Journal of Neurochemistry, 2007, **103**, 303–311 doi:10.1111/j.1471-4159.2007.04741.x

Rotenone selectively kills serotonergic neurons through a microtubule-dependent mechanism

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Abstract

As a major co-morbidity of Parkinson's disease (PD), depression is associated with the loss of serotonergic neurons. Our recent study has shown that midbrain dopaminergic neurons are particularly vulnerable to microtubule-depolymerizing agents including rotenone, an environmental toxin linked to PD. Here we show that rotenone also selectively killed serotonergic neurons in midbrain neuronal cultures. Its selective toxicity was significantly decreased by the microtubule-stabilizing drug taxol and mimicked by microtubule-depolymerizing agents such as colchicine and nocodazole. Microtubule depolymerization induced by rotenone or colchicine caused vesicle accumulation in the soma and killed serotonergic neurons through a mechanism dependent on serotonin metabolism in the cytosol. Blocking serotonin synthesis or degradation, as well as application of antioxidants, significantly reduced the selective toxicity of rotenone or colchicine. Inhibition of vesicular sequestration of serotonin exerted a selective toxicity on serotonergic neurons that was mitigated by blocking serotonin metabolism. Over-expression of parkin, a protein-ubiquitin E3 ligase that strongly binds to microtubules, greatly attenuated the selective toxicity of rotenone or colchicine. The protective effects of parkin were abrogated by its PD-linked mutations. Together, our results suggest that rotenone and parkin affect the survival of serotonergic neurons by impacting on microtubules in opposing manners.

Keywords: depression, microtubules, parkin, Parkinson's disease, rotenone, serotonergic neuron.
J. Neurochem. (2007) **103**, 303–311.

Depression is one of the most common comorbid symptoms of Parkinson's disease (PD), occurring in 40–50% of patients with PD (Dooneief *et al.* 1992). Although some studies suggest that depression in PD may be a reactive and secondary response to the psychological and social stress of an incurable, debilitating disease, many different lines of evidence converge on the notion that comorbid depression stems from neurodegeneration associated with PD (McDonald *et al.* 2003). The 'reactive hypothesis' has difficulty in explaining the much higher incidence of depression in PD compared with other serious chronic diseases (Ehmann *et al.* 1990). There is also no significant correlation between the severity of depression and the degree of disability caused by PD (Menza and Mark 1994). In contrast, post-mortem and imaging studies have shown that PD patients with depression have smaller subcortical nuclei than PD patients without depressive symptoms (Lisanby *et al.* 1993) and the decrease is similarly seen in depression patients without PD (McDonald and Krishnan 1992). In addition to the degeneration of dopaminergic (DA) neurons in substantia nigra, many other subcortical neuronal populations also exhibit variable degrees of loss. In particular, a significant decrease of serotonergic neurons in the dorsal raphe nucleus is found in PD patients with depression (Paulus and Jellinger 1991). Decreased levels of the serotonin metabolite 5-hydroxyindoleacetic acid in the cerebrospinal fluid is associated with comorbid depression in PD and are reversely correlated with the severity of depression symptoms

Received February 7, 2007; revised manuscript received May 15, 2007; accepted May 15, 2007.

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Abbreviations used: DA, dopaminergic; GAT1, GABA transporter-1; PD, Parkinson's disease; TH, tyrosine hydroxylase; TMR, tetramethylrhodamine; TpH, tryptophan hydroxylase; vGlut2, vesicular glutamate transporter 2; VMAT2, vesicular monoamine transporter 2.

(Mayeux *et al.* 1984). Furthermore, a polymorphism in the promoter region of the serotonin transporter gene has been linked to depression in PD (Mossner *et al.* 2001). Thus, degeneration of serotonergic neurons may be a significant etiological factor for comorbid depression symptoms of PD.

On the other hand, many epidemiological studies have implicated exposure to agricultural pesticides as a significant risk factor for PD (Langston 2002). Long-term, systemic administration of rotenone, a widely-used pesticide, produces degeneration of nigral DA neurons and PD-like locomotor symptoms in animal models (Betarbet *et al.* 2000). Rotenone has two sites of action in the cell; it inhibits complex I in the mitochondrial respiratory chain (Chance *et al.* 1963) and depolymerizes microtubules (Brinkley *et al.* 1974; Marshall and Himes 1978). Our recent study has shown that the microtubule-depolymerizing activity of rotenone plays a significant role in determining its selective toxicity on DA neurons (Ren *et al.* 2005). Microtubule depolymerization induced by rotenone or other drugs such as colchicine or nocodazole disrupts vesicular transport and leads to accumulation of vesicle in the soma. Increased oxidation of dopamine leaked from vesicles to the cytosol jeopardizes the survival of DA neurons. Glutamatergic and GABAergic neurons in the same culture are spared because their neurotransmitters cannot be oxidized, even though microtubule depolymerization induces a similar level of vesicle accumulation (Ren *et al.* 2005). The results predict that all monoaminergic neurons with long projection are much more vulnerable to microtubule depolymerization than non-monoaminergic neurons. Our previous study has examined tyrosine hydroxylase-positive (TH^+) neurons in midbrain neuronal cultures, which include DA, epinephrinergic and norepinephrinergic neurons. Here, we expand the study to serotonergic neurons to test the validity of our model.

Materials and methods

Antibodies, reagents, and constructs

Sheep anti-tryptophan hydroxylase (TpH), Mouse anti-NeuN or anti-glutamic acid decarboxylase, guinea pig anti-vesicular glutamate transporter 2 (vGlut2), and rabbit anti-GABA transporter 1 (GAT1) were from Chemicon (Temecula, CA, USA). Mouse antibodies against FLAG or synaptophysin, as well as FITC- or rhodamine-conjugated secondary antibodies against mouse, rabbit or sheep were from Sigma (St. Louis, MO, USA). Cy5-conjugated anti-mouse antibody was from GE Bioscience (Piscataway, NJ, USA). Alexa Fluor 488-conjugated anti-guinea pig IgG was from Molecular Probes (Eugene, OR, USA). Apoptosis was detected by TUNEL staining using a kit from Roche (Indianapolis, IN, USA). In some experiments, tetramethylrhodamine (TMR)-conjugated dUTP was replaced with BODIPY-conjugated dUTP (Molecular Probes), which emits fluorescence in the Cy5 channel. FLAG-tagged wild-type and mutant parkin were generated previously (Ren *et al.* 2003).

