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Increased expression of the dopamine transporter leads to loss of dopamine neurons, oxidative stress and L-DOPA reversible motor deficits

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Abstract

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The dopamine transporter is a key protein responsible for regulating dopamine homeostasis. Its function is to transport dopamine from the extracellular space into the presynaptic neuron. Studies have suggested that accumulation of dopamine in the cytosol can trigger oxidative stress and neurotoxicity. Previously, ectopic expression of the dopamine transporter was shown to cause damage in non-dopaminergic neurons due to their inability to handle cytosolic dopamine. However, it is unknown whether increasing dopamine transporter activity will be detrimental to dopamine neurons that are inherently capable of storing and degrading dopamine. To address this issue, we characterized transgenic mice that over-express the dopamine transporter selectively in dopamine neurons. We report that dopamine transporter over-expressing (DAT-tg) mice display spontaneous loss of midbrain dopamine neurons that is accompanied by increases in oxidative stress markers, 5-S-cysteinyl-dopamine and 5-S-cysteinyl-DOPAC. In addition, metabolite-to-dopamine ratios are increased and VMAT2 protein expression is decreased in the striatum of these animals. Furthermore, DAT-tg mice also show fine motor deficits on challenging beam traversal that are reversed with L-DOPA treatment. Collectively, our findings demonstrate that even in neurons that routinely handle dopamine, increased uptake of this neurotransmitter through the dopamine transporter results in oxidative damage, neuronal loss and LDOPA reversible motor deficits. In addition, DAT over-expressing animals are highly sensitive to MPTP-induced neurotoxicity. The effects of increased dopamine uptake in these transgenic mice could shed light on the unique vulnerability of dopamine neurons in Parkinson's disease.

Keywords

Dopamine transporter; Parkinson's disease; Transgenic mice; Cytosolic dopamine; Dopamine neuron loss; Oxidative stress; Motor deficits; L-DOPA; MPTP

INTRODUCTION

Malfunction of the dopamine system is implicated in several disease states including schizophrenia, addiction and Parkinson's disease (PD) (Howes and Kapur, 2009; Volkow et al., 2007; Fahn 2003). In particular, PD is characterized by a profound loss of nigrostriatal dopamine neurons leading to reduced dopamine levels in the basal ganglia (Fahn 2003). One of the key proteins involved in regulating dopaminergic tone is the dopamine transporter (DAT). DAT is located on the cell membrane of dopaminergic neurons and functions to rapidly take up dopamine from the extracellular space into the presynaptic neuron. DAT not only controls the magnitude and duration of extracellular dopamine signaling, but also acts to maintain intracellular dopamine levels. In DAT knock-out (DAT-KO) mice, stored dopamine levels are reduced by 95% despite an increase in the rate of dopamine synthesis (Giros et al., 1996; Jones et al., 1998). This dramatic decrease in dopamine tissue content is largely due to lack of uptake since the number of dopaminergic neurons in DAT-KO mice are mostly preserved (Giros et al., 1996; Jaber et al., 1999). These findings from DAT-KO animals highlight the critical role of DAT in loading the presynaptic neuron with dopamine, which has important physiological consequences.

Indeed, if cytosolic dopamine is not appropriately sequestered into vesicles, it can produce reactive oxygen species, quinones and toxic intermediates through metabolism, autooxidation

and enzyme-dependent reactions (Stokes et al., 1999; Graham et al., 1978; Ramkissoon and Wells, 2011; Goldstein et al., 2012). Several studies have shown that intracellular accumulation of dopamine can lead to oxidative stress and neurotoxicity. For instance, direct injection of dopamine into the rat striatum resulted in loss of dopaminergic cells - an effect that was rescued by antioxidant co-injection (Hastings et al., 1996). Notably, Mosharov et al. (2009) demonstrated that cytosolic dopamine levels directly impact toxicity in cultured midbrain neurons. They showed that blocking dopamine degradation led to accumulation of cytosolic dopamine and caused neurotoxicity, whereas inhibiting the conversion of L-3,4-dihydroxyphenylalanine (L-DOPA) to dopamine, reduced cytosolic dopamine levels and prevented neurotoxicity (Mosharov et al., 2009). Additionally, over-expression of vesicular monoamine transporter 2 (VMAT2), a protein that sequesters intracellular dopamine into vesicles and reduces cytosolic dopamine levels, has been shown to have protective effects against neuronal damage both in cultured midbrain neurons and mice (Mosharov et al., 2009; Lohr et al., 2014). Conversely, we have previously reported that genetic knockdown of VMAT2 in mice leads to oxidative stress and progressive degeneration of nigrostriatal neurons (Caudle et al., 2007). These studies suggest that amplifying the cytosolic pool of dopamine can aggravate oxidative damage and negatively impact neuronal survival.

In a previous study, Chen et al. (2008) showed that ectopic expression of DAT in GABAergic striatal neurons leads to progressive cell loss and oxidative protein modifications. These results indicated that ectopic DAT expression in non-dopaminergic neurons is deleterious since these cells do not possess the capacity to efficiently metabolize or store dopamine in vesicles. In the current study, we investigated whether increased DAT-mediated uptake of dopamine can produce damage in dopaminergic cells that routinely handle this neurotransmitter and are equipped to sequester and degrade it. In particular, we used bacterial artificial chromosome (BAC) transgenic mice that selectively over-express DAT in dopaminergic cells (DAT-tg mice, Salahpour et al., 2008). Previously, we reported that total DAT protein levels were increased in DAT-tg mice and expression of DAT was restricted to dopaminergic neurons (Salahpour et al., 2008). Functionally, transgenic animals showed a 46% increase in the rate of dopamine uptake and a 40% decrease in extracellular dopamine levels, which led to up-regulation of post-synaptic dopamine receptors (Salahpour et al., 2008; Kile et al., 2012; Calipari et al., 2013; Ghisi et al., 2009).

