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Parkinsonism without dopamine neuron degeneration in aged DOPA-responsive dystonia knockin mice

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Abstract

Background—Recent neuroimaging studies implicate nigrostriatal degeneration as a critical factor in producing late-onset parkinsonism in patients with DRD-causing mutations. However, postmortem anatomical studies do not reveal neurodegeneration in DRD patients. These contrasting findings make it unclear how parkinsonism develops in DRD-mutation carriers.

Methods—We prospectively assessed motor dysfunction, responses to dopaminergic challenge and dopamine neuron degeneration with aging in a validated knockin mouse model bearing a DRD-causing mutation found in humans.

Results—As DRD mice aged, dystonic movements waned while locomotor activity decreased and initiation of movements slowed. Despite the age-related reduction in movement, there was no evidence for degeneration of midbrain dopamine neurons. Presynaptically-mediated dopaminergic responses did not change with age in DRD mice, but responses to D1 dopamine receptor agonists decreased with age.

Conclusions—We demonstrate for the first time the co-occurrence of dystonia and Parkinson-like features (mainly consisting of hypokinesia) in a genetic mouse model. We show, in this model, that these features evolve without dopaminergic neurodegeneration, suggesting that postsynaptic plasticity, rather than presynaptic degeneration may contribute to the development of parkinsonism in patients with DRD.

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Authors' roles

SJR, HAJ, and EJH designed the research project

SJR, PH, CD, XF, VJ, and MGT executed the research project

SJR and EJH prepared the first draft of the manuscript

SJR, PH, CD, XF, VJ, MGT, HAJ, and EJH reviewed and critiqued the manuscript

Keywords

basal ganglia; dopamine; tyrosine hydroxylase; dystonia; Parkinson's disease

Introduction

The bradykinesia and tremor associated with adult-onset Parkinson's disease results from a deficit in striatal dopamine (DA) caused by the degeneration of nigrostriatal DA neurons. On the other hand, the deficit in striatal DA caused by mutations in genes critical for catecholamine synthesis, including *GCHI* and *TH*, causes DOPA-responsive dystonia (DRD), which is characterized by childhood-onset generalized dystonia. Although DRD patients may exhibit parkinsonian features in addition to dystonia [1, 2], autopsy findings suggest that DRD is not associated with nigrostriatal DA neuron degeneration [3, 4]. However, this assertion has been challenged by recent studies describing a reduction in dopamine transporter (DAT) binding, an indirect indication of nigrostriatal DA neuron degeneration, in patients with DRD-causing mutations but adult-onset parkinsonism [5–7]. Thus, it was suggested that the presence of DRD-causing mutations predisposes some patients to neurodegenerative Parkinson's disease.

Because it is unclear how the same genetic variant causes DRD in some patients but predisposes others to neurodegeneration and Parkinson's disease, we examined the relationship between these movement disorders and neurodegeneration in a knockin mouse bearing the human DRD-causing p.381Q>K mutation in tyrosine hydroxylase [8]. DRD mice exhibit the core features of DRD in humans including reduced striatal DA concentrations, dystonic movements and amelioration in response to L-DOPA [9; includes video of the dystonia]. Here, we demonstrate that dystonia decreases while akinesia increases with age in these mice. Further, we demonstrate that the age-related change in motor dysfunction was associated with functional changes to DA receptor signaling but not nigrostriatal DA neuron degeneration.

Methods

Animals

Mice homozygous for the c.1160C>A *Th* mutation (DRD mice) and normal littermates on a mixed C57BL/6J and DBA/2J background were bred and genotyped as previously described [9]. DRD/+ were crossed and the pregnant dam's drinking water was supplemented with 100 µg/mL isoproterenol, 20 µg/mL phenylephrine and 2.5 mg/mL ascorbic acid. From postnatal days 9.5 to 16.5, the dams' drinking water was supplemented with 1.5 mg/mL L-DOPA, 0.5 mg/mL benserazide, and 2.5 mg/mL ascorbic acid. Thereafter, all mice received a daily injection (s.c.) of 5 mg/kg L-DOPA, 5 mg/kg benserazide and 2.5 mg/mL ascorbic acid in saline. All compounds were obtained from Sigma-Aldrich, St. Louis, MO except isoproterenol (TCI America). L-DOPA supplementation was terminated >24 h prior to all experiments. Housing conditions were in accordance with Emory University's institutional animal care and use committee. Mice were group housed with nestlets and 'igloos' for environmental enrichment and fed Mouse Diet 5015 (www.labdiet.com). Male and female

mice were used because we have previously found no differences between the sexes [9]. The light period was 7 am to 7 pm.

