

Activation of Group III Metabotropic Glutamate Receptors Attenuates Rotenone Toxicity on Dopaminergic Neurons through a Microtubule-Dependent Mechanism

Qian Jiang, Zhen Yan, and Jian Feng

Department of Physiology and Biophysics, State University of New York at Buffalo, Buffalo, New York 14214

Systemic administration of rotenone, a widely used pesticide, causes selective degeneration of nigral dopaminergic (DA) neurons and Parkinson's disease-like symptoms in animal models. Our previous study has shown that the microtubule-depolymerizing activity of rotenone plays a critical role in its selective toxicity on tyrosine hydroxylase-positive (TH⁺) neurons in rat embryonic midbrain neuronal cultures. Here, we show that application of group III metabotropic glutamate receptor (mGluRIII) agonists (e.g., L-AP-4) significantly reduced rotenone toxicity on midbrain TH⁺ neurons in culture. The protective effect of L-AP-4 was abolished by pharmacological inhibition of the microtubule-associated protein (MAP) kinase kinase (MEK) or overexpression of dominant-negative MEK1, suggesting its dependence on the MAP kinase cascade. We found that L-AP-4 induced a rapid and transient activation of the MAP kinase extracellular signal-regulated kinase (ERK) through a pathway mediated by dynamin, β -arrestin 2, and Src. ERK activated in this manner targeted cytosolic rather than nuclear substrates. Consistent with this, L-AP-4 significantly attenuated rotenone- or colchicine-induced microtubule depolymerization in an MEK-dependent manner. Moreover, L-AP-4 decreased colchicine toxicity on TH⁺ neurons in an MEK-dependent manner as well. The protective effect of L-AP-4 against rotenone toxicity was occluded by the microtubule-stabilizing agent Taxol. Together, these results suggest that activation of group III metabotropic glutamate receptors attenuates the selective toxicity of rotenone on DA neurons by activating the MAP kinase pathway to stabilize microtubules. These findings may offer a novel neuroprotective approach against rotenone-induced parkinsonism.

Key words: rotenone; metabotropic glutamate receptor; microtubules; MAP kinase; β -arrestin; Src; dynamin

Introduction

Parkinson's disease (PD) is characterized by several pathological hallmarks such as the relatively selective degeneration of nigral dopaminergic (DA) neurons, intracellular inclusions (Lewy bodies) and dystrophic processes (Lewy neurites) (Lang and Lozano, 1998). Long-term epidemiological studies, especially those performed on twins, strongly suggest that environmental toxins play a critical role in the common, late-onset forms of PD (Tanner et al., 1999; Langston, 2002). It has been shown recently that systemic administration of rotenone, a naturally occurring substance widely used as a pesticide, produces specific degeneration of nigral DA neurons and PD-like locomotor symptoms in rats (Betarbet et al., 2000).

Rotenone acts directly on two targets in the cell; it inhibits complex I in the mitochondrial respiratory chain (Chance et al., 1963) and depolymerizes microtubules (Marshall and Himes, 1978). Our previous study has shown that the microtubule-depolymerizing activity of rotenone is critical in determining its selective toxicity on DA neurons (Ren et al., 2005). Microtubule

depolymerization disrupts vesicular transport, which leads to accumulation of vesicles in the soma. In dopaminergic neurons, leakage of dopamine from the vesicles greatly increases oxidative stress induced by DA oxidation, which triggers cell death (Ren et al., 2005). Consistent with these results, defects in microtubule-based transport have been shown to play a major role in many neurodegenerative disorders (Goldstein, 2003). Thus, microtubule dysfunction is not only critically involved in neurodegeneration, it may also provide a unique cellular mechanism that leads to the death of specific neuronal population. Enhancing microtubule stability would offer a novel approach to protect against environmental PD toxins such as rotenone.

Metabotropic glutamate receptors (mGluRs) are a group of G-protein-coupled receptors (GPCRs) for glutamate. Based on their sequence homology, signal transduction, and pharmacological profiles, eight different mGluR genes are classified into three subfamilies (Conn and Pin, 1997). Group I metabotropic glutamate receptors (mGluRI) include mGluR1 and mGluR5, which couple to the G_q or G_o class of G-proteins to activate phospholipase C (PLC). Group II mGluRs (mGluRII) consist of mGluR2 and mGluR3, which inhibit adenylyl cyclase by activating G_i. Group III mGluRs (mGluRIII) comprise mGluR4, mGluR6, mGluR7, and mGluR8, which also inhibit adenylyl cyclase through G_i. The unique expression pattern and subcellular localization of different mGluR enables them to perform very specific

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Correspondence should be addressed to Dr. Jian Feng, Department of Physiology and Biophysics, State University of New York at Buffalo, 124 Sherman Hall, Buffalo, NY 14214. E-mail: jianfeng@buffalo.edu.

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functions in the brain (Conn, 2003). For example, various mGluRs are differentially expressed in basal ganglia and play critical roles in modulating neuronal activities in the motor circuits that are severely affected in Parkinson's disease (Rouse et al., 2000; Conn et al., 2005). On the other hand, increasing evidence has suggested that mGluRs have diverse neuroprotective functions (Nicoletti et al., 1996; Flor et al., 2002). For instance, activation of mGluRIII attenuates excitotoxic neuronal death (Bruno et al., 2000), hypoxic/hypoglycemic neuronal damages (Sabelhaus et al., 2000), and $\alpha\beta$ toxicity (Copani et al., 1995). In the present study, we show that mGluRIII agonists attenuated rotenone toxicity on TH⁺ neurons in midbrain neuronal cultures by activating the microtubule-associated protein (MAP) kinase pathway to stabilize microtubules.

Materials and Methods

Antibodies. Rabbit polyclonal antibodies against extracellular signal-regulated kinase 1/2 (ERK1/2) and Thr²⁰²/Tyr²⁰⁴ phosphorylated ERK1/2 were purchased from Cell Signaling (Beverly, MA). A rabbit antibody against Tyr⁴¹⁸ phosphorylated Src was purchased from Biosource (Camarillo, CA). Rabbit anti-TH was from Affinity BioReagents (Golden, CO). Mouse anti-NeuN was from Chemicon (Temecula, CA). Mouse anti-HA was from Roche (Nutley, NJ). Mouse anti- α -tubulin was from Sigma (St. Louis, MO). Mouse anti- β -arrestin 1 was from BD Biosciences (San Jose, CA). Mouse anti- β -arrestin 2 and rabbit anti-c-Src were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-MAP2 was from Upstate Biotechnology (Lake Placid, NY). Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 594-conjugated anti-mouse were purchased from Invitrogen (Carlsbad, CA).

Preparation of midbrain cultures. All reagents were from Invitrogen unless otherwise specified. Midbrain neuronal cultures were prepared from embryonic day 17 fetuses of pregnant Sprague Dawley rats (Harlan, Indianapolis, IN) as described previously (Ren et al., 2005), with minor modifications. Briefly, ventral mesencephalons were meticulously dissected and freed of meninges in ice-cold DMEM. Midbrain cells were dissociated by digestion in 0.25% trypsin-EDTA for 30 min at 37°C and subsequent addition of DNaseI (Roche) to a final concentration of 0.1 mg/ml. Cells were then incubated at room temperature for another 5 min, followed by addition of seeding medium (DMEM 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 seeded onto poly-D-lysine-coated (Sigma) wells or coverslips in multi-well plates at a density of $0.5\text{--}2 \times 10^5$ cells/cm². Cultures were incubated at 37°C in 5% CO₂ and maintained in Neurobasal medium supplemented with B-27 and 0.5 mM glutamine. Cytosine arabinoside (2.5 μM) was added to culture media from the fourth day to inhibit glia growth and the medium was half changed every 4–5 d.

Drug treatments. 4-Amino-1-*tert*-butyl-3-(1'-naphthyl)pyrazolo[3,4-*d*]pyrimidine (PP1), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), and 4-amino-7-phenylpyrazolo[3,4-*d*]pyrimidine (PP3) were purchased from Calbiochem (La Jolla, CA). L-(+)-2-Amino-4-phosphonobutyric (L-AP-4), O-phospho-L-serine (L-SOP), (RS)- α -cyclopropyl-4-phosphonophenylglycine (CPPG), (RS)- α -methylserine-O-phosphate (MSOP), *trans*-azetidine-2,4-dicarboxylic acid (tADA), (RS)-3,5-dihydroxyphenylglycine (DHPG), (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (APDC), (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (LCCG), 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt), 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), (2S)- α -ethylglutamic acid (EGLU), (RS)-1-amino-5-phosphonoinidan-1-carboxylic acid (APICA), NMDA, AMPA, Gln-Val-Pro-Ser-Arg-Pro-Asn-Arg-Ala-Pro (dynamin inhibitory peptide, P4), and myristoylated P4 (myr-P4) were obtained from Tocris (Ellisville, MO). All drugs were made as 1000 \times stocks in water, except for L-AP-4 and CPPG, which were made as 1000 \times stocks in 0.1N NaOH, and PP1, PP2, and PP3, which were made as 1000 \times stocks in DMSO (Sigma). All 1000 \times stocks were diluted to 10 \times stocks with culture medium immediately before addition to the neuronal cultures, which were main-

tained *in vitro* for 14 d. All inhibitors were added 30 min before the primary treatment and were present for the whole duration of the primary treatment.

