Published in final edited form as:

Neurobiol Dis. 2012 October ; 48(1): 66–78. doi:10.1016/j.nbd.2012.05.009.

Functional Analysis of Dopaminergic Systems in a DYT1 Knock-in Mouse Model of Dystonia

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Abstract

The dystonias are a group of disorders characterized by involuntary twisting movements and abnormal posturing. The most common of the inherited dystonias is DYT1 dystonia, which is due to deletion of a single GAG codon (ΔE) in the *TOR1A* gene that encodes torsinA. Since some forms of dystonia have been linked with dysfunction of brain dopamine pathways, the integrity of these pathways was explored in a knock-in mouse model of DYT1 dystonia. In DYT1(ΔE) knock-in mice, neurochemical measures revealed only small changes in the content of dopamine or its metabolites in tissue homogenates from caudoputamen or midbrain, but microdialysis studies revealed robust decreases in baseline and amphetamine-stimulated extracellular dopamine in the caudoputamen. Quantitative stereological methods revealed no evidence for striatal or midbrain atrophy, but substantia nigra neurons immunopositive for tyrosine hydroxylase were slightly reduced in numbers and enlarged in size. Behavioral studies revealed subtle abnormalities in gross motor activity and motor coordination without overt dystonia. Neuropharmacological challenges of dopamine systems revealed normal behavioral responses to amphetamine and a minor increase in sensitivity to haloperidol. These results demonstrate that this DYT1(ΔE) knock-in mouse model of dystonia harbors neurochemical and structural changes of the dopamine pathways, as well as motor abnormalities.

Keywords

Mouse mutant; behavior; biochemistry; anatomy; neuropharmacology; microdialysis

Introduction

The dystonias are a group of disorders characterized by involuntary muscle contractions that often lead to twisting movements or odd postures (Fahn, 1988; Tarsy and Simon, 2006). There are many different etiologies, both genetic and acquired. The pathogenesis of the abnormal movements is thought to involve dysfunction of motor circuits of the basal ganglia, cerebellum, thalamus, and cerebral cortex (Neychev et al., 2011). Several dystonic

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disorders have been linked with dysfunction of dopaminergic pathways in the brain (Perlmutter and Mink, 2004; Wichmann, 2008). For example, dystonia is a prominent feature of inherited disorders that disrupt dopamine synthesis, known as the dopa-responsive dystonias (Segawa et al., 2008). Dystonia also may occur in degenerative disorders that affect dopamine neurons, such as Parkinson's disease (Tolosa and Compta, 2006; Wickremaratchi et al., 2011). Additionally, dystonia may occur as an acute or chronic side effect of therapy with dopamine receptor antagonists (Cardoso, 2008).

Although some dystonias are linked with dysfunction of dopaminergic pathways, the nature of the dysfunction and how it may cause dystonia is not clear. Furthermore, other forms of dystonia are not clearly linked with any obvious dopaminergic defect. The most frequent genetic form of early-onset dystonia, DYT1 dystonia, is a dominantly inherited disorder caused by a common GAG deletion in the *TOR1A* gene, which encodes torsinA (Tanabe et al., 2009). PET studies have described small reductions in striatal D2 dopamine receptors (Asanuma et al., 2005; Carbon and Eidelberg, 2009), and postmortem studies of autopsied brains have revealed subtle neurochemical and histological changes in the dopaminergic pathways (Augood et al., 2004; Furukawa et al., 2000; Rostasy et al., 2003). However, the reported abnormalities always have been quite small, and individuals with DYT1 dystonia do not respond to medications alter dopamine transmission. Although early studies suggested that torsinA might be expressed at particularly high levels in dopamine neurons (Augood et al., 1999; Augood et al., 1998), subsequent studies revealed a relatively broad pattern of expression that is not particularly enriched in these neurons (Konakova et al., 2001; Konakova and Pulst, 2001; Rostasy et al., 2003; Shashidharan et al., 2000; Xiao et al., 2004). At the cellular level, torsinA functions as a molecular chaperone with no specific mechanistic links to dopamine neurons (Granata et al., 2009). Taken together, these observations provide only circumstantial evidence for the hypothesis that dopamine dysfunction is responsible for abnormal movements in DYT1 dystonia.

DYT1 dystonia is relatively rare, and the types of studies required to implicate dopamine pathways are not easily accomplished in humans. In this situation, animal models can be valuable for exploring dopamine pathways (Jinnah et al., 2005). Fortunately, several genetic mouse models of DYT1 dystonia have been developed (Table 1). Multiple transgenic lines have been created, with mutant human torsinA being driven by the cytomegalovirus promoter for global expression (CMV-transgenics) (Sharma et al., 2005), the neuron-specific enolase (NSE-transgenics) (Shashidharan et al., 2005) or prion-protein promoters (PrP-transgenics) (Grundmann et al., 2007) for broad expression among neurons, or the tyrosine hydroxylase (TH) promoter to restrict expression to midbrain dopamine neurons (TH-transgenics) (Page et al., 2010). Although mice in which the gene is knocked out globally are not viable, there are knock-down mice (Dang et al., 2006) as well as mice in which the gene has been selectively deleted in the cerebral cortex (Yokoi et al., 2008), striatum (Yokoi et al., 2011), or cerebellum (Yokoi et al., 2012; Zhang et al., 2011). Finally, two different knock-in lines have been created, with the homologous mutation in the mouse *Tor1A* gene driven by its endogenous promoter (Dang et al., 2005; Goodchild et al., 2005).

Even though overt signs of dystonia are lacking in these mouse models of DYT1 dystonia, they provide useful tools for detailed investigations of how mutant torsinA may influence dopamine neurons (Wichmann, 2008). Prior biochemical studies of several of these models have shown small but inconsistent changes of total striatal dopamine levels (Table 1). However, microdialysis studies have revealed robust decrements in striatal dopamine release in response to dopamine-releasing agents in the CMV-transgenics and the TH-transgenics (Balcioglu et al., 2007; Page et al., 2010). Although Nissl stains have revealed no overt anatomical abnormalities, precise histological studies of dopamine neurons are lacking. In the current studies the integrity of dopamine pathways was probed in a heterozygous mutant

DYT1(Δ E) knock-in model using a combination of neurochemical, anatomical, and behavioral methods.

Materials & Methods

Mice

Heterozygous DYT1(Δ E) mutant knock-in mice (Goodchild et al., 2005) were maintained congenically with C57BL/6J mice from the Jackson Laboratories (Bar Harbor ME). Mice were housed with a 12 hr light/dark cycle. Food and water were provided *ad libitum*. Tail DNA was genotyped using a primer pair to detect the 34 base pair *loxP* site in the DYT1 mutant (forward primer, AGTCTGTGGCTGGCTCTCCC; reverse primer, CCTCAGGCTGCTCACAACCAC). Because some prior studies have suggested that abnormalities may be dependent on sex or age (Dang et al., 2005; Grundmann et al., 2007; Page et al., 2010; Shashidharan et al., 2005), both male and female mutant (Dyt1 Δ E/+) and littermate controls (Dyt1+/+) were evaluated at 3, 6 and 10 months of age. Unless otherwise noted, all behavioral studies and tissue collections were performed during daylight hours. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Emory University.

Locomotor activity

Gross levels of spontaneous motor activity were examined using automated 29 $\times$ 50 cm photocell activity cages equipped with 12 infrared beams arranged in a 4 $\times$ 8 grid (San Diego Instruments, San Diego CA) as previously described (Jinnah et al., 1992). Each animal was acclimated to the test cage from 10:00h to 17:00h on 3 separate days. On the test day, the mice were allowed a 4 hr habituation period, and gross motor activity was recorded at 10 min intervals for 24 hr. Mice had access to food and water *ad libitum* during the entire test.

Amphetamine-induced stimulation of gross motor activity was recorded at 10 min intervals for 4 hr following an intraperitoneal injection of 2, 4 or 8 mg/kg amphetamine (Jinnah et al., 1992). Acclimation was conducted as described above, with a one-week interval between each dose. Haloperidol-induced suppression of gross motor activity was recorded similarly after a subcutaneous injection of 0.25 mg/kg in a separate cohort of mice.

