Parkin protects human dopaminergic neuroblastoma cells against dopamine-induced apoptosis

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Parkinson's disease (PD) is characterized by the selective degeneration of dopaminergic (DA) neurons in substantia nigra pars compacta (SNpc). A combination of genetic and environmental factors contributes to such a specific loss. Among the five PD-linked genes identified so far, parkin, a protein-ubiquitin E3 ligase, appears to be the most prevalent genetic factor in PD. Although a variety of substrates have been identified for parkin, none of them is selectively expressed in nigral DA neurons. It remains unclear how accumulation of these substrates in the absence of functional parkin may cause the selective death of DA neurons in SNpc. Here, we show that overexpression of parkin protected human DA neuroblastoma cell line (SH-SY5Y) against apoptosis induced by DA or 6-OHDA, but not by H_2O_2 or rotenone. Parkin significantly attenuated dopamine-induced activation of c-Jun N-terminal kinase (JNK) and caspase-3. It also decreased the level of reactive oxygen species (ROS) and protein carbonyls in the cell. Inhibiting DA uptake through dopamine transporter or treating the cell with antioxidants significantly reduced oxidative stress and dopamine toxicity. Furthermore, PD-linked mutations of parkin significantly abrogated the protective effect of wild-type parkin, as well as its ability to suppress ROS and protein carbonylation. These results suggest that parkin protects against dopamine toxicity by decreasing oxidative stress and ensuing activation of apoptotic programs such as the JNK/caspase pathway. This protective function of parkin, which is greatly attenuated by its PD-linked mutations, may be uniquely important for the survival of DA neurons, as they are constantly threatened by oxyradicals produced during dopamine oxidation.

INTRODUCTION

Parkinson's disease (PD) is one of the most common neuro-degenerative disorders. It is characterized by the progressive and selective loss of dopaminergic (DA) neurons in substantia nigra pars compacta (SNpc). A variety of genetic and environmental factors contribute to the degeneration of nigral DA neurons and PD. Exposures to agricultural pesticides, heavy metals and neurotoxins have been implicated as significant risk factors (1). On the other hand, mutations of several genes have been linked clearly to familial forms of PD. Among the five PD genes identified so far, mutations of parkin appear to be most prevalent, whereas mutations of α-synuclein, UCH-L1, DJ-1 and NR4A2 have only been found in rare cases (2). In addition, parkin mutations are also found in many patients with idiopathic PD, especially those with an early age at onset (3). In a genome-wide scan

for PD-linked genes, a very high degree of linkage has been found for parkin [with a logarithm of odds (LOD) score of 5.47], in contrast to other loci with much lower LOD scores (4). These lines of evidence strongly suggest that parkin is critically involved in the survival of nigral DA neurons and PD.

Parkin encodes for a protein-ubiquitin E3 ligase (5), which is responsible for catalyzing ubiquitination of many proteins to facilitate their degradation by the 26S proteasome. Although a number of substrates with diverse functions have been identified for parkin, none of them is specifically expressed in DA neurons (6). It remains unclear how their accumulation in many types of cells in the absence of parkin would lead to selective degeneration of nigral DA neurons. An alternative possibility is that parkin may be involved in physiological processes unique to DA neurons. One key culprit for the selective death of DA neurons in PD may be dopamine itself. Previous studies have shown that injection of dopamine into rat striatum

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causes selective death of DA neurons, and the effect is significantly attenuated by antioxidants (7). Both auto-oxidation of dopamine and its enzymatic catabolism by monoamine oxidase produce reactive oxygen species (ROS). It has been shown that dopamine treatment of HEK293 cell or rat striatal neuronal cultures induces apoptosis through a mechanism dependent on ROS and the activation of c-Jun N-terminal kinase (JNK) (8). Here, we provide evidence that parkin protected human DA neuroblastoma cells by decreasing the production of ROS and the activation of apoptotic pathways emanated from JNK. As both the protective effect and the ability to decrease oxidative stress were significantly abrogated by PD-causing mutations of parkin, our results may provide a link between parkin—the most prevalent genetic factor in PD—and dopamine-induced oxidative stress, which plays a critical role in the selective death of DA neurons.

RESULTS

Overexpression of parkin protects human DA neuroblastoma cells against dopamine-induced apoptosis

As loss-of-function mutations of parkin are linked to degeneration of DA neurons and PD, we hypothesize that overexpressing parkin may protect DA cells from toxins such as DA itself. To test this, we generated stable lines overexpressing FLAG-tagged parkin. In order to minimize clonal variation, we examined pooled positive clones (SH/PKN) and an individual clone (E8), which exhibited similar characteristics. As shown in Figure 1A, parkin was overexpressed in SH/PKN and E8 cells, at a much higher level than the endogenous parkin in parental cells (SH). Expression level of tyrosine hydroxylase (TH) was unaffected. These cell lines had similar growth rates and maintained normal morphological features, such as long, fine, neurite-like processes (Fig. 1B–D).

We treated the cells with various concentrations of DA for 12 h, and assessed apoptosis using TUNEL staining. Dopamine caused apoptosis of SH-SY5Y cells in a dose-dependent manner. The effect was significantly attenuated in SH/PKN cells at all dopamine concentrations used in the study (Fig. 1E). A similar effect was observed in E8 cells (data not shown). We also examined the time-course of dopamine-induced apoptosis in these cell lines. A significant level of apoptosis was seen after 8 h of dopamine treatment (75 μM), and cell death at this time point or later (monitored up to 20 h) was greatly reduced in SH/PKN cells, compared with SH cells ($P < 0.05$, data not shown). Representative images from cells treated with 75 μM of dopamine (12 h) showed that the percentage of nuclei (red signals) with broken DNA ends (green or yellow signals) was markedly lower in SH/PKN or E8 cells, compared with SH cells (Fig. 1F–H). These results showed that overexpression of parkin in SH-SY5Y cells significantly attenuated dopamine-induced apoptosis. Because the individual clone E8 behaved in the same way as the pooled clones (SH/PKN), we used SH/PKN cells in all subsequent experiments. The protective effect of parkin was also observed when we quantified cell death with

trypan blue staining, which only distinguishes dead cells from live ones (data not shown).

Parkin attenuates dopamine-induced apoptotic signaling in SH-SY5Y cells

Previous studies have demonstrated that dopamine-induced apoptosis is mediated by JNK (8), which is required for stress-induced cytochrome c release from mitochondria (9) and for ensuing activation of caspase-3 (10). One substrate of active caspase-3 is poly(ADP-ribose) polymerase (PARP), a DNA damage sensor activated by broken DNA ends produced during apoptosis (11). To evaluate the impact of parkin overexpression on dopamine-activated apoptotic pathways, we treated SH or SH/PKN cells with dopamine, and examined the activation of JNK, c-Jun, caspase-3 and PARP by western blotting total cell lysates with antibodies that recognize the activated forms of these enzymes. With a treatment of 75 μM of DA for 12 h, significantly less phospho-JNK, phospho-c-Jun (either at Ser63 or Ser73), cleaved caspase-3 and cleaved PARP was observed in SH/PKN cells, compared with SH cells (Fig. 2). When dopamine treatment was increased to 150 μM, levels of the active forms of these enzymes were still lower in SH/PKN cells than in SH cells (Fig. 2). These results suggest that parkin overexpression decreases the activation of the JNK/caspase pathway, which is critical for DA-induced apoptosis (8). They are consistent with the significant attenuation of apoptosis in SH/PKN cells, compared with SH cells (Fig. 1). We also examined the activation of another stress-activated MAP kinase, p38. The level of phospho-p38 was similar in both cell lines, when they were treated with 75 or 150 μM of dopamine (Fig. 2). It indicates that overexpression of parkin has no significant effect on dopamine-induced activation of p38 MAP kinase. Western blots of the same set of samples using antibodies that recognize total JNK, total c-Jun, uncleaved caspase-3 or total p38 showed that dopamine treatment did not affect expression levels of these proteins (Fig. 2).

