Parkin-deficient Mice Exhibit Nigrostriatal Deficits but Not Loss of Dopaminergic Neurons*

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Loss-of-function mutations in parkin are the major cause of early-onset familial Parkinson’s disease. To investigate the pathogenic mechanism by which loss of parkin function causes Parkinson’s disease, we generated a mouse model bearing a germline disruption in parkin. Parkin−/− mice are viable and exhibit grossly normal brain morphology. Quantitative in vivo microdialysis revealed an increase in extracellular dopamine concentration in the striatum of parkin−/− mice. Intracellular recordings of medium-sized striatal spiny neurons showed that greater currents are required to induce synaptic responses, suggesting a reduction in synaptic excitability in the absence of parkin. Furthermore, parkin−/− mice exhibit deficits in behavioral paradigms sensitive to dysfunction of the nigrostriatal pathway. The number of dopaminergic neurons in the substantia nigra of parkin−/− mice, however, is normal up to the age of 24 months, in contrast to the substantial loss of nigral neurons characteristic of Parkinson’s disease. Steady-state levels of CDCrel-1, synphilin-1, and α-synuclein, which were identified previously as substrates of the E3 ubiquitin ligase activity of parkin, are unaltered in parkin−/− brains. Together these findings provide the first evidence for a novel role of parkin in dopamine regulation and nigrostriatal function, and a non-essential role of parkin in the survival of nigral neurons in mice.

Parkinson’s disease (PD) is an age-related movement disorder characterized by bradykinesia, rigidity, resting tremor, and postural instability. The neuropathologic hallmarks of PD are the loss of dopaminergic neurons in the substantia nigra (SN) and the presence of intraneuronal cytoplasmic inclusions known as Lewy bodies. The clinical manifestations of PD are due to progressive degeneration of dopaminergic neurons in the pars compacta of the SN that give rise to the nigrostriatal pathway, causing dopamine (DA) depletion in the striatum, where it is required for normal motor function. Little is known about the mechanisms of PD pathogenesis and nigral degeneration, although DA neurons have been shown to be susceptible to oxidative stress (1), mitochondrial defects (2), and environmental toxins (3).

The recent identification of genes linked to familial forms of PD (FPD) makes it possible to investigate the pathogenic mechanism by employing genetic approaches (4–6). Over fifty recessively inherited mutations, including deletion, frameshift, nonsense, and missense mutations, have been identified in parkin in large numbers of families, making parkin the major gene responsible for early-onset FPD (7–10). Although the first report linked parkin mutations to autosomal recessive juvenile parkinsonism (AR-JP) with atypical clinical features (5), many more cases identified subsequently were considered typical early-onset FPD with symptoms often indistinguishable from sporadic PD (9, 11). Autopsies of limited numbers of patients showed selective loss of dopaminergic neurons in the SN either in the absence (12–15) or in the presence (16) of Lewy bodies. The recessive inheritance mode and variety of parkin mutations indicate a loss-of-function pathogenic mechanism. Parkin is widely expressed in most tissues including brain and heart (5). Although its transcripts are equally abundant in various brain sub-regions, parkin protein is enriched in the SN (5, 17–20). In vivo studies have shown that parkin can function as an E3 ubiquitin ligase, mediating the covalent transfer of ubiquitin to protein substrates subject to proteasomal degradation (21–23). However, it is unclear how loss of parkin function leads to nigral degeneration and PD.

To investigate the pathogenic mechanism of PD in an animal model and to elucidate the normal physiological role of parkin in vivo, we created a mouse model bearing a targeted germline disruption of parkin. Molecular, histological, neurochemical, electrophysiological, behavioral, and biochemical analyses of parkin−/− mice reveal a novel role for parkin in dopamine regulation and nigrostriatal function in vivo, and a non-essential role of parkin in the survival of nigral neurons in mice.

