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ORIGINAL ARTICLE

JNK3 Mediates Paraquat- and Rotenone-Induced Dopaminergic Neuron Death

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Abstract

Mechanistic studies underlying dopaminergic neuron death may identify new drug targets for the treatment of Parkinson disease. Epidemiological studies have linked pesticide exposure to increased risk for sporadic Parkinson disease. Here, we investigated the role of c-*Jun*-N-terminal kinase 3 (JNK3), a neural-specific JNK isoform, in dopaminergic neuron death induced by the pesticides rotenone and paraquat. The role of JNK3 was evaluated using RNA silencing and gene deletion to block JNK3 signaling. Using an antibody that recognizes all isoforms of activated JNKs, we found that paraquat and rotenone stimulate JNK phosphorylation in primary cultured dopaminergic neurons. In cultured neurons transfected with *Jnk3*-specific siRNA and in neurons from $Jnk3^{-/-}$ mice, JNK phosphorylation was nearly abolished, suggesting that JNK3 is the main JNK isoform activated in dopaminergic neurons by these pesticides. Paraquat- and rotenone-induced death of dopaminergic neurons was also significantly reduced by *Jnk3* siRNA or *Jnk3* gene deletion, and deletion of the *Jnk3* gene completely attenuated paraquat-induced dopaminergic neuron death and motor deficits in vivo. Our data identify JNK3 as a common and critical mediator of dopaminergic neuron death induced by paraquat and rotenone, suggesting that it is a potential drug target for Parkinson disease treatment.

Key Words: Dopaminergic neuron, JNK3, Paraquat, Parkinson disease, Reactive oxygen species, Rotenone.

INTRODUCTION

Parkinson disease (PD) is the second most common aging-related neurodegenerative disorder and characterized by selective and progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the midbrain (1). Although several gene mutations have been linked to familial PD, the etiology of sporadic PD, which accounts for 90% to 95% of all PD cases, is largely undefined. The prevailing hypothesis is that increased risk for sporadic PD is most likely associated with multiple factors including exposure to environmental toxicants and a complex interaction between genetic factors and environmental exposure to neurotoxicants. At the time of PD diagnosis, approximately 80% of dopaminergic neuron terminals in the striatum and 60% of dopaminergic neurons in the SNpc are already lost in PD brains (2). Current therapy focuses on symptomatic relief, and there is a great need to identify neuroprotective therapies that will slow disease progression. The elucidation of cell death mechanisms common to several models of PD may identify new drug targets for PD treatment.

Epidemiological studies have suggested a potential link between increased risk for PD and occupational exposure to pesticides, including paraquat (3). Alarmingly, a recent study based on exposure data from the Central Valley of California suggests that residential exposure to paraquat and maneb, 2 commonly used pesticides in that region, increases the risk for PD (4). Paraquat and rotenone are pesticides used worldwide (5) that selectively kill dopaminergic neurons in primary cultures (6,7). Chronic administration of paraquat or rotenone to rodents in vivo induces many key features of PD, including motor deficits, loss of dopaminergic neurons, and the presence of α-synuclein–containing inclusion bodies (8–16). Thus, treatment of rodents or cultured cells with paraquat or rotenone provides a useful model to study mechanisms of dopaminergic neuron death associated with PD.

The c-*Jun*-N-terminal kinases (JNKs) are mitogen-activated protein kinases that are preferentially activated by cell stress including environmental stress and toxic chemical insults (17,18). Notably, JNKs are activated by oxidative stress, a key mechanism underlying dopaminergic neuron death associated with PD pathogenesis (2,19–22). Increasing in vitro and in vivo evidence suggests a role for JNK in the pathogenesis of dopaminergic neuron death in PD models (23). For example, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) activates JNK and the JNK kinase MKK4 in the nigrostriatal system (24). Gene transfer of the JNK interacting

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protein-1, which interferes with JNK signaling, protects dopaminergic neurons in the MPTP model of PD (25). CEP-1347, a pharmacological inhibitor of mixed lineage kinases that are upstream kinase kinases of JNK, attenuates MPTP-mediated nigrostriatal dopaminergic neuron loss and MPTP-induced JNK activation (26). In addition, dopaminergic neuron death induced by rotenone, paraquat, and 6-hydroxydopamine all require JNK activation (7,27–30). There are 3 *Jnk* genes: *Jnk1*, *Jnk2*, and *Jnk3* (31). The goal of this study was to determine whether JNK3, the only neural-specific JNK isozyme, is critical for dopaminergic neuron death induced by paraquat or rotenone.

