Loss of Parkin contributes to mitochondrial turnover and dopaminergic neuronal loss in aged mice

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A B S T R A C T

Parkinson's disease (PD), the second most common neurodegenerative disorder, is characterized by the loss of nigrostriatal dopamine neurons. PARK2 mutations cause early-onset Parkinson's disease (EO-PD). PARK2 encodes an E3 ubiquitin ligase, Parkin. Extensive in vitro studies and cell line characterization have shown that Parkin is required for mitophagy, but the physiological pathology and context of the pathway remain unknown. In general, monogenic Parkin knockout mice do not accurately reflect human PD symptoms and exhibit no signs of dopaminergic (DA) neurodegeneration. To assess the critical role of Parkin-mediated mitophagy in DA neurons, we characterized Parkin knockout mice over a long period of time. At the age of 110 weeks, Parkin knockout mice exhibited locomotor impairments, including hindlimb defects and neuronal loss. In their DA neurons, fragmented mitochondria with abnormal internal structures accumulated. The age-related motor dysfunction and damaged mitochondria pathology in Parkin-deficient mice suggest that impairment of mitochondrial clearance may underlie the pathology of PD.

1. Introduction

Parkinson's disease (PD), the second most common neurodegenerative disorder, is characterized by the loss of nigrostriatal dopamine neurons and the formation of intracellular Lewy bodies (LBs), which consist primarily of α-synuclein (Spillantini et al., 1997). Interactions between genetic predisposition and environmental factors are likely to be primarily responsible for mitochondrial dysfunction and oxidative damage, which result in oligomerization and aggregation of synuclein; however, the underlying molecular mechanisms remain poorly understood. The vast majority of PD is sporadic, and inherited familial forms of the disease accounting for only 5% of all cases (Dauer and Przedborski, 2003). The identification of PD-related genes and risk factors has implicated several pathways in PD etiology, with accumulating evidence suggesting a link between dysfunctional mitochondrial clearance and PD pathogenesis. In PARK2-linked PD, intrinsically disordered mutant Parkin initiates PD pathology (Kitada et al., 1998).

Since the discovery of Parkin, the protein has received special interest because it has ubiquitin–protein ligase activity. Early studies assumed that proteolytic dysfunction causes substrate accumulation, resulting in massive loss of dopaminergic (DA) neurons (Chung et al., 2001; Shimura et al., 2000). Subsequently, several lines of Parkin-null mice were analyzed, but the causative mechanisms underlying early-onset PD (EO-PD) remain largely unknown, in part because most substrates of Parkin reported thus far remain unchanged in the disease state. Furthermore, loss of DA neurons is not observed in Parkin-deficient mice (Goldberg et al., 2003; Periquet et al., 2005; Sato et al., 2006).

Given that Parkin is associated with mitochondrial degradation in vitro (Matsuda et al., 2010; Narendra et al., 2008), it is likely that a dysfunction of mitophagy (i.e., autophagy of the mitochondria) contributes to the neurodegenerative process. Macroautophagy (hereafter,
autophagy) is a highly conserved bulk protein degradation pathway in eukaryotes. Cytoplasmic proteins and organelles, including mitochondria, are engulfed within autophagosomes; these bodies eventually fuse with the lysosome, where they are degraded along with their cargo (Levine and Klionsky, 2004). Insights regarding the functions of PINK1 and Parkin (Pickrell and Youle, 2015), which shed light on the mitochondria, have also enhanced our understanding of EO-PD pathogenesis. Specifically, experiments in which cultured cells were treated with the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), which causes mitochondrial depolarization, revealed that the PINK1/parkin pathway is associated with mitochondrial quality control.

Several lines of evidence indicate that damaged mitochondria are predominantly degraded by mitophagy in vitro. In addition, Parkin mutations implicate induction of mitophagy in PD, and suggest that accumulation of damaged mitochondria contributes to DA neurodegeneration. However, the phenotypes of mice harboring Parkin deletion have not been fully examined. To elucidate the critical role of PINK1/Parkin-mediated mitophagy in DA neurons, we characterized the age-related pathological and motor phenotypes in Parkin knockout mice.