EXPERIMENTAL PROCEDURES

Generation of parkin−/− Mice—A targeting vector was constructed using 1.8- and 3.5-kb DNA fragments as the 5′ and 3′ homologous sequences, respectively (Fig. 1A). A negative selection cassette, PGK-dt, which encodes the diphtheria toxin and has been shown to enhance

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sclerin assay for homologous recombination. Six clones were identified by the presence of the expected 3.7-kb band corresponding to the targeted allele. Using the 3’ external probe and a probe specific for the neo sequence, two clones were confirmed to carry the desired homologous recombination events without random insertion. ES cells of both clones were injected into C57BL/6 and Balb/c blastocysts. Chimeric offspring were crossed with C57BL/6 mice to obtain germline transmission, which was confirmed by Southern analysis with the 5’ probe shown in Fig. 1A.

Histology and Neuron Count—Mouse brains were dissected, Formalin fixed for 2 h, processed for paraffin embedding, and sectioned in the coronal plane at 16-μm thickness. Each paraffin block contained 4 parkin−/− and 4 wild-type brains. Deparaffinized sections were stained with cresyl violet or tyrosine hydroxylase (TH) antibodies. The immunoreactive neurons in coronal sections of four brains per genotype were counted using unbiased stereology under a Leica DMRB microscope equipped with an image analysis software. Values are reported as means ± S.E. Statistical differences were assessed by Student’s t test.

Behavioral Tests—All tests were performed by investigators blind to the genotypes. For the open field test, individual mice were placed in 42 × 42 cm acrylic animal cages for 15 min during which their horizontal and vertical movements were monitored by 3 arrays of 16 infrared light beam sensors (AccuScan Instruments) and analyzed using AccuScan VersaMax software.

For the rotarod test, mice were placed 4 at a time on an EconomeX accelerating rotarod (Columbus Instruments) equipped with individual timers for each mouse. Mice were initially trained to stay on the rod at 20 rpm for 5 min. After a 2-min rest, each mouse was trained for 2 days to stay on the rotating rod for at least 2 min. Following training, mice were subsequently tested by placing them on the rod at a rotation speed of 5 rpm, as the rod accelerated by 0.2 rpm/sec, the latency to fall was measured. Mice were tested for a total of 3 trials.

For the beam traversal task, a Plexiglas beam (Plastics Zone Inc., Woodland Hills, CA) consisting of four sections (25 cm each, 1 m total length) of varying width (3.5, 2.5, 1.5, and 0.5 cm) was used. To increase the difficulty of the task, a wire mesh cover (1 cm²) of corresponding width was placed on the beam surface. Mice were trained for 2 days to traverse the beam without the wire mesh to their home cages. On the third day, for each test, mice were trained for 5 min further with two trials with the grid overlay and two trials with the wire grid placed on the beam. Mice were then tested for 3 trials by traversing the grid-surfaced beam, and their performance was videotaped. The numbers of steps and slips (a limb slipped through the wire grid during a forward movement) were measured and input-output relationships were plotted. From each cell, the averaged PSPs and peak amplitude were measured, and input-output relationships were plotted. From each cell, the averaged PSPs and peak amplitude were measured, and input-output relationships were plotted. From each cell, the averaged PSPs and peak amplitude were measured, and input-output relationships were plotted.
counted by viewing the videotapes in slow motion. Fisher’s LSD was used for planned comparisons between genotypes.

For the adhesive removal test, small adhesive stimuli of five increasing sizes were placed on the forehead, out of view for the mice. The stimuli consisted of 0.25 and 0.5 inch Avery labels cut in half or whole or combined. To remove the stimulus, mice raised both forelimbs toward their head and swiped off the stimulus with both forepaws within a 60-s trial, after which the experimenter removed the adhesive. Each mouse was given a score equal to the largest size adhesive it was unable to sense and remove, averaged over two trials. Scores were compared between genotypes using a Mann-Whitney U test. All mice were able to sense and remove the largest size adhesive, but none could sense and remove the smallest adhesive.