Behavioral Assessments

Assessments were performed prospectively from 1 to 15 months of age. An abnormal movement inventory was used to identify the type of abnormal movement [10]. Abnormal movements included tonic flexion, tonic extension, clonus, twisting, and tremor. Abnormal movements were scored for 30 s in 10 min intervals for 60 min in a novel open field at 2 pm and the scores were summed. Locomotor activity was recorded in automated activity cages (San Diego Instruments) at 2 pm for 60 min. To test for the ability to initiate voluntary movement, mice were tested at 8 am whereby the forepaws were gently placed on a horizontal bar (0.5 cm diameter), 3 cm above the cage floor. Time to remove both forepaws from the bar was recorded. Cutoff time was 1 min and 3 consecutive trials were averaged [11]. This test is similar to the test that is classically used to assess catalepsy in response to antipsychotic drugs. However, because antipsychotic drugs were not tested here, the results provide an estimate for latency to initiate movements, similar to akinesia observed in parkinsonism. SKF 81297 and amphetamine (Sigma) were administered (10 ml/kg, s.c.) in saline in pseudorandom order 10 min prior to behavioral tests.

Histochemical Analyses

Brain tissue was prepared by perfusion fixation with paraformaldehyde solution. For stereological counting, every 6th section was immunostained for using TH antibody (1:1000, Pel-Freez, Rogers, AR), reacted with biotinylated goat α -rabbit antibody (1:500, Jackson ImmunoResearch, West Grove, PA) and processed with 3,3-diaminobenzadine (Sigma) (Song et al., 2012). An Olympus BX51 light microscope equipped with a motorized stage (MACC500, Ludl Electronic Products, Hawthorne, NY) coupled to a computer with StereoInvestigator software (MicroBrightField, Williston, VT) was used for stereological cell counts of TH-positive cells, which were outlined using a mouse brain atlas as reference [14] to define the borders of the SN and VTA. TH-positive cells in every 6th section were counted at 40 $\times$ using the optical fractionator probe with a 55,185 μm^2 counting grid and a 10,000 μm^2 frame with 15 μm depth and 1 μm guard zone (Song et al., 2012). For DAT immunostaining, 3 sections per animal at similar rostral-caudal positions were treated with Citra antigen retrieval reagent (BioGenex, Fremont, CA) for 1 hr at 70 $^{\circ}\text{C}$, prior to incubation with rat α -DAT antibody (1:1000, Millipore, Temecula, CA) and then processed with 3,3-diaminobenzadine [12]. DAT immunohistochemistry was quantified using ImageJ software (NIH, Bethesda, MD). For each DAT staining experiment, DRD and normal mice were processed in parallel; staining was normalized to normal mouse DAT staining within experiments and expressed as a percent of control. For Fluoro-Jade histochemistry, 6-hydroxydopamine-treated (6OHDA) rat tissue was used as a positive control for neurodegenerating terminals and cell bodies. Briefly, 20 μg of 6OHDA (Sigma, St. Louis MO) was infused into the striatum and sacrificed 35 days later, producing ~44% dopaminergic nigral degeneration [13]. Sections were incubated in 0.06% potassium permanganate for 13 min, developed in 0.0002% Fluoro-Jade B solution (Millipore) for 20 min and imaged with a Nikon 80i fluorescent microscope; results were replicated in a second experiment.