The differential effects of parkin towards JNK and p38 activation, together with previous studies on the requirement of JNK in dopamine-induced apoptosis in other systems, suggest that the death of SH-SY5Y cells caused by dopamine may depend on the activation of JNK, rather than p38. To test this, we treated SH-SY5Y cells with or without selective inhibitors of JNK (SP600125) or p38 (SB203580) for 30 min at 10 μM, before a co-treatment with vehicle or 75 μM dopamine for 12 h. Co-application of the JNK inhibitor with dopamine induced apoptosis in $18.87 \pm 0.81\%$ of the cell, which was significantly lower than that caused by dopamine treatment alone ($28.64 \pm 2.1\%$, $P < 0.01$, $n = 4$ experiments). In contrast, the effect of the p38 inhibitor in combination with dopamine ($24.11 \pm 0.37\%$) was not significantly different from dopamine treatment alone ($P > 0.05$, $n = 4$). Without dopamine, both inhibitors had significant, but very mild toxicity on SH-SY5Y cells ($2.85 \pm 0.46\%$ for the JNK inhibitor and $2.35 \pm 0.51\%$ for the p38 inhibitor), in comparison with vehicle control ($0.99 \pm 0.02\%$). These data suggest that JNK, rather than p38, is specifically required for dopamine-induced apoptosis, although both stress-activated MAP kinases were activated by dopamine treatment.
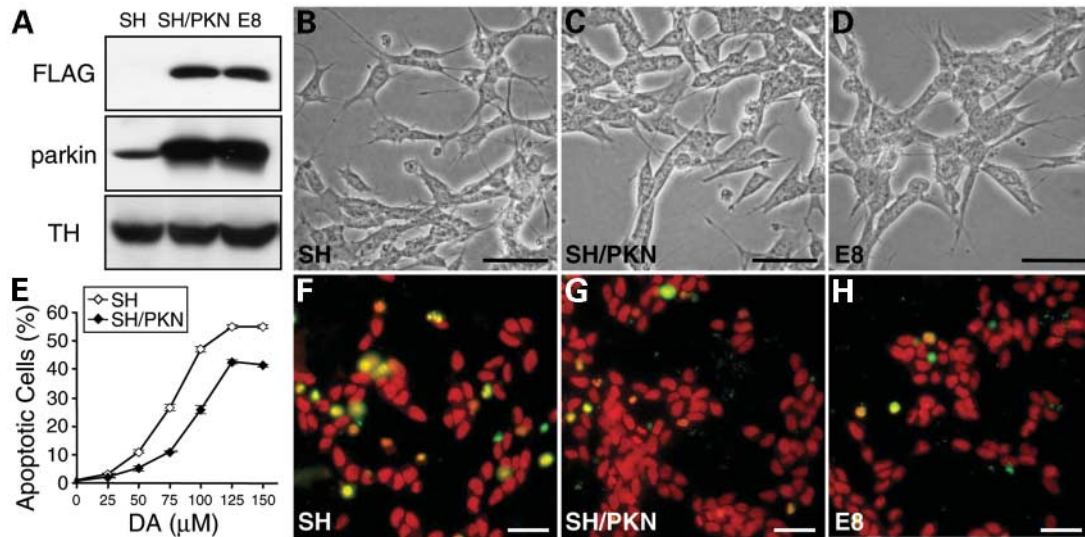
Figure 1. Overexpression of parkin in SH-SY5Y cells protects against dopamine-induced apoptosis. (**A**) Western blots of total cell lysates from SH-SY5Y cells (SH), pooled clones (SH/PKN) or an individual clone (E8) of SH-SY5Y cells stably expressing FLAG-tagged parkin, using antibodies against FLAG, parkin or tyrosine hydroxylase (TH). (**B**–**D**) Phase-contrast images of these stable cell lines showed that morphology was unaffected by exogenous parkin. (**E**) Dopamine (DA) treatment (12 h) of SH or SH/PKN cells caused apoptosis in a dose-dependent manner. Significant decrease of apoptosis was observed for cells overexpressing parkin when dopamine concentration was at or above 25 μM ($P < 0.05$, SH versus SH/PKN cells). The experiments were repeated three times, with two cover slips for each condition, and 600–800 nuclei were counted for each cover slip. (**F**–**H**) Representative images of cells treated with dopamine (75 μM for 12 h). After TUNEL staining (green), cells were counter-stained with the DNA-binding dye TO-PRO-3 (red). In some cells, the nucleus was so condensed that only TUNEL label was observed. Bars, 40 μm.

Parkin expression decreases ROS in the cell

As intracellular oxidation of dopamine produces ROS, a trigger for apoptosis, we would like to test whether parkin expression reduces the level of ROS. The membrane-permeable probe CM-H$_2$DCFDA [5(6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate, acetyl ester] was used to measure ROS produced in live cells. We treated SH-SY5Y cells or SH/PKN cells with or without dopamine (75 μM for 4 h) and stained them with CM-H$_2$DCFDA (1 μM for 15 min). Fluorescence images and corresponding DIC images were acquired and merged in Figure 3A–D. Without dopamine treatment, the amount of ROS was significantly less in SH/PKN cells (Fig. 3A versus Fig. 3C). After dopamine treatment, ROS production was increased in both cell lines, however, the amount of ROS was still much less when parkin was overexpressed (Fig. 3B versus Fig. 3D). As quantified in Figure 3E, a significant difference was found between SH and SH/PKN cells, without or with dopamine treatment ($P < 0.01$ in both cases). These results indicate that overexpression of parkin reduces ROS level in the cell.

Parkin attenuates dopamine-induced protein oxidation

ROS produced during dopamine oxidation modifies many cellular proteins by forming carbonyl groups (12). Thus, the level of protein carbonyls in the cell is indicative of the amount of ROS. To test whether parkin overexpression in SH-SY5Y cells reduces protein oxidation, we treated SH or SH/PKN cells with 75 or 150 μM of dopamine for 4 h and measured the protein carbonyls in total cell lysates. As shown in

Figure 4A, the amount of oxidized proteins was similar between the two cell lines in the absence of dopamine treatment. However, after the cells were treated with dopamine, much fewer protein carbonyls were formed in SH/PKN cells, compared with SH cells. The lack of signal from samples not derivatized with 2,4-dinitrophenylhydrazine (DNPH) confirmed that the 2,4-dinitrophenyl (DNP) antibody specifically recognized DNPH-derivatized proteins. Anti-actin immunoblot of the same set of samples was used as loading control to quantify protein carbonyls (Fig. 4B). The significant difference ($P < 0.01$) between the two cell lines at both DA concentrations suggests that oxidative stress is reduced by parkin overexpression in the cell.

