

1.3. Support for a DAT connection to ADHD: brain imaging studies

Positron emission tomography (PET) methods have afforded a direct inspection of DAT levels in the brain of human ADHD subjects (Varrone and Halldin, 2010; Zimmer, 2009). However, the findings with this approach have been mixed, possibly due to prior drug exposure in some studies (Fusar-Poli et al., 2012). Thus, whereas DAT binding in the basal ganglia of both children (Cheon et al., 2005) and adults (Dougherty et al., 1999; Dresel et al., 2000; Krause et al., 2000) has been reported to be increased (Spencer et al., 2007) in ADHD, others have seen no change (van Dyck et al., 2002) or decreased DAT density in ADHD (Volkow et al., 2007).

Subsequent studies have focused on brain imaging abnormalities within specific domains of ADHD. Volkow and colleagues correlate reduced DAT and D2-like receptor availability in ADHD subjects with motivational deficits stemming from dysfunction in dopamine reward pathways (Volkow et al., 2011). However, other groups report that increased DAT availability contributes to impulsivity (Costa et al., 2013a; Forbes et al., 2009). Studies examining DAT variability (namely the DAT 3' VNTR, discussed in Section 2.4) have similarly disparate findings, with a study suggesting an association between the DAT VNTR 10-repeat allele and frontal, medial, and parietal activation during a response inhibition task (Braet et al., 2011), and meta-analysis of SPECT studies reporting no effect of the DAT VNTR on striatal DAT availability (Costa et al., 2011).

Some of the conflicting reports of DAT availability in ADHD may reflect a differential role for DAT in specific ADHD traits. However, methodological limitations, including inadequate sensitivity and imaging ligands affected by endogenous DA (Weyandt et al., 2013), may restrict our ability to interpret imaging studies. Further research is required to improve DAT imaging approaches and clarify our understanding of DAT in ADHD.

1.4. Support for a DAT connection to ADHD: genetic studies

Many lines of evidence implicate DAT and DA receptors in ADHD. For example, genetic studies have repeatedly demonstrated an association between ADHD and D1 (Bobb et al., 2005; Ribases et al., 2012), D2 (Nyman et al., 2007), D4 (Roman et al., 2001; Bidwell et al., 2011) and D5 (Manor et al., 2004) receptors, though how the genetic variants perturb receptor function in ADHD is unclear. Several studies have also observed association between core components of ADHD and variation in the DAT gene, including spatial working memory function (Brehmer et al., 2009; Li et al., 2012; Shang and Gau, 2013), attentional asymmetry (Bellgrove et al., 2005; Newman et al., 2012), impulsivity (Costa et al., 2013a; Forbes et al., 2009; Paloyelis et al., 2010), response inhibition (Cornish et al., 2005; Cummins et al., 2012), and reward-related striatal responsivity (Hahn et al., 2011; Hoogmann et al., 2013; Wittmann et al., 2013). In addition, DAT genotype is linked to how well a patient responds to psychostimulant treatment (Costa et al., 2013b; Gilbert et al., 2006; Kambeitz et al., 2013; Pasini et al., 2013).

The DAT messenger RNA (mRNA) possesses a large, 3' untranslated region (UTR) that contains a variable number tandem repeat (VNTR) polymorphism (Vandenbergh et al., 1992a, 1992b) (Fig. 1). The DAT VNTR is a 40-base pair sequence that repeats 3 to 13

times, with 9- and 10-repeats being the most common in human populations (Doucette-Stamm et al., 1995; Gerlernter et al., 1998; Kang et al., 1999). Multiple studies have found a link between the 10-repeat VNTR allele and ADHD (Faraone et al., 2013; Kambeitz et al., 2013), however this finding has not been replicated in all studies (Holmes et al., 2000; Palmer et al., 1999; Todd et al., 2001). Interestingly, recent studies have linked the 10-repeat VNTR allele to risk for childhood ADHD and the 9-repeat VNTR allele with persistent adult ADHD symptoms (Dresler et al., 2010; Franke et al., 2010).

1.5. Support for a DAT Connection to ADHD: search for functional coding variation

Although DAT plays a significant role in normal DA signaling, is a prominent drug target, and has been repeatedly implicated by genetic association studies in disorders such as ADHD, only limited efforts have sought evidence for functional coding variation associated with the DAT gene (*SLC6A3*) that could contribute to ADHD risk. Thus far, several studies have sought evidence for *SLC6A3* coding polymorphisms, identifying multiple subjects both heterozygous (Cargill et al., 1999; Grünhage et al., 2000; Hamilton et al., 2013; Mazei-Robison et al., 2005; Mergy MA and Blakely RD, unpublished data; Puffenberger et al., 2012; Sakrikar et al., 2012; Vandenberg et al., 2000), and homozygous (Kurian et al., 2009, 2011) for nonsynonymous variants. Screening of ADHD subjects for new DAT coding variants is also ongoing in various diseases including bipolar disorder and autism with comorbid ADHD (Davis and Kollins, 2012; Mahajan et al., 2012; Rommelse et al., 2011). Disease-associated DAT coding variants are listed in Table 1 and shown on the DAT structure in Fig. 2. Other heterozygous coding variants that have been characterized for functional deficits (V24M, V55A, R237Q, and E602G) have yet to demonstrate changes in transporter protein expression or DA transport function *in vitro*. V382A DAT, which has yet to be mapped to a specific clinical phenotype, demonstrated upon *in vitro* expression a reduction in transporter protein levels, reduced capacity for both DA transport, and a reduced capacity for phorbol ester (PMA)-induced trafficking (Mazei-Robison and Blakely, 2005). The authors suggest that DAT Ala382 stabilizes an inactive conformation in the plasma membrane such that the loss of uptake exceeds that expected by PKC-induced internalization. Sakrikar and colleagues identified the variant R615C in an ADHD subject and found the Cys615 variant to display a significant reduction in cell surface DAT levels (approximately 50% of WT DAT) (Sakrikar et al., 2012), a commensurate reduction in DA transport velocity, and a shift in surface distribution from regulated to constitutive recycling. Accompany these changes was an insensitivity to the endocytic acceleration of DAT endocytosis by AMPH or protein kinase C (PKC) activation. The constitutive endocytic phenotype of DAT Cys615 was accompanied by hyperphosphorylation, increased association with calcium/calmodulin-dependent protein kinase II (CaMKII), decreased flotillin-1 association, and a disrupted localization to GM1 ganglioside-enriched membrane microdomains. These findings led Sakrikar and colleagues to propose a model whereby the distal DAT C-terminus dictates targeting to surface membrane microdomains where the transporter acquires its post-translational regulatory influences.

