Parkin-Mediated Protection of Dopaminergic Neurons in a Chronic MPTP-Minipump Mouse Model of Parkinson Disease

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Abstract

Loss-of-function mutations in the ubiquitin ligase parkin are the major cause of recessively inherited early-onset Parkinson disease (PD). Impairment of parkin activity caused by nitrosative or dopamine-related modifications may also be responsible for the loss of dopaminergic (DA) neurons in sporadic PD. Previous studies have shown that viral vector-mediated delivery of parkin prevented DA neurodegeneration in several animal models, but little is known about the neuroprotective actions of parkin in vivo. Here, we investigated mechanisms of neuroprotection of overexpressed parkin in a modified long-term mouse model of PD using osmotic minipump administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Recombinant adeno-associated viral vector-mediated intranigral delivery of parkin prevented motor deficits and DA cell loss in the mice. Ser129-phosphorylated α-synuclein-immunoreactive cells were increased in the substantia nigra of parkin-treated mice. Moreover, delivery of parkin alleviated the MPTP-induced decrease of the active phosphorylated form of Akt. On the other hand, upregulation of p53 and mitochondrial alterations induced by chronic MPTP administration were barely suppressed by parkin. These results suggest that the neuroprotective actions of parkin may be impaired in severe PD.

INTRODUCTION

Parkinson disease (PD) is a progressive neurodegenerative disorder characterized clinically by resting tremor, rigidity, akinesia, and postural instability (1). The pathologic hallmarks of PD are loss of dopaminergic (DA) neurons in the substantia nigra (SN) pars compacta (SNpc) and intraneuronal protein inclusions termed Lewy bodies, which are composed mainly of α-synuclein (αSyn) (2). Both environmental and genetic factors are considered to be involved in PD pathogenesis (1, 3). Sporadic cases represent more than 90% of total patients with PD, but there are several inherited forms caused by mutations in single genes (1). Among these familial forms of PD, approximately 50% of recessively inherited early-onset parkinsonism is caused by loss-of-function mutations in the parkin gene PARK2 (4).

PARK2 encodes a 465-amino acid protein that functions as a ubiquitin ligase (5). Most PARK2 patients seem to lack Lewy bodies (6–9), suggesting an important role for parkin in Lewy body formation (10). Several putative substrates of parkin have been reported and can be divided into 2 subgroups: those that are destined for proteasomal degradation by receiving canonical K48-linked polyubiquitination (11) and others that acquire multiple physiological or pathophysiological functions by receiving monoubiquitination or K63-linked polyubiquitination. The latter may be involved in inclusion formation (12–16). In animal models, loss of parkin increases mitochondrial dysfunction and oxidative damage (17), impairment of evoked dopamine release (18), and vulnerability to inflammation-related neurodegenerative insult (19). S-Nitrosylation or covalent binding of dopamine-related compounds may be responsible for parkin inactivation and subsequent DA cell death in sporadic PD (20–22). Moreover, there is increasing evidence that ectopically overexpressed parkin provides neuroprotective effects in genetic and environmental PD models, including LRRK2-transgenic (23) and PINK1-knockdown fruit flies (24), 6-hydroxydopamine–lesioned rats (25), and mice treated transiently with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (26). We previously reported that recombinant adeno-associated viral (rAAV) vector-mediated delivery of parkin...
prevented αSyn-induced DA neuronal loss in rat and monkey brains (27, 28).

Recent studies indicate that there are functional interactions of parkin with PINK1, which is involved in mitochondrial quality control (29–33). However, the neuroprotective actions of parkin against long-term mitochondrial insult are relatively unknown in vivo. This study was designed to dissect the parkin-mediated neuroprotection in a long-term environmental model of PD. We generated modified high-dose and long-term MPTP mice using Alzet osmotic minipumps and investigated the impact of rAAV-mediated parkin delivery.

MATERIALS AND METHODS

Mice

Normal male C57BL/6J mice were purchased from Charles River Laboratories (Kanagawa, Japan). All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Juntendo University and by the Animal Experimentation and Ethics Committee of the Kitasato University School of Medicine.

Preparation of rAAV Vector

The plasmid pAAV-MCS (CMV promoter; Stratagene, La Jolla, CA) carrying human parkin complementary DNA (named pAAV-MCS-parkin) or human αSyn complementary DNA (pAAV-MCS-αSyn) was constructed as previously reported (27, 34). High-titer serotype-1 rAAV (rAAV1) vector stocks were prepared using the plasmid pAAV-MCS-parkin, pAAV-MCS-αSyn, or pAAV-hrGFP (humanized recombinant green fluorescent protein; Stratagene), as described (28, 35). The rAAV1 vectors were purified by ultracentrifugation in a gradient density of OptiPrep solution (Axis-Shield PoC AS, Oslo, Norway), which was then removed by ultrafiltration using Centricon Plus-20 (10,000 MWCO; Millipore Corp, Temecula, CA). The titers of rAAV1 to produce parkin (named rAAV1-parkin), αSyn (rAAV1-αSyn), or hrGFP (rAAV1-hrGFP) were 5 × 10^{11} genomes per milliliter.