Experimental Design and Statistical Analysis

Blinding and randomization was performed by second party concealment with coded syringes. *A priori* power analyses using means, standard deviations, and effect sizes from Rose et al. (2015) were used to determine sample sizes that achieved 80% statistical power at $\alpha = 0.05$. Statistical analyses were performed using SigmaStat (Systat Software). For two group comparisons, Student t tests were performed; one-tailed t tests were performed based on previously published results [9]. For more than two groups, one-way or two-way repeated measures ANOVAs were performed. *Post hoc* effects were tested with the Holm-Sidak test or Tukey's *post hoc* test if there was no obvious baseline condition. Detailed statistics are presented in the figure legends.

Results

Age-related changes in motor symptoms are sometimes observed in DRD patients [2, 15, 16]. Therefore, we prospectively quantified abnormal movements, including tonic flexion or extension and clonic movements, in DRD mice from 1 month of age, a juvenile developmental stage, through 15-months-old, late in the typical lifespan of a mouse. Abnormal movements increased during adolescence, peaked at 2.5 to 4 months of age and ebbed in 12–15-month-old mice (Fig. 1A; $p < 0.05$ for 2.5 or 3 months vs. 1, 1.5, 12, and 15 months and $p < 0.05$ for 4 months vs. 12 and 15 months Tukey's *post hoc* test). Normal littermates never exhibited dystonic movements (not shown). Some reports suggest that prolonged L-DOPA treatment in DRD patients induces dyskinetic side effects [17] whereas others suggest that the response is stable over time [18]. Like patients, DRD mice receive daily L-DOPA treatments throughout life. L-DOPA abolished abnormal movements in 15-month-old DRD mice (Fig. 1B). Abnormal movements in DRD mice were also significantly reduced by both the D1R agonist SKF 81297 and the D2-type DA receptor (D2R) agonist quinpirole (Fig. 1B). Thus, dyskinetic movements were not observed in response to either indirect-or direct-acting agonists, despite chronic life-long L-DOPA treatment.

We also prospectively assessed locomotor activity in normal and DRD mice. Overall, locomotor activity decreased with age in both DRD and normal mice. However, based on the significant genotype $\times$ age interaction effect (Fig. 2A), the age-related changes in locomotor activity of normal and DRD mice did not occur in parallel. After an initial early increase in locomotor activity, normal mouse activity gradually declined with age. In contrast, 1-month-old DRD mice were hyperactive, similar to the hyperactivity in rats deprived of DA as neonates [19]. Thereafter, DRD and normal mouse locomotor activity was comparable until 12 months of age when DRD mice exhibited a reduction in locomotor activity compared to age-matched normal littermates. To test the ability to initiate voluntary movements, we tested the ability of a mouse to escape an imposed abnormal posture. Normal mice immediately escaped, regardless of age. DRD mice showed a mean latency to move of ~12 sec at 3 months of age, and the mean latency to move increased to ~28 sec at 15 months of age. (Fig. 2B). L-DOPA treatment abolished the deficit in the ability to initiate voluntary movement in DRD mice.

To determine if the hypokinetic motor dysfunction in aged DRD mice is associated with nigrostriatal degeneration, we assessed midbrain DA neurons in 15-month-old normal and

DRD mice using immunostaining for TH. We have previously demonstrated that the number of midbrain DA neurons in 3-month-old DRD mice is comparable to normal mice [9]. Consistent with results in 3-month-old DRD mice [9], TH staining in 15-month-old DRD mice was weaker than in normal mice (Fig. 3A), likely due to the instability of the p.381Q>K mutant TH protein [20]. However, there was no difference in the number of midbrain TH-positive neurons between aged normal and DRD mice (Fig. 3D). DAT immunostaining, a marker of DA terminals, was also similar in 15-month-old DRD and normal mice (Fig. 3B, 3C). Finally, a direct assessment of neurodegeneration using Fluoro-Jade staining did not reveal Fluoro-Jade-labeled cells in 15-month-old normal or DRD mice (Fig. 3D), whereas many Fluoro-Jade-positive cells were observed in the 6OHDA-lesioned positive control tissue (Fig. 3E). Taken together, these results suggest that overt neurodegeneration does not likely explain the age-related decline in motor activity in DRD mice.