Here we determined the effects of increased DAT expression on dopamine homeostasis, neuronal survival, oxidative stress and motor behavior of DAT-tg mice. We also evaluated the response these animals to the PD-inducing neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al., 1983). Our results demonstrate that DAT-tg mice have 30-36% loss of midbrain dopamine neurons that is accompanied by evidence of oxidative stress. These animals also show fine motor deficits that are reversed by L-DOPA, the main treatment for motor symptoms of PD. In addition, DAT-tg mice are particularly vulnerable to dopaminergic damage induced by MPTP. These findings demonstrate that even in dopaminergic cells that endogenously express DAT, an increase in DAT-mediated uptake of dopamine leads to basal neurotoxicity and heightened sensitivity to exogenous insults.

METHODS

Mice

Generation of DAT-tg mice using BAC transgenesis has been described in Salahpour et al., 2008. Briefly, transgenic animals were created by pronuclear injection of a BAC containing the DAT locus and 80kb of upstream and downstream genomic sequences. Adult (3-5 month old) DAT-tg mice and their wild-type (WT) littermates (C57BL/6J background) were age and sex-matched across groups. All experiments were conducted in accordance with the Canadian Council on Animal Care and approved by the Faculty of Medicine Animal Care Committee at the University of Toronto.

Western blots

Western blots were used to quantify DAT, VMAT2, TH, GAPDH and α -tubulin protein expression in striatal tissue. Western blots were performed as previously described (Caudle et al., 2007; Salahpour et al., 2008). For DAT and its corresponding loading control, GAPDH, striatal tissue (pooled from 6 mice per sample) was homogenized in 320mM sucrose, 4mM HEPES buffer with protease and phosphatase inhibitors. These homogenized samples were then used to analyze protein concentration (BCA protein assay, Pierce). For TH and α -tubulin, striatal tissue was mechanically homogenized in RIPA buffer with protease inhibitors. Samples were centrifuged at 15,000rpm for 15 minutes and the supernatant was used to analyze protein concentration (BCA protein assay, Pierce). For VMAT2 and its corresponding loading control, GAPDH, striatal tissue was mechanically homogenized in 320mM sucrose, 5mM HEPES buffer with protease inhibitors. Homogenized samples were centrifuged at 3500rpm for 5 minutes and the supernatant was again centrifuged at 14,000rpm for 1 hour. The pellet was resuspended in homogenization buffer and used to analyze protein concentration (BCA protein assay, Pierce).

Protein extracts (20-30ug) were separated by 8.5% or 10% SDS/PAGE and transferred onto polyvinylidene difluoride membranes. Nonspecific binding was blocked using either 5-7.5% milk (TH, VMAT2 and corresponding loading controls) or Rockland blocking buffer (DAT and GAPDH loading control). Immunoblots were incubated overnight at 4°C with the following primary antibodies: rat anti-DAT (1:750, Millipore) rabbit anti-TH (1:3000, Millipore), mouse anti-GAPDH (1:4000, Sigma), rabbit anti-VMAT2 (1:20,000, obtained from Miller lab, Lohr et al., 2014) and mouse anti- α -tubulin (1:2000, Hybridoma Bank). Appropriate secondary antibodies (1:5000, Alexa Fluor 680 or IRDye 800CW, Rockland) were used and blots were developed using the LI-COR Odyssey Imaging System (LI-COR). Densitometric analysis of protein bands were performed using Image-J software (National Institutes of Health). GAPDH and α -tubulin immunoblots were used to normalize protein loading across samples.

HPLC with electrochemical detection

For measurement of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), dissected striata were homogenized in 0.1M perchloric acid and centrifuged ($9,400 \times g$ for 10 min at 4°C). The supernatant was filtered through a 0.22 μ m membrane (Millipore). Samples were analyzed using a Hypersil Gold C18 column (150 $\times$ 3mm; 5 μ m;

Thermo Scientific) and a LC-4C Amperometric Detector (BASi) set at an oxidizing potential of +0.75V. The mobile phase contained 24mM Na₂HPO₄, 3.6mM 1-octanesulfonic acid, 30mM citric acid, 0.14mM EDTA in 19% methanol, adjusted to pH 4.7 using concentrated NaOH. 5-S-Cysteinyldopamine and 5-S-cysteinyldopamine were measured as described previously (Hatcher et al., 2007; Caudle et al., 2007). Briefly, striatal samples were sonicated in 0.1M perchloric acid containing 347μM sodium bisulfite and 134μM EDTA. Homogenates were centrifuged, filtered and separated on a C18 column. The electrochemical detector was set at an oxidizing potential of +0.65V. The mobile phase was MD-TM (ESA) containing 2mM NaCl and adjusted to pH 2.1 using concentrated HCl. Quantification of all neurochemicals was conducted by referring to calibration curves constructed from pure standards (purity >98%; dopamine, DOPAC and HVA from Sigma Aldrich; 5-S-cysteinyldopamine and 5-S-cysteinyldopamine from NIMH Chemical Repository).