Stereotaxic Injection of rAAV1 Vectors

Mice were anesthetized with sodium pentobarbital (50 mg/kg body weight, intraperitoneally [i.p.]) and positioned in a stereotaxic frame. For immunohistochemistry (IHC) and measurement of the striatal dopamine and its metabolites, rAAV1 vector was injected unilaterally. For Western blotting, the rAAV1 vector was injected bilaterally. The skull was exposed, and a small portion of the skull over the SN was removed with a dental drill. Subsequently, the rAAV1 vector was injected into the SN (2 μL; 2.8 mm posterior and 1.3 mm lateral from the bregma, 4.4 mm below the dural surface; tooth bar = –2 mm) through a 5-μL Hamilton microsyringe, as previously described (35).

MPTP Infusion

The 13-week-old mice (~30 g body weight) were implanted i.p. with the Alzet osmotic minipumps (Model 2004, releasing rate = 0.25 μL/h, reservoir volume = 200 μL; Durect Corp, Cupertino, CA), filled with saline (control group), 250 mg/mL MPTP-HCl (50-mg/kg-per-day group; dissolved in saline; Sigma-Aldrich Corp, St Louis, MO), or 500 mg/mL MPTP-HCl (100-mg/kg-per-day group). For bolus injection, mice were injected i.p. with MPTP at 30 mg/kg per day for 5 consecutive days (designated as subacute; n = 4).

For rAAV1-injected mice, the same Alzet minipumps filled with saline (control group) or 250 mg/mL MPTP-HCl (50-mg/kg-per-day group) were implanted at 14 days after injection of the rAAV1 vectors. MPTP was handled in accordance with guidelines reported by Przedborski et al (36).

Behavioral Analysis

To evaluate behavioral changes, the mice were analyzed by a rotarod test 25 days after implantation of the osmotic minipump (i.e. 3 days before death). Mice were kept for 300 seconds twice on a rotarod apparatus accelerating from 0 to 32 rpm at 45-minute intervals (Model 7650, rota-rod for mice; Ugo Basile Biological Research Apparatus, Comerio VA, IT). The latency times (seconds) to fall were measured by acceleration from 0 to 32 rpm in 300 seconds.

rAAV1-injected mice were analyzed by apomorphine-induced rotation test 25 days after the minipump implantation (3 days before death). Mice were habituated in a circular chamber (16 cm in diameter) for 10 minutes. Then, after the injection of apomorphine-HCl (0.5 mg/kg i.p., dissolved in saline containing 30% ascorbic acid; Sigma-Aldrich Corp), rotation behavior was monitored for 40 minutes using a video recorder. In a previous report, apomorphine-challenged rats rotated toward the side with weaker DA neurotransmission (37), and the number of contralateral full body turns was

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**TABLE. Numbers of Mice Used for rAAV1 Injection Experiments**

<table>
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<th>Immunohistochemistry and Other Studies</th>
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<td>Group</td>
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<td>rAAV1-parkin/saline</td>
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<td>4/4</td>
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<td><strong>Western</strong></td>
<td>rAAV1-hrGFP/saline</td>
<td>rAAV1-parkin/saline</td>
</tr>
<tr>
<td>No. mice analyzed/injected</td>
<td>4/4</td>
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Numbers of mice injected with the rAAV1 vector (injected) and used for data analyses (analyzed) are shown. For immunohistochemistry and measurements of dopamine and its metabolites, mice that exhibited foreign protein expression in more than ~80% of the area of the entire rostrocaudal region of the substantia nigra pars compacta were used for the analyses. In Western blotting analysis, the protein samples that showed an intense foreign protein expression were used for the analyses. *One mouse died before minipump implantation.

hrGFP, humanized recombinant green fluorescent protein; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; rAAV1, recombinant adeno-associated viral vector.
counted by playing back the recorded videotape. A full-body turn was defined as continuous and pivotal turning exceeding 180 degrees.

**Tissue Processing**

At 7 or 28 days after implantation of the minipumps, or 25 days after the first injection of MPTP (subacute group), mice were deeply anesthetized with sodium pentobarbital (250 mg/kg, i.p.) and perfused transcardially with phosphate-buffered saline (PBS). The brains were removed en bloc from the skull and cut coronally along the anterior tangent to the median eminence. The striatal tissues were then dissected and immediately frozen on dry ice. For Western blotting, the brain blocks including the entire rostrocaudal extent of the SN were cut coronally at 2-mm thickness (Figure, Supplemental Digital Content 1, parts A, B, http://links.lww.com/NEN/A252). After removal of the cortical and hippocampal tissues, the ventral midbrains were cut horizontally along the ventral end close to the SN to remove the tissues including the median eminence and pontine nucleus. Then, ventral parts of midbrain tissues (~1.2 mm from the ventral end) were dissected horizontally, from which the ventrolateral tissues including SN pars reticulata were removed (Figure, Supplemental Digital Content 1, parts A, B, http://links.lww.com/NEN/A252), and immediately frozen on dry ice. For IHC, the posterior parts of brain blocks, including the entire rostrocaudal extent of the SN, were fixed overnight in 4% paraformaldehyde in PBS and immersed in PBS containing 30% sucrose until sinking. Coronal sections of the SN were cut serially at 20-μm thickness by a cryostat (CM1900; Leica Microsystems, Wetzlar, Germany).