It is possible that age-related changes in DA release [21] account for the change in motor behaviors. Because the striatal DA concentration in DRD mice is too low to assess extracellular DA using microdialysis or fast scan cyclic voltammetry, we instead exploited the locomotor response to amphetamine, which stimulates presynaptic DA release, as an indirect measure of presynaptic function. Both 3- and 15-month-old normal and DRD mice exhibited a significant increase in locomotor activity in response to 4 mg/kg amphetamine compared to saline (Fig. 4A). Consistent with studies in aging rats [22], amphetamine-induced locomotor activity was attenuated in normal 15-month-old mice compared to 3-month-old mice ($p<0.01$; Fig. 4B). In contrast, the locomotor response to amphetamine was comparable in young and aged DRD mice (Fig. 4A), suggesting that a change in presynaptic dopaminergic tone does not likely account for the reduction in locomotor activity and deficit in the ability to initiate movement in aged DRD mice. Because we have previously demonstrated that postsynaptic D1R responses are supersensitive in young DRD mice [9], we hypothesized that a decrement in the D1R receptor response may be associated with the age-related motor dysfunction. Therefore, we assessed locomotor activity in response to the D1R agonist SKF 81297 in young and aged mice. Although overall locomotor activity was attenuated in 15-month-old normal mice compared to 3-month-old normal mice (Fig. 4D), the EC_{50} for SKF 81297-induced motor activity was ~ 1 mg/kg in both ages. In contrast, the EC_{50} for SKF 81297 in 3-month-old DRD mice was ~ 0.08 mg/kg, but increased to ~ 0.2 mg/kg in 15-month-old DRD mice (Fig. 4C). The highest dose tested in young DRD mice was 0.2 mg/kg SKF 81297 because doses as low as 0.5 mg/kg sometimes caused seizures, a phenomenon normally associated with 10-fold higher doses [23]. In contrast, 1 mg/kg SKF 81297 never induced seizures in aged DRD mice, another indication that D1R sensitivity is reduced with age.

Discussion

Recent genetic and imaging studies implicate nigrostriatal degeneration as a critical factor in producing late-onset parkinsonism in patients with DRD-causing mutations [5–7]. Although these observations have led to speculation that these mutations cause degenerative Parkinson's disease, postmortem anatomical studies have not found evidence of neurodegeneration in DRD patients [3, 4]. Using a mouse model of DRD, which facilitated

prospective behavioral, functional and anatomical analyses that are not feasible in patients, we demonstrate that as dystonic movements waned with age, hypokinesia and akinesia, increased in DRD mice. The hypokinesia and akinesia combined with the dopamine deficiency in aged DRD mice resemble some aspects of parkinsonism, though it must be acknowledged that parkinsonism is associated with specific signs and symptoms in humans that are difficult to translate precisely in mice. DRD patients also appear to exhibit a similar phenotypic shift with age [2, 15, 24], though to our knowledge, no controlled, longitudinal cohort study akin to the one performed here with DRD mice has been performed with DRD patients. Despite the age-related increase in hypokinesia, decrements in midbrain DA neuron numbers and presynaptically-mediated dopaminergic responses were not observed in aged DRD mice, demonstrating that the hypokinetic features evolved without the neurodegeneration and concomitant reduction in presynaptically-mediated DA neurotransmission associated with classic Parkinson's disease. In contrast, we identified an age-related postsynaptic decrement in D1R agonist sensitivity that could, at least in part, account for the behavioral changes. While a more extensive analysis of postsynaptic changes is needed to reveal detailed mechanisms, our results suggest that neurodegeneration may not be required to produce the age-related parkinsonism that is often observed in DRD and suggest the alternative hypothesis that postsynaptic plasticity contributes to the development of parkinsonism in DRD-mutation carriers.

