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Neurotrophic Factors Stabilize Microtubules and Protect against Rotenone Toxicity on Dopaminergic Neurons*

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Parkinson disease is characterized by the selective degeneration of dopaminergic (DA) neurons in substantia nigra. Long term epidemiological studies have implicated exposure to agricultural pesticides as a significant risk factor. Systemic administration of rotenone, a widely used pesticide, causes selective degeneration of nigral DA neurons and Parkinson disease-like symptoms in rats. Our previous study has shown that the microtubule depolymerizing activity of rotenone plays a critical role in its selective toxicity on DA neurons. Rotenone toxicity is mimicked by the microtubule-depolymerizing drug colchicine and attenuated by the microtubule-stabilizing agent taxol. Here we show that nerve growth factor (NGF) significantly reduced rotenone toxicity on TH^+ neurons in midbrain neuronal cultures. The protective effect of NGF was completely abolished by inhibiting the microtubule-associated protein kinase kinase (MEK) and partially reversed by blocking phosphatidylinositol 3-kinase. In addition, NGF decreased colchicine toxicity on TH^+ neurons in a manner dependent on MEK but not phosphatidylinositol 3-kinase. The protective effect of NGF against rotenone toxicity was occluded by the microtubule-stabilizing drug taxol. In a MEK-dependent manner, NGF significantly attenuated rotenone- or colchicine-induced microtubule depolymerization and ensuing accumulation of vesicles in the soma and elevation in protein carbonyls. Moreover, other neurotrophic factors such as brain-derived neurotrophic factor and glia cell line-derived neurotrophic factor also reduced rotenone- or colchicine-induced microtubule depolymerization and death of TH^+ through a MEK-dependent mechanism. Thus, our results suggest that neurotrophic factors activate the microtubule-associated protein kinase pathway to stabilize microtubules, and this action significantly attenuates rotenone toxicity on dopaminergic neurons.

Parkinson disease (PD)[2] is a movement disorder associated with a characteristic pattern of neurodegeneration that includes progressive losses of selected dopaminergic neurons in substantia nigra, selected catecholaminergic and serotonergic neurons in brain stem, some cholinergic neurons in nucleus basalis, and certain small cortical neurons (1). The most prevalent form of PD occurs sporadically, with no obvious inheritance pattern. A large number of epidemiological studies (2), especially those performed on twins (3), have demonstrated strong association of environmental factors to PD. The concordance rates are virtually identical in monozygotic and dizygotic twins with age at onset older than 50. However, in PD twin pairs with age at onset younger than 50, the concordance rate in monozygotic twins (100%) is significantly higher than that in dizygotic twins (17%) (3). The study provides very clear evidence that the common, sporadic forms of late onset PD are highly influenced by environmental factors, whereas the early onset forms of Parkinson disease have a strong genetic influence.

Among the environmental factors studied, exposure to pesticides and herbicides has been found to be a significant risk factor for PD (4). Recent studies have demonstrated that, rotenone, a wildly used natural pesticide, induces PD-like symptoms and selective neurodegeneration in animals (5, 6). In addition to its well characterized inhibitory activity on complex I of the mitochondria respiratory chain (7), rotenone is also a strong microtubule-depolymerizing agent (8). It depolymerizes purified microtubules *in vitro*, as well as microtubules in the cell (8–10), by binding to the colchicine site on tubulin heterodimers (8, 10). Our previous study has shown that TH^+ neurons in midbrain neuronal cultures are much more vulnerable than TH^- neurons to the toxicity of microtubule-depolymerizing agents such as rotenone, colchicine, and nocodazole (10). Microtubule depolymerization abolishes vesicle transport and results in vesicle accumulation in the soma. Leakage of dopamine from the vesicles significantly increases dopamine oxidation in the cytosol, which appears to trigger the selective death of dopaminergic neurons (10). Consistent with this model, the microtubule-stabilizing agent taxol significantly attenuates the selective toxicity of rotenone on TH^+ neurons (10). Thus, microtubule stabilization appears to be a promising strategy to protect DA neurons from environmental PD toxins such as rotenone.

Neurotrophic factors include neurotrophins, glia cell line-derived neurotrophic factor (GDNF) family ligands and neurokines (11, 12). The neurotrophin family, which consists of nerve

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[2] The abbreviations used are: PD, Parkinson disease; DA, dopamine; TH, tyrosine hydroxylase; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; GDNF, glia cell line-derived neurotrophic factor; MAP, microtubule-associated protein; PI, phosphatidylinositol; PLC, phospholipase C; PKA, protein kinase A; MEK, MAP kinase kinase; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; wt, wild type; dn, dominant-negative; MES, 4-morpholineethanesulfonic acid; TUNEL, terminal deoxynucleotidyltransferase dUTP nick end labeling.

growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4/5, exert their diverse actions in the nervous system through interaction with the common neurotrophin receptor p75 and the ligand-specific Trk family receptor tyrosine kinases (13). The GDNF family ligands include GDNF, artemin, neurturin, and persephin. They share the receptor tyrosine kinase RET as their common signaling receptor and utilize different ligand-binding subunits (GFRα1 through GFRα4) that are selective for each GDNF family ligand (12). Neurotrophic factors such as NGF, BDNF, and GDNF, as well as their receptors have been localized by immunocytochemistry studies in dopaminergic neurons in substantia nigra (14–16), suggesting both autocrine and paracrine functions for these neurotrophic factors. A variety of studies in cell cultures and animal models have shown that these neurotrophic factors effectively protect against the degeneration of nigral dopaminergic neurons induced by PD toxins such as MPTP or 6-hydroxydopamine (17, 18).

In the present study, we show that neurotrophic factors such as NGF, BDNF, and GDNF attenuated the selective toxicity of rotenone against cultured midbrain TH^+ neurons. The ability of these neurotrophic factors to activate the MAP kinase pathway appears to be critical for microtubule stabilization and attenuation of rotenone toxicity on DA neurons.

EXPERIMENTAL PROCEDURES

Antibodies—Rabbit polyclonal antibodies against ERK1/2 and Thr^{202}/Tyr^{204} phosphorylated ERK1/2 were purchased from Cell Signaling (Beverly, MA). The neutralizing antibody against $p75^{NTR}$ (192-IgG) was from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit anti-TH was from Affinity BioReagents (Golden, CO). Mouse anti-NeuN was from Chemicon (Temecula. CA). Mouse anti-HA was from Roche Applied Science. Mouse antibodies against α-tubulin and synaptophysin were from Sigma. Alexa Fluor 488-conjugated anti-rabbit or anti-mouse IgG, Alexa Fluor 568-conjugated anti-rabbit IgG, and Alexa Fluor 594-conjugated anti-mouse IgG were purchased from Invitrogen.

Preparation of Midbrain Cultures—Midbrain neuronal cultures were prepared from embryonic day 17 fetuses of pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) as previously described (10), with minor modifications. Briefly, ventral mesencephalons were meticulously dissected and free of meninges in ice-cold Dulbecco's modified Eagle's medium. Midbrain cells were dissociated by digestion in 0.25% trypsin-EDTA for 30 min at 37 °C and subsequent addition of DNaseI (Roche Applied Science) to a final concentration of 0.1 mg/ml. The cells were then incubated at room temperature for another 5 min, followed by the addition of seeding medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum) and gentle trituration. After the cells were pelleted at 200 g for 5 min, they were resuspended in seeding medium and placed onto poly-D-lysine-coated (Sigma) wells or coverslips in multi-well plates at a density of $0.5–2 \times 10^5$ cells/cm^2. The cultures were incubated at 37 °C in 5% CO_2 and maintained in neurobasal medium supplemented with B-27 and 0.5 mM glutamine. Cytosine arabinoside (2.5 μM) was added to culture medium from the fourth day to inhibit glia growth, and the medium was half-changed every 4–5 days.