Transfection of siRNA or plasmid DNA. Double-stranded small interfering RNA (siRNA) targeting rat β -arrestin 1 (NM_012910) and β -arrestin 2 (NM_012911) were purchased from Ambion (Austin, TX). Their sequences are 5'-GGCGAGUCUACGUGACACUtt-3' and 5'-GGACCGGAASGUGUUUGUGtt-3', respectively. Plasmid constructs for HA-tagged wild-type MAP kinase kinase (MEK) 1 (wt-MEK) and dominant-negative MEK1 (dn-MEK) (K97M mutant) were described previously (Yuen et al., 2005). At 10–12 d *in vitro*, midbrain neuronal cultures were transfected with 1 μg of siRNA per 3.5 cm dish or transfected with 1.6 μg of plasmid DNA per well in 12-well plates, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Subsequent treatments were performed 48 h after transfection.

Preparation of cell extracts. For each treatment, one 3.5 cm dish that was initially seeded with $2 \times 10^5/\text{cm}^2$ midbrain neurons was washed three times with PBS. The culture was lysed in 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 Na₃VO₄) for 20 min on ice. The lysates were centrifuged at 4°C at 16,000 \times g for 20 min, and the supernatant was analyzed by Western blotting. Subcellular fractionation studies were performed as described previously (Wong et al., 2002), with minor modifications. Briefly, PBS-washed neuronal cultures were harvested by scraping in 200 μl cold-buffer A (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM MgCl₂, 0.1 mM EDTA, 15% glycerol, 0.1 mM Na₃VO₄, 50 mM NaF, 1 mM DTT, 0.5 mM PMSF, and protease inhibitors). Cell lysates were incubated on ice for 10 min with gentle shaking. After 5 min spin at 200 \times g at 4°C, supernatant S1 and precipitant P1 were collected. S1 was centrifuged at 16,000 \times g for 15 min and the supernatant S2 was used as the cytosol cell extract. P1 was suspended in 100 μl ice-cold buffer B (10 mM HEPES, pH 7.4, 10 mM KCl, 1 mM MgCl₂, 0.1 mM Na₃VO₄, 50 mM NaF, 0.1% NP40, 1 mM DTT, 0.5 mM PMSF, and protease inhibitors) and incubated on ice for 30 min with gentle shaking. Then the suspension was centrifuged for 15 min at 16,000 \times g at 4°C, and supernatant S3 was saved as the nuclear cell extract. In parallel experiments, cultured neurons were washed thrice in PBS, scraped off the plate and lysed in 200 μl 1% hot SDS with glass homogenizer in a boiling water bath. After the lysate was centrifuged at 16,000 \times g for 15 min, the supernatant was used as the total cell lysate.

Immunocytochemistry and quantification of cell death. Immunocytochemistry was performed as described previously (Ren et al., 2003), with minor modifications. After drug treatments, cells growing on coverslips were fixed in 4% PFA for 20 min at room temperature, followed by three washes in PBS, 15 min permeabilization in 0.1% Triton X-100 in PBS, and 1 h incubation with 3% BSA in PBS. Subsequently, cells were incubated with primary antibodies for 2 h at room temperature and then for 16 h at 4°C. After washing in PBS, cells were incubated with appropriate secondary antibodies (Alexa Fluor 488-conjugated anti-rabbit IgG or Alexa Fluor 594-conjugated anti-mouse IgG) for 2 h at room temperature. In some experiments, cells were stained with the DNA-binding dye TOPRO-3 (Invitrogen) for 20 min at room temperature at this point to highlight the nucleus. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed after secondary antibody incubation using a kit from Roche. For quantification of cell death, at least five coverslips from independent experiments were examined for each condition. All TH⁺ neurons on a coverslip (100–200) were counted, together with a random selection of 120–250 TH⁻ neurons. All fluorescence images were acquired on a confocal microscope from Bio-Rad (Hercules, CA). Monochrome images (512 \times 512 pixels) were pseudocolored and merged with the National Institutes of Health (NIH) Image J software.

Measurement of free or polymerized tubulin in the cell. Free or polymerized tubulin from midbrain neuronal cultures was extracted as described previously (Ren et al., 2005). Briefly, midbrain neuronal cultures maintained in 3.5 cm dishes *in vitro* for 14 d were washed twice at 37°C with 1 ml Buffer A containing the following: 0.1 M MES (2-[N-morpholino]ethanesulfonic acid), pH 6.75, 1 mM MgSO₄, 2 mM EGTA, 0.1 mM EDTA, and 4 M glycerol. After the cultures were incubated at 37°C

for 5 min in 600 μ l 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 16,000 \times 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 μ l of 25 mM Tris, pH 6.8, plus 0.5% SDS, and contained tubulin originally in a polymerized state (i.e., as 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 imaging.

Results

mGluRIII agonists attenuate the selective toxicity of rotenone on cultured midbrain TH⁺ neurons

Our previous study has shown that rotenone exhibits much greater toxicity on TH⁺ neurons than on TH⁻ neurons in rat embryonic midbrain neuronal cultures (Ren et al., 2005). To identify neuroprotective agents against the selective toxicity of rotenone on dopaminergic neurons, we treated midbrain neuronal cultures with rotenone (100 nM for 12 h) in the presence or absence of various metabotropic glutamate receptor agonists (all at 100 μ M for 12.5 h, added 0.5 h before rotenone application). Treated cultures were costained for TH (green), TUNEL (red), and NeuN (blue). Rotenone induced significant apoptosis of TH⁺ neurons, but had much less toxicity on TH⁻ neurons (Fig. 1B). In addition to the positive TUNEL staining in the nucleus, which signified cell death, TH⁺ neurons treated with rotenone also exhibited prominent segmentation of the processes that was often associated with neuronal death (Park et al., 1996). The selective toxicity of rotenone on TH⁺ neurons was markedly reduced by L-AP-4, a selective mGluRIII agonist (Fig. 1C), but not by the selective mGluRI agonist tADA (Fig. 1D) or the mGluRII agonist APDC (Fig. 1E). The protective effect of L-AP-4 was blocked by the selective mGluRIII antagonist CPPG (40 μ M for 13 h, added 30 min before L-AP-4) (Fig. 1F). As summarized in Figure 1G, rotenone toxicity on TH⁺ neurons was significantly attenuated only by the selective mGluRIII agonists L-AP-4 and L-SOP ($p < 0.001$; $n = 7$ coverslips), but not by mGluRI agonists (tADA and DHPG) or mGluRII agonists (APDC and the less selective LCCG). Furthermore, the protective effects of mGluRIII agonists were blocked by the selective mGluRIII antagonists CPPG and MSOP (Fig. 1G). These mGluRIII antagonists per se had no significant effect on rotenone toxicity, and neither did mGluRI antagonists such as CPCCOEt (100 μ M) and MPEP (2 μ M), nor mGluRII antagonists such as EGLU (100 μ M) or APICA (100 μ M) (data not shown). When added alone, none of these mGluR agonists or antagonists had any significant toxicity on TH⁺ neurons (data not shown).