**RESULTS**

**Generation of Parkin-deficient Mice**—parkin is a large gene (~2 Mb), which contains 12 exons and encodes a protein of 465 amino acid residues (5, 33). The exon 3 deletion mutation is one of the most common mutations in AR-JP and results in absence of parkin protein (8, 18, 20). Exon 3 contains a non-integral number of codons, thus, deletion of exon 3 results in a frame-shift after amino acid 57 and premature termination at a stop codon in exon 4 following 39 additional out-of-frame amino acid residues in humans (8, 18, 20). We therefore chose to target exon 3 to generate a parkin-null mutant mouse. A targeting vector was constructed in which most of exon 3 was replaced in-frame by the coding sequence of EGFP, followed by translation and transcription termination sequences and the PGK-neo cassette (Fig. 1A). The protein sequences predicted to result from wild-type and mutant transcripts are depicted in Fig. 1B. Two clones of ES cells carrying the proper homologous recombination events without random integration of the targeting vector were injected into blastocysts. Germine transmission of the targeted allele was confirmed by Southern analysis (Fig. 1C). Interbreeding of heterozygous mice gave rise to wild-type, heterozygous, and homozygous knockout (parkin−/−) mice at the expected Mendelian ratio.

To determine whether our targeted mutation causes skipping of exon 3, we performed Northern and RT-PCR analyses. Northern analysis of total RNA using a probe specific for exons 4–12 showed a smaller parkin transcript in parkin−/− brains (Fig. 1D), RT-PCR analysis using primers specific for exons 2 and 5 followed by sequencing confirmed that in parkin−/− brains exon 2 was spliced to exon 4, skipping exon 3 entirely (Fig. 1E). Exon 3 skipping causes a reading frameshift after amino acid 57 and premature termination at a stop codon in exon 5 following 49 additional out-of-frame amino acid residues in mice (Fig. 1B). The sensitivity of RT-PCR confirmed the absence of intact parkin transcripts in parkin−/− mice. Sequencing also revealed an aberrant splice product, which results from the use of a cryptic splice acceptor site 3 bases into exon 4, leading to addition of 48 rather than 49 out-of-frame amino acid residues (Fig. 1B). Although these truncated parkin transcripts are present in parkin−/− mice, it is unlikely that functional parkin fragments can be produced from these truncated transcripts. Western analysis using an antisera raised against the C-terminal region of parkin confirmed the absence of parkin in parkin−/− mice (Fig. 1F), and ruled out the presence of possible parkin fragments initiated from in-frame ATGs downstream of exon 3, consistent with the notion that reinitiation of translation following a sizable open reading frame is highly unlikely (34).

Since we introduced the EGFP cDNA fused in-frame into parkin exon 3, which was intended for a reporter system for the parkin promoter activity, we also performed Northern analysis using an EGFP-specific probe and confirmed the presence of EGFP transcripts in parkin−/− mice (data not shown). RT-PCR followed by sequencing confirmed that the EGFP coding sequence is intact and fused in-frame to parkin exon 3. However, the parkin-EGFP fusion protein was barely detectable by Western analysis (data not shown), perhaps due to the presence of the ubiquitin-like domain of parkin (35).

parkin−/− mice are viable and fertile without obvious abnormalities. Open field tests of parkin−/− mice revealed no significant alterations in their general behavior and exploratory anxiety (Fig. 2). Nissl staining revealed normal brain morphology in parkin−/− mice (Fig. 3, A and B). Immunohis-
tochemical analysis of parkin−/− brains using antibodies specific for synaptophysin, Munc-18 and calbindin showed grossly normal synaptic staining and striatum formation (data not shown). No inclusions were observed in any brain sub-regions, including the SN, using antibodies specific for α-synuclein and ubiquitin (data not shown).

Normal Neuroanatomy of DA Neurons in parkin−/− Mice—It has been proposed that the reduction in DA neurons in AR-JP could be due to impaired generation and maturation of these neurons, based on the observation that the remaining DA neurons in autopsies of AR-JP patients appear immature (18). Recently, parkin-null flies have also been shown to exhibit smaller dopaminergic neurons (36). Immunohistochemical analysis of parkin−/− mice using an antibody specific for TH, however, revealed normal morphology of DA neurons in the SN (Fig. 3, C–F) and noradrenaline neurons in the locus ceruleus (Fig. 3, G and H).