The rAAV1-αSyn-injected (n = 3) and rAAV1-hrGFP-injected mice (n = 3) were killed at 4 weeks after injection. Brain tissues including the entire rostrocaudal extent of the SN were fixed overnight in 4% paraformaldehyde in PBS and processed for IHC as described previously.

**Antibodies**

The primary antibodies used for IHC were rabbit anti-hrGFP (diluted at 1:500; Stratagene), rabbit anti-parkin (no. 2132; 1:500; Cell Signaling Technology, Inc, Danvers, MA), mouse anti-parkin (no. 4211, clone Park8; 1:200; Cell Signaling Technology), mouse anti-tyrosine hydroxylase (TH) (1:10000; Calbiochem, San Diego, CA), rabbit anti-TH (1:5000; Calbiochem), sheep anti-TH (1:1000; Calbiochem), mouse anti-glial fibrillary acidic protein (clone ab10062; 1:500; Abcam, Cambridge, MA), mouse anti-human αSyn (clone LB509; 1:200; Invitrogen Corp, Carlsbad, CA), rabbit anti–Ser129-phosphorylated αSyn (clone ab59264; 1:500; Abcam), and rabbit anti-translocase of the outer membrane 20 (Tom20) antibodies (FL-145; 1:500; Santa Cruz Biotechnology, Inc, Santa Cruz, CA).

The primary antibodies used for Western blotting were as follows: rabbit anti-hrGFP (1:500), mouse anti-parkin (1:500), rabbit anti–phospho-Akt (Ser473) (no. 4060, clone D9E; 1:1000; Cell Signaling Technology), rabbit anti-Akt (no. 9272; 1:500; Cell Signaling Technology), mouse anti-p53 (Pab 1801; 1:100; Santa Cruz Biotechnology), mouse anti-Bax (B-9; 1:100; Santa Cruz Biotechnology), mouse anti–phospho-stress-activated protein kinase/c-Jun N-terminal kinase (JNK) (Thr183/Tyr185) (no. 9255, clone G9; 1:1000; Cell Signaling Technology), mouse anti-TH (1:500; Calbiochem), rabbit anti-PINK1 (NB100-493; 1:500; Novus Biologicals, Littleton, CO), rabbit anti-DJ-1 (NB100-483; 1:500; Novus Biologicals, Littleton, CO), and rabbit anti-translocase of the outer membrane 20 (Tom20) antibodies (FL-145; 1:500; Santa Cruz Biotechnology, Inc, Santa Cruz, CA).

![FIGURE 1. Long-term and high-dose MPTP models generated using Alzet osmotic minipumps. (A–F) Substantia nigra (SN) sections of saline- (Saline) and MPTP-minipump mice (50 mg/kg per day, MPTP [50] or 100 mg/kg per day MPTP [100]) immunostained for tyrosine hydroxylase (TH) and counterstained with Cresyl violet. (G–J) Numbers of dopaminergic (DA) cell bodies in the SN pars compacta (G), and levels of dopamine (H), 2-(3,4-dihydroxyphenyl)acetic acid (DOPAC) (I), and homovanillic acid (HVA) in the striatum (J). The implantation of MPTP-minipump caused a significant degeneration of the nigrostriatal DA neurons. Numbers of mice analyzed in each group are indicated within the bars. Data are mean ± SEM. **, p < 0.01; and ***, p < 0.001 (1-way analysis of variance followed by Tukey-Kramer post hoc test). Scale bars = (A) 500 μm (applicable to A–C); (D) 500 μm (D–F).](http://jnen.oxfordjournals.org/)

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Novus Biologicals), rabbit anti-Tom20 (FL-145; 1:200; Santa Cruz Biotechnology), rabbit anti-Ser129-phosphorylated αSyn (clone ab42906; 1:1000; Wako Pure Chemical Industries, Ltd, Osaka, Japan), mouse anti-αSyn (clone 42; 1:500; BD Biosciences, Franklin Lakes, NJ), and mouse anti-actin antibodies (clone C4; 1:500; Millipore Corp).