Rotarod test

Motor skills were assessed with an Economex accelerating Rotarod apparatus (Columbus Instruments, Columbus OH) (Jones and Roberts, 1968). This apparatus measures motor coordination, with different paradigms that are preferable for revealing problems with endurance or adaptive motor learning. Fixed speeds for long periods are preferable for evaluating endurance, while short periods of progressively increasing speeds are preferable for adaptive motor learning. To focus on potential defects in motor coordination and especially motor learning, mice were acclimated for 30 sec at 4 rpm, and rotation speed increased by 6 rpm/min. Mice naive to the test apparatus were tested for 5 consecutive days, with an initial maximum recording time of 150 sec. The recording time increased by 30 sec each successive day. The mean latency to fall was determined from 3 trials per test session separated by 5–10 min for each mouse.

Beam walking test

Motor coordination and balance were assessed by examining the ability of mice to traverse a wooden beam 100 cm long (Dang et al., 2005; Grundmann et al., 2007; Zhao et al., 2008). Each mouse was individually trained to traverse a square beam 2 cm wide by allowing 3 trials separated by 5–10 min on each of 3 consecutive days, for a total of 9 training trials.

The mice were encouraged to cross the beam by starting them at an exposed platform on one end, with the home cage on the other end. Following the training period, each mouse was tested on a 1 cm wide beam. The number of foot slips and the time required to traverse the beam were recorded for 3 separate trials for each mouse.

Gait pattern analysis

Detailed kinematic analyses of the gait were done using the DigiGait imaging system (Hampton et al., 2004; Kale et al., 2004). The apparatus consists of a motorized treadmill with a digital camera positioned below a transparent belt (Mouse Specifics Inc., Boston MA). For each mouse, the location and timing of each paw contact on the belt was automatically recorded at a belt speed of 35 cm/sec for 5–10 sec after acclimation at 20 cm/sec for 2 min. A minimum of 500 video frames collected at 100 frames/sec was digitized and calculated by the accompanying software for each animal.

This system has the ability to measure multiple aspects of the gait. A stride cycle represents the combination of stance and swing phases for each limb. The stance phase is defined by the period when the paw is in contact with the floor, and is divided into braking and propulsion phases. The swing phase represents the time that the paw is lifted. Since the treadmill operated at a fixed speed of 35 cm/sec, gait parameters for a stride cycle are inter-dependent. Thirteen core gait parameters examined included stance time, swing time, propulsion time, braking time, stride time, stride length, stride frequency, stance width, step angle, paw angle, paw area, overlap distance and paw placement. Many additional derived variables available through this system, such as ratios of various core measurements or coefficients of variation, were not analyzed.

Tissue monoamines

For micro-dissection of brain tissues, mice were killed by decapitation and the brain was rapidly removed, placed into ice-cold saline for 60 sec, and dissected on an ice-cooled platform as previously described (Jinnah et al., 1994). Regional dissections focused on the frontal cortex, accumbens, caudoputamen, midbrain and cerebellum. Tissue was stored frozen at -80°C until analysis. For analysis, tissue was homogenized in 100 mM perchloric acid by probe sonication at 4°C , and centrifuged at 10,000 g for 10 min. The supernatant was filtered through 0.45 μm membranes (Micro-Spin Centrifuge Filter Tubes, Grace Davison Discovery Sciences, Deerfield IL) before monoamine analysis. The insoluble precipitate was dissolved in 2% SDS to measure protein levels with the BCA assay kit (Pierce, Rockford IL).

Monoamines were examined by high performance liquid chromatography with electrochemical detection as described previously (Jinnah et al., 1994). The system included an ESA MD-150 $\times$ 3.2 mm column, an ESA 5020 guard cell, and an ESA 5600A Coularray detector with an ESA 6210 detector cell (ESA, Bedford MA). The guard cell potential was 475 mV; and the analytic cell potentials were set at -175 , 100, 350 and 425 mV. Samples were eluted at a flow rate of 0.4 mL/min with a mobile phase consisting of 1.7 mM 1-octanesulfonic acid sodium, 75 mM NaH_2PO_4 , 0.25% triethylamine, and 8% acetonitrile at pH 2.9. Monoamines were identified by retention time and electrochemical profile in comparison with known standards consisting of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 3-methoxytyramine (3MT) from Sigma Chemical Co. (St. Louis MO).

In vivo microdialysis

Microdialysis was performed as described previously to measure monoamines released in the caudoputamen (Fan and Hess, 2007). Briefly, a concentric microdialysis probe was

constructed and calibrated with 100 ng/mL dopamine in artificial cerebrospinal fluid consisting of 147 mM NaCl, 3.5 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, and 1 mM NaH₂PO₄ at pH 7.0–7.4. After anesthetization with 2,2,2-tribromoethanol, the probe was implanted into the caudoputamen (anterior 0.6 mm, lateral 1.7 mm, and ventral 4.5 mm from bregma), and perfused at a flow rate of 0.6 μ L/min while the mice habituated to the microdialysis environment overnight. The following day, the mouse was subjected to no-net flux or conventional microdialysis. For no-net flux microdialysis, 0, 2, 10 and 20 nM dopamine (DA_{in}) including 250 μ M ascorbic acid were perfused in random order at 0.6 μ L/min, and 3 samples (DA_{out}) were collected at 40 min intervals allowing dopamine equilibration. The basal dopamine level was determined using linear regression analysis of the gain or loss of dopamine from the perfusate (DA_{in} - DA_{out}) versus DA_{in}. For conventional microdialysis, consecutive samples were collected at 40 min intervals in tubes containing 1 μ L of 6.25 mM ascorbic acid for the determination of basal monoamines. Mice then received 4 mg/kg amphetamine in saline via intraperitoneal injection, and 6 additional samples were collected every 20 min for 2 hr. The samples were stored at -80 °C until analysis for monoamines as described above. After sample collection, the probe location was confirmed by a perfusion of 3% bromophenol blue, and only samples from mice with the probe in the appropriate region of the caudoputamen were included in the final analyses.

Histological analyses

Histological stains were conducted in serial sections from the frontal pole of the cerebral cortex through the caudal brainstem and cerebellum. The mice were anesthetized with 2,2,2-tribromoethanol, and perfused through the heart first with 50 mL of 137 mM NaCl, 22.2 mM dextrose, 23.4 mM sucrose, 2 mM CaCl₂ and 1.6 mM sodium cacodylate at pH 7.2. They then were perfused with 4% paraformaldehyde containing 117 mM sucrose and 67 mM sodium cacodylate for 16 hr followed by 67 mM sodium cacodylate solution (pH 7.2). Fixed brains were sent to Neuroscience Associates (Knoxville TN) for coronal sectioning at 40 μ m. Every 6th section was stained with thionine to identify Nissl substance.

Parallel series of sections at the same intervals were immunostained for TH (Pelfreez, Rogers AR) or calbindin (Swant, Bellinzona, Switzerland). Sections were viewed using an Olympus BX51 light microscope (Melville NY), equipped with a motorized stage (MAC5000, Ludl Electronic Products, Hawthorne NY) and coupled to a computer with StereoInvestigator for stereological measures (MicroBrightField, Williston VT). The TH-positive region of the midbrain was outlined using a 4X objective, and adjacent Nissl and calbindin-stained sections were used as a guide to estimate borders separating the substantia nigra (area A9), ventral tegmental area (area A10) and retrorubral field (area A8). TH-positive cells were counted at 100X using an optical fractionator with a 14,400 μ m² counting grid and a 2500 μ m² frame with 15 μ m depth and 1 μ m top guard zone as described previously (Egami et al., 2007). The volume of the TH-positive cells was determined using the optical rotator with a 3 μ m focal plane separation and a 3 grid line separation of 7 μ m. The TH-positive region of the striatum also was outlined using a 4X objective, and the borders of the caudoputamen and accumbens estimated by following cytoarchitectonic borders. The volumes of the caudoputamen and accumbens were estimated via the Cavalieri method with 30 μ m spacing. For all measures, the Gundersen coefficient of error was below 0.1.

Tissue culture model

Rat PC6-3 clonal cell lines (PC6-parent) with inducible expression of normal human torsinA (PC6-nor) or mutant human torsinA (PC6-mut) were kindly provided by Dr. Gonzalez-Alegre. They were grown as previously described, with addition of varied concentrations of doxycycline to induce expression of the transgenes (Gonzalez-Alegre et al., 2005).