The most prominent neuropathological feature of AR-JP and PD is the selective loss of dopaminergic neurons in the SN (12–15). We therefore quantified the number of DA neurons in the SN of parkin−/− (n = 4) and control mice (n = 4) at 12, 18, and 24 months using unbiased stereological methods (27). Similar numbers of TH-positive neurons were found in the SN of parkin−/− and wild-type mice at 12 (+/+: 8520 ± 540; −/−: 8720 ± 710; p > 0.05), 18 (+/+: 8960 ± 450; −/−: 9360 ± 470; p > 0.05) and 24 months (+/+: 10500 ± 720; −/−: 10300 ± 1180; p > 0.05). We also measured the volume of DA neurons (+/+: n = 47; −/− n = 50) in the SN of 4 mice per genotype at age 24 months, and found similar neuron volumes between parkin−/− (2702 ± 150 μm³) and wild-type (2674 ± 173 μm³; p > 0.05) mice.

Increased Extracellular DA in the Striatum of parkin−/− Mice—We then looked for alterations in dopamine neurotransmission, which may occur prior to frank loss of dopaminergic neurons. Dopaminergic projections revealed by TH immunoreactivity appear normal in the striatum of parkin−/− mice (Fig. 3, I and J). Striatal levels of DA and its major metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) are similar between parkin−/− and control mice at 6, 12, 18, and 24 months (p > 0.05) (Fig. 4A). To determine whether there are alterations in dopamine release or reuptake, we performed no-net-flux microdialysis (37, 38) in the striatum of parkin−/− and wild-type mice at 8–9 months. When perfused with ACSF containing no DA, the dialysate DA concentration in parkin−/− mice (n = 10) (10.8 ± 1.2 nM) was significantly higher than that in wild-type mice (n = 10) (7.7 ± 0.4 nM; p < 0.02) (Fig. 4B).

The concentration of DA at the interpolated point of no-net-flux, a measure of extracellular DA concentration, was also significantly higher in parkin−/− mice (n = 10) (24.2 ± 1.2 nM) relative to wild-type controls (n = 9) (20.2 ± 0.9 nM; p < 0.02) (Fig. 4, C and D). The data from one wild-type mouse (indicated by an arrow in Fig. 4C), which met the statistical standard as an outlier (39), was excluded. The extraction fraction, a measure of DA reuptake, was not significantly different between wild-type (0.39 ± 0.04) and parkin−/− (0.46 ± 0.05; p > 0.05) mice (Fig. 4E). The elevated level of extracellular DA in parkin−/− mice, therefore, likely results from increased release of dopamine.

Unchanged Levels and Binding Affinities of D1 and D2 Receptors in the parkin−/− Striatum—We then examined whether the increase in extracellular DA results in an alteration of striatal DA receptors. Similar total receptor binding was found for both D1 and D2 receptors in the striatum of
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parkin−/− and control mice (Bmax [fmol/mg], D1: 308 ± 65 versus 236 ± 34, D2: 347 ± 82 versus 328 ± 54 in +/+ and −/−, respectively; n = 6 for D1, p = 0.35; n = 3 for D2, p = 0.85).

Examination of binding kinetics for D1 and D2 receptors similarly revealed no alterations in the affinity of either receptor (Kd [nM], D1: 1.60 ± 0.34 versus 1.06 ± 0.21, D2: 1.80 ± 0.46 versus 1.64 ± 0.32 in +/+ and −/−, respectively; n = 6 for D1, p = 0.21; n = 3 for D2, p = 0.95). These results suggest that there are no significant changes in DA receptor levels or binding affinities in parkin−/− mice, despite the increased extracellular concentration of DA.

Loss of parkin Decreases Synaptic Excitability of Striatal Neurons—The significant increase in extracellular DA in the striatum of parkin−/− mice prompted us to examine the electrophysiological responses of striatal neurons, as previous studies have demonstrated that the excitability of these neurons is strongly influenced by DA levels (40, 41). We focused our analysis on the medium-sized spiny neurons, which are the major neuronal subtype in the striatum. Major inputs received by these neurons include glutamatergic excitatory inputs from the cortex and dopaminergic inputs from the SN. Acute coronal corticostriatal slices were prepared from parkin−/− mice (n = 7) and wild-type controls (n = 6) at 6–9 months of age and striatal neurons (−/−: n = 13, +/+: n = 10) were examined by injecting current pulses intracellularly into each neuron (Fig. 5A). No significant differences in passive and active membrane properties were observed between the genotypes and all values were in the range of those reported in the literature for medium-sized spiny neurons (RMP: −78 ± 3 versus −80 ± 2 mV; input resistance: 29 ± 5 versus 37 ± 5 MOhms; AP amplitude: 66 ± 3 versus 68 ± 3 mV; AP width at 1/2 amplitude: 0.76 ± 0.03 versus 0.75 ± 0.07 ms; AHP amplitude: 11.7 ± 1.4 versus 12.1 ± 1.0 mV in +/+ versus −/− neurons, respectively) (Fig. 5A).