2. Materials and methods

2.1. Generation of Parkin knockout mice

A targeting vector was constructed using 1.5- and 7-kb DNA fragments as the 5′ and 3′ homologous sequences, respectively. A negative selection cassette, DTA, which encodes the diphtheria toxin, was also included. The linearized targeting vector was transfected into TT2 ES cells. After selection in G418, clones were screened for homologous recombination by Southern blotting. Using the 5′ external probe and probe specific for neo sequence, we confirmed that the clones carried the desired homologous recombination. ES cells derived from these clones were injected into C57BL/6 J mice to obtain germline transmission, which was confirmed by Southern analysis with the 5′ probe. Heterozygous mice were then interbred to obtain homozygous knockout and wild-type control mice. Mice were subsequently genotyped by PCR using primers specific for the wild type and the targeted allele (Saito et al., 2006). All animals were kept in a pathogen- and odor-free environment, which was maintained under a 12 h light/dark cycle at ambient temperature. Procedures were approved by the Animal Experimental Committee of the Juntendo University Graduate School of Medicine, and performed in accordance with the guidelines of NIH and the Juntendo University Graduate School of Medicine.

2.2. Behavioral tests

Locomotor behavior was assessed in mice from 100 to 125 weeks of age. Accelerating rotarod tests were performed on a rotarod machine with automatic timers and falling sensors (MK-660D, Muromachi Kikai, Japan). Male Parkin knockout mice and wild-type mice (n = 10 for each genotype) were placed on a 3-cm diameter rotating rod covered with rubber, and rotation was accelerated from 3 to 40 rpm over 5 min. Fall latency was recorded, and the first fall latency of the third trial was used for analysis. The runway test was performed using a narrow, horizontally fixed beam (3 cm wide, 80 cm long, held at a height of 40 cm from the table). The animal was placed at one end of the beam and urged to move toward the opposite end, where an escape platform was located. Each genotype mice were randomly selected and behavioral tests were conducted in a blinded fashion.

2.3. Histological analyses

A combination anesthetic (Kawai et al., 2011) was prepared with 0.3 mg/kg of medetomidine hydrochloride (DO-0003; Nippon Zenyaku Kogyo, Japan), 4.0 mg/kg of midazolam (001541582; Astellas Pharma, Japan), and 5.0 mg/kg of butorphanol (DO-0023; Meiji Seika Pharma, Japan). The anesthetics were administered to mice by intraperitoneal injection. Mice were perfused with 4% paraformaldehyde (PFA), and their brains were immersion-fixed at 4 °C for 36 h. The fixed samples were cryoprotected with 20% sucrose and sliced on a freezing microscope to obtain 40-μm-thick floating sections. For immunohistochemistry of TH, sections were incubated with anti-TH antibody (657,012; Calbiochem, Germany) and visualized with DAB (Diaminobenzidine), which generates brown precipitates. The sections were observed on a VS120 (Olympus, Japan). The counting of dopaminergic neurons was done with unbiased stereology. For double immunofluorescence staining for cytochrome c (green) and TH (red), floating sections were incubated with anti–cytochrome c antibody (ab90529; Abcam, UK) and anti-TH (clone TH-16; Sigma-Aldrich, USA) antibody. The sections were then incubated with anti–guinea pig IgG conjugated with Alexa Fluor 488 and anti-rabbit IgG conjugated with Alexa Fluor 546. Fluorescence signals were captured on an FV3000 confocal microscope (Olympus). Using these fluorescence data, the number and area of green stained mitochondria within red stained dopaminergic neurons were measured using “cellsens” analysis software (Olympus, Japan).