Dopamine-induced apoptosis is dependent on oxidation of imported dopamine

To ascertain that dopamine-induced apoptosis in SH-SY5Y cells was indeed caused by ROS, we treated SH or SH/PKN cells with the antioxidant N-acetyl-L-cysteine (NAC, 10 mM), 30 min before dopamine treatment (75 μM for 12 h). Co-treatment of the antioxidant almost abolished dopamine-induced apoptosis in both SH cells and SH/PKN cells (Supplementary Material, Fig. S1A). The significant effect ($P < 0.001$) suggests that ROS is the major cause of dopamine-induced apoptosis. ROS is produced by oxidation of dopamine, which can be either taken up from the media through dopamine transporter (DAT) or synthesized by TH in the cell. To distinguish the two possibilities, we treated SH or SH/PKN cells with the DAT inhibitor GBR-12909 (10 μM) or the TH inhibitor α-MT (1 mM), 30 min before
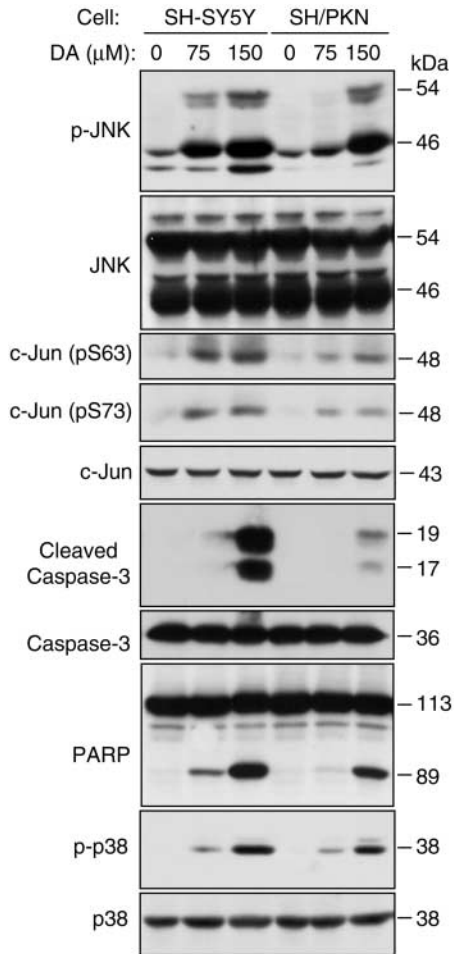
Figure 2. Parkin suppresses dopamine-activated apoptotic pathway. SH-SY5Y or SH/PKN cells were treated with different concentrations of dopamine for 12 h. Equal amounts of proteins (100 µg) from total cell lysates were analyzed by western blotting with antibodies against phospho-JNK, total JNK, phospho-c-Jun (Ser63), phospho-c-Jun (Ser73), c-Jun, cleaved caspase-3, total caspase-3, PARP, phospho-p38 and total p38, respectively. Both JNK and phospho-JNK antibodies recognized splicing isoforms of JNK1, 2, 3, which migrate around 54 or 46 kDa. Phosphorylation of c-Jun at either Ser63 or Ser73 causes a significant upward shift of its apparent molecular weight from 43 to 48 kDa. Cleaved caspase-3 had two major fragments, migrating at 19 and 17 kDa. Anti-PARP recognized both uncleaved (113 kDa) and cleaved (89 kDa) PARP. Similar results were obtained from at least three independent experiments.

Figure 3. Parkin expression reduces the level of ROS. Merged pictures of phase-contrast and fluorescence images taken from (**A** and **B**) SH-SY5Y or (**C** and **D**) SH/PKN cells treated (A and C) without or (B and D) with dopamine (75 µM for 4 h) and stained with CM-H_2DCFDA (1 µM for 15 min). ROS in the cell converted the non-fluorescent dye into fluorescein, which emitted green fluorescence. (**E**) Average fluorescence intensity was significantly reduced in SH/PKN cells, compared with SH cells, with or without dopamine treatment. *$P < 0.01$, SH versus SH/PKN cells. Images from six coverslips were analyzed for each condition. At least 100 cells were measured for each coverslip.

the addition of dopamine (75 µM for 12 h). DAT inhibitor significantly ($P < 0.05$) attenuated apoptosis in both SH and SH/PKN cells, whereas TH inhibitor only had a small and insignificant effect ($P > 0.05$, Supplementary Material, Fig. S1A). Treatments with any of these inhibitors alone did not have significant effect on cell viability (data not shown). Inhibiting dopamine synthesis by α-MT did not significantly reduce protein carbonyls in either cell lines. In contrast, blocking dopamine uptake from the media by GBR-12909 greatly decreased protein carbonyls in SH cells (Supplementary Material, Fig. S1B and C). These data are consistent with the significant protective role of the DAT, but not the TH inhibitor, against dopamine-induced apoptosis, and suggest
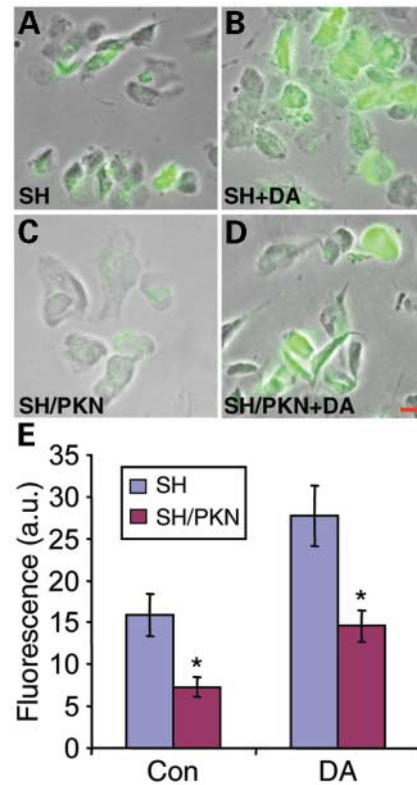
that dopamine taken up from the media, rather than that synthesized in the cell, is the source of toxicity. As DAT inhibition already reduced protein carbonyls to the basal level in the absence of exogenous dopamine, overexpression of parkin in SH/PKN cells did not cause any further decrease. On the other hand, co-application of the antioxidant NAC significantly reduced protein carbonyls in both cell lines (Supplementary Material, Fig. S1B and C). It suggests that ROS produced by dopamine oxidation is the direct killer.

Parkin protects against the toxicity of 6-OHDA, but not H_2O_2 or rotenone, on SH-SY5Y cells

As there was no significant difference in dopamine-induced apoptosis or protein carbonyls between SH and SH/PKN cells when DAT was inhibited, parkin appears to act on dopamine toxicity caused by DAT. If this is true, parkin should exhibit differential effects on various toxins. We treated SH or SH/PKN cells with two concentrations of 6-OHDA, H_2O_2 or rotenone for 12 h, and quantified apoptosis under these conditions. As shown in Figure 5, parkin overexpression significantly reduced apoptosis induced by 6-OHDA at both 10
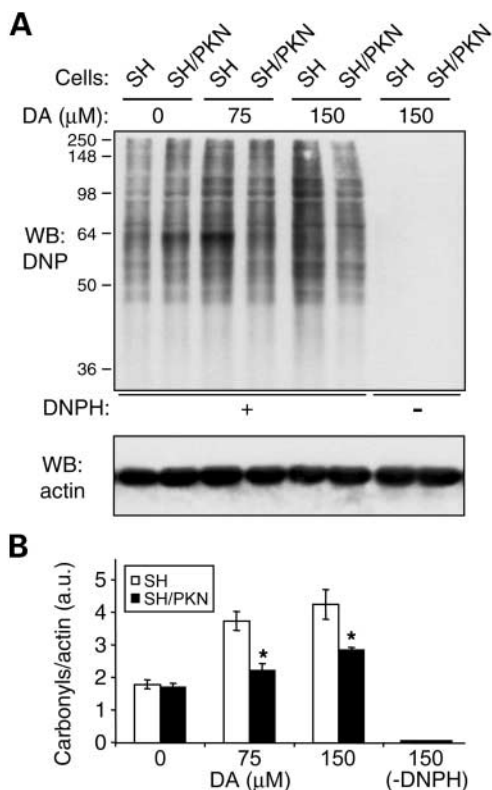
Figure 4. Parkin decreases protein oxidation caused by dopamine metabolism. (**A**) SH or SH/PKN cells were treated with different concentrations of dopamine for 4 h. Protein carbonyls in the total cell lysates were derivatized with or without DNPH, and analyzed by western blotting with anti-DNP. Actin immunoblot was used as loading controls. (**B**) Levels of protein carbonyls were quantified against the amount of actin in the same lysates from three separate experiments. $*P < 0.01$, SH/PKN versus SH cells at the corresponding DA concentration.

