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Mice expressing markedly reduced striatal dopamine transporters exhibit increased locomotor activity, dopamine uptake turnover rate and cocaine responsiveness

Anjali Rao^{1,*}, Alexander Sorkin², and Nancy R. Zahniser¹

¹Department of Pharmacology, School of Medicine, University of Colorado - Anschutz Medical Campus, Aurora, CO 80045

²Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

Abstract

Variations in the expression levels of the dopamine transporter (DAT) can influence responsiveness to psychostimulant drugs like cocaine. To better understand this relationship, we studied a new DAT-low expresser (DAT-LE) mouse model and performed behavioral and biochemical studies with it. Immunoblotting and [³H]WIN 35,428 binding analyses revealed that these mice express ~35% of wildtype (WT) mouse striatal DAT levels. Compared to WT mice, DAT-LE mice were hyperactive in a novel open-field environment. Despite their higher basal locomotor activity, cocaine (10 or 20 mg/kg, i.p.) induced greater locomotor activation in DAT-LE mice than in WT mice. The maximal velocity (V_{max}) of DAT-mediated [³H]DA uptake into striatal synaptosomes was reduced by 46% in DAT-LE mice, as compared to WT. Overall, considering the reduced number of DAT binding sites (B_{max}) along with the reduced V_{max} in DAT-LE mice, a 2-fold increase in DA uptake turnover rate (V_{max}/B_{max}) was found, relative to WT mice. This suggests that neuroadaptive changes have occurred in the DAT-LE mice that would help to compensate for their low DAT numbers. Interestingly, these changes do not include a reduction in tyrosine hydroxylase levels, as was previously reported in DAT knockout homozygous and heterozygous animals. Further, these changes are not sufficient to prevent elevated novelty- and cocaine-induced locomotor activity. Hence, these mice represent a unique model for studying changes of *in vivo* DAT function and regulation that result from markedly reduced levels of DAT expression.

Keywords

SLC6; neurotransmitter transporter; low-expresser; mouse model; psychostimulant; [³H]WIN 35,428

Introduction

The dopamine (DA) transporter (DAT) is essential for clearance of extracellular DA, resulting in termination of DA neurotransmission, and is a primary target for psychostimulant drugs like cocaine (Giros et al., 1996). Thus, knock-in mice that express a cocaine-insensitive DAT with ~50% of normal DA uptake activity exhibit no acute cocaine-induced increases in extracellular DA in nucleus accumbens or locomotor activation (Chen

*Corresponding author: Dr. Anjali Rao, Department of Pharmacology MS 8303, School of Medicine, University of Colorado - Anschutz Medical Campus, Aurora, CO 80045. Phone: 303-532-6532; anjali.rao@ucdenver.edu.

et al., 2005 & 2006). Likewise, in these mice, repeated cocaine exposure does not result in cocaine-induced locomotor sensitization, cocaine reward as measured by conditioned place preference (CPP), cocaine reinforcement as measured by self-administration, or increases in dendritic spine density in nucleus accumbens (Chen et al., 2006; Martin et al., 2011; Thomsen et al., 2009). It is known that cocaine-induced locomotion and reward, as well as other functions of DA-mediated neurotransmission, are influenced by variations in the number of DATs (Uhl et al., 2002). Studies have helped to identify the prevalence of individual differences in levels of DAT in humans (Drgon et al., 2006; Kurian et al., 2011) and strain-related differences in mice (Janowsky et al., 2001). At least 47% of DATs need to be occupied by cocaine before addicts report subjective effects of the drug, suggesting the existence of a 'spare' pool of DATs (Volkow et al., 1997). Additionally, abuse of cocaine has been shown to alter DAT numbers in humans (Little et al., 1993; Mash et al., 2002).

Several studies have shown that variations in DAT expression levels are associated with alterations in psychostimulant drug effects. It was found that in human embryonic kidney (HEK293) cells, over-expression of DAT resulted in reduced potency of cocaine, as well as other DAT inhibitors (Chen and Reith, 2007). On the other hand, in DAT knockout mice, serotonin transporter (SERT) and norepinephrine transporter (NET) inhibitors are capable of eliciting reward, unlike in wild type (WT) mice (Hall et al., 2002). Since a complete null mutation of DAT leads to extensive developmental and neurochemical changes (Bosse et al., 1997; Jones et al., 1998), studying mouse models with altered DAT expression levels may offer several advantages for understanding the function of the transporter and how it affects the actions of psychostimulants like cocaine. Previously, mouse models with DAT levels reduced to ~ 10% and ~ 50% of WT have been generated and were shown to have elevated levels of extracellular DA due to slower clearance rates, along with retention of cocaine-induced hyperlocomotion and cocaine reward (Giros et al., 1996; Tilley et al., 2007; Zhuang et al., 2001). On the other hand, transgenic mice over-expressing DAT by 20 – 30 %, compared to WT, exhibit increased cocaine reward (Donovan et al., 1999).

Our past research using outbred Sprague-Dawley rats showed that these animals exhibit individual differences in their maximal number of striatal DAT binding sites (B_{max}; Nelson et al., 2009). Rats with an average of 33% lower DAT B_{max} than the group mean are more responsive to the DAT inhibiting and locomotor activating effects of acute low dose cocaine but also more rapidly up-regulate their DAT function (Mandt and Zahniser, 2010; Nelson et al., 2009; Sabeti et al., 2002). In response to repeated cocaine administration, we found that the rats that initially had fewer striatal DATs are less likely to develop locomotor sensitization, exhibit lower cocaine CPP and appear less motivated to self-administer cocaine (Allen et al., 2007; Mandt et al., 2008). However, given the variability in DAT numbers and behavioral responsiveness among individual animals, it is difficult to understand the exact relationship. Here we report results obtained from behavioral and biochemical studies using a novel genetically altered DAT low-expresser (DAT-LE) mouse model that has ~30% of WT striatal DAT protein levels (Rao et al., 2012).