MATERIALS AND METHODS

Animals

Generation and characterization of the $Jnk3^{-/-}$ mice were described (32). The JNK3 heterozygotes ($Jnk3^{+/-}$) were bred to generate littermates of $Jnk3^{+/+}$, $Jnk3^{+/-}$, and $Jnk3^{-/-}$ embryos for culture or adult mice for in vivo paraquat administration.

Primary Mesencephalic Neuron Cultures and Drug Treatments

Primary cultured dopaminergic neurons were prepared from mesencephalon of E14 C57/BL6 mouse embryos (Charles Rivers, Wilmington, MA) or $Jnk3^{+/+}$, $Jnk3^{+/-}$, and $Jnk3^{-/-}$ individual embryos, as described (33). For single-embryo cultures, polymerase chain reaction (PCR) genotyping of the embryos was performed after the culture, and the results were matched to each embryo at the end of the experiment. Therefore, all experiments were performed blinded regarding the status of *Jnk3* genotype. Cells were plated ($3–5 \times 10^4$ cells in 100 μL) on 9-mm-diameter Aclar embedding film (Electron Microscopy Sciences, Fort Washington, PA) that had been precoated with 100 μg/mL poly-d-lysine and 4 μg/mL laminin (BD Bioscience, Bedford, MA). The cultures were maintained at 37°C in a humidified 7% carbon dioxide atmosphere. After overnight incubation, fresh culture medium was added. Thereafter, half of the medium was changed every 48 hours.

Rotenone (Sigma, St Louis, MO) was dissolved in dimethyl sulfoxide as 10-mmol/L stock solution, and paraquat (Sigma) was dissolved in water as 400 mmol/L stock. Drugs were diluted in N2 medium (Invitrogen, Carlsbad, CA) right before the drug treatments. When cell cultures were treated with rotenone, the final concentration of dimethyl sulfoxide did not exceed 0.0001%. All drug treatments were performed in defined serum-free N2 medium. Half of the medium was replaced with N2 medium on the day before drug treatment and then again at the time of drug treatment. Cultures treated with vehicle were used as controls.

Immunoblot Analysis

After treatments, protein lysates were prepared from cells and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis gel electrophoresis and Western blotting, as described (6). Anti–active caspase-3 and anti–phospho-JNK antibodies (p-Thr183 and p-Tyr185) were purchased from Cell Signaling Technology (Beverly, MA). Anti–β-actin antibody was from Sigma.

siRNA

Small interfering RNA (siRNA) against *Jnk1*, *Jnk2*, *or Jnk3*, and scrambled control nonsilencing siRNA were described (34) and purchased from Qiagen (Valencia, CA). *Jnk1* siRNA sequence is 5′ GAAGCUCAGCCGGCCAUUUdTdT 3′; *Jnk2* siRNA, 5′ GCCUUGCGCCACCCGUAUAdTdT 3′; *Jnk3* siRNA, 5′ GCCAGGGACUUGUUGUCAAdTdT 3′; scrambled siRNA, 5′ UUCUCCGAACGUGUCACGUdTdT 3′. E14 Sprague-Dawley rat mesencephalic primary neurons were plated on 24-well or 48-well plates at 80% density and transfected with siRNA using TransMessenger Transfection Reagent (Qiagen) according to the manufacturer's protocol. The final siRNA concentration was 2.5 μg/mL. An enhanced green fluorescent protein expression vector was cotransfected to identify transfected cells (4:1 for siRNA:enhanced green fluorescent protein).

Immunocytochemistry and Quantification of Neurons and JNK Phosphorylation

Neuron cultures were fixed with 4% paraformaldehyde/4% sucrose for 30 minutes at room temperature and blocked for 1 hour in blocking buffer (PBS containing 5% bovine serum albumin, 5% normal goat serum, and 0.1% Triton X-100). Cells were then incubated with primary antibodies in blocking buffer at 4°C overnight. Primary antibodies included mouse monoclonal antibody against tyrosine hydroxylase ([TH] 1:500; Sigma), rabbit polyclonal antibody against TH (1:50,000; Pel-Freez, Rogers, AR), and rabbit polyclonal antibody against phospho-JNK (1:100; Cell Signaling). After 3 washes with PBS, cells were incubated at room temperature for 1 hour with appropriate secondary antibodies: Alexa Fluor-488, -568 or -660 goat anti-rabbit IgG and Alexa Fluor-488, -568, or -660 goat anti-mouse IgG (1:200; Molecular Probes, Eugene, OR). Cells incubated as above but without primary antibodies were used as negative controls for staining specificity (data not shown).