**Immunohistochemistry**

Free-floating sections were washed in a PBS medium containing 0.05% Triton X-100 (PBS-T). When the rabbit and sheep primary antibodies were used, the sections were soaked with 10% Block Ace (Yukijirushi-Nyugyo Co, Sapporo, Japan) in PBS-T and then incubated with the primary antibodies dissolved in PBS-T containing 2% Block Ace at 4°C for 48 hours. When the mouse primary antibody was used, Vector M.O.M. Immunodetection Kit (Vector Laboratories, Inc, Burlingame, CA) was used for blocking and antibody dilution according to the instructions provided by the manufacturer. Subsequently, for fluorescent visualization of the antigens, the sections were incubated for 2 hours in fresh medium containing fluorescein isothiocyanate–conjugated anti-mouse or rabbit IgG and Cy3-conjugated anti-mouse, rabbit, or sheep IgG secondary antibodies (1:200–500; Jackson ImmunoResearch Laboratories, Inc, West Grove, PA). The sections were mounted on slide glass and coverslipped with Vectashield Mounting Medium with DAPI (Vector Laboratories). Images were captured using a confocal laser scanning microscope (LSM510; Zeiss, Jena, Germany). For colorimetric visualization of the antigen, the sections were incubated for 2 hours in fresh medium containing biotinylated

![FIGURE 2](image-url). Immunoreactivity for Ser129-phosphorylated α-synuclein (p-αSyn) in the substantia nigra (SN) pars compacta of MPTP-minipump mice. (A, B) SN sections of saline- (A) or MPTP-minipump mice (50 mg/kg per day, MPTP [50]) (B) were coimmunostained for the p-αSyn (dark brown/purple) and tyrosine hydroxylase (TH) (brown). p-αSyn-positive cells (arrowhead, enlarged in [B] inset) were found in the SN of MPTP-minipump mice. (C–J) SN sections of saline- (C–F) and MPTP-minipump mice (G–J) coimmunostained for the p-αSyn (C, D, F, G, H, J, green) and TH (C, E, G, I, J, red), merged with anti- p-αSyn in (C, F, G, J, yellow) and visualized by fluorescence. Boxed areas in (C) and (G) are enlarged in (D–F) and (H–J), respectively. Scale bars = (A) 50 μm (applicable to B), (C) 50 μm (applicable to G), (J) 10 μm (applicable to D–F, H–J).
anti-mouse or rabbit IgG secondary antibody (1:500), followed by avidin-biotin-peroxidase complex (ABC Elite) (both from Vector Laboratories, Inc) for 1 hour. Then the sections were reacted in 0.05 mol/L Tris-HCl buffer (pH 7.6) containing 0.04% diaminobenzidine and 0.002% H2O2 with (dark brown/purple color) or without (brown color) 0.04% nickel chloride. Images were captured using a light microscope (ACT-1; Nikon Corp, Tokyo, Japan).

Western Blotting

Ventral midbrain tissues were sonicated in chilled CellLytic-MT mammalian tissue lysis/extraction reagent (Sigma), mixed with protease inhibitor cocktail set I (Calbiochem) and phosphatase inhibitor cocktail set V (Calbiochem). The protein concentration in the lysate was determined using BCA protein assay kit (Pierce, Rockford, IL). Each protein sample (10 µg) was resolved by SDS-PAGE by means of Compact-PAGE-twin (ATTO Corp, Tokyo, Japan) and then electro-transferred to Clear Blot Membrane-P (ATTO Corp) using powered BLOTmini (ATTO Corp). The membrane was washed in PBS, incubated for 1 hour in a PBS medium containing 50% ChemiBLOCKER (Millipore Corp) and 0.05% Tween-20, and then incubated for 24 hours with primary antibody in the same fresh medium. Subsequently, the membrane was incubated for 2 hours in fresh medium containing horseradish peroxidase–linked anti-mouse or rabbit IgG secondary antibody (1:10000; GE Healthcare Bio-Sciences, Uppsala, Sweden), followed by development of chemiluminescence using Amersham ECL Plus Western Blotting Detection System (GE Healthcare Bio-Sciences). The image was captured using LAS-3000 (Fujifilm, Tokyo, Japan) and quantified by Image Gauge software. Samples that showed intense protein expression of hrGFP or parkin were used for the subsequent investigations (Table).

Cell Counts

Every eighth 20-µm-thick serial section of the brain was immunostained for parkin (for mice injected with rAAV1-parkin) (Figure, Supplemental Digital Content 1, parts C–J, http://links.lww.com/NEN/A252) or hrGFP (for mice injected with rAAV1-hrGFP). Coimmunostaining for parkin or hrGFP and TH was also performed (Figure, Supplemental Digital Content 1, parts K–M, http://links.lww.com/NEN/A252). Mice that exhibited foreign protein expression in most DA cells were more than ~80% of the area of the entire rostrocaudal region of the SNpc were used for the subsequent investigations (Table). The rostrocaudal area of the SNpc immunopositive for foreign protein was determined in each mouse and used for DA cell counting and phosphorylated αSyn (p-αSyn)–positive cell counting. In every fourth serial section, the numbers of TH- and Nissl-double-positive cells in the SNpc were counted both in the rAAV1-injected and noninjected sides using a stereological method and in a blind manner, as previously reported (35, 38). In brief, SNpc cells with nuclei optimally visible by TH immunostaining and with nuclei, cytoplasm, and nucleoli prominently stained by Nissl staining were counted. To avoid