Drug Treatments and Transfection of Plasmid DNA—Rotenone was purchased from Sigma. Recombinant human β-NGF (NGF) was from R & D Systems (Minneapolis, MN). 2′-Amino-3′-methoxyflavone (PD98059), 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene (U0126), wortmannin, 2-[4-morpholinyl]-8-phenyl-4H-1-benzopyran-4-one (LY294002), myristoylated protein kinase A inhibitor 14–22 Amide (myr-PKI), and cpt-cAMP were purchased from EMD Biosciences Inc. (San Diego, CA). 1-[6-[((17β)-3-Methoxyestra-1,3,5 (10)-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione (U73122) and K252a were from Tocris (Ellisville, MO). All of the drugs were made as 1000× stocks in water, except for rotenone, PD98059, U0126, wortmannin, K252a, and U73122, which were made as 1000× stocks in Me_2SO (Sigma). All of the 1000× stocks were diluted to 10× stocks with culture medium immediately before addition to the neuronal cultures maintained *in vitro* for 14 days. All of the inhibitors were added 30 min before the primary treatment and were present for the whole duration of the primary treatment. Plasmid constructs for HA-tagged wild type MEK1 (wt-MEK) and dominant-negative MEK1 (K97M mutant) (dn-MEK) were described before (19). At 10–12 days *in vitro*, midbrain neuronal cultures were transfected with 1.6 μg of plasmid DNA/well in 12-well plates, using LipofectAMINE2000 (Invitrogen) according to the manufacturer's protocol. Subsequent treatments were performed 48 h after transfection.

Quantification of Cell Death and Microtubules Depolymerization—Immunocytochemistry was performed as previously described (10). For quantification of cell death, at least five coverslips from independent experiments were examined for each condition. All of the TH^+ neurons on a coverslip (100–200) were counted, together with a random selection of 120–250 TH^- neurons. For quantification of microtubules depolymerization, the midbrain neurons were cultured at low density (50,000 cells/cm^2) to allow clear visualization of microtubules in TH^+ neurons. At least 10 coverslips from independent experiments were examined for each condition. All of the TH^+ neurons (20–30 neurons) were counted for each coverslip. The fluorescence images were acquired on a confocal microscope from Bio-Rad. Monochrome images (512 × 512 pixels) were pseudocolored and merged with the software NIH Image J. All of the error bars represented standard errors, and Student's *t* tests were used for statistical analysis.

Determination of ERK Activation—At 14 days *in vitro*, midbrain cultures initially seeded at 2×10^5/cm^2 in 3.5-cm dishes were washed three times with phosphate-buffered saline and lysed with cold-buffer T (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_3VO_4) for 20 min on ice. The lysates were centrifuged at 4 °C at 16,000 × *g* for 20 min, and the supernatant was analyzed by Western blotting with anti-ERK and anti-phospho-ERK antibodies, respectively.

Quantification of Synaptophysin Intensity—Immunocytochemistry and quantification was performed as previously described using NIH Image J (10). The contour of the soma was drawn manually. The degree of synaptophysin accumulation in

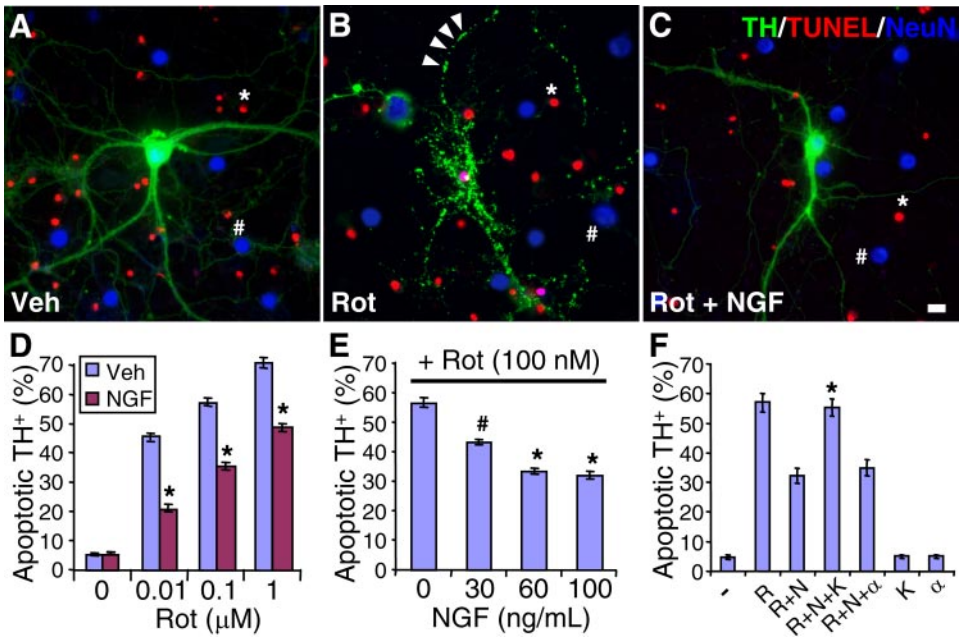

FIGURE 1. **NGF attenuates rotenone-induced apoptosis in TH$^+$ neurons in rat embryonic midbrain neuronal cultures.** Rat midbrain neurons cultured *in vitro* for 14 days were treated for 12 h with vehicle (*Veh*, *A*), rotenone (*Rot*, 100 nM) (*B*), and rotenone plus NGF (100 ng/ml, added 30 min before and during rotenone treatment) (*C*). Fixed neurons were co-stained for TH (*green*), TUNEL (*red*), and NeuN (*blue*). *Bar*, 10 μM. #, TH$^-$ neurons; *, dead glial cells (killed by AraC in the media); *arrowheads*, broken processes. *D*, statistical summary showing that NGF (100 ng/ml) attenuated the toxicity induced by various concentrations of rotenone (0.01 ~ 1 μM). *, $p < 0.001$ *versus* vehicle. *E*, statistical summary showing that NGF protected against the selective toxicity of rotenone (100 nM) on TH$^+$ neurons in a dose-dependent manner. #, $p < 0.05$; *, $p < 0.001$, all *versus* 0 ng/ml, $n = 6$. *F*, the protective effect of NGF was dependent on TrkA, but not p75^{NTR}. Inhibiting TrkA with K252a (*K*) completely reversed the protective effect of NGF (*N*) against rotenone (*R*), while blocking p75^{NTR} with a neutralizing antibody (α) did not significantly change the protective effect of NGF. *, $p > 0.70$ *versus* rotenone, $n = 3$.

the cell body was calculated by dividing the intensity of background-subtracted signal within the border by its area. At least 10 randomly selected TH$^+$ neurons were analyzed from each coverslip, which contained ~20–30 TH$^+$ neurons. At least three coverslips were used for each treatment.

Measurement of Free or Polymerized Tubulin in the Cell—Free or polymerized tubulin from midbrain neuronal cultures was extracted as described previously (10). Briefly, midbrain neuronal cultures maintained in 3.5-cm dishes *in vitro* for 14 days were washed twice at 37 °C with 1 ml of Buffer A containing 0.1 M MES (pH 6.75), 1 mM $MgSO_4$, 2 mM EGTA, 0.1 mM EDTA, and 4 M glycerol. After the cultures were incubated at 37 °C for 5 min in 600 μl of free tubulin extraction buffer (Buffer A plus 0.1% (v/v) Triton X-100 and protease inhibitors), the extracts were centrifuged at 37 °C for 2 min at 16000 × *g*. The supernatant fractions contained free tubulin extracted from the cytosol. The pellet fraction and lysed cells in the culture dish were dissolved in 600 μl of 25 mM Tris (pH 6.8) plus 0.5% SDS and contained tubulin originally in microtubules. Equal amounts of total proteins from free or polymerized tubulin fractions were analyzed by Western blotting with anti-α-tubulin antibody. The intensity of tubulin bands was quantified from three different experiments with the software NIH Image.

Measurement of Protein Carbonyls—After treatments, midbrain neuronal cultures were washed three times in phosphate-buffered saline and lysed in cold lysis buffer (0.5% v/v Nonidet P-40, 1 M Tris, pH 8.0, 150 mM NaCl, 50 mM NaF, 10% (v/v) glycerol, 0.5 M EDTA, and 0.1 M Na_3VO_4). Protein carbonyls were measured with the Oxyblot protein oxidation detection kit (Chemicon) as described before (10). Intensities of the dots were quantified with NIH Image J by measuring signals in a preset circle of the same size.