2.4. Stereology quantification

For stereological quantification, three areas (VTA: ventral tegmental area; SNcc: center area of substantia nigra pars compacta; SNcl: lateral area of substantia nigra pars compacta) were selected. Every other 40-μm section of serial coronal brain slices for each genotype were stained for DAB. Quantification was performed with design-based stereology system (Stereo-Investigator version 2019, MBF Bioscience, Williston, VT, USA). The sampling parameters were set up according to the software guide to achieve the coefficient of error ranged between 0.07 and 0.09 using the Gundersen test, normally with a counting frame size 30 × 30μm, optical dissector an average of 10 sampling sites per section.

2.5. Electron microscopy

For conventional electron microscopy, mice were fixed by cardiac perfusion with 2.5% glutaraldehyde in 0.1 mol/L PB (pH 7.2). Brain slices were embedded in epoxy resin, and ultrathin sections (70 nm thick) were cut and observed on an H7700 electron microscope (Hitachi, Japan). Using these electron microscopy data, the number and area of mitochondria within dopaminergic neurons were manually measured using “cellsens” analysis software (Olympus, Japan).

2.6. HPLC analysis

Dorsal striata from 120-week-old mice were dissected, quickly frozen on dry ice, and then homogenized with 0.5 mL of 0.2 M perchloric acid containing 100 μM EDTA per 100 mg wet tissue. Samples were centrifuged at 20,000 g for 15 min at 4 °C, and the supernatant was collected and analyzed by HPLC.

2.7. Statistical analysis

Statistical significance was determined by Student’s t-test (STATVIEW; SAS Institute). Data are expressed as means ± S.E. P < .05 was considered significant.
Fig. 1. Behavioral examinations of aged Parkin knockout mice. (A) Runway test of wild-type mice (a) and Parkin knockout mice (b). In contrast to wild-type mice, which exhibited well-coordinated movement and almost no slips of the forepaw or hindpaw from the beam, Parkin knockout mice could hardly move on the beam, and the hindpaw slipped frequently (see Video S1, Parkin KO; Video S2, Parkin WT). These experiments were performed using mice over 110 weeks of age. In this figure, the hindpaw of the Parkin knockout mouse has slipped off the beam. (c) The number of hindlimb slips was recorded from 80-week-old (Parkin wild-type, n = 5; Parkin knockout, n = 5) and 120-week-old (Parkin wild-type, n = 5; Parkin knockout, n = 5) mice crossing the challenge beam. Data are means ± SE (error bars); statistical significance was evaluated using Student’s t-test. ** $P < .01$. (B) Footprint of wild-type mice and Parkin knockout mice. Red footsteps indicate forepaws; black footsteps indicate hindpaws. Each stride length was recorded. Data are means ± SE (error bars); statistical significance was evaluated using Student’s t-test. ** $P < .01$. (C) In the accelerating rotarod assay, rotation was accelerated from 3 rpm to 40 rpm over 5 min, and fall latency was recorded. The experiments were performed using mice from 100 to 125 weeks of age (Parkin wild-type, n = 5; Parkin knockout, n = 5). Red line: Parkin knockout mice; blue line: wild-type mice. Data are means ± SE (error bars); statistical significance was evaluated using Student’s t-test. ** $P < .01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3. Results

3.1. Characterization of the locomotor impairments of aged Parkin knockout mice

Previously, we generated mice deficient in exon 2 of Parkin (Parkin knockout mice) and characterized them in the early part of life. When young, the mice exhibited very little phenotypes other than down-regulation of DA release and upregulation of D1 and D2 dopamine receptors in the striatum. However, the abundance of DA neurons was not decreased in 48-week-old mice (Sato et al., 2006). Hence, we observed Parkin knockout mice over longer period of time.