FIGURE 3. Recombinant adeno-associated viral (rAAV1) vector-parkin–mediated prevention of behavioral deficit and dopaminergic (DA) cell loss in MPTP-minipump mice. (A) Time schedule for gene delivery experiment with rAAV1-parkin. At day 14 after intranigral injection of rAAV vector, Alzet osmotic minipumps were implanted i.p. to deliver saline or MPTP at a dose of 50 mg/kg per day for 7 (Western blotting) or 28 days (immunohistochemistry and dopamine measurement). Apomorphine-induced behavioral change was analyzed at day 25 after implantation. (B) Apomorphine-induced contralateral turns were counted in rAAV1-humanized recombinant green fluorescent protein (hrGFP)-injected (GFP) or rAAV1-parkin–injected (parkin) saline- (Saline) or MPTP-minipump mice. There was a significant increase in the number of contralateral turns in rAAV1-parkin/MPTP mice. (C–F) Substantia nigra (SN) sections of rAAV1-parkin/MPTP mice immunostained for parkin (C, brown, D, green), and tyrosine hydroxylase (TH) (E, red, merged with anti-parkin in F, yellow). (G–N) Representative photomicrographs of TH- and Nissl-double-positive cells in the ipsilateral (rAAV1-parkin, G, I, K, M), and contralateral (noninjected) sides of the SN pars compacta (H, J, L, N), in saline- (G, H, K, L) or MPTP-minipump mice (I, J, M, N). (O) Transduction efficiencies of the rAAV1 vectors, expressed as percent of the entire rostrocaudal region of the SN. There were no significant differences among the groups. (P) Counts of DA cell bodies in the SNpc. Data are expressed as percentage of the contralateral side (% of contra); that is, the cell number in the rAAV1-injected side over that in the noninjected side. rAAV1-parkin ameloriated MPTP-induced DA cell loss. Numbers of mice in each group are indicated within the bars. Data are mean ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001; and N.S., not significant (1-way analysis of variance followed by Tukey-Kramer post hoc test). Scale bars = (C) 500 µm (applicable to G–J); (D) 10 µm (applicable to F–I). (K) 50 µm (applicable to K–N).
rAAV1-parkin/MPTP

A

B

C

D

E

F

G

H

merged

p-αSyn

TH

p-αSyn-positive cell number in SN

30

20

10

0

(4)

(4)

(4)

(5)

ipsi cont

ipsi cont

ipsi cont

ipsi cont

GFP

parkin

GFP

parkin

Saline

MPTP

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double counting of neurons with unusual shapes, TH- and Nissl-double-positive cells were counted only when their nuclei and nucleoli were optimally visualized. Data were expressed as percentage of the contralateral side, that is, the cell number in the rAAV1-injected side over that in the noninjected side. The numbers of the p-αSyn-positive cells visualized by dianibenzidine with nickel chloride were counted in every eighth serial section of the SN.

**Determination of the Striatal Levels of Dopamine and its Metabolites by High-Performance Liquid Chromatography**

Frozen striatal tissues were sonicated in chilled 0.1 mol/L perchloric acid. The samples were centrifuged (20,000 × g for 10 minutes at 4°C), and the resulting supernatants were used for the measurement of dopamine, 2-(3,4-dihydroxyphenyl)acetic acid (DOPAC), and homovanillic acid (HVA) concentrations. The high-performance liquid chromatography (HPLC) system equipped with an 8-electrode coulometric electrochemical detection system (ESA-400; ESA, Inc, Chelmsford, MA) and a reverse-phase C18 column (150 × 4.6 mm; ODS-100s; Tosoh, Tokyo, Japan) was used. The concentrations of dopamine, DOPAC, and HVA were determined in nanomoles per gram of tissue.

**Statistical Analysis**

All data are expressed as mean ± SEM. Two-tailed Student t-test (for 2 groups) and 1-way analysis of variance (ANOVA), followed by Tukey-Kramer post hoc test (for ≥3 groups) were applied. A p value less than 0.05 denoted statistically significant differences.