Fast-scan cyclic voltammetry (FSCV)

FSCV was performed according to Salahpour et al. 2008. Briefly, mice were anesthetized, decapitated, and coronal slices (300μm) from the striatum were cut and maintained in cold artificial cerebral spinal fluid (pH 7.4, 95% O₂ / 5% CO₂). Recordings were performed in a slice perfusion chamber at 37°C (Warner Instruments). DA release was electrically stimulated by biphasic (2ms/phase) constant-current (350μA) pulses via a tungsten bipolar electrode. The detector microelectrode was placed 75-100μm into the slice and 100-200μm away from the stimulating electrode. Release measurements were performed with and without 2β-propanoyl-3β-(4-tolyl)-tropane (PTT, 200nM, applied for 35mins).

Stereology

Mice were perfused with 4% paraformaldehyde. Brains were removed, stored in 10% sucrose/ 4% paraformaldehyde and coronally sectioned (60μm) with a Leica CM3050S cryostat (Leica). Coronal brain sections containing the substantia nigra and the ventral tegmental area (−2.92 to −3.64 mm from the bregma; Paxinos and Franklin, 2001) were kept at 4°C in phosphate buffered saline (PBS) and processed for free-floating immunofluorescence. Sections were incubated in permeabilizing solution containing 1.2% Triton X-100 in PBS followed by blocking solution containing 10% normal goat serum in PBS to avoid non-specific binding. Sections were then incubated with primary antibodies (rabbit anti-TH, 1:500; mouse anti-NeuN, 1:200, Millipore), secondary antibodies (fluorescein (FITC)-conjugated goat anti-mouse IgG, 1:500; DyLight 594-conjugated goat anti-rabbit IgG, 1:500; Jackson ImmunoResearch Laboratories) and Hoechst 33342 (1:10,000 in PBS, Invitrogen). Sections were mounted in Vectashield medium (Vector Laboratories) on Superfrost slides for visualization under a confocal spinning disk microscope (Olympus).

Number of NeuN and TH-positive cells in the SNc or VTA were determined by unbiased stereological quantification using the optical fractionator of Stereo Investigator software (MBF Bioscience). Five coronal sections containing the SNc and VTA were considered per animal: −2.92, −3.10, −3.28, −3.46 and −3.64 mm from the bregma (Paxinos and Franklin, 2001). Borders of the SNc or VTA were determined by TH-immunostaining using 2X objective. Cells were counted with a 60X PlanApo oil-immersion objective and 1.4

numerical aperture attached to an Olympus BX51 microscope. A systematic sampling of the outlined area was made from a random starting point. Counts were made at predetermined intervals ($x = 250$, $y = 150$) and a counting frame ($50 \mu\text{m} \times 50 \mu\text{m}$) was superimposed on live images of the brain sections. Section thickness was measured by focusing on the top of the section, zeroing the z-axis and focusing on bottom of the section (average section thickness was $60 \mu\text{m}$ with a range of $58.9 - 61.1 \mu\text{m}$). The dissector height was set at $50 \mu\text{m}$. Immunoreactive neurons were only counted if the recognizable profile came into focus within the counting frame. This method certified a uniform, random and systematic cell count. Focusing through the z-axis revealed that NeuN and TH antibodies penetrated the full depth of tissue sections.

Behavioral assessments

Baseline motor behavior of untreated animals was assessed using open field locomotion, stereotypy and wire-hang test. Open field locomotor activity and stereotypy were measured using the VersaMax Animal Activity Monitoring System (Omnitech Electronics). Mice were placed in acrylic chambers ($20\text{cm} \times 20\text{cm} \times 45\text{cm}$) and infrared light sensors were used to track movement. Locomotor activity was measured as total distance traveled. Stereotypic behavior was detected when the animal broke the same beam or set of beams repeatedly. Total distance traveled and stereotypy counts were recorded in five minute intervals over a two hour period. The wire-hang test was conducted by placing a mouse on a wire cage lid and shaking the lid slightly to make the animal grip the wires. Then the lid was inverted and suspended above a clean cage containing bedding. The latency of the mouse to fall off the grid was measured. Trials were stopped if the mouse remained on the lid for over 10 minutes. Average values were calculated from two trials (at least 15 minutes apart).

For challenging beam traversal, the effect of L-DOPA treatment on motor ability was evaluated. The challenging beam traversal was conducted as previously described by Fleming et al., 2004. Animals were trained to traverse the length of a Plexiglas beam consisting of four sections (25 cm each, 1 m total length) decreasing in width from 3.5 cm to 0.5 cm by 1 cm increments. Untreated mice were trained for two days to traverse the beam that led to the animal's home cage. On the third day (test day), a mesh grid (1 cm squares) of corresponding width was placed over the beam surface. A space of approximately 1 cm separated the grid from the surface of the beam. On the test day, animals were treated with 12.5 mg/kg of benserazide (i.p.), followed 20 minutes later by 25 mg/kg of L-DOPA (i.p.). In the control group, animals received two 0.9% saline injections separated by 20 minutes. Testing on the challenging beam began 10 minutes after the second injection. This treatment regimen has previously been used to assess L-DOPA effects on challenging beam motor behavior (Hwang et al., 2005). Animals were videotaped while traversing the grid-surfaced beam over three trials. Video was viewed in slow motion to detect 1) errors including paw slips through the mesh-grid and paws placed on the side rather the top of the grid during forward motion and 2) steps taken to traverse the beam. Errors per step were calculated and time to traverse the beam was also recorded. Number of errors, number of steps, errors per step and time to traverse the beam were quantified per trial and averaged over three trials for each animal.