Midbrain neuronal culture, drug treatment and transfection of parkin constructs

Primary midbrain neuronal cultures were prepared from rat embryos at E18 (Ren *et al.* 2005). Cultures on coverslips were maintained in 24-well plates in Neurobasal media supplemented with 2% B27 (Invitrogen, Carlsbad, CA, USA) and AraC (5 μmol/L, Sigma) for 14 days before they were treated with indicated drugs. We chose 12 h as the primary time point after the initial time course study on DA neurons (Ren *et al.* 2005). The time course for rotenone toxicity on serotonergic neurons was very similar. The 12 h treatment regimen offers a big differential in rotenone toxicity between monoaminergic and non-monoaminergic neurons. For some experiments, 3 μg of parkin constructs were transfected per well on day 10 using Lipofectamine 2000 (Invitrogen) before drug treatment 4 days later. Wild-type or mutant parkin was expressed in pCMV-Tag2 (Stratagene, La Jolla, CA, USA) in primary midbrain neuronal cultures. The proteins were expressed at similar levels as judged by immunostaining. Our previous studies have shown that expression levels of the parkin constructs used in this study are very similar in many cell lines (Ren *et al.* 2003). Treated neuronal cultures were fixed and stained as previously described (Zhao *et al.* 2003). In experiments with TUNEL staining, the reaction was performed for 1 h at 37°C after incubation with antibodies.

Image acquisition and quantification

Green, red, or blue signals in all images represented fluorescence from FITC, rhodamine, or Cy5 channels, respectively. Fluorescence images of apoptosis were acquired on a Nikon fluorescence microscope with a CCD camera (Diagnostic Instrument, Sterling Heights, MI, USA), and merged using the software SPOT (Diagnostic Instrument). For quantification of neuronal death, *n* equals the number of coverslips for each condition. All TpH^+ neurons (200–250) on a coverslip were counted, together with a random selection of 250–300 TpH^- neurons, or about 200 $GAT1^+$ or $vGlut2^+$ neurons. Our midbrain neuronal cultures typically contained 0.3% TH^+ neurons, 0.2% TpH^+ neurons, ~50% $GAT1^+$ neurons, and ~50% $vGlut2^+$ neurons.

For transfection experiments with FLAG-parkin, 24 coverslips were examined in eight independent experiments for each condition. All of the 20–30 transfected TpH^+ neurons and 120–250 untransfected TpH^+ neurons on each coverslip were counted, together with a random selection of 120–250 transfected or untransfected TpH^- neurons. Quantification of synaptophysin or syntaxin intensity was performed using NIH Image J. The contour of the soma was drawn manually. The degree of synaptophysin or syntaxin accumulation in the cell body was calculated by dividing the intensity of background-subtracted signal within the border by its area. At least 20 TpH^+ and 20 TpH^- neurons were analyzed for each coverslip and nine coverslips from three independent experiments were examined for each condition. All data were expressed as means ± SEM Statistical analyses were performed with unpaired *t*-test using the software Origin (Origin Lab, Northampton, MA, USA).

Results

Serotonergic neurons are much more vulnerable than non-serotonergic neurons to rotenone

Rat embryonic midbrain neuronal cultures maintained *in vitro* for 14 days were treated with various concentrations of rotenone for 12 h. Fixed cultures were co-stained with antibodies against the neuronal nuclear marker NeuN and TpH, a marker for serotonergic neurons, to highlight non-serotonergic (TpH^-) and serotonergic (TpH^+) neurons, respectively. TUNEL staining was used to label cells undergoing apoptosis. Rotenone induced apoptosis and broken processes in TpH^+ neurons, whereas most of the TpH^- neurons were unaffected (Fig. 1b). As shown in Fig. 1(c), rotenone caused significant death of TpH^+ neurons at all concentrations tested ($p < 0.01$, vs. control, $n \geq 6$), but produced much less toxicity to TpH^- neurons (e.g. 84.44 ± 3.11% in TpH^+ neurons vs. 18.36 ± 2.09% TpH^- neurons at 10 μmol/L rotenone). To confirm the specificity of rotenone toxicity, we examined apoptosis of major neuronal populations in the same cultures by using antibodies against GAT1 or vGlut2 to label GABAergic or glutamatergic neurons, respectively. Rotenone (e.g. 10 μmol/L) had very mild toxicity on GABAergic (14.27 ± 0.73%) or glutamatergic (14.55 ± 1.06%) neurons, which was similar to that on TpH^- neurons (14.58 ± 1.87%), but was much lower than that on TpH^+ neurons (82.43 ± 5.23%) (Fig. 1d). Furthermore, we examined rotenone toxicity on cortical neuronal cultures, which contained mostly glutamatergic or GABAergic neurons, but very few TpH^+ neurons. As shown in Fig. 1(e), the toxicity of rotenone (e.g. 10 μmol/L) on TpH^- neurons in cortical cultures was very mild (13.56 ± 0.75%) and almost the same as TpH^- neurons in midbrain cultures (14.58 ± 1.87%), which was much lower than that on midbrain TpH^+ neurons (82.43 ± 5.23%). Together, our results above illustrated the much higher toxicity of rotenone on TpH^+ neurons compared with TpH^- neurons including GABAergic or glutamatergic neurons. Similar results were obtained when neuronal death was quantified by cell counts using propidium iodide (data not shown).

The selective toxicity of rotenone on serotonergic neurons is dependent on its microtubule-depolymerizing activity

Rotenone (10 μmol/L for 12 h) depolymerized microtubules in all neurons, producing shrunk and broken processes (Fig. 2b). Co-application of the microtubule-stabilizing agent taxol (10 μmol/L) significantly attenuated rotenone toxicity and restored normal morphology of the processes (Fig. 2c). As summarized in Fig. 2(g), the selective toxicity of rotenone at 10 nmol/L (61.41 ± 2.75%) or 10 μmol/L (78.21 ± 3.65%) was significantly reduced by 100 nmol/L (31.80 ± 1.25%) or 10 μmol/L (52.43 ± 0.86%) taxol, respectively ($p < 0.01$, $n \geq 6$). Compared to vehicle control (2.82 ± 0.25%), taxol had no significant toxicity on TpH^+ neurons (4.54 ± 0.34%, $p > 0.05$, $n \geq 6$). For TpH^- neurons, the effect of taxol (10 μmol/L) and rotenone (10 μmol/L) co-treatment (8.08 ± 1.67%) was not significantly different from the mild toxicity of 10 μmol/L rotenone alone (12.12 ± 2.91%, $p > 0.20$, $n \geq 6$).