For immunoblotting, approximately 1×10^6 cells were seeded in 6-well culture plates, and treated with different concentrations of doxycycline (Sigma Chemical Co, St. Louis MO) for 1 to 3 days. The cells were scraped from the plates, centrifuged at 400 g for 3 min at 4 °C, and the pellet was incubated for 30 min at 4 °C in 150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris buffer at pH 8.0. Following centrifugation to remove insoluble debris, the supernatant was subjected to protein assay or immunoblotting after dissolving in 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, and 0.008% bromophenol blue in 125 mM Tris buffer at pH 6.8. Electrophoretic separation of proteins was performed using 12% Tris-HCl ready gels (Bio-Rad, Hercules CA), and the protein was transferred onto Immobilon-P PVDF membranes (Amersham Pharmacia Biotech, Piscataway NJ) with a Transblot apparatus (Bio-Rad, Hercules CA). After blocking in 5% nonfat milk in phosphate-buffered saline (PBS) including 0.1% Tween-20 (PBST) for 30 min, the membrane was incubated with primary antibodies in 1% nonfat milk-PBST for 1 hr at a room temperature. Antibodies included rabbit polyclonal for human torsinA (1:1000, kindly provided by Dr. Gonzales-Alegre) and mouse monoclonal antibody for α -tubulin (1:1000, Sigma Chemical Co., St. Louis MO) as a control. After washing with PBST, the membrane was incubated with horseradish-peroxidase-labeled secondary antibodies for rabbit IgG (1:3000; Cell Signaling, Beverly MA) or mouse IgG (1:3000; Bio-Rad, Hercules CA) for 1 hr at room temperature, and visualized by chemiluminescence (Bio-Rad, Hercules CA) according to the manufacturer's instructions. The bands were quantified using a LAS-3000 phosphorimager with Image Reader software version 2.2 (Fuji Film, Tokyo, Japan). TorsinA expression was quantified using Fuji Film, Multi Gauge Ver 3.0 (Fuji Film, Tokyo, Japan), and normalized to α -tubulin levels.

For cell growth and TH enzymatic assays, cells were seeded at a density of 1×10^4 in 96-well plates in triplicate. Cell growth was determined with Cell Counting kit 8 (Dojindo Molecular Technologies, Gaithersburg MD) according to the manufacturer's instructions using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale CA). TH activity was measured on the microplate reader with the fluorogenic peptide substrate, tyrosine-7-amino-4-methylcoumarin (Tyr-AMC, Bachem, Torrance CA), with emission at 460 nm following excitation at 380 nm. Each culture experiment was repeated three times.

Data analysis

The strategy for data analysis varied according to the different experimental designs employed. The general approach was to use Analysis of Variance (ANOVA), with repeated measures where appropriate for measurements that were highly inter-dependent. Experiments were repeated in animals of both sexes and across different age groups where prior studies suggested abnormalities may depend on sex or age, where prior studies yielded conflicting results, or when subtle or borderline differences between normal and mutant animals were found in initial studies. Replication of experiments with different age groups was not conducted where results showed obvious abnormalities with a single cohort of animals. Because the main focus was to delineate differences between the DYT1(ΔE) knock-in mutants and normal mice, the statistics of interest were main effects for genotype or interactions between genotype and another factor. If the initial ANOVA failed to reveal sex or age to be significant factors, the relevant groups were combined for the final analysis. For all analyses, $p < 0.05$ determined statistical significance.

Results

Gross motor activity

To identify changes in gross motor activity, baseline locomotion was compared in mutant and normal mice of both sexes over the entire 24 hr light/dark cycle in 2 independent

cohorts of mice at 3 and 6 months of age. The circadian pattern of motor activity appeared grossly similar in normal and mutant mice (Figure 1A). The results were analyzed by ANOVA with main factors of genotype, sex and age. Recording time bin was treated as a repeated measure. There was a significant effect of recording time reflecting the normal circadian pattern of activity ($F=33.9$; $p<0.01$), but no interaction between the time bin and genotype ($F=0.7$; $p>0.10$). There were significant main effects of sex showing higher overall activity in females compared with males ($F=7.1$; $p<0.05$), but no effects for genotype ($F=1.9$; $p>0.10$) or age ($F=0.01$; $p>0.10$). There were no significant interactions between genotype and sex ($F=0.03$; $p>0.10$) or genotype and age ($F=1.6$, $p>0.10$).

In a second type of analysis used in some previously published studies, individual time bins were summed to estimate total daytime and nighttime activities, and the results were analyzed by ANOVA with main factors of genotype, sex and age (Figure 1B). In this analysis, activity appeared 22% higher in mutant animals compared with controls during the daylight period ($F=7.3$; $p<0.05$). These results suggest a small increase in baseline locomotor activity in the mutant animals that may be dependent upon the type of analysis employed.

Motor skills & motor learning

To delineate potential impairments in motor learning or coordination, mutant and control mice of both sexes were compared for 5 consecutive days on the accelerating Rotarod in 3 independent cohorts at 3, 6 and 10 months of age (Figure 2A). The results were analyzed by ANOVA with main factors of genotype, sex and age. Test day was treated as a repeated measure and used as a surrogate for motor learning. There was a significant main effect for age ($F=10.0$; $p<0.01$), but not for genotype ($F=1.2$; $p>0.10$) or sex ($F=0.1$; $p>0.10$). There was a significant effect of test day ($F=198.4$; $p<0.01$), but no interaction between genotype and test day ($F=2.0$; $p=1.0$). There were no interactions between genotype and sex ($F=3.2$; $p=0.08$) or genotype and age ($F=0.1$; $p>0.10$). These results imply no abnormalities of motor skills or motor learning in the mutant mice.

However, careful inspection of the data suggested that mutants of each age group showed shorter latencies to fall on the first test day (Figure 2B). The results for the first test day therefore were examined independently by ANOVA with main factors of genotype, sex and age. In this analysis, there was a main effect of genotype ($F=8.9$; $p<0.01$) and age ($F=5.6$; $p<0.01$), but no effect of sex ($F=0.6$; $p>0.10$). There were no interactions between genotype and sex ($F=0.05$; $p>0.10$) or genotype and age ($F=0.4$; $p>0.10$). These results suggest a minor deficit in motor skills of mutant animals for the first day of testing only.

As a further test of motor skills, mutant and control mice of both sexes were compared for 3 consecutive days on the beam-walking test at 3 months of age. This test was not repeated across ages, because of the robust effect measurable in a single cohort (Figure 2C–D). Results for foot slips were examined separately by ANOVA with genotype and sex as main factors, and test day was treated as a repeated measure. For foot slips, there were significant main effects for genotype ($F=20.1$; $p<0.01$) and test day ($F=55.5$; $p<0.01$), but not for sex ($F=0.3$; $p>0.10$). There also was a significant interaction between genotype and test day ($F=5.3$; $p<0.05$), but no interaction between genotype and sex ($F=0.3$; $p>0.10$). These results imply an impairment in the mutant mice with increased slipping of the foot from the beam, although both mutants and controls were able to improve performance with repeated testing.

The same statistical strategy was used to examine beam traversal times, with results similar to those for foot slips. There were significant main effects for genotype ($F=5.9$; $p<0.05$), sex ($F=5.7$; $p<0.05$), and test day ($F=27.9$; $p<0.01$). There were no significant interactions between genotype and sex ($F=0.002$; $p>0.10$) or genotype and test day ($F=2.0$; $p>0.10$).

These results show an impairment in the mutant mice, with longer times to traverse the beam, but improved performance with repeated testing. All together, the results from Rotarod and beam-walking tests provide evidence for abnormalities in motor coordination, but relatively similar rates of improved performance with repeated testing.

Gait kinematics

A detailed assessment of various aspects of the gait was performed in mutant and control mice of both sexes using the DigiGait apparatus in 3 independent cohorts of mice at 3, 6 and 10 months of age (Table 1). This study generated a very large dataset in which 13 core gait parameters were analyzed by MANOVA with main factors of genotype, sex and age. The effect of genotype was significant only for stance time ($F=7.8$; $p<0.01$), but not any of the other measures. There were significant effects of sex for swing time ($F=8.9$; $p<0.01$), stride-time ($F=6.2$; $p<0.05$), stride lengths ($F=6.0$; $p<0.05$) and stride frequency ($F=7.1$; $p<0.01$). No interaction between genotype and sex was found for any parameter. There were significant effects of age for braking time ($F=2.7$; $p<0.05$), propulsion time ($F=3.1$; $p<0.05$), stance time ($F=27.3$; $p<0.01$), swing time ($F=10.8$; $p<0.01$), stride-time ($F=33.1$; $p<0.01$), stride lengths ($F=33.2$; $p<0.01$) and stride frequency ($F=30.7$; $p<0.01$). There were significant interactions between genotype and age for swing time ($F=6.8$; $p<0.01$), stride time ($F=6.3$; $p<0.01$), stride lengths ($F=6.7$; $p<0.01$), stride frequency ($F=5.2$; $p<0.01$) and paw overlap ($F=3.8$; $p<0.05$). Results of post-hoc testing are shown in Table 1.