We then examined the synaptic response of the medium-sized spiny neuron by stimulation of corticostriatal afferents, the major glutamatergic excitatory pathway into the striatum. Although parkin−/− (n = 13) and wild-type (n = 10) neurons exhibited similar mean amplitudes and durations of evoked synaptic responses (peak amplitude: 9.9 ± 0.8 versus 10.5 ± 0.7 mV; peak duration at 1/2 amplitude: 9.05 ± 0.46 versus 9.22 ± 0.56 ms for +/+ versus −/− neurons, respectively) (Fig. 5B), higher currents were needed to evoke similar responses in parkin−/− neurons relative to the wild-type, as indicated by the significant rightward shift in the input-output relationships (p < 0.02) (Fig. 5C). The current required to evoke action potentials synthetically in parkin−/− neurons (n = 10; 720 ± 163 μA) was also significantly higher than in wild-type controls (n = 12; 202 ± 53 μA) (p < 0.004). These findings suggest that medium-sized striatal neurons are less excitable synthetically in parkin−/− mice. There were no differences in paired-pulse facilitation (50 ms interpulse interval) between wild-type (0.98 ± 0.06) and parkin−/− (1.02 ± 0.57) neurons, suggesting that the observed synaptic defect in parkin−/− mice may be caused by a postsynaptic alteration.

Behavioral Impairments in parkin−/− Mice—We further tested parkin−/− mice for possible alterations in motor activities using a beam traversal task, which is sensitive to impairment in the nigrostriatal pathway (42–45). Planned comparisons revealed that parkin−/− mice performed significantly worse than wild-type controls with higher numbers of slips and slips per step at all age groups (p < 0.05), while the number of steps was similar between the genotypic groups (Fig. 6, A–C).

We further evaluated these mice in the rotarod task (46), which is commonly used to score severe motor impairments in rodents. parkin−/− and control mice exhibited similar latencies for remaining on the rotating rod (Fig. 6D).

Another test sensitive to nigrostriatal dysfunction in rodent models is the adhesive removal test, which assesses somatosensory abilities (47–49). parkin−/− mice at 2–4 and 7 months performed significantly worse compared with wild-type controls (p < 0.05), though the difference at 18 months was not as marked, likely due to the age-dependent decline of wild-type mice (Fig. 6E). Only 63% of wild-type mice at 18 months removed the middle size stimulus, compared with 100% at 4 months. This result is consistent with a previous report showing poorer performance by older animals (50).

Unchanged Levels of parkin Substrates—Recent in vitro studies have shown that parkin functions as an E3 ubiquitin ligase (21–23,26,51–54). Several proteins, such as CDCrel-1, synphilin-1, and glycosylated α-synuclein, have been shown to be ubiquitinylated by parkin in vitro. We therefore examined whether loss of parkin function results in accumulation of these proteins in parkin−/− mice. Western analysis showed similar levels of CDCrel-1, synphilin-1, and α-synuclein in parkin−/− and wild-type control brains (Fig. 7, A–C). Immunoprecipitation of α-synuclein followed by Western blotting using a second α-synuclein-specific antibody revealed the presence of an additional low abundance isoform at ~22 kDa, consistent with the apparent molecular weight of the glycosylated isoform of α-synuclein previously reported as a parkin substrate (26). No differences were observed in the steady state level of this higher molecular weight species between the genotypes (Fig. 7D). These results indicate that loss of parkin function does not affect the steady state level of any of these proteins in the mouse brain.
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Despite a large body of evidence linking loss-of-function mutations of parkin to FPD, the normal physiological role of parkin and the mechanism by which parkin mutations cause nigral degeneration are unknown. Here we present three independent lines of evidence supporting a novel role of parkin in the nigrostriatal pathway. First, the extracellular DA concentration is significantly increased in the striatum of parkin−/− mice, which is likely due to an increase in dopamine release from nigral neurons. Second, the synaptic excitability of medium-sized spiny striatal neurons is reduced, as evidenced by increased currents required to evoke synaptic responses and action potentials. Lastly, parkin−/− mice exhibit deficits in behavioral tasks previously shown to be sensitive to nigrostriatal dysfunction (42–45, 47–49, 55, 56), although we cannot exclude the possibility that alterations in other neural circuits may also contribute to the observed behavioral deficits.