Cells immunostained for TH and having neurites twice the length of the soma were scored as TH-positive cells under a fluorescence microscope (Leica, Heidelberg, Germany). All TH-positive cells on a 9-mm-diameter Aclar embedding film were scored. The total number of TH-positive cells in vehicle control–treated cultures of each genotype or siRNA-transfected group was defined as 100% survival. The number of TH-positive cells in rotenone- or paraquat-treated cultures was divided by that in the respective vehicle control cultures and presented as percentage survival.

To quantify the extent of JNK phosphorylation in TH-positive neurons, cells were double stained with anti–p-JNK and anti-TH antibody. Images of 8 random fields from each well were captured using a fluorescent microscope equipped with a digital camera (Axiovert 200M; Zeiss, Thornwood, NY). The staining intensity of p-JNK in the transfected TH-positive neuron population was quantified using National Institutes of Health ImageJ program (http://rsb.info.nih.gov/ij/). All transfected TH-positive neurons in captured images were scored, and the average p-JNK level per TH-positive neuron was calculated. At least 50 TH-positive cells were used to quantify p-JNK per data point. The p-JNK staining units were arbitrary, and the relative intensity was normalized to the vehicle

control–treated group. All cell counting was performed in a blinded manner.

Drug Administration In Vivo

Ten-week-old male and female $Jnk3^{+/+}$ (n = 11) and $Jnk3^{-/-}$ (n = 13) littermates were group housed (4–5 per cage) in a room maintained under constant temperature (72°F–74°F) and humidity and a 12-hour light/dark cycle (light on at 6:00 AM) with ad libitum access to food and water. Mice were habituated to the vivarium for at least 1 week before commencement of experiments. Mice were injected intraperitoneally with either saline (vehicle) (n = 5 for $Jnk3^{+/+}$ and n = 6 for $Jnk3^{-/-}$) or 10 mg/kg paraquat dichloride hydrate (Sigma) dissolved in saline (n = 6 for $Jnk3^{+/+}$ and n = 7 for $Jnk3^{-/-}$) twice per week for 6 weeks for a total of 12 doses, a procedure modified from previous reports (13, 14, 35). Body weights were determined before each injection to ensure that there was no marked weight loss. Animals were cared for and treated in accordance with National Institutes of Health and the University of Washington Animal Care Committee guidelines.

Behavioral Tests and Data Analysis

At 24 hours after the last paraquat injection, mice were subjected to an open-field test and then rotarod test. All behavioral tests were carried out by the researcher without knowledge of the genotype of the animals. Data from both male and female mice were pooled and analyzed together because no statistically significant difference was observed between males and females.

For the open-field test, mice were tested in a rectangular open-field arena (48 × 48 cm, 30 cm high) made of white hard plastic board (36, 37). Locomotor activity was recorded with a video camera placed on the ceiling above the arena and connected to an automated video tracking system (Polytrack system; San Diego Instruments, San Diego, CA). Locomotor activity was measured and analyzed as total distance traveled (in centimeters) during the first 30 minutes.

At 3 days after the open-field test, mice were tested on a rotarod apparatus as described (36, 38). Briefly, mice were placed on the stationary cylinder of the rotarod apparatus (San Diego Instruments). Before the test, mice were habituated on the apparatus for at least 3 consecutive trials in which the rod was kept at constant speed (first trial at 0 rpm and the rest at 4 rpm). Once the animals were able to stay on the rod rotating at 4 rpm for at least 60 seconds, they were subjected to the rotarod test. Mice were placed individually on the rod rotating at an accelerating speed from 4 to 29 rpm in 300 seconds. The time before animals fell off the rod was recorded with a maximum cutoff of 300 seconds. Mice were tested for 8 consecutive trials with at least 5-minute intervals. The data from the last 4 trials were averaged as the latency to fall.

