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

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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 peoxyreredoxin 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 clarification.

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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 pathogenesis 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 knockdown or knock-out of 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 adeno viruses that expressed sh-Pink1 or scrambled DNA as control. Adeno viruses (2 μl; 1 × 10⁷ 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 × 10⁷ 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,
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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 adeno virus 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 rostral 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 ×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. 1B). 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. 1F).

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-
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. 2F, results similar to TH counts were obtained. Taken together, these results clearly indicate the critical role of endogenous Pink1 in
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 Parkin-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
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 Parkin deficiency, but also provides further protection even in the absence of Parkin. This in agreement with the recent reports showing that ectopic expression of AAV Parkin was protective against MPTP under WT backgrounds (34, 35).

**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, Parkin 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 Parkin in Survival**—One critical aspect of Parkin function has to do with its potential role in survival. As stated previously, it has been reported that WT Parkin 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 Parkin (23). However, the endogenous role of Parkin, 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.

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**FIGURE 3.** Expression of AAV Parkin at the SNc area rescues DA neurons of Parkin 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. Values are means ± S.E. (error bars; n = 3–4), except GFP-injected saline-treated WT control (n = 2).

**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).
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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 ΔPink1 (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 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 peroxiredoxin 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.

REFERENCES

1. Olanow, C. W., Stern, M. B., and Sethi, K. (2009) The scientific and clinical basis for the treatment of Parkinson disease. Neurology 72, S1–136
2. Martin, I., Dawson, V. L., and Dawson, T. M. (2011) Recent advances in the genetics of Parkinson’s disease. Annu. Rev. Genomics Hum. Genet. 12, 301–325
3. Palacino, J. J., Sagi, D., Goldberg, M. S., Krauss, S., Motz, C., Wacker, M., Klose, J., and Shen, J. (2004) Mitochondrial dysfunction and oxidative damage in Parkin-deficient mice. J. Biol. Chem. 279, 18614–18622
4. Narendran, D. P., Jin, S. M., Tanaka, A., Suen, D. F., Gautier, C. A., Shen, I., Cookson, M. R., and Youle, R. J. (2010) PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol. 8, e1000298
5. Jin, S. M., Lazarou, M., Wang, C., Kane, L. A., Narendran, D. P., and Youle, R. J. (2010) Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. J. Cell Biol. 191, 933–942
6. Narendran, D. P., and Youle, R. J. (2011) Targeting mitochondrial dysfunc-
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7. Andres-Mateos, E., Perier, C., Zhang, L., Blanchard-Fillion, B., Greco, T. M., Thomas, B., Ko, H. S., Sasaki, M., Ischiropoulos, H., Przedborski, S., Dawson, T. M., and Dawson, V. L. (2007) DJ-1 gene deletion reveals that DJ-1 is an atypical peroxidorexin-like peroxidase. Proc. Natl. Acad. Sci. U.S.A. 104, 14807–14812

8. Kim, R. H., Smith, P. D., Aleyasin, H., Hayley, S., Mount, M. P., Pownall, S., Wakeham, A., You-Ten, A. J., Kalia, S. K., Horne, P., Westaway, D., Lozano, A. M., Anisman, H., Park, D. S., and Mak, T. W. (2005) Hypersensitivity of DJ-1-deficient mice to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and oxidative stress. Proc. Natl. Acad. Sci. U.S.A. 102, 5215–5220

9. Aleyasin, H., Rousseaux, M. W., Phillips, M., Kim, R. H., Bland, R. J., Callaghan, S., Slack, R. S., During, M. J., Mak, T. W., and Park, D. S. (2007) The Parkinson’s disease gene DJ-1 is also a key regulator of stroke-induced damage. Proc. Natl. Acad. Sci. U.S.A. 104, 18748–18753

10. Valente, E. M., Abou-Sleiman, P. M., Caputo, V., Mueg, M. M., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A. R., Healy, D. G., Albanese, A., Nussbaum, R., González-Maldonado, R., Deller, T., Salvi, S., Cortelli, P., Gilks, W. P., Latchman, D. S., Harvey, R. J., Dallapiccola, B., Auburger, G., and Wood, N. W. (2004) Hereditary early-onset Parkinson’s disease caused by mutations in PINK1. Science 304, 1158–1160

11. Rymar, V. V., Sasseville, R., Luk, K. C., and Sadikot, A. F. (2004) Neurogenesis and stereological morphometry of calretinin-immunoreactive GABAergic interneurons of the neostriatum. J. Comp. Neurol. 469, 325–339

12. Gundersen, H. I., Bagger, P., Bentsen, T. F., Evans, S. M., Korbo, L., Marcusson, N., Müller, A., Nielsen, K., Nyengaard, J. R., Pakkenberg, B., and et al. (1988) The new stereological tools: dissector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APJMS 96, 857–881

13. Hayley, S., Sasseville, R., Luk, K. C., and Sadikot, A. F. (2004) Neuronal degeneration and stereological morphometry of calretinin-immunoreactive GABAergic neurons of the neostriatum. J. Comp. Neurol. 469, 325–339

14. Paterna, J. C., Leng, A., Weber, E., Feldon, J., and Büeler, H. (2007) DJ-1 and Parkin modulate dopamine-dependent behavior and inhibit MPTP-induced nigral dopamine neuron loss in mice. Mol. Ther. 15, 698–704