Parkin knockout mice were viable at birth, indistinguishable in appearance from their littersates, and had a normal survival rate. Although these mice have not yet been observed over their entire lifespan, they began to show motor behavioral deficits at 110 weeks. These clinical abnormalities could be observed by the runway (Fig. 1A a, b, and c; Video S1, Parkin KO; Video S2, Parkin WT), footprint (Fig. 1B), and rotarod tests (Fig. 1C). In the runway test, wild-type mice exhibited well-coordinated movement and almost no slips of the forepaw or hindpaw from the beam; by contrast, Parkin knockout mice could hardly move on the beam, and frequently slipped. In particular, the hindpaws of Parkin knockout mice often slipped off the beam (Fig. 1A b and c). Next, we conducted the footprint test, because a short stride is a characteristic of PD, including patients with PARK2 mutations. Parkin knockout mice had a shorter foot range than wild-type mice (Fig. 1B). Furthermore, in the accelerating rotarod test, fall latency was reduced in Parkin knockout mice (Fig. 1C). There were no effects on weight (Supplementary. Fig. S2. A) and lifespan (Supplementary. Fig. S2. B). Gait disturbance progressed, and by the terminal stage (beyond 120 weeks), the majority of affected mice could hardly move.

3.2. Loss of Parkin leads to mitochondrial fragmentation in DA neuron

Accumulating knowledge regarding PINK1 and Parkin, both of which are associated with mitochondrial, has increased our understanding of the proteins’ cellular functions. The PINK1/Parkin pathway is associated with mitochondrial quality control, but it remains unclear how Parkin influences mitochondrial function or structure in vivo.

To evaluate its in vivo function, we analyzed mitochondria in DA neurons in 110-week-old Parkin-deficient mice, which exhibit locomotor impairments. To identify DA neuron, we performed immunofluorescence staining for tyrosine hydroxylase (TH) and cytochrome C (mitochondria inner membrane protein). Specifically, after fixation with 4% paraformaldehyde (PFA), 6-μm paraffin-embedded tissue sections were cut and stained for TH and cytochrome c. For immunofluorescence, secondary antibodies were coupled to Alexa fluorophores (Invitrogen). In Parkin knockout mice (Fig. 2A d–f), small mitochondria accumulated in the substantia nigra (SN) to a greater extent than in wild-type mice (Fig. 2A a–c). These mitochondria were observed not only in the cytosol, but also outside the cell bodies. We speculated that the phenomenon would also be observed in axon and dendrites. To evaluate mitochondria in DA neurons, we analyzed mitochondrial area in the TH-positive cell bodies. In Parkin knockout DA neurons, mean mitochondrial area was smaller than in wild-type mice (Fig. 2B). Next, we investigated the percentage of the cytosolic area occupied by mitochondria. DA cells in Parkin knockout mice trended toward a higher ratio, but did reach significant difference from wild type mice (Fig. 2C). Together, these observations suggest that mitochondrial fragmentation might be facilitated in the DA neurons of aged mice. We supposed that damaged mitochondria were fragmented and the number of mitochondria increased.

3.3. Mitochondria with damaged internal structure accumulate in Parkin knockout mice

To further characterize these mitochondria, we performed ultrastructural analysis in DA neurons of 110-week-old mice (Fig. 3A a–c, wild-type control; Fig. 3A d–f, Parkin knockout mouse sample 1; Fig. 3A g–i, Parkin knockout mouse sample 2). We observed small, round, fragmented mitochondria in Parkin-deficient DA neurons (Fig. 3A d–i), but not in controls (Parkin wild-type) (Fig. 3A a–c). Precise quantification revealed that mitochondria area was reduced (Fig. 3B left panel) and the number of fragmented mitochondria per unit of cytosolic area was elevated (Fig. 3B right panel) in Parkin-deficient DA neurons. In order for damaged mitochondria to be degraded by autophagy, they must be segregated by fission (Twig et al., 2008). If Parkin-mediated mitophagy might contribute to mitochondrial degradation systems, our in vivo results are reasonable.