Brain monoamine levels

Monoamines and their metabolites were examined in tissue homogenates from 5 different brain regions of mutant and control littermates of both sexes in independent cohorts at 3, 6 and 10 months of age (Tables 2–3). These studies generated a large dataset that was examined separately according to the brain region by MANOVA with genotype, sex and age as main factors. In the caudoputamen, there were significant overall effects of age for dopamine ($F=20.2$; $p<0.01$) and its metabolites DOPAC ($F=121.1$; $p<0.01$), 3MT ($F=8.7$; $p<0.01$) and HVA ($F=46.8$; $p<0.01$). There was also a significant effect of sex for DOPAC ($F=5.7$; $p<0.05$). There was a significant effect of genotype for HVA ($F=4.9$; $p<0.05$), as well as a significant interaction between genotype and age for HVA ($F=4.6$; $p<0.05$). Post-hoc analyses suggested a 22% increase in caudoputamen HVA levels in the mutants at 10 months of age only ($F=5.2$; $p<0.05$).

In the midbrain, there were significant main effects of age for dopamine ($F=33.3$; $p<0.01$), HVA ($F=38.0$; $p<0.01$), DOPAC ($F=21.9$; $p<0.01$), and 3MT ($F=25.0$; $p<0.01$). There were no significant effects of genotype for dopamine ($F=3.1$; $p=0.09$), HVA ($F=0.01$; $p>0.10$), DOPAC ($F=0.7$; $p>0.10$) or 3MT ($F=1.1$; $p>0.10$). However, there was a significant interaction between genotype and age for dopamine ($F=4.2$; $p<0.05$). Dopamine levels were normal in mutants at 3 months of age ($F=2.5$; $p>0.10$), but reduced by 31% at 6 months of age ($F=4.6$; $p<0.05$) and by 18% at 10 months of age ($F=5.2$; $p<0.05$). There were no corresponding age-dependent differences for the associated dopamine metabolites (HVA, DOPAC or 3MT) between mutant and control in the midbrain. There were no significant abnormalities of dopamine or its metabolites in the accumbens, frontal cortex or cerebellum of mutants (not shown). Overall, these results suggest the DYT1 mutants may have some small and potentially age-dependent changes in total tissue monoamine levels.

Microdialysis studies of caudoputamen dopamine release

Extracellular dopamine release in the caudoputamen was compared in mutant and control mice by both no-net flux and conventional microdialysis after amphetamine challenge. In no-net flux studies, extracellular dopamine was 43% lower in mutant mice compared with controls (3.5 ± 0.6 nM in mutants vs 6.1 ± 1.1 nM in controls, $p<0.05$ by independent t-test)

(Figure 3A–B). However, the slope of the no-net flux function was not significantly altered (0.58 ± 0.04 in mutants vs 0.61 ± 0.03 in controls, $p > 0.10$ by independent t-test). These results reveal lower basal dopamine levels, but they do not suggest any change in dopamine uptake by the dopamine transporter as suggested in a prior study of the CMV-transgenic model (Hewett et al., 2010).

In conventional microdialysis, baseline and amphetamine-stimulated dopamine release both appeared to be lower in mutants compared with controls (Figure 3C). The dopamine level was analyzed by ANOVA with genotype as a main factor and sample number as a repeated measure. The results showed a significant effect of genotype ($F=5.2$; $p < 0.05$) and sample number ($F=35.2$; $p < 0.01$), as well as a significant interaction between genotype and sample number ($F=4.6$; $p < 0.05$). The cumulative data revealed that baseline striatal dopamine was 41% lower in mutants compared to controls (3.0 ± 0.6 ng/ml for mutants vs 5.1 ± 0.3 ng/ml for controls, $p < 0.05$ by independent t-test) (Figure 3D). Additionally, dopamine release following a challenge with 4 mg/kg amphetamine was 51% lower in the mutants compared with controls (98.7 ± 21.3 ng/ml for mutants vs 201.9 ± 39.4 ng/ml for controls, $p < 0.05$ by independent t-test). No differences were detected between mutants and controls for any other monoamines or the dopamine metabolites, DOPAC, 3MT and HVA (not shown). The results from microdialysis demonstrate relatively large reductions in caudoputamen baseline and amphetamine-stimulated dopamine release in mutant animals, despite little or no change in total dopamine levels in caudoputamen homogenates.

Midbrain dopamine neurons

To determine if the neurochemical changes in dopamine might be associated with morphological changes in midbrain dopamine neurons, serial sections through the entire striatum and midbrain were examined at intervals of 240 μ m via Nissl stains in both male and female mutant and control mice at 3 and 6 months of age. In agreement with prior studies, Nissl stains failed to reveal any obvious structural abnormalities in any brain region of the mutant animals (Figure 4A–B, E–F and I–J).

To more precisely characterize midbrain dopaminergic neurons, serial sections immunostained for TH also were examined through the entire striatum and midbrain (Figure 4C–D, G–H and K–L). Morphologically, there were no obvious abnormalities of TH-positive axonal fields in the striatum of the mutant animals. Stereological measures for volume of TH-positive caudoputamen or accumbens were examined in 2 separate cohorts of mutant and normal animals of both sexes at 3 and 6 months of age. Overall results showed no differences between mutants and controls for the volume of either the caudoputamen (8.56 ± 0.15 mm³ vs 8.43 ± 0.06 mm³) or the accumbens (1.04 ± 0.04 mm³ vs 1.09 ± 0.03 mm³). Results were analyzed by ANOVA with main factors of genotype, sex and age. For the caudoputamen volume, there were significant effects of sex ($F=6.7$; $p < 0.05$) and age ($F=22.7$; $p < 0.01$), but no effects of genotype ($F=1.6$; $p > 0.10$). There were no interactions between genotype and sex ($F=3.3$; $p=0.09$) or genotype and age ($F=3.1$; $p=0.10$). For the accumbens, there were no main effects of genotype ($F=1.6$; $p > 0.10$), sex ($F=0.1$; $p > 0.10$) or age ($F=3.4$; $p=0.08$). These results suggest that the volume of TH-stained neuropil was not significantly affected in the mutant animals for either the caudoputamen or accumbens.

Morphologically there was no obvious loss or structural abnormalities of TH-positive neurons in the substantia nigra (area A9), ventral tegmental (area A10) area or retrorubral field (area A8). However, some of the neurons in the substantia nigra appeared slightly larger in the mutant mice. Therefore, the numbers and sizes for TH-positive cells were stereologically measured in the substantia nigra and ventral tegmental area, and the data were analyzed by ANOVA with main factors of genotype, sex and age (Table 4). In the substantia nigra, TH-positive cell numbers showed significant effects of genotype ($F=17.9$;

$p < 0.01$), but not age ($F = 1.8$; $p > 0.10$) or sex ($F = 0.2$; $p > 0.10$). Overall, nigral cell numbers were reduced by 6.9% in the mutants. TH-positive cells sizes showed significant effects of genotype ($F = 34.4$; $p < 0.01$) and age ($F = 8.8$; $p < 0.01$), but not sex ($F = 0.9$; $p > 0.10$). Overall, nigral cell sizes were increased by 15.8% in the mutants, with a frequency histogram suggesting overall enlargement of these cells rather than an abnormally large subpopulation ($p < 0.01$ by independent t-test) (Figure 4M).

In the ventral tegmental area, there were no significant effects of genotype ($F = 1.3$, $p > 0.10$), sex ($F = 0.1$, $p > 0.10$), or age ($F = 0.4$, $p > 0.10$). Ventral tegmental cell sizes showed significant effects of age ($F = 7.3$; $p < 0.05$) and sex ($F = 11.2$; $p < 0.01$), but no significant effect of genotype ($F = 1.3$; $p > 0.10$). Thus abnormalities of cell numbers and sizes detectable in the substantia nigra of mutant animals were not evident in the ventral tegmental area.