We also examined the protective effect of L-AP-4 against var-

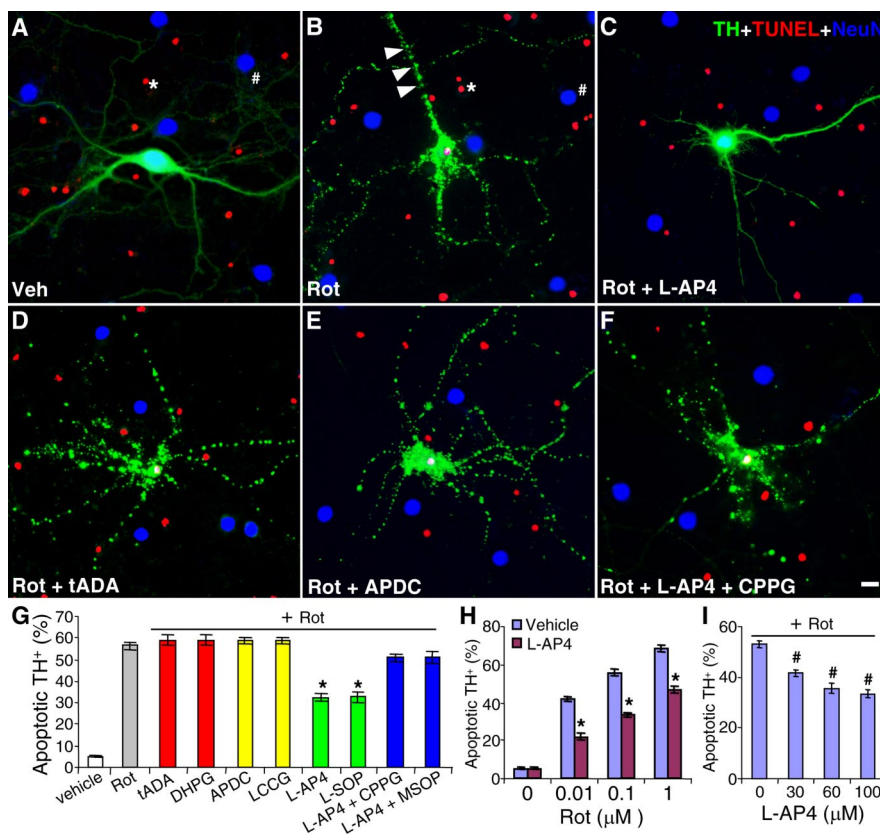


Figure 1. Activation of group III metabotropic glutamate receptors attenuates rotenone toxicity on TH⁺ neurons in rat embryonic midbrain neuronal cultures. **A–F**, Midbrain neuronal cultures were treated for 12 h with vehicle (Veh) (**A**), rotenone (Rot, 100 nM) (**B**), rotenone plus L-AP-4 (mGluRIII agonist, 100 μ M) (**C**), rotenone plus tADA (mGluRI agonist, 100 μ M) (**D**), rotenone plus APDC (mGluRII agonist, 100 μ M) (**E**), and rotenone plus L-AP-4 and CPPG (mGluRIII antagonist, 40 μ M) (**F**). Fixed neurons were costained for TH (green), TUNEL (red), and NeuN (blue). Arrowheads, Broken process. *Condensed nuclei of dead glial cells (killed by AraC in the media). #Nucleus of TH⁺ neuron. Scale bar: (in **F**) **A–F**, 10 μ m. **G**, Statistical summary of data represented in **A–F** showed that rotenone toxicity on TH⁺ neurons was selectively attenuated by activation of group III metabotropic glutamate receptors. tADA (100 μ M) and DHPG (200 μ M) are mGluRI agonists, APDC (100 μ M) and LCCG (200 μ M) are mGluRII agonists, L-AP-4 (100 μ M) and L-SOP (100 μ M) are mGluRIII agonists, and CPPG (40 μ M) and MSOP (200 μ M) are mGluRIII antagonists. * $p < 0.001$ ($n = 7$) versus rotenone alone. **H**, mGluRIII agonist L-AP-4 (100 μ M) significantly reduced the toxicity of rotenone at different concentrations. * $p < 0.001$ ($n = 7$) versus rotenone at the corresponding concentration. **I**, L-AP-4 decreased the toxicity of rotenone (100 nM) in a dose-dependent manner. # $p < 0.001$ ($n = 6$) versus 0 μ M L-AP-4. Error bars indicate SE.

ious concentrations of rotenone and found that the mGluRIII agonist significantly reduced the toxicity of rotenone from 10 nM to 1 μ M ($p < 0.001$; $n = 7$) (Fig. 1H). The protective effect of L-AP-4 against rotenone (100 nM) was dose-dependent, reaching a plateau at 100 μ M (Fig. 1I). Thus, all subsequent experiments used 100 nM rotenone and 100 μ M L-AP-4.

The protective effect of mGluRIII agonists against rotenone toxicity is dependent on the MAP kinase pathway

To study the mechanism underlying the protective effect of mGluRIII agonists, we treated midbrain neuronal cultures with rotenone and L-AP-4 in the absence or presence of various inhibitors that block signaling cascades emanating from the activated mGluRIII receptors. The protective effect of L-AP-4 against rotenone-induced apoptosis of TH⁺ neurons (Fig. 1C) was blocked by the MEK inhibitor 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) (20 μ M for 13 h, added 30 min before L-AP-4) (Fig. 2A). This was confirmed by another MEK inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto) butadiene (U0126) (Fig. 2F). Both MEK inhibitors significantly abrogated the protective effect of L-AP-4 ($p <$

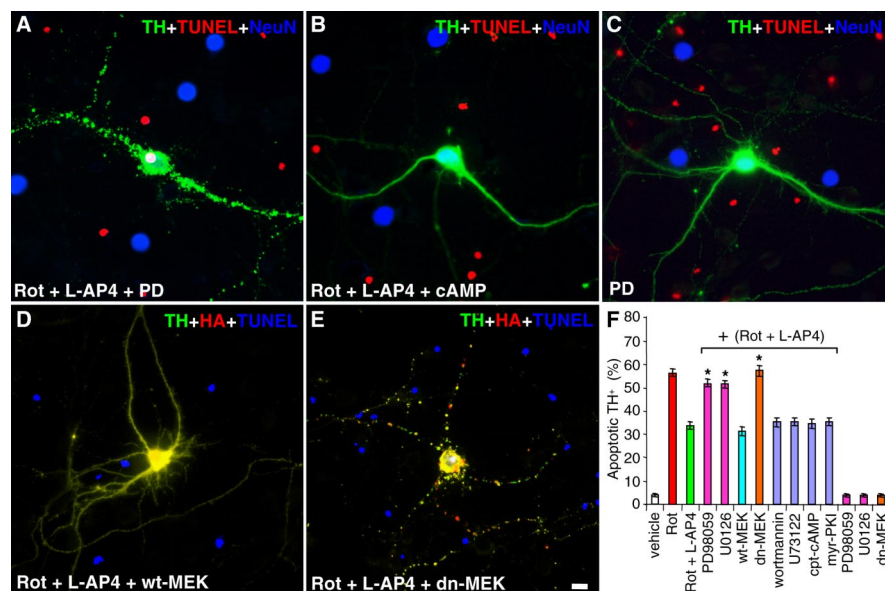


Figure 2. The protective effect of mGluRIII agonists against rotenone toxicity is dependent on the MAP kinase pathway. **A–C**, Midbrain neuronal cultures were treated with rotenone (Rot; 100 nM), L-AP-4 (100 μ M), and the MEK inhibitor PD98059 (PD; 20 μ M) (**A**), rotenone, L-AP-4, and cpt-cAMP (50 μ M) (**B**), or PD98059 alone (**C**). Costaining for TH (green), TUNEL (red), and NeuN (blue) showed that the protective effect of L-AP-4 against rotenone toxicity on TH⁺ neurons was reversed by the MEK inhibitor. **D, E**, Midbrain neuronal cultures transfected with HA-tagged wild-type MEK (**D**) or dominant-negative MEK (**E**) were treated with rotenone (100 nM) plus L-AP-4 (100 μ M). Costaining for TH (green), HA (red), and TUNEL (blue) showed that the protective effect of L-AP-4 against rotenone was not obviously affected by wild-type MEK1, but was abrogated by dominant-negative MEK1. Scale bar: (in **E**) **A–E**, 10 μ m. **F**, A bar plot showing that the protective effect of L-AP-4 against rotenone was significantly blocked by the MEK inhibitor PD98059 or U0126 (both at 20 μ M), or overexpression of dn-MEK, but was not significantly affected by overexpression of wt-MEK, the PI-3 kinase inhibitor wortmannin (0.5 μ M), the phospholipase C inhibitor U73122 (1 μ M), the PKA activator cpt-cAMP (50 μ M), or myr-PKI (1 μ M). By itself, PD98059 (20 μ M), U0126 (20 μ M), or overexpression of dn-MEK had no significant toxicity on TH⁺ neurons. * $p < 0.001$; $n = 5$ versus Rot plus L-AP-4. Error bars indicate SE.

0.001; $n = 5$) and had no appreciable toxicity on TH⁺ neurons (Fig. 2C,F). To substantiate the involvement of MEK in the protective effect of L-AP-4, we transfected midbrain neuronal cultures with HA-tagged wild-type or dominant-negative MEK1 (Mansour et al., 1994). When overexpressed in cells, the catalytically inactive MEK1 mutant (K97M) acts dominantly to suppress the function of endogenous MEK (Mansour et al., 1994). Expression of the dominant-negative construct abolished the protective effect of L-AP-4 (Fig. 2E,F). In contrast, wild-type MEK1 did not significantly change the protective effect of L-AP-4 ($p > 0.60$ vs rotenone plus L-AP-4; $n = 5$) (Fig. 2D,F).

On the other hand, inhibition of phosphatidylinositol-3 (PI-3) kinase by wortmannin (0.5 μ M for 13 h) did not significantly change the effect of L-AP-4 (Fig. 2F). Similar results were obtained with 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) (10 μ M for 13 h), another specific inhibitor of PI-3 kinase (data not shown). Blocking PLC with U73122 (1 μ M for 13 h) did not significantly alter the effect of L-AP-4 (Fig. 2F). We also examined the involvement of protein kinase A (PKA) by using its activator chlorphenylthio (cpt)-cAMP (50 μ M for 13 h) or myristoylated PKA inhibitory peptide (myr-PKI; 1 μ M for 13 h). Neither PKA reagent significantly changed the effect of L-AP-4 (Fig. 2B,F). Application of PD98059, U0126, wortmannin, LY294002, U73122, cpt-cAMP, or myr-PKI by itself had no significant toxicity on TH⁺ or TH⁻ neurons, neither did transfection of wild-type or dominant-negative MEK1 per se (data not shown). Together, these results suggest that activation of mGluRIII attenuates rotenone toxicity through a mechanism dependent on the MAP kinase pathway.

mGluRIII agonists induce rapid and transient activation of the MAP kinase ERK

To examine the impact of mGluRIII agonists on MAP kinase, we treated midbrain neuronal cultures with L-AP-4 and blotted total cell lysates with an antibody that recognizes activated ERK1/2, which are doubly phosphorylated on the Thr-Glu-Tyr motif in the activation loop of the kinases (Payne et al., 1991). As shown in Figure 3A, L-AP-4 (100 μ M) induced a rapid activation of ERK1 and ERK2, which returned to the basal levels within 30 min. The amounts of total ERK1/2 were not changed by the treatment. We also examined the dose-response of L-AP-4-induced ERK activation. A 3 min treatment with L-AP-4 at various concentrations activated ERK1/2 in a dose-dependent manner (Fig. 3B). Because ERK1/2 were efficiently activated in response to 100 μ M L-AP-4 treatment for 3 min, all subsequent mechanistic studies used this regimen.

