THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 27, pp. 23162–23170, June 29, 2012
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Inactivation of *Pink1* Gene *in Vivo* Sensitizes Dopamine-producing Neurons to 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and Can Be Rescued by Autosomal Recessive Parkinson Disease Genes, *Parkin* or *DJ-1*

Received for publication, January 26, 2012, and in revised form, March 21, 2012 Published, JBC Papers in Press, April 17, 2012, DOI 10.1074/jbc.M112.346437

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Background: Mutations in *Pink1* are associated with Parkinson disease.
Results: Mouse *Pink1* deficiency results in hypersensitivity to MPTP-induced dopaminergic neuronal loss, which can be rescued with expression of human *Parkin* or *DJ-1*.
Conclusion: *Pink1* gene can regulate response to exogenous stress.
Significance: These results indicate how endogenous *Pink1* plays an important role in management of exogenous stress in mouse brain.

Mutations in the mitochondrial PTEN-induced kinase 1 (*Pink1*) gene have been linked to Parkinson disease (PD). Recent reports including our own indicated that ectopic *Pink1* expression is protective against toxic insult *in vitro*, suggesting a potential role for endogenous *Pink1* in mediating survival. However, the role of endogenous *Pink1* in survival, particularly *in vivo*, is unclear. To address this critical question, we examined whether down-regulation of *Pink1* affects dopaminergic neuron loss following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the adult mouse. Two model systems were utilized: virally delivered shRNA-mediated knockdown of *Pink1* and germ line-deficient mice. In both instances, loss of *Pink1* generated significant sensitivity to damage induced by systemic MPTP treatment. This sensitivity was associated with greater loss of dopaminergic neurons in the Substantia Nigra pars compacta and terminal dopamine fiber density in the striatum region. Importantly, we also show that viral mediated expression of two other recessive PD-linked familial genes, *DJ-1* and *Parkin*, can protect dopaminergic neurons even in the absence of *Pink1*. This evidence not only provides strong evidence for the role of endogenous *Pink1* in neuronal survival, but also supports a role of *DJ-1* and *Parkin* acting parallel or downstream of endogenous *Pink1* to mediate survival in a mammalian *in vivo* context.

Parkinson disease (PD)[3] is a neurodegenerative disorder characterized by loss of dopamine neurons and movement deficits (1). Several recessive genes (*Pink1*, *Parkin*, and *DJ-1*) have been linked with familial forms of the disease (2). How mutation of these genes leads to PD pathology is unknown. *Parkin* possesses an E3 ubiquitin ligase activity, and its loss is associated with mild mitochondrial defects (3). Expressed *Parkin* translocates to defective mitochondria in response to the loss of the mitochondrial membrane potential and mediates mitophagy (4–6). In contrast to *Parkin*, *DJ-1* has an atypical peoxyredoxin like peroxidase activity (7). Its expression or loss has been associated with cell survival, particularly in response to oxidative stress (8, 9).

Pink1, the third and most recent recessive PD gene identified, contains a mitochondrial targeting motif and a serine-threonine kinase domain (10). The physiological substrate(s) of *Pink1* are not fully defined. *Pink1* can phosphorylate two mitochondrial proteins, *Trap1* and *HtrA2* (11, 12). However, the physiological relevance of this phosphorylation needs clarifica-

[1] Recipient of Canadian Institute of Health Research Training Program in Neurodegenerative Lipidomics.
[2] Supported by grants from Neuroscience Canada, Canadian Institute of Health Research, Heart and Stroke Foundation, Ontario, Centre for Stroke Recovery, Parkinson's Disease Foundation, Parkinson's Society Canada, Parkinson's Research Consortium, Centres of Excellence in Neurodegeneration (COEN), and World Class University program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology, South Korea, Grant R31-2008-000-20004-0. Holder of a Career Scientist award from the Heart and Stroke Foundation Ontario. To whom correspondence should be addressed: Dept. of Cellular and Molecular Medicine, University of Ottawa, 451 Smyth Rd., Ottawa, ON K1H 8M5, Canada. Tel.: 613-562-5800 (ext. 8816); Fax: 613-562-5403; E-mail: dpark@uottawa.ca.

[3] The abbreviations used are: PD, Parkinson disease; AAV, adeno-associated virus; DA, dopamine; DAT, dopamine transporter; ISH, *in situ* hybridization; MPP^+, 1-methyl-4 phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTN, medial terminal nucleus; *Pink1*, PTEN-induced kinase 1; SNc, Substantia Nigra pars compacta; TH, tyrosine hydroxylase.

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tion. Growing evidence also suggests that *Pink1* is required for the proper maintenance and regulation of mitochondrial morphology and function (13–16). Germ line deletion of *Pink1* in *Drosophila* causes mitochondrial defects in indirect flight muscles and results in complete disruption of mitochondrial cristae (13, 14). However, this does not occur with *Pink1* germ line-deficient mice. Similarly, *Pink1* has been shown to be essential for the translocation of expressed *Parkin* to the mitochondria in response to mitochondrial depolarization (17). However, whether this pathway of mitochondrial quality control is critical for the pathology of PD is ultimately unknown.