Materials and Methods

Animals

Details regarding the generation and maintenance of DAT-LE mice have been previously published (Rao et al., 2012). DAT-LE heterozygous (DAT-LE HET) mice were back-crossed for 5 generations with C57/B16 mice obtained from Jackson Laboratories (Bar Harbor, ME). These DAT-LE HET mice were then crossed to obtain homozygous mice (referred to as DAT-LE). Their WT littermates were used for all control experiments. Mice were housed in groups of 5 on a 14:10 lights on:off cycle with food and water available *ad libitum*. All male and female mice were 2-4 months old at the time of the experiments. Prior

to brain dissections, the mice were quickly euthanized by cervical dislocation. All experimental procedures involving the generation and use of the mice were conducted in accordance with National Institutes of Health guidelines as approved by the Institutional Animal Care and Use Committee at the University of Colorado -Anschutz Medical Campus.

Immunoblotting

For immunoblotting, tissue samples were prepared from mice of all 3 genotypes (WT, DAT-LE HET, and DAT-LE). To examine striatal DAT, striatal tissue was dissected out and solubilized in Triton X-100/glycerol/HEPES lysis buffer, and subsequently denatured in sample buffer at 75°C for 10 min. Lysates were then electrophoresed and blotted with the following antibodies: HA.11 (1:1000 dilution; Covance, Princeton, NJ), rat monoclonal N-terminal DAT (1:1000 dilution; Chemicon, Temecula, CA), C-terminal DAT (1:1000 dilution; Santa Cruz Biotechnology Inc, Santa Cruz, CA.), tyrosine hydroxylase (TH; 1:5000 dilution; Chemicon, Temecula, CA) or synaptophysin (1:10,000 dilution; Santa Cruz Biotechnology Inc.). Quantitative analysis of band intensity was performed using densitometry and Image J software (National Institutes of Health, Bethesda, MD).

To investigate the less abundant mesencephalic DAT protein levels, midbrain was dissected out and the transporter was concentrated from the mesencephalic lysates via immunoprecipitation. Briefly, mesencephalic lysates were incubated with polyclonal goat DAT antibody (1:1000; Santa Cruz Biotechnology Inc.) at 4°C overnight. The resulting supernatant was incubated with Protein G beads (ThermoFisher Scientific, Rockford, IL) for 1 hr at 4°C. Subsequently, the beads were washed and eluted using 200 mM glycine. The eluted mesencephalic DAT was then prepared for immunoblotting as described above.

Quantitative Real time PCR

To investigate the total mRNA levels of DAT in mesencephalon, the ventral tegmental area and substantia nigra of the DAT-LE and WT mice were dissected out in cold RNA lysis buffer (Qiagen), and tissue was frozen on dry ice. Total RNA extraction and subsequent DAT mRNA measurements by real-time quantitative RT-PCR were done in the Endocrinology PCR Core, University of Colorado Denver, using an ABI Prism 7900 Sequence detector and TaqMan gene expression assay (Mm00438396_m1; Slc6a3) purchased from Applied Biosystems (Foster City, CA). DAT mRNA quantities in all samples were normalized to the corresponding 18S ribosomal RNA levels.

[³H]WIN 35,428 Binding assays

Indirect saturation binding assays were performed using a previously described protocol (Hanania et al., 2004). Briefly, striata were dissected out from the DAT-LE mice and their WT littermates, homogenized in ice-cold assay buffer (30 mM NaH₂PO₄, 15 mM Na₂HPO₄, and 0.32 M sucrose; pH 7.4) using a sonicator, and centrifuged at 20,000g for 20 min at 4°C. The pellet was re-suspended in assay buffer and subsequently incubated with [³H]WIN 35,428 (~ 4.2 nM; Perkin-Elmer Life Sciences, Boston, MA) and 10 concentrations of unlabeled WIN 35,428 (1×10^{-5} – 3.16×10^{-10} M) on ice for 60 min. Incubation with 30 μM benztrapine (Sigma-Aldrich, St. Louis, MO) was used to obtain the non-specific binding. Vacuum filtration with 2 washes was performed using Whatman GF/C glass microfiber filters (Brandel Inc., Gaithersburg, MD), and the resulting radioactivity on the filters was measured by liquid scintillation spectrometry. The amount of protein in each sample was determined (Bradford, 1976). Nonlinear curve fitting of the indirect saturation curves with Prism 5 (GraphPad Software, La Jolla, CA) was used to determine the values for the apparent binding affinity (IC₅₀, Kd) and the total number of binding sites (B_{max}; DeBlasi et al., 1989).

Behavioral testing

Locomotor activity was tested in open-field chambers (16" ×16" ×15") surrounded by an 8 × 8 photobeam frames (San Diego Instruments, San Diego, CA). The mice were divided into 3 groups based on the cocaine doses to be administered on day 2. Each group consisted of 6 DAT-LE and 6 WT littermates. Both males and females were used, and the genders were equally distributed in all of the groups. All testing was performed between 9 AM-12 PM. On day 1, mice were transferred to the testing room and kept in their home cages for a 30-min habituation period. Subsequently, mice were placed in the novel open-field chambers, and their locomotor activity was recorded for 90 min. Locomotor activity was determined from the number of consecutive horizontal photobeam breaks per 10 min. Thereafter, all mice were injected with saline (1 ml/kg; i.p.) and re-placed in the chamber. Their activities were recorded for another 60 min, after which all mice were returned to their home cages and the colony room. On day 2, the same set of mice was subjected to the same protocol but were injected with a single dose of cocaine (5, 10, or 20 mg/kg; i.p.). These doses of cocaine were chosen because they have been previously shown to elicit a linearly increasing locomotor response in C57/Bl6 mice (Tilley et al., 2007). At the end of the day 2 session, all mice were euthanized 150 min after the cocaine injection; and their striatal tissue was used immediately for [³H]DA uptake measurements. This time period was chosen because it is 5 times the half-life of cocaine; thus, tissue cocaine levels should be negligible (Maisonneuve and Kreek, 1994).