To ascertain that L-AP-4-induced ERK activation was mediated selectively by group III metabotropic glutamate receptors, we treated midbrain neuronal cultures with specific mGluRIII antagonists for 30 min before the addition of L-AP-4 (100 μ M for 3 min). L-AP-4-induced activation of ERK1/2 was completely blocked by the specific mGluRIII antagonist CPPG (40 μ M) and greatly diminished by another antagonist, MSOP (200 μ M) (Fig. 3C). To confirm the effect of L-AP-4, we treated midbrain neuronal cultures with another selective mGluRIII agonist L-SOP. Application of L-SOP (100 μ M for 3 min) induced strong ERK activation, which was completely blocked by the selective mGluRIII antagonist CPPG (40 μ M) and greatly attenuated by another antagonist MSOP (200 μ M) (Fig. 3D). Together, these data showed that activation of group III metabotropic glutamate receptors activated the MAP kinase ERK.

mGluRIII-induced ERK activation is dependent on Src, β -arrestin 2, and dynamin-mediated endocytosis

A previous study in human embryonic kidney 293 (HEK293) cells overexpressing mGluR4, one member of the group III metabotropic glutamate receptors, has indicated the involvement of Src in L-AP-4-induced activation of ERK1/2 (Iacovelli et al., 2004). To examine whether L-AP-4-induced ERK activation is dependent on Src in midbrain neuronal cultures, we treated the cultures with the selective Src kinase inhibitors PP1 and PP2, or the inactive structural homolog PP3 (all at 20 μ M) for 30 min before the addition of L-AP-4 (100 μ M for 3 min). As shown in Figure 4A, L-AP-4-induced ERK activation was completely blocked by the Src inhibitor PP1 or PP2, but not by the inactive homolog PP3. By themselves, these Src-targeted compounds had no significant effect on ERK. Using an antibody that recognizes activated Src, which is phosphorylated on Tyr⁴¹⁸ or equivalent in the catalytic domain (Cooper and MacAuley, 1988), we found that L-AP-4 (100 μ M) also activated Src in a rapid and transient manner similar to that of ERK, reaching a plateau around 2–5

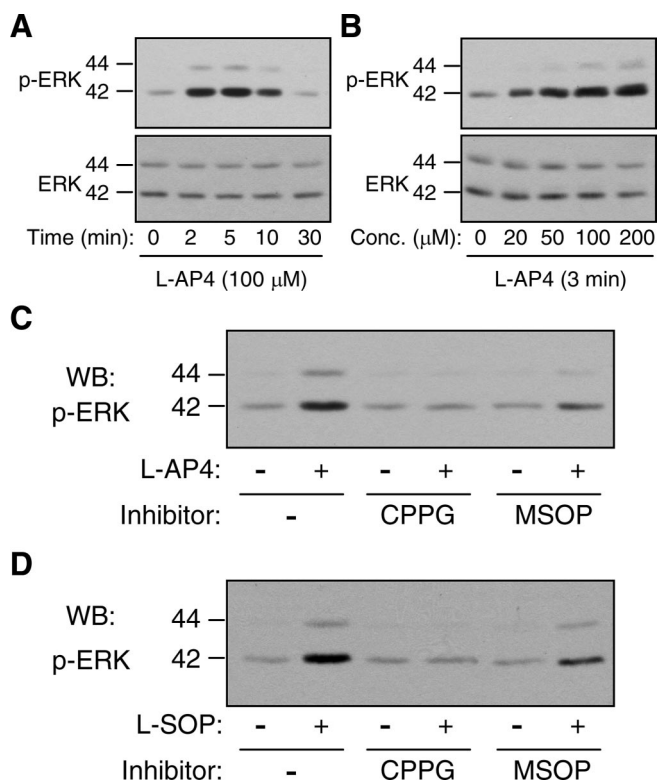


Figure 3. mGluRIII agonists induce rapid and transient activation of ERK in midbrain neuronal cultures. **A, B**, Rat embryonic midbrain neuronal cultures were treated with 100 μM L-AP-4 for the indicated durations (**A**) or 3 min with L-AP-4 at the indicated concentrations (**B**). Total cell lysates were analyzed by Western blotting with anti-phospho-ERK1/2 (top panels) or anti-ERK1/2 (bottom panels). The experiments were repeated five times with similar results. **C, D**, Midbrain neuronal cultures were treated without or with the selective mGluRIII agonist L-AP-4 (**C**) or L-SOP (**D**) (both at 100 μM for 3 min) in the presence or absence of the specific mGluRIII antagonists CPPG (40 μM) or MSOP (200 μM), which was added 30 min before the agonist. Total cell lysates were immunoblotted with anti-phospho-ERK1/2. Activation of ERK1/2 by L-AP-4 (**C**) or by L-SOP (**D**) was blocked by either mGluRIII antagonist. Similar results were obtained from three independent experiments.

min and diminishing to the basal level at 30 min (Fig. 4B). When midbrain neuronal cultures were treated with L-AP-4 for 3 min at various concentrations, a plateau effect was seen at 100 μM L-AP-4 (Fig. 4C). Thus, both the time course and dose–response of L-AP-4-induced Src activation parallel those of ERK1/2 (Fig. 3A, B).

Group III metabotropic glutamate receptors couple to G_i/G_o classes of G-proteins (Conn and Pin, 1997), which normally do not activate Src kinase. An alternative mechanism that may lead to Src activation is mediated by the association of ligand-bound GPCRs with β -arrestin, which recruits and activates Src (Lefkowitz and Shenoy, 2005). To examine this possibility, we transfected midbrain neuronal cultures with siRNA against β -arrestin 1 or β -arrestin 2 to knock down their expression, respectively. As shown in Figure 4D, the level of β -arrestin 1 or β -arrestin 2 was greatly reduced by the corresponding siRNA, but not the other (top two panels). L-AP-4-induced activation of ERK1/2 (third panel) and Src (bottom panel) was blocked by knockdown of β -arrestin 2, but not β -arrestin 1. The expression level of total ERK1/2 or total Src was unaffected by either siRNA (data not shown). These data suggest that L-AP-4-induced ERK activation, which is downstream of Src, is dependent on β -arrestin 2.

β -arrestin-mediated GPCR signaling relies on clathrin-dependent endocytosis (Lefkowitz and Shenoy, 2005), which re-

quires dynamin to pinch off clathrin-coated vesicles (Sever, 2002). To examine whether mGluRIII-induced ERK activation is dependent on clathrin/dynamin-mediated endocytosis, we treated midbrain neuronal cultures with myr-P4 or the unmyristoylated, membrane-impermeable control peptide of the same sequence (P4). Once inside the cell, the P4 peptide disrupts the binding between dynamin and amphiphysin (Gout et al., 1993) and thus blocks clathrin-dependent endocytosis (Kittler et al., 2000). As shown in Figure 4E, myristoylated P4 peptide completely blocked L-AP-4-induced activation of ERK1/2, whereas the unmyristoylated control peptide had no significant effect. Neither peptide affected the expression level of ERK1/2 (data not shown). Together, the above data suggest that mGluRIII agonists activate ERK through clathrin/dynamin-mediated endocytosis of the receptors and ensuing β -arrestin 2-dependent activation of Src.

mGluRIII-activated ERK targets cytosolic rather than nuclear substrates

To study the cellular consequence of L-AP-4-induced ERK activation, we examined the subcellular localization of activated ERK1/2, as well as total ERK1/2, in midbrain neuronal cultures treated with L-AP-4 (100 μM for 3 min). Interestingly, ERK1/2 activated by L-AP-4 stimulation were largely localized in the cytoplasm and did not translocate to the nucleus (Fig. 5, compare A, B). This was in sharp contrast to the rapid nuclear translocation of ERK induced by the activation of ionotropic glutamate receptors with a 3 min treatment of 100 μM NMDA plus 100 μM AMPA (N+A) (Fig. 5, compare A, C). Similar results were obtained when the treated cultures were stained for total ERK, which did not show any significant nuclear translocation in response to L-AP-4 (Fig. 5, compare D, E) but exhibited marked accumulation in the nucleus after the treatment with NMDA plus AMPA (Fig. 5, compare D, F). We also used the microtubule-associated protein MAP2 as a marker for the cytosolic compartment. Costaining with anti-MAP2 and anti-phospho-ERK showed that L-AP-4-activated ERK remained in the cytoplasm, with very little in the nucleus (Fig. 5, compare G, H), whereas ERK activated by NMDA plus AMPA was prominently translocated into the nucleus (Fig. 5, compare G, I). This was confirmed by costaining with anti-MAP2 and anti-ERK (Fig. 5J–L).