At a more fundamental level, *Pink1* may be an important modulator of cell survival (18). *Pink1* deficiency by itself does not appear to induce neuronal loss (19, 20). However, whether *Pink1* is essential in response to exogenous stress is an intriguing possibility. Indeed, it has been shown that expression of *Pink1 in vitro* is protective in response to exogenous stress such as 1-methyl-4 phenylpyridinium (MPP^{+}), rotenone, and staurosporine (21–23). However, these studies suffer from the caveats of overexpression, and the role of endogenous *Pink1* in dopamine neurons *in vivo* is unknown. Here, we show that knockdown or knock-out of *Pink1* in the SNc area is more sensitive to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) insults. We also find that this sensitization is reversed by other PD-associated genes such as *Parkin* or *DJ-1* and that these genes can protect even in the absence of *Pink1*. This evidence suggests that there are functional interactions among these three recessive PD genes that have a demonstrable impact on environmental stress.

EXPERIMENTAL PROCEDURES

Mice—All procedures were approved by the University of Ottawa Animal Care Committee, and the animals were maintained in strict accordance with the Guidelines for the Use and Treatment of Animals put forth by the Animal Care Council of Canada and endorsed by the Canadian Institutes of Health Research. Germ line-deleted *Pink1* mice were a generous gift from Dr. J. Shen and were back-crossed to C57BL/6 for more than seven generations (19).

Generation of Mouse sh-Pink1 Adenovirus—The *Pink1* siRNA oligonucleotides (Ambion ID nos. 180640, 180641, and 180642) (directed to silence mouse *Pink1* as well as negative control siRNA (nontargeting siRNA) were purchased from Ambion and cloned into the pSilencer 3.0-H1 siRNA vector as reported previously (23). The shRNA fragment, including the H1 promoter, was subcloned to pAdTrack-CMV vector to generate adenovirus as previously described and validated (23). We used the siRNA (ID no. 180640) in the subsequent experiment because it showed the highest knockdown efficiency.

Generation of Human DJ-1 Adenovirus—We generated adenovirus-harboring human *DJ-1* as mentioned earlier (8). Briefly, adenovirus vector-expressing human *DJ-1* were generated by subcloning into pAdTrack-CMV vector (8) in which the expression of GFP and *DJ-1* is driven by two separate CMV promoters. Adenovirus was produced and titered as described previously (8).

Generation of Human Parkin Adeno-associated Virus (AAV)—GFP-*Parkin* AAV was a generous gift from Dr. Edward A. Fon (University of McGill, Canada). AAV was generated and purified as described previously (24).

Viral Gene Delivery in Vivo—Male, 8–10 week-old C57BL/6 mice were purchased from Charles River Laboratories. The mice were individually housed and were acclimated to the new environment before receiving the recombinant adenoviruses that expressed sh-*Pink1* or scrambled DNA as control. Adenoviruses (2 μl; 1×10^7 particles/μl per construct) were stereotaxically injected into the striatum (coordinates from bregma: anterior-posterior, +0.5 mm; medial-lateral, −2.2 mm; dorsal-ventral, −3.4 mm) at an infusion rate of 0.5 μl/min using a syringe pump (PHD2000; Harvard Apparatus). The reduction of *Pink1* message is as described before (23). In addition, we performed *in situ* hybridization (ISH) to show the down-regulation of *Pink1* in the virus-injected area especially in the SNc area as described below. Similarly, human *DJ-1* or GFP control adenoviruses were injected to the midbrain of *Pink1* KO and WT mice. The mice that received sh-*Pink1* or *DJ-1* were challenged with MPTP or saline as mentioned in the MPTP injection section.

AAVs harboring human *Parkin* or GFP control were diluted with 20% mannitol to obtain 1.3×10^5 virus particles/injection. The mannitol premixed viruses (2 μl) were stereotaxically injected into the SNc area (coordinates from bregma: anterior-posterior, −3.0 mm; medial-lateral, −1.6 mm; dorsal-ventral, −4.1 mm) at an infusion rate of 0.5 μl/min using a syringe pump as mentioned earlier. The mice were kept for 2 weeks for complete expression of *Parkin* or GFP, and then the animals were challenged with MPTP as mentioned below.

ISH of Mouse Pink1—ISH was performed as described previously (25) using digoxigenin-labeled antisense RNA riboprobes prepared by *in vitro* transcription from linearized plasmids containing partial sequence of the mouse *Pink1* gene. In brief, mouse *Pink1* cDNA was generated from mouse RNA by RT-PCR. The generated cDNA was used as a template to synthesize a 500-bp DNA fragment by PCR and cloned into pcDNA 3.1(+) vector in sense and antisense direction. Sense and antisense vectors were then used to generate digoxigenin-labeled RNA probes where sense is used as a negative control. Mouse brains were sectioned at 14-μm thickness and incubated with the above mentioned probe overnight and then processed for staining. The stained sections were analyzed on an Axioskop 2 microscope and images were captured using a Microfire camera. The ISH signal was assessed by densitometric analyses using ImageJ software (National Institutes of Health). Briefly, the average densities of striatal fields or individual neurons in the SNc area in both contralateral and ipsilateral sites were determined. Percentage signal was then calculated as ratio of the signal in the ipsilateral *versus* contralateral site and multiplied by 100.