RESULTS

NGF Attenuates the Selective Toxicity of Rotenone on TH$^+$ Neurons in Midbrain Neuronal Cultures—Our previous study has shown that rotenone exhibited much greater toxicity on TH$^+$ neurons than TH$^-$ neurons in midbrain neuronal cultures (10). To identify neuroprotective agents against this environmental PD toxin, we treated rat embryonic midbrain neuronal cultures maintained *in vitro* for 14 days with rotenone (100 nM for 12 h) and NGF (100 ng/ml for 12.5 h, added 0.5 h before rotenone). Fixed cultures were co-stained for TH (*green*), the neuronal nuclear marker NeuN (*blue*), and TUNEL (*red*, for detection of apoptosis). Rotenone-induced selective death of TH$^+$ neurons (Fig. 1, *B versus A*) was markedly reduced by co-application of NGF (Fig. 1*C*). The protective effect of NGF was observed for various concentrations of rotenone (Fig. 1*D*). In addition, NGF attenuated rotenone toxicity in a dose-dependent manner (Fig. 1*E*). Because the protective effect of NGF against rotenone (100 nM) reached a plateau at ~100 ng/ml, we used this condition for subsequent experiments.

To investigate which NGF receptor is involved in the protective effect, we used the selective TrkA inhibitor K252a (400 nM) and the p75^{NTR} neutralizing antibody 192-IgG (1:500). As shown in Fig. 1*F*, the protective effect of NGF was completely reversed by K252a ($p > 0.70$ *versus* rotenone alone, $n = 3$) but was not significantly altered by the antibody against p75^{NTR} ($p > 0.50$, *versus* rotenone plus NGF, $n = 3$). Neither K252a nor p75^{NTR} antibody *per se* had any significant toxicity on TH$^+$ neurons.

The Protective Effect of NGF against Rotenone Toxicity Is Dependent on MEK and Partially on PI 3-Kinase—To study the mechanism by which NGF protects against rotenone toxicity on TH$^+$ neurons, we examined the involvement of the MAP kinase pathway, which is potently activated by neurotrophic factors such as NGF (11, 12). Midbrain neuronal cultures were treated with rotenone (100 nM for 12 h), NGF (100 ng/ml for 12.5 h, added 0.5 h before rotenone), and PD98059 (20 μM for 13 h, added 1 h before rotenone). The selective MEK inhibitor PD98059 completely abolished the protective effect of NGF (Fig. 2, *A* and *E*). Another specific MEK inhibitor, U0126 (20 μM

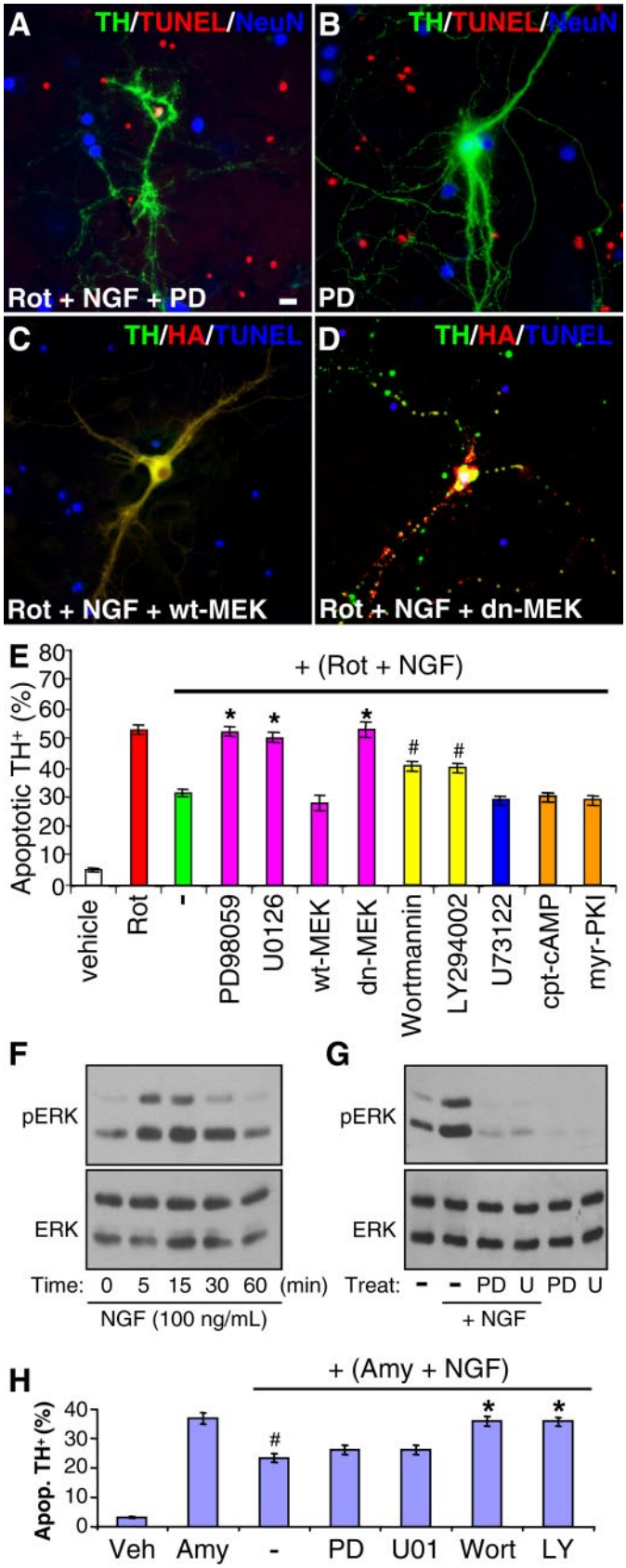

FIGURE 2. **The protective effect of NGF against rotenone is dependent on MEK and partially on PI 3-kinase.** *A* and *B*, midbrain neuronal cultures were treated for 12 h with rotenone (*Rot*, 100 nM), NGF (100 ng/ml, added 30 min before rotenone) and the MEK inhibitor PD98059 (*PD*, 20 μM, added 1 h before rotenone) (*A*), or PD98059 alone (*B*). *Veh*, vehicle. The cultures were co-stained for TH (*green*), TUNEL (*red*), and NeuN (*blue*). PD98059 blocked the protective effect of NGF against rotenone toxicity. *C* and *D*, midbrain neuronal cultures transfected with HA-tagged wt-MEK-1 (*C*) or a dn-MEK-1 mutant (K97M) (*D*) were treated for 12 h with rotenone (100 nM) and NGF (100 ng/ml, added 30 min before rotenone). Fixed cultures were co-stained for TH (*green*), HA (*red*), and TUNEL (*blue*). The dominant-negative mutant, but not the wild type MEK1, abolished the protective effect of NGF against rotenone. *Bar*, 10 μm. *E*, statistical analyses showed that the protective effect of NGF against rotenone was almost completely reversed by inhibition of MEK with PD98059 or U0126 (20 μM) or expression of dn-MEK, partially reversed by PI 3-kinase inhibitors wortmannin (0.5 μM) and LY294002 (20 μM), but not affected by wt-MEK, PLCγ inhibitor U73122 (1 μM), PKA activator cpt-cAMP (50 μM), or PKA inhibitor myr-PKI (1 μM). *, $p < 0.001$, *versus* Rot plus NGF, $n = 5$. These inhibitors and expression constructs had no significant toxicity by themselves ($p > 0.5$, $n = 5$) and did not significantly change rotenone toxicity ($p > 0.1$, $n = 6$). *F* and *G*, total cell lysates from treated midbrain cultures were blotted with anti-phospho-ERK (*top panels*) or anti-ERK (*bottom panels*). NGF (100 ng/ml) induced rapid and transient activation of ERK (*F*), which was blocked by MEK inhibitor PD98059 (*PD*, 20 μM) or U0126 (*U*, 20 μM) added 30 min before and during NGF (*G*). *H*, NGF significantly attenuated the toxicity of complex I inhibitor amytal (*Amy*) on TH$^+$ neurons, and the protective effect was almost completely reversed by the PI 3-kinase inhibitor wortmannin (*Wort*, 0.5 μM) or LY294002 (*LY*, 20 μM) but not affected by the MEK inhibitor PD98059 (*PD*, 20 μM) or U0126 (*U01*, 20 μM). #, $p < 0.001$ *versus* amytal, $n = 6$; *, $p > 0.70$, *versus* amytal, $n = 6$. None of these inhibitors significantly changed the toxicity of amytal on TH$^+$ neurons ($p > 0.40$, $n = 5$).

for 13 h), also totally eliminated the protective effect of NGF (Fig. 2*E*). To substantiate the dependence of MEK in the protective effect of NGF against rotenone, we transfected midbrain neuronal cultures with HA-tagged wild type MEK1 or a dominant-negative MEK1 mutant (K97M), which blocks the activation of endogenous MEK1 (19). Two days after transfection, the cultures were treated with rotenone and NGF in the same manner. Overexpression of the dominant-negative mutant (Fig. 2*D*), but not the wild type MEK1 (Fig. 2*C*), completely abrogated the protective effect of NGF (Fig. 2, *C–E*). Thus, inhibition of MEK by pharmacological blockers or dominant-negative protein had the same consequence, which suggests that the protective effect of NGF is dependent on the activation of MEK.