All baseline motor behaviors were tested in both males and females. Sex-stratified results are shown in Supplementary Figure 1.

MPTP treatment

MPTP hydrochloride (Sigma Aldrich) was dissolved in PBS and administered (i.p. 0.1ml/g body weight) twice, 10 hours apart, at a dose of 15 or 30 mg/kg of body weight. Animals were sacrificed after seven days and brains were harvested for toxicity analyses.

Immunohistochemistry

Mice were perfused using 4% paraformaldehyde. Brains were removed, stored in 30% sucrose and coronally sectioned (50 μ m, Leica cryostat). Striatal sections were incubated with primary rabbit anti-TH antibody (1:500, Millipore) and appropriate secondary antibody (1:5000, Rockland Inc.). Immunofluorescence was visualized using the LI-COR Odyssey Imaging System (LI-COR).

Statistics

Data shown are means $\pm$ SEM. Data were statistically analyzed by two tailed t-tests, one-way ANOVA with Bonferroni post hoc tests, or two-way ANOVA with Bonferroni post hoc tests, as appropriate. GraphPad Prism and SPSS software were used for graphs and statistical analyses. Significance is reported at $p < 0.05$.

RESULTS

Altered dopamine homeostasis in DAT-tg mice

First, we confirmed over-expression of DAT protein in the striatum of DAT-tg mice using western blots ($p < 0.05$, Fig. 1). Then, we investigated the effect of increased DAT expression on dopamine homeostasis by assessing dopamine tissue content in the striatum of transgenic animals. DAT-tg mice showed a 33% reduction in dopamine tissue levels in comparison to wild-type (WT) animals ($p < 0.05$, Fig. 2A). To confirm this decrease in dopamine tissue content, we measured the electrically-evoked release of dopamine from striatal slices. The amount of dopamine released was reduced by 72% in striatal slices from DAT-tg animals ($p < 0.001$, Fig. 2B). To eliminate the confounding effect of enhanced dopamine uptake in DAT-tg mice, the dopamine release experiments were repeated in the presence of 2 β -propanoyl-3 β -(4-tolyl)-tropane (PTT), a quasi-irreversible DAT blocker (Bennett et al., 1995). Even with DAT blockade, there was a 46% reduction in dopamine release in DAT-tg mice ($p < 0.001$, Fig. 2B), which is in line with the lower dopamine tissue content observed in these animals. Next, we assessed dopamine metabolism in the striatum by measuring tissue levels of 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), the major metabolites of dopamine. DAT-tg animals showed a 60% increase in the DOPAC/dopamine ratio ($p < 0.01$, Fig. 2C) and a 38% increase in the HVA/dopamine ratio ($p < 0.01$, Fig. 2D), suggesting a higher turnover of dopamine in these animals.

Spontaneous loss of midbrain dopamine neurons and increased oxidative stress in DAT-tg mice

The reductions in dopamine tissue content (Fig. 2A) and release (Fig. 2B) could indicate compromised integrity of dopamine neurons in DAT-tg mice. To verify if these changes resulted from dopaminergic cell loss, we performed stereological counts of dopamine neurons in two major dopaminergic centers of the midbrain: 1) the substantia nigra pars compacta (SNc) and 2) the ventral tegmental area (VTA) (Fig. 3A). Using NeuN as a general neuronal marker, a 32% reduction in the overall number of neurons was observed in the SNc of DAT-tg mice ($p < 0.01$, Fig. 3B). Tyrosine hydroxylase (TH) was used as a specific marker of dopamine neurons and a 36% loss of TH-positive cells was detected in the SNc of transgenic animals ($p < 0.01$, Fig. 3B). In the VTA, the numbers of NeuN- and TH-immunoreactive neurons were reduced by 30% and 28% respectively, in DAT-tg animals (NeuN, $p < 0.05$; TH $p < 0.01$, Fig. 3C). These results indicate that increased DAT activity leads to loss of dopaminergic neurons in the midbrain.

Since previous studies have suggested that cytosolic dopamine is highly reactive and can induce oxidative stress, we investigated whether the neuronal death in DAT-tg mice might be associated with increased oxidative damage (Graham et al., 1978; Stokes et al., 1999; Hastings et al., 1996). The formation of cysteinyl adducts on dopamine and its metabolites are indicative of oxidative damage occurring specifically within dopaminergic neurons (Graham et al., 1978; Fornstedt and Carlsson, 1989; Hastings and Zigmond, 1994). Therefore, we measured 5-S-cysteinyl-dopamine and 5-S-cysteinyl-DOPAC tissue content as markers of oxidative stress in the striatum of DAT-tg mice. Despite a reduction in overall dopamine tissue levels (Fig. 2A), DAT-tg mice exhibit a 35% increase in 5-S-cysteinyl-dopamine ($p < 0.05$, Fig. 4A), in addition to a 62% increase in 5-S-cysteinyl-DOPAC levels ($p < 0.01$, Fig. 4B). Elevated tissue content of cysteinyl-dopamine and cysteinyl-DOPAC suggests that oxidative stress may underlie the dopaminergic cell loss observed in these mice.

