Disorders of the Nervous System

The *Polg* Mutator Phenotype Does Not Cause Dopaminergic Neurodegeneration in *DJ-1*-Deficient Mice

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Abstract

Mutations in the *DJ-1* gene cause autosomal recessive parkinsonism in humans. Several mouse models of *DJ-1* deficiency have been developed, but they do not have dopaminergic neuron cell death in the substantia nigra pars compacta (SNpc). Mitochondrial DNA (mtDNA) damage occurs frequently in the aged human SNpc but not in the mouse SNpc. We hypothesized that the reason *DJ-1*-deficient mice do not have dopaminergic cell death is due to an absence of mtDNA damage. We tested this hypothesis by crossing *DJ-1*-deficient mice with mice that have similar amounts of mtDNA damage in their SNpc as aged humans (*Polg* mutator mice). At 1 year of age, we counted the amount of SNpc dopaminergic neurons in the mouse brains using both colorimetric and fluorescent staining followed by unbiased stereology. No evidence of dopaminergic cell death was observed in *DJ-1*-deficient mice with the *Polg* mutator mutation. Furthermore, we did not observe any difference in dopaminergic terminal immunostaining in the striatum of these mice. Finally, we did not observe any changes in the amount of GFAP-positive astrocytes in the SNpc of these mice, indicative of a lack of astrogliosis. Altogether, our findings demonstrate the *DJ-1*-deficient mice, *Polg* mutator mice, and *DJ-1*-deficient *Polg* mutator mice have intact nigrostriatal pathways. Thus, the lack of mtDNA damage in the mouse SNpc does not underlie the absence of dopaminergic cell death in *DJ-1*-deficient mice.

Key words: *DJ-1*; mtDNA; neurodegeneration; parkinsonism; *Polg* mutator; substantia nigra

Significance Statement

Parkinson’s disease research has been hampered by the absence of animal models that replicate the disease phenotypes observed in humans. We hypothesized that the reason mice lacking *DJ-1*, a gene that causes parkinsonism when mutated, do not replicate the human phenotype is because mice do not have the same levels of mtDNA damage that humans do. We tested this hypothesis by crossing *DJ-1*-deficient mice with mice that develop similar amounts of mtDNA damage as humans. We found that the added stress of mtDNA damage does not cause the *DJ-1*-deficient mice to replicate the human phenotype. These data should be informative for the development of future animal models of Parkinson’s disease.

Introduction

Early onset autosomal recessive parkinsonism is caused by mutations in the *parkin*, *PINK1*, and *DJ-1* genes (Kitada

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et al., 1998; Bonifati et al., 2003; Valente et al., 2004). PINK1 and parkin have been shown to maintain mitochondrial quality control (Corti and Brice, 2013). While the precise biological function of DJ-1 is unknown, it is known to respond to oxidative stress and defend against mitochondrial damage (Wilson, 2011). Since mitochondrial dysfunction and oxidative stress are features of Parkinson’s disease (PD) (Hauser and Hastings, 2013), studying the functions of PINK1, parkin, and DJ-1 may lead to insights about the pathogenesis of sporadic Parkinson’s disease.

In the last decade, several independent lines of DJ-1 knockout mice have been generated and characterized by multiple groups (Chen et al., 2005; Goldberg et al., 2005; Kim et al., 2005; Manning-Bog et al., 2007; Chandran et al., 2008; Pham et al., 2010; Rousseaux et al., 2012). In most cases, there was no evidence of dopaminergic cell death in the substantia nigra, with the exception of one of the more recent studies that observed it in a subset of DJ-1−/− mice (Rousseaux et al., 2012). The general lack of dopaminergic neuronal degeneration has also been reported for parkin knockout (Goldberg et al., 2003; Itier et al., 2003), PINK-1 knockout (Kitada et al., 2007), and triple DJ-1/parkin/PINK-1 knockout mice (Kitada et al., 2009).