Recent advances in whole-exome and whole-genome sequencing (1000 Genomes Project Consortium, 2010) have resulted in a plethora of new *SLC6A3* nonsynonymous variants. Since these exome screening projects target either subjects without a clinical diagnosis or

community samples with no available clinical history, variants identified are generally not associated with a particular disorder. Reported DAT coding variants (D = found in dbSNP database, G = found by 1000 Genomes, N = found in National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project) without known disease association are as follows: K3N (D, G, N), M11I (D, G, N), M11V (D, G), S12P (D, G, N), V14M (D, G), A16T (G), P17L (G,N), E20(stop) (D), E20V (G, N), I32M (G, N), V24A (G), G39R (G), L42F (D, G), P50L (D, G, N), S53R (D, G, N), V73I (D, G, N), L104I (D, G, N), G121S (G), V131I (D, G), L138P (G), L138R (G), A161T (G), A163V (G), A192T (D, G), S198T (G), S202L (D, G), S202W (D, G, N), G209R (G, N), V221M (D, G, N), R237W (G, N), V245A (D, G), I268V (G, N), T271N (G), V275L (D, G, N), L281P (G), G289R (G), G293S (G), V300I (D, G, N), E307K (D, G), A308V (G), A314V (G), D345G (G, N), A346T (G, N), A346V (D, G, N), F362L (G), L368Q (G), Q373R (D, G, N), G380R (G, N), P395L (G) G433R (G), D436N (G), R445G (G), A455V (G, N), V464I (D, G, N), V471I (D, G), I490V (D, G, N), V501A (G, N), Q509H (D, G), R515W (G), S517T (G), G538A (D), V538I (G, N), R544S (G), P545T (G, N), H547Q (D, N), A559T (D, G), A576E (G), K579R (G, N), R588Q (G, N), G607W (G), R610H (D, G), T613M (G, N), K619N (D, G, N). The reader is referred to the 1000 Genomes website (<http://www.1000genomes.org>) for information regarding the estimated frequencies of *SLC6A3* variants, which generally are quite low, consistent with the existence of only rare nonsynonymous variation in the genes of the *SLC6* transporter family. Interestingly, of the variants in DAT identified in disease-targeted studies, V24M, L167F, R237Q, A346V, V464I, A559V, and E602G are present in these whole genome efforts, though with no clinical information. Given the frequency of mental illness, both diagnosed and undiagnosed, it is likely that a significant fraction of the carriers of these variants meet criteria for a DSM-IV disorder, including ADHD.

1.6. Support for a DAT connection to ADHD: DAT Val559 and anomalous DA efflux

Our efforts to identify functional variation in DAT that impact risk for brain disorders originated with a screening of subjects diagnosed with ADHD for variations in DAT coding sequences and *SLC6A3* exon splice junctions (Mazei-Robison et al., 2005). These efforts identified A559V in a pair of brothers, both diagnosed with ADHD. Interestingly, the DAT Val559 variant had been identified previously in a female diagnosed with bipolar disorder, though information related to the mode of transmission or shared traits could not be pursued in the parents (Grünhage et al., 2000). Given the association of DAT with both ADHD and bipolar disorder (Greenwood et al., 2013), it is possible that DAT Val559 variant may have contributed to this subject's disorder.

Heterologous expression studies revealed that DAT Val559 supports comparable total and cell surface DAT protein expression, as well as DA transport kinetics, as DAT Ala559. However, the variant transporter induces an anomalous, DAT-mediated, outward "leak" of cytoplasmic DA when cells are pre-loaded with DA, which we have termed anomalous DA efflux (ADE) (Fig. 3A). The ADE of DAT Val559 was shown to be voltage-dependent, with DA efflux more prominent at depolarized membrane potentials (Fig. 3B). AMPH has been shown to trigger a rise in intracellular Na⁺ that contributes to the mechanism supporting DA efflux (Khoshbouei et al., 2003). In DAT Val559 transfected cells, the transporter's sensitivity to intracellular Na⁺ increases, providing one mechanism by which ADE may be

facilitated. Surprisingly, we found that AMPH lacked an ability to induce DA efflux in DAT Val559-transfected cells, instead acting only as a DAT antagonist, blocking ADE (Fig. 3C). These findings led to the development of a synaptic model for the ADHD of Val559 carriers, described in Figs. 4A-C. As illustrated, at wild-type (WT) DAT Ala559 containing synapses, DA is released by fusion of synaptic vesicles, triggered by afferent electrical stimulation (Fig. 4A) followed by efficient DAT-mediated DA uptake that inactivates DA signaling. In contrast, at DAT Val559 expressing synapses, ADE exists in parallel with vesicular DA release, mimicking the affects of AMPH on WT synapses, and leading to a loss of a tight coupling of DA release to afferent input (Fig. 4B). Although psychostimulant treatment reduces DA clearance by blocking DAT, this interaction also prevents ADE, restoring a more normal relationship between DA neuron activity and extracellular DA levels (Fig. 4C). This mechanism also provides an explanation for why only a single Val559-encoding transporter could lead to ADE and elevate risk for ADHD.

Subsequent studies of the DAT Val559 variant (Bowton et al., 2010) revealed that ADE is sustained by tonic D2 receptor (D2R) signaling, as well as by CaMKII action. CaMKII-mediated phosphorylation of the WT DAT N-terminus is known to support reverse transport of DA in response to AMPH treatment (Fog et al., 2006). In keeping with a role for CaMKII in supporting ADE, Bowton and colleagues found that the N-terminus of DAT Val559 to be hyperphosphorylated (Fig. 5) (Bowton et al., 2010), suggesting that the DAT Val559N-terminus is more accessible to CamKII, consistent with elevated Val559 association with the kinase, and the ability of CamKII inhibitors to block ADE. Finally, the DAT Val559 variant induces an increase in outward channel states, previously associated with AMPH action (Kahlig et al., 2005). These studies support a model whereby DAT Val559 exhibits an abnormal residence of the transporter in an “efflux-willing” state as if being constantly stimulated with AMPH.

Recently, Hamilton and colleagues characterized a functional *SLC6A3* variant, T356M, identified in a subject with autism spectrum disorder (ASD) (Hamilton et al., 2013). Key to the findings is the *de novo* status of the variant. ASD has been found to exhibit an increased number of *de novo* variants (Kenny et al., 2013; Jiang et al., 2013), including copy number variants (CNVs) (Griswold et al., 2012; Holt et al., 2012; Krumm et al., 2013; Menashe et al., 2013; Poultney et al., 2013; Vaishnavi et al., 2013) as well as single nucleotide polymorphisms (SNPs). Because *de novo* coding mutations are low in probability, particularly in the coding regions of genes, geneticists consider *de novo* variations to be of particular significance in establishing genetic risk. Approximately one third of subjects with ASD meet DSM-IV criteria for ADHD and subjects with ADHD are more likely to have ASD traits than found in the general population (Kotte et al., 2013; Leyfer et al., 2006). Functional studies indicate that the DAT Met356 variant exhibits significantly reduced DA uptake, as well as ADE. Structural evidence suggests that the variant may induce a more prominent outward-facing conformation which may be more conducive to ADE. Finally, in keeping with the reduced activity of DAT Met356, the mutant fails to reverse the hyperactivity of flies that lack DAT.

Our discovery of two cases of ADHD-associated, functional variation in DAT (Val559 and Cys615), along with several other variants being characterized, as well as the recent

discovery of DAT Met356, provides both rationale and opportunity for the generation of construct-valid, transgenic mouse models of ADHD. Below, we describe our initial efforts to capture this opportunity using the DAT Val559 variant, followed by a discussion of our efforts in the context of existing rodent models of ADHD.