[³H]DA uptake assays

Striatal synaptosomes were prepared according to Rao et al. (2012) from mice on day 2 after the behavioral testing (see above) and in another control group of WT and DAT-LE mice (N = 4/genotype) that had received saline injections on days 1 and 2, but no behavioral testing. Briefly, striata were homogenized with a Teflon glass homogenizer in ice-cold phosphate buffer (3.3 mM NaH₂PO₄, 12.7 mM Na₂HPO₄, 0.32 M sucrose; pH 7.4) and subsequently centrifuged at 1,000g for 12 min at 4°C. The resulting supernatant was centrifuged at 12,500g for 15 min to obtain the synaptosomal fraction. Synaptosomes were re-suspended at 40 mg/ml (wet weight of tissue) in assay buffer (134 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.4 mM MgSO₄, 3.3 mM NaH₂PO₄, 12.7 mM Na₂HPO₄, 11 mM glucose, and 1 mM ascorbic acid; pH 7.4). 1 μM pargyline (Sigma-Aldrich) was added to inhibit monoamine oxidase before pre-incubation of the synaptosomes with assay buffer for 10 min at 37°C. Next, 0.5 nM [³H]DA (Perkin Elmer Life Sciences; Boston, MA) and one of eight concentrations of unlabeled DA (0, 10, 50, 75, 100, 175, 250, or 500 nM) was added before incubation for 3 min at 37°C. Non-specific uptake was determined in the presence of 100 μM cocaine (gift from the National Institute on Drug Abuse, Research Triangle Institute International, Research Triangle Park, NC). The samples were filtered through Whatman GF/C glass microfiber filters with a cell harvester and washed 3 times with ice-cold 0.32 M sucrose solution. Radioactivity and protein concentrations were determined as described above. Nonlinear curve fitting using GraphPad Prism 5 software was performed to obtain values for apparent affinity (K_m) and maximal velocity (V_{max}).

Statistical analysis

Data are expressed as mean values ± SEM. Prism 5 (GraphPad Software) was used to determine the independent sample t-test, as well as the radioligand binding and uptake kinetic parameters. SPSS, version 16.0 (SPSS Inc., Chicago, IL) was used to perform the repeated measures analysis of variance (ANOVA) of the behavioral experiments. Post-hoc pair wise comparison was carried out with either Bonferroni or Tukey HSD test. The level of significance was set at p<0.05.

Results

Characterization of the DAT-LE mouse model

We generated a knock-in mouse model, DAT-LE, with a modified endogenous DAT locus (Rao et al., 2012). These mice contain an altered DAT allele with a floxed neomycin cassette in intron 3 and an HA11 (haemagglutinin) epitope sequence cloned into exon 4 of the DAT gene. Western blotting revealed that there is a marked reduction of DAT protein levels in the striatum of DAT-LE and DAT-LE HET mice (Fig. 1A). We have previously demonstrated that crossing DAT-LE mice with β -actin driven cre mice results in the elimination of the floxed neomycin cassette from the DAT locus and a subsequent rescue of the DAT low expression phenotype (Rao et al., 2012). These results suggest that the HA11 epitope in exon 4 does not interfere with DAT expression levels and that the insertion of the floxed neomycin cassette in intron 3 is the likely cause of this aberrant DAT expression..

Densitometry was used to quantify levels of striatal and mesencephalic DAT protein (~75 KDa) that were then normalized to TH levels (TH levels were not altered in these mutant mice; Fig. 1A). We found that DAT-LE mice expressed $42.3 \pm 8.7\%$ (N = 4) of the WT striatal DAT protein levels (Fig. 1B). In contrast, DAT-LE HET mice expressed $83.4 \pm 17.7\%$ (N = 4) of WT striatal DAT protein levels (Fig. 1B). Similar results were obtained using N- and C-terminal DAT antibodies (data not shown). In the mesencephalic tissue, immunoprecipitation of DAT (see Methods) revealed a truncated form of the DAT protein (~60 KDa) that was not delivered to the DA axons in striatum (Fig. 1A). The signal intensity of this DAT short form (S; 9.9 arbitrary units (AU)) was similar to that of the full-length (FL) WT DAT (8.1 AU) in mesencephalon, suggesting that it is not degraded in the soma and is relatively stable in the endoplasmic reticulum (ER) or post-ER compartments. Additionally, total mesencephalic DAT mRNA levels were not significantly different in DAT-LE and WT mice (DAT-LE: 55.2 ± 12.1 fg/ng 18S, N = 3; WT: 137.3 ± 62.9 fg/ng 18S, N = 4; p value = 0.2848).

DAT is known to be important for development (Bosse et al., 1997; Jones et al., 1998). Thus, we examined the total body weight of the DAT-LE mice. We found that there was a significant 10% reduction in body weight of both adult female and male DAT-LE mice, as compared to their WT littermates (Figs. 1C & D).