Neuropharmacological challenges of dopamine pathways

Drugs that interact with brain dopamine pathways can provide useful probes for subclinical defects in dopamine pathways in mutant mice (Jinnah et al., 1991; Jinnah et al., 1992). Therefore, the effects on locomotion in 6 month old mutant and normal mice were compared after administration of a dopamine-releasing drug, amphetamine (Figure 5). In both mutant and control mice, doses of 2 and 4 mg/kg amphetamine led to progressively increased locomotor activity, while a dose of 8 mg/kg led to a biphasic activity profile due to the emergence of focused stereotyped behavior at peak doses, as previously described (Jinnah et al., 1991; Jinnah et al., 1992). The data were analyzed by ANOVA with genotype, dose, and sex as main factors. Time was treated as a repeated measure. There were significant main effects of dose ($F = 28.1$; $p < 0.01$) and sex ($F = 4.7$; $p < 0.05$). There were no significant main effects of genotype ($F = 0.01$; $p > 0.10$), and no significant interactions involving genotype (Figure 5A–C).

A challenge with 0.25 mg/kg haloperidol reduced motor activity in both mutant and control mice. When the activity was analyzed by ANOVA with genotype and sex as main factors and recording time as a repeated measure, the results showed a significant effect of genotype ($F = 5.0$; $p < 0.05$) and sex ($F = 24.5$; $p < 0.01$), but no interaction between genotype and sex ($F = 0.1$; $p > 0.10$). However, the magnitude of the effect appeared quite small (Figure 5D), so the biological relevance of this effect is questionable.

The administration of extremely high doses or repeated administration of methamphetamine damages nigrostriatal dopamine axons and neurons in rodents by a mechanism that involves oxidative stress (Visser et al., 2002). To probe the vulnerability of dopamine neurons to this mechanism, normal and mutant mice were given 4 doses of 5 mg/kg methamphetamine at 3 hr intervals, and their caudoputamens were collected to measure residual monoamines 2 weeks later. A parallel group of mutant and control mice received repeated doses of saline. The results were examined by ANOVA with main factors of genotype and pre-treatment (saline or methamphetamine). Compared to saline-treated mice, the toxic regimen of methamphetamine markedly reduced the levels of dopamine and its metabolites DOPAC and 3MT in both mutant and control mice (not shown). However, there were no differences between mutant and normal mice, suggesting no change in the vulnerability of DYT1(ΔE) knock-in mice to methamphetamine toxicity.

PC6-3 cell model

The studies of the knock-in mouse model of DYT1 dystonia reveal several similarities and differences in comparison to prior studies of other genetic DYT1 mouse models. One potential contributor to the differences is that knock-in mice express mutant torsinA at physiologically normal levels and locations, while transgenic models express it at

abnormally high levels and sometimes in ectopic locations. This difference raises the possibility for potential non-specific deleterious effects of over-expressing the mutant protein in the wrong regions in the transgenic mouse models. To determine if over-expression of torsinA might be detrimental to cells, we took advantage of PC6-3 cell lines in which expression of normal or mutant torsinA is under the control of a doxycycline-inducible promoter (Gonzalez-Alegre and Paulson, 2004). Normal and mutant torsinA expression was induced by doxycycline in a dose-dependent and time-dependent manner (Figure 6A–B). No morphological abnormalities were detected after induction of torsinA for up to 3 days in culture. ANOVA with genotype as the main variable and days in culture as a repeated measure showed a significant effect of cell line ($F=6.2$, $p<0.05$), and post-hoc testing revealed reduced numbers of cells from cultures expressing either normal or mutant torsinA ($p<0.05$) (Figure 6C). Although the technique employed to estimate cell numbers cannot discriminate between reduced cellular proliferation versus actual cell death, the results imply that over-expression of either mutant or normal torsinA impairs the growth of cells in culture.

TH enzyme activity also was assessed in each of the PC6-3 cell lines with and without NGF exposure for differentiation. ANOVA with main factors of genotype and NGF showed a significant main effect of genotype ($F=7.1$; $p<0.01$), but the effect of NGF failed to reach significance ($F=3.8$; $p=0.07$). TH activities in PC6-3 expressing either normal or mutant torsinA both were lower than non-expressing PC6-3 cells ($p<0.05$). These results show that over-expression of either normal human or mutant torsinA has a negative effect on TH enzyme function as well as cell growth.

Dopamine content and release were examined after exposure of the PC6-3 cells to varying concentrations of doxycycline over periods from 1–7 days. The time after plating and final cell density on the test day had a strong influence on dopamine content and release in these cells, but there were no consistent effects after induction of either normal or mutant torsinA (not shown). These results therefore fail to provide evidence that torsinA can induce a cell-intrinsic defect in dopamine content or releasability.

Discussion

These studies demonstrate subtle but reproducible differences between DYT1(ΔE) knock-in mice and their normal littermate controls relating to motor behavior, the neurochemistry of caudoputamen dopamine, and the number and size of substantia nigra TH-positive neurons. Although studies of other mouse models of DYT1 dystonia have examined some of these features, such studies have not so far been reported for the knock-in model examined here. The results from this knock-in mouse are consistent with prior studies of other DYT1 mouse models showing similar defects, although some differences are apparent. A more important question is how these abnormalities may relate to each other, and what they mean for the concept that dysfunction of dopamine pathways may be relevant for dystonia in humans with the similar mutation.

Motor function in DYT1 mouse models

The current studies showing subtle abnormalities of motor behavior without overt dystonia are consistent with prior studies of other DYT1 mouse models. Most of the models have been tested in automated activity chambers that measure gross levels of locomotor behavior. Similar to results shown here (Figure 1), several of the other models have exhibited small increases in locomotor activity (Dang et al., 2005; Grundmann, 2007 #3737; Dang et al., 2006; Yokoi et al., 2008). The NSE-transgenics differed in exhibiting marked hyperactivity and circling behavior (Shashidharan et al., 2005), but this phenotype disappeared in subsequent generations (Lange et al., 2011). The CMV-transgenics also were unusual in that

they showed markedly reduced activity in one study (Hewett et al., 2010), and normal motor behavior in another (Sharma et al., 2005). Thus the most consistent effect across models appears to be a small increase in activity.

Motor skills also have been examined in most of the models either by examining performance on the Rotarod apparatus or performance while traversing a narrow beam. The CMV-transgenics initially were reported to fail to improve Rotarod performance with repeated testing (Sharma et al., 2005), a finding that was not replicated in a second study of this model (Zhao et al., 2008). One of the PrP-transgenic lines exhibited impaired Rotarod performance (Grundmann et al., 2007), while most other studies have not revealed any significant abnormalities (Dang et al., 2005; Dang et al., 2006; Lange et al., 2011; Page et al., 2010). The current studies revealed nearly normal Rotarod performance, although a subtle defect could be detected when results from large numbers of animals were pooled (Figure 2A–B). Motor deficits were more readily apparent on the beam-walking test (Figure 2C–D), in agreement with other studies (Dang et al., 2012; Dang et al., 2005; Dang et al., 2006; Page et al., 2010; Yokoi et al., 2011; Yokoi et al., 2008; Zhao et al., 2008). Subtle gait abnormalities can also be detected by detailed analysis of the gait using the DigiGait apparatus (Table 1) or manual tracing of footprints (Dang et al., 2005; Yokoi et al., 2008; Zhao et al., 2008). Taken together, these results suggest a subtle problem with motor skills, with the beam-walking test being more sensitive than other tests so far employed.

Interpreting the relevance of the motor abnormalities is challenging because the nature of the defects has not been entirely consistent across the different mouse lines, and different results were sometimes obtained even in the same line. Furthermore, some studies have suggested that abnormalities may be dependent on sex or age (Dang et al., 2005; Page et al., 2010; Shashidharan et al., 2005), yet few studies have specifically addressed gender or replicated studies across ages (Table 1). Other possible reasons for the different results across the different models include the location in which mutant torsinA is expressed in the brain, the levels at which mutant torsinA are expressed, whether mutant torsinA is mouse or human, and the genetic background of the mouse strain used. Perhaps the most important reason for differences among the mouse lines is a very small effect sizes in relation to normal test variance, so that abnormalities on some tests are readily missed when small numbers of animals are tested without replication. This problem of statistical power is evident when comparing the non-significant differences in circadian locomotor activity (Figure 1A) with the significant differences when these same activities are aggregated for daytime or nighttime periods (Figure 1B). The problem of statistical power also is evident when comparing non-significant differences in Rotarod behavior across 3 cohorts of mice at different ages (Figure 2A), versus aggregate data for all 3 groups combined (Figure 2B). Overall, behavioral studies of the DYT1 mouse models suggest subtle changes in motor behavior, but detecting these changes depend on the statistical power of the experimental design and the motor test employed.