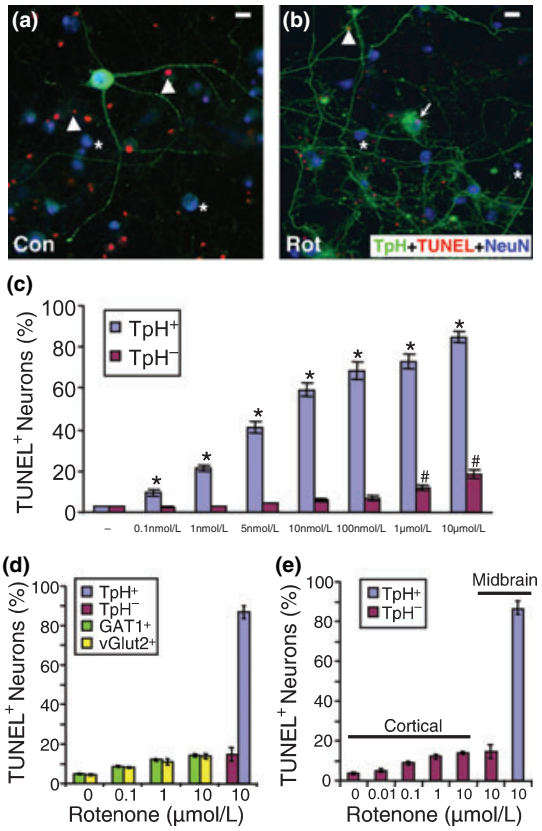

Fig. 1 Rotenone selectively killed serotonergic neurons in rat embryonic midbrain neuronal cultures in a dose-dependent manner. (a, b) Cultures treated with various concentrations of rotenone for 12 h were co-stained for TpH (green), TUNEL (red) and NeuN (blue). Representative images at 0 (a) or 10 μmol/L (b) rotenone were shown. Arrow, dead serotonergic (TpH^+) neuron; *non-serotonergic (TpH^-) neurons; arrowheads, dead glial cells killed by AraC in the media; bars, 10 μm. (c) Rotenone induced apoptosis of serotonergic (TpH^+) neurons in a dose-dependent manner, but caused minimal death of non-serotonergic (TpH^-) neurons only at high concentrations. *,#$p < 0.01$, $n \geq 6$, versus control for TpH^+ or TpH^- neurons, respectively. (d) Rotenone toxicity on GABAergic ($GAT1^+$), glutamatergic ($vGlut2^+$) or TpH^- neurons was very similar, but much lower than that on TpH^+ neurons. (e) The mild toxicity of rotenone was very similar on TpH^- neurons from cortical or midbrain cultures, but was much lower than that on TpH^+ neurons from midbrain cultures ($p < 0.001$, $n \geq 6$). For quantification of neuronal death, n equals the number of coverslips for each condition. All TpH^+ neurons (200–250) on a coverslip were counted, together with a random selection of 250–300 TpH^- neurons, or about 200 $GAT1^+$ or $vGlut2^+$ neurons.

To confirm the role of microtubule depolymerization in the specific death of TpH^+ neurons, we treated midbrain cultures with colchicine, a well-recognized microtubule-depolymer-