To substantiate our findings, we performed subcellular fractionation of midbrain neuronal cultures treated for 3 min with the vehicle control, N+A, or 100 μM L-AP-4. Nuclear and cytosolic fractions as well as total cell lysates were blotted with anti-phospho-ERK or anti-ERK. The amount of activated ERK or total ERK in the nuclear fraction was not significantly increased by L-AP-4, but was greatly elevated by NMDA plus AMPA (Fig. 5M, left). On the other hand, both N+A and L-AP-4 treatments induced a marked increase of phospho-ERK, but not total ERK, in the cytosolic fraction (Fig. 5M, middle). When total cell lysates were examined, L-AP-4 and N+A treatment induced similar increases in activated ERK, but not total ERK (Fig. 5M, right). Thus, subcellular fractionation studies confirmed the immunostaining results. Both readouts suggest that L-AP-4-activated ERK1/2 target cytosolic rather than nuclear substrates.

mGluRIII agonist attenuates rotenone- or colchicine-induced microtubule depolymerization in an MEK-dependent manner

Because activated ERK may phosphorylate microtubule-associated proteins and stabilize microtubules (Cassimeris and Spittle, 2001), we examined the involvement of microtubules in the protective effect of mGluRIII agonists against rotenone tox-

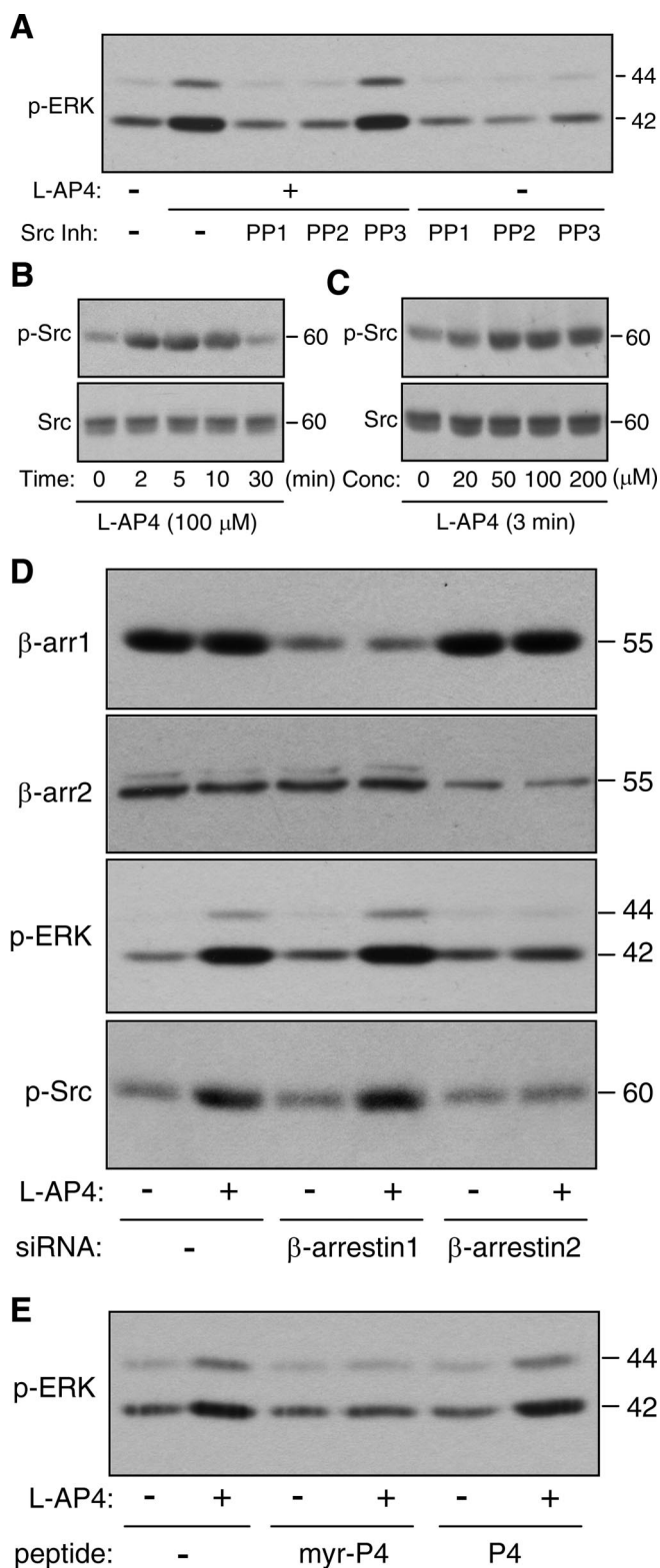


Figure 4. mGluRIII-induced ERK activation is mediated by Src, β-arrestin 2, and dynamin. **A**, Midbrain neuronal cultures were treated with the selective Src kinase inhibitors PP1 and PP2 or their inactive homolog PP3 (all at 20 μM) for 30 min before and during L-AP-4 treatment (100 μM for 3 min). Total cell lysates were immunoblotted with anti-phospho-ERK1/2. L-AP-4-induced ERK activation was blocked by Src inhibitors, but not their inactive homolog. **B, C**, Total cell lysates from midbrain neuronal cultures treated with L-AP-4 were blotted with anti-phospho-Src (top) or anti-Src (bottom). L-AP-4 induced rapid and transient activation of Src in the same time (**B**) and dose (**C**) courses as those of ERK1/2. **D**, Midbrain neuronal cultures were transfected with siRNA of β-arrestin 1 or β-arrestin 2 and treated without or with L-AP-4 (100 μM for 3 min). Total cell lysates were blotted with antibodies against β-arrestin 1, β-arrestin 2,

icity, which is dependent on microtubules (Ren et al., 2005). Midbrain cultures were treated with rotenone (100 nM for 3 h) and costained 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. 6, compare A, B), as well as in TH⁻ neurons (data not shown). Frank dendritic segmentation or retraction was seen in rotenone-treated neurons. Coapplication of L-AP-4 (100 μM for 3.5 h, added 0.5 h before rotenone) greatly attenuated rotenone-induced microtubule depolymerization, as well as dendritic segmentation and retraction (Fig. 6, compare B, C). The effect of L-AP-4 was abrogated by the MEK inhibitor PD98059 (20 μM for 4 h, added 0.5 h before L-AP-4) (Fig. 6, compare C, D). Similar results were found when we treated the cultures with colchicine (10 μM for 3 h) instead of rotenone. Colchicine-induced microtubule depolymerization (Fig. 6F) was attenuated by L-AP-4 (Fig. 6G). The rescuing effect of L-AP-4 (Fig. 6G) was abolished by PD98059 (Fig. 6H).

We counted the number of neurons with at least one obvious microtubule (defined operationally by a continuous length of at least 10 μm) and calculated the percentage of neurons without any microtubule to quantify the degree of microtubule depolymerization. The situations in TH⁺ neurons and TH⁻ neurons were essentially the same. Thus, we only showed data on TH⁺ neurons in Figure 6I. Rotenone completely depolymerized microtubules in 51.8 ± 1.3% of TH⁺ neurons. Coapplication of the mGluRIII agonist L-AP-4 significantly mitigated the effect of rotenone (33.6 ± 1.9%; *p* < 0.001; *n* = 5 coverslips). The effect of L-AP-4 was totally blocked by the MEK inhibitor PD98059 (49.0 ± 2.0%; *p* > 0.20 vs rotenone alone; *n* = 5). Similar results were found with colchicine (Fig. 6I). We also used another selective inhibitor of MEK, U0126 (20 μM), and obtained similar results as those of PD98059 in experiments using rotenone or colchicine (data not shown). By itself, L-AP-4, PD98059 (Fig. 6E, I), or U0126 (data not shown) had no significant effect on microtubules, in comparison with the vehicle control.

To substantiate these results, 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 top panel of Figure 6J, the amount of free tubulin was greatly increased in response to rotenone (100 nM for 30 min). L-AP-4 (100 μM for 60 min, added 30 min before rotenone) significantly reduced the level of free tubulin. This effect was abolished by the MEK inhibitor PD98059 (20 μM for 90 min, added 30 min before L-AP-4). Similar results were obtained with colchicine (Fig. 6J). Reciprocal changes in response to these agents were observed in the amount of polymerized tubulin (Fig. 6J, bottom). By itself, L-AP-4 or PD98059 had no significant effect on the amount of free tubulin or polymerized tubulin, in comparison to the vehicle control (Fig. 6J). We also used U0126 and obtained similar results as those from PD98059 (data not shown). Results from three

phospho-ERK1/2 or phospho-Src, respectively. Knockdown of β-arrestin 2 but not β-arrestin 1 blocked L-AP-4-induced activation of ERK and Src. Similar results were obtained from four independent experiments. **E**, Midbrain neuronal cultures were treated without or with L-AP-4 (100 μM for 3 min). Myristoylated dynamin inhibitory peptide P4 or unmodified P4 (both at 25 μM) was added 30 min before and during the L-AP-4 treatment. Total cell lysates were blotted with antibodies against phospho-ERK1/2. L-AP-4-induced ERK activation was blocked by myristoylated dynamin inhibitory peptide, but not by the unmodified P4 peptide, which is membrane impermeable. Similar results were obtained from at least three independent experiments.

independent experiments were quantified in Figure 6*K*. Together, these two different readouts on the polymerization state of microtubules suggest that activation of mGluRIII attenuates rotenone- or colchicine-induced microtubule depolymerization through an MEK-dependent mechanism.

mGluRIII agonist reduces colchicine toxicity on TH⁺ neurons in an MEK-dependent manner