Together, these results indicate that parkin loss-of-function mutations cause defects in both DA release from nigral neurons and synaptic excitability of medium-sized spiny striatal neurons, which are the major target of nigral dopaminergic projections. Previous studies have shown that DA decreases glutamate release from nigral neurons, possibly contributing to the observed behavioral deficits in parkin−/− mice.
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mate release and striatal excitability via activation of pre- and post-synaptic D2 family of dopamine receptors (41, 57), suggesting that the decreased excitability of striatal neurons in parkin−/− mice could be a consequence of the increased extra-cellular DA level in the striatum. The observation that parkin interacts with CASK via its PDZ-domain binding motif and associates with components of the glutamate receptor-signal-ing complex, such as PSD-95, NMDAR NR2B subunit, and CaMKII (58), also suggests a possible role for parkin in the regulation of synaptic transmission and plasticity. Several proteins that are enriched in presynaptic fractions, including CD-Crel-1 (23), synphilin-1 (51), and α-synuclein (26), have been described in vitro as substrates for parkin-mediated ubiquiti-nation. Although we did not observe an increase in the abundance of these substrates in parkin−/− brains (Fig. 7), parkin-mediated ubiquitylation may regulate their activities in the synapse, possibly resulting in the observed synaptic phenotypes.

The cardinal neuropathological feature of PD, profound loss of dopaminergic neurons in the SN, is absent in parkin−/− mice. Thus, the mechanism underlying the mild motor deficits observed in parkin−/− mice may differ from the movement disorder characteristic of PD, which results primarily from substantial loss of DA neurons. Our results, however, are consistent with other mutant mice that exhibit decreased performance in beam traversal and increased concentrations of striatal DA (43). Mouse models of neurodegenerative diseases have previously been observed to recapitulate some aspects of the disease in the absence of substantial neuronal loss in the affected brain subregions. Transgenic mice overexpressing wild-type and FPD-linked mutant human α-synuclein exhibit motor deficits in the absence of loss of DA neurons (59–63). Similarly, genetically engineered mouse models of Alzheimer’s (64) and Huntington’s diseases (65) have successfully reproduced behavioral and neuropathological aspects of these diseases largely without recapitulating the cortical and striatal neuronal loss, respectively.

The fact that loss of parkin function alone in mice is insufficient to cause substantial loss of dopamine neurons suggests that other factors also contribute to nigral degeneration. One possibility is that parkin protects neurons from various insults, which has been observed by overexpressing parkin in transfected cells (66, 67) and transgenic flies (68). However, no decrease in DA neuron survival has been observed in Drosophila in which endogenous parkin has been inactivated (36) or partially inactivated by RNAi (68), consistent with our findings in parkin−/− mice. Together, these findings suggest that additional events may be required for neuronal degeneration. This notion is also consistent with the fact that the age of onset for FPD patients carrying parkin mutations ranges from juvenile to elderly and varies by as much as 20 years even within single families (9). Other possibilities, including higher tolerance to neuronal degeneration in the mouse DA neuron, shorter life span of mice, and the well-controlled environment in which these mice are housed, may also contribute to the absence of the profound nigral degeneration characteristic of PD brains.

In summary, our findings provide important insights into the normal physiological role of parkin in dopamine regulation and nigrostriatal function, which will facilitate the exploration of the mechanisms of PD pathogenesis and may assist the identification of novel preventative and therapeutic strategies.

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