2. Materials and methods

2.1. Generation of DAT Val559 knock-in mouse

All procedures with animals were performed according to a protocol approved by the Vanderbilt Institutional Animal Care and Use Committee (IACUC). Transgenic mice were produced using a linearized construct, where 5' and 3' arms were derived from 129S6 genomic DNA that was mutated in the 5' arm to encode Val at amino acid 559 by oligonucleotide mediated site-directed mutagenesis (Stratagene Quik-Change Mutagenesis Kit, Agilent Technologies, Santa Clara, CA). Our construct includes self-excising Cre recombinase and neomycin-resistance cassettes for positive selection and a thymidine kinase cassette for negative selection. The construct was electroporated into 129S6/SvEvTac-derived embryonic stem cells (TL-1) and successful homologous recombination was confirmed by Southern blotting, with the presence of the Val559 substitution confirmed directly by Sanger sequencing. Targeted stem cells were then injected into C57BL/6J blastocysts and implanted in pseudo-pregnant females. Chimeric offspring were mated with WT C57BL/6J mice to test for germline transmission of the DAT Val559 allele. Thereafter, genotypes of all mice were determined by PCR of genomic DNA isolated from tail snips using the REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, MO) using as forward primer, CAG CAT GGA AAA AAT CCA TGA A and as reverse primer, AGC TAT ATT CAC CAT CAA AAG G. Products generated in this analysis are WT, 490 base pair (bp), homozygous Val559, 561 bp product and both 490 and 561 bp products for heterozygous animals. A male founder was then crossed with a 129S6 female to generate the initial colony for breeding. Mice were maintained under standard housing conditions on a 12-h light/dark cycle (lights on 0600–1800 for breeding colony; lights off 0200–1400 for all animals used in experiments) with food (irradiated LabDiet 5L0D chow, PMI Nutrition International, St. Louis, MO) and water provided *ad libitum*.

2.2. Assessment of growth and reproduction of DAT Val559 mice

To assess growth, male mice of the three genotypes were weighed weekly from 3 to 12 weeks of age. Data were plotted and analyzed statistically by a two-way analysis of variance (ANOVA) using Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA), with $P < .05$ taken as indication of statistical significance. To assess reproduction, the number and sex of pups generated were tabulated over a course of 33 months. All animals studied derived from heterozygous breedings (75% 129S6, 25% C57BL/6J). Assessment of genotype and gender distributions were compared to expectations derived from Hardy–Weinberg equilibrium using χ^2 tests, implemented in Prism 5.0 software, with $P < .05$ taken as indication of statistical significance.

2.3. Analysis of sensorimotor function in DAT Val559 mice

To assess sensorimotor function in the DAT Val559 mice, we implemented a modified version of the Irwin test battery (Irwin, 1968). Mice were observed and recorded over 2 consecutive days during the light phase (1600–2000).

2.3.1. General physical condition—The presence of whiskers, quality of fur, general coat condition, bald patches, piloerection, limb tone, and body tone were observed in a clean cage containing corncob bedding.

2.3.2. Motor abilities and reflexes—*Trunk curl*: Each mouse was lifted up 12–18 inches by the base of the tail followed by assessment of the presence of nose-to-tail curling. *Forepaw reaching*: Each mouse was lifted by the base of the tail and moved horizontally towards a metal wire food bin/cage insert. The degree to which the mouse extended its forepaws as it approached the wire food bin was scored (no reaching = 0, reaching upon nose contact = 1, reaching upon whisker contact = 2, reaching before whisker contact (18–20 mm) = 3, early, vigorous reaching (>25 mm) = 4). *Wire hang*: Two to four mice were placed on a metal grid screen (10 × 14 cm) into individual compartments. After placement, mice were given time to establish a grip on a wire screen before it was inverted 60 cm over a clean plastic cage containing fresh corncob bedding. The latency to fall was recorded up to 60 s, at which point the mice were removed from the apparatus and returned to the home cage. *Positional passivity*: Each mouse was subjected to sequential handling and the reaction to handling assessed. Mice were first restrained by the tail (score = 0), then gently restrained by the neck (score = 1), restrained by the nape of the neck (scruffed) and held supine (score = 2), and finally restrained by the hind legs (score = 3). Data are presented as the number of mice showing any positional passivity. *Rotarod*: Motor coordination and balance were assessed using an accelerating Rotarod apparatus (Ugo Basile, Comerio VA, Italy). Mice were placed on the rotating cylinder (3 cm in diameter) and confined to a segment of the cylinder approximately 6 cm wide by gray plastic dividers. The rotational speed of the cylinder was increased from 5 to 40 rpm over the 5-min testing period. The latency at which mice fell off of the rotating cylinder was measured. Each mouse was given a trial on the Rotarod before performance was assessed. *Grip strength*: Grip strength was measured using a force gauge attached to a small metal grate (8 × 8 cm). Each mouse was allowed to grip the metal grate with its forepaws, then gently pulled backwards by the base of the tail until it released the grate. Grip strength was recorded with a digital gauge that returned the maximum force during each trial. The average grip strength, given in Newtons, is the average of three trials. *Righting reflex*: Mice were inverted to a supine position in the researcher's hand. The mouse was released and the ability of the mouse to right itself assessed. *Air righting reflex*: Mice were inverted to a supine position in the researcher's hand while being held approximately 30 cm above a cage containing 8–10 cm of clean corncob bedding. Mice were then released and allowed to drop into the cage and bedding below. The ability of the mouse to right itself while falling was assessed. Data for all measures of motor function were analyzed using a one-way ANOVA and Bonferroni's *post hoc* test for multiple comparisons, with $P < .05$ taken as indication of statistical significance.

2.3.3. Sensory testing—Ear twitch: Mice were gently restrained and the auditory meatus gently touched with the tip of a size 6.1 Touch Test (Von Frey) Sensory Evaluator (Stoelting, Wood Dale, IL). The presence of a reaction (active retraction and/or flick of the ear) was assessed. *Petting escape:* Mice were stroked down the length of the body starting with a light touch and gradually increased pressure as the researcher approached the mouse's tail. The escape reaction was assessed as follows: no response (score = 0), mild (escape from firm pressure; score = 1), moderate (escape from light pressure; score = 2), or vigorous (escape from approach; score = 3). Data are presented as the number of mice showing an escape response. Data for all measures of sensory function were analyzed using a one-way ANOVA and Bonferroni's *post hoc* test for multiple comparisons, with $P < .05$ taken as indication of statistical significance.

3. Results

3.1. Generation of DAT Val559 mice

In order to express the DAT Val559 variant from the endogenous mouse *Slc6a3* locus, we pursued a homologous recombination, knock-in strategy with a construct bearing an altered codon encoding the Val substitution in exon 13 as described in Materials and Methods, along with a self-excising loxP-flanked Cre recombinase and neomycin resistance (Neo^R) cassettes in the 3' intron (Fig. 6A). Following the screening of embryonic stem cell clones that passed both positive and negative selection by Southern blots, we identified two clones that appeared to be successfully targeted. Subsequent sequencing verified that one of the two stem cell clones possessed the sequence encoding DAT Val559 and no evidence of other coding mutations.