**RESULTS**

**Generation of a High-Dose and Long-Term MPTP Infusion Model Using Alzet Osmotic Miniumps**

The 50- and 100-mg/kg-per-day MPTP-minipump mice had 61.3% ± 6.3% (p = 0.003262; df = 9; 1-way ANOVA followed by Tukey-Kramer post hoc test) and 46.1% ± 5.6% (p = 0.006822) of DA cell bodies in the SNpc found in the saline controls (Figs. 1A–G); they had 48.1% ± 5.1% (p = 6.64 × 10^-5; df = 22) and 31.2% ± 5.1% (p = 9.80 × 10^-5) of dopamine in the striatum found in the saline group, respectively (Fig. 1H). Dopamine metabolites, DOPAC and HVA, were also decreased (Figs. 1I, J). Levels of striatal MPP^7 (the active metabolite of MPTP) were 4.23 ± 1.46 nmol/g tissue (p < 0.05 vs the saline control group) and 7.05 ± 0.64 nmol/g tissue (p < 0.001 vs control) for the 50- and 100-mg/kg-per-day regimens, respectively (p < 0.05 and p < 0.001, by 1-way ANOVA followed by Tukey-Kramer post hoc test). Despite the loss of nigrostriatal DA neurons 28 days after the implantation, behavioral changes were not evident by rotarod test (even in the 100-mg/kg-per-day MPTP group) at 25 days after implantation of minipumps (latency time to fall: 198.3 ± 26.0 seconds in the saline group and 229.7 ± 17.2 seconds in the 100-mg/kg-per-day MPTP group; p = 0.3461).

**Immunoreactivity for the Ser129-Phosphorylated αSyn in the SN of MPTP-Minipump Mice**

Fornai et al (39) previously observed electron-dense and fibrillar neuronal inclusions containing αSyn in the SN of MPTP-minipump model mice. One of the critical pathogenic modifications of αSyn is the phosphorylation at Ser129 residue (40); therefore, we examined Ser129-p-αSyn immunoreactivity in the SN of our MPTP-minipump mice. In preliminary studies, the anti-p-αSyn antibody was evaluated using nigral sections of mice that had received a stereotoxic intranigral injection of rAAV1 vector encoding human αSyn (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A253). The antibody reacted specifically with DA cell bodies in the ipsilateral side of the SN (Figure, Supplemental Digital Content 2, parts B-E, I-K, http://links.lww.com/NEN/A253) in which human αSyn is overexpressed (Figure, Supplemental Digital Content 2, parts 2 A, D, J, http://links.lww.com/NEN/A253). We found a small number of p-αSyn-immunopositive cells in the SN of MPTP-minipump (50 mg/kg per day) mice (Figs. 2B, G–J; see also Fig. 5I). These cells were not seen in saline-minipump mice (Figs. 2A, C–F; also see Fig. 5I).

**Amelioration of Nigral DA Cell Loss by rAAV1-Mediated Parkin Overexpression in MPTP-Minipump Mice**

We next investigated the effect of rAAV1-mediated overexpression of parkin on the survival of DA neurons in MPTP-minipump mice. High-titer rAAV1-parkin or rAAV1-hrGFP was injected unilaterally into the SN of C57BL/6 mice.
FIGURE 6. Proapoptotic and antiapoptotic molecules influenced by parkin overexpression in MPTP-minipump mice. (A) Western blotting was performed using midbrain tissues of mice killed 7 days after minipump implantation. Phosphorylated Akt (p-Akt) was reduced in the rAAV1-humanized recombinant green fluorescent protein (hrGFP)-injected side (GFP) but not in the rAAV1-parkin-injected side (parkin) of MPTP-minipump mice. (B–G) Data are presented as percentage of control (GFP/saline group) for p-Akt/Akt/Actin (B), Akt/Actin (C), p53/Actin (D), Bax/Actin (E), the phosphorylated JNK (p-JNK)/Actin (F), tyrosine hydroxylase (TH)/Actin (G), p-αSyn/αSyn/Actin (H), and αSyn/Actin (I). Reduction of p-Akt was significant in the rAAV1-hrGFP/MPTP group but not in the rAAV1-parkin/MPTP group. Total protein amount of Akt was unchanged. The protein levels of p53 and p-αSyn were significantly increased in the MPTP groups, and there were nonsignificant differences between the rAAV1-hrGFP/MPTP and rAAV1-parkin/MPTP groups. Numbers of mice analyzed in each group are indicated within the bars. Data are mean ± SEM. *, p < 0.05; and N.S., not significant (1-way analysis of variance followed by Tukey-Kramer post hoc test).
At 14 days after injection of the rAAV1 vectors, mice were implanted with minipumps to deliver MPTP at dose of 50 mg/kg per day (Fig. 3A; Table). Mice were examined for an apomorphine-induced rotation behavior 25 days after the treatment with MPTP. The injection of rAAV1-parkin resulted in increased contralateral turns in MPTP-minipump mice compared with rAAV1-parkin/saline (p = 0.0163; df = 16) and rAAV1-hrGFP/MPTP mice (p = 0.02843) (Fig. 3B). This suggests functional preservation of the nigrostriatal pathways provided by parkin delivery. Immunohistochemistry revealed overexpression of parkin in TH-positive DA cells in the SNpc (Figs. 3C–F; Figure, Supplemental Digital Content 1, parts 1C–M, http://links.lww.com/NEN/A252). Next, we counted TH- and Nissl-double-positive cells in the SNpc of these mice. As shown in Figures 3G to N and P, the injection of rAAV1 vector itself (i.e. in saline-treated groups) caused a minor decrease of DA cell number (15.1% ± 2.9% decrease in the rAAV1-hrGFP–injected mice and 7.9% ± 2.6% decrease in the rAAV1-parkin–injected mice). Importantly, rAAV1-parkin delivery promoted the survival of DA cell bodies in MPTP-minipump mice (109.0% ± 2.9% relative to the contralateral noninjected side) compared with parkin-overexpressed saline-minipump mice (p = 0.005143; df = 16) and hrGFP-overexpressed MPTP-minipump mice (88.0% ± 3.2%; p = 0.0008401) (Fig. 3P). The transduction efficiency of the rAAV1 vectors (i.e. the area immunopositive for parkin or hrGFP over the entire rostrocaudal area of the SNpc) varied from 87.5% ± 2.6% to 93.75% ± 3.6%, which had no statistical difference among the groups (Fig. 3O; Figure, Supplemental Digital Content 1, parts 1C–M, http://links.lww.com/NEN/A252). On the other hand, the striatal level of dopamine was not preserved with the injection of rAAV1-parkin, the same as with rAAV1-hrGFP (Fig. 4A). There was no influence on the striatal levels of dopamine metabolites, DOPAC and HVA, in these mice (Figs. 4B, C).