MPTP Administration in Vivo—Mice were challenged with MPTP once a day for 5 consecutive days (25 mg/kg, intraperitoneal, measured as free base; MPTP-HCl; Sigma-Aldrich) 1 week after adenovirus injection to permit sufficient time for retrograde transport and expression of the adenoviral-derived proteins (26, 27). In the case of AAV,

MPTP injection started 2 weeks after initiation of viral injection. Mice used as control received an equivalent volume of saline (0.9%) once daily. Assessment of dopamine neuron survival was performed 2 weeks after the start of the MPTP dosing regimen.

Immunohistochemical Analysis of Tyrosine Hydroxylase (TH)-positive Neurons—Brain tissues from mice injected with MPTP or saline were collected for immunohistochemical analyses as described previously (26, 27). Antibodies used were TH (1:10,000; Immunostar), and immunoreactivity was visualized by using an avidin-biotin complex peroxidase reaction.

Assessment of Dopamine Neuron Loss in Vivo—For viral experiments, the loss of neurons in the SNc was determined by serial section analysis of the total number of TH neurons in the medial terminal nucleus (MTN) region. Intrastriatal administration of adenovirus results in the highest retrograde expression of the gene at this level (26). Briefly, mouse brains were collected and sectioned into 14-μm slices for TH staining. The total numbers of TH positive neurons in the MTN region (-3.08 to -3.28 mm of bregma) in the ipsilateral and contralateral hemispheres were counted separately from at least three sections for each animal. The average numbers of TH neurons in each site of the brain were calculated and presented in the graph. We counted the same subpopulations of TH neurons in the case of AAV injections because they were directly delivered in the MTN region. Cresyl violet staining and counting were similarly performed to validate the result of TH immunostaining as reported previously (26). Briefly, sections at the levels of the MTN as described above were assessed for healthy cells with the morphology and size of dopamine neurons. Quantitation is similar as described for TH counts.

For the evaluation of TH neurons of *Pink1* KO mice only, we employed optical fractionation (28) using Stereo Investigator (version 6; MicroBrightField, Williston, VT), as described previously (29). In brief, 40-μm brain sections were examined within the rostal and caudal limits of the SNc (-2.54 to -3.88 mm of bregma). For each brain, seven coronal sections were examined. After immunoblotting, mounting, defatting, and coverslipping, the thickness of the sections was measured with a *z*-axis microcreator according to the manufacturer's instructions. Sections were analyzed using a $\times 100$ lens. Total number of TH-positive neurons was determined using the optical fractionator. Cresyl violet staining for *Pink1* KO mice was performed at one of the levels of the SNc area (-2.54 to -3.88 mm of bregma) where TH population is highest. The results are presented in number and percentage.

HPLC Analysis of MPP$^+$—Analysis of MPP$^+$ was carried as described previously (30). Briefly, *Pink1* KO and WT mice were injected with a single dose of MPTP (25 mg/kg, intraperitoneal, measured as free base; MPTP-HCl; Sigma-Aldrich). 90 min after MPTP injection, the mice were killed, and striata were collected and processed for HPLC analysis (31).

Statistical Analyses—Data analysis was carried out using independent two-tailed *t* test. Significance was marked by * when $p < 0.05$, ** when $p < 0.01$.

RESULTS

Dopamine Neurons of Pink1 Knock-out Mice Are Sensitive to DA Toxin MPTP in Vivo—We demonstrated previously that ectopic expression of WT *Pink1* in the SNc area protected the TH neurons against the dopaminergic toxin MPTP (23). The metabolite of MPTP, MPP$^+$, is transported to dopaminergic neurons by the dopamine transporter (DAT) where it blocks mitochondrial complex I function and results in degeneration (32). However, to demonstrate the role of *Pink1* in degeneration more definitely in the adult *in vivo* context, loss-of-function studies are required. Our first approach in this regard was to utilize *Pink1*-deficient mice. Recently, several groups generated germ line-deleted *Pink1* mice (19, 33). These *Pink1* KO mice do not show basal loss of DA neurons. However, subtle defects such as decrease in DA release have been observed (19). Accordingly, we initially challenged either WT or *Pink1*-deficient animals with a subchronic paradigm of MPTP as described under "Experimental Procedures" and examined dopaminergic neuron survival. We evaluated the entire SNc region (-2.54 to -3.88 mm of bregma) by optical fractionation/stereology for healthy TH-immunopositive neurons. Importantly, DA neurons of *Pink1*-deficient mice were more sensitive to MPTP than their WT littermates as shown in Fig. 1, *A* and *B*. We also performed cresyl violet staining at one of the level of SNc area as mentioned under "Experimental Procedures" to corroborate the loss of dopamine neurons further. We observed a trend similar to that obtained for TH immunohistochemical analysis (Fig. 1*B*). The TH-positive neurons of SNc project their processes to the striatum. These terminal fibers are enriched with the DAT. Therefore, we examined whether the loss of DA neurons in the SNc area correlated with terminal loss as evaluated by DAT staining. As expected, we found a significant loss of DAT staining in *Pink1*-deficient animals compared with littermate controls (54.76% striatal density in MPTP-treated WT animal *versus* 31.80% in *Pink1* KO animals when normalized with saline-treated WT animals) (Fig. 1, *D* and *E*). To ascertain that the greater loss of DA neurons or decrease in DAT density is not due to the increased production of MPP$^+$ from MPTP, we measured the MPP$^+$ in the striatum. Indeed, we found that equal amounts of MPP$^+$ available in both WT/KO mouse striatum (Fig. 1*F*).