We also examined the involvement of other signaling pathways activated by NGF, such as PI 3-kinase, phospholipase Cγ (PLCγ), or protein kinase A (PKA). As shown Fig. 2*E*, PI 3-kinase inhibitors such as wortmannin (0.5 μM) and LY294002 (20 μM) partially reversed the protective effect of NGF, whereas PLCγ inhibitor U73122 (1 μM), PKA activator cpt-cAMP (50 μM), or PKA inhibitor myr-PKI (1 μM) did not significantly change the effect of NGF. These results suggest that in addition to the MAP kinase pathway, activation of the PI 3-kinase pathway by NGF also contributes partially to its protective effect against rotenone toxicity.

None of the above inhibitors or MEK expression constructs had any significant toxicity on TH$^+$ neurons ($p > 0.50$ *versus* vehicle, $n = 5$), nor did they significantly change the toxicity of rotenone ($p > 0.10$ *versus* rotenone alone, $n = 6$). To show that NGF indeed induced MAP kinase activation, we treated midbrain neuronal cultures with NGF (100 ng/ml) for various durations and analyzed the total cell lysates by Western blotting with anti-phospho-ERK, which recognizes activated ERK1/2 or anti-ERK, which recognize total ERK1/2. As shown in Fig. 2*F*, NGF induced rapid and transient activation of ERK1/2. This effect was almost completely blocked by MEK inhibitors such as PD98059 or U0126 (Fig. 2*G*). When applied alone, both MEK inhibitors lowered the basal level of ERK activation (Fig. 2*G*).

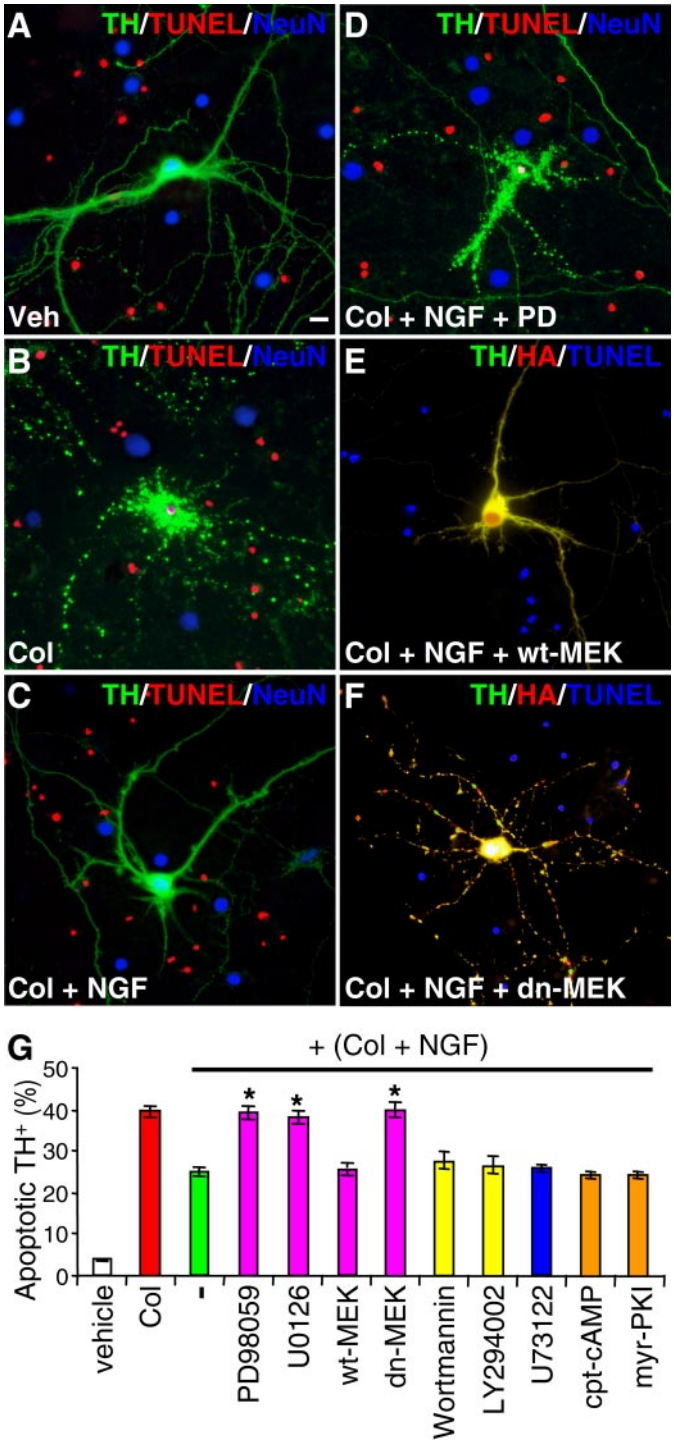

FIGURE 3. **NGF attenuates the selective toxicity of colchicine on TH$^+$ neurons in a MEK-dependent manner.** *A–D*, midbrain neuronal cultures were treated for 12 h with vehicle (*A*), colchicine (*Col*, 10 μM) (*B*), colchicine and NGF (100 ng/ml, added 30 min before and during colchicine treatment) (*C*), and colchicine and NGF plus PD98059 (PD 20 μM, added 60 min before and during colchicine treatment) (*D*). Fixed cultures were co-stained for TH (*green*), TUNEL (*red*), and NeuN (*blue*). *E* and *F*, midbrain neuronal cultures transfected with HA-tagged wt-MEK or its dn-MEK were treated with for 12 h with colchicine (10 μM) and NGF (100 ng/ml, added 30 min before colchicine). Cultures were co-stained for TH (*green*), HA (*red*), and TUNEL (*blue*). *Bar*, 10 μM. *G*, statistical summary showed that the protective effect of NGF against colchicine toxicity was almost completely reversed by inhibition of MEK with PD98059, U0126 (20 μM), or dn-MEK but was not significantly affected by wild type MEK1, PI 3-kinase inhibitor wortmannin (0.5 μM) or LY294002 (20 μM), PLCγ inhibitor U73122 (1 μM), PKA activator cpt-cAMP (50 μM), or PKA inhibitor myr-PKI (1 μM). *, $p < 0.001$ *versus* colchicine plus NGF, $n = 5$. None of the inhibitors or expression constructs significantly changed colchicine toxicity on TH$^+$ neurons ($p > 0.8$, $n = 6$).

Our previous study has shown that the selective toxicity of rotenone on TH$^+$ neurons is contributed by both complex I inhibition and microtubule depolymerization (10). To assess whether NGF attenuates toxicity caused by complex I inhibition, we used amytal, a pure complex inhibitor without any significant effect on microtubules (10). As shown in Fig. 2*H*, NGF significantly reduced amytal toxicity on TH$^+$ neurons. The protective effect of NGF was almost completely reversed by PI 3-kinase inhibitors such as wortmannin and LY294002 but not significantly affected by MEK inhibitors PD98059 and U0126. Thus, the protective effect of NGF against complex I inhibition by amytal or rotenone may be mediated by activation of the PI 3-kinase pathway, whereas toxicity induced by the microtubule depolymerizing activity of rotenone may be mitigated by activation of the MAP kinase cascade.