Our results demonstrating that hypokinetic behavior associated with a DA deficit can emerge without nigrostriatal neurodegeneration contrast with recent studies suggest that neurodegeneration underlies late-onset parkinsonism in DRD-mutation carriers [5–7]. Species differences might account for the discrepant findings. It is also possible that the specific gene defect plays a significant role in determining the fate of dopaminergic neurons; studies in humans all focused on the GCH1 gene for DRD, whereas our studies focused on the TH gene. Approaches used to quantify DA neurons may also account for the different findings. We directly quantified DA neurons in mice. In the human studies, DA neuron integrity was assessed using DAT imaging, which is often used to infer neurodegeneration in Parkinson's disease but does not necessarily correlate with nigral neuron loss [25]. Similarly, loss of DAT occurs in methamphetamine abusers without DA terminal loss [26]. Further, DAT availability is highly regulated [27, 28] and correlated with synaptic DA levels whereby high DAT expression is associated with high synaptic DA concentrations [26]. Therefore, it is possible that the low DAT availability observed in DRD mutation carriers with adult onset parkinsonism reflects homeostatic DAT downregulation in response to lifelong low synaptic DA concentrations, considering that these patients have not benefitted from chronic dopamine replacement therapy, unlike DRD patients and mice. Postmortem analyses will be useful to discern degeneration, DAT plasticity or both in these patients.

In both DRD and Parkinson's disease, it is not uncommon for patients to exhibit both dystonia and/or parkinsonism, but the precise mechanism(s) governing the proclivity toward one motor sign or the other is not fully understood, although both are mediated by low synaptic DA concentrations. Here, we demonstrate for the first time the co-occurrence of dystonia and some features reminiscent of parkinsonism in a mouse model carrying a human disease-causing mutation, which will greatly facilitate the identification of both shared pathophysiologies and abnormalities unique to each disorder. Understanding both shared and

unique pathomechanisms has significance not only for DRD but also for Parkinson's disease in which dystonia is often a co-morbid feature.

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Abbreviations

D1R	D1-type dopamine receptor
D2R	D2-type dopamine receptor
L-DOPA	L-3,4-dihydroxyphenylalanine
DRD	L-DOPA-responsive dystonia
DA	dopamine
DAT	dopamine reuptake transporter
GCH1	GTP cyclohydrolase
6OHDA	6-hydroxydopamine
TH	tyrosine hydroxylase
s.c.	subcutaneous
SN	substantia nigra
VTa	ventral tegmental area

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Figure 1.

Dystonic movements decrease with age in DRD mice. (A) Abnormal movements were examined prospectively in DRD mice ($n=5$ [2 male, 3 female]/time point). Age significantly affected the expression of abnormal movements ($F_{4,32} = 6.91$, $p<0.001$, repeated measures ANOVA). (B) Peripheral administration of 5 mg/kg L-DOPA, 0.1 mg/kg SKF 81297, and 0.1 mg/kg quinpirole significantly reduced dystonic movements in 15 month-old DRD mice ($n=4$ [4 male], Student's paired one tailed t test). Values represent mean $\pm$ SEM; * $p<0.05$, ** $p<0.01$.

Figure 2.

DRD mice become hypokinetic with age. (A) Locomotor activity in a novel open field was assessed prospectively in DRD ($n=5$ [2 male, 3 female]/time point) and normal mice ($n=6$ [4 male, 2 female]/time point) for one hour. There was a significant genotype $\times$ age interaction effect ($F_{7,63} = 5.85$, $p < 0.001$, two-way repeated measures ANOVA) reflecting a shift from hyperactivity at 1 month of age to hypoactivity at 12 months of age in DRD mice ($p < 0.001$, Tukey's *post hoc* test within DRD mice). (B) The ability to initiate voluntary movements was assessed in DRD and normal mice using the mouse test of catalepsy at 3 months of age ($n = 7$ [3 male, 4 female] DRD mice; $n = 7$ [1 male, 6 female] normal mice) and 15 months of age ($n = 7$ [3 male, 4 female] DRD mice; $n = 10$ [4 male, 6 female] normal mice). There was a significant genotype $\times$ age interaction effect ($F_{1,27} = 8.31$, $p < 0.01$, two-way ANOVA) with DRD mice showing increased latency to move at 15 months compared to 3 months ($p < 0.001$, Tukey's *post hoc* test within DRD mice). Peripheral administration of 5 mg/kg L-DOPA significantly reduced the latency to move in 15-month-old DRD mice ($p < 0.001$; paired Student's *t* test). Values represent mean $\pm$ SEM; *** $p < 0.001$.