Accumulation of Ser129-Phosphorylated αSyn Promoted by Parkin Overexpression

There were increased numbers of p-αSyn–positive cell bodies in the rAAV1-parkin–injected side of the SN in MPTP-minipump mice (Figs. 5A–I). Thus, parkin delivery enhanced accumulation of the p-αSyn in DA cells in MPTP-minipump mice.

Alleviation of MPTP-Induced Inactivation of Akt by Parkin Delivery

On the basis of our previous report that showed upregulation of Bax 6 to 8 days after the first treatment with MPTP (30 mg/kg per day for 5 consecutive days) (41), we performed Western analyses at 7 days after minipump implantation (Fig. 3A). As shown in Figure 6, TH protein was slightly but nonsignificantly reduced by MPTP treatment and parkin counteracted the effect (Figs. 6A, G). There were no significant influences on the protein amounts of proapoptotic Bax (Figs. 6A, E) and the phosphorylated active form of JNK (Figs. 6A, F) in this model. The level of pS3 was increased significantly in MPTP-minipump mice but had no significant difference between the rAAV1-hrGFP and rAAV1-parkin groups (Figs. 6A, D). Importantly, phosphorylated Akt, an active form of a prosurvival kinase Akt, was reduced in the rAAV1-hrGFP–injected hemisphere of MPTP-minipump mice (p = 0.02829; df = 15; compared with rAAV1-hrGFP/saline); the decrease was alleviated by rAAV1-parkin (p = 0.8434; compared with rAAV1-parkin/saline) (Figs. 6A, B). The level of total Akt protein was not changed by MPTP (Figs. 6A, C), indicating that parkin diminished the MPTP-induced dephosphorylation of p-Akt. p-αSyn was increased in response to MPTP (p = 0.0001902; df = 15; compared between rAAV1-hrGFP/saline and rAAV1-hrGFP/MPTP mice; and p = 0.0009918; compared between rAAV1-parkin/saline and rAAV1-parkin/MPTP groups) (Figs. 6A, H). A similar result was obtained with another anti-p-αSyn antibody (clone pSyn/64; Wakо; Figure, Supplemental Digital Content 3, part A, http://links.lww.com/NEN/A254). The total amount of αSyn protein was not changed by MPTP (Figs. 6A, I).

Effects of Parkin Overexpression on Mitochondrial Alterations

Finally, we addressed the effect of intranigral parkin delivery on the protein levels of PINK1 and a mitochondrial protein marker Tom20. Western blotting analysis demonstrated that both MPTP treatment and parkin expression rendered PINK1 to increase slightly but nonsignificantly (Figure, Supplemental Digital Content 3, parts A, B, http://links.lww.com/NEN/A254). The protein amounts of Tom20 and DJ-1 were not changed at this time point (Figure, Supplemental Digital Content 3, parts A, C, D, http://links.lww.com/NEN/A254). At day 28 after implantation of the minipumps, there was increased immunoreactivity for Tom20 in the SNpc of MPTP-minipump mice (Figure, Supplemental Digital Content 3, parts E–J, K–Z, http://links.lww.com/NEN/A254); however, this phenomenon was not influenced by overexpression of parkin (Figure, Supplemental Digital Content 3, parts K–Z, http://links.lww.com/NEN/A254). The overexpressed parkin was found scarcely colocalized with the mitochondrial Tom20 (Figure, Supplemental Digital Content 3, parts L–N′, P–R′, T–V′, and X–Z′, http://links.lww.com/NEN/A254).