Immunohistochemistry and Stereological Quantification of Neurons

After motor behavior tests, animals were intracardially perfused with heparinized saline followed by 4% paraformaldehyde. Brains were harvested and postfixed in 4% paraformaldehyde overnight and then cryoprotected in PBS with 30% sucrose for more than 2 days. Fixed brains were cut into 40-μm sections and collected in cryoprotectant. Sections were stored at −20°C before staining.

Sections were washed with PBS, blocked for nonspecific binding, and incubated with antibody against TH (1:1000; Sigma) in PBS containing 0.1% Triton X-100, 5% bovine serum albumin, and 5% goat serum. Sections were washed and incubated with avidin-biotin solution using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Sections were treated with 0.3% H_2O_2 for 10 minutes to quench endogenous peroxidase activity and then developed in 3-3′-diaminobenzidine tetrachloride for 2 to 5 minutes. Sections were rinsed several times in tap water and coverslip mounted.

The TH-positive neurons were counted stereologically and in a blinded manner, as described (39). Briefly, analysis was carried out with a Zeiss Axiovert 200M microscope equipped with a motorized stage in 3 axes. The boundaries of SNpc were outlined using the set of anatomical landmarks according to the mouse brain atlas (40). The number of TH-positive neurons was counted at high power (60×) at randomly chosen fields of the SNpc, and the number of total TH-positive neurons on each slide was calculated following optical dissector rules using dedicated software (Stereology Module of Slidebook, Olympus). We began cell counting at the first slide of the SNpc section when TH-positive neurons were visible, and then on every fourth slide throughout the entire SNpc. The estimate of total number of TH-positive neurons in each brain was calculated using the optical dissector method (39). Brains of 4 representative animals from each genotype/treatment group were analyzed for the stereology cell counting, and data were presented as percentage of TH-positive neurons. The total number of TH-positive cells in vehicle control–treated mice of each genotype was defined as 100% survival, and the number of TH-positive cells in paraquat-treated cultures was divided by that in the respective vehicle control mice and presented as percentage survival. The remaining brains were analyzed by nonstereological TH cell counting that yielded similar results (data not shown).

Statistical Analysis

Data were from at least 3 independent experiments, each with at least duplicate or triplicate determinations. Statistical analysis of data was performed using 2-way analysis of variance and post hoc Student *t*-test.

RESULTS

Paraquat and Rotenone Induce JNK Phosphorylation and Cell Death in Primary Cultured Dopaminergic Neurons

Paraquat and rotenone dose-response curves for death of TH-positive neurons were determined using primary neurons cultured from E14 mouse mesencephalon after 6 days in culture. Both paraquat and rotenone induced dopaminergic neuron death in a dose-dependent manner (Figs. 1A, B). Furthermore, caspase-3 was activated by both paraquat and rotenone (Figs. 1C, D), implicating apoptosis. To evaluate the contribution of JNK to cell death, we monitored JNK activity using an antibody that recognizes all isoforms of phosphorylated and