NGF Attenuates the Selective Toxicity of Colchicine on TH$^+$ Neurons in a MEK-dependent Manner—Our previous study has shown that, like rotenone, the microtubule-depolymerizing agent colchicine also selectively kills TH$^+$ neurons in midbrain neuronal cultures (10). To examine whether NGF attenuates the selective toxicity of colchicine, we treated midbrain neuronal cultures with colchicine (10 μM for 12 h) in the absence or presence of NGF (100 ng/ml for 12.5 h, added 0.5 h before colchicine). Co-application of NGF markedly reduced the selective toxicity of colchicine on TH$^+$ neurons (Fig. 3, *C versus B*). This effect was completely blocked by the MEK inhibitor PD98059 or U0126 (both at 20 μM for 13 h, added 1 h before colchicine) (Fig. 3, *D* and *G*). To confirm these results, we transfected midbrain neuronal cultures with HA-tagged wild type or dominant-negative MEK1 and treated the cultures with colchicine and NGF in the same manner. Overexpression of the dominant-negative MEK1, but not the wild type, completely reversed the protective effect of NGF (Fig. 3, *E–G*). Furthermore, the protective effect of NGF against colchicine was not significantly affected by PI 3-kinase inhibitors such as wortmannin and LY294002, PLCγ inhibitor U73122, PKA activator cpt-cAMP, or PKA inhibitor myr-PKA (Fig. 3*G*). None of these inhibitors or MEK constructs significantly changed the toxicity of colchicine on TH$^+$ neurons ($p > 0.80$, $n = 6$). Together, these results indicate that NGF attenuates the selective toxicity of colchicine on TH$^+$ neurons, and the effect is dependent on MEK.

The Protective Effect of NGF against Rotenone Toxicity Is Occluded by the Microtubule-stabilizing Drug Taxol—The ability of NGF to protect against the selective toxicity of both rotenone and colchicine suggests that it is acting on microtubules, which play a critical role in the survival of dopaminergic neurons (10). Because the microtubule-stabilizing drug taxol significantly attenuates the selective toxicity of rotenone and completely eliminated the selective toxicity of colchicine (10), we compared the protective effects of NGF, taxol, and their combination against rotenone-induced death of TH$^+$ neurons. Midbrain neuronal cultures were treated for 12 h with rotenone (100 nM), rotenone plus NGF (100 ng/ml, added 0.5 h before

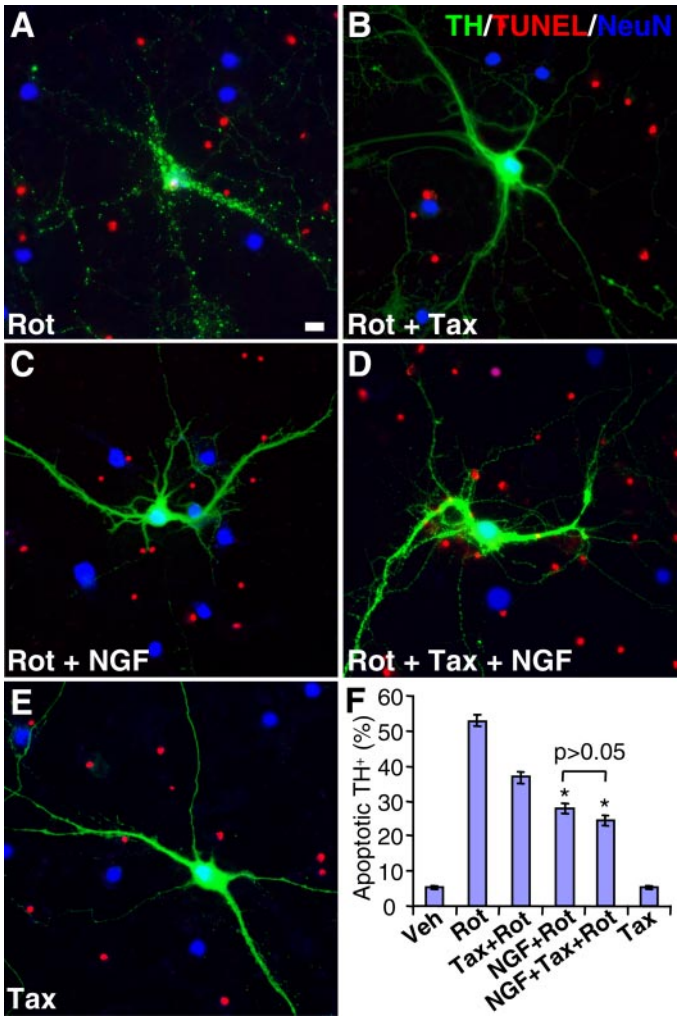

FIGURE 4. **The protective effect of NGF against the selective toxicity of rotenone is occluded by the microtubule-stabilizing drug taxol.** *A–E*, midbrain neuronal cultures treated with rotenone (*Rot*, 100 nM) (*A*), rotenone and taxol (*Tax*, 10 μM) (*B*), rotenone and NGF (100 ng/ml) (*C*), rotenone, NGF and taxol (*D*), or taxol alone (*E*) were co-stained for TH (*green*), TUNEL (*red*), and NeuN (*blue*). *Bar*, 10 μm. *F*, statistical summary of data represented in *A–E* showed that the protective effects of NGF or NGF plus taxol was significantly better than that of taxol (*, $p < 0.05$ *versus* Tax+Rot, $n = 6$), but NGF plus taxol was not significantly better than NGF alone ($p > 0.05$, $n = 6$). *Veh*, vehicle.

rotenone), rotenone plus taxol (10 μM, added 0.5 h before rotenone), and rotenone and NGF plus taxol (both added 0.5 h before rotenone). The protective effect of NGF (27.8 ± 1.2%) was significantly better than that of taxol (36.8 ± 1.7%, $p < 0.05$, $n = 6$; Fig. 4*F*), suggesting that other mechanisms in addition to microtubule stabilization (*e.g.* PI 3-kinase) are involved in the protective effect of NGF against rotenone toxicity. On the other hand, the protective effect of NGF (27.8 ± 1.2%) was not significantly enhanced by the addition of taxol (24.4 ± 1.6%, $p > 0.05$, $n = 6$). The occlusive effects of NGF and taxol suggest that they are acting on the same target in the cell.

NGF Attenuates Rotenone- or Colchicine-induced Microtubule Depolymerization in a MEK-dependent Manner—The ability of NGF to protect against the selective toxicity of rotenone or colchicine, as well as the occlusion of the protective effects of NGF and taxol, suggests that NGF is acting on microtubules, whose depolymerization renders midbrain TH^+ neurons particularly vulnerable (10). To investigate the role of microtubules in the protective effect of NGF, we treated midbrain neuronal cultures with rotenone (100 nM for 3 h) and co-stained the cultures with anti-TH and anti-α-tubulin to examine microtubules in TH^+ and TH^- neurons. Microtubules were strongly depolymerized in response to rotenone in TH^+ neurons (Fig. 5, *B versus A*), as well as in TH^- neurons (data not shown). Co-application of NGF (100 ng/ml for 3.5 h, added 0.5 h before rotenone) greatly reduced rotenone-induced microtubule depolymerization (Fig. 5, *C versus B*). The effect of NGF was abolished by the MEK inhibitor PD98059 (20 μM for 4 h, added 0.5 h before NGF) (Fig. 5, *D versus C*). Similar results were found when we treated the cultures with colchicine (10 μM for 3 h) instead of rotenone. Colchicine-induced microtubule depolymerization (Fig. 5*F*) was strongly attenuated by NGF (Fig. 5*G*). The effect of NGF was abrogated by the MEK inhibitor PD98059 (Fig. 5*H*).