shRNA-mediated Knockdown of Mouse Pink1 in SNc Area Sensitizes DA Neurons to MPTP—The use of germ line deficiencies brings up potential concerns of unforeseen confounds brought on by developmental compensation that may have little to do with the original function of the ablated gene. Because of these concerns, a more transient approach to *Pink1* knockdown was explored in conjunction with the deficient mice. We first generated shRNA viral vectors to *Pink1* which could be used in our *in vivo* adult MPTP model. We employed the shRNA sequence which we had previously shown to be effective in down-regulating mouse *Pink1* expression *in vitro* (23). We subsequently generated sh-*Pink1* adenovirus or a control shRNA virus (scrambled sequence with no known homology with mouse gene). The effectiveness of our sh-*Pink1* vector to silence *Pink1* in the SNc was evaluated utilizing ISH. This method was chosen due to the absence of reliable *Pink1* anti-

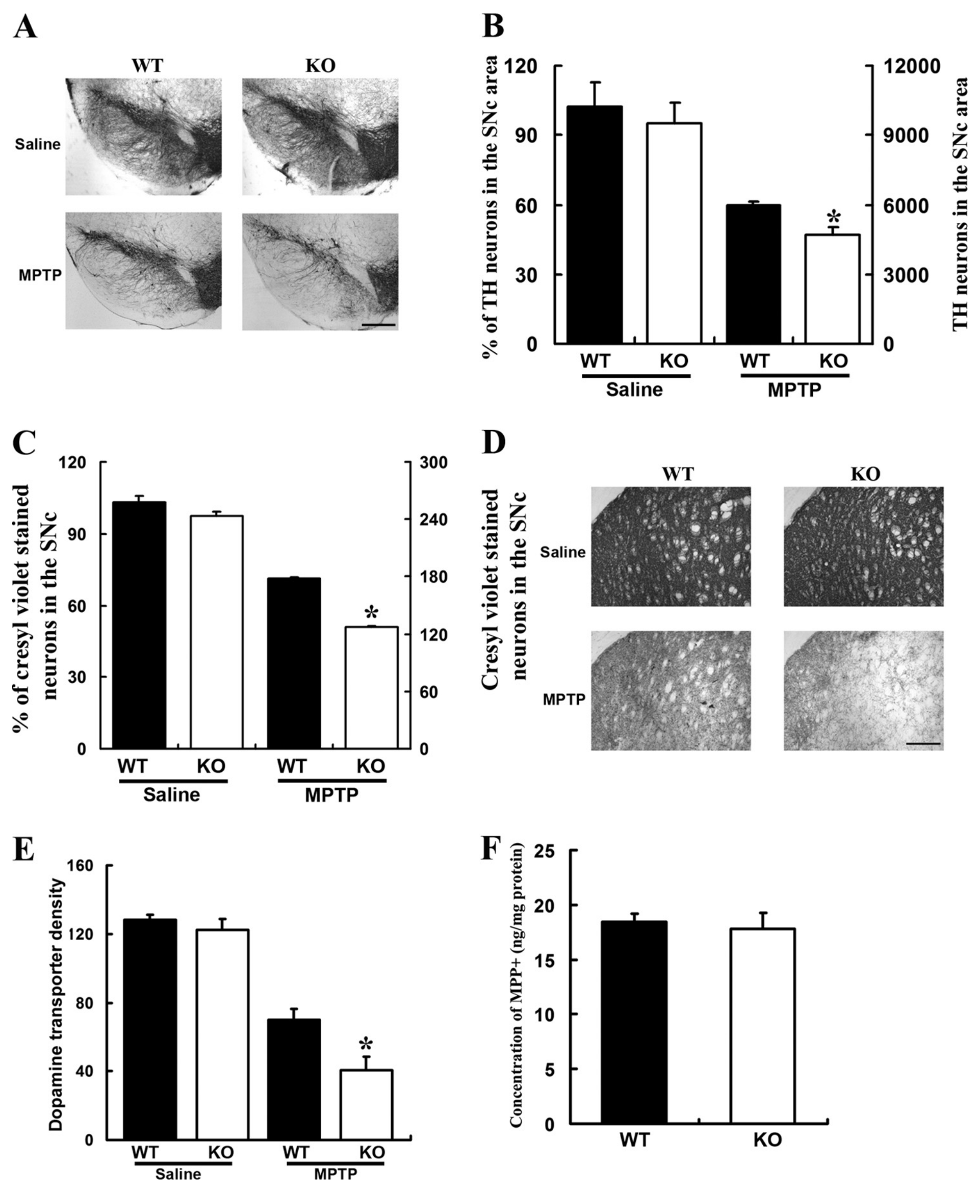

FIGURE 1. **Dopamine neurons of germ line-deficient *Pink1* mice are more sensitive to neurotoxin MPTP.** *A*, representative photomicrographs illustrating TH immunoreactivity in the ventral midbrain SNc. *Scale bar*, 250 μm. *B*, quantification of TH-immunoreactive neurons in SNc area of *Pink1* WT/KO animals by stereology, as described under "Experimental Procedures." *C*, cresyl violet-stained neurons in SNc area. *D*, representative photomicrographs illustrating DAT immunoreactivity in the striatum of *Pink1* WT/KO animals. *Scale bar*, 500 μm. *E*, quantification of optical density of striatal DAT-stained fiber density of *Pink1* WT/KO animals. *F*, HPLC analysis of striatal MPP^+ levels in *Pink1* WT/KO animals. Values are means $\pm$ S.E. (*error bars*; $n = 3$–5). *, $p < 0.05$ compared with WT animals treated with MPTP.