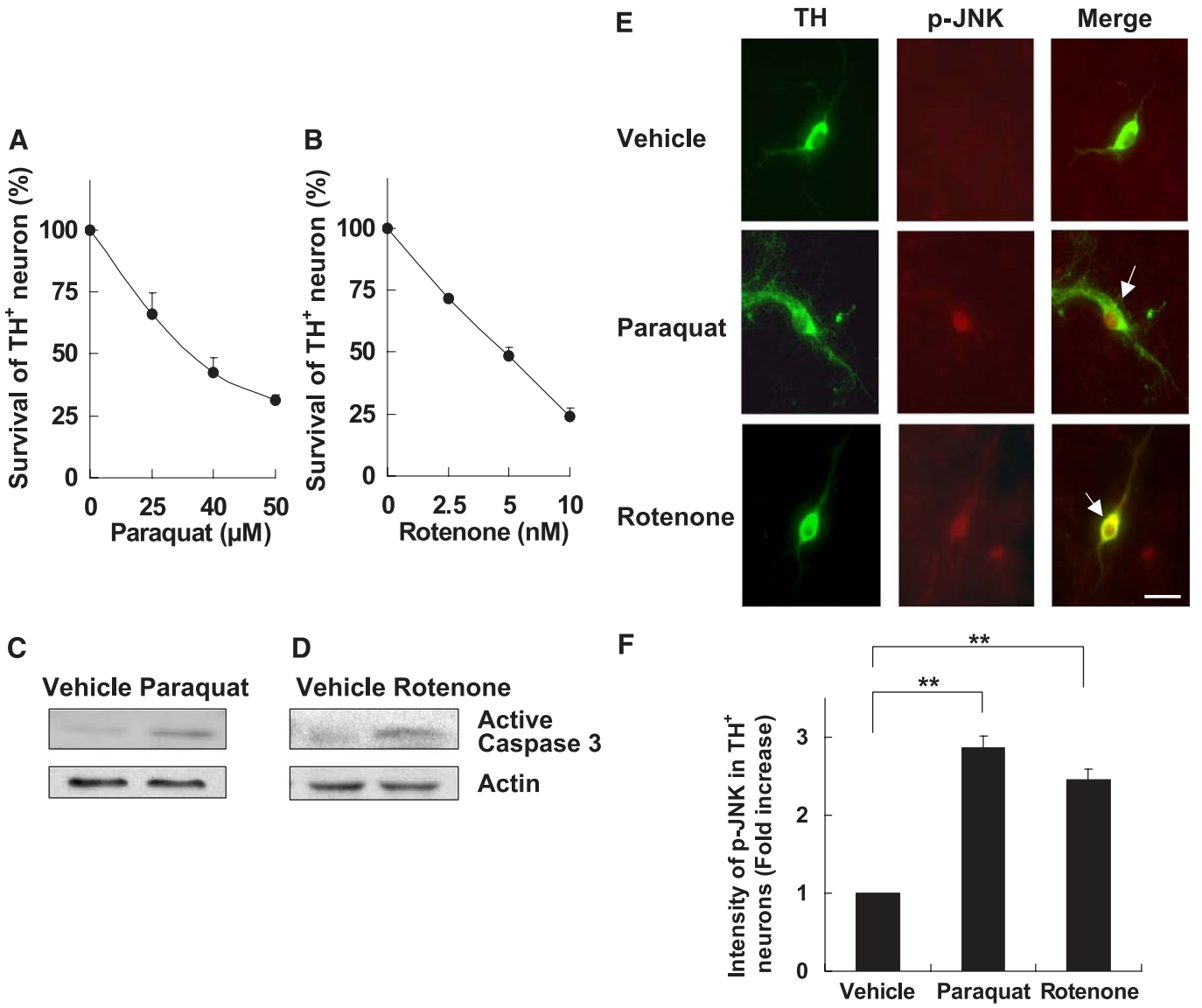

FIGURE 1. Treatment of paraquat and rotenone induces c-*Jun*-N-terminal kinase (JNK) phosphorylation and death of dopaminergic neurons. Primary ventral mesencephalic cultures were prepared from E14 mice and treated with paraquat, rotenone, or vehicle control. **(A)** Dose-response of paraquat (24 hours)-induced dopaminergic neuron death. **(B)** Dose-response of rotenone (24 hours)-induced dopaminergic neuron death. **(C, D)** Caspase-3 activation induced by paraquat (40 μmol/L, 12 hours) or rotenone (5 nmol/L, 12 hours). Actin was used as a loading control. **(E)** Paraquat (40 μmol/L, 8 hours) and rotenone (5 nmol/L, 8 hours) induce JNK phosphorylation in dopaminergic neurons. Images are representative photomicrographs of cells immunostained for tyrosine hydroxylase (TH) and for phosphorylated JNK (p-JNK). Arrows point to dopaminergic neurons with JNK phosphorylation, indicative of JNK activation. Scale bars = 20 μm. **(F)** Quantification of data in **(E)** for JNK phosphorylation in TH-positive dopaminergic neuron; **$p < 0.01$.

activated JNK (p-JNK). Treatment with either paraquat or rotenone stimulated JNK phosphorylation in primary dopaminergic neurons (Figs. 1E, F). The JNK phosphorylation after paraquat treatment is consistent with data in a previous study (29).

siRNA Silencing of *Jnk3* Attenuates Paraquat- and Rotenone-Induced JNK Phosphorylation and Dopaminergic Neuron Death