We counted the number of neurons with at least one obvious microtubule (operationally defined by a continuous length of 10 μm or longer) and calculated the percentage of neurons without microtubule to quantify the degree of microtubule depolymerization. Because the situations in TH^+ neurons and TH^- neurons were very similar, we only showed data on TH^+ neurons in Fig. 5*I*. Rotenone completely depolymerized microtubules in 51.8 ± 1.3% of TH^+ neurons. Co-application of NGF significantly reduced the effect of rotenone (37.6 ± 1.4%, $p < 0.001$, $n = 5$ coverslips). The effect of NGF was totally blocked by the MEK inhibitor PD98059 (50.8 ± 1.3%, $p > 0.20$ *versus* rotenone alone, $n = 5$). Similar results were found when we treated the cultures with colchicine (Fig. 5*I*). We also used the MEK inhibitor U0126 (20 μM) and obtained the same results as those of PD98059 in experiments using rotenone or colchicine (data not shown). By itself, NGF, PD98059 (Fig. 5, *E* and *I*) or U0126 (data not shown) had no significant effect on microtubules, in comparison with the vehicle control.

To substantiate these results with another independent assay, we measured the amount of free tubulin and polymerized tubulin in midbrain neuronal cultures by gently lysing the neurons at 37 °C in a low concentration of detergent (0.1% Triton X-100) to extract free tubulin while not disturbing polymerized tubulin in microtubules. As shown in the *upper panel* of Fig. 5*J*, the amount of free tubulin was markedly increased in response to rotenone (100 nM for 30 min). NGF (100 ng/ml for 60 min, added 30 min before rotenone) greatly reduced the level of free tubulin. This effect was abolished by the MEK inhibitor PD98059 (20 μM for 90 min, added 30 min before NGF). Similar results were obtained with colchicine (10 μM for 30 min) (Fig. 5*J*). Reciprocal changes in response to these agents were observed in the amount of polymerized tubulin (Fig. 5*J*, *bottom panel*). By itself, NGF or PD98059 had no obvious effect on the amount of free tubulin or polymerized tubulin, in comparison with the vehicle control (Fig. 5*J*). We also used U0126 and obtained similar results as those from PD98059 (data not shown). Results from three independent experiments were quantified in Fig. 5*K*. Together, these two different readouts on the polymerization state of microtubules suggest that NGF decreases rotenone- or colchicine-induced microtubule depolymerization through a MEK-dependent mechanism.

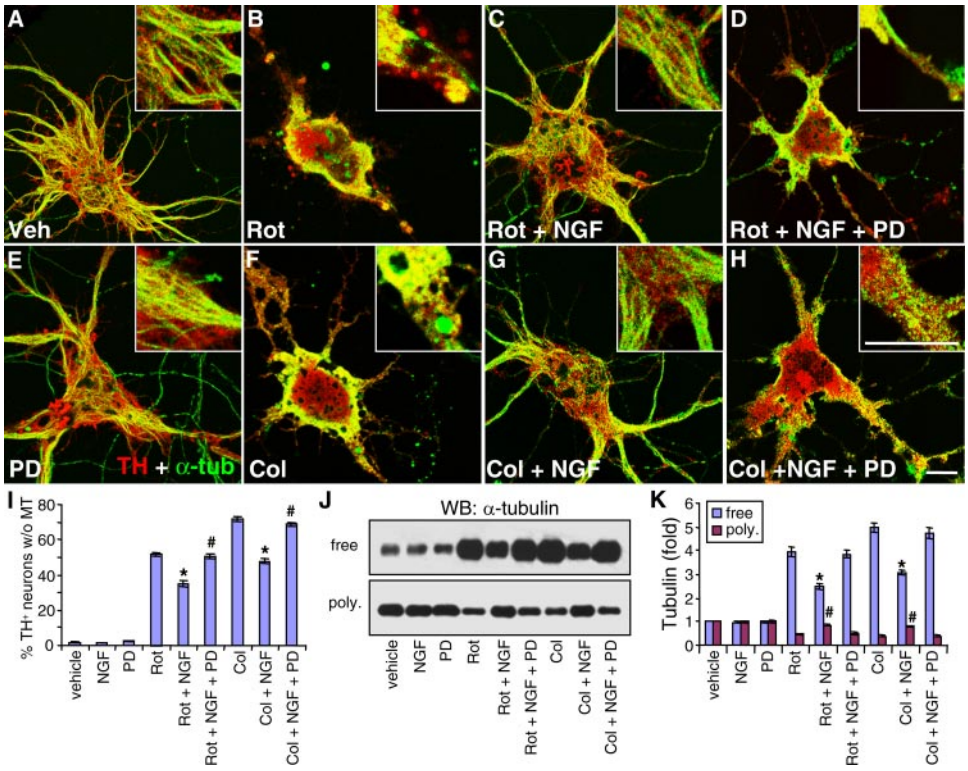

FIGURE 5. **NGF attenuates rotenone- or colchicine-induced microtubule depolymerization in a MEK-dependent manner.** *A–H*, the polymerization state of microtubules in midbrain TH^+ neurons treated with vehicle (*A*); rotenone (*Rot*, *B*); rotenone and NGF (*C*); rotenone, NGF, and PD98059 (*PD*, *D*); PD98059 (*E*); colchicine (*Col*, *F*); colchicine and NGF (*G*); and colchicine, NGF and PD98059 (*H*). PD98059 (20 μM) was added 30 min before NGF (100 ng/ml), which was applied 30 min before and during the treatment with rotenone (100 nM for 3 h) or colchicine (10 μM for 3 h). Cultures were co-stained with anti-TH (*red*) and anti-α-tubulin (*green*). *Insets*, enlarged portion of proximal processes. *Bars*, 10 μm. *I*, statistical summary of data represented in *A–H* showed that the effect of NGF against microtubule depolymerization induced by rotenone or colchicine was abolished by the MEK inhibitor PD98059. *, $p < 0.001$; #, $p > 0.20$, all *versus* rotenone or colchicine alone, respectively ($n = 5$). *J*, the amount of free or polymerized tubulin extracted from midbrain neuronal cultures treated as above, except that the duration for rotenone or colchicine was 30 min. NGF greatly attenuated rotenone- or colchicine-induced increase of free tubulin and concomitant decrease of polymerized tubulin. The effects were abolished by the MEK inhibitor PD98059. *K*, statistical summary of three separate experiments shown in *J*. *, $p < 0.05$ for free tubulin; #, $p < 0.05$ for polymerized tubulin, all *versus* rotenone or colchicine alone, respectively. *WB*, Western blot; *Veh*, vehicle.

NGF Attenuates Rotenone- or Colchicine-induced Vesicle Accumulation in the Soma and Increase in Protein Carbonyls—Disruption of the microtubule network by rotenone or colchicine causes vesicle accumulation in the soma and increase in oxidative stress caused by oxidation of dopamine leaked from the vesicles (10). We examined whether NGF mitigated these consequences of microtubule depolymerization, which are directly linked to the death of dopaminergic neurons. In the cell body, rotenone induced accumulation of synaptophysin-positive puncta that are indicative of vesicles (Fig. 6, *B versus A*). This response was greatly attenuated by NGF (Fig. 6, *C versus B*), whose effect was reversed by the MEK inhibitor PD98059 (Fig. 6*D*). Similarly, colchicine-induced vesicle accumulation in the soma (Fig. 6*E*) was markedly reduced by NGF (Fig. 6*F*). The effect of NGF was reversed by PD98059, which induced no significant change in vesicle distribution by itself (Fig. 6*H*). The statistical summary in Fig. 6*I* showed that NGF significantly attenuated rotenone- or colchicine-induced vesicle accumulation in the soma ($p < 0.001$, $n = 3$). The effects of NGF were significantly reversed by the MEK inhibitor PD98059 ($p < 0.005$, $n = 3$). Neither NGF nor PD98059 *per se* had any significant effect on vesicle distribution ($p > 0.90$, $n = 3$).

To assess the impact of NGF on oxidative stress induced by rotenone or colchicine, we measured the amounts of protein carbonyls in midbrain neuronal cultures treated the same way as above for 4 h. As shown in Fig. 6*J*, rotenone- or colchicine-induced increases in protein carbonyls were significantly reduced by NGF ($p < 0.005$, $n = 4$). The effects of NGF were significantly blocked by the MEK inhibitor PD98059 ($p < 0.005$, $n = 4$). NGF or PD98059 alone did not significantly change the amount of protein carbonyls ($p > 0.90$, $n = 4$).