The reasons underlying the phenotypic discrepancies between mouse models of autosomal recessive parkinsonism and the humans that have these diseases are not known. It is possible that mouse substantia nigra pars compacta (SNpc) neurons deficient for the autosomal recessive PD genes do not degenerate because they are not exposed to the same types of stressors that human SNpc neurons are. One such stressor is mitochondrial DNA (mtDNA) damage, which accumulates with age at high levels in the human SNpc (Bender et al., 2006; Kraytsberg et al., 2006). Polg mutant mice develop mtDNA damage as they age due to a knock-in proofreading-deficient version of the mtDNA polymerase gamma (Kujoth et al., 2005). By the time they reach 1 year of age, ~50% of mtDNA molecules found in SNpc neurons of the Polg mutant mice have deletions, which is comparable to that observed in the aged human SNpc (Bender et al., 2006; Kraytsberg et al., 2006; Perier et al., 2013). This increased mtDNA damage results in a decrease in the abundance of mitochondrial respiratory chain complex I subunits in Polg mutant brains (Hauser et al., 2014). Since DJ-1 protects against complex I inhibition both in vitro (Mullett and Hinkle, 2011) and in vivo (Kim et al., 2005), we hypothesized that increasing mtDNA damage in the DJ-1 knockout mouse SNpc would result in neurodegeneration. We tested this hypothesis by crossing DJ-1 knockout mice with Polg mutant mice.

Materials and Methods

**DJ-1;Polg mice**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committees of the US National Institute of Child Health and Human Development (Animal study protocol number 12-059).

The Polg mutator mice used in this study were originally described by Kujoth and colleagues (2005). DJ-1 knockout mice were generated and originally characterized by Chandran and colleagues (2008) and given to us after having been backcrossed at least two generations into C57BL/6J. We backcrossed the DJ-1 mouse for an additional three generations into C57BL/6J prior to mating one DJ-1−/− mouse with one PolgWT/MT mouse. DJ-1−/−;PolgWT/MT mice were then bred with each other to produce the cohorts of mice used in this study. All of the mice were given access to food and water ad libitum.

Four genotypes (DJ-1−/−;PolgWT/WT, DJ-1−/−;PolgMT/MT, DJ-1+/−;PolgWT/WT, and DJ-1+/−;PolgMT/MT) of mice were aged to at least 1 year (365–391 d, median 377 d) before transcardial perfusion. One male DJ-1+/−;PolgMT/MT mouse and one male DJ-1−/−;PolgMT/MT mouse were euthanized at the ages of 340 and 352 d, respectively, at the request of the veterinarians due to the severity of their phenotype. One female DJ-1−/−;PolgMT/MT mouse was euthanized at 365 d at the request of the veterinarians due to an ear infection. These mice were not used for weight analysis or the pole test but were used for immunohistochemistry.

**Pole Test**

We performed the pole test as previously described (Ogawa et al., 1985; Matsuura et al., 1997). A wooden dowel (1 cm diameter, 0.5 m height) was mounted into a wooden base and the entire apparatus was placed into an empty mouse cage and covered with fresh bedding. The mice were placed at the top of the pole and video recorded as they descended. Several pretrials were done before a series of four to seven trials were recorded for each animal. Some animals were given an intraperitoneal injection of L-DOPA (25 mg/kg) and benserazide (5 mg/kg) after their first set of trials and then subjected to four to seven more trials 30 min following the injection. After all the mice had been tested, an operator that was blinded to both the genotype and drug treatment of the mice scored the video files. The operator recorded the time it took the mice to reach the floor of the cage after being placed atop the pole along with their method of doing so (Walk, Slide, Walk/Slide, or Fall). A mouse was judged to have fallen if it fell to the cage floor at any point of its descent.

**Immunohistochemistry**

Mice were transcardially perfused using PBS (1 min) and then 4% PFA in PBS (5 min). After perfusion, brains were removed and postfixed overnight in 4% PFA in PBS at 4 °C. The brains were then transferred into a solution of 30% w/v sucrose in PBS that was supplemented with 30% w/v sucrose in PBS that was supplemented with...
0.05% sodium azide and stored at 4 °C until the brains had sunk to the bottom of the containers. Each brain was then bisected along the longitudinal fissure and the left hemisphere was sectioned on a cryostat into 40-μm-thick sections. Slices that included the midbrain were collected and stored individually, while sections rostral and caudal to the midbrain were stored in groups. For stereology, every fourth section through the midbrain was stained for glial fibrillary acidic protein (GFAP) and/or tyrosine hydroxylase (TH) immunoreactivity using a free-floating procedure in which all steps were performed on a rotating shaker (~250 rpm).