DAT Val559-positive stem cells were injected into blastocysts, producing four chimeric males. Three of the chimeras achieved germline transmission, as assessed by genotyping of pups (Fig. 6B). One of the chimeras positive for germline transmission of both the Val559 variant as well as Neo^f excision was selected to establish a breeding colony. We also backcrossed to C57BL/6 and 129S6 strains to generate congenic lines. (data not shown). All experiments to date have been performed with animals maintained on a 75% 129S6/25% C57BL/6 hybrid background.

3.2. Breeding of DAT Val559 mice

Considering 514 separate breeding events of heterozygous DAT Val559 parents, we observed 5.2 ± 0.1 pups per litter, assessed at 3 weeks of age when animals were weaned and genotyped. This value is in line with the categorization of 129 strains as good breeders with three to seven pups per litter (<http://jax-mice.jax.org/support/husbandry/breeding-considerations.html>). When the genotypes of male and female offspring within these litters were pooled, a significant deviation from Hardy–Weinberg distribution was detected (χ^2 test: $P < .05$). This effect did not arise from an alteration in the genotype distribution of females ($P > .05$). Rather, males drove the effect, demonstrating a significant overall deviation ($P < .05$) arising from a significant reduction in the presence of homozygous Val559 males (Table 2).

3.3. Growth of DAT Val559 mice

After weighing male mice weekly from 3 to 12 weeks of age, we assessed the growth relationships between WT, heterozygous and homozygous DAT Al559 mice. No significant genotype, time, or genotype \times time interactions were observed ($P > .05$, two-way ANOVA) (Fig. 6C). Similarly, no genotype effects were evident in comparing body length (rump to nose, cm): WT, 8.10 ± 0.39 ($n = 10$); het, 8.36 ± 0.23 ($n = 11$); homo, 8.10 ± 0.32 ($n = 10$).

3.4. Sensorimotor characteristics of DAT Val559 mice

General sensorimotor assessment of mice at 7 weeks revealed no significant genotype differences in health, reflexes or basic motor functions (Table 3). Although the overall ANOVA did not detect a genotype difference in time to fall in the wire hang test, this measure displayed a trend for a progressive genotype decrease in latency, with an ~50% reduction occurring in homozygous DAT Val559 vs. WT animals ($P = .06$, unpaired Student's *t*-test).

4. Discussion

Our successful generation and breeding of the DAT Val559 mice indicates that the introduction of the variant results in viable offspring that grow normally. In contrast, DAT KO mice demonstrate reduced weight gain and survival (Giros et al., 1996). These findings likely speak to the relatively conservative nature of the Val for Ala substitution, as compared to full elimination of DAT. DAT is expressed in the mouse during embryonic development (Chung et al., 2012) and full loss of the transporter leads to profound changes in a number of biochemical and physiological processes including more than a 90% reduction in tyrosine hydroxylase (TH) expression (Jones et al., 1998), as well as significant reductions in D2R mRNA levels (~50%) (Giros et al., 1996) and protein expression (~55%) (Jones et al., 1999). DAT KO mice also demonstrate a reduction in striatal volume that includes a fewer number of neurons (Cyr et al., 2005), as well as a loss of dendritic spines in proximal portions of dendrites (Berlanga et al., 2011). Although the profound hyperactivity evident in the DAT KO model (Giros et al., 1996) initially suggested that these animals might prove a relevant ADHD model, recent findings indicate that humans homozygous for DAT loss-of-function alleles display a complex motor phenotype characterized by infantile dystonia and Parkinsonian symptoms (Kurian et al., 2011). Interestingly, during early postnatal life, these subjects display hyperkinetic features characterized by twitching and frequent limb movements. These observations suggest that the DAT KO mice may be a useful model for a movement disorder rather than ADHD. Additionally, these studies indicate that hyperactivity may be a misleading expectation for animal models of the latter disorder.

Although we did not note differences in the growth profiles of the DAT Val559 mice, our measures were confined to ages post-weaning, after our animals were genotyped. Thus, an impact of the variant on embryonic growth or early neonatal survival could have been missed. This point may relate to our finding of a small, but significant reduction in the number of males homozygous for the Val559 variant. How this reduction could arise is a matter of speculation, though we note that sex-specific differences in the expression of genes that determine a dopaminergic phenotype, including DAT, have been detected in the

mouse (Tao et al., 2012). A further investigation of this issue may be important given the reported male-bias in ADHD diagnoses (Gaub and Carlson, 1997; Getahun et al., 2013). We also note that this change is only evident in homozygous DAT Val559 mice whereas the two boys from whom we identified the allele are heterozygous carriers of the Val559 allele (Mazei-Robison et al., 2005). Genetic background can have a profound impact on the penetrance of modifications introduced into the mouse genome (see Kerr et al., 2013, for a recent example), and are expected for modest alterations that modulate risk versus determinant genetic changes. In relation to our efforts, we also note that a number of the phenotypes of the DAT KO mice vary by genetic background (Morice et al., 2004). As such, one cannot always predict whether phenotypes will emerge from heterozygous or homozygous animals, nor whether all traits will be exhibited on all genetic backgrounds.

In keeping with the relatively benign influence of the DAT Val559 variant, no significant changes were noted in initial sensorimotor tests. Certainly, future studies should examine these aspects in greater depth, particularly given the role of DA in basal ganglia control of motor function and in specific sensory modalities, such as the initial stages of visual perception (Feldkaemper and Schaeffel, 2013; Reis et al., 2007; Witkovsky, 2004; Xi et al., 2002). We did detect a trend toward a genotype-dependent reduction in latency to fall in the wire hang test. A reduction in latency is generally taken as a sign of motor weakness, though we saw no reductions in performance on the grip strength test. The wire-hang test however is likely more taxing than the grip strength test given that the animal is asked to support the full weight of the body. Alternatively, DAT Val559 animals may simply lack motivation for performance in this test, something that obviously the basic measures implemented to date do not assess. Other tests that have been reported to measure “behavioral despair” such as the Forced Swim Test or the Tail Suspension Test, may provide further insights.

The absence of gross changes in growth or sensorimotor function lays an important foundation for future tests of these animals which involve cue detection and motor engagement. These tests are often utilized to assess face and predictive validity of rodent disease models. Many such ADHD models with face and/or predictive validity have been advanced (Table 4), though none to date derive from genetic variation present in ADHD subjects, as introduced with the creation of the DAT Val559 model. Our *in vitro* studies with the DAT Val559 variant predict a reduction in sensitivity to AMPH given that the psychostimulant loses the ability to produce DAT-dependent, DA efflux. A number of the prior animal models display a reduced sensitivity to AMPH as well as MPH. We do not expect to lose sensitivity to AMPH completely, as the ability of AMPH to antagonize DA uptake is retained (Mazei-Robison et al., 2008), as well as the compound’s MAO inhibitory actions, should be intact. MPH sensitivity relies on vesicular DA release. The degree to which vesicular DA release or postsynaptic DA responses are impacted *in vivo* cannot be inferred from in the *in vitro* models we have used to date to characterize DAT Val559. It would not be surprising therefore to find that *in vivo* DA ADE could lead to compensations in vesicular DA release, such that the psychostimulant properties of MPH are also altered.