Neurochemical changes in DYT1 mouse models

The current studies showed a small and potentially age-dependent decrease of dopamine in the midbrain and increase of the dopamine metabolite HVA in the striatum (Table 3–4). Although many studies have shown abnormalities of brain monoamine levels (Dang et al., 2005; Dang et al., 2006; Grundmann et al., 2007; Page et al., 2010; Shashidharan et al., 2005; Yokoi et al., 2008; Zhao et al., 2008), interpreting the physiological relevance of these changes is challenging because the nature and direction of changes are not consistent across different studies (Table 1). The reasons for these differences among the models are similar to those outlined above for the behavioral studies. Again, perhaps the most important reason for the discrepancies may be that the reported changes in dopamine or its metabolites are

often quite small in relation to experimental variance, so that differences may reach statistical significance only when large numbers of samples are examined.

A more consistent and robust abnormality in the DYT1 mouse models is a marked reduction in baseline and amphetamine-stimulated striatal dopamine release as measured by in vivo microdialysis or voltammetry. Although caudoputamen tissue dopamine levels were normal, no-net flux microdialysis revealed 43% reduced basal extracellular dopamine in the caudoputamen of mutant mice (Figure 3A–B). Conventional microdialysis also showed a ~51% decrement in striatal dopamine release following 4 mg/kg amphetamine in a DYT1(Δ E) knock-in model (Figure 3C–D). These results are strikingly similar to prior studies of two different transgenic models. A ~55% reduction in striatal dopamine release in response to 5 mg/kg amphetamine was reported for the CMV transgenics (Balcioglu et al., 2007). A follow-up study of these mice suggested a defect in dopamine uptake (Hewett et al., 2010), a finding not supported by the current results. Another study showed almost complete lack of dopamine release in response to 20 mg/kg cocaine in the TH-transgenics (Page et al., 2010). Because of the restricted expression of the mutant protein in midbrain dopamine neurons, these authors argued that mutant torsinA may cause a cell-autonomous defect in dopamine release. However, the possibility of non-specific dysfunction of these neurons caused by over-expression of the transgene limits the strength of this conclusion. Although there are some differences in the interpretation of the results, the robustness and consistency of the defect in dopamine release measured by microdialysis in the 3 different models warrants further attention.

Neuroanatomical changes in the DYT1 mouse model

The current studies are the first to identify anatomical anomalies of dopamine neurons in the substantia nigra of DYT1(Δ E) knock-in mice. Although the TH stained striatal and midbrain tissue volume was normal, the mutant mice showed a small reduction in the number of TH-positive substantia nigra cells, and the remaining cells were larger than controls (Table 5). These changes were limited to the substantia nigra and not seen in ventral tegmental TH-positive neurons, suggesting a selective nigrostriatal abnormality. These anomalies likely have been overlooked in prior anatomical studies of DYT1 mouse models, because they are subtle and measurable only with rigorous quantitative measures. Interestingly, a prior study of 3 DYT1 human brains collected at autopsy described subjective impressions of enlarged nigral neurons without obvious cell loss (Rostasy et al., 2003). However, the interpretation of these findings was limited by the challenges inherent in performing quantitative studies in relatively scarce human autopsy material. Further studies of human DYT1 brains clearly are needed.

Although there may be small reductions in the number of dopamine neurons in the DYT1(Δ E) knock-in mice, it seems unlikely that this reduction could be responsible for any significant motor deficits. Among different normal mouse strains, the numbers of midbrain dopamine neurons vary naturally by nearly 2-fold (Zaborszky and Vadasz, 2001). Additionally, reductions of more than 50% of nigrostriatal neurons are required to provoke motor deficits, and even greater reductions are required if the loss occurs in a developmental context (Bruno et al., 1998; Joyce et al., 1996). These results imply a large reserve capacity in the dopamine neuron population whereby substantial cell loss is required to see any functionally relevant behavioral change. The relevance of enlarged soma in the DYT1(Δ E) knock-in mice is more difficult to interpret. While soma enlargement may reflect a pathological process, it may also reflect a compensatory effect, since this soma enlargement is a well-characterized secondary consequence of increased activity among neurons throughout the nervous system.

Consequences of over-expression of mutant torsinA

Some of the differences among the DYT1 models may be attributable to the way the abnormal gene is expressed. Knock-in mice express mutant torsinA at physiologically normal levels and locations, while transgenic models express it at abnormally high levels, often in ectopic locations. Transgenic models therefore may exhibit phenotypic abnormalities that reflect non-specific deleterious consequences of over-expressing the mutant protein in the wrong regions. This possibility was examined indirectly by taking advantage of PC6-3 cell lines in which normal or mutant torsinA is under the control of a doxycycline-inducible promoter (Gonzalez-Alegre and Paulson, 2004). Studies of these lines revealed that over-expression of both normal and mutant torsinA negatively influenced cell growth and expression of TH (Figure 6). These studies echo concerns raised in a prior study, that over-expression of torsinA, whether normal or mutant, may have non-specific harmful effects (Grundmann et al., 2007). The results do not invalidate the transgenic models, but they imply that phenotypic defects reported for transgenic models must be interpreted with caution, since these effects may reflect artifacts of over-expression that are not relevant to the human disease.

Relevance to the concept of dopamine dysfunction in human DYT1 dystonia

As previously noted, several forms of human dystonia have been linked with dysfunction of basal ganglia dopamine pathways. In this setting, it is tempting to speculate that the robust decrement in dopamine release observed in microdialysis studies of the DYT1 mouse models may relate to the pathogenesis of dystonia. If this is the case, the reduced dopamine release in the DYT1(ΔE) knock-in mice should have a behavioral correlate such as altered sensitivity to the ability of amphetamine or haloperidol to influence motor activity (Jinnah et al., 1992). Unfortunately, the mutants showed normal behavioral responses to 3 doses of amphetamine. They showed a statistically significant increased sensitivity to haloperidol, but the magnitude of the effect was so small that it seems unlikely to be biologically relevant. In contrast, the CMV-transgenics showed a markedly attenuated motor response to amphetamine, although interpretation of this result is confounded by a markedly reduced spontaneous level of activity (Hewett et al., 2010). The TH-transgenics did not show a consistent change in motor behavior after cocaine administration (Page et al., 2010). No clear abnormalities were observed in the NSE-transgenics with a battery of 6 different dopaminergic drugs used at different doses (Lange et al., 2011). All together, these studies do not provide compelling evidence to support the concept that the lower dopamine release observed in microdialysis studies has a behaviorally relevant correlate in the DYT1 mouse models.

There are two potential explanations for the discrepancy between the reduced dopamine release observed in microdialysis and the relatively normal behavioral responses to pharmacological challenges of the dopamine systems. One explanation is that the mice have developed compensatory mechanisms during development that erase the consequences of their low dopamine release, such as augmented postsynaptic responsiveness. This explanation is not supported by other studies showing normal responses to dopamine receptor agonists (Lange et al., 2011). Another explanation is that the low dopamine release reflects a compensatory response to some other defect. Studies of the CMV-transgenics (Martella et al., 2009; Pisani et al., 2006) and a knock-in model (Dang et al., 2012) have suggested defects in striatal cholinergic neurotransmission, raising the possibility that changes in dopamine release may reflect compensations for chronically abnormal cholinergic activity. Other studies have suggested a defect in cortico-striatal signaling pathways (Yokoi et al., 2008), again raising the possibility that changes in dopamine release may reflect compensations for chronically abnormal glutamatergic tone. Finally, changes in dopamine release may be secondary to a more distant defect elsewhere. Two of the DYT1

mouse models have been shown to harbor metabolic and structural defects in cerebellar circuits (Ulug et al., 2011; Zhang et al., 2011; Zhao et al., 2011), and prior studies have shown that abnormal cerebellar signaling can alter striatal dopamine release (Neychev et al., 2008), presumably via subcortical connections between the cerebellum and basal ganglia (Neychev et al., 2011). These studies raise the possibility that the marked reductions of dopamine release observed in microdialysis studies do not reflect defects intrinsic to dopamine neurons, but instead reflect compensatory responses to defects arising elsewhere in the motor circuit.