DISCUSSION

In the present study, we generated a modified high-dose and long-term mouse model of PD using Alzet osmotic minipump administration of MPTP. In our preliminary experiments, we tried to produce an MPTP-minipump model according to the regimen of Fornai et al (39) but were unsuccessful. Alvarez-Fischer et al (42) recently demonstrated that an Alzet minipump-mediated infusion of MPTP alone (40 mg/kg per day for 3 weeks) caused only a transient depletion of the striatal dopamine and no DA cell loss in the SN. They further indicated that minipump-mediated infusion of MPTP (40–80 mg/kg per day for 2–4 weeks) in combination with the uricosuric agent probenecid caused moderate degeneration of DA neurons (42). We did not attempt to
inhibit renal excretion and/or brain efflux clearance of MPTP/MPP+ but could generate a novel MPTP-minipump model by simply increasing the dose of MPTP to 50 and 100 mg/kg per day.

In this long-term environmental model of PD, we first evaluated the therapeutic effect of parkin. The rAAV vector was chosen because of its ability for long-term stable gene expression in postmitotic neurons with low accompanying cytotoxicities (43, 44). These properties are preferable for recent clinical trials to treat neurodegenerative disorders including PD (44, 45). Paterna et al (26) reported that rAAV vector–mediated transduction of parkin protected DA neurons of mice that were treated transiently with low dose of MPTP (20 mg/kg per day for 4 days). Our present data are in line with those results and indicate further that parkin gene therapy might be effective in a more severe and continuous condition causing PD. In 6-hydroxydopamine–lesioned rats, Vercammen et al (25) reported that lentiviral vector-parkin delivery resulted in a significant preservation of DA cell bodies and nerve terminals with corresponding behavioral improvement; by contrast, another group demonstrated that rAAV-parkin delivery ameliorated motor deficits but had no protection on the striatal DA innervation and nigral TH-positive neurons (46). In the present study, the MPTP-induced decrease of striatal dopamine was not prevented by rAAV1-parkin, whereas motor deficits and DA cell loss were ameliorated. We speculate that this discrepancy might be a result of an enhanced dopamine release of the surviving DA neurons that overexpress parkin (46), in consideration with an impaired dopamine release in parkin knockout mice (18).

We observed more the p-αSyn–immunopositive cells in the parkin-overexpressed SN of MPTP-minipump mice. There have been conflicting reports about the neurotoxicity of the p-αSyn in αSyn overexpression PD models; alteration of Ser129 to nonphosphorylated Ala or a phospho-mimetic Asp resulted in enhanced, eliminated, or unchanged the neurotoxicity of αSyn (47–51). Our present data imply that parkin delivery promoted DA neuronal survival in part by increasing the accumulation of the p-αSyn. This is consistent with the report by Gorbatyuk et al (48), who demonstrated that rAAV-mediated overexpression of αSyn Ser129Asp (which seemed to form punctate inclusions) caused no pathologic change in the SN. It has been speculated that parkin promoted accumulation of αSyn through catalyzing a nonclassic polyubiquitination of modified αSyn and/or αSyn-interacting proteins (13).

We found that MPTP-induced reduction of the phosphorylated active form of Akt was prevented by parkin overexpression. Recent work indicated that parkin potentiates epidermal growth factor (EGF)–induced activation of Akt signaling through interfering with Eps15, a negative regulator of the EGF/EGF receptor pathway (52). It is known that rAAV vector–mediated transduction of constitutively active form of Akt can provide DA neuroprotection in 6-hydroxydopamine mice (53). Moreover, Aleyasin et al (54) recently reported that DJ-1 (the loss-of-function mutations of which cause another form of recessively inherited PD) is necessary for Akt-mediated neuronal protection against MPTP. In agreement with these reports, our results suggest that maintenance of Akt signaling by parkin is important for the promotion of DA neuronal survival. On the other hand, da Costa et al (55) demonstrated that parkin elicits ubiquitin ligase-independent transcriptional repression of p53 gene. In our present experiments, however, we did not find that ectopic parkin counteracted against the MPTP-induced upregulation of p53.

Parkin acts in concert with PINK1 in mitochondrial quality control (29–33). Overexpressed parkin interacts directly with and stabilizes PINK1 (56). Mitochondrial impairment also stabilizes PINK1, and recruitment of parkin to the damaged mitochondria is dependent on PINK1 in mitophagy (30, 31, 33). In the present study, the virally expressed parkin seemed not to affect the clearance of mitochondria that were damaged with MPTP treatment. These results suggest that a long-term insult makes it difficult for parkin to be effective in eliminating potentially harmful accumulated mitochondria.

In conclusion, the present study lends support to the hypothesis that the rAAV vector–mediated parkin gene therapy may have clinical benefits for advanced patients with idiopathic PD (16, 45, 57) and provides a new insight into the neuroprotective actions of multifunctional parkin in animal PD models.

ACKNOWLEDGMENT

The authors thank Hideki Shimura, MD, PhD, Department of Neurology, Juntendo University Urayasu Hospital, for his excellent advice.

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