Evidence of dopaminergic oxidative stress in DAT-tg mice implicates cytosolic dopamine as a potential source of oxidative damage. Aside from DAT-mediated uptake of dopamine, another important regulator of dopamine accumulation in the cytosol is VMAT2-mediated vesicular storage. Therefore, we evaluated VMAT2 protein levels in the striatum of DAT-tg mice. As shown in Figure 5, transgenic animals displayed lower VMAT2 protein levels ($p < 0.01$), suggesting that reduced vesicular storage could contribute to buildup of cytosolic dopamine in these animals.

Fine motor deficits in DAT-tg mice are reversed by L-DOPA treatment

Since the nigrostriatal dopamine pathway is heavily involved in controlling motor activity (Albin et al., 1989; Zhou and Palmiter, 1995), we assessed whether dopaminergic cell loss in DAT-tg mice had any influence on their baseline motor behavior. First, we measured open-field locomotion for two hours and found no changes in total distance traveled (Fig. 6A) or stereotypy (Fig. 6B) in DAT-tg mice. Next, animals were assessed using the wire-hang test, a measure of motor strength in rodent models (Luk et al., 2012; Oaks et al., 2013). DAT-tg

mice showed a 36% decrease in the latency to fall off the wire ($p < 0.05$, Fig. 6C), demonstrating compromised motor ability.

Furthermore, we evaluated the effects of L-DOPA treatment on the motor behavior of animals using the challenging beam traversal. This task is particularly sensitive to motor deficits that arise from nigrostriatal dopamine dysfunction (Drucker-Colín et al., 1991; Fleming et al., 2004). Saline-treated DAT-tg mice showed a 50% increase in number of errors (slips and misplaced paws) and a 47% increase in errors per step while traversing the beam ($p < 0.01$, Fig. 7A and $p < 0.01$, Fig. 7C, respectively). However, when treated with L-DOPA, DAT-tg animals performed significantly better as demonstrated by decreased errors, fewer steps taken and lower errors per step in comparison to saline-treated transgenic mice ($p < 0.01$, Fig. 7A; $p < 0.05$, Fig. 7B and $p < 0.05$, Fig. 7C, respectively). Across all groups, there were no differences in time to traverse the beam (Fig. 7D). Collectively, results from these behavioral tests indicate that although DAT-tg mice do not show any changes in gross locomotion, they display significant deficits in fine motor coordination that can be reversed with L-DOPA treatment.

DAT-tg mice are highly sensitive to MPTP-induced dopaminergic toxicity

Sensitivity of DAT-tg mice to exogenous toxicant insult was measured using two doses of MPTP, 15 and 30 mg/kg of body weight. We evaluated dopaminergic damage in the striatum by investigating expression of TH, a marker of dopaminergic cells. TH protein expression was assessed qualitatively by immunohistochemistry (Fig. 8A) and quantitatively by western blots (Fig. 8B,C). At 15 mg/kg of MPTP, DAT-tg mice displayed lower TH immunofluorescence (Fig. 8A) and protein levels ($p < 0.05$, Fig. 8C) than WT animals (Fig. 8A,B). Indeed, in WT mice, this dose of MPTP did not elicit any significant change in TH immunoreactivity (Fig. 8A) or protein levels (Fig. 8B) when compared to saline treatment. At 30mg/kg of MPTP, TH immunofluorescence was decreased in both WT and DAT-tg mice (Fig. 8A) however, the extent of reduction was greater in DAT-tg mice as quantified by western blot analysis (Fig. 8B,C). In particular, TH levels were reduced by 65% in transgenic animals ($p < 0.001$, Fig. 8C) in contrast to only 28% in WT animals ($p < 0.01$, Fig. 8B), when compared to saline treatment. These results demonstrate that DAT-tg mice are more vulnerable to MPTP treatment and exhibit sensitivity at doses that do not significantly affect WT animals.

Next, striatal dopamine tissue content was measured to assess the integrity of dopaminergic nerve terminals in MPTP-treated mice. At both 15 and 30 mg/kg of MPTP, the respective reductions in dopamine tissue content were greater in DAT-tg mice compared to WT controls, indicating that increased DAT levels exacerbate MPTP-induced neurotoxicity (15 mg/kg MPTP, $p < 0.05$; 30 mg/kg MPTP, $p < 0.01$; Fig. 9). A difference in striatal dopamine content was also detected between saline-treated WT and DAT-tg mice ($p < 0.01$, Fig. 9), corroborating the basal reduction in dopamine tissue levels previously observed in untreated transgenic animals (Fig. 2A).

DISCUSSION

In this study, we report that over-expression of DAT is capable of triggering oxidative stress, dopamine neuron loss and L-DOPA reversible motor deficits in DAT-tg mice. Previously, ectopic expression of DAT was shown to cause death of non-dopaminergic cells, presumably due to their inability to properly handle cytotoxic dopamine (Chen et al., 2008). However, we demonstrate that even in dopamine cells that are inherently equipped with the molecular machinery to properly store, metabolize and release dopamine, an increase in DAT expression can lead to higher dopamine uptake and damaging consequences. Aside from our work, previous studies using plasmid and lentiviral techniques have also reported that DAT over-expression can increase dopamine uptake and alter downstream behaviors (Martres et al., 1998, Adriani et al., 2009). Transgenic mice expressing DAT under the TH promoter showed higher DAT levels, greater dopamine uptake and modest, but significant reductions in striatal dopamine tissue content (Donovan et al., 1999), similar to DAT-tg mice. In comparison to these studies, our BAC transgenic approach to over-express DAT has several important advantages including: 1) robust, long-term DAT expression, 2) selectivity for dopaminergic neurons using the DAT promoter and 3) lack of injection and transfection-related complications. Collectively, this body of work shows that increased DAT activity can significantly impact and change dopamine homeostasis.