The results presented so far suggest that mGluRIII agonists attenuate rotenone toxicity by stabilizing microtubules against rotenone-induced depolymerization. If so, mGluRIII agonists such as L-AP-4 should also protect against the selective toxicity of colchicine on TH⁺ neurons (Ren et al., 2005). To test this, we treated midbrain neuronal cultures with or without colchicine (10 μ M for 12 h) in the absence or presence of L-AP-4 (100 μ M for 12.5 h, added 0.5 h before colchicine). The selective toxicity of colchicine on TH⁺ neurons (Fig. 7*B*) was greatly attenuated by L-AP-4 (Fig. 7*C*). This protective effect was abolished by coapplication of the MEK inhibitor PD98059 or U0126 (20 μ M for 13 h, added 0.5 h before L-AP-4) (Fig. 7*D, G*). To substantiate the involvement of MEK, midbrain neuronal cultures transfected with HA-tagged wild-type or dominant-negative MEK1 were treated with colchicine (10 μ M for 12 h) and L-AP-4 (100 μ M for 12.5 h, added 0.5 h before L-AP-4). Expression of dominant-negative MEK1 abolished the protective effect of L-AP-4 (Fig. 7*F*), whereas the wild-type construct had no significant impact (Fig. 7*E*). Results from five different experiments were quantified in Figure 7*G*, which suggests that, through an MEK-dependent mechanism, mGluRIII agonists reduce the death of TH⁺ neurons induced by microtubule depolymerization.

The protective effect of L-AP-4 against rotenone toxicity is occluded by Taxol

If the protective effect of L-AP-4 against rotenone-induced apoptosis is mediated through microtubule stabilization, then coapplication of Taxol should occlude the effect of L-AP-4, because both of them would act on the same target. To test this, we treated midbrain neuronal cultures with various combinations of rotenone (100 nM for 12 h), Taxol (10 μ M for 12.5 h, added 0.5 h before rotenone) and L-AP-4 (100 μ M for 12.5 h, added 0.5 h before rotenone). Rotenone-induced apoptosis of TH⁺ neurons (Fig. 8*A*) was significantly reduced by Taxol (Fig. 8*B*), L-AP-4 (Fig. 8*C*) or Taxol plus L-AP-4 (Fig. 8*D*). Application of Taxol (Fig. 8*E*) or L-AP-4 alone (data

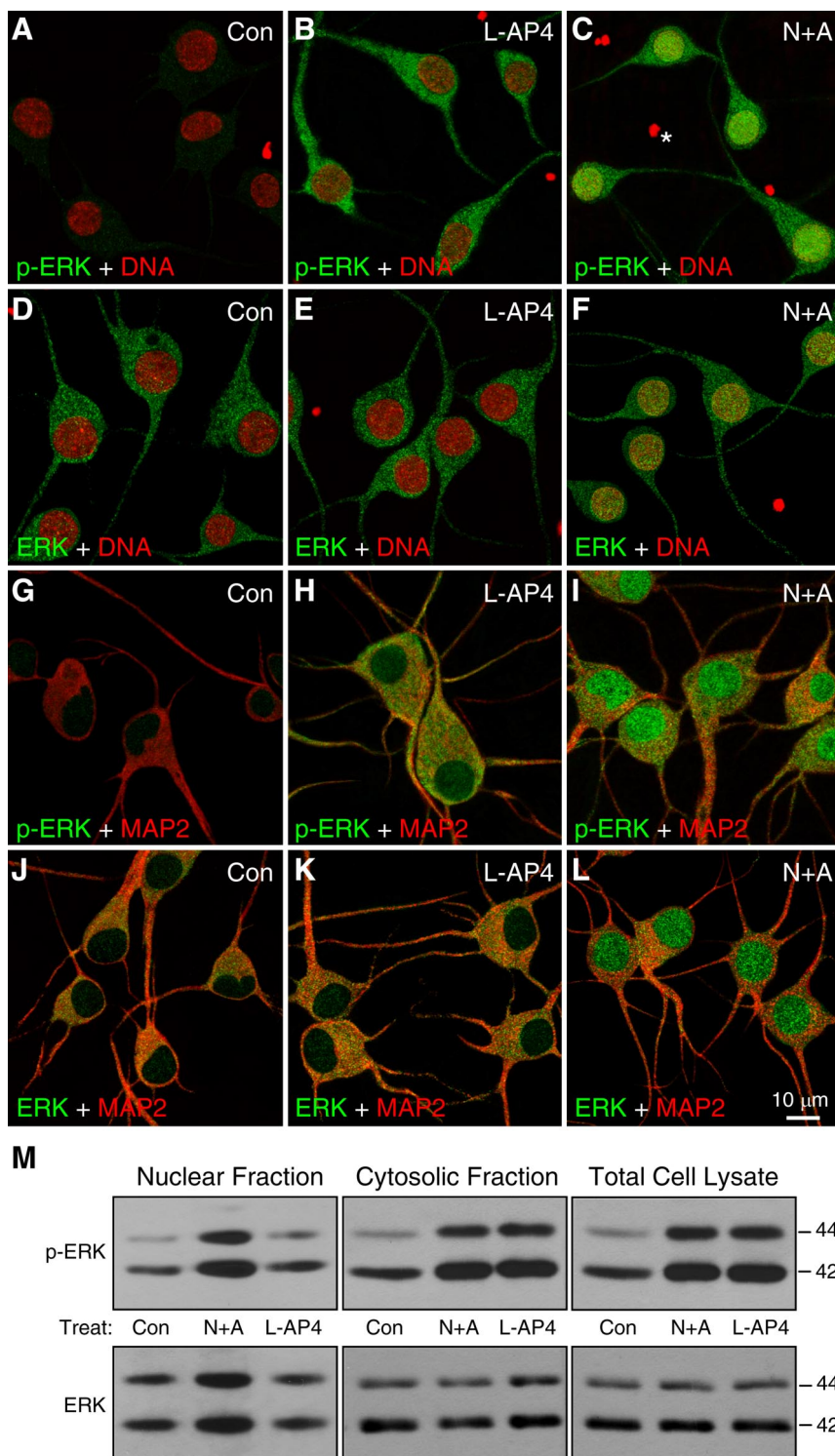


Figure 5. mGluRIII-activated ERK targets cytosolic rather than nuclear substrates. **A–L**, Midbrain neuronal cultures were treated for 3 min with vehicle control (Con), 100 μ M L-AP-4, or N+A, respectively. Fixed cultures were costained with the DNA-binding dye TOPRO-3 (red; DNA) and anti-phospho-ERK (green; **A–C**) or anti-ERK (green; **D–F**). Cultures were also costained with anti-MAP2 (red) and anti-phospho-ERK (green; **G–I**) or anti-ERK (green; **J–L**). Scale bar: (in **L**) **A–L**, 10 μ m. *Condensed nuclei of dead glial cells (killed by AraC in the media). **M**, Nuclear or cytosolic fractions, as well as total cell lysates, from midbrain neuronal cultures treated as above were immunoblotted with anti-phospho-ERK or anti-ERK. Similar results were obtained from four independent experiments.

not shown) did not significantly affect the survival of TH⁺ neurons. Results from five separate experiments showed that the effects of L-AP-4 (32.4 \pm 1.9%) or Taxol (36.8 \pm 1.7%) on rotenone toxicity were similar. The combination of L-AP-4 and Taxol