We next determined the affinity and number of DATs in striatal membranes by indirect [3 H]WIN 35,428 saturation binding analysis (Fig. 2). There were no statistical differences in the affinities for the radioligand in the mutant DAT and WT DAT mice (Kd values, DAT-LE: 16.0 ± 4.3 and WT: 5.4 ± 1.1 nM). In contrast, DAT-LE mice had a significantly lower number of striatal DATs: 25.6 % of WT mice (Bmax values, DAT-LE: 0.9 ± 0.2 and WT: 3.7 ± 0.8 pmol [3 H]WIN 35,428/WIN 35,428 specifically bound/mg protein). Together, the immunoblotting and binding results indicate that striatal DATs in the DAT-LE mice were reduced to ~35% of those in WT mice.

DAT-LE mice display increased locomotor activity

We next analyzed basal locomotor activity of age- and sex-matched naïve DAT-LE and WT mice when exposed to a novel open-field environment (Fig. 3). On day 1, locomotor activity differed significantly between the two genotypes [F(1, 34) = 14.90, p < 0.001]. Post-hoc analysis revealed that during the initial 50 min in the novel environment DAT-LE mice displayed significantly higher locomotor activity (p < 0.001; Fig. 3A). However, towards the end of the initial 90 min-session, mice of both genotypes exhibited similar lower levels of locomotor activation. On day 2, differences between the two genotypes were maintained [F(1, 34) = 14.75, p < 0.01]. In this case, DAT-LE mice displayed higher locomotor activity for the entire 90 min, as compared to the WT mice (p < 0.001). Furthermore, on both days,

DAT-LE mice traveled ~70% longer distances during the first 30 min in the open-field ($p < 0.001$), indicating hyperactivity in the DAT-LE mice (Fig. 3B).

Greater locomotor activation of DAT-LE mice by cocaine

After the 90 min-habituation period in the open field box, DAT-LE and WT mice were injected with saline on day 1 and with one of three doses of cocaine on day 2. Locomotor activity was recorded for 60 min post-injection on both days (Fig. 4). Fig. 4A shows the entire time course of the experiment for locomotor responses on day 1 to saline (1 ml/kg, i.p.) and on day 2 to the three doses of cocaine (5, 10 and 20 mg/kg; i.p.) in the DAT-LE and WT mice. Upon comparing the responses of the mice on days 1 and 2 within each group ($N = 6/\text{group}$), we found that there was a significant effect of drug [$F(2, 30) = 5.24, p < 0.05$] and a difference in genotype [$F(1, 30) = 10.77, p < 0.01$], but the genotype \times drug interaction did not reach significance [$F(2, 30) = 1.38, p = 0.27$]. Upon post hoc analysis using Tukey HSD test, we found no differences between genotypes after the injection of saline on day 1 (Fig. 4A). In contrast, on day 2, 10 mg/kg cocaine potentiated locomotor activity of the DAT-LE mice to a greater extent than the WT mice ($p < 0.05$; Fig. 4A). Interestingly, over the entire 60 min after injection, 5 and 20 mg/kg cocaine produced similar effects in both genotypes (Fig. 4A). However, the majority of cocaine-induced activation occurs within the first 30 min after injection. Thus, we analyzed the drug effect as a function of distance traveled during first 30 min and found that both 10 and 20 mg/kg cocaine stimulated DAT-LE mice to a much greater extent than the WT mice ($p < 0.001$ and $p < 0.05$ respectively; Fig. 4B). A similar trend was observed for the 5 mg/kg dose of cocaine. Overall, in DAT-LE mice all doses of cocaine elicited higher locomotor activity, as compared to saline within their respective treatment groups ($p < 0.05$), while in the WT mice only 10 and 20 mg/kg cocaine increased locomotor activity over saline-treatment ($p < 0.05$).

Lower V_{\max} and higher V_{\max}/B_{\max} values for DA uptake into striatal synaptosomes from DAT-LE mice

We next examined the functionality of the mutant DAT in DAT-LE mice by measuring [^3H]DA/DA uptake kinetics in striatal synaptosomes (Fig. 5). We measured uptake kinetics in groups of control DAT-LE and WT mice that had been injected with saline on two consecutive days but never tested behaviorally ($N = 4/\text{genotype}$). Following the behavioral experiments, striatal synaptosomes were also prepared from DAT-LE and WT mice at 150 min post cocaine injection, a time when any residual cocaine in the tissue should be negligible. To account for day-to-day variations in absolute uptake values, we compared V_{\max} and K_m values for the DAT-LE mice as a percentage of the WT mouse values obtained on the same assay day. No significant genotype differences were detected in either parameter following saline or any of the doses of cocaine tested, suggesting no persistent regulation of the DAT following cocaine exposure (data not shown). Thus, we pooled the striatal DA uptake results within the two genotypes, resulting in $N = 22/\text{genotype}$ (Fig. 5).

Relative to the V_{\max} value of 38.3 ± 7.1 pmol [^3H]DA/DA taken up/mg protein/min in WT mice, the DAT-LE mice showed a significantly lower V_{\max} value of 20.8 ± 4.0 pmol/mg protein/min (Fig. 5B). Taking into account the reduced number of DAT binding sites in the DAT-LE mouse striatum (Fig. 2) an overall increase in uptake turnover rate (V_{\max}/B_{\max}) was revealed. This rate in DAT-LE mice was 23.1 ± 4.4 DA taken up/DAT binding site/min, as compared to 10.4 ± 1.9 in WT mice (Fig. 5C). On the other hand, K_m values for uptake in DAT-LE mice did not differ significantly from those for WT mice (Fig. 5D). K_m for WT mice was 62.51 ± 6.77 nM, while DAT-LE mice showed K_m of 83.63 ± 13.89 nM (p value = 0.1789).