One of the most widely studied rodent models of ADHD is the spontaneously hypertensive rat (SHR). Originally derived from selective breeding of Wistar rats (Okamoto and Aoki, 1963) and used to study essential hypertension (Yamori, 1977), researchers soon recognized

the spontaneous hyperactivity of SHRs (McCarty and Kopin, 1979). Investigators then demonstrated that AMPH (Myers et al., 1982) and MPH (Wultz et al., 1990) reduce the motor hyperactivity of the SHRs, versus triggering motor activation, often denoted as a central feature of the psychostimulant response of subjects with ADHD. Further studies have demonstrated increased impulsivity (Fox et al., 2008; Sagvolden et al., 1992; Sagvolden, 2011), impaired attention (Sagvolden and Xu, 2008; Sagvolden, 2011), and cognitive deficits (Brackney et al., 2012; Clements and Wainwright, 2006; Kantak et al., 2008). Accordingly, the SHR has been lauded as the best validated model of ADHD (Sagvolden, 2011). However, as no genetic alterations have been identified that account for ADHD-like traits in SHR animals, the model cannot be said to possess construct validity. Additionally, although the SHR model displays a number of ADHD-like characteristics, these traits are not all improved by psychostimulant treatment. Moreover, although psychostimulants improve SHR performance on attention tasks in some studies (Kantak et al., 2008; Sagvolden, 2011) results of other studies are discordant (van den Bergh et al., 2006; Ferguson et al., 2007). Similarly, although AMPH typically reduces locomotor hyperactivity in the model (Sagvolden and Xu, 2008), the drug has been shown to potentiate locomotor behavior in some studies (Calzavara et al., 2011). Finally, SHRs display deficits in sensorimotor gating, as measured by pre-pulse inhibition tests (Levin et al., 2011; Li et al., 2007) Humans with ADHD, however do not typically display this phenotype (Feifel et al., 2009; Hanlon et al., 2009; Holstein et al., 2011), unless the task requires sustained attention (Hawk et al., 2003). The PPI deficits observed in SHRs suggest that these animals could be a better model for schizophrenia than ADHD (Levin et al., 2011).

Another concerning aspect of the SHR model of ADHD is the lack of evidence that subjects with ADHD demonstrate a propensity for hypertension. One study did identify such an association, but when data were adjusted for body mass index (BMI), this effect was eliminated, indicating that the main relationship in the study was one between ADHD and obesity (Fuemmeler et al., 2011). Furthermore, neither hypertension nor hypotension arise with chronic psychostimulant treatment of ADHD subjects (Vitiello et al., 2012), adding further concern that a model with both cardiovascular and cognitive features fails to pass a test of face validity.

Having established the DAT Val559 model, what *in vivo* phenotypes may be predicted from *in vitro* studies? As noted above in our discussion of DAT KO animals, overt hyperactivity in a mouse model likely signals a profound disruption of DA signaling such as occurs with complete loss of the transporter. *In vitro* studies indicate that although DAT Val559 exhibits ADE, the variant supports normal total and surface expression, as well as DA recognition and uptake (Mazei-Robison et al., 2008). How, in a rodent, this combination of functional properties plays out in terms of biochemical, physiological and behavioral changes can only be determined empirically. Even ADE, the most conspicuous phenotype of the Val559 variant in transfected cells is capable of being suppressed by alterations in D2 DA receptor and CamKII signaling (Bowton et al., 2010), which could well occur as a compensation to constitutive DA efflux. If such compensations do not occur, reasonable *in vivo* phenotypes include an elevation in basal extracellular DA levels and a lack of AMPH-induced DA efflux in the context of normal DAT protein expression. Elevations in extracellular DA may

be derived from both ADE and elevated vesicular DA release if presynaptic D2 DA receptors that normally limit vesicular release become desensitized by maintained, high levels of synaptic DA. Our preliminary *in vivo* microdialysis and brain slice release studies are consistent with these ideas (Mergy et al., 2012).

Beyond constitutive elevations in extracellular DA and a reduction in AMPH-evoked DA efflux, predictions of circuit and behavioral level phenotypes in the DAT Val559 model are more conjectural and await experimental evaluation. No model generated to our knowledge provides for elevations in extracellular DA throughout development in the context of normal DA uptake capacity. Thus, whether these animals should exhibit overt hyperactivity is unclear, though it seems likely that such behavior will be milder than that seen in the DAT KO, if evident at all. Similarly, the degree of impulsivity and inattention evident in these mice cannot be predicted. However, it seems reasonable to consider that a constitutive elevation in extracellular DA will reduce the dynamic range across which DA release can signal changes in presynaptic excitation and may therefore reduce cue saliency (Gowrishankar et al., 2013). At a behavioral level, a diminished dynamic range of DA signaling may lead to inattention, poor reward recognition, and diminished motivation. In such a context, restlessness and impulsivity may arise as subjects fail to constrain behavior as a result of learning the connections between appropriate response patterns and reward. Finally, we anticipate that constitutive changes in DA signaling will perturb molecular networks that support synaptic structure, strength and plasticity. Although a demonstration of behavioral alterations that mimic changes seen in ADHD subjects appears, on the surface, a test of the relevance of an ADHD animal model, we suspect that we will more often than not find difficulty with aspects of face validity, owing to differences in the mouse and human brain. Perhaps a better test of the value of the DAT Val559 model is the degree to which these animals afford insights into pathophysiological mechanisms, subsequently subjected to testing in human subjects. Although the DAT Val559 variant is rare, we propose that some ADHD subjects exhibit ADE derived from changes not in DAT, but in the cellular signaling pathways that insure a biased inward flow of DA across the transporter at the presynaptic membrane.