Next, we observed mitochondrial microstructure in Parkin knockout mice. Electron microscopy revealed that the fragmented mitochondria had normal outer membrane structure (Fig. 3A c inset), but displayed irregular inner structure, i.e., the normal structures of matrix and cristae were broken. This observation indicates that damaged mitochondria accumulate due to abrogation of Parkin-mediated mitophagy. Finally, we confirmed the presence of Lewy bodies, but we could not find them in Parkin knockout mice.

3.4. The number of TH neurons decreases in aged Parkin knockout mice, contributing to locomotor dysfunction

Although the molecular components required for mitophagy have been identified through extensive in vitro work, the physiological pathology and context of these pathways remains largely unknown (Rodger et al., 2018). Many genetically modified mice have been developed as PD models, but most of them do not exhibit neuronal loss. To assess the contribution of the accumulation of damaged mitochondria, we compared the number of TH neurons between aged Parkin knockout and control mice. As demonstrated in the rotarod test (Fig. 1C), Parkin knockout mice exhibited locomotor dysfunction at ages above 110 weeks. We sacrificed these mice (Parkin knockout, n = 5; wild type, n = 5) and counted TH neurons in three sections (VTA: ventral tegmental area; SNcc: center area of substantia nigra pars compacta; SNc: lateral area of substantia nigra pars compacta) (Fig. 4). Parkin knockout mice had fewer TH neurons than wild-type control mice. The reduction in TH cell number was most prominent in SNcc (Fig. 4B). We conducted stereological quantification of the area of cell bodies, but we did not observe a difference between Parkin WT (mean 374.48 ± 10.1 μm²) and Parkin KO (mean 312.85 ± 33.6 μm²). Furthermore, we conducted stereological quantification of striatal DA fibers (Supplementary. Fig. S1. B). Parkin KO mice demonstrate significant loss of dopamine fibers in the striatum. The possibility that the behavioral changes are due to Parkin deficiency in other parts of the brain must be elucidated. Then, we conducted further pathological examination in cortex, cerebellum and striatum. But, we found no obvious pathological changes including Lewy pathology (Supplementary. Fig. S1).

No neuronal loss was observed in young mice, which did not exhibit motor dysfunction (data not shown). Consistent with our results, a reduction in the abundance of TH neurons also occurs in PD, primarily in the SNcc. Loss of DA neurons may contribute to the motor impairment observed in the late stages of disease. In addition, we tested DA physiology in these 120-week-old Parkin knockout mice by neurochemical analysis of the dorsal striata. High-performance liquid chromatography (HPLC) revealed a reduction in striatal DA levels in Parkin knockout mice relative to control mice (Fig. 4C). Thus, DA content is affected by loss of DA neurons. Intriguingly, the significant decrease in dopamine is not accompanied by concomitant reductions in the dopamine catabolites DOPAC and HVA. Currently, we have not clarified the reason, but...
abnormal mitochondrial accumulations may induce a partially modified DA metabolism (Thiffany et al., 2000).

4. Discussion

Great progress has been made toward understanding the pathogenesis of familial PD, mainly due to the discovery of PINK1/Parkin-mediated mitophagy. However, little is understood about the in vivo functions underlying PD pathogenesis. In this study, we carried out detailed observations of old Parkin-deficient mice, which exhibit motor dysfunction and loss of DA neurons in association with aging.

In general, the phenotypes of Parkin knockout mice do not accurately reflect the symptoms of PD (Goldberg et al., 2003; Periquet et al., 2005; Sato et al., 2006). In 2006, we performed the first examination of the status of DA metabolism in the brain, focusing on DA release and DA receptors using in vivo autoradiography. In vivo autoradiography using [11C]raclopride revealed a clear decrease in endogenous DA release after methamphetamine challenge in Parkin-deficient mice. Furthermore, Parkin deficiency was associated with considerable upregulation of dopamine D1 and D2 receptor binding in the striatum, as well as elevated DA levels in the midbrain. These results suggested that DA neurons behave abnormally prior to their death; this event may be the initial change underlying the development of EO-PD (Sato et al., 2006).