bodies needed for immunohistochemical analysis of endogenous *Pink1 in vivo*. The adenovirus was injected into the striatum area which retrogradely transports to the SNc. A week after the injection, the brain tissues were prepared for ISH to detect the *Pink1* signal. As shown in Fig. 2, *A–C*, the *Pink1* message signal was significantly lower (71%) in the striatum of the ipsilateral side, where viral injection was carried out, relative to the contralateral side. Most importantly, this reduction was clear in the area of the SNc in cells with the shape and size of DA neurons (68% reduction). One week after viral transduction with either sh-*Pink1* vector or control, animals were challenged with the same subchronic regimen of MPTP utilized with the *Pink1*-deficient animals as described earlier. Two weeks after the initial MPTP or saline dose, animals were killed, and the midbrain was sectioned. The number of TH-immunopositive neurons in both virus-injected (ipsilateral) and virus-uninjected (contralateral) in the SNc at the level of the MTN was assessed. Consistent with the germ line deficiency data, we found that silencing of *Pink1* in the SNc area led to increased loss of DA neurons in response to MPTP (Fig. 2, *D* and E). Silencing of *Pink1* by itself did not significantly affect TH neuron numbers, at least in the 3-week time frame examined. This result is in line with recent reports indicating that *Pink1* knockdown or knockout does not cause any DA neuron loss (19, 20, 33). To further ensure that the loss of TH neurons was not due to simple loss of TH marker expression and not degeneration, we also carried out cresyl violet staining and analyses of the SNc in adjacent sections at the level of the MTN. As shown in Fig. 2*F*, results similar to TH counts were obtained. Taken together, these results clearly indicate the critical role of endogenous *Pink1* in