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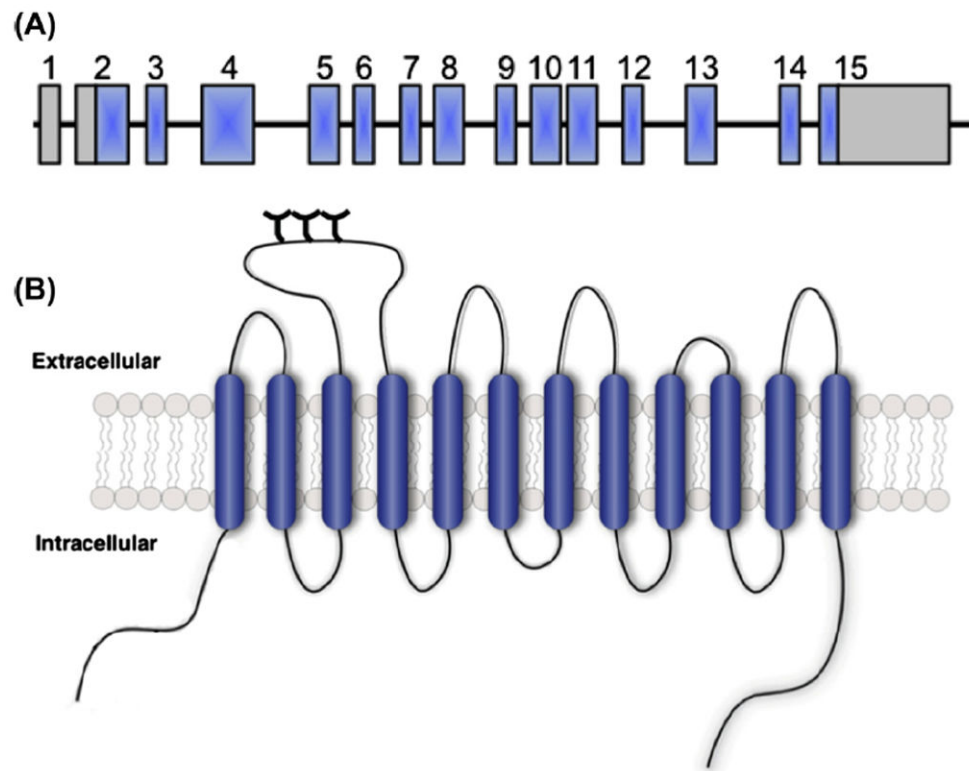


Fig. 1. Gene and protein structure of the *SLC6A3* gene encoding DAT. (A) The *SLC6A3* gene consists of 15 exons (blue boxes), spanning 60 kb of genomic DNA sequence, with both 5' and 3' untranslated regions (gray boxes). Please note, gene structure is not depicted to scale. (B) Schematic representation of the DAT protein, a 620 amino acid protein with 12 transmembrane domains and intracellular N- and C-termini. Figure reproduced from Mazei-Robison and Blakely, 2006.

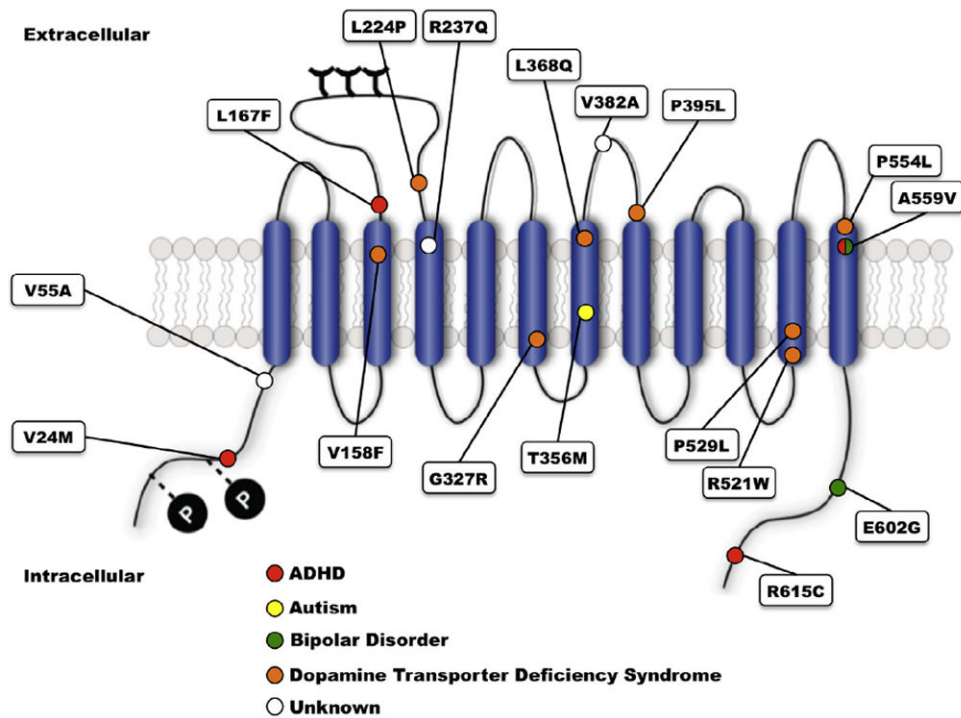
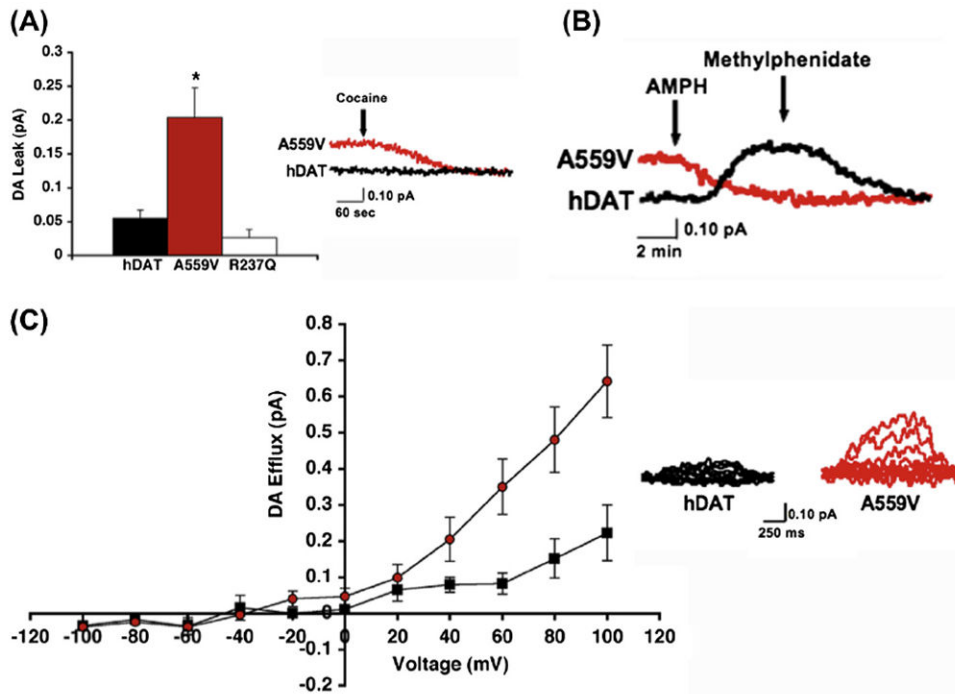


Fig. 2. Location of disease-associated hDAT variants. ADHD-associated variants are marked with red circles, ASD-associated variants with yellow circles, bipolar disorder-associated variants with green circles, DAT deficiency syndrome-associated variants with orange circles, and variants without disease association with white circles.