Parkin knockout mice tend to anticipate phenotypes including DA neuronal loss (Pickrell et al., 2015; Pinto et al., 2018), but monogenic Parkin knockout mice do not accurately reflect human PD symptom and show no signs of DA neurodegeneration. The most parsimonious explanation for the lack of DA neurodegeneration in genetic PD models is...
A

B

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the existence of a compensatory mechanism resulting from adaptive changes during development, making it hard to observe the degenerative phenotype over the lifespan of the mouse. To identify the phenotypes associated with Parkin deficiency, we observed motor and pathological phenotypes in mice older than 100 weeks. Interestingly, PD model mice began to exhibit a motor phenotype (Fig. 1C) and neuronal loss (Fig. 4B) around the age of 110 weeks. In 2018, in an effort to create a PD model, we generated DA neuron–specific autophagy-deficient mice (Atg7fl/fl;TH-Cre), and we observed the same motor dysfunction and neurodegeneration phenotypes in these animals. Specifically, Atg7fl/fl;TH-Cre mice began to exhibit impairment in motor coordination tasks around 100 weeks and apparent motor behavioral deficits around 110 weeks. In contrast to control mice (Atg7fl/fl), which exhibited well-coordinated movement and almost no slips of the forepaw or hindpaw from the beam, Atg7fl/fl;TH-Cre mice could hardly move on the beam and slipped frequently. In particular, the hindpaws of Atg7fl/fl;TH-Cre mice often slipped off the beam. Furthermore, in the accelerating rotated test, fall latency was reduced in Atg7fl/fl;TH-Cre mice. Moreover, aged Atg7fl/fl;TH-Cre mice exhibited synuclein accumulation in neurites and/or cell bodies, as well as loss of DA neurons (Sato et al., 2018). These features may represent a common motor phenotype in PD models. Long-term disruption of autophagy results in widespread synuclein accumulation in DA neurons. In a previous study, Friedman et al. (Friedman et al., 2012) reported that DA axons developed synuclein aggregates at the age of 20 months, and suggested that autophagy is involved in synuclein homeostasis in mice. The protein catabolic pathways that regulate synuclein degradation have been discussed by multiple authors (Ebrahimi-Fakhari et al., 2011; Rideout et al., 2004; Webb et al., 2003). Additionally, autophagy plays essential roles in the clearance of organelles, including mitochondria (Twig et al., 2008).

Many mutations at the PARK2 locus induce autosomal recessive Parkinsonism (Hattori et al., 1998; Lucking et al., 1998), as do mutations of the PINK1 (PTEN-induced kinase 1) gene at the PARK6 (Valente et al., 2004). PINK1 contains an N-terminal mitochondrial targeting signal and a highly conserved serine/threonine kinase domain, in which many missense and nonsense mutations have been reported. The activity of PINK1 kinase is crucial for the phosphorylation of ubiquitin (Kane et al., 2014) (Kazlauskaite et al., 2014) (Koyano et al., 2014) and Parkin (Shiba-Fukushima et al., 2012), which in turn are required for mitochondrial maintenance. Loss of PINK1/Parkin function may increase the cell’s vulnerability to various stresses. In Drosophila models, PINK1 and parkin ensure stable mitochondrial function. Parkin-null mutants exhibit severe mitochondrial pathology in association with reduced lifespan, apoptosis, and muscle degeneration. The PINK1 mutant phenotype can be rescued by overexpression of Parkin, suggesting that Parkin and PINK1 act in a common pathway to maintain mitochondrial integrity. PINK1 loss-of-function decreases mitochondrial membrane potential, and the PINK1/parkin pathway is associated with mitochondrial elimination. To date, the exact mechanism leading to mitochondrial autophagy has not been fully elucidated (Pickrell and Youle, 2015).