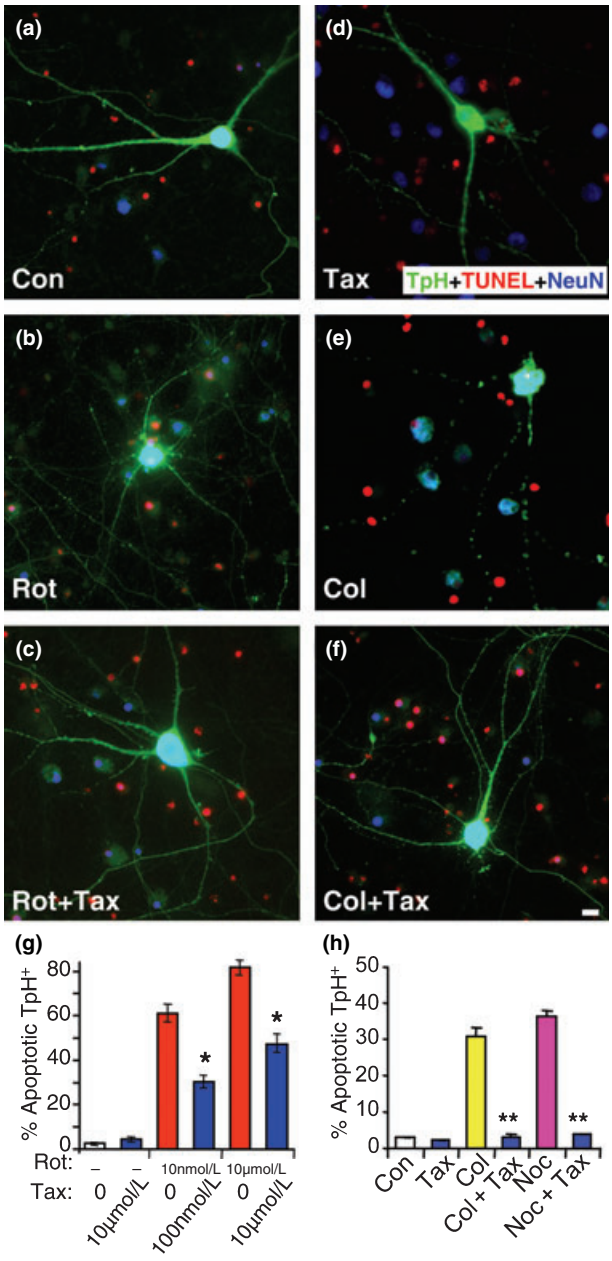

Fig. 2 The selective toxicity of rotenone on serotonergic neurons was significantly reduced by the microtubule-stabilizing agent taxol, and mimicked by microtubule-depolymerizing drugs. (a–f) Midbrain neuronal cultures were treated for 12 h without (a) or with 10 μmol/L of: rotenone (b) or rotenone plus taxol (c), taxol (d), colchicine (e) or colchicine plus taxol (f), and co-stained for TpH (green), TUNEL (red) and NeuN (blue). Bar, 10 μm. (g) The selective toxicity of rotenone on serotonergic (TpH^+) neurons at low (10 nmol/L) or high (10 μmol/L) concentration was significantly attenuated by co-application of taxol (Tax, 100 nmol/L or 10 μmol/L, respectively). *$p < 0.01$, $n \geq 6$, versus rotenone alone for the corresponding concentration. (h) The selective toxicity of microtubule-depolymerizing agent colchicine (Col) or Nocodazole (Noc) mimicked that of rotenone and was almost totally blocked by taxol (Tax). **$p < 0.001$, $n \geq 7$, versus colchicine or nocodazole.

izing agent. Colchicine (10 μmol/L for 12 h) induced significant apoptosis in TpH^+, but not TpH^- neurons (Fig. 2e). Co-application of taxol (10 μmol/L) greatly reduced colchicine toxicity (Fig. 2f). Colchicine-induced microtubule depolymerization led to shrunk and broken processes (Fig. 2e), very similar to the situation with rotenone treatment (Fig. 2b). Co-treatment with taxol restored normal morphology of the processes (Fig. 2f). As summarized in Fig. 2(h), colchicine-induced apoptosis of TpH^+ neurons (31.03 ± 3.55%) was almost completely blocked by taxol (3.16 ± 0.34%, $p < 0.001$, $n \geq 7$). On the other hand, colchicine, taxol, or their combination (all at 10 μmol/L) had no significant toxicity on TpH^- neurons, in comparison to the vehicle control (data not shown).

To further substantiate the results, we used another structurally different microtubule-depolymerizing agent, nocodazole. As shown in Fig. 2(h), nocodazole (10 μmol/L for 12 h) induced apoptosis of TpH^+ neurons (36.17 ± 1.96%) to a similar extent as that by colchicine (31.03 ± 3.55%, $p > 0.05$, $n \geq 7$). The toxicity of nocodazole (36.17 ± 1.96%) was almost totally blocked by 10 μmol/L taxol (3.59 ± 0.35%, $p < 0.001$, $n \geq 7$). In contrast, the toxicity of nocodazole on TpH^- neurons (4.30 ± 0.43%) was much less than that on TpH^+ neurons, and was not significantly different from the dimethyl sulfoxide vehicle control (2.92 ± 0.48%, $p > 0.10$, $n \geq 7$). Together, these lines of evidence suggest that microtubule depolymerization poses much greater toxicity in serotonergic neurons than in non-serotonergic neurons.

Rotenone toxicity is mimicked by the combination of microtubule depolymerization and complex I inhibition

As rotenone has two sites of action in the cell, we tested whether the combination of a pure complex I inhibitor and a pure microtubule-depolymerizer might mimic the potent toxicity of rotenone on serotonergic neurons. Amytal (10 μmol/L for 12 h), a complex I inhibitor with no known effect on microtubules, induced a moderate level of apoptosis in TpH^+ neurons (Fig. 3b). Co-application of amytal and colchicine (10 μmol/L for 12 h) markedly increased apoptosis in TpH^+, but not in TpH^-, neurons (Fig. 3c). The toxicity of amytal was not significantly affected by taxol (10 μmol/L) (Fig. 3d). As shown in Fig. 3(e), the combination of amytal and colchicine (60.96 ± 2.46% apoptotic TpH^+ neurons) was significantly more toxic than either agent alone (29.28 ± 1.91% for colchicine; 24.88 ± 1.06% for amytal; $p < 0.001$, $n \geq 6$, double vs. single treatments), and was similar to rotenone at 10 nmol/L (63.59 ± 6.46%, $p > 0.05$, $n \geq 6$), but less than the toxicity of 10 μmol/L rotenone (83.48 ± 2.66%). The additive effect in the dual treatment suggests that colchicine and amytal are acting on different sites that independently contribute to cell death. This was corroborated by the result that taxol did not significantly alleviate amytal toxicity on TpH^+ neurons [$p > 0.15$,

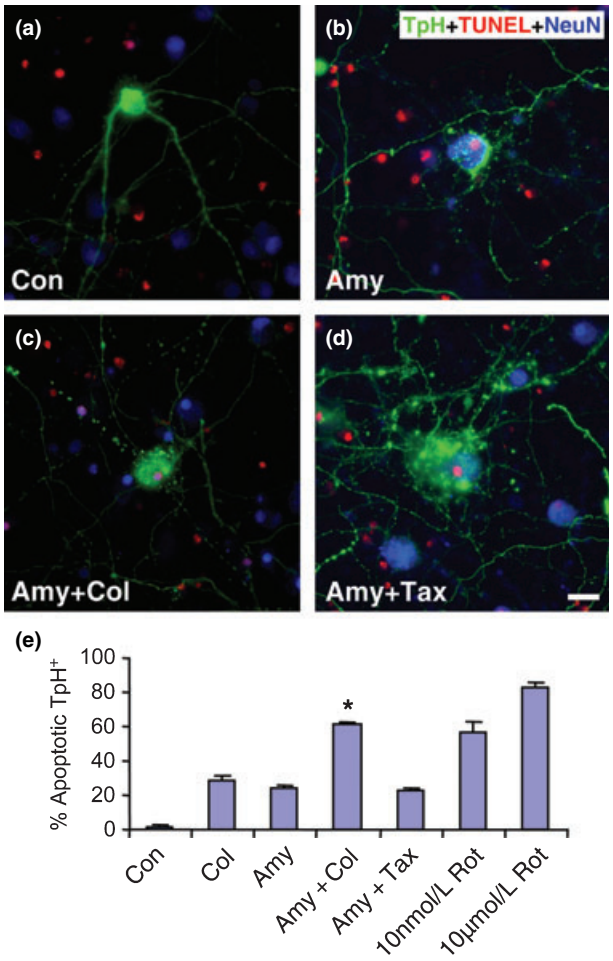

Fig. 3 The selective toxicity of rotenone on serotonergic neurons was recapitulated by the combination of complex I inhibition and microtubule depolymerization. (a–d) Midbrain cultures were treated for 12 h without (a) or with 10 μmol/L of: amytal (b), amytal plus colchicine (c), or amytal plus taxol (d), and co-stained for TpH (green), TUNEL (red) and NeuN (blue). Bar, 10 μm. (e) The selective toxicity of amytal (Amy) plus colchicine (Col) on serotonergic neurons was significantly greater than that seen with either agent alone (*$p < 0.001$, $n \geq 6$, Amy + Col vs. Amy or Col alone). Amytal toxicity was not significantly changed by taxol ($p > 0.15$, $n \geq 5$).

amytal plus taxol (23.07 ± 1.10%) vs. amytal alone (24.88 ± 1.06%)].