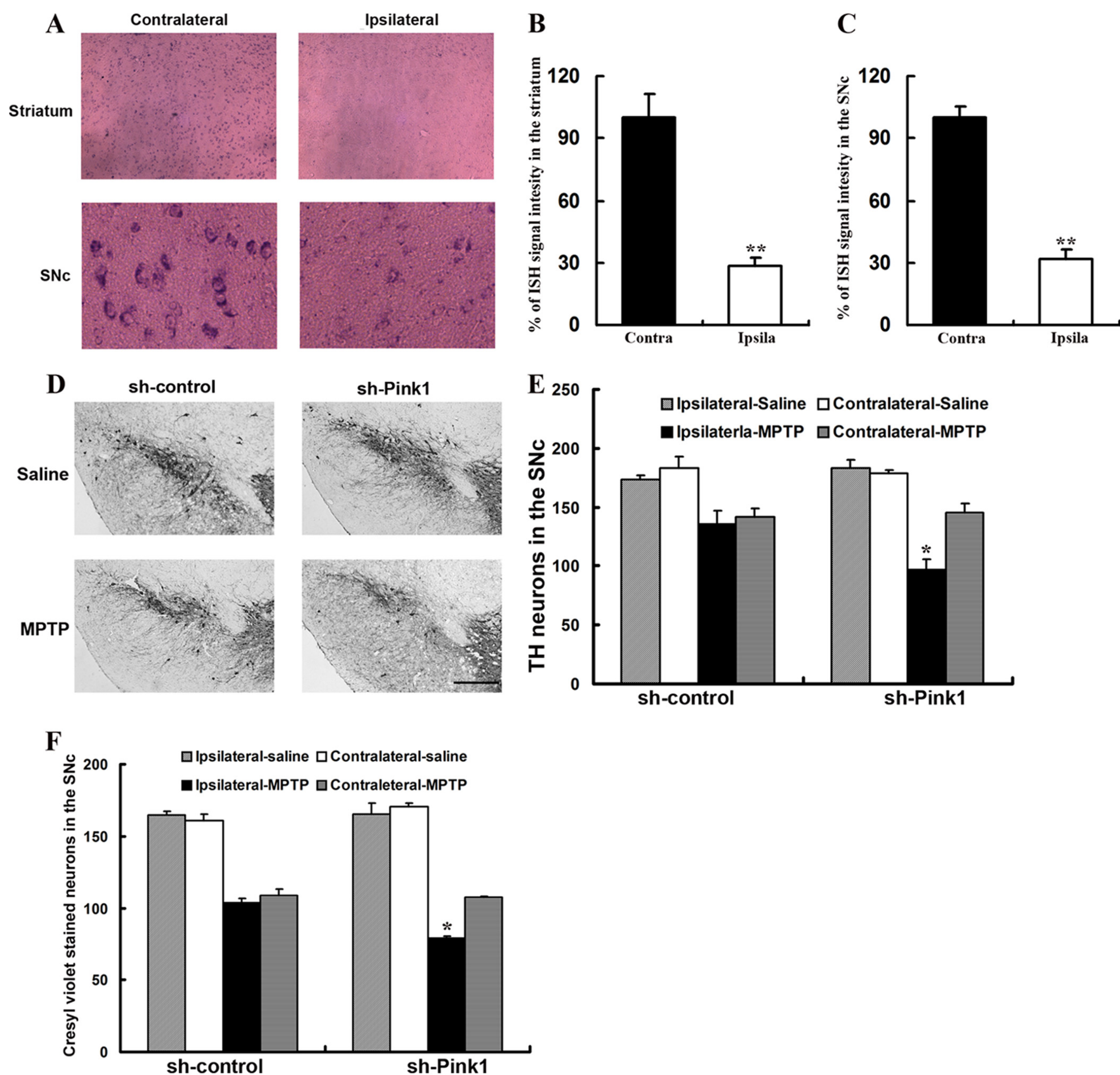

FIGURE 2. **shRNA-mediated knockdown of *Pink1* results in greater loss of dopamine neurons upon MPTP treatment.** *A*, adenoviruses (2 μl, 1×10^7 particles/μl) expressing *Pink1* shRNA or control injected directly into the striatum (ipsilateral side) of the animals. A week after viral injection, the brains were collected, fixed, and sectioned. The brain sections were processed for ISH to examine *Pink1* transcript in striatum and SNc area. *B* and *C*, percentage of ISH signal intensity in the striatum (*B*) and SNc (*C*). **, $p < 0.01$ *versus* contralateral site. *D*, adenovirus-injected mice challenged with MPTP as described under "Experimental Procedures." Brains were collected and sectioned into 14-μm slices for TH immunostaining. Representative images of TH-immunoreactive neurons of the ipsilateral side of the animals, 2 weeks after treatment with MPTP or saline, are shown. *Scale bar*, 250 μm. *E* and *F*, quantifications of TH-immunoreactive neurons from the ipsilateral or the contralateral region of SNc area (*E*) or cresyl violet-stained neurons (*F*). All values are means ± S.E. (*error bars*; $n = 3–5$). *, $p < 0.05$ compared with control virus-injected (ipsilateral) side of MPTP-treated animals.

neurons in mediating neuronal survival to exogenous stress in the adult animal *in vivo*.

Role of Parkin and DJ-1 in Modulating Sensitization Observed with Pink1 Loss—A functional relationship between *Pink1* and the other recessive PD genes, *Parkin* and *DJ-1*, has been suggested. For example, studies in *Drosophila* indicated that *Parkin* acts downstream of *Pink1* to rescue the abnormal wing and mitochondrial defects in indirect flight muscles, a phenotype associated with *Pink1* loss of function, at least in flies. Similarly, *Parkin* accumulates in damaged mitochondria and enhances mitophagy in a *Pink1*-dependent manner (4, 6, 17). However, in this case, the role of this translocation in mediating survival is unknown. Importantly, the capacity of *Parkin* to compensate for *Pink1* loss-mediated sensitization to death or to protect in the absence of *Pink1* is unknown. To test this, we examined whether *Parkin* expression in dopaminergic neurons can also rescue sensitization effects of MPTP due to absence of *Pink1*, *in vivo*. We delivered *Parkin*

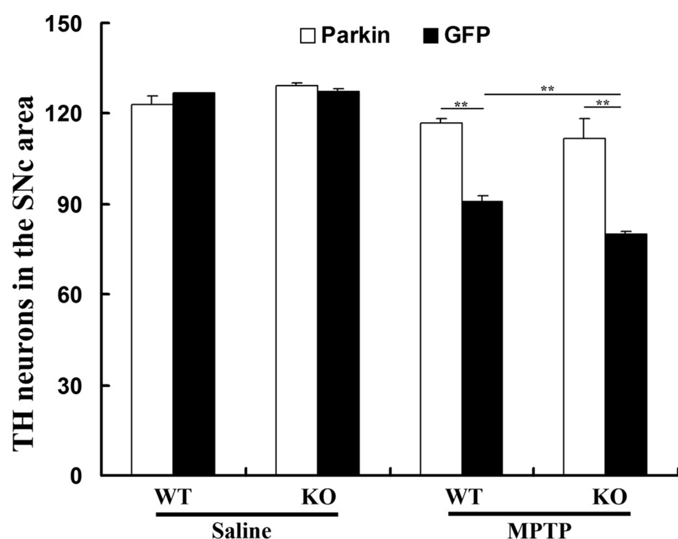

FIGURE 3. **Expression of AAV *Parkin* at the SNc area rescues DA neurons of *Pink1* KO mice from MPTP sensitization.** The AAVs expressing human *Parkin* or control were injected directly into the SNc area of animals 2 weeks before the initiation of MPTP treatment. Brains were collected and sectioned into 14-μm slices for TH immunostaining. Quantifications of TH-immunoreactive neurons from the ipsilateral region of SNc area of mice brain of different groups are shown. All values are means ± S.E. (*error bars*; $n = 3–4$), except GFP-injected saline-treated WT control ($n = 2$).

or GFP control AAV to the SNc 2 weeks prior to a challenge with MPTP. We found that expression of *Parkin* is effective in rescuing the sensitization induced by *Pink1* loss. It is important to note that in this case, the sensitization induced by *Pink1* deficiency was slightly lower (although still significant) than that observed in Fig. 1*B*. This may be due to the effects of viral transduction. Interestingly, *Parkin* provided the same extent of neuroprotection in the absence or presence of endogenous *Pink1* (Fig. 3). This result suggests that *Parkin* not only compensates for *Pink1* deficiency, but also provides further protection even in the absence of *Pink1*. This in agreement with the recent reports showing that ectopic expression of AAV *Parkin* was protective against MPTP under WT backgrounds (34, 35).