Semiquantitative reverse transcription–PCR confirmed that *Jnk3*-specific siRNA, but not the control siRNA, significantly reduced *Jnk3* mRNA levels (Fig. 2A). Importantly, transfection of the *Jnk3* siRNA significantly attenuated JNK phosphorylation after paraquat (Fig. 2B) or rotenone (Fig. 2C) treatment. However, JNK phosphorylation was not completely abolished by siRNA to JNK3. To assess the possibility that other JNK isoforms, JNK1 and JNK2, may also contribute, siRNA to JNK1 or JNK2 were used. The effectiveness and specificity of these siRNAs have been well documented (34). siRNA to JNK1 or JNK2 partially suppressed JNK phosphorylation, although the effect was much smaller than

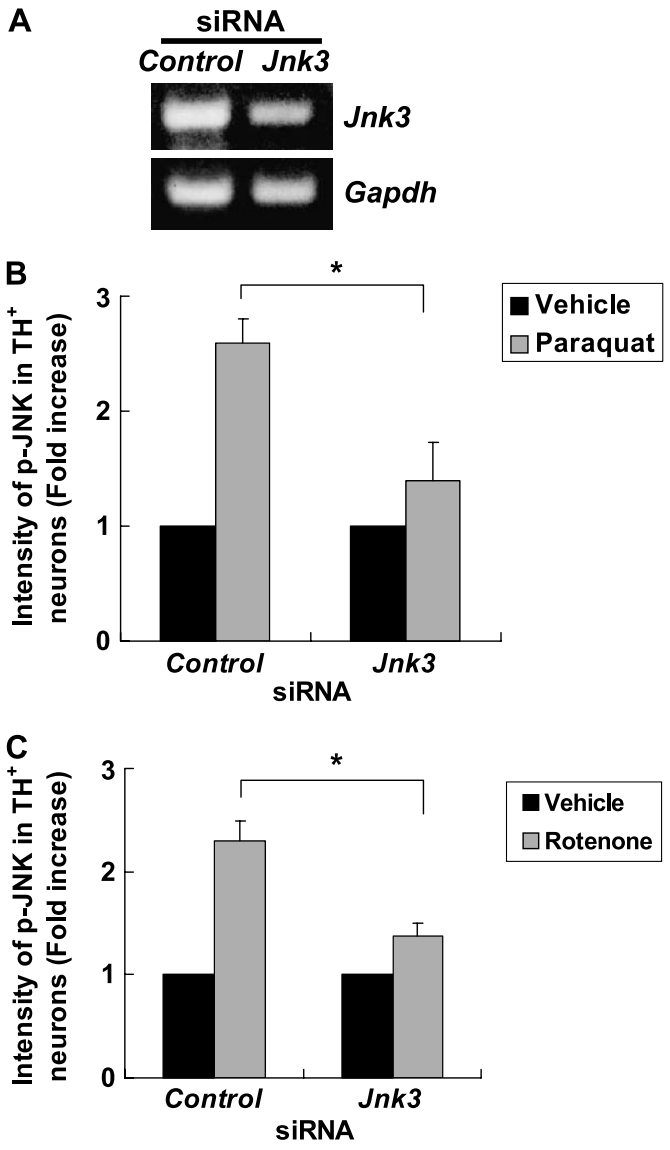

FIGURE 2. c-*Jun*-N-terminal kinase 3 (JNK3) is the major JNK isoform in dopaminergic neurons activated by paraquat or rotenone. **(A)** Transfection of siRNA specific for *Jnk3* reduces *Jnk3* mRNA levels. E14 mesencephalic neurons were transfected with *Jnk3* siRNA or control siRNA. Total mRNA was purified 24 hours later and analyzed by semiquantitative reverse transcription–polymerase chain reaction. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. **(B, C)** The *Jnk3* siRNA reduced paraquat **(B)**- or rotenone **(C)**-induced JNK activation in dopaminergic neurons. At 24 hours after siRNA transfection, cultures were treated with 40 μmol/L paraquat, 5 nmol/L rotenone, or their respective vehicle controls for another 8 hours. Cells were fixed and stained for tyrosine hydroxylase (TH) and p-JNK. The intensity of JNK phosphorylation in TH-positive dopaminergic neurons was quantified. *$p < 0.05$.

siRNA to JNK3 (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A110). These data suggest that JNK3 is the main JNK isozyme activated by paraquat and rotenone in dopaminergic neurons. Furthermore, transfection of the *Jnk3* siRNA almost completely blocked paraquat- or rotenone-induced dopaminergic neuron death (Fig. 3), suggesting that JNK3 activation plays a major role in these forms of cell death.