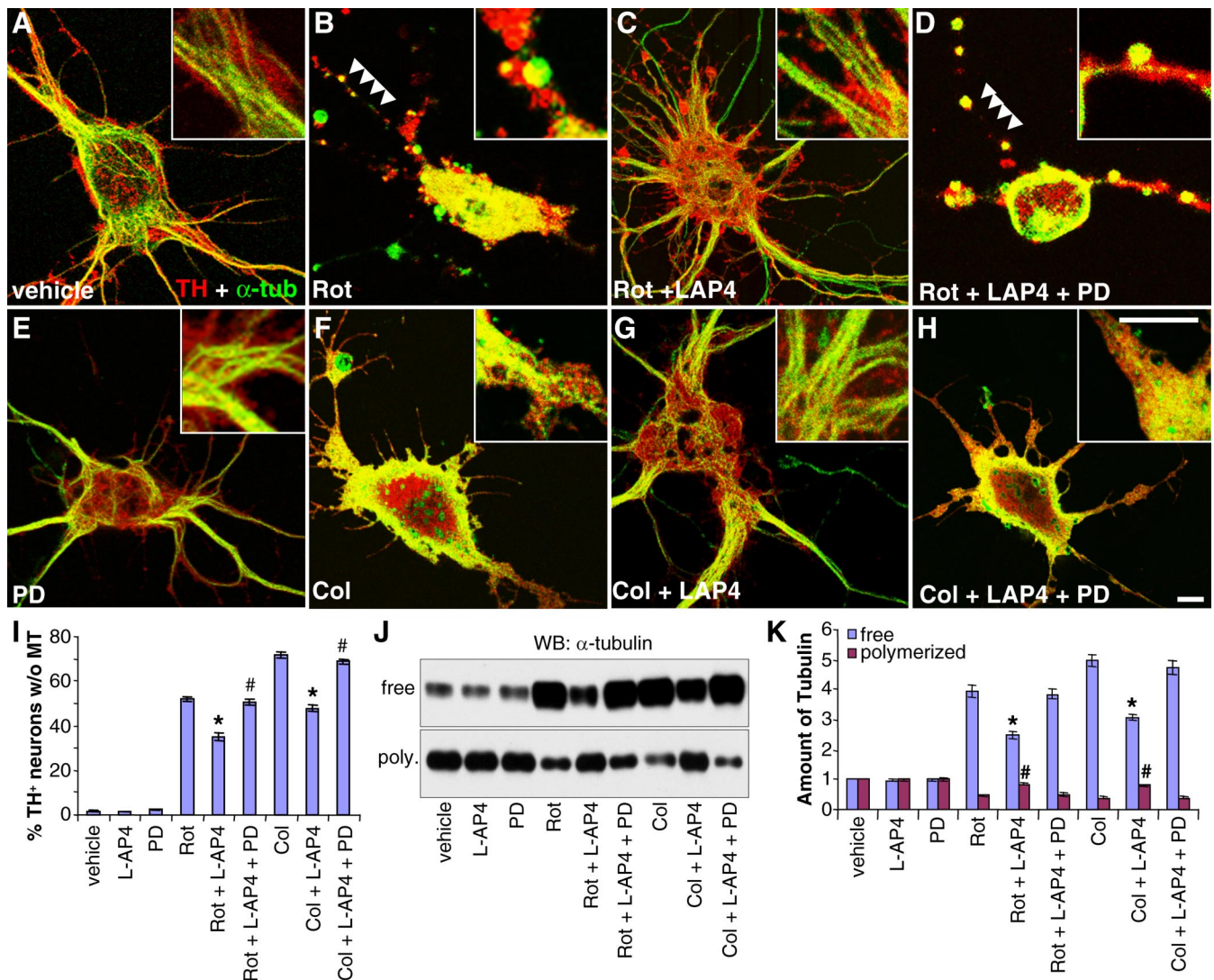


Figure 6. L-AP-4 attenuates rotenone- or colchicine-induced microtubule depolymerization in an MEK-dependent manner. **A–H**, The polymerization states of microtubules in TH⁺ neurons in midbrain neuronal cultures treated with vehicle (**A**), rotenone (**B**), rotenone and L-AP-4 (**C**), rotenone, L-AP-4, and PD98059 (**D**), PD98059 (**E**), colchicines (**F**), colchicine and L-AP-4 (**G**), and colchicine, L-AP-4, and PD98059 (**H**). PD98059 (20 μM) was added 30 min before L-AP-4 (100 μM), 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 costained with anti-TH (red) and anti-α-tubulin (green). Insets, enlarged portion of proximal processes. Scale bars: (in **H**) **A–H**, insets, 10 μm. Arrowheads, Dendritic segmentation. **I**, Statistical summary of data represented in (**A–H**) showed that the effect of L-AP-4 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. L-AP-4 greatly attenuated the 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. Error bars indicate SE.

(30.8 ± 2.0%) had an effect similar to the individual agents (*p* > 0.05, among the three conditions) (Fig. 8*F*). Thus, the data suggest that L-AP-4 and Taxol are acting on the same target, microtubules, by stabilizing them against rotenone-induced depolymerization.

Discussion

The present study is based on our previous finding that the microtubule-depolymerizing activity of rotenone plays a critical role in its selective toxicity on dopaminergic neurons (Ren et al., 2005). Microtubule depolymerization disrupted vesicular transport, which relies on intact microtubules and motor proteins (Goldstein, 2003). This leads to accumulation of vesicles in the cell body and increased cytosolic concentration of the neurotransmitter leaked from the vesicles (Ren et al., 2005). In the case

of dopaminergic neurons, elevated cytosolic concentration of dopamine causes greater oxidative stress because of dopamine oxidation (Ren et al., 2005), which seems to explain the selectivity of rotenone toxicity. Thus, microtubule stabilization appears to be an effective strategy to protect against rotenone toxicity on DA neurons.

We found in this study that activation of group III metabotropic glutamate receptors (mGluRIII) attenuated rotenone toxicity by stabilizing microtubules. The protective effect was highly selective among agonists and antagonists of metabotropic glutamate receptors (mGluR); only mGluRIII agonists significantly reduced the selective toxicity of rotenone (Fig. 1) or colchicine (Fig. 7) on midbrain TH⁺ neurons in culture. Many lines of evidence showed that this protective effect was dependent on microtubules. The observation that mGluRIII agonists such as

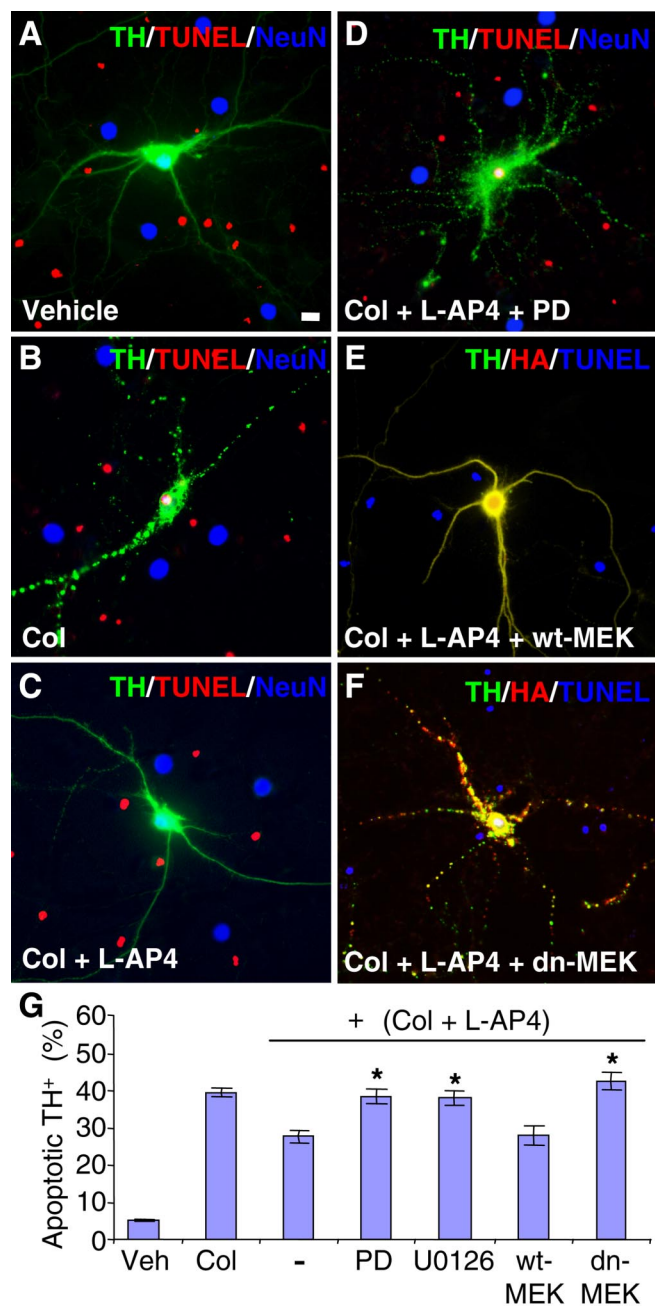


Figure 7. L-AP-4 reduces colchicine-induced death of TH⁺ neurons in a MEK-dependent manner. **A–D**, Midbrain neuronal cultures treated with vehicle (**A**), colchicine (Col, 10 μ M) (**B**), colchicine and L-AP-4 (100 μ M) (**C**), or colchicine, L-AP-4, and PD98059 (20 μ M) (**D**) were costained for TH (green), TUNEL (red), and NeuN (blue). L-AP-4 markedly reduced the selective toxicity of colchicine on TH⁺ neurons. The effect was reverted by the MEK inhibitor PD98059. **E**, **F**, Midbrain neuronal cultures transfected with HA-tagged wt-MEK1 (**E**) or dn-MEK1 (**F**) were treated with colchicine and L-AP-4. Costaining of the cultures for TH (green), HA (red), and TUNEL (blue) showed that the protective effect of L-AP-4 against colchicine toxicity was unaffected by wt-MEK1 (**E**) and was abolished by dn-MEK1 (**F**). **G**, The protective effect of L-AP-4 against colchicine was abolished by the MEK inhibitors PD98059 or U0126 (both at 20 μ M), or overexpression of dominant-negative MEK1, but was not significantly affected by overexpression of wild-type MEK1. * $p < 0.05$ versus Col plus L-AP-4; $n = 5$. Scale bar: (in **A–F**), 10 μ m. Error bars indicate SE.