BDNF and GDNF Attenuate Rotenone- or Colchicine-induced Death of TH^+ Neurons and Microtubule Depolymerization through a MEK-dependent Mechanism—To assess whether the protective effect of NGF against the selective toxicity of rotenone is common for other neurotrophic factors, we treated midbrain neuronal cultures with rotenone (100 nM for 12 h) in the presence or absence of BDNF (50 ng/ml for 12.5 h, added 0.5 h before rotenone) or GDNF (20 ng/ml for 12.5 h, added 0.5 h before rotenone). Application of BDNF or GDNF greatly attenuated rotenone-induced selective death of TH^+ neurons in midbrain neuronal cultures (Fig. 7, *main images*). The protective effect of BDNF (Fig. 7*C*) or GDNF (Fig. 7*E*) was reversed by co-application of the MEK inhibitor PD98059 (20 μM for 13 h, added 0.5 h before either neurotrophic factor) (Fig. 7, *D* and *F*). When the results were quantified, we found that both BDNF and GDNF significantly attenuated rotenone toxicity on TH^+ neurons to the similar extent as that achieved by NGF (Fig. 7*G*). Similarly, the protective effects of both BDNF and GDNF were completely blocked by MEK inhibitor PD98059 (Fig. 7*G*) or U0126 (data not shown). We also used colchicine (10 μM for 12 h) instead of rotenone and obtained similar results (Fig. 7*G*). Thus, neurotrophic factors including NGF, BDNF, and GDNF protected against the selective toxicity of microtubule-depolymerizing agents such as rotenone or colchicine through a mechanism dependent on MEK.

To examine the impact of BDNF and GDNF on microtubule depolymerization induced by rotenone, we treated midbrain neuronal cultures in the same way as above for 3 h and co-stained the cultures with anti-TH (*red*) and anti-α-tubulin (*green*) to observe the polymerization state of microtubules in

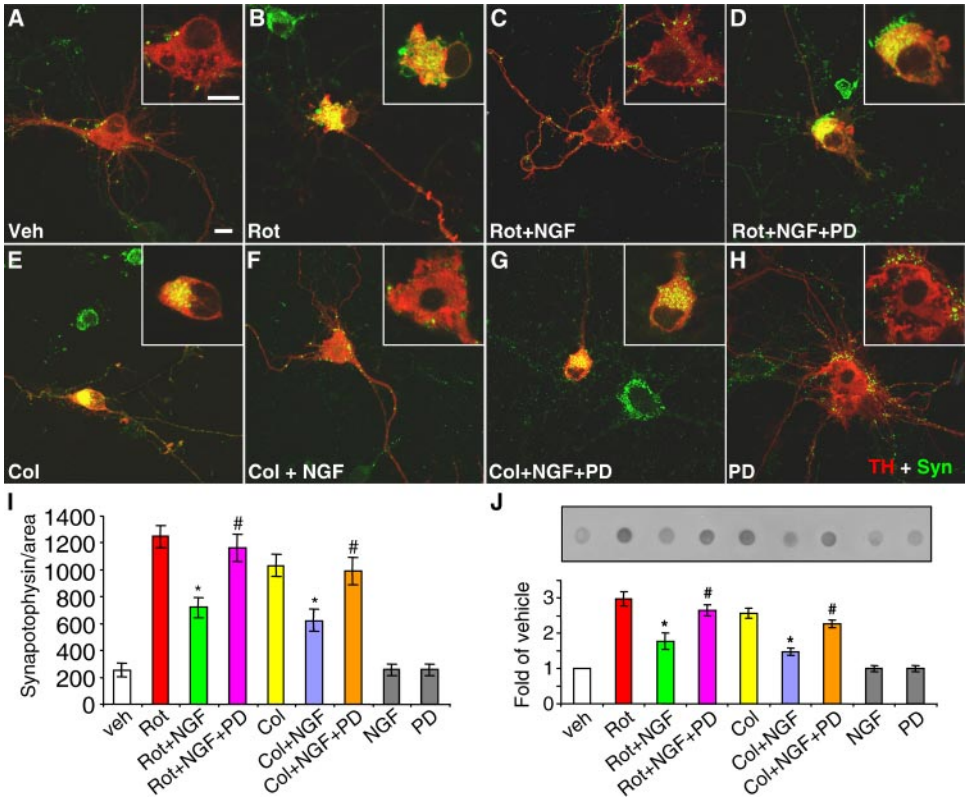

FIGURE 6. **NGF attenuates rotenone- or colchicine-induced vesicle accumulation in the soma and increase in protein carbonyls.** *A–I*, NGF attenuated rotenone- or colchicine-induced vesicle accumulation in the soma. Midbrain neuronal cultures were treated for 12 h with vehicle (*Veh*, *A*), rotenone (*Rot*, 20 nM) (*B*), Rot plus NGF (100 ng/ml, added 30 min before Rot) (*C*), Rot plus NGF plus PD98059 (*PD*, 20 μM) (*D*), colchicine (*Col*, 10 μM) (*E*), colchicine plus NGF (100 ng/ml, added 30 min before Col) (*F*), colchicine plus NGF plus PD98059 (20 μM) (*G*), or PD98059 alone (*H*). Fixed neurons were co-stained with anti-TH (*red*) and anti-synaptophysin (*green*). A stack of confocal images along the *z* axis was merged to show the accumulation of synaptophysin puncta in the soma. One section in the middle of the cell body was displayed in *insets* to show synaptophysin puncta inside the soma. *Bars*, 10 μm. *I*, the intensity of synaptophysin signals divided by the area of the cell body was plotted for each treatment in *A–H*. *, $p < 0.001$ *versus* Rot or Col; #, $p < 0.005$ *versus* Rot+NGF or Col+NGF. At least three coverslips were examined for each condition, with more than 10 TH^+ neurons analyzed for each coverslip. *J*, NGF attenuated rotenone- or colchicine-induced increase in protein carbonyls. Midbrain neuronal cultures were treated for 4 h with the same panel of drugs as above. The amounts of protein carbonyls were measured by the Oxyblot method (*top panel*) and quantified from four independent experiments (*bottom panel*). *, $p < 0.005$ *versus* Rot or Col; #, $p < 0.005$ *versus* Rot+NGF or Col+NGF.

TH^+ neurons (Fig. 7, *insets*). Rotenone-induced microtubule depolymerization (Fig. 7*B*, *inset*) was markedly reduced by co-application of BDNF (Fig. 7*C*, *inset*) or GDNF (Fig. 7*E*, *inset*). The effects of BDNF and GDNF were abolished by the MEK inhibitor PD98059 (Fig. 7, *D* and *F*, respectively, *insets*). Using the method described above, we quantified the degree of microtubule depolymerization by calculating the percentage of TH^+ neurons without an obvious microtubule. As shown in Fig. 7*H*, both BDNF and GDNF significantly attenuated rotenone-induced microtubule depolymerization ($p < 0.001$, $n = 5$ coverslips). These effects were completely reversed by the MEK inhibitor PD98059 (Fig. 7*H*) or U0126 (data not shown). Similar results were also obtained when we used colchicine, instead of rotenone (Fig. 7*H*). Thus, like NGF, both BDNF and GDNF significantly attenuated microtubule depolymerization induced by rotenone or colchicine in a MEK-dependent manner.

DISCUSSION

One of the pathological hallmarks of Parkinson disease is the relative selective degeneration of dopaminergic neurons in substantia nigra pars compacta. A variety of environmental and genetic factors are involved in the progressive loss of nigral DA neurons and ensuring motor symptoms typically found in Parkinson disease. Many long term epidemiological studies, particularly those performed on twins, have strongly implicated the influence of environmental toxins in idiopathic Parkinson disease, especially in cases with a late age at onset (2, 3). This notion is supported by the observation that systemic administration of the widely used pesticide rotenone in animal models recapitulates characteristic features of Parkinson disease: selective degeneration of nigral DA neurons, PD-like locomotor problems, and Lewy body-like intracellular inclusions enriched with α-synuclein (5, 6).