The dopamine system is notoriously sensitive to endogenous and exogenous challenges (Hastings et al., 1996; Mosharov et al., 2009; Langston et al., 1983). Therefore, 46% higher dopamine uptake in DAT-tg mice (Salahpour et al., 2008) produces dramatic effects on dopamine homeostasis, cell survival, oxidative stress and motor behaviors, as noted in this study. These results highlight the physiological importance of tightly regulating cytosolic dopamine levels since moderate deviations in dopamine compartmentalization can directly impact neuronal survivability. Another example of this is the VMAT2-knockdown (VMAT2-kd) mice. These animals express only 5% of normal VMAT2 protein and display decreased dopamine tissue content, nigrostriatal neurodegeneration and increased levels of cysteinyl-catechols (Caudle et al., 2007), similar to DAT-tg mice. Physiologically, VMAT2-kd mice are deficient in sequestering intracellular dopamine into vesicles while DAT-tg mice have excess dopamine uptake (Caudle et al., 2007; Salahpour et al., 2008). In addition to higher uptake, DAT-tg mice also have reduced VMAT2 expression, suggesting that vesicular storage of dopamine could also be compromised. Taken together, the genetic manipulations in VMAT2-kd and DAT-tg mice effectively act to increase the cytosolic pool of dopamine. This buildup of cytosolic dopamine could be a common pathway that is responsible for the basal loss of dopamine neurons and oxidative stress evident in both VMAT2-kd and DAT-tg mice.

There are several observations supporting the hypothesis that accumulation of cytosolic dopamine results in loss of dopaminergic neurons in DAT-tg mice. First, results from DAT-KO animals highlight the critical role of DAT in loading the presynaptic neuron with dopamine (Giros et al., 1996; Jones et al., 1998; Sotnikova et al., 2005). In DAT-KO mice, lack of uptake leads to 5-times higher extracellular dopamine levels and extremely low dopamine tissue content (5%), indicating depleted intracellular stores. Conversely, in DAT-tg mice, higher levels of functional DAT leads to a 46% increase in dopamine uptake and a

40% decrease in extracellular dopamine, suggesting that the neurotransmitter is accumulating in the presynaptic neuron (Salahpour et al., 2008). However, despite the likely buildup of dopamine within each dopaminergic cell, DAT-tg mice display a 33% reduction in overall dopamine tissue content as a direct consequence of 30-36% loss of dopamine neurons. Secondly, we report higher metabolite-to-dopamine ratios in DAT-tg mice. Since DOPAC is a direct product of cytosolic dopamine metabolism, a 60% increase in the DOPAC/dopamine ratio could indicate that a greater proportion of dopamine is present in the cytosol and not sequestered into vesicles (Di Monte et al., 1996). Elevated metabolite-to-dopamine ratios also imply enhanced dopamine turnover that could be a compensatory mechanism to tackle the buildup of intracellular DA (Zigmond et al., 2002). Thirdly, increased levels of 5-S-cysteinyldopamine and 5-S-cysteinyldopamine were detected in the striatum of DAT-tg mice. These cysteinyl-modified adducts have been suggested to arise from the oxidation of cytosolic dopamine and its metabolites (Hastings and Zigmond, 1994; Fornstedt and Carlsson, 1989; Graham et al., 1978). Not only are cysteinyl adducts a direct consequence of cytosolic dopamine reactivity, they are also capable of independently inducing further neuronal damage (Spencer et al., 2002). Next, lower VMAT2 protein expression in DAT-tg mice also suggests potential buildup of cytosolic dopamine. Although this decrease may be a reflection of dopaminergic cell loss per se, nonetheless, reduced VMAT2 levels can negatively impact vesicular storage, thus disabling these mice from handling increased dopamine uptake from DAT over-expression. Lastly, accumulation of cytosolic dopamine has been suggested to have deleterious effects on cell survival (Chen et al., 2008; Caudle et al., 2007; Mosharov et al., 2009) that is clearly reflected in the loss of dopamine neurons in DAT-tg mice. Collectively, these observations suggest that DAT over-expression most likely leads to high cytosolic levels of dopamine, thereby producing the downstream detrimental effects observed in DAT-tg mice.

We also demonstrated that DAT-tg mice are highly sensitive to MPTP-induced neurotoxicity. Indeed, when treated with MPTP, DAT-tg mice showed greater reductions in striatal TH levels and dopamine tissue content compared to WT animals. MPP⁺, the toxic metabolite of MPTP, is a substrate for DAT and therefore, causes selective damage to dopaminergic cells (Gainetdinov et al., 1997; Langston et al., 1984; Chiba et al., 1985; Ramsay et al., 1986; Schober et al., 2004). While the dependence of MPTP neurotoxicity on DAT function has previously been demonstrated (Gainetdinov et al., 1997; Bezard et al., 1999; Miller et al., 1999; Schober 2004), our results indicate a synergistic interaction between environmental and genetic risk factors that could have broader implications for complex pathological conditions such as PD (Cannon and Greenamyre, 2013). In PD, both genetic mutations and environmental conditions have been documented to increase disease risk (Hardy et al., 2006; Priyadarshi et al., 2000; Cannon and Greenamyre, 2013; Bezard et al., 2013; Martin et al., 2011). Moreover, animal models that depend on a single type of insult seldom recapitulate the full spectrum of the disorder (Beal, 2010). Although genes such as PINK1, DJ1 and PARK2 (parkin) have been implicated in familial forms of PD, mutating or knocking-out these essential genes in most animal models does not reproduce dopaminergic cell loss (Gispert et al., 2009; Yamaguchi and Shen, 2007; Goldberg et al., 2003). Conversely, while acute toxicant treatment (e.g. MPTP or 6-hydroxydopamine) can produce abrupt neurodegeneration, it does not address the underlying disease mechanism of