Figure 3.

Lack of nigrostriatal degeneration in 15-month-old DRD mice. (A, B) Representative sections immunostained for TH (A) or DAT (B) from striatum or midbrain of 15 month-old normal and DRD mice (scale bars = 1 mm, striatum; 200 μ m, midbrain). (C) DAT immunointensity (n=4 DRD [4 male] mice; n=4 [4 male] normal mice) expressed as a percentage of normal. There was no significant difference ($p>0.05$) between normal and DRD mice in DAT immune-intensity. (D) Stereological cell counts of TH-positive neurons (n=6 [6 male] DRD mice; n= 6 [5 male, 1 female] normal mice). There was no significant difference in the number of TH-positive cells in normal and DRD mice in either the substantia nigra pars compacta (SNc; $p>0.1$, Student's *t* test) or ventral tegmental area (VTA; $p>0.1$, Student's *t* test). Values represent mean $\pm$ SEM. (E) DRD mice (n=3), normal mice (n=3) and a single matched nigral section from a 6-OHDA rat were stained for Fluoro-Jade B to detect ongoing degeneration. Fluoro-Jade staining in the SNc of normal and DRD mice show no Fluoro-Jade-positive cells, whereas Fluoro-Jade abundantly label cells in 6-OHDA lesioned SNc (scale bar = 40 μ m).

Figure 4.

Dopamine receptor agonist-induced responses in young and aged DRD and control mice. (A, B) Locomotor response to 4 mg/kg amphetamine in 3- and 15 month-old DRD mice (A; n=6 [3 male, 3 female] 3 month-old; n = 6[2 male, 4 female] 15 month-old) and normal mice (B; n=6 [5 male, 1 female] 3 month-old; n = 6[1 male, 5 female] 3 month-old). In DRD mice, there was a significant main effect of treatment in DRD mice ($F_{1,10} = 17.6$, $p < 0.01$, two-way repeated measures ANOVA), but not age. In contrast, there was a significant treatment $\times$ age interaction effect in normal mice ($F_{1,10} = 5.7$, $p < 0.05$, two-way repeated measures ANOVA) whereby 15-month-old normal mice exhibited a significantly reduced response to amphetamine compared to 3-month-old normal mice ($p < 0.01$, Tukey's *post hoc* test). (C, D) Locomotor response to the D1R agonist SKF 81297 in 3 month-old (n = 8 [3 male, 5 female]) and 15-month-old DRD mice (C; n=6 [2 male, 4 female]) and normal mice (D; n = 8 [3 male, 5 female] 3 month-old; n = 6 [1 male, 5 female] 15 month-old). A significant age $\times$ dose interaction effect was observed in DRD mice ($F_{3,36} = 7.86$, $p < 0.001$, two-way repeated measures ANOVA), where a significant difference between saline and 0.08 mg/kg SKF 81297 was observed in 3-month-old DRD mice ($p < 0.001$, Holm-Sidak *post hoc* test) but not in 15-month-old DRD mice ($p > 0.1$, Holm-Sidak *post hoc* test). In normal mice, there was a significant main effect of dose ($F_{5,60} = 34.6$, $p < 0.001$, two-way repeated measures ANOVA) and age ($F_{1,12} = 21.8$, $p < 0.001$, two-way repeated measures ANOVA),

but there was not an age $\times$ dose interaction effect ($F_{5,60} = 1.91$, $p > 0.1$, two-way repeated measures ANOVA). Saline baseline and maximum SKF 81297-stimulated locomotor activity was not significantly different between normal and DRD mice at either 3 or 15 months of age (Student's t test). Values represent mean $\pm$ SEM; ** $p < 0.01$, *** $p < 0.001$.