Discussion

We found that mice expressing ~35% of WT striatal DATs, as measured with both immunoblotting and radioligand binding, were hyperactive in a novel open-field environment and showed higher cocaine-induced locomotor activation at several doses. These results are all consistent with a loss of DAT function, which was confirmed by a 46% reduction in the maximal DA uptake velocity into striatal synaptosomes from DAT-LE mice, as compared to WT mice. Taking into account both V_{max} and B_{max} values for DATs, however, revealed that the DA uptake turnover rate of the DAT-LE mice was increased by 2-fold; suggesting a compensatory functional up-regulation of maximal DA uptake. Nonetheless, our behavioral findings are consistent with the conclusion that such a marked reduction in DAT levels contributes to higher levels of extracellular DA, thereby resulting in both greater baseline and cocaine-induced activation. We do not know how DA release may be altered in these mice. Extracellular DA reflects the combination of both DA release and uptake, and previous studies have shown that cocaine promotes stimulation-evoked DA release preferentially in the nucleus accumbens (Wu et al., 2001). Thus, in order to understand the relative contributions of DA uptake and release to cocaine responsiveness in this model, it will be informative to investigate cocaine-mediated striatal DA release in DAT-LE mice in the future.

Transgenic mouse models with reduced DAT levels offer several advantages for studying the function of the transporter and its regulation by psychostimulants like cocaine. Several mouse models with low DAT levels have been studied in the past. These models have either used ES cell-targeted genetic manipulations (Giros et al., 1996; Zhuang et al., 2001) or transient knock-down approaches via RNA interference (RNAi; Salahpour et al., 2007; Thakker et al., 2004). In making a HA-DAT mouse model for studies of DAT endocytosis, our first knock-in mouse unexpectedly had reduced DAT protein expression (Rao et al., 2012). Here, we took advantage of this unique DAT-LE mouse model in order to perform behavioral and functional studies, comparing it to age- and sex-matched WT littermates.

Although the DAT-LE mice were able to breed normally, the body weights of the adult (2-4 months) female and male mice were reduced by ~10% (Figs. 1C & D). The body weights of DAT knockout mice were reduced to a greater extent, by 43% (Blizotes et al., 2000; Bosse et al., 1997). Interestingly, however mice expressing 10% (Beeler et al., 2012; Zhuang et al., 2001) or ~50% (Giros et al., 1996) of WT DAT did not show any abnormalities in body weight.

Western blotting revealed a 58% reduction in striatal DAT protein levels in DAT-LE mice (Figs. 1A & B) whereas [3 H]WIN 35,428 binding studies showed a 73% reduction in the striatal DAT number (B_{max} ; Fig. 2). It is likely that these 15% differences are due to the different methods of tissue preparations (solubilization in detergent vs. homogenization in buffer). Also, the antibody and radioligand likely bind to different sites on DAT. In any case, the DAT-LE mice appear to have ~35% of the normal compliment of striatal DATs. In the mesencephalic region of these mice, lower molecular weight DAT protein was found (Fig. 1A). This DAT species is recognized by N- and C-terminal specific antibodies but not HA11 antibody (Fig. 1A), suggesting this DAT form has an internal deletion encompassing a part of the extracellular loop 2 and HA sequence. Since we did not find this mutant DAT in the striatal region, it is likely that this DAT variant was mis-folded and retained in the ER in the somatodendritic compartment of the DA neurons. Numerous previous mutagenesis studies have shown the high susceptibility of ER retention of DAT to even small changes in extracellular loops of the DAT molecule (Miranda et al., 2004). Alternatively, short DAT form could be delivered to the axons but then turned over very rapidly. This explanation is less likely because axons of DA neurons lack lysosomes, the site of degradation of

membrane proteins (Rao et al., 2011, 2012). Regardless of the nature of the trafficking defect of the short DAT form, this defect is rescued upon excision of the floxed neomycin cassette from the intron 3 (Rao et al., 2012), suggesting that the defect is not due to the insertion of the HA sequence.

DAT homozygous and heterozygous knockout mice have been shown to have reduced TH levels but the normal physiological activity of TH is preserved (Jones et al., 1998). In contrast, DAT-LE mice had normal TH protein expression (Fig. 1A). The level of TH activity in these mice remains to be tested. Nonetheless, our observations indicate that distinct neuroadaptive changes have occurred in the DAT-LE mice that differ from the DAT knockout animals. Hence, these mice presented us with a unique model to study the transporter function and sensitivity to cocaine.

Monitoring locomotor activity in the open-field showed that in a novel environment the DAT-LE mice were hyperactive as compared to WT littermates (Fig. 3). These results are similar to a previously studied mouse model with 10% DAT protein levels (Zhuang et al., 2001). Interestingly, DAT heterozygous knockout mice with a Bmax of 55% of WT did not show markedly different locomotor responsiveness to a novel environment (Giros et al., 1996; Jones et al., 1998). This suggests that the threshold for DAT expression level for manifestation of the novelty-induced hyperactive phenotype falls between 35% and 55% of WT levels.