and 20 μM. In contrast, cell death caused by H_2O_2 or rotenone at two different doses was very similar between SH and SH/PKN cells. Thus, our studies suggest that in SH-SY5Y cells, parkin selectively protects against the toxicity of dopamine or its analog 6-OHDA, but not against the toxicity of general inducers of oxidative stress, such as H_2O_2 or rotenone.

Parkin clears ubiquitinated proteins caused by dopamine treatment

As parkin is a protein-ubiquitin E3 ligase (5), we tested the effect of parkin on dopamine-induced protein ubiquitination in SH-SY5Y cells. ROS produced during dopamine oxidation would oxidize many proteins and increase their ubiquitination. SH or SH/PKN cells were treated with various concentrations of dopamine for 12 h. Total cell lysates were analyzed by western blotting with anti-ubiquitin to examine the amount of ubiquitinated proteins in the cell. As shown in Figure 6A, dopamine treatment significantly increased protein ubiquitination in both SH and SH/PKN cells. However, such an increase was significantly attenuated by parkin overexpression (Fig. 6B, $P < 0.05$, SH/PKN versus SH at 75 or 150 μM dopamine). When the cells were treated with lactacystin (1 μM) and
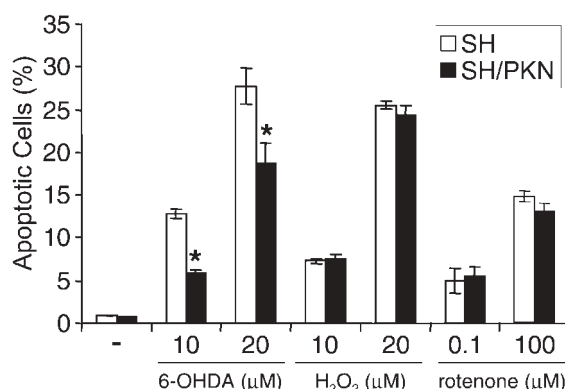

Figure 5. Parkin suppresses cell death induced by 6-OHDA, but not by H_2O_2 or by rotenone. SH or SH/PKN cells were treated with 6-OHDA, H_2O_2 or rotenone for 12 h at the indicated doses. The percentage of apoptotic cells was calculated for each condition by co-staining the sample with TUNEL and TO-PRO-3. $*P < 0.05$, versus SH cells under the same condition. At least five coverslips were examined for each condition, with at least 1000 cells counted for each coverslip.

various concentrations of dopamine together, there were significantly more ubiquitinated proteins in SH/PKN cells than in SH cells, at 0 or 75 μM of dopamine, but not when dopamine concentration was raised to 150 μM (Fig. 6B). These data showed that dopamine indeed increased protein ubiquitination, some of which may be ubiquitinated by parkin. They also suggest that, as an E3 ligase, parkin facilitates the degradation of its ubiquitinated substrates. Consequently, in the absence of proteasome inhibition, the steady-state concentration of ubiquitinated proteins was reduced by parkin overexpression. When proteasomes are inhibited, the E3 ligase activity of parkin is manifested by the accumulation of its ubiquitinated substrates. The combined treatment with 150 μM of dopamine and 1 μM of lactacystin (both for 12 h) produced significant cell death, which may explain the net reduction in ubiquitinated proteins in SH and SH/PKN cells, compared with the situation at 75 μM of dopamine (Fig. 6B).

PD-linked mutations abrogate the protective function of parkin and its ability to suppress oxidative stress

As parkin overexpression appears to accelerate the clearance of ubiquitinated proteins induced by dopamine treatment (Fig. 6), it suggests that the protective function of parkin against dopamine-induced cell death may be mediated by its E3 ligase activity. If such protection is critical to PD, then PD-causing mutations of parkin should abrogate the effect. To test this, we generated SH-SY5Y cell lines stably expressing K161N, T240R or R42P mutant parkin, as well as a control cell line stably expressing FLAG-luciferase. Expression levels of these constructs, in relation to that of endogenous parkin, were shown by western blotting with anti-FLAG and anti-parkin (Fig. 7A). K161N and T240R mutants, with reduced E3 ligase activity on several substrates (13,14), were expressed at levels similar to the FLAG-tagged wild-type parkin in SH/PKN cells, which were markedly higher than the amount of endogenous parkin. The expression level of
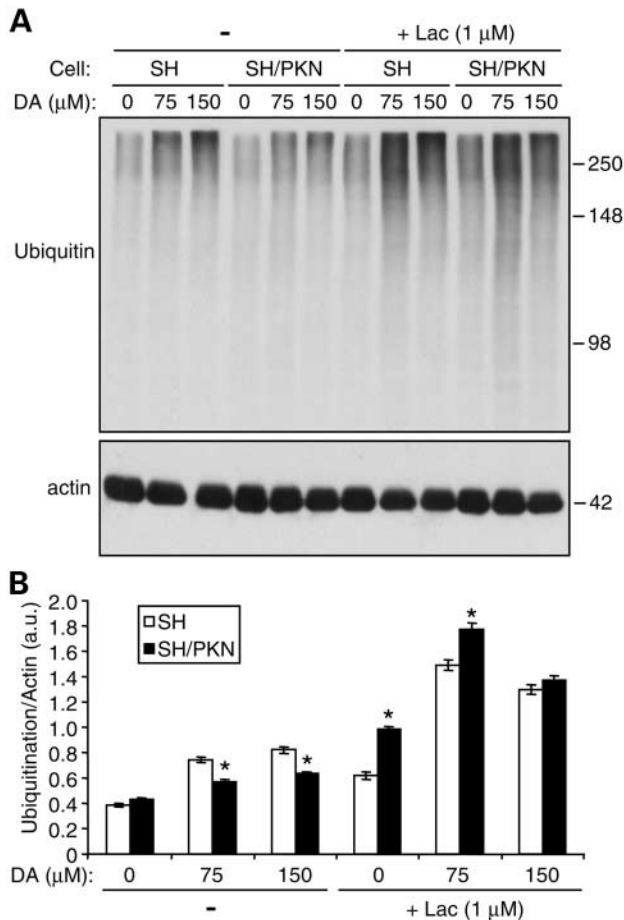
Figure 6. Dopamine-induced protein ubiquitination is attenuated by parkin overexpression. SH or SH/PKN cells were treated with the indicated concentrations of dopamine for 12 h in the absence or presence of 1 μM lactacystin. (**A**) Total cell lysates were analyzed by western blotting with anti-ubiquitin or anti-actin (loading control). (**B**) The amount of ubiquitinated proteins for each condition was quantified against actin, from three separate experiments. Parkin overexpression significantly reduced the level of protein ubiquitination elevated by dopamine treatment in the absence of lactacystin, as ubiquitinated proteins were quickly degraded. When 26S proteasomes were inhibited by lactacystin, increased protein ubiquitination caused by parkin was seen with 0 or 75 μM of dopamine treatments. The lack of further increase with 150 μM of dopamine treatment was probably due to significant cell death caused by lactacystin and high concentration of dopamine. *$P < 0.05$, versus SH cells under the same condition.