**Fig. 3.**

The hDAT A559V mutation supports anomalous DA efflux. (A) DAT Val559 mediates enhanced outward DA leak, reported as mean amperometric current \pm SEM. (B) DAT Val559-transfected cells display enhanced voltage-dependent DA amperometric currents; *left*: mean amperometric current \pm SEM, *right*: representative amperometric traces from WT DAT and DAT Val559 cells. (C) Representative amperometric trace showing that AMPH-evoked DA release can be blocked by methylphenidate in WT DAT-transfected cells (black trace), whereas AMPH blocks basal DA efflux in DAT Val559-transfected cells (red trace). No further reduction in amperometric current is observed upon application of MPH with AMPH. Figure reproduced from Mazei-Robison et al., 2008.

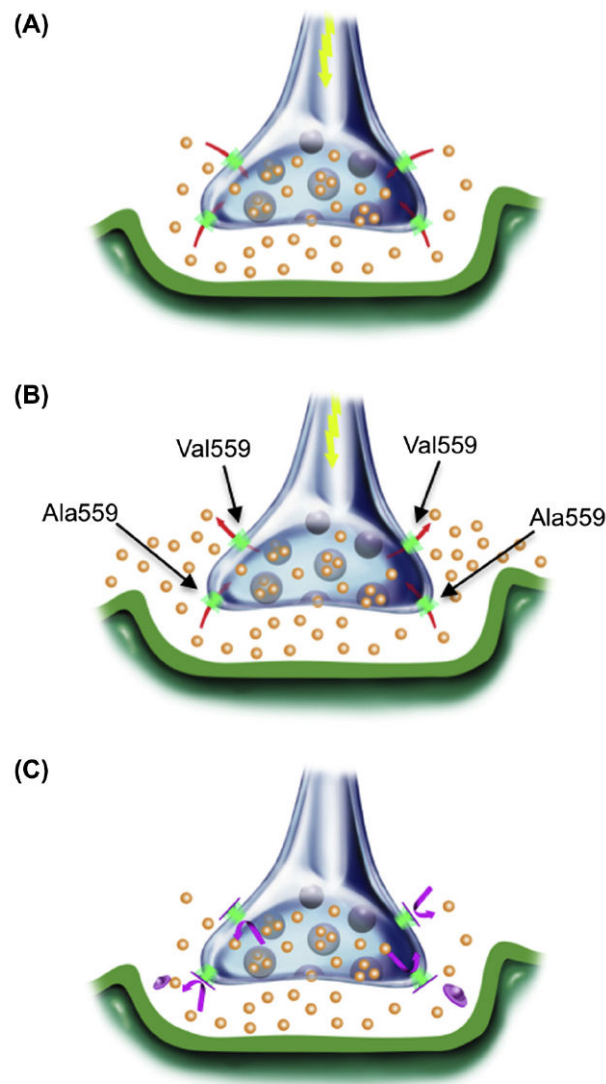


Fig. 4. Illustration of disrupted synaptic transmission as a result of DAT-mediated ADE in ADHD subjects (A) Under normal circumstances, presynaptic DAT (green) inactivates and recycles DA (orange) released via vesicular fusion. (B) AMPH (red) acts both as a DAT substrate and as a reuptake inhibitor, eliciting reverse transport and blocking normal DA reuptake, thereby increasing synaptic DA levels. (C) At heterozygous DAT Val559 synapses, basal DAT-mediated DA efflux is a second, unexpected and temporally distinct mechanism for DA release, although normal DA reuptake can still occur. (D) Drugs such as MPH or cocaine (magenta) that block DAT-mediated ADE limit DA released only to that arising from vesicular fusion. Figure reproduced from Mazei-Robison et al., 2008.

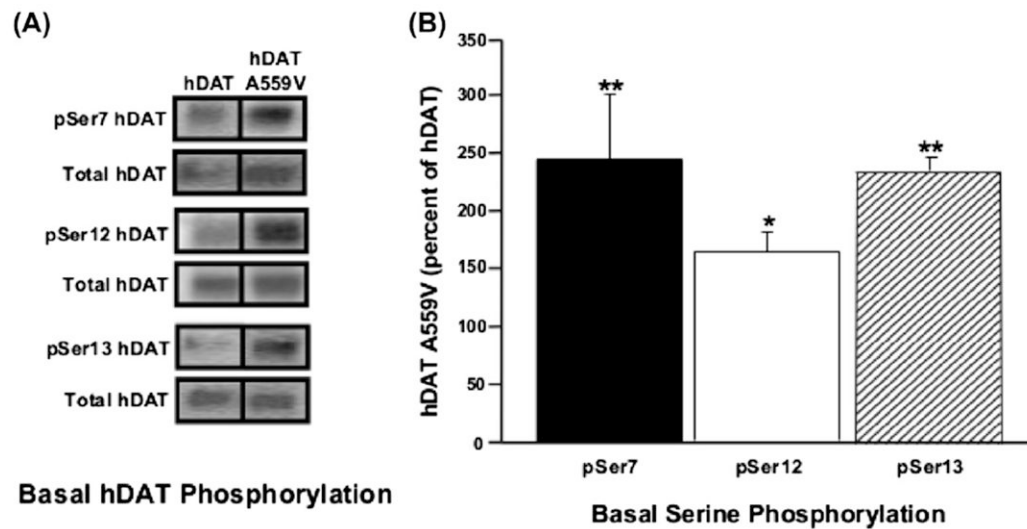


Fig. 5.

CaMKII-mediated hyperphosphorylation of the DAT Val559N-terminus. (A) Representative immunoblots of WT DAT and DAT Val559-transfected cells for phosphorylated serines at amino acids 7, 12, and 13 (pSer7, pSer12, and pSer13, respectively), and for total DAT.

Inset: immunoblot for dopamine D₂ receptor (top) and CaMKII (bottom), confirming their presence in the heterologous cell system. (B) Quantification of pSer DAT band intensities, normalized to total DAT. Figure reproduced from Bowton et al., 2010.