For the 3,3′-diaminobenzadine (DAB) staining protocol, the sections were incubated in 0.3% hydrogen peroxide in PBS for 20 min at room temperature (RT) then washed three times with PBS. Sections were blocked for 1 h at RT in blocking buffer [PBS supplemented with 1% w/v bovine serum albumin (BSA), 0.3% Triton X-100, and 1% v/v donkey serum], which was also used to dilute primary and secondary antibodies in later steps. Sections were then incubated overnight in primary TH antibody (PelFreez # P40101, rabbit polyclonal, 1:2000 dilution) and GFAP antibody (BD Pharmingen #556329, mouse monoclonal, 1:1000 dilution) at 4 °C. The following day, sections were rinsed in 1× PBS before being mounted on glass slides using Prolong Gold mounting media.

For the fluorescent staining protocol, the sections were washed with 1× PBS three times each for 10 min at room temperature on shaker. Sections were blocked for 1 h at RT in blocking buffer (PBS supplemented with 1% w/v BSA, 0.3% Triton X-100, and 1% v/v donkey serum), which was used to dilute primary and secondary antibodies in later steps. Sections were then incubated overnight in primary TH antibody (PelFreez # P40101, rabbit polyclonal, 1:2000 dilution) and GFAP antibody (BD Pharmingen #556329, mouse monoclonal, 1:1000 dilution) at 4 °C. The sections were then washed three times for 10 min each in 1× PBS before being mounted on glass slides using Prolong Gold mounting media.

Stereology was performed on a Zeiss Axio Imager A1 microscope running Stereo Investigator software (MBF Biosciences). An operator blinded to the genotype of each sample operated the microscope and performed stereology. Unbiased counting of the SNpc TH- and GFAP-positive cells was accomplished using the software’s optical fractionator protocol. As only the SNpc of the left hemisphere was analyzed, the cell counts were multiplied by two to estimate whole-brain SNpc cell numbers.

In order to determine striatal TH terminal density, three sections through the striatum were stained per animal, with one DJ-1+/−;PolgMT/MT animal removed from this analysis because its striatum was sectioned at a different thickness than all other animals. The sections were stained for TH as described above, except in this case a different secondary antibody was used (Jackson ImmunoResearch #711-655-152, Alexa-Fluor 790 AffiniPure Donkey anti-rabbit IgG, 1:1000).

| Data structure | Type of test | Power (f = 0.25) |
|----------------|-------------|------------------|
| a N too small to determine if normally distributed | $ \chi^2 $ | 0.999 |
| b N too small to determine if normally distributed | One-way ANOVA | 0.0001 |
| c N too small to determine if normally distributed | One-way ANOVA | 0.471 |
| d N too small to determine if normally distributed for 3 of 4 genotypes. The fourth genotype with 8 animals is normally distributed. | One-way ANOVA | 0.274 |
| e N too small to determine if normally distributed for 3 of 4 genotypes. The fourth genotype with 8 animals is normally distributed. | One-way ANOVA | 0.632 |
| f N too small to determine if normally distributed for 3 of 4 genotypes. The fourth genotype with 8 animals is normally distributed. | One-way ANOVA | 0.493 |
| g N too small to determine if normally distributed for 3 of 4 genotypes. The fourth genotype with 8 animals is normally distributed. | One-way ANOVA | 0.350 |
To quantitatively image the sections, all of the slides were scanned at once using an Odyssey CLx imaging system. The highest resolution (21 μm) and scan quality settings were used, and the system’s automatic intensity feature was employed to avoid pixel saturation. The signal intensity was measured inside an equally sized circle placed approximately in the same area of the dorsal striatum of each slice. The mean intensity of the sections from each animal was used for comparisons.

Statistics

Post hoc power analysis was done using the “pwr” package in R (http://www.R-project.org/). Sample sizes were the minimum group size, the effect size was 0.25 (Cohen, 1988), and the p values calculated from the ANOVA or χ² tests were used to determine post hoc power values. These values are reported in Table 1.