R42P mutant was significantly lower than those of the other two mutants or the wild-type. This is consistent with the critical role of the ubiquitin-like domain in the stability of parkin (15). Furthermore, the R42P mutant exhibits defective interaction with the Rpn10 subunit of the 26S proteasome (16).

As shown in Figure 7B, T240R and R42P mutants provided no protection against dopamine-induced apoptosis, just like the control cell line expressing luciferase and the parental SH-SY5Y cells. The K161N mutant exhibited moderate protection, which was significantly different from the parental cell line ($P < 0.05$). However, all three mutants, as well as the luciferase control cell line, were significantly different from the cell line expressing wild-type parkin ($P < 0.05$). These data showed
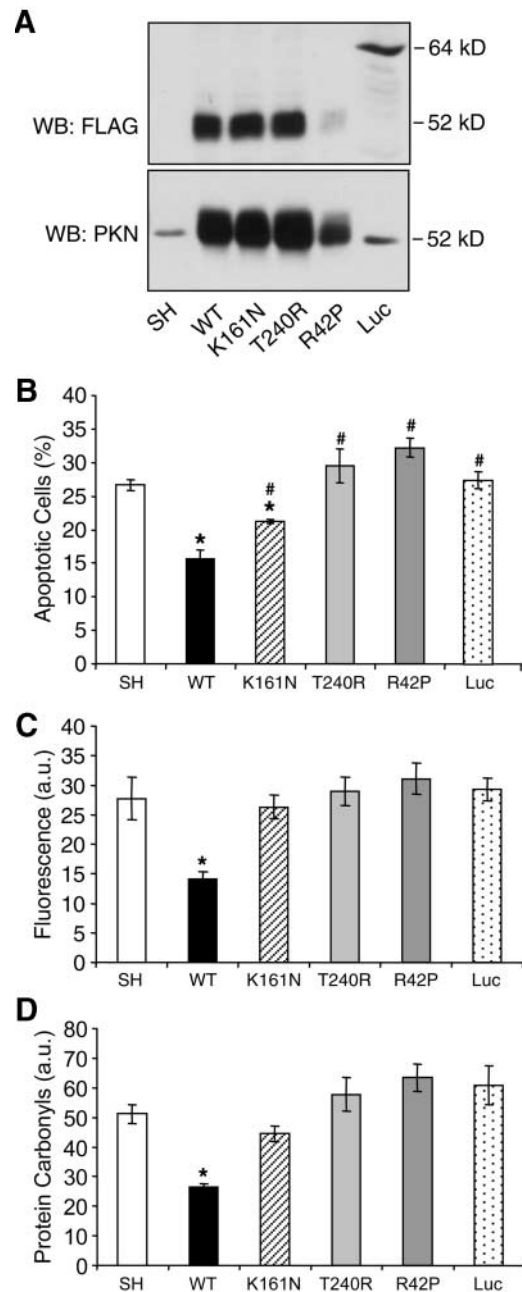

Figure 7. The protective role of parkin against dopamine toxicity is attenuated by mutations linked to PD. SH-SY5Y cell lines stably expressing FLAG-tagged wild-type (WT), mutant parkin (K161N, T240R, R42P), or luciferase were generated. (**A**) Total lysates from these cell lines were analyzed by western blotting with anti-FLAG or anti-parkin to compare the expression level of each construct against endogenous parkin. (**B**) They were treated with dopamine (75 μM for 12 h) in parallel with the parental SH-SY5Y cells (SH). Percentage of apoptotic cells was calculated and plotted. *$P < 0.05$, versus SH cells; #$P < 0.05$, versus WT cells. Experiments were repeated three times, with two coverslips for each cell line, and 600–800 nuclei were counted for each coverslip. (**C**) After the cell lines were treated with dopamine (75 μM for 4 h), they were stained with CM-H$_2$DCFDA (1 μM for 15 min). Average fluorescence intensity was measured to quantify ROS in the cell. *$P < 0.01$, versus SH cells. Three independent experiments were performed with two coverslips for each cell line, and at least 100 cells were measured for each coverslip. (**D**) The amount of protein carbonyls was measured from total cell lysates by dot blot after the cell lines were treated with 75 μM of DA for 4 h. *$P < 0.01$, versus SH cells, from three separate experiments.

that PD-linked mutations of parkin significantly attenuated its ability to protect against dopamine toxicity.

To investigate the impact of parkin mutations on oxidative stress in the cell, we measured the level of ROS using CM-H$_2$DCFDA and the amount of protein carbonyls using the Oxyblot method. As shown in Figure 7C, only wild-type parkin was able to significantly reduce ROS in the cell. Compared with the parental SH-SY5Y cells, none of the parkin mutant cell lines, or the luciferase control, exhibited a significant difference in cellular ROS level. Consistently, the amount of protein carbonyls was significantly reduced by overexpression of wild-type, but not mutant, parkin (Fig. 7D). The luciferase control cell line behaved in the same way as the parental SH-SY5Y cells in all three parameters (Fig. 7B–D), indicating that the procedure of generating a stable cell line *per se* did not produce any significant effect. Taken together, these data suggest that parkin protects against dopamine toxicity by reducing oxidative stress, which is a critical factor in dopamine-induced apoptosis (7,8).

DISCUSSION

The most prevalent and unifying feature of PD is the selective degeneration of nigral DA neurons. Although neurodegeneration also occurs in other regions of the brain in certain PD cases, the loss of DA neurons in SNpc is far more pervasive and appears to contribute most significantly to symptoms associated with PD (17). The obligatory role of nigral DA neurons in PD is further substantiated by dopamine replacement therapy, which is currently the most effective treatment of the disease (18); as well as lesion studies in animal models using 6-hydroxydopamine (6-OHDA) (19), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (20) or rotenone (21). However, it is still unclear what causes the selective degeneration of nigral DA neurons in PD. Among the many etiological factors implicated in PD, mutations in parkin clearly cause degeneration of DA neurons and associated PD symptoms (22). Yet it is puzzling how loss of parkin, which is expressed in many tissues, results in such a specific phenotype.

One possibility is that parkin has a unique function in the survival of DA neurons. Our data showed that overexpression of parkin in human DA neuroblastoma cells slowed down, but not entirely prevented dopamine-induced apoptosis. In addition, dopamine-activated apoptotic pathways were significantly attenuated by parkin overexpression. Previous studies have demonstrated that dopamine-induced apoptosis is dependent on ROS (7), which are produced in dopamine oxidation. Through an unknown mechanism, ROS, like many stress stimuli, activates the JNK family of MAP kinases, which play a critical role in dopamine-induced apoptosis (8). The specific requirement of JNK, rather than p38, in dopamine-induced apoptosis is further substantiated by our studies using selective inhibitors of JNK or p38. JNK activation triggers mitochondrial release of cytochrome *c* (9), which, in combination with Apaf-1, cleaves and activates the initiator caspase, capase-9 (23). Activated caspase-9 cleaves and activates effector caspases including caspase-3, which execute the apoptotic program (24). Indeed, the cleavage and activation of PARP, a well-established caspase-3 substrate

required for MPTP-induced apoptosis of DA neurons (25), was significantly reduced by parkin overexpression. Obviously, ROS produced during dopamine oxidation may activate other pro-apoptotic pathways besides the JNK/caspase cascade. This is evidenced by the less pronounced effect of the JNK inhibitor in comparison with that of parkin. With 75 μM of dopamine treatment (12 h), JNK inhibitor reduced apoptosis from $28.64 \pm 2.10\%$ to $18.87 \pm 0.81\%$ (9.7% reduction). In contrast, parkin overexpression decreased cell death from $26.65 \pm 0.95\%$ to $11.10 \pm 0.20\%$ (15.6% reduction). This difference suggests that either JNK activation alone does not fully account for dopamine toxicity or parkin may impact on other pathways in addition to JNK to reduce dopamine-induced apoptosis.