Since DAT is essential for the action of the drug cocaine, we examined its effect in our mouse model. Despite the higher basal locomotor activity of DAT-LE mice, cocaine-induced hyperactivity was preserved and even magnified in the DAT-LE mice (Fig. 4). These mice displayed significantly greater locomotor activity in response to an i.p. injection of 5, 10 or 20 mg/kg cocaine, as compared to saline. Additionally, 10 and 20 mg/kg cocaine had a greater locomotor stimulating effect in these mice than in the WT mice. Hence, our results suggest that a reduction in DAT protein levels is associated with greater acute cocaine-induced hyperactivity, consistent with what we have seen in Sprague-Dawley rats (Nelson et al., 2009). Interestingly, in mice with 10% residual DAT, 5 and 10 mg/kg cocaine produced a greater locomotor activation than in WT; but there was no genotype difference to a dose of 20 mg/kg cocaine (Tilley et al., 2007). On the other hand, in DAT heterozygous mice, 10 and 40 mg/kg cocaine elicited locomotor responses that were similar to the WT mice (Giros et al., 1996; Jones et al., 1998). Again, our DAT-LE mice with 35% striatal DATs exhibit an intermediate phenotype compared to these two previously studied mouse models. It will also be interesting to study the response of our intermediate DAT expression mouse to different doses of amphetamine, which have been studied in other DAT mutant models.

Functional studies of [³H]DA uptake into striatal synaptosomes revealed a 46% decrease in maximal DAT-mediated uptake velocity in the DAT-LE mice, as compared to WT. On the other hand, Km values reflecting affinity for substrate in the DAT-LE mice were similar to WT, as were Kd values for the cocaine congener [³H]WIN 35,428. Considering the reduced number of DATs along with the reduced DAT function in the DAT-LE mice, an overall 2-fold increase in DA uptake turnover (Vmax/Bmax) was found. Since membrane binding measures the majority of cellular DATs, this number likely under-estimates the function per transporter. We also do not know if the cellular distribution of DATs differs between DAT-LE and WT mice. Nevertheless, our results strongly suggest that neuroadaptive changes have occurred in the DAT-LE mice that have either up-regulated DAT activity or that involve other biogenic amine transporters to compensate for the low DAT numbers. Further, cocaine also increases evoked striatal DA release (Oleson et al., 2009; Wu et al., 2001), and we do not know what neuroadaptive changes in DA release may have occurred in the DAT-

LE mice during development. In any case, our results suggest that the increased DAT activity in the DAT-LE mice is insufficient to reduce extracellular DA levels to those in WT mice, resulting in novelty-induced hyperactivity and greater behavioral activation in the DAT-LE mice in response to moderate doses of cocaine that would be expected to only partially inhibit normal striatal DAT activity.

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Abbreviations

ANOVA	analysis of variance
AU	arbitrary units
Bmax	maximal number of binding sites
CPP	conditioned place preference
DA	dopamine
DAT	dopamine transporter
ER	endoplasmic reticulum
HA11	haemagglutinin
HET	heterozygous
Kd	binding affinity
Km	uptake affinity
LE	low expresser
NET	norepinephrine transporter
RNAi	RNA interference
SERT	serotonin transporter
TH	tyrosine hydroxylase
Vmax	maximal uptake velocity
WT	wildtype

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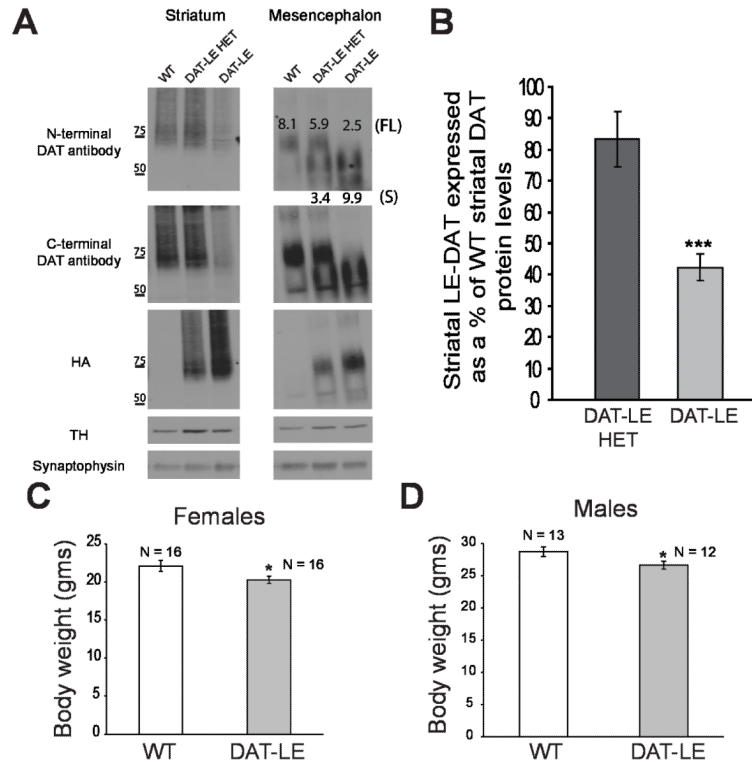


Fig. 1. Characterization of DAT protein levels and body weights of DAT-LE mice
(A) Representative western blots of striatal (left panel) and mesencephalic tissue (right panel) from mice of all three genotypes (WT, DAT-LE HET and DAT-LE). Striatal lysates or mesencephalic immunoprecipitates using DAT-specific antibodies were electrophoresed and probed by blotting with antibodies to DAT (both against the N- and C-termini), HA, TH and synaptophysin (loading control). The molecular weight markers in kilodaltons are indicated to the left of the striatal blot. The values shown on the mesencephalic blots are the calculated signal intensity (in AU) of mesencephalic DAT protein relative to TH protein levels. The values at the top correspond to the higher molecular weight full-length (FL) DAT (~75 KDa) while the values at the bottom correspond to the short (S) form of DAT (~60 KDa), denoted as FL and S, respectively, on the right side of the blot. **(B)** Relative intensity of striatal tissue DAT western blot signals, as detected by the N-terminal DAT antibody, in the DAT-LE HET and DAT-LE animals expressed as a percentage of the WT animals. The DAT signal intensity was normalized to that of the TH levels. Mean values \pm SEM (N = 4; *** $p < 0.001$ DAT-LE vs. DAT-LE HET). **(C & D)** Body weights of adult (2-4 mo) female **(C)** and male **(D)** DAT-LE mice compared to their WT littermates. Mean values \pm SEM (N = 12-16; * $p < 0.05$, DAT-LE vs WT).