Results

We bred double heterozygous DJ-1+/−;PolgWT/MT mice and analyzed the birth rates of the resulting nine genotypes (Fig. 1A,B). All of the genotypes were born at the anticipated Mendelian ratios (Fig. 1B). From the nine possible genotypes of mice, we used the four double homozygous genotypes for subsequent analysis. We aged a cohort of 27 mice (Table 2) for 1 year in order to maximize the aging effect of the Polg phenotype. We note that this approaches the maximum lifespan of these animals as the Polg genotype causes severe weight loss as the animals approach 1 year of age (Kujoth et al., 2005). We weighed our animals after they had reached a year of age to determine if the loss of DJ-1 had any effect on the weight loss phenotype caused by Polg mutation (Fig. 1C,D). In both males and females, we observed weight loss in the Polg mutator animals consistent with previous results (Kujoth et al., 2005). However, in the females, we observed no difference between the Polg mutator mice with and without DJ-1 (Fig. 1C). Our cohort did not have enough males to allow for statistical analysis, but the trend of no difference was also apparent in the males (Fig. 1D).

In order to determine if any of the mice had motor impairments that could be indicative of dopamine cell loss, we tested them using the pole test. During this test, the mouse is placed atop a vertical pole and observed as...
Mice with SNpc dopamine cell loss caused by 6-hydroxydopamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine spend significantly longer amounts of time at the top of the pole (Ogawa et al., 1985; Matsuura et al., 1997). Importantly, the behavior of mice lesioned by either drug can be rescued by administration of L-DOPA, which demonstrates that this test is sensitive to dopamine levels (Ogawa et al., 1985; Matsuura et al., 1997). When we tested our mice using this assay, we noticed that mice with the Polg mutator mutation tended to slide down or fall off of the pole but did not freeze at the top (Fig. 2A). Videos of a DJ-1+/+;PolgWT/WT mouse and a DJ-1-/-;PolgWT/WT mouse performing the task correctly by reorienting themselves and walking down the pole are shown in Movies 1 and 2, respectively. A DJ-1+/+;PolgMT/MT mouse sliding down the pole and a DJ-1-/-;PolgMT/MT falling from the pole can be seen in Movies 3 and 4. When we compared the duration it took the mice to descend, regardless of falls and slides, we found that there was no difference between the genotypes (Fig. 2B). This likely reflects the different means by which the animals descended. To determine if dopamine depletion was...
the underlying reason that the Polg mutator genotypes tended to slide and fall, we gave several of them L-DOPA to increase dopamine levels in their brains and tested them again. We found that L-DOPA did not prevent the mice from sliding or falling during the task (Movies 5,6; Fig. 2C). Thus, the inability of Polg mutator mice, regardless of DJ-1 genotype, to perform the pole test correctly was likely not due to dopamine deficiency.

After all of the mice had been perfused, we determined the integrity of their nigrostriatal axis using several measures. First, we counted the number of dopaminergic neurons in the SNpc using unbiased stereology. The staining and counting was done blindly, and a prospective power analysis calculated that our study design had a power of 95.5% to detect a 25% change in SNpc TH-positive cells. We performed the stereology experiment twice, once with a colored DAB stain (Fig. 3A,B) to mark TH-positive neurons and then once with fluorescent detection (Fig. 3C,D) using a separate group of tissue sections. We reasoned that doing the stereology using two different methods would decrease the likelihood of de-
In some instances of damage to the nigrostriatal axis, such as methamphetamine toxicity, the cell bodies of SNpc dopaminergic neurons remain alive while their nerve terminals in the striatum degenerate (Ricaurte et al., 1982). To determine if there was any dopaminergic terminal degeneration in any of our mice, we immunostained sections through their striata for TH and quantified the stain intensity using an infrared imaging system. Using this assay, we were unable to detect any changes in striatal TH intensity amongst any of our groups of mice (Fig. 4).

Altogether, our data demonstrates that the nigrostriatal axis is intact in aged Polg mutator mice with DJ-1 deficiency. Since DJ-1 is known to be expressed in astrocytes (Bandopadhyay et al., 2004), we considered whether or not our mice would have phenotypes that manifest themselves in astrocytes. To determine this, we chose to examine the SNpc for astrogliosis as indicated by increased GFAP immunoreactivity. We found no difference when we compared the numbers of GFAP-positive astrocytes in the SNpc between the genotypes (Fig. 5).
Therefore, the Polg mutator mutation in DJ-1-deficient mice does not cause increased astrogliosis in the SNpc.