The protective effect of parkin appears to be afforded by its ability to suppress oxidative stress. Overexpression of parkin in SH-SY5Y cells significantly reduced intracellular level of oxyradicals, as determined by the ROS probe CM-H$_2$DCFDA (Fig. 3), and the level of protein carbonyls (Fig. 4). Application of antioxidants such as NAC greatly diminished toxicity and protein carbonyls induced by dopamine (Supplementary Material, Fig. S1), suggesting that ROS produced during dopamine oxidation is indeed the direct killer. Furthermore, parkin mutants that did not protect against dopamine toxicity also did not reduce ROS levels or protein carbonyls in the cell (Fig. 7). As these mutations cause PD, our results suggest that the ability of wild-type parkin to protect against dopamine toxicity may be critically involved in the survival of DA neurons. It may also provide a clue to the specificity issue. As the oxidation of dopamine produced in DA neurons generates ROS that could potentially kill the cells, these neurons would heavily rely on mechanisms that control intracellular level of ROS in order to survive. Thus, when parkin is mutated, it may no longer suppress ROS production in DA neurons, which would lead to their selective degeneration.

As parkin mutants used in the study exhibit either no significant E3 ligase activity towards known substrates (13,14,26,27), or defective interaction with the 26S proteasome (16), it appears that the protective function of parkin against dopamine toxicity is dependent on its E3 ligase activity or its interaction with proteasome. Among the many substrates of parkin identified so far, none appears to be directly related to oxidative stress in DA neurons (6). It seems likely that unknown substrate(s) involved in unique physiological processes of DA neurons may underlie the protective effect of parkin against dopamine-induced apoptosis. The mildly protective effect of K161N mutant (Fig. 7B) may be due to a partial loss of E3 ligase activity of this mutant towards such substrate(s). It has been shown that the K161N mutation dose not significantly affect the E3 ligase activity of parkin on the p38 subunit of the amino-acyl tRNA synthetase complex (28). Nevertheless, the significant difference between wild-type parkin and K161N mutant (Fig. 7B) showed that only wild-type parkin could provide full protection. The variable effects of parkin mutations in this regard may explain the different severity of clinical symptoms seen in PD patients with various mutations of the parkin gene (29).

Consistent with a role of parkin in suppressing dopamine toxicity and oxidative stress, it has been found that

3,4-dihydroxyphenylacetic acid (DOPAC) level is significantly increased in parkin-deficient mice (30). As DOPAC is produced along with H_2O_2 in the enzymatic oxidation of dopamine, the rise in DOPAC level suggests that ROS production may be increased in these parkin mutant mice. On the other hand, overexpression of parkin in NT-2 or SK-N-MC cells significantly decreases basal levels of protein carbonyls and 3-nitrotyrosine; whereas expression of several mutants, including T240R, increases these markers of oxidative stress (31). These results are in agreement with our findings that ROS and protein carbonyls were reduced by wild-type, but not mutant, parkin in SH-SY5Y cells (Fig. 7). The protective role of parkin against DA toxicity in DA neuroblastoma cells is consistent with the selective protection of midbrain catecholaminergic neurons by parkin against proteasomal inhibitors or mutant α-synuclein (32).

A recent study using SH-SY5Y cells shows that stable expression of parkin does not attenuate cell death induced by 125 μM of dopamine treatment for 24 h (33), which is different from our results. The discrepancy may be due to the difference in SH-SY5Y cells and the way they were maintained. Our cells appear to be more sensitive to dopamine. In the presence of 125 μM of dopamine, most of the cells were killed and floated up around 20 h (data not shown). In addition, the media turned black around 12 h, because of dopamine oxidation by the air. However, if our SH-SY5Y cells were not properly maintained, they very easily lost their normal morphology (with long, fine, neurite-like processes shown in Fig. 1B–D) and became quite resistant to dopamine. At this condition, there was no significant difference in dopamine-induced apoptosis between SH and SH/PKN cells.

The lack of protection by parkin against H_2O_2 or rotenone (Fig. 5) is consistent with similar studies done in PC12 cells (34). However, in our SH-SY5Y cells, parkin selectively attenuated the toxicity of 6-OHDA (10 or 20 μM for 12 h), which is not observed in PC12 cells treated with 100 μM of 6-OHDA for 24 h (34). This may be due to different cell types and the relative sensitivity of the two cell lines towards 6-OHDA. Our results on dopamine and 6-OHDA are consistent with each other and suggest that the protective role of parkin is selective on DA drugs.

In summary, our studies have provided evidence that parkin protects against dopamine-induced apoptosis in SH-SY5Y cells by decreasing ROS produced during dopamine metabolism. This protective function of parkin is dependent on its E3 ligase activity or its interaction with the 26S proteasome. Further investigations are necessary to identify parkin substrate(s) involved in the process. As intracellular oxidation of dopamine plays a critical role in the degeneration of DA neurons (12), our studies using DA neuroblastoma cells strongly suggest that parkin may impact on specific targets in DA cells.

MATERIALS AND METHODS

Generation of stable cell lines

SH-SY5Y cells were purchased from ATCC (Manassas, VA). As undifferentiated SH-SY5Y cells were very difficult to transfect with high efficiency, we generated stable cell lines by transfecting linearized constructs with LipofectAmine 2000 (Invitrogen, Carlsbad, CA), and selecting for positive clones with limited dilution in the presence of 600 μg/ml of Geneticin (Invitrogen), which completely killed untransfected cells. Individual clones were confirmed by western blot analysis with anti-FLAG (Sigma, St Louis, MO) and anti-parkin (13). Wild-type and mutant human parkin cDNAs (13) were subcloned into pCMV-Tag2B (Stratagene, La Jolla, CA), which contains the FLAG-tag. A control cell line was generated by transfecting pCMV-Tag2B/Luciferase (a control plasmid from Stratagene) into SH-SY5Y cells and selecting in the same manner by anti-FLAG immunoblots.

All cell lines were maintained in DMEM with 10% fetal calf serum, 100 μg/ml penicillin and 100 μg/ml streptomycin (Invitrogen). They exhibited similar growth rates and morphological features. Great care was taken in maintaining the cells in an undifferentiated state. Otherwise, they very easily lost their normal morphology (with long, fine, neurite-like processes shown in Fig. 1) and became more resistant to dopamine toxicity. This occurred quite readily when the cells reach 80–90% confluency just once during subcultures, or after the cells were maintained for more than 2 months. At such conditions, there was no significant difference with regard to dopamine toxicity between SH and SH/PKN cells.