Based on previous findings, we carefully monitored Parkin-deficient mice and observed accumulation of fragmented mitochondria (Fig. 2B, 3B) in the DA neurons and livers of aged mice (data not shown). In Parkinson knockout mice, small mitochondria accumulated in DA neurons (Fig. 2A d–f). Interestingly, these mitochondria were observed not only within the cytosol, but also outside the cell. Mean mitochondrial area was smaller in Parkinson-deficient DA neurons than in wild-type mice (Fig. 2B). To further characterize these mitochondria, we conducted ultrastructural analysis. In Parkinson knockout mice, DA neurons were filled with small, round mitochondria (Fig. 3); mean mitochondrial area was smaller than in wild-type mice, and the number of mitochondria per unit of cytosolic area as elevated (Fig. 3B). Careful observation of the internal structure of mitochondria in Parkinson-deficient mice revealed that the matrix and cristae structure were broken (Fig. 3A). In aged Parkinson-deficient DA neurons, fragmented and damaged mitochondria accumulated.

As to how Parkinson deficiency is linked to mitophagy and fission/fusion, recent studies of the substrates of mitochondrial ubiquitin ligases including MULAN, MARCH5, and Parkin, identify them as potential critical mediators that govern selective fusion and mitophagy. Parkin was shown to translocate selectively to energetically impaired mitochondria. Studies in Drosophila have shown that upon recruitment to depolarized mitochondria, Parkin ubiquitinates Mfn (Ziviani et al., 2010). And the knockout of Parkin leads to an accumulation of Mfn and to mitochondrial elongation (Poole et al., 2010). These results in Drosophila suggest that in the absence of Parkin, damaged mitochondria are rescued by fusion instead of being removed by mitophagy. This compromises mitochondrial quality maintenance. It should be mentioned that the effects of manipulations on Parkin had an ambiguous effect on mitochondrial morphology between Drosophila and mouse. In our report, we observed that Parkinson KO led to mitochondria fragmentation in Parkinson-deficient mice. Other reports on Parkinson KO mice support the observation of fragmented mitochondrial phenotypes and demonstrate no Mfn accumulation (Stevens et al., 2015) (Pinto et al., 2018). The reasons for these conflicting reports remain to be fully elucidated, but, species-specific differences may be a contributing factor.

Considering mitochondria degradation in cultured cells, depolarized mitochondria were clustered with p62 (Narendra et al., 2010) (Okatsu et al., 2010). Specifically, depolarized mitochondria are ubiquitylated by Parkin, recognized by p62 (Bartlett et al., 2011), transported via microtubules to the cytosol, and degraded by autophagy (Komatsu et al., 2007). Loss of Parkin may inhibit physiological clustering of depolarized mitochondria and microtubule transport to the cytosol. An electron microscopic analysis by Gai et al. revealed that many mitochondria are concentrated in the early forms of Lewy bodies (LBs) (Gai et al., 2000), and Bedford et al. also reported deposits of mitochondria in early LBs in PD patients (Bedford et al., 2008). We speculate that parkin-mediated clustering of p62 may be a key process involved in LB formation. Consistent with this, LBs are usually absent in EO-PD caused by Parkin mutations (Mori et al., 1998).

In general, Parkinson KO mice show a rare phenotype, namely, that they do not demonstrate DA neuronal loss and movement dysfunction. Previously, we have reported no loss of neurons in Parkinson exon 2 deletion mutant mice at ages up to 12 months (48 weeks) (Sato et al., 2006) and Perez and Palmer claimed no loss of neurons at ages up to 22 months (88 weeks) (Perez and Palmer, 2005). Specifically, Parkinson KO mice did not recapitulate central signs of EO-PD. But in our
Fig. 4. Delayed degeneration of DA neurons in Parkin knockout mice.