a chronic and progressive disorder like PD (Schober 2004). Given the shortcomings of these individual approaches, the convergence of genetic as well as environmental insults may be more representative of idiopathic PD that is hypothesized to arise from multiple hits (Cannon and Greenamyre, 2013; Sulzer, 2007). Our results lend support to this idea by showing that genetic over-expression of DAT combined with exogenous exposure to MPTP, aggravates toxicity to dopamine neurons. Although the effect of genetic mutations on DAT expression is unclear in humans, a correlation study reports that DAT genetic variants in combination with exposure to exogenous compounds (e.g. pesticides) can potentiate the risk of developing PD by 3 or 4 fold (Ritz et al., 2009). This highlights the significance of genetic and environmental interactions in the pathology of PD.

The cellular, neurochemical and behavioral changes observed in DAT-tg mice recapitulate important features of PD. Firstly, loss of midbrain dopamine neurons and reduced dopamine tissue content in the striatum of DAT-tg mice capture the major pathological characteristics of PD (Dauer and Przedborski, 2003). However, it should be noted that PD is characterized by selective nigrostriatal degeneration, whereas DAT-tg mice also demonstrate loss of VTA dopamine neurons. This is probably due to transgenic over-expression of DAT in the VTA, which enhances the vulnerability of this region in DAT-tg mice. Physiologically, VTA neurons do not express as much DAT as SNc neurons and therefore, the VTA is relatively spared from damage in PD (Blanchard et al., 1994). The relationship between DAT expression and neurodegeneration is supported by a study in PD patients showing that brain regions containing the highest levels of DAT protein – the caudate and putamen – are also the most sensitive to damage (Miller et al., 1997). In addition, a recent meta-analysis has identified the DAT gene as a risk factor for PD in certain populations (Zhai et al., 2014). Secondly, oxidative stress has long been postulated to be involved in the development of PD (Fahn and Cohen, 1992) and we report that DAT-tg mice display increased levels of cysteinyl-dopamine and cysteinyl-DOPAC, two markers that are also elevated in the SN of PD patients (Spencer et al., 1998). Thirdly, increased dopamine turnover in the transgenic mice mirrors elevated metabolite-to-dopamine ratios that have been reported in PD patients (Zigmond et al., 2002; Rabey and Burns, 2002). In addition, both DAT-tg mice and PD patients show reductions in VMAT2 protein expression in comparison to control samples (Miller et al., 1999). Behaviorally, DAT-tg mice do not exhibit any deficits in gross locomotion, probably because the level of cell loss in these animals is not sufficient to cause major motor disturbances. In PD patients, motor deficits are only evident when greater than 70% of dopaminergic tone is lost in the striatum (Bernheimer et al., 1973). However, results from the wire-hang test and challenging beam traversal clearly demonstrate that fine motor coordination, balance and strength are compromised in DAT-tg mice similar to PD patients. Other studies on dopaminergic dysfunction have shown that these two tests are sensitive to motor impairment even in the absence of gross locomotor changes (Hwang et al., 2005; Luk et al., 2011). Furthermore, not only do DAT-tg mice display motor disturbances on the challenging beam traversal; these deficits are also reversed by L-DOPA, the principal treatment for motor symptoms of PD. This suggests that dopamine neuronal loss in DAT-tg mice leads to motor deficits that can be reversed by restoring dopaminergic tone. Hence, parallel to PD patients, DAT-tg mice also demonstrate motor behaviors that are responsive to L-DOPA treatment. Given these overlapping results, we postulate that the mishandling of

cytosolic dopamine exhibited by DAT-tg mice could provide important insights on the unique vulnerability of dopamine cells in PD.

In conclusion, we used transgenic mice that selectively over-express DAT in dopaminergic neurons to investigate the effects of cytosolic dopamine accumulation *in vivo*. As shown by our results, moderate increases in DAT function cause spontaneous dopaminergic cell loss, oxidative stress and fine motor impairment that is reversed by L-DOPA treatment. These results suggest that the integrity of dopamine neurons depends heavily on the ability of DAT to maintain proper homeostatic control of presynaptic dopamine. Since dopaminergic cells are selectively damaged by a broad variety of genetic and environmental insults, it demonstrates that these cells are inherently at risk. Our results imply that buildup of cytosolic dopamine, a highly reactive and potentially toxic molecule, may underlie the cell-specific vulnerability of dopaminergic neurons to damage. We propose that dopamine uptake through DAT, maintains a constant cytosolic pool of this neurotransmitter that can propagate oxidative stress in dopamine cells. This type of chronic damage may render these neurons vulnerable to degeneration, especially if coupled with other genetic or environmental insults that are linked with the pathogenesis of PD. Since DAT-tg mice display spontaneous neuronal loss and heightened toxicity in response to MPTP, these mice provide a useful tool to study the effects of endogenous and exogenous challenges on dopamine cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