L-AP-4 attenuated the toxicity of two structurally different microtubule-depolymerizing agents, rotenone and colchicine, suggests that the common target is microtubules. This notion is corroborated by the results that the protective effect of L-AP-4

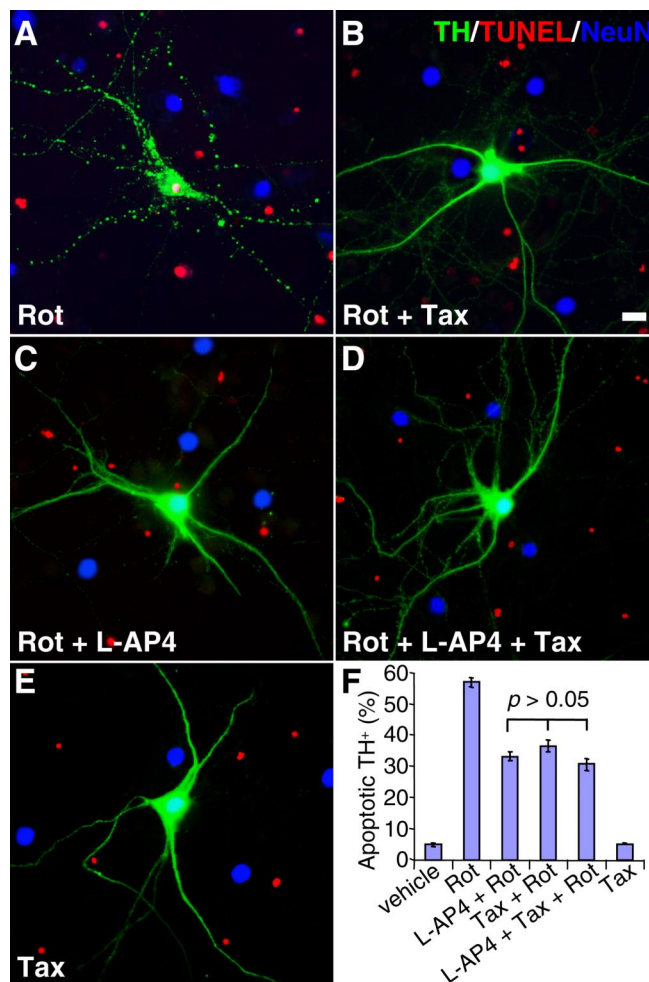


Figure 8. The protective effect of L-AP-4 against rotenone toxicity is occluded by the microtubule-stabilizing drug Taxol. **A–E**, Midbrain neuronal cultures treated with rotenone (Rot; 100 nM) (**A**), rotenone and Taxol (Tax; 10 μ M) (**B**), rotenone and L-AP-4 (100 μ M) (**C**), rotenone, L-AP-4, and Taxol alone (**E**) were costained for TH (green), TUNEL (red), and NeuN (blue). Scale bar: (in **B–E**), 10 μ m. **F**, Statistical summary of data represented in (**A–E**) showed that the protective effects of L-AP-4, Taxol, and L-AP-4 plus Taxol were not significantly different from each other ($p > 0.05$; $n = 5$). Error bars indicate SE.

was occluded by the microtubule-stabilizing drug Taxol (Fig. 8). The direct evidence that L-AP-4 stabilized microtubules against depolymerizing agent such as rotenone or colchicine is provided in Figure 6. We quantified microtubule depolymerization by directly counting neurons without an obvious microtubule and by measuring the amount of free or polymerized tubulin in the cell (Ren et al., 2005). Both methods yielded the same conclusion that activation of mGluRIII reduced microtubule depolymerization induced by rotenone or colchicine.

The ability of mGluRIII agonists to attenuate the selective toxicity (Figs. 2, 7) and the microtubule-depolymerizing effect (Fig. 6) of rotenone or colchicine appears to be dependent on activation of the MAP kinase pathway. We used pharmacological agents and molecular manipulations to demonstrate this point. MEK inhibitors such as PD98059 and U0126 completely blocked the protective effect of L-AP-4 against rotenone (Fig. 2) or colchicine (Fig. 7). This is substantiated by transfection studies using the dominant-negative MEK1 mutant, which also abolished the protection of L-AP-4 (Figs. 2, 7). Furthermore, the microtubule-stabilizing effect of L-AP-4 against rotenone- or colchicine-

induced depolymerization was totally abrogated by inhibiting MEK (Fig. 6). It should be noted that neither MEK inhibitors nor MEK1 expression constructs had any significant effect on the survival of TH⁺ neurons (Fig. 2) or the polymerization state of microtubules inside (Fig. 6). These reagents exhibited their effects only in the presence of mGluRIII agonists such as L-AP-4, which activated the MAP kinase pathway through a novel mechanism.

Our results showed that L-AP-4 induced a rapid and transient activation of the MAP kinase ERK in midbrain neurons (Fig. 3). This effect is apparently mediated by the β -arrestin pathway, a novel paradigm of GPCR signaling that relies on clathrin/dynamin-dependent endocytosis of the activated GPCR (Lefkowitz and Shenoy, 2005). Independent of the canonical G_i-coupled pathway activated by mGluRIII agonists (Conn and Pin, 1997), the β -arrestin pathway leads to MAP kinase activation either directly (Luttrell et al., 2001) or through Src (Luttrell et al., 1999). Previous studies have shown that L-AP-4-induced ERK activation in HEK293 cells transfected with mGluR4 (an mGluRIII) is independent of adenylyl cyclase but is dependent on Src (Iacovelli et al., 2004). Our studies in native midbrain neurons showed that L-AP-4 activated ERK in a Src-dependent manner (Fig. 4A). In addition, L-AP-4-induced activation of ERK or Src was dependent on β -arrestin 2 (Fig. 4D) and clathrin/dynamin-dependent endocytosis (Fig. 4E). ERK activated through this novel mechanism apparently targeted cytosolic, rather than nuclear, substrates. Both immunostaining and subcellular fractionation studies showed that L-AP-4-activated ERK largely remained in the cytoplasm and exhibited no significant nuclear translocation (Fig. 5). This is in sharp contrast to the situation of ERK activated by many other stimuli, such as agonists of ionotropic glutamate receptors, which induce rapid and robust nuclear translocation of ERK (Fig. 5). Increasing evidence has suggested that the MAP kinase cascade activated through the β -arrestin pathway may regulate events in the cytosol that are distinct from the canonical transcription-related effects of MAP kinase in the nucleus (Lefkowitz, 2004).

Microtubule-associated protein kinase is also known as mitogen-activated protein kinase. In postmitotic neurons, many stimuli that activate MAP kinase do not serve any mitogenic function. L-AP-4-activated ERK, which was retained in the cytosol, may stabilize microtubules against rotenone- or colchicine-induced depolymerization by phosphorylating MAPs. The stability of microtubules, particularly those in neurons, is strongly influenced by many microtubule-associated proteins, some of which are exclusively or highly expressed in neurons. For example, tau is a neuron-specific MAP that is highly enriched in axons, whereas MAP2 is a neuronal MAP found in dendrites. Overexpression of microtubule-associated protein such as tau or MAP2 bundles and stabilizes microtubules in distinct manners (Takemura et al., 1992). The interactions of MAPs with microtubules are highly regulated by the phosphorylation states of the MAPs, which are controlled by a variety of kinases including MAP kinases such as ERK (Cassimeris and Spittle, 2001). Although additional studies are necessary to pinpoint which microtubule-associated protein(s) are involved in the microtubule-stabilizing effect of mGluRIII agonists, it seems reasonable to assume that L-AP-4-activated ERK may stabilize microtubules by phosphorylating MAPs. It is noteworthy that activated Src kinase exhibits punctate localization on cytoskeleton (Weernink and Rijkse, 1995; Luttrell et al., 1999). The β -arrestin complex recruited by the activated mGluRIII, which includes Src and components of the MAP kinase path-

way, may serve to efficiently direct the activated ERK kinase to substrates associated with cytoskeleton.

Apoptosis is a cellular program that takes many hours. We examined the protective effect of mGluRIII agonists against rotenone-induced apoptosis of TH⁺ neurons in the time frame of 12 h. The protective effect could be correlated to the rapid and transient activation of ERK (Fig. 3) because activated ERK targeted cytosolic substrates (e.g., microtubule-associated proteins) (Fig. 5) to stabilize microtubules (Fig. 6). Once microtubules are stabilized, apoptosis of TH⁺ neurons is significantly attenuated (Ren et al., 2005). The activation of ERK does not need to be persistent to trigger MAP phosphorylation and microtubule stabilization.

The ability of mGluRIII agonists to attenuate rotenone toxicity on dopaminergic neurons adds further proof to the neuroprotective effects of metabotropic glutamate receptors (Nicoletti et al., 1996; Flor et al., 2002). Recent studies increasingly suggest that manipulation of the metabotropic system in basal ganglia may be an effective therapeutic strategy for Parkinson's disease (Conn et al., 2005), which is characterized by a relatively selective loss of nigral DA neurons. Because the rest of basal ganglia nuclei are largely intact, rebalancing the activities of the basal ganglia network by selective activation of certain metabotropic glutamate receptors appears to significantly alleviate PD-like symptoms in animal models (Conn et al., 2005). For example, intracerebroventricular injection of L-AP-4 markedly reduces locomotor deficiencies in various animal models of PD (Valenti et al., 2003; Macinnes et al., 2004). The neuroprotective effect of L-AP-4 on DA neurons (this study) may work synergistically with its ability to modulate synaptic transmission in non-DA neurons in basal ganglia (Conn et al., 2005), especially at the early stage of PD before the heavy loss of nigral DA neurons.

In conclusion, results presented in the present study showed that agonists of group III metabotropic glutamate receptors attenuated the selective toxicity of rotenone on midbrain TH⁺ neurons by activating the MAP kinase pathway to stabilize microtubules. This neuroprotective effect of mGluRIII agonists may provide a useful strategy in developing novel therapies for Parkinson's disease.

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