Jnk3 Gene Deletion Prevents Paraquat- and Rotenone-Induced JNK Phosphorylation and Dopaminergic Neuron Death

We used *Jnk3*-null mice to provide further genetic evidence supporting a role for JNK3 in dopaminergic neuron death. The $Jnk3^{+/-}$ mice were bred to generate $Jnk3^{-/-}$ embryos so that $Jnk3^{+/+}$ and $Jnk3^{+/-}$ littermates could be used

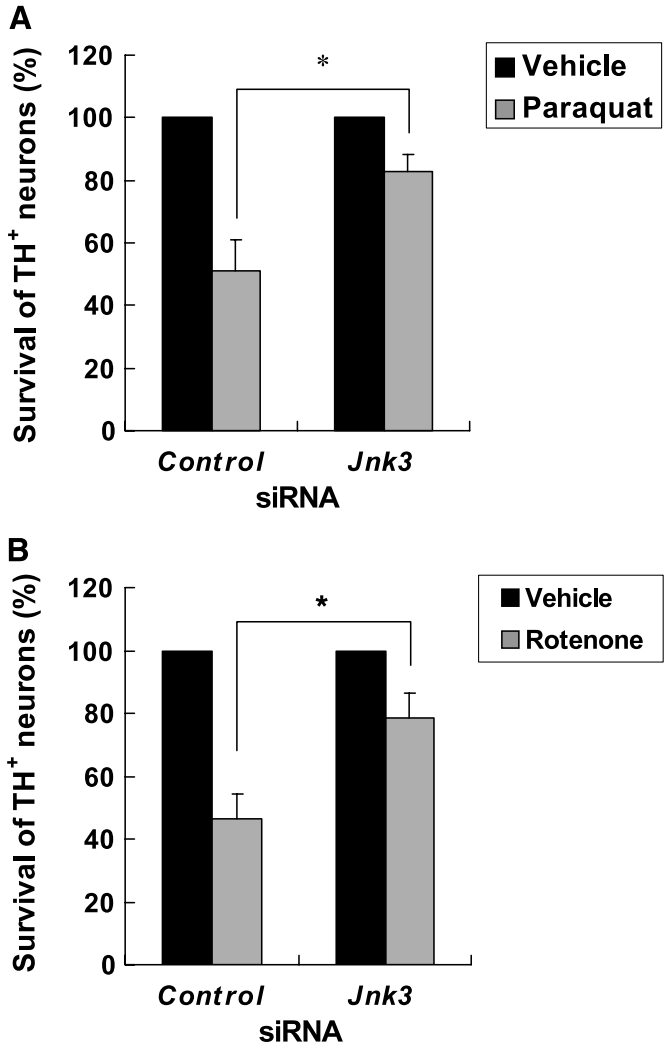

FIGURE 3. Small interfering RNA (siRNA) silencing of c-*Jun*-N-terminal kinase 3 (JNK3) protects dopaminergic neurons from paraquat and rotenone toxicity. **(A, B)** At 24 hours after transfection of siRNA, primary cultured E14 mesencephalic neurons were treated with 40 μmol/L paraquat **(A)**, 5 nmol/L rotenone **(B)**, or their respective vehicle control for another 24 hours. The cells were fixed and stained for tyrosine hydroxylase (TH). The number of TH-positive neurons was normalized to vehicle control–treated groups. *$p < 0.05$.

as controls. Mesencephalic neurons were cultured from individual E14 embryos obtained from breeding *Jnk3*$^{+/-}$ mice. The genotype of each embryo was determined by PCR using residual tissue and matched to each culture after cell counting at the completion of each experiment. Therefore, the experiments were performed blind without knowledge of the genotype of each culture. Consistent with the *Jnk3* siRNA results, deletion of *Jnk3* abrogated JNK phosphorylation in dopaminergic neurons (Fig. 4) and the death of these neurons caused by paraquat or rotenone (Fig. 5).