DAT	dopamine transporter
PD	Parkinson's disease
DAT-tg	dopamine transporter over-expressing transgenic
DAT-KO	dopamine transporter knock-out
VMAT2	vesicular monoamine transporter 2
VMAT2-kd	vesicular monoamine transporter 2 knock-down
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
DOPAC	3,4-dihydroxyphenylacetic acid
HVA	homovanillic acid

BAC	bacterial artificial chromosome
FSCV	fast-scan cyclic voltammetry
PTT	2 β -propanoyl-3 β -(4-tolyl)-tropane
TH	tyrosine hydroxylase
SNc	substantia nigra pars compacta
VTA	ventral tegmental area
L-DOPA	L-3,4-dihydroxyphenylalanine

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HIGHLIGHTS

Dopamine transporter (DAT) over-expression leads to loss of midbrain dopamine neurons.

Neuronal loss is accompanied by oxidative stress and fine motor deficits.

Motor deficits on challenging beam traversal are reversed by L-DOPA treatment.

DAT transgenic mice are highly sensitive to MPTP-induced neurotoxicity.

Deleterious effects of DAT over-expression may be due to increased cytosolic dopamine.

Figure 1.

Over-expression of DAT protein in DAT-tg mice. DAT western blot and densitometry analysis of striatal tissue from WT and DAT-tg mice (pooled striata from 6 mice per sample, n=18 mice in total per genotype). DAT levels were corrected for loading using GAPDH and normalized to WT expression. Data shown are means $\pm$ SEM. * $p<0.05$.

Figure 2.

Altered dopamine homeostasis in DAT-tg mice. A- Striatal dopamine tissue content (n=7-9). B- Stimulated dopamine release assessed in brain slices in the absence or presence of PTT, a potent DAT blocker (n=4). C- Ratio of DOPAC-to-dopamine tissue content in the striatum (n=10-11). D- Ratio of HVA-to-dopamine tissue content in the striatum (n=10-11). Data shown are means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3.

Dopaminergic cell loss in the SNc and VTA of DAT-tg mice. A- Representative midbrain images double-labeled for NeuN (green) and TH (red). IF, immunofluorescence; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; VTA, ventral tegmental area. Scale bar = 100 μ m. Stereological counts of NeuN and TH-positive neurons are shown in the B- SNc and C- VTA. Significant differences (denoted by lines) are in comparison to corresponding WT animals. Data shown are means $\pm$ SEM (n= 3-7). *p<0.05 and **p<0.01.

Figure 4.

Increased markers of oxidative stress in the striatum of DAT-tg mice. HPLC quantification of A- 5-S-cysteinyldopamine and B- 5-S-cysteinyldopamine-DOPAC tissue content in the striatum of WT and DAT-tg mice (n=9-10). Data shown are means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$.

Figure 5.

Reduced VMAT2 protein expression in DAT-tg mice. VMAT2 western blot and densitometry analysis of striatal tissue from WT and DAT-tg mice (N=4). VMAT2-knockdown (VMAT2-kd) samples were used as a negative control to identify the specific VMAT2 band. VMAT2 levels were corrected for loading using GAPDH and normalized to WT expression. Data shown are means $\pm$ SEM. ** $p < 0.01$.

Figure 6.

Locomotor behavior of DAT-tg mice. A- Total distance traveled and B- stereotypy counts from WT and DAT-tg mice tested in open field activity monitors for two hours (n=25-28). Stereotypy counts are defined as the number of beam breaks detected on the infrared monitor during stereotypic behavior. C- Average latency of mice to fall off the wire in the wire-hang test (n=37-40). Data shown are means $\pm$ SEM. *p<0.05.

Figure 7.

Challenging beam deficits in DAT-tg mice are reversed by L-DOPA administration. Animals were injected with benserazide (12.5 mg/kg), followed 20 minutes later by L-DOPA (25 mg/kg). Control animals were injected with 0.9% saline separated by 20 minutes. Mice were tested on the challenging beam traversal task (3 trials) 10 minutes after the second injection. A- Number of errors (including slips and misplaced paws) made while traversing the beam. B- Number of steps taken to traverse beam. C- Number of errors per step taken. D- Time to traverse the beam. (n=8-13). Data shown are means $\pm$ SEM. *p<0.05, **p<0.01

Figure 8.

Reduced tyrosine hydroxylase (TH) protein levels in MPTP-treated DAT-tg mice. A- Immunohistochemical analysis of TH in the striatum of WT and DAT-tg mice treated with saline, 15 or 30 mg/kg of MPTP. Representative TH-labeled (black) coronal sections are shown. Western blot analysis of TH protein expression in the striatum of B- WT and C- DAT-tg mice treated with saline, 15 or 30 mg/kg of MPTP (n=3-4). TH levels were corrected for loading using α-tubulin and normalized to WT expression. Data shown are means ± SEM. Differences are in comparison to saline-treated animals. * p<0.05, **p<0.01, ***p<0.001.

Figure 9.

Increased dopaminergic damage in response to MPTP treatment in DAT-tg mice. Relative striatal dopamine tissue content is shown for mice treated with saline, 15 or 30 mg/kg of MPTP (n=7-9). Levels are represented as percent of WT saline-treated mice. Significant differences are in comparison to WT mice at each dose. Data shown are means $\pm$ SEM.

*p<0.05; **p<0.01.