(A) Histological analyses of SN in 120-week-old Parkin knockout mice and wild mice. Paraffin sections were immunostained for TH. SNcl: lateral area of substantia nigra pars compacta; SNcc: center area of substantia nigra pars compacta; VTA: ventral tegmental area. Scale bars: 20 µm. (B) DA neurons in the SN were identified by TH immunoreactivity. Data are means ± SE (Parkin knockout mice, n = 5; wild mice, n = 5); ** P < .01 (Student’s t-test). (C) HPLC analysis of DA (left), DOPAC (middle), and HVA (right) levels in dorsal striatum at 120 weeks. Data are means ± SE (Parkin knockout mice, n = 3; wild mice, n = 3); * P = .05 (Student’s t-test).
investigation, the long-term observation enabled us to find dopaminergic neuronal loss at 120 weeks. Previous reports indicated that Parkin-deficient mice display inconsistent phenotypes (Itier et al., 2003), (Goldberg et al., 2003), (Von Coelln et al., 2004), (Palacino et al., 2004). One of these reasons is differences in the genetic background between the mouse strains tested. Thus, we performed further examinations in mice with the same genetic background. Intriguingly, our Parkin KO mice are B6;129 genetic background, the same as in Perez's study. Our results demonstrated that Parkin exon 2 deletion mutant mice exhibited age-dependent DA neuronal loss at 120 weeks. In addition to the genetic background, the type of Parkin mutation could explain the phenotypic differences in various Parkin KO mice, because these finding could be due to the loss of Parkin function or expression of a mutant Parkin. Although the phenotypes of Parkin-deficient mice could be modified by genetic background or the mutation, carefully controlled studies are required to rule out the possibility that these phenotypes are not the result of Parkin deletions and instead reflect artifacts due to several technical confounding factors of gene targeting in mice (Gerlai, 1996), (Gerlai, 2001). Further long-term observation beyond 110 weeks will be needed to identify Parkin KO phenotypes.

Identifying the reasons, why highly aged Parkin KO mice demonstrate Parkinsonism, could provide us with important clues to elucidate the pathogenesis of PD. For example, an analysis of PARIS (ZNF746) levels using a conditional knockout of Parkin in adult animals may reveal to progressive loss of dopaminergic neurons in a PARIS-dependent manner (Shin et al., 2011).

In our investigation, the lack of Parkin leads to an increase in the small size of mitochondria, which occupy the same or greater proportion of cytosolic area of DA neurons in aged mice. Stevens et al. reported that conditional knockout of Parkin in the adult mice ventral midbrain leads to a decrease in mitochondria size and number with dopaminergic neuronal loss (Stevens et al., 2015). On the other hand, Pinto et al. showed that Parkin-deficient mice, where mitochondrial DNA undergoes double-strand breaks in the dopaminergic neurons only, exhibit a small but significant decrease in the number of mitochondria in the dopaminergic axons only (Pinto et al., 2018). Fragmented small mitochondria are commonly observed in Parkin KO mice. In general, damaged mitochondria are fragmented. Considering the pathophysiology of Parkin loss, it was assumed that the accumulation of damaged mitochondria might be derived from defects in Parkin-mediated mitophagy. However, given the formation of damaged mitochondria, other factors remain to be elucidated. Currently, mutant mtDNA levels and levels of proteins associated with mitochondrial dynamics, such as Mfn2, Opa1, Drp1, and a repressor of PGC-1 called PARIS, may contribute.

The age-related motor dysfunction and pathology we observed in Parkin-deficient mice suggests that impairment of mitochondrial clearance may underlie PD pathology. Our PD model mice exhibited accumulation of damaged mitochondria and neuronal loss. Furthermore, DA levels were affected by DA neuronal loss (Fig. 4B). These PD models will provide insight into mitochondria-associated PD pathology, and will be crucial for the development of novel therapeutic targets.

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Authors contributions

Authors' contributions SS, NH designed the study, and wrote the initial draft of the manuscript. SN, TF contributed to analysis and interpretation of data, and assisted in the preparation of the manuscript. All other authors have contributed to data collection and interpretation, and critically reviewed the manuscript. All authors approved the final version of the manuscript, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declaration of Competing Interest

The author declares no conflicts of interest.

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