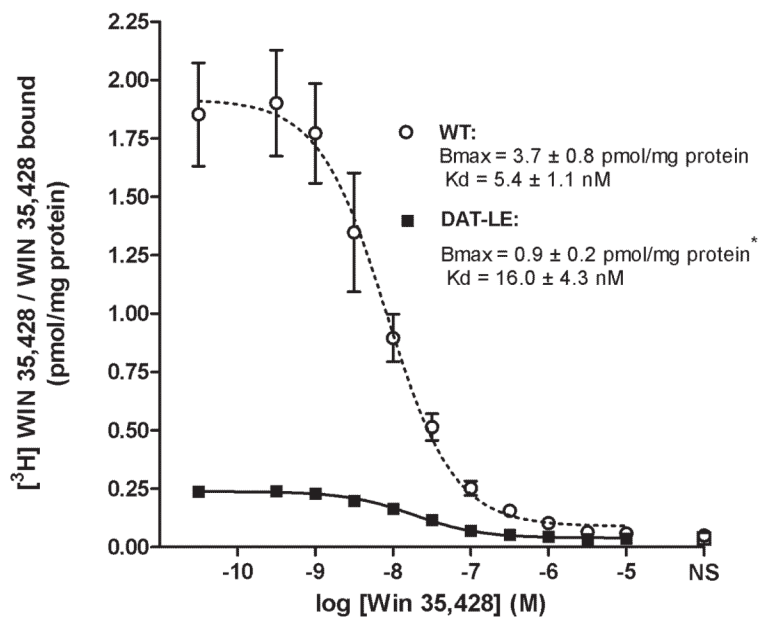


Fig. 2. $[^3\text{H}]$ WIN 35,428/WIN 35,428 indirect saturation binding isotherms to DATs in striatal membranes of DAT-LE and WT mice
 Kd and Bmax values for both genotypes derived from this analysis are indicated. NS = nonspecific binding. Mean values \pm SEM (N = 4 mice/genotype; * $p < 0.05$, DAT-LE vs WT mice).

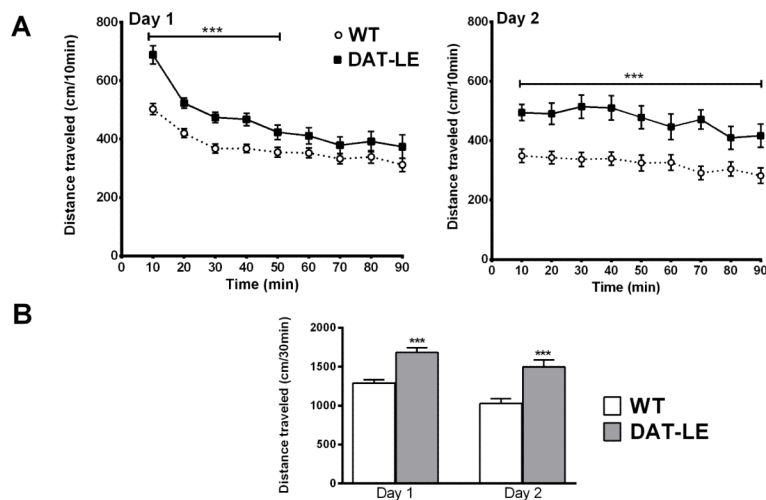


Fig. 3. Effects of novelty on locomotor activity in DAT-LE and WT mice

Naïve animals were placed in open-field chambers on day 1, and their locomotor activity in the novel environment was recorded for 90 min (mean values \pm SEM; N = 18/genotype). Subsequently, all of the mice were injected with saline; and their behavior was monitored for an additional 60 min (see Fig. 4). On day 2 their activity in the open-field was again monitored for the initial 90 min, prior to being injected with either saline or cocaine (see Fig. 4). **(A)** Time course of distance traveled during the first 90 min in the chamber by WT and DAT-LE mice day 1 (left panel) and day 2 (right panel). *** $p < 0.001$, DAT-LE vs WT **(B)** Total distance traveled by the two genotypes in the first 30 min (novelty) in the chamber on days 1 and 2. *** $p < 0.001$, DAT-LE vs WT.