DJ-1 is an another PD gene involved in scavenging oxidative stress in multiple model systems (8, 9, 36). We have previously shown that loss of *DJ-1* sensitizes animals to multiple environmental stressors such as ischemia and MPTP *in vivo* (8, 9). Given its roles in processes such as mitochondrial function (37) and survival (9) which are also implicated for *Parkin* and *Pink1*, we next tested whether *DJ-1* might also compensate for *Pink1* loss. In this case, *DJ-1* or GFP controlled adenovirus was delivered stereotaxically to the striatum in either WT or KO *Pink1* animals. We have previously shown *DJ-1* to be effective in promoting survival *in vivo* with this virus (8). As shown previously, GFP-injected *Pink1* KO animals showed hypersensitization to death compared with WT controls in response to MPTP. Importantly, exogenous *DJ-1* expression was effective at overcoming this sensitization. Similar to *Parkin* expression, *DJ-1*-expressing animals showed the same level of survival at the SNc whether or not *Pink1* was present (Fig. 4). These results indicate that the other two recessive PD genes *Parkin* and *DJ-1* can protect even in the absence of *Pink1*. This suggests that they are

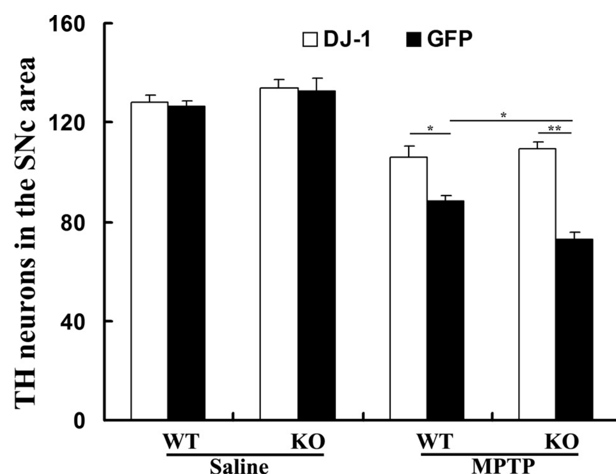

FIGURE 4. **Delivery of adenovirus-expressing *DJ-1* at striatum area rescues DA neurons of *Pink1* KO mice from MPTP sensitization.** The adenoviruses (2 μl, 1×10^7 particles/μl) expressing human *DJ-1* or control were injected directly into the striatum of animals 7 days before the initiation of MPTP treatment. Brains were sectioned into 14-μm slices for TH immunostaining. Quantifications of TH-immunoreactive neurons from the ipsilateral region of SNc area of mice brain of different groups are shown. Values are means ± S.E. (*error bars*; $n = 3–4$).

either downstream of *Pink1* or on parallel pathways to regulate survival.

DISCUSSION

Recent discoveries of several genes associated with familial PD have given us the opportunity to enhance our molecular understanding of this devastating disease. Among these genes, *Pink1* has received much attention due to its clear mitochondrial targeting motif, its well defined kinase domain, and involvement in mitochondrial quality control pathways. Yet, the mechanism by which its loss leads to PD in humans is still unclear.

Role of Pink1 in Survival—One critical aspect of *Pink1* function has to do with its potential role in survival. As stated previously, it has been reported that WT *Pink1* expression blocks death against a number of death stimuli including MPP^+, rotenone, staurosporine and MG-132 (10, 21–23). We likewise observed this phenomenon in primary neurons expressing WT human *Pink1* (23). However, the endogenous role of *Pink1*, particularly in the adult mammalian brain *in vivo*, is less clear. This question is made more significant given recent questions on the relevance of seemingly critical biological processes such as *Parkin*-mediated mitochondrial quality control in the adult animal (38). This reflects a broader question of relevance of *in vitro* findings in the adult *in vivo* context.

Pink1 deficiency in mice does not lead to increased basal DA neuronal loss as observed in human PD. Our present data demonstrating that shRNA knockdown of *Pink1*, at least in the time frame examined, did not lead to increased basal DA loss is consistent with these results. In the fly, *Pink1* deficiency does lead to demonstrable DA loss, but the reason for this difference is unclear. One explanation may be that in the mammalian system, there are other survival pathways that are sufficient to prevent basal DA loss at least under basal conditions in the lifetime of the mouse. These compensatory pathways likely do

not involve *DJ-1* and *Parkin* because *Pink1/DJ-1/Parkin* triple KO mice also do not show basal DA loss (39).