Rotenone-induced microtubule depolymerization leads to vesicle accumulation in the somal

Microtubule depolymerization disrupts vesicular transport and would cause accumulation of vesicles in the soma. To examine the impact of rotenone on the subcellular localization of vesicles, we treated midbrain neuronal cultures with or without rotenone (20 nmol/L for 12 h) and co-stained them with antibodies against TpH (green) and synaptophysin (red), a vesicle marker. In control condition, small amount of synaptophysin puncta were observed in the soma and in the processes (Fig. 4a). After rotenone treatment, a marked increase in synaptophysin puncta was seen in the cell body in both TpH^+ and TpH^- neurons (Fig. 4b). Co-application of taxol (10 μmol/L) blocked rotenone-induced accumulation of synaptophysin puncta (Fig. 4c). Application of colchicine (10 μmol/L for 12 h) also increased synaptophysin puncta in the cell body (Fig. 4e). This effect was greatly attenuated by taxol co-treatment (Fig. 4f), while taxol alone (Fig. 4d) did not cause any obvious change, compared to the control. We quantified the effects on synaptophysin localization by the ratio of its fluorescence signal in the soma to the area of the cell body. As there was no significant difference between TpH^+ and TpH^- neurons regarding this parameter, we pooled the data together. As shown in Fig. 4(g), rotenone induced a marked increase in this ratio (2.75 ± 0.19), in comparison to the control (0.73 ± 0.04, $p < 0.001$, $n = 9$). The effect was significantly attenuated by taxol (1.09 ± 0.07, $p < 0.001$, $n = 9$). Colchicine had an effect similar to that of rotenone (2.47 ± 0.13), which was also blocked by taxol (0.80 ± 0.05, $p < 0.001$, $n = 9$, vs. colchicine alone). To confirm our results, we used another vesicle maker, syntaxin I. As shown in Fig. 5(h), rotenone or colchicine induced a significant increase of syntaxin I puncta in the soma ($p < 0.001$, $n = 9$, vs. control). The effects were significantly blocked by the co-application of taxol ($p < 0.001$, $n = 9$ vs. the corresponding treatment without taxol). Thus, when microtubules were depolymerized by rotenone or colchicine, there was a significant accumulation of vesicles in the soma because microtubule-based transport was disrupted.

Rotenone toxicity is dependent on serotonin metabolism

Accumulation of vesicles, which is constitutively leaky, would increase cytosolic concentration of the neurotransmitter. In serotonergic neurons, elevated level of serotonin in the cytosol may trigger cell death due to oxidative degradation of serotonin, which produces reactive oxygen species. To examine the involvement of serotonin in the selective toxicity of rotenone on TpH^+ neurons, we treated midbrain neuronal cultures with rotenone alone, or in combination with inhibitors of serotonin metabolism or antioxidants. As shown in Fig. 5(a), co-application of the TpH inhibitor *p*-chlorophenylalanine (PC, 10 μmol/L) significantly attenuated rotenone toxicity (32.40 ± 7.15%, $p < 0.01$, $n = 6$). A similar effect was observed for NSD-1015 (NS, 1 μmol/L, 19.8 ± 2.00%, $p < 0.001$, $n = 6$), an inhibitor of L-aromatic amino acid decarboxylase, the second enzyme in serotonin synthetic pathway. Rotenone toxicity was also significantly reduced by clorgyline (Cl, 10 μmol/L, 34.57 ± 2.50%, $p < 0.01$, $n = 6$) or pargyline (Pa, 10 μmol/L, 30.92 ± 2.82%, $p < 0.01$, $n = 6$), inhibitor of monoamine oxidase A or B, which catalyzes the oxidative deamination of serotonin. Furthermore, application of the antioxidant *N*-acetyl-L-cysteine (NA, 1 mmol/L) or 2-methyl-*N*-(phenylmethylene)-2-propanamine *N*-oxide (PB, 1 mmol/L) signifi-