15. Yasuda, T., Hayakawa, K., Nihira, T., Ren, Y. R., Nakata, Y., Nagai, M., Hattori, N., Miyake, K., Takada, M., Shimada, T., Mizuno, Y., and Mochizuki, H. (2011) Parkin-mediated protection of dopaminergic neurons in a chronic MPTP-minipump mouse model of Parkinson disease. J. Neurochem. Exp. Neurol. 70, 686–697

16. Aleyasin, H., Rousseaux, M. W., Marcusgole, P. C., Hewitt, S. J., Ircrter, L., Joselin, A. P., Parsanejad, M., Kim, R. H., Rizzu, P., Callaghan, S. M., Slack, R. S., Mak, T. W., and Park, D. S. (2010) DJ-1 protects the nigrostrial area from the neurotoxin MPTP by modulation of the AKT pathway. Proc. Natl. Acad. Sci. U.S.A. 107, 3186–3191
37. Irrcher, I., Aleyasin, H., Seifert, E. L., Hewitt, S. J., Chhabra, S., Phillips, M., Lutz, A. K., Rousseaux, M. W., Bevilacqua, L., Jahani-Asl, A., Callaghan, S., MacLaurin, J. G., Windkofler, K. F., Rizzu, P., Rippstein, P., Kim, R. H., Chen, C. X., Fon, E. A., Slack, R. S., Harper, M. E., McBride, H. M., Mak, T. W., and Park, D. S. (2010) Loss of the Parkinson’s disease-linked gene DJ-1 perturbs mitochondrial dynamics. *Hum. Mol. Genet.* 19, 3734–3746
38. Sterky, F. H., Lee, S., Wibom, R., Olson, L., and Larsson, N. G. (2011) Impaired mitochondrial transport and Parkin-independent degeneration of respiratory chain-deficient dopamine neurons in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 108, 12937–12942
39. Kitada, T., Tong, Y., Gautier, C. A., and Shen, J. (2009) Absence of nigral degeneration in aged Parkin/DJ-1/PINK1 triple knockout mice. *J. Neurochem.* 111, 696–702
40. Lin, W., and Kang, U. J. (2010) Structural determinants of PINK1 topology and dual subcellular distribution. *BMC Cell Biol.* 11, 90
41. Takatori, S., Ito, G., and Iwatsubo, T. (2008) Cytoplasmic localization and proteasomal degradation of N-terminally cleaved form of PINK1. *Neurosci. Lett.* 430, 13–17
42. Zhou, C., Huang, Y., Shao, Y., May, J., Prou, D., Perier, C., Dauer, W., Schon, E. A., and Przedborski, S. (2008) The kinase domain of mitochondrial PINK1 faces the cytoplasm. *Proc. Natl. Acad. Sci. U.S.A.* 105, 12022–12027
43. Dagda, R. K., Cherra, S. J., 3rd, Kulich, S. M., Tandon, A., Park, D., and Chu, C. T. (2009) Loss of PINK1 function promotes mitophagy through effects on oxidative stress and mitochondrial fission. *J. Biol. Chem.* 284, 13843–13855
44. Gautier, C. A., Kitada, T., and Shen, J. (2008) Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11364–11369
45. Goldberg, M. S., Pisani, A., Haburcak, M., Vortherms, T. A., Kitada, T., Costa, C., Tong, Y., Martella, G., Tschetter, A., Martins, A., Bernardi, G., Roth, B. L., Pothos, E. N., Calabresi, P., and Shen, J. (2005) Nigrostriatal dopaminergic deficits and hypokinesia caused by inactivation of the familial parkinsonism-linked gene DJ-1. *Neuron* 45, 489–496
46. Kitada, T., Pisani, A., Karouani, M., Haburcak, M., Martella, G., Tschetter, A., Platania, P., Wu, B., Pothos, E. N., and Shen, J. (2009) Impaired dopamine release and synaptic plasticity in the striatum of parkin−/− mice. *J. Neurochem.* 110, 613–621
47. Clements, C. M., McNally, R. S., Conti, B. J., Mak, T. W., and Ting, J. P. (2006) DJ-1, a cancer- and Parkinson’s disease-associated protein, stabilizes the antioxidant transcriptional master regulator Nrf2. *Proc. Natl. Acad. Sci. U.S.A.* 103, 15091–15096
48. Kuroda, Y., Mitsui, T., Kunishige, M., Shono, M., Akaike, M., Azuma, H., and Matsumoto, T. (2006) Parkin enhances mitochondrial biogenesis in proliferating cells. *Hum. Mol. Genet.* 15, 883–895
49. Hao, L. Y., Giasson, B. I., and Bonini, N. M. (2010) DJ-1 is critical for mitochondrial function and rescues PINK1 loss of function. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9747–9752
50. Thomas, K. J., McCoy, M. K., Blackinton, J., Beilina, A., van der Brug, M., Sandebring, A., Miller, D., Maric, D., Cedazo-Minguez, A., and Cookson, M. R. (2011) DJ-1 acts in parallel to the PINK1/Parkin pathway to control mitochondrial function and autophagy. *Hum. Mol. Genet.* 20, 40–50