Rather than focus on basal processes, the effects of exogenous environmental stress might better illuminate the functional role of *Pink1* in survival. Because of the role of *Pink1* in mitochondrial function/quality control and its localization, the effect of *Pink1* loss on mitochondrial stress is particularly interesting. This is even more critical given the increased association of mitochondrial defects in PD and the links of variety of PD genes to mitochondrial processes. Accordingly, we examined how *Pink1* loss may affect DA survival in response to the mitochondrial complex I toxin MPTP, to address the central question of whether endogenous *Pink1* plays a role in DA survival in the adult mammalian brain. Our results support the veracity of these hypotheses as follows: (i) germ line-deficient *Pink1* animals are more susceptible to MPTP and (ii) this is likely not due to developmental compensatory changes because transient knockdown of *Pink1* also shows similar results.

The mechanism(s) by which *Pink1* regulates survival has not been identified as yet. The localization of *Pink1* alone is complex. It has been reported to localize to the inner mitochondrial membrane basally and at the outer mitochondrial membrane following mitochondrial membrane depolarization (4, 6). It is suggested that *Pink1* can exist also in the cytoplasm (23, 40, 41). This last point is particularly intriguing given the fact that although the kinase activity of *Pink1* is critical for its protective effects when expressed, its mitochondrial localization sequence is not required either *in vitro* or *in vivo* in response to mitochondrial stress (23). This finding suggests that although *Pink1* may play a role in mitochondrial quality control, this may not be the primary pathway by which survival is mediated. Alternatively, *Pink1* may act on substrates in the cytoplasm to mediate survival as it is known that when *Pink1* is localized to the outer mitochondrial membrane, its kinase domain faces the cytoplasm (42). Interestingly, it was shown that stable knockdown of *Pink1* in cell culture system promotes autophagy. This autophagic event due to the absence of *Pink1* can be reversed by overexpression of WT *Pink1* or Δ*Pink1* (43). However, the link between autophagy and survival is unclear. In this regard, autophagy has been shown either to promote or inhibit death processes. Finally, *Pink1* has also been shown to phosphorylate *Trap1*, and this phosphorylation event is necessary for survival against oxidative stress (11). Whether this is critical in the adult DA system is unknown.

Interaction of Pink1 with Other Autosomal Recessive PD Genes, DJ-1 and Parkin—There are several common features of the three recessive PD genes *DJ-1*, *Parkin*, and *Pink1*. Loss of all of three genes has in some way been associated with mitochondrial deficits in the mouse tissue (3, 37, 44). Deficiencies also appear to mediate defects in DA uptake and turnover (19, 45, 46). These three genes are also associated with management of free radical damage (7, 9, 23, 34, 35). For example, several reports indicated that *DJ-1* expression protects and its loss sensitizes to oxidative inducers (8). Similar observations have been shown for *Parkin* and *Pink1* (23, 34, 35). In fact, *DJ-1* itself has atypical peroxyredoxin activity, was modified by reactive oxygen species (7), and was shown to regulate the stability of one of the master antioxidant regulator *Nrf2* (47). *Parkin* is also reported to induce genes such as *TFAM*, which is critical for mitochondrial biogenesis (48). How these parameters relate to the direct mechanism of cell-mediated survival is not known. Likewise, whether or not these three recessive genes act together to mediate survival *in vivo* remains to be established. As mentioned previously, ablation of all three recessive genes does not lead to DA neuron loss *in vivo* (39). This argues against the notion that these genes act in parallel to mediate basal DA survival *in vivo*. However, our present work supports the notion that modulation of either *DJ-1* or *Parkin* levels can compensate for the loss of *Pink1*. This is consistent with reports indicating that *Parkin* and *DJ-1* can also compensate for *Pink1*-mediated defects in the fly (49). Moreover, it supports a recent report that *DJ-1* acts in parallel with the *Pink1/Parkin* pathways (50).

Our observation that *Parkin* can compensate for *Pink1* deficiency is also interesting in light of the recent observations that *Pink1* is required for *Parkin* translocation to the mitochondria upon mitochondrial depolarization (17). Therefore, one interpretation of this finding is that *Parkin* acts downstream of *Pink1*, and thus the ability of *Parkin* expression to rescue *Pink1* deficiency make sense. However, there are some caveats regarding this interpretation. First, it is thought that *Pink1* is an absolute requirement for *Parkin* translocation. In this regard, our results demonstrating *Parkin*-mediated protection in the absence of *Pink1* are pertinent. The manner by which *Parkin* is mediating survival must be independent of its presence in the mitochondria. In addition, a mouse model defective in respiratory chain components which results in fragmented mitochondria and dissipation of membrane potential did not demonstrate *Parkin* accumulation at the mitochondria (38). They also found that mitophagy in these mice was not impacted in the presence or absence of *Parkin*, arguing against the role of *Parkin* in clearing defective mitochondria. Taken together, these observations call into some question whether or not mitochondrial quality control pathways mediated by *Pink1/Parkin* may be critical for DA survival. Much more work is clearly needed to address these essential questions. Nevertheless, our work clearly demonstrates that endogenous *Pink1* is an essential component that prevents neuronal loss in the adult SNc in response to environmental stress. We also provide evidence that modulation of either *Parkin* or *DJ-1* can protect even in the absence of *Pink1*, suggesting that *Pink1* is not a required factor for *DJ-1/Parkin*-mediated protection.

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