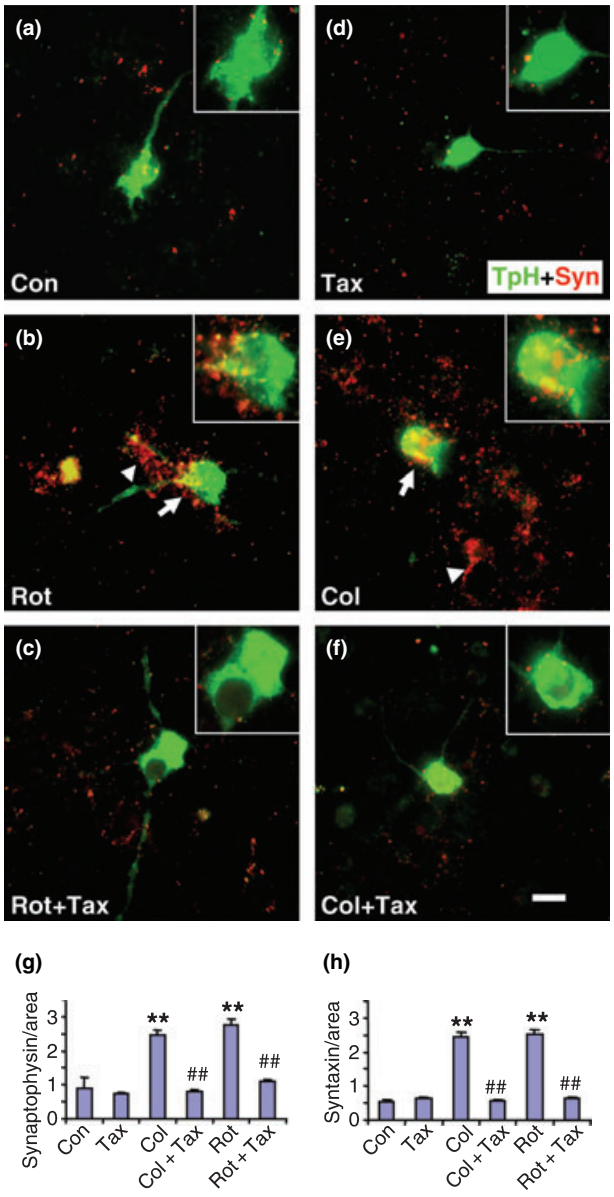

Fig. 4 Rotenone-induced microtubule depolymerization led to vesicle accumulation in the soma. (a–f) After midbrain neuronal cultures were treated for 12 h without (a) or with 20 nmol/L rotenone (b), 20 nmol/L rotenone plus 10 μmol/L taxol (c), 10 μmol/L taxol alone (d), 10 μmol/L colchicine (e) or 10 μmol/L colchicine and taxol (f), they were co-stained with anti-TpH (green) and anti-synaptophysin (red). Insets, enlarged portion of the main image showing vesicle accumulation in the soma. Arrows and arrowheads, synaptophysin puncta in TpH^+ or TpH^- neurons, respectively; bar, 10 μm. (g) The intensity of synaptophysin signal divided by the area of the cell body was plotted for each treatment in (a–f). $^{**}p < 0.001$, $n = 9$, versus control (Con); $^{\#\#}p < 0.001$, $n = 9$, versus the corresponding single treatment without taxol. (h) The intensity of syntaxin signal divided by the area of the cell body was plotted for parallel experiments using the same treatments in (a–f). $^{**}p < 0.001$, $n = 9$, versus control (Con); $^{\#\#}p < 0.001$, $n = 9$, versus the corresponding single treatment without taxol.

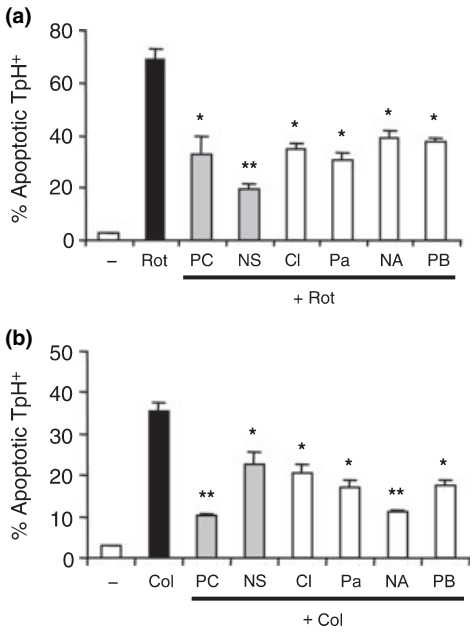

Fig. 5 Rotenone toxicity was dependent on serotonin metabolism. (a) Midbrain neuronal cultures were treated for 12 h without (–) or with 1 μmol/L rotenone (Rot) in the absence or presence of: 10 μmol/L PCPA (TpH inhibitor, PC), 1 μmol/L NSD-1015 (L-aromatic amino acid decarboxylase inhibitor, NS), 10 μmol/L Clorgyline (monoamine oxidase-A inhibitor, Cl), 10 μmol/L Pargyline (monoamine oxidase-B inhibitor, Pa), 1 mmol/L NAC (antioxidant, NA), or 1 mmol/L PBN (antioxidant, PB). Fixed cultures were stained for TpH, NeuN, and TUNEL. Percentage of apoptotic TpH^+ neurons was plotted for each treatment. $^{*}p < 0.01$, $n = 6$; $^{**}p < 0.001$, $n = 6$, all versus rotenone alone (Rot). (b) The same experiments were performed with 10 μmol/L colchicine (Col) replacing rotenone. $^{*}p < 0.01$, $n = 6$; $^{**}p < 0.001$, $n = 6$, all versus colchicine alone (Col).

cantly decreased rotenone toxicity (38.65 ± 2.91% or 37.88 ± 1.41%, respectively, $p < 0.01$, $n = 6$ for both). The same panel of agents also significantly attenuated the selective toxicity of colchicine on TpH^+ neurons (Fig. 5b). These results suggest that microtubule depolymerization exerts selective toxicity on TpH^+ neurons through serotonin metabolism and ensuing production of reactive oxygen species.

Inhibiting vesicular uptake of serotonin leads to selective death of TpH^+ neurons

The results above suggest that oxidation of cytosolic serotonin is critical for the specificity of rotenone toxicity. To test this idea further, we used reserpine to block vesicular monoamine transporter 2 (VMAT2), which is responsible for vesicular uptake of cytosolic serotonin (Liu and Edwards 1997). This would significantly increase cytosolic serotonin concentration, while depleting vesicular 5-HT pool (Liu and Edwards 1997). As shown in Fig. 6(a), reserpine caused significant apoptosis of TpH^+ neurons (36.98 ± 3.24%, $p < 0.01$, $n = 6$). The selective toxicity was significantly attenuated by inhibitors of serotonin synthesis [PC

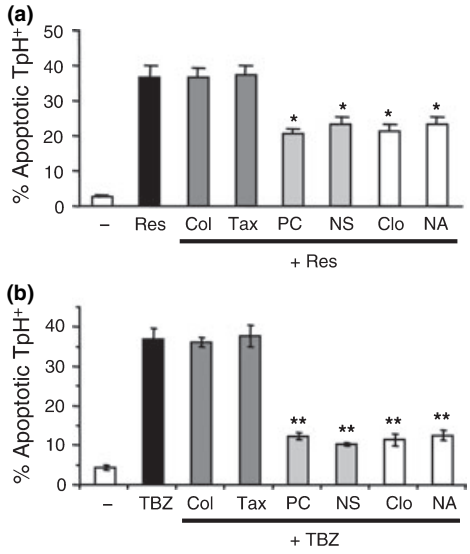

Fig. 6 Inhibition of vesicular uptake of serotonin induced selective death of TpH^+ neurons. (a) Inhibition of vesicular serotonin uptake by reserpine (Res, 10 μmol/L for 12 h) caused selective apoptosis of TpH^+ neurons, which was not affected by co-treatment with colchicine or taxol, but was significantly reduced by blocking serotonin synthesis, oxidation or ROS production. $^*p < 0.01$, $n = 6$, versus reserpine alone. (b) Similar effects were observed with another VMAT2 inhibitor, tetrabenazine (TBZ, 100 μmol/L for 12 h). $^{**}p < 0.01$, $n = 4$, versus tetrabenazine alone.