Detection of apoptosis

Cells were plated on coverslips coated with 0.01% poly-D-lysine and collagen at a density of 5×10^4 cells/cm^2 in 12-well plates. After being treated with various concentrations of dopamine for different durations, they were washed with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde. TUNEL staining was performed according to the manufacturer's protocol (Roche, Indianapolis, IN). Nuclei were stained with the DNA-binding dye To-Pro-3 (Molecular Probes, Eugene, OR) as described before (35). DNA stained with To-Pro-3 (1 μM for 5 min) emits fluorescence in the Cy5 channel, which is well-separated from FITC fluorescence used for TUNEL staining. Images were acquired under a Nikon fluorescence microscope with a CCD camera (Diagnostic Instrument, Sterling Heights, MI), and merged using the SPOT software (Diagnostic Instrument). To test the effects of various inhibitors on dopamine-induced apoptosis, cells were pre-treated with the inhibitors for 30 min and then 75 μM of dopamine was added to the culture media for 12 h. DAT inhibitor GBR-12909, antioxidant NAC and TH inhibitor α-MT were purchased from Sigma. JNK inhibitor SP600125 was from Calbiochem (La Jolla, CA). SB203580, a selective inhibitor of p38, was purchased from AG Scientific (San Diego, CA).

Dopamine was freshly prepared every time in boiled water to minimize its oxidation in the air. A 12 h treatment regiment was used because media containing more than 100 μM of DA turned black around that time, probably due to dopamine oxidation by the air. In addition, most of the cells were killed and floated up around 20 h in the presence of 100 μM or higher DA. Once dopamine is oxidized to dopamine-quinones and their adducts, which are dark-colored, it is hard to say whether the toxicity is due to dopamine *per se* or due to its auto-oxidation products. To maintain consistency, treatment

with all toxins (DA, 6-OHDA, H$_2$O$_2$ and rotenone) was performed for 12 h.

Western blot analysis

Cells were treated with various concentrations of dopamine for 12 h with or without lactacystin (1 μM), washed three times in cold PBS buffer and lysed in cold lysis buffer (1% Triton X-100, 10 mM Tris pH 7.6, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 mM EDTA and 0.1 mM Na$_3$VO$_4$) for 20 min. Lysates were centrifuged at 4°C at 16 000g. Supernatant fractions containing equal amounts of total proteins were separated on sodium dodecyl sulfate (SDS)–polyacrylamide gels and analyzed by western blotting with antibodies against JNK, phospho-JNK (Thr183/Tyr185), phospho-c-Jun (Ser63), phospho-c-Jun (Ser73), caspase-3, cleaved caspase-3 (Asp175), p38 MAP kinase, phospho-p38 MAP kinase (Thr180/Tyr182) (all from Cell Signaling Technology, Beverly, MA), c-Jun, ubiquitin, actin (all from Santa Cruz Biotechnology, Santa Cruz, CA) or PARP (from Roche). ECL detection was performed according to the manufacturer's protocol (Amersham, Piscataway, NJ).

Detection of ROS

Cells treated with or without dopamine (75 μM for 4 h) were incubated at 37°C for 15 min with 1 μM of CM-H$_2$DCFDA (Molecular Probes). Once the compound enters the cell, it is cleaved by intracellular esterases and is retained inside the cell due to the loss of its hydrophobic moieties. Upon oxidation by ROS, its fluorescein moiety is unmasked and can be detected by fluorescence microscopy. The amount of fluorescence emitted correlates with the quantity of ROS in the cell. This method has been widely used to measure ROS in living cells (36). After being washed in cold PBS for three times, cells were imaged under a Nikon fluorescence microscope with a CCD camera. Fluorescence images were acquired with a single 300 ms exposure, followed by acquisition of a DIC image of the same field. Un-retouched images were merged using the SPOT software (Diagnostic Instrument). Fluorescence intensity for each cell was measured in a 60 × 60 pixel box by using NIH Image J. Values from at least 100 cells were averaged for each coverslip.

Measurement of protein carbonyls

Cells were treated with various concentrations of dopamine with or without inhibitors for 4 h, washed three times in PBS and lysed in cold lysis buffer. Protein carbonyls were measured with the Oxyblot protein oxidation detection kit (Chemicon, Temecula, CA). Briefly, 15–20 μg of proteins from total cell lysates were incubated with an equal volume of 12% SDS and 2 vol of DNPH solution for 15 min at room temperature, and then with 1.5 vol of neutralization solution to stop the reaction. The samples were either separated on SDS-polyacrylamide gel or spotted on a nitrocellulose membrane, dried and crosslinked with a UV Crosslinker (Stratagene, La Jolla, CA). Membranes were western blotted with an antibody against DNP to detect protein carbonyls that are derivatized by DNPH.

REFERENCES

1. Langston, J.W. (2002) Parkinson's disease: current and future challenges. *Neurotoxicology*, **23**, 443–450.
2. Dekker, M.C., Bonifati, V. and van Duijn, C.M. (2003) Parkinson's disease: piecing together a genetic jigsaw. *Brain*, **126**, 1722–1733.
3. Oliveira, S.A., Scott, W.K., Martin, E.R., Nance, M.A., Watts, R.L., Hubble, J.P., Koller, W.C., Pahwa, R., Stern, M.B., Hiner, B.C. *et al.* (2003) Parkin mutations and susceptibility alleles in late-onset Parkinson's disease. *Ann. Neurol.*, **53**, 624–629.
4. Scott, W.K., Nance, M.A., Watts, R.L., Hubble, J.P., Koller, W.C., Lyons, K., Pahwa, R., Stern, M.B., Colcher, A., Hiner, B.C. *et al.* (2001) Complete genomic screen in Parkinson disease: evidence for multiple genes. *J. Am. Med. Assoc.*, **286**, 2239–2244.
5. Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K. *et al.* (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat. Genet.*, **25**, 302–305.
6. Feng, J. (2003) Genetic factors in Parkinson's disease and potential therapeutic targets. *Curr. Neuropharm.*, **1**, 301–313.
7. Hastings, T.G., Lewis, D.A. and Zigmond, M.J. (1996) Role of oxidation in the neurotoxic effects of intrastriatal dopamine injections. *Proc. Natl Acad. Sci. USA*, **93**, 1956–1961.
8. Luo, Y.Q., Umegaki, H., Wang, X.T., Abe, R. and Roth, G.S. (1998) Dopamine induces apoptosis through an oxidation-involved SAPK/JNK activation pathway. *J. Biol. Chem.*, **273**, 3756–3764.
9. Tournier, C., Hess, P., Yang, D.D., Xu, J., Turner, T.K., Nimnual, A., Bar-Sagi, D., Jones, S.N., Flavell, R.A. and Davis, R.J. (2000) Requirement of JNK for stress-induced activation of the cytochrome *c*-mediated death pathway. *Science*, **288**, 870–874.
10. Budihardjo, I., Oliver, H., Lutter, M., Luo, X. and Wang, X. (1999) Biochemical pathways of caspase activation during apoptosis. *Annu. Rev. Cell Dev. Biol.*, **15**, 269–290.
11. Wang, H., Shimoji, M., Yu, S.W., Dawson, T.M. and Dawson, V.L. (2003) Apoptosis inducing factor and PARP-mediated injury in the MPTP mouse model of Parkinson's disease. *Ann. NY Acad. Sci.*, **991**, 132–139.
12. Jenner, P. and Olanow, C.W. (1996) Oxidative stress and the pathogenesis of Parkinson's disease. *Neurology*, **47**, S161–S170.
13. Ren, Y., Zhao, J.H. and Feng, J. (2003) Parkin binds to alpha/beta tubulin and increases their ubiquitination and degradation. *J. Neurosci.*, **23**, 3316–3324.
14. Shimura, H., Schlossmacher, M.G., Hattori, N., Frosch, M.P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K.S. and Selkoe, D.J. (2001) Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. *Science*, **293**, 263–269.
15. Finney, N., Walther, F., Mantel, P.Y., Stauffer, D., Rovelli, G. and Dev, K.K. (2003) The cellular protein level of parkin is regulated by its ubiquitin-like domain. *J. Biol. Chem.*, **278**, 16054–16058.
16. Sakata, E., Yamaguchi, Y., Kurimoto, E., Kikuchi, J., Yokoyama, S., Yamada, S., Kawahara, H., Yokosawa, H., Hattori, N., Mizuno, Y. *et al.* (2003) Parkin binds the Rpn10 subunit of 26S proteasomes through its ubiquitin-like domain. *EMBO Rep.*, **4**, 301–306.
17. Poewe, W.H. and Wenning, G.K. (1998) The natural history of Parkinson's disease. *Ann. Neurol.*, **44**, S1–S9.
18. Carlsson, A. (2001) A paradigm shift in brain research. *Science*, **294**, 1021–1024.