Conclusions

The current studies of a knock-in mouse model of DYT1 dystonia reveal changes in basal ganglia dopamine neurochemistry, nigrostriatal dopamine neurons, and motor behavior. These results add to a relatively large body of evidence pointing to alterations of dopamine systems in animal models of DYT1 dystonia. However, the motor abnormalities, neuroanatomical changes, and most of the neurochemical changes appear relatively subtle. The microdialysis studies showing decrements in dopamine release are more robust, but a consistent and relevant behavioral correlate seems lacking. At least two different interpretations could be applied to explain the lack of a cohesive conceptual model that can accommodate the available results regarding the dopamine systems. The first is that dysfunction of dopamine pathways plays a central role in the pathogenesis of the disorder, but that the right animal models and exact studies needed to formulate a cohesive mechanistic explanation have not yet been conducted. The second is the various abnormalities of dopamine systems that have been uncovered so far are indirect reflections of a primary defect in other parts of the relevant motor circuit such as cortical glutamatergic, striatal cholinergic, or cerebellar pathways. Further studies of both the dopamine systems and other neural pathways will be needed to dissect these possibilities.

Acknowledgments

We thank Dr. William Dauer for making the DYT1 knockin mice available for these studies, and Dr. Pedro Gonzalez-Alegre for providing the PC6-3 cells. This work was supported in part by The Dystonia Medical Research Foundation, Bachmann-Strauss Dystonia & Parkinson Foundation, and NIH grants NS040470 and NS033592.

Abbreviations

ΔE	deletion of a single GAG codon in <i>Tor1A</i> gene
CMV	cytomegalovirus
NSE	neuron-specific enolase
PrP	prion-protein
TH	tyrosine hydroxylase
DOPAC	3,4-dihydroxyphenylacetic acid
HVA	homovanillic acid
3MT	3-methoxytyramine
DA_{in}	inflow of dopamine
DA_{out}	outflow of dopamine
TA	torsinA
PBS	phosphate-buffered saline

PBST	PBS including 0.1% Tween-20
Tyr-AMC	tyrosine-7-amino-4-methylcoumarin
ANOVA	Analysis of Variance
AMPH	amphetamine
HAL	haloperidol
Dox	doxycycline
NGF	nerve growth factor

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Highlights

- Subtle abnormalities in motor skills were found in a DYT1 knock-in model of dystonia with multiple motor tasks.
- Striatal microdialysis showed reduced baseline and amphetamine-stimulated dopamine release.
- Anatomical studies revealed a small reduction in TH-immunopositive nigrostriatal neurons, with a small increase in soma size.

Figure 1.

Gross locomotor activity. Spontaneous activity was assessed at 10 min intervals for 24 hr in a total of 14 normal (control) and 17 DYT1(ΔE) knock-in (mutant) mice of both sexes at 3 or 6 months of age. Panel A shows the pattern of circadian activity. The bar above the graph represents daytime and nighttime periods. No differences were detected between control (closed circles) and mutant (open circles) mice. Panel B shows total cumulative beam breaks for daytime in mutant (white bars) compared with controls (black bars). All data represent average values $\pm$ SEM, and asterisks denote statistical significance at $p < 0.05$.

Figure 2.

Coordinated motor skills on Rotarod and beam walking tests. Panel A shows motor learning rates in control (closed circles) and mutant (open circles) mice on the accelerating Rotarod. The mean time to fall from the Rotarod was determined from 27 controls and 26 mutants at 3 months of age, 31 controls and 27 mutants at 6 months of age and 27 controls and 30 mutants at 10 months of age. A mean for each mouse was determined from 3 trials for each test day, with 5 consecutive days of testing. Panel B shows the initial motor skills in control (black bars) and mutant (white bars) mice on the first test day, highlighted by boxes in panel A at each age. Panels C–D show foot slips and beam traversal times in 12 control (black bars) and 13 mutant (white bars) mice of both sexes at 3 months of age. All data represent average values $\pm$ SEM. Single asterisks denote statistical significance at $p < 0.05$, while double asterisks denote $p < 0.01$.

Figure 3.

Microdialysis measures of dopamine in the striatum. Microdialysis was performed in alert and freely moving control (n=9) and mutant (n=9) mice at 10 weeks of age. Results for the no-net flux method are shown in panels A–B, while those for the conventional method and amphetamine challenge are shown in panels C–D. A) In no-net flux studies, no difference was detected between control (closed circles) and mutant (open circles) for the extraction fraction of the slope, as calculated from the ratio of [DA (in) - DA (out)] versus DA (in)]. B) Basal extracellular dopamine when equilibrated [DA (in) - DA (out) = 0] was significantly reduced in the mutants (white bars) compared to controls (black bars). C) In conventional studies, the first 6 consecutive samples were collected for basal levels, and the following 6 samples were collected after 4 mg/kg amphetamine for both controls (closed circles) and mutants (open circles). D) Post-hoc analyses revealed significantly reduced basal and amphetamine-stimulated (AMPH) dopamine levels in mutants (white bars) compared with controls (black bars) ($p < 0.05$). All data represent average values $\pm$ SEM, and asterisks denote statistical significance at $p < 0.05$.

Figure 4.

Histological analysis of the nigrostriatal dopamine pathways. Nissl stains revealed no obvious structural abnormalities in the striatum (A–B) or midbrain (E–F).

Immunohistochemical stains for tyrosine hydroxylase (TH) revealed a normal striatal volume and morphology (C–D). There were no obvious differences in the midbrain tissue volume (G–H) and cell morphology (K–L) between mutant and control, although some TH-positive neurons in the substantia nigra appeared slightly larger in the mutants. A histogram of size distributions for TH-positive cells from normal ($n=723$, black curves) and mutant mice ($n=627$, gray curves) suggested a shift of the entire population to larger sizes, rather than a small subpopulation of very large cells. Double asterisks denote statistical significance at $p < 0.01$.

Figure 5.

Locomotor activity induced by dopamine-related drugs. Panels A–C show amphetamine-induced motor activity recorded for 11 control (closed circles) and 15 mutant (open circles) mice in 10 min bins for 4 hr following injection of 2, 4 or 8 mg/kg amphetamine (AMPH) with a week interval between each dose. Panel D shows haloperidol-induced suppression of motor activity recorded for 20 mutant and 20 control mice in 10 min bins for 2.5 hr following injection of 0.25 mg/kg haloperidol (HAL). Mutants (open circles) showed slightly reduced activity compared with controls (closed circles). All data represent average values $\pm$ SEM, and asterisks denote statistical significance at $p < 0.05$.

Figure 6.

Cell growth and TH enzymatic activity in PC6-3-cells. Panels A shows torsinA (TA) expression via immunoblots with α -tubulin as a control after 3 days exposure to varying doses of doxycycline in PC6-nor cells expressing normal TA or PC6-mut cells expressing mutant TA. Panel B shows TA expression after exposure to 10 ng/mL doxycycline for 1–3 days in PC6-nor and PC6-mut cells. Panels C–D show TA expression normalized to α -tubulin as percent of control, corresponding to panels A–B. Gray bars depict PC6-nor cells and white bars depict PC6-mut cells. Panel E shows cell viability in PC6-parent (black bars), PC6-nor (gray bars) and PC6-mut (white bars) treated with 10 ng/mL doxycycline for 1 to 3 days. Panel F shows TH activity in PC6-parent (black bars), PC6-nor (gray bars) and PC6-mut (white bars) treated with 10 ng/mL doxycycline and 100 ng/ml nerve growth factor (NGF) for 3 days as percent of control PC6-parent cells. Single asterisks denote statistical significance at $p < 0.05$, while double asterisks denote $p < 0.01$.

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Mouse models for DYT1 dystonia