(20.74 ± 1.43%) and NS (23.22 ± 2.39%)] or degradation (Clo, 21.45 ± 1.95%), and by antioxidant (NA, 23.13 ± 2.38%) ($p < 0.01$, $n = 6$, vs. reserpine alone for all these treatments). In contrast, microtubule drugs (colchicine or taxol) had no significant effect on reserpine toxicity. As reserpine had depleted serotonin in the vesicles, microtubule depolymerization or stabilization would no longer contribute to cell death. Reserpine had no significant toxicity on TpH^- neurons (2.40 ± 0.20%), because VMAT2 is only expressed in monoaminergic neurons (Liu and Edwards 1997).

To confirm these results, we used another structurally unrelated, selective inhibitor of VMAT2, tetrabenazine. As shown in Fig. 6(b), tetrabenazine (TBZ, 100 μmol/L for 12 h) exerted on TpH^+ neurons a selective toxicity that was not significantly affected by co-application of colchicine (10 μmol/L) or taxol (10 μmol/L). In contrast, the selective toxicity of tetrabenazine was significantly reduced by the TpH inhibitor PCPA (PC, 10 μmol/L), the L-aromatic amino acid decarboxylase inhibitor NSD-1015 (NS, 1 μmol/L), the monoamine oxidase-A inhibitor clorgyline (Clo, 10 μmol/L) or the antioxidant NAC (NA, 1 mmol/L) ($p < 0.01$, $n = 4$ for all these co-treatments, vs. TBZ alone).

Parkin, but not its PD-linked mutants, protects against the selective toxicity of rotenone on TpH^+ neurons

Microtubule depolymerization induces rapid degradation of tubulin (Drubin *et al.* 1988). As our previous studies have

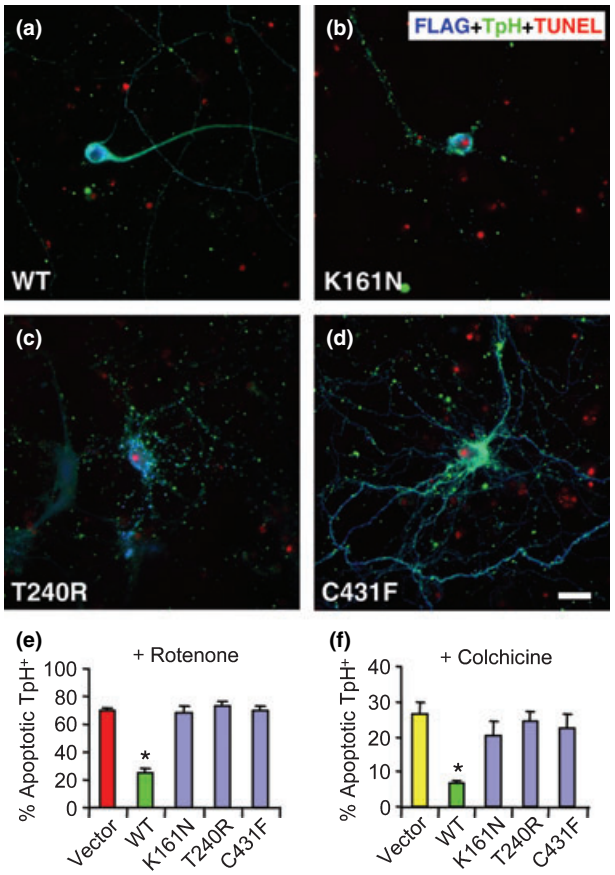

Fig. 7 Parkin, but not its PD-linked mutants, protected against the selective toxicity of rotenone or colchicine on serotonergic neurons. (a–d) Midbrain neuronal cultures were transfected with FLAG-tagged human wild-type parkin (a) or three PD-linked point mutants, K161N (b), T240R (c), or C431F (d). After the cultures were treated with 100 nmol/L rotenone for 12 h, they were co-stained for FLAG (Blue), TpH (green), and TUNEL (red). (e–f) Transfection of parkin, but not its mutants, in serotonergic neurons significantly attenuated apoptosis induced by rotenone (e) or colchicine (f), compared to TpH^+ neurons transfected with empty vector. $^*p < 0.01$, $n = 24$ coverslips (eight experiments), versus empty vector.

shown that parkin ubiquitinates α- and β-tubulins and accelerates their degradation (Ren *et al.* 2003), we tested whether over-expression of parkin or its PD-linked mutants had any effect on rotenone toxicity against TpH^+ neurons, which was dependent on microtubule depolymerization. Midbrain neuronal cultures were transfected with FLAG-tagged parkin or its PD-linked mutants (K161N, T240R, and C431F) in the pCMV-Tag2 vector (Ren *et al.* 2003). After the cultures were treated with 100 nmol/L rotenone for 12 h, they were co-stained with anti-FLAG (blue), anti-TpH (green), and TUNEL (red). As shown in Fig. 7(a–d) and summarized in Fig. 7(e), expression of wild-type parkin (24.31 ± 4.10%) in TpH^+ neurons significantly reduced rotenone-induced apoptosis (70.77 ± 1.70%, $p < 0.01$, $n = 24$ coverslips), while none of the three point mutants

had a significant protective effect (68.34 ± 5.46%, 73.51 ± 2.76%, 69.52 ± 3.21%, respectively, $p > 0.05$, $n = 24$).