Our recent study has shown that the microtubule depolymerizing activity of rotenone plays a critical role in determining the selectivity of its toxicity on dopaminergic neurons (10). To identify neuroprotective agents against the selective toxicity of rotenone, we examined neurotrophic factors such as NGF, BDNF, and GDNF in the present study. The results showed that these neurotrophic factors exhibited similar levels of protection against the selective toxicity of rotenone on cultured midbrain TH^+ neurons (Figs. 1 and 7). Several lines of evidence suggest that their protective effects against rotenone toxicity are dependent on microtubules. First, all three neurotrophic factors significantly attenuated the selective toxicity of colchicine (Figs. 3 and 7*G*), a well characterized microtubule-depolymerizing agent that has no other known targets in the cell except tubulin and microtubules. Second, the protective effect of NGF (Fig. 4), or BDNF and GDNF (data not shown) was occluded by the microtubule-stabilizing drug taxol. It suggests that these neurotrophic factors are acting on the same target that taxol does. Third, all of these neurotrophic factors significantly attenuated microtubule depolymerization induced by rotenone or colchicine (Figs. 5 and 7). We examined the polymerization state of microtubules in the cell by two independent assays: counting the percentage of neurons without an obvious microtubule and directly measuring the amount of free or polymerized tubulin in the cell. Both methods led to the same conclusion that these neurotrophic factors stabilized microtubules against depolymerizing agents such as rotenone or colchicine. Fourth, both the protective effect of these neurotrophic factors and their

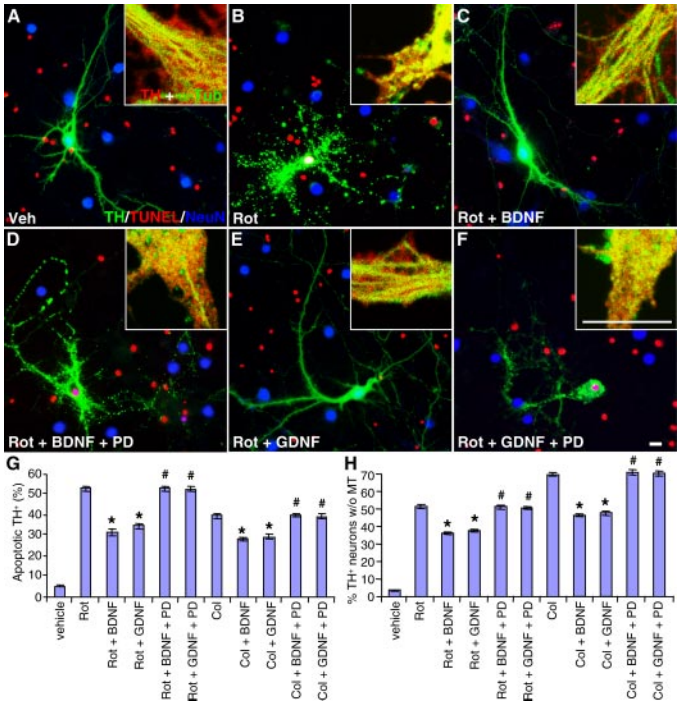

FIGURE 7. **BDNF and GDNF attenuate rotenone- or colchicine-induced death of TH$^+$ neurons and microtubules depolymerization through a MEK-dependent mechanism.** *A–F*, midbrain neuronal cultures were treated for 12 h with vehicle (*Veh*, *A*); rotenone (*Rot*, 100 nM) (*B*); rotenone and BDNF (50 ng/ml) (*C*); rotenone, BDNF and PD98095 (*PD*, 20 μM) (*D*); rotenone and GDNF (20 ng/ml) (*E*); and rotenone, GDNF and PD98095 (*F*) and were co-stained for TH (*green*), TUNEL (*red*) and NeuN (*blue*) to examine the death of TH$^+$ neurons (*main images*). Cultures were also treated in the same way for 3 h and co-stained for TH (*red*) and α-tubulin (*green*) to examine the status of microtubules in TH$^+$ neurons (*insets*). *Bars*, 10 μM. *G*, statistical summary showed that BDNF or GDNF significantly reduced the toxicity of rotenone or colchicine (*Col*) on TH$^+$ neurons in a MEK-dependent manner. *, $p < 0.001$; #, $p > 0.20$ *versus* rotenone or colchicine alone, respectively ($n = 5$). *H*, statistical summary showed BDNF or GDNF significantly attenuated microtubule depolymerization induced by rotenone or colchicine, and the effects were totally blocked by PD98059. *, $p < 0.001$, #, $p > 0.20$ *versus* rotenone or colchicine alone, respectively ($n = 5$).

microtubule-stabilizing effect were dependent on activation of the MAP kinase pathway. When the MAP kinase kinase MEK was inhibited either by selective blockers (PD98059 and U0126) or by overexpression of a dominant-negative MEK1 construct, the protective effects of all three neurotrophic factors against rotenone or colchicine were completely abolished (Figs. 2, 3, and 7) and so were their microtubule-stabilizing effects (Figs. 5 and 7). Thus, it appears that neurotrophic factors such as NGF, BDNF, or GDNF may activate the MAP kinase pathway to stabilize microtubules against depolymerizing agents like rotenone or colchicine.

In addition to the MAP kinase pathway, PI 3-kinase pathway also contributed partially to the protective effect of NGF on rotenone (Fig. 2*E*) but not colchicine (Fig. 3*G*). This difference is most likely due to the complex I inhibiting activity of rotenone, which can be mimicked by amytal. Indeed, the protective effect of NGF against amytal toxicity was almost completely blocked by PI 3-kinase inhibitors but was not significantly affected by MEK inhibitors (Fig. 2*H*). Thus, neurotrophic factors such as NGF are very effective in protecting against rotenone toxicity because they activate both the MAP kinase pathway and the PI 3-kinase pathway to suppress the dual components of rotenone toxicity: microtubule depolymerization and complex I inhibition.

Because microtubule depolymerization leads to the selective death of TH$^+$ neurons in midbrain neuronal cultures (10), the ability of these neurotrophic factors to induce microtubule stabilization is translated into neuroprotection against rotenone toxicity. How does activation of the MAP kinase pathway results in microtubule stabilization? Before MAP kinase is known as mitogen-activated protein kinase, it initially stood for microtubule-associated protein (MAP) kinase because of its high efficiency in phosphorylating MAPs such as MAP2 (20, 21). Through binding to microtubules, a variety of MAPs strongly influence the stability and dynamic properties of microtubules in distinct manners (22). The binding between MAPs and microtubules are highly regulated by the phosphorylation state of MAPs (23), which are good substrates of MAP kinase. Although further investigations are needed to identify the MAP that is involved in the microtubule-stabilizing effect of the neurotrophic factors, it seems plausible that these neurotrophic factors stabilize microtubules by activating the MAP kinase pathway to phosphorylate MAPs. Once microtubules were stabilized, rotenone- or colchicine-induced vesicle accumulation in the soma was greatly attenuated, and oxidative stress in the cell was reduced accordingly (Fig. 6). The dependence of all of these effects on MEK indicates the central role of MAP kinase activation in the protection afforded by neurotrophic factors.

Numerous studies using cell culture systems or animal models have demonstrated the efficacy of neurotrophic factors in protecting dopaminergic neurons against PD toxins such as MPTP and 6-hydroxydopamine (17). Our results showed that NGF, BDNF, and GDNF significantly reduced rotenone toxicity on cultured midbrain TH$^+$ neurons through a common mechanism that is largely dependent on activation of the MAP kinase pathway and microtubule stabilization. Although recent clinical trials of GDNF on PD patients produce mixed results (18, 24), it is clear that more studies are necessary to improve the therapeutic use of neurotrophic factors for the treatment of Parkinson disease. Because the MAP kinase pathway plays a major role in the protective effects of all three neurotrophic factors that we tested, it may be possible to design small molecule compounds to manipulate this critical pathway to mimic the protective effects of these neurotrophic factors on DA neurons. Such a strategy would obviate the difficulty in delivering macromolecules inside the brain while maintaining the protective effect.

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