19. Jeon, B.S., Jacksonlewis, V. and Burke, R.E. (1995) 6-Hydroxydopamine lesion of the rat substantia-nigra—time-course and morphology of cell-death. *Neurodegeneration*, **4**, 131–137.

20. Langston, J.W., Ballard, P., Tetrud, J.W. and Irwin, I. (1983) Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science*, **219**, 979–980.

21. Betarbet, R., Sherer, T.B., MacKenzie, G., Garcia-Osuna, M., Panov, A.V. and Greenamyre, J.T. (2000) Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat. Neurosci.*, **3**, 1301–1306.

22. Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y. and Shimizu, N. (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*, **392**, 605–608.

23. Hakem, R., Hakem, A., Duncan, G.S., Henderson, J.T., Woo, M., Soengas, M.S., Elia, A., de la Pompa, J.L., Kagi, D., Khoo, W. *et al.* (1998) Differential requirement for caspase 9 in apoptotic pathways *in vivo. Cell*, **94**, 339–352.

24. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*, **91**, 479–489.

25. Mandir, A.S., Przedborski, S., Jackson-Lewis, V., Wang, Z.Q., Simbulan-Rosenthal, C.M., Smulson, M.E., Hoffman, B.E., Guastella, D.B., Dawson, V.L. and Dawson, T.M. (1999) Poly(ADP-ribose) polymerase activation mediates 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism. *Proc. Natl Acad. Sci. USA*, **96**, 5774–5779.

26. Zhang, Y., Gao, J., Chung, K.K., Huang, H., Dawson, V.L. and Dawson, T.M. (2000) Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. *Proc. Natl Acad. Sci. USA*, **97**, 13354–13359.

27. Chung, K.K., Zhang, Y., Lim, K.L., Tanaka, Y., Huang, H., Gao, J., Ross, C.A., Dawson, V.L. and Dawson, T.M. (2001) Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. *Nat. Med.*, **7**, 1144–1150.

28. Corti, O., Hampe, C., Koutnikova, H., Darios, F., Jacquier, S., Prigent, A., Robinson, J.C., Pradier, L., Ruberg, M., Mirande, M. *et al.* (2003) The p38 subunit of the aminoacyl-tRNA synthetase complex is a Parkin substrate: linking protein biosynthesis and neurodegeneration. *Hum. Mol. Genet.*, **12**, 1427–1437.

29. Khan, N.L., Graham, E., Critchley, P., Schrag, A.E., Wood, N.W., Lees, A.J., Bhatia, K.P. and Quinn, N. (2003) Parkin disease: a phenotypic study of a large case series. *Brain*, **126**, 1279–1292.

30. Itier, J.M., Ibanez, P., Mena, M.A., Abbas, N., Cohen-Salmon, C., Bohme, G.A., Laville, M., Pratt, J., Corti, O., Pradier, L. *et al.* (2003) Parkin gene inactivation alters behaviour and dopamine neurotransmission in the mouse. *Hum. Mol. Genet.*, **12**, 2277–2291.

31. Hyun, D.H., Lee, M., Hattori, N., Kubo, S., Mizuno, Y., Halliwell, B. and Jenner, P. (2002) Effect of wild-type or mutant Parkin on oxidative damage, nitric oxide, antioxidant defenses, and the proteasome. *J. Biol. Chem.*, **277**, 28572–28577.

32. Petrucelli, L., O'Farrell, C., Lockhart, P.J., Baptista, M., Kehoe, K., Vink, L., Choi, P., Wolozin, B., Farrer, M., Hardy, J. *et al.* (2002) Parkin protects against the toxicity associated with mutant alpha-synuclein: proteasome dysfunction selectively affects catecholaminergic neurons. *Neuron*, **36**, 1007–1019.

33. Muqit, M.M., Davidson, S.M., Payne, S., MacCormac, L.P., Kahns, S., Jensen, P.H., Wood, N.W. and Latchman, D.S. (2004) Parkin is recruited into aggresomes in a stress-specific manner: over-expression of parkin reduces aggresome formation but can be dissociated from parkin's effect on neuronal survival. *Hum. Mol. Genet.*, **13**, 117–135.

34. Darios, F., Corti, O., Lucking, C.B., Hampe, C., Muriel, M.P., Abbas, N., Gu, W.J., Hirsch, E.C., Rooney, T., Ruberg, M. *et al.* (2003) Parkin prevents mitochondrial swelling and cytochrome *c* release in mitochondria-dependent cell death. *Hum. Mol. Genet.*, **12**, 517–526.

35. Zhao, J., Ren, Y., Jiang, Q. and Feng, J. (2003) Parkin is recruited to the centrosome in response to inhibition of proteasomes. *J. Cell Sci.*, **116**, 4011–4019.

36. Larsen, K.E., Fon, E.A., Hastings, T.G., Edwards, R.H. and Sulzer, D. (2002) Methamphetamine-induced degeneration of dopaminergic neurons involves autophagy and upregulation of dopamine synthesis. *J. Neurosci.*, **22**, 8951–8960.

SUPPLEMENTARY MATERIAL

Figure lengend

Figure S1. The effect of dopamine transporter inhibitor, tyrosine hydroxylase inhibitor or antioxidant on dopamine-induced apoptosis and oxidative stress. (**A**) SH-SY5Y or SH/PKN cells were treated with DA alone (75 μM, 12 hr) or together with the DAT inhibitor GBR-12909 (10μM), the TH inhibitor α-MT (1mM), or the antioxidant NAC (10 mM). Treatment with inhibitors started 30 min before the addition of DA and lasted 12.5 hr. Percentage of apoptotic cells was plotted for each treatment. DAT inhibitor or antioxidant significantly reduced apoptosis in both cell lines (*****, $p < 0.05$, DA+GBR vs. DA alone; #, $p < 0.001$, DA+NAC vs. DA alone). However, TH inhibitor did not provide significant protection in either cell line ($p > 0.05$). The experiments were repeated three times, with two coverslips for each condition, and 600-800 nuclei were counted for each coverslip. (**B-C**) Cells treated without or with 75 μM of dopamine for 4 hr in the absence or presence of various inhibitors were assayed for the amount of protein carbonyls by the dot blot method (B). Quantification of results from three independent experiments showed that protein carbonyls elevated by dopamine in SH-SY5Y cells were significantly reduced by the DAT inhibitor or the antioxidant. In SH/PKN cells, dopamine-induced protein carbonyls were already suppressed by parkin overexpression and could be attenuated further only by the antioxidant (C). *****, $p < 0.05$, vs. dopamine alone in SH or SH/PKN cells, respectively.