If the protective function of wild-type parkin is acting on the microtubule-depolymerizing activity of rotenone, then parkin should be able to protect against colchicine-induced apoptosis of TpH^+ neurons. To test it, the same experiments were repeated with colchicine (10 μmol/L for 12 h). As summarized in Fig. 7(f), colchicine toxicity on TpH^+ neurons (26.77 ± 2.89%) was significantly attenuated by transfection of wild-type parkin (6.71 ± 0.96%, $p < 0.01$, $n = 24$ coverslips), but not by any of the three PD-linked mutants (20.43 ± 3.70%, 24.20 ± 2.99%, 22.34 ± 4.23%, respectively, $p > 0.05$, $n = 24$). The significant difference between wild-type parkin and its PD-linked mutants suggest that this protective function is important for PD. It also suggests that this effect of parkin is dependent on its E3 ligase activity, because all three mutants lost their E3 ligase activity on tubulin (Ren *et al.* 2003). Thus, it seems that by ubiquitinating tubulin, parkin protects against the selective toxicity of rotenone or colchicine, which depolymerizes microtubules and triggers rapid degradation of tubulin (Drubin *et al.* 1988).

Discussion

Our previous study has shown that cultured midbrain catecholaminergic (TH^+) neurons are much more vulnerable than TH^- neurons to microtubule-depolymerizing agents such as rotenone, colchicine, or nocodazole (Ren *et al.* 2005). Microtubule depolymerization disrupts vesicular transport and causes vesicle accumulation in the soma (Ren *et al.* 2005). The constitutive leakage of neurotransmitters from vesicles wrecks havoc in catecholaminergic neurons, because increased cytosolic concentration of catecholamine elevates oxidative stress from oxidation of catecholamine. GABAergic and glutamatergic neurons in the same culture are largely spared, as their neurotransmitters cannot be oxidized. The study predicts that all monoaminergic neurons with long processes are vulnerable to microtubule depolymerization. To test this idea, we examined serotonergic neurons in midbrain neuronal cultures in the present study.

The results provide clear evidence that, like catecholaminergic neurons, serotonergic (TpH^+) neurons are much more susceptible to rotenone toxicity than non-serotonergic (TpH^-) neurons (Fig. 1). The selective toxicity of rotenone was mimicked by microtubule-depolymerizing agents (colchicine or nocodazole) and was attenuated by the microtubule-stabilizing drug taxol (Fig. 2). These data suggest that the microtubule-depolymerizing activity of rotenone plays a key role in determining its selective toxicity on monoaminergic neurons. Not surprisingly, when we combined a pure microtubule-depolymerizing drug (colchicine) and a pure mitochondrial complex I inhibitor (amytal), the toxic effect was much greater than either agent alone and approached that of rotenone (Fig. 3). Thus, the results confirm that the potent toxicity of rotenone on monoaminergic neurons has two components – one from its complex I-inhibiting activity and one from its microtubule-depolymerizing activity. The former results in a general inhibition of mitochondria complex I in all types of cells (Betarbet *et al.* 2000) and would not readily explain the selectivity of rotenone toxicity. In contrast, the microtubule-depolymerizing effect of rotenone causes the selective death of monoaminergic neurons because it impinges on the vulnerable neurochemistry of these cells. Consistent with this, the toxicity of rotenone or colchicine on serotonergic neurons was dependent on the metabolism of serotonin in the cytosol (Fig. 5 and 6). Similar findings are observed in catecholaminergic neurons (Ren *et al.* 2005).

This study implies that environmental toxins such as rotenone may kill serotonergic neurons in addition to DA neurons in PD patients. Accelerated degeneration of serotonergic neurons is more frequently seen in PD patients with comorbid depression than in PD patients without depression (Paulus and Jellinger 1991). Although the prevalence of depression in PD is very high, it is unclear why some PD patients develop depressive symptoms while others remain unaffected. The great variability in environmental exposure to PD toxins may be a contributing factor worthy of examination. Our results also suggest that long-projection monoaminergic neurons, including nigral DA neurons and serotonergic neurons from dorsal raphe, share a common vulnerability to microtubule-depolymerizing agents. Thus, toxins that trigger the degeneration of DA neurons may also threaten the survival of serotonergic neurons. In the environment, there is a plethora of microtubule-destabilizing chemicals including natural products made in many plants (Jordan and Wilson 2004), synthetic herbicides such as 2,4-Dichlorophenoxyacetic acid (Rosso *et al.* 2000), dinitroaniline (Zeng and Baird 1999), Chlorpropham (Holy 1998), as well as antifungal or antiparasitic agents such as nocodazole, thiabendazole (Kiso *et al.* 2004), and benzimidazole (Lacey and Gill 1994). The diversity and abundance of microtubule-depolymerizing agents in the environment call for further studies on their involvement in PD and comorbid depression.

Our results also provide evidence that microtubule is a common target for rotenone and parkin, two prominent environmental and genetic factors in PD. Expression of parkin strongly protected serotonergic neurons against apoptosis induced by microtubule-depolymerizing agents such as rotenone or colchicine (Fig. 7). The protective effects appear to be dependent on the protein-ubiquitin E3 ligase activity of parkin towards tubulin. First, PD-linked mutations that abrogate the E3 ligase activity also abolished the

protection afforded by wild-type parkin (Fig. 7), indicating that the effect is dependent on the E3 ligase activity of the enzyme. Second, our previous study has shown that parkin promotes the ubiquitination and degradation of α- and β-tubulins (Ren *et al.* 2003). As microtubule depolymerization induces rapid degradation of tubulin protein (Drubin *et al.* 1988), it seems likely that parkin, as an E3 ligase for tubulin (Ren *et al.* 2003), may be responsible for the degradation. As over-expression of tubulin is toxic to yeast cells (Burke *et al.* 1989; Weinstein and Solomon 1990), the prompt removal of excess tubulin depolymerized from microtubules by rotenone may help to maintain the dynamic properties of microtubules. On the other hand, reactive oxygen species generated by rotenone in DA neurons would increase protein oxidation. As an abundant protein that requires perhaps the most complicated folding reactions (Lewis *et al.* 1997), tubulin would be susceptible to oxidation and ensuing misfolding. By removing misfolded tubulin, parkin may prevent it from interfering with microtubule assembly by native tubulin, and thus maintain the normal functions of the microtubule network, on which the survival of monoaminergic neurons is critically dependent (Feng 2006).

Acknowledgements

We would like to acknowledge supports from NIH grant NS4172 and the New York State Center of Excellence in Bioinformatics and Life Sciences (to J. F.).

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