Table 1

Study	Mouse	Ages	Sexes	Tissue MA	Striatal microdialysis	Locomotor activity	Rotarod	Beam walkin	Anatomical phenotype	Others
Sharma et al., 2005	CMV-tg	4, 6, 9 mo	M	NR	NR	normal (9 mo)	↓ motor learning(9 mo)	NR	no TA/ubiquitin+ PN inclusions	NR
Balcioglu et al., 2007	CMV-tg	6 mo	M/F	normal in striatum (M ^f)	normal basal DA, but DA ↓ to AMPH (M ^f)	NR	NR	NR	NR	normal DAT, VMAT2, and D1R, D2R (F ^f)
Zhao et al., 2008	CMV-tg	3–4 mo	M	↑ striatal DOPAC/DA and HVA/DA	NR	NR	normal	↑ time and foot slips	no TA/ubiquitin+ PN inclusions	↑ hindlimb width
Hewett et al., 2010	CMV-tg	6 mo	M	NR	↓ DAT function	↓ locomotion in basal and response to AMPH	NR	NR	NR	NR
Pisani et al., 2006	CMV-tg	9 mo	NR	NR	NR	NR	NR	NR	NR	abnormal cholinergic response
Martella et al., 2009	CMV-tg	2 mo	NR	NR	NR	NR	NR	NR	NR	↑ AChE activity
Shashidharan et al., 2005	NSE-tg	1.5 mo	NR	↓ striatal DA ² , and ↓ DOPAC/DA	NR	hyperactivity and circling ²	NR	NR	TA/ubiquitin+ PN inclusions in PPNi, pontine and PAG	NR
Lange et al., 2011	NSE-tg	3, 6, 9, 12 mo	NR	NR	NR	↓ rearing (3 mo)	normal	NR	NR	↓ forelimb and hindlimb stride (9 mo)
Grundmann et al., 2007	PrP-tg	5–6 mo	M/F	↑ BS DOPAC, 5-HT and 5-HIAA	NR	mild ↑ locomotion	↓ motor skill and learning	normal	TA/ubiquitin+ inclusions in BS and abnormal nuclear envelope in striatum and BS	abnormal DT-MRI in cortex, striatum and cerebellum
Page et al., 2010	TH-tg	2–10 mo	M/F	normal in striatum (2.5 mo, F ^f)	normal basal DA, but DA ↓ to cocaine (M ^f ,3)	normal (4–10 mo, M ^f)	normal (2, 4, 6 mo)	↑ time and foot slips	NR	NR
Yokoi et al., 2008	cortical TA KO	4–12 mo	M/F	normal in striatum (9–12 mo)	NR	↑ locomotion (4–7 mo)	normal (4–7 mo)	↑ foot slips (4–7 mo)	NR	NR
Yokoi et al., 2010	striatal TA KO	2, 5.5 mo	M/F	normal in striatum	NR	normal	normal	↑ foot slips	normal nuclear envelope in striatal medium spiny neuron	↓ D2R binding
Dang et al., 2006	TA KD	6–9 mo	M/F	↓ striatal DOPAC (8–9 mo, M ^f)	NR	↑ horizontal locomotion (6 mo, M only)	normal (6 mo)	↑ foot slips (6 mo, M only)	NR	NR
Dang et al., 2005	DYT1(ΔE) KI	3–11 mo	M/F	↓ striatal HVA (11 mo, M only)	NR	mild ↑ locomotion (6 mo, M only)	normal (6 mo)	↑ foot slips (6 TA/ubiquitin+ inclusions in BS	TA/ubiquitin+ inclusions in BS	NR
Zhang et al., 2011	DYT1(ΔE) KI & PC TA KO	3, 8 mo	NR	NR	NR	NR	NR	NR	↓ PC dendrites length (8 mo) and ↓ spine number (3 and 8 mo)	NR
Goodchild et al., 2005	DYT1(ΔE) KI	E18, P14	NR	NR	NR	NR	NR	NR	normal nuclear envelope ⁴	NR
Ulug et al., 2011	DYT1(ΔE) KI	4 mo	NR	NR	NR	NR	NR	NR	NR	↓ CbTC connectivity

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Table 2

Kinematic analysis of the gait

	Brake (ms)	Propulsion (ms)	Stance (ms)	Swing (ms)	Stride (ms)	Stride (cm)	Stride (steps/sec)	Paw overlap (cm)
<i>3 month</i>								
control (11)	39.4 ± 1.5	71.2 ± 1.8	110.3 ± 0.8	92.2 ± 1.0	202.4 ± 1.4	7.09 ± 0.05	5.09 ± 0.04	0.58 ± 0.21
mutant (11)	39.1 ± 1.7	72.5 ± 1.9	111.6 ± 0.8	92.2 ± 1.0	203.8 ± 1.4	7.14 ± 0.05	5.07 ± 0.04	0.36 ± 0.19
<i>6 month</i>								
control (6)	38.0 ± 4.7	75.8 ± 3.6	115.2 ± 1.3	94.3 ± 1.7	209.4 ± 2.1	7.33 ± 0.07	4.90 ± 0.06	0.75 ± 0.28
mutant (7)	41.5 ± 3.1	78.5 ± 3.0	119.8 ± 1.5	99.9 ± 1.0**	219.8 ± 1.6**	7.70 ± 0.06**	4.66 ± 0.04**	0.69 ± 0.22
<i>10 month</i>								
control (11)	45.7 ± 2.5	72.9 ± 2.8	118.5 ± 1.3	101.6 ± 1.9	220.1 ± 2.2	7.71 ± 0.08	4.67 ± 0.05	0.13 ± 0.33
mutant (15)	42.2 ± 2.1	77.9 ± 2.3	120.2 ± 1.0	96.8 ± 1.3**	217.0 ± 1.6	7.60 ± 0.06	4.74 ± 0.04	0.96 ± 0.10**
<i>All age groups</i>								
control (28)	41.8 ± 1.3	72.4 ± 1.4	113.4 ± 0.7	95.1 ± 0.9	208.5 ± 1.2	7.30 ± 0.04	4.94 ± 0.03	0.48 ± 0.15
mutant (33)	40.8 ± 1.2	75.4 ± 1.3	116.0 ± 0.7**	95.1 ± 0.7	211.2 ± 1.1	7.39 ± 0.04	4.88 ± 0.03	0.63 ± 0.11

Results are expressed as average values ± SEM. The 13 core parameters were analyzed by MANVOA with main factors of genotype, sex and age. Double asterisks indicate $p < 0.01$ from post-hoc testing.

Table 3

Tissue monoamines in the caudoputamen

	Dopamine	DOPAC	3MT	HVA
<i>3 month old</i>				
control (6♂ & ♀)	95.0 ± 3.2	13.1 ± 0.9	13.0 ± 0.2	18.3 ± 0.6
mutant (6♂ & ♀)	96.7 ± 3.0	13.5 ± 0.9	12.6 ± 0.3	18.0 ± 0.5
<i>6 month old</i>				
control (6♂ & ♀)	72.0 ± 4.0	74.0 ± 5.9	10.1 ± 0.5	24.0 ± 1.2
mutant (6♂ & ♀)	76.2 ± 5.6	72.3 ± 4.5	11.3 ± 0.7	24.2 ± 0.7
<i>10 month old</i>				
control (3♂ & ♀)	68.9 ± 4.6	52.3 ± 3.6	10.9 ± 0.7	26.4 ± 1.6
mutant (6♂ & ♀)	72.4 ± 6.6	68.2 ± 6.7	12.3 ± 0.8	32.1 ± 1.9*

Results are expressed as average ng/mg tissue ± SEM. Abbreviations; DOPAC (3,4-dihydroxyphenylacetic acid), 3MT (3-methoxytyramine) and HVA (homovanillic acid). Asterisk indicates $p < 0.05$.

Table 4

Tissue monoamines in the midbrain

	Dopamine	DOPAC	3MT	HVA
<i>3 month old</i>				
control (6♂ & 6♀)	1.2 ± 0.1	0.8 ± 0.0	0.0 ± 0.0	1.5 ± 0.1
mutant (6♂ & 6♀)	1.4 ± 0.1	0.8 ± 0.0	0.1 ± 0.0	1.7 ± 0.1
<i>6 month old</i>				
control (6♂ & 6♀)	1.3 ± 0.2	1.9 ± 0.2	0.0 ± 0.0	1.3 ± 0.1
mutant (6♂ & 6♀)	0.9 ± 0.1*	1.6 ± 0.2	0.0 ± 0.0	1.4 ± 0.1
<i>Both age groups</i>				
control (3♂ & 3♀)	2.2 ± 0.1	2.0 ± 0.4	0.3 ± 0.1	2.7 ± 0.2
mutant (6♂ & 6♀)	1.8 ± 0.1*	1.9 ± 0.1	0.3 ± 0.1	2.3 ± 0.1

Results are expressed as average ng/mg tissue ± SEM. Abbreviations; DOPAC (3,4-dihydroxyphenylacetic acid), 3MT (3-methoxytyramine) and HVA (homovanillic acid). Asterisks indicate $p < 0.05$.

Table 5

Numbers and sizes of TH stained cells in the midbrain

	Control	Mutant	% control
<i>Substantia nigra</i>			
Total cells	10125 ± 111	9430 ± 130	93.1**
Cell size (μm ³)	2826 ± 48	3273 ± 72	115.8**
<i>Ventral tegmental area</i>			
Total cells	8185 ± 102	8049 ± 94	98.3
Cell size (μm ³)	2222 ± 40	2265 ± 51	101.9

Data are average numbers or sizes ± SEM of TH stained cells in the midbrain of 12 mutant and 12 control mice with equal numbers of mice from both sexes and two age groups (3 and 6 months of age). Double asterisks indicate $p < 0.01$.