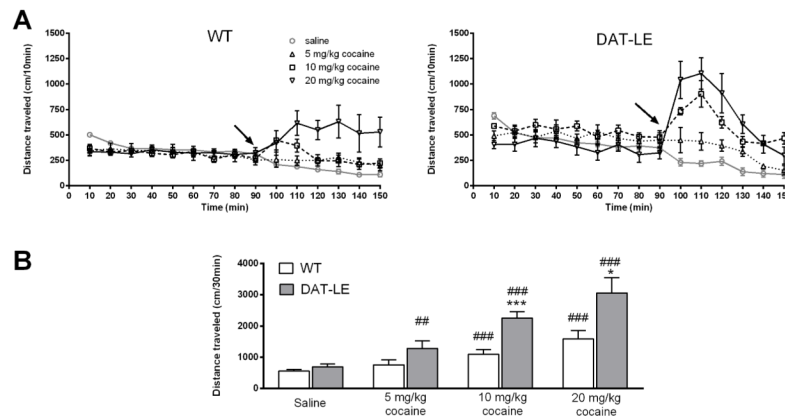


Fig. 4. Dose-effect of acute cocaine exposure on locomotor activity in DAT-LE and WT mice
 Following the 90-min habituation of the mice to the open field chamber on day 2 (see Fig. 3), WT (**A**, left panel) and DAT-LE (**A** right panel) mice were injected with a single dose of cocaine (arrow; 5, 10 or 20 mg/kg; mean values \pm SEM, $N = 6$ for each dose and each genotype). Following injection, their locomotor activity was monitored for an additional 60 min. See Results for statistical analyses. Locomotor activity on day 1 following saline treatment is shown as a single group only for reference ($N = 18$). (**B**) Total distance traveled in the first 30 min after saline or cocaine injection (mean values \pm SEM; * $p < 0.05$, *** $p < 0.001$, DAT-LE vs WT; ## $p < 0.01$, ### $p < 0.001$, cocaine vs saline within the respective genotype).

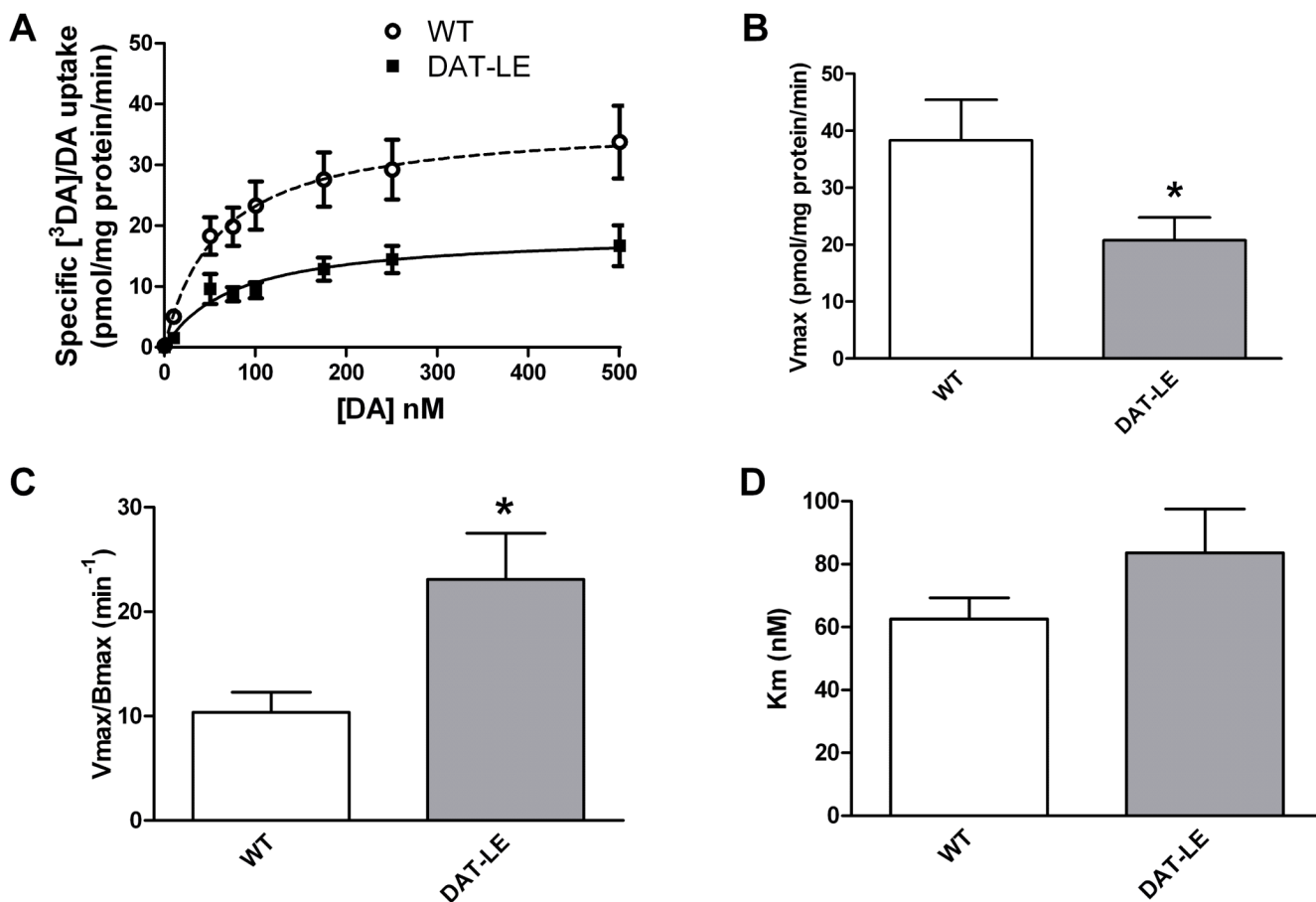


Fig. 5. DA uptake kinetics in striatal synaptosomes of DAT-LE and WT mice

(A) DA uptake levels were measured in the presence of increasing [³H]DA/DA concentrations in striatal synaptosomes prepared from WT and DAT-LE mice injected with saline (N = 4/genotype) or cocaine (N = 6/genotype) 150 min after injection. Since no treatment group differences in the uptake parameters were found within each genotype, the results were pooled (mean values ± SEM, N = 22/genotype). Values for Vmax (B), uptake turnover rate (C; Vmax/Bmax) and Km (D) were determined from the curves in panel A. The group mean Bmax values (Fig. 2) were used to calculate uptake turnover rate in panel C. * p<0.05, DAT-LE vs WT.