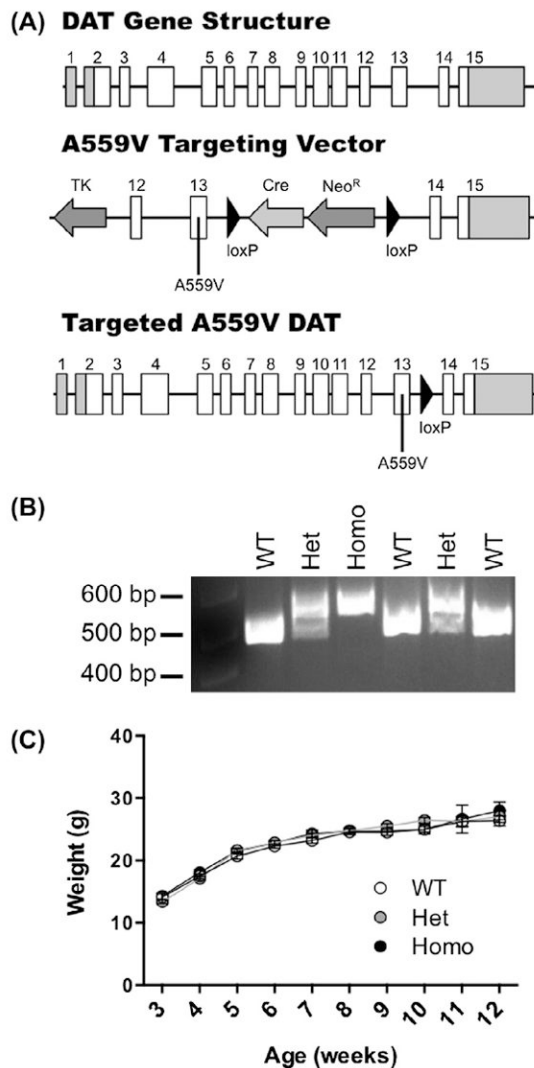


Fig. 6. Generation of DAT Val559 mice by homologous recombination. (A) Structure of the *Slc6a3* gene (top), DAT Val559 targeting construct (middle), and successfully recombined DAT gene (bottom). Coding exons are displayed as white boxes and non-coding exonic sequence as gray boxes. Following successful recombination and excision of Cre recombinase and neomycin resistance (Neo^R) cassettes, (B) genotypes of all mice are determined by PCR (490 base pair (bp) band = WT, 561 bp band = DAT Val559 homozygote, 490 and 561 bp bands = DAT Val559 heterozygote). (C) Growth and development of mice post-weaning does not differ among DAT genotypes.

Table 1

Disease-associated DAT coding variants. ADHD = attention deficit hyperactivity disorder; DTDS = dopamine transporter deficiency syndrome (juvenile dystonia/Parkinsonism).

Variant	Disease Association	References
Val24Met	ADHD	M. Mergy, unpublished findings
Val55Ala	?	Vandenbergh et al. (2000)
Val158Phe	DTDS	Kurian et al., 2011
Leu167Phe	ADHD	M. Mazei-Robison, unpublished findings
Leu224Pro	DTDS	Kurian et al. (2011)
Arg237Gln	?	Cargill et al., 1999
Gly327Arg	DTDS	Kurian et al. (2011)
Leu368Gln	DTDS	Kurian et al. (2009, 2011)
Val382Ala	?	Vandenbergh et al. (2000)
Pro395Leu	DTDS	Kurian et al. (2009, 2011)
Arg521Trp	DTDS	Kurian et al. (2011)
Pro529Leu	DTDS	Kurian et al. (2011)
Pro554Leu	DTDS	Kurian et al. (2011)
Ala559Val	Bipolar, ADHD	Grünhage et al. (2000); Mazei-Robison et al. (2005)
Glu602Gly	Bipolar	Grünhage et al. (2000)
Arg615Cys	ADHD	Sakrikar et al. (2012)

Table 2

Expected and observed allele frequencies of DAT Val559. The DAT Val559 allele is significantly under-represented in the total population of post-weaning mice ($P < 0.01$, χ^2 test), driven primarily by a reduction in male mice possessing the DAT Val559 allele ($P = 0.001$, χ^2 test). Males were obtained from 446 litters, females were obtained from 367 litters.

	Male	Female	Total
<i>Expected</i>			
WT	332 (25%)	286.5 (25%)	618.5 (25%)
Het	664 (50%)	573 (50%)	1237 (50%)
Homo	332 (25%)	286.5 (25%)	618.5 (25%)
	Male***	Female	Total**
<i>Observed</i>			
WT	386 (29.07%)	299 (26.09%)	685 (27.69%)
Het	650 (48.95%)	561 (48.95%)	1211 (48.95%)
Homo	292 (21.99%)	286 (24.96%)	578 (23.36%)

**
 $P = 0.0057$

 $P = 0.0010$,

Table 3

Sensorimotor evaluation of DAT Val559 mice. DAT Val559 hetero- and homozygous mice display no deficiencies in measures of general health, motor capability, or reflexes (each test analyzed by one-way ANOVA, $P > .05$ for all measures).

	WT	Het	Homo
<i>General</i>			
Missing whiskers	1/8	0/8	0/8
Poor coat condition	0/8	0/8	0/8
Piloerection	0/8	0/8	0/8
Bald patches	0/8	0/8	1/8
Body tone (# with normal tone)	8/8	8/8	8/8
Limb tone (# with normal tone)	8/8	8/8	8/8
Respiration rate (# abnormal)	0/8	0/8	0/8
Heart rate (# abnormal)	0/8	0/8	0/8
Tremor	0/8	0/8	0/8
Gait (# abnormal)	0/8	0/8	0/8
<i>Motor ability</i>			
Trunk curl	1/8	0/8	3/8
Forepaw reaching	8/8	8/8	8/8
Horizontal wire hang	8/8	8/8	8/8
Vertical wire hang	8/8	8/8	8/8
Pole climb	8/8	8/8	8/8
Inverted screen (latency to fall, sec)	34.9 ± 8.1	22.9 ± 8.3	16.7 ± 3.7
Positional passivity	0/8	0/8	0/8
Rotorod (latency to fall, sec)	171.9 ± 25.8	204.0 ± 10.1	166.9 ± 23.5
Grip strength (N)	1.71 ± 0.17	1.43 ± 0.04	1.71 ± 0.13
<i>Reflexes</i>			
Righting reflex	8/8	8/8	8/8
Air righting reflex	8/8	8/8	8/8
Ear twitch	5/8	7/8	8/8
Petting escape	7/8	6/8	8/8

Table 4

Rodent models of ADHD. + = phenotype observed in model; – = phenotype absent from model; ND = phenotype not determined for model.

	Hyperactivity	Inattention	AMPH/MPH response
<i>Lesion/insult models</i>			
6-OHDA (rat)	+	ND	+
Prenatal nicotine exposure (mouse)	+	ND	+
Prenatal BrdU exposure (rat)	+	ND	–
Prenatal ethanol exposure (rat)	ND	+	ND
Neonatal hypoxia (rat)	+	–	ND
Neonatal PCB exposure (rat)	+	ND	ND
Neonatal BPA exposure (rat)	+	ND	–
Neonatal X-ray exposure (rat)	ND	+	+
Neonatal chronic GBR 12909 (rat)	+	+	–
<i>Genetic KOs</i>			
DAT (mouse)	+	+	–
Steroid sulfatase (mouse)	+	–	ND
GIT1 (mouse)	+	+	+
NK1 receptor (mouse)	+	+	+
SNAP-25/coloboma (mouse)	+	ND	+
p35 (mouse)	+	ND	+
Guanylyl cyclase C (mouse)	+	ND	+
<i>Other genetic manipulations</i>			
Grin1 (missense mutant mouse)	+	ND	+
SynCAM1 dominant negative	+	ND	+
Cocaine-insensitive DAT	+	ND	+
DAT knock-down	+	–	+
CK1-delta overexpression	+	ND	+
Thyroid hormone receptor beta-PV	+	+	+
<i>Selective breeding models</i>			
SHR	+	+	+
Genetically Hypertensive (GH) rat	+	ND	ND
NHE rat	+	+	ND
WKHA rat	+	ND	–
Wig rat	+	ND	–
I/LnJ acallosal mouse	+	+	ND
<i>Developmental models</i>			
Social Isolation	+	+	+
Dirupted habenula development	+	+	+
Maternal separation	+	+	ND