**Discussion**

We hypothesized that crossing DJ-1-deficient mice with Polg mutator mice in order to increase mtDNA damage in their substantia nigra would result in the degeneration of dopaminergic neurons. Using a cohort of mice designed to test this hypothesis with sufficient statistical power, we were able to demonstrate that our hypothesis was false. We also found that the loss of DJ-1 had no effect on the weight phenotype of the Polg mutator mice, that none of the nine possible combinations of DJ-1 and Polg genotypes were embryonic lethal, and that there was not increased astrogliosis in the Polg mutator DJ-1-deficient mouse SNpc.

Our results are similar to other studies that have crossed DJ-1/parkin/PINK-1 had no effect on SNpc cell numbers in mice up to 24 months of age (Kitada et al., 2009). Similarly, crossing DJ-1/parkin knockout mice with GPx1 knockout mice did not result in SNpc degeneration at 18 months of age (Hennis et al., 2014). Likewise, no effect on dopaminergic cell numbers was observed when DJ-1/parkin knockout mice were crossed with mice deficient for either SOD1 or SOD2 and aged to at least 16 months (Hennis et al., 2013).

Two studies have analyzed the nigrostriatal axis in aged Polg mutator mice (Dai et al., 2013; Perier et al., 2013). While both studies found no degeneration of SNpc dopaminergic neuron cell bodies, they reported conflicting results for striatal TH terminal density. One reported a decrease in striatal TH staining in aged Polg mutator mice (Dai et al., 2013), while the other did not observe a change in striatal TH (Perier et al., 2013). In our cohort of animals,

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**Figure 4** Dopaminergic terminal density in the striatum. A, Representative TH-stained tissue sections through the striatum. The sections were immunostained using an infrared fluorescent dye conjugated secondary antibody and imaged using an infrared imaging system. The sections are pseudo-colored using a heat map, with warmer colors indicating strong TH immunoreactivity. B, Striatal TH staining intensity calculated from infrared imaged tissues. Individual data points represent animals and the mean and SEM are also displayed (three sections per animal were averaged, n = 5-8 animals, ANOVA $F_{(3,22)} = 1.189, p = 0.3369$).

**Figure 5** Detection of astrogliosis in the SNpc. A, GFAP-positive astrocytes were immunostained in the SNpc (outlined in white) and surrounding tissue (GFAP = red; scale bars, 500 µm). Unbiased stereology was used to count GFAP-positive cells in the SNpc simultaneously with the TH cells counts shown in Figure 3B. B, GFAP-positive SNpc cell counts per animal (red circles) along with mean and SEM are displayed in the graph (ANOVA $F_{(3,23)} = 1.744, p = 0.1860$).
the Polg mutator genotype did not cause SNpc cell loss nor did it cause the loss of striatal TH terminals.

Previous studies have shown that aged Polg mutator mice accumulate SNpc mtDNA deletions to a similar extent as that found in the SNpc in both PD patients and aged neurologically normal controls (~50% of mtDNA molecules harboring deletions) (Bender et al., 2006; Kraytsberg et al., 2006; Perier et al., 2013). In addition, our analysis of the brains of aged Polg mutator mice from our own colony demonstrated a loss of respiratory chain proteins, which is indicative of mtDNA damage (Hauser et al., 2014). Since the experiments reported here required the use of fixed tissue, measuring the amount of mtDNA damage in the SNpc of our mice could not be done and is an important future experiment. We note that the Polg mutator mouse with and without DJ-1 all developed the premature aging phenotype and the body weights between these two groups were similar (Fig. 1C,D). This suggests that the absence of DJ-1 was unlikely to have strongly accelerated mtDNA damage caused by the Polg mutation, although we cannot exclude a more subtle effect. Regardless, whatever the level of mtDNA damage that had occurred in these animals, it was not sufficient to induce dopaminergic cell death. Whether or not other genetic manipulations combined with the loss of DJ-1 lead to SNpc degeneration in mice should be the subject of future studies.

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