Dopaminergic Neuron Death Induced by Oxidative Stress Is Also Prevented by *Jnk3* Deletion

Oxidative stress has been implicated as a major underlying mechanism responsible for death of dopaminergic

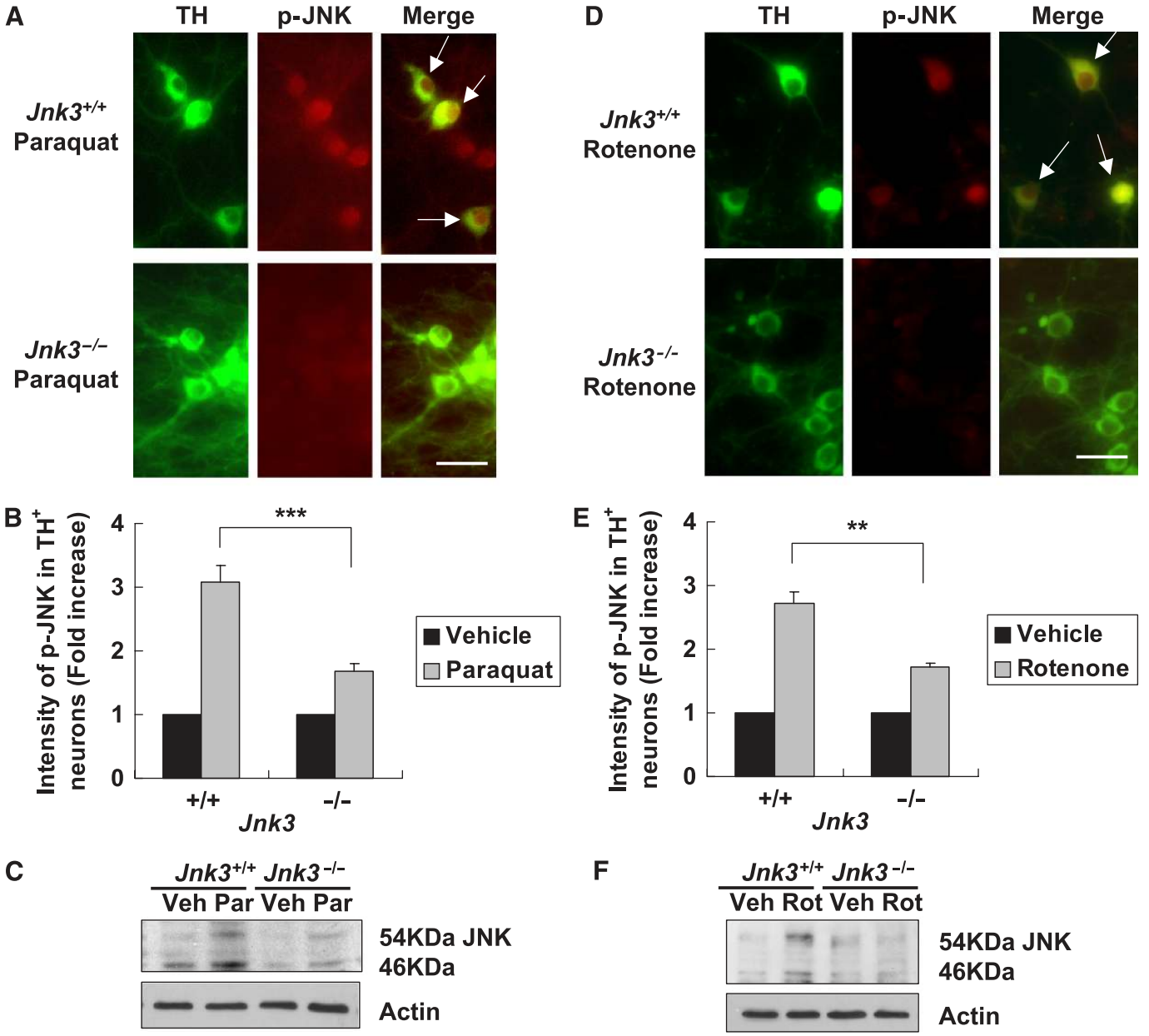

FIGURE 4. Deletion of the c-*Jun*-N-terminal kinase 3 (*Jnk3*) gene diminishes JNK activation in culture in response to paraquat and rotenone. The *Jnk3* heterozygotes (*Jnk3*$^{+/-}$) were mated, and primary mesencephalic neurons were cultured separately from each E14 mouse fetus. At 6 days, cells were treated with 40 μmol/L paraquat, 5 nmol/L rotenone, or vehicle control for another 8 hours. Cells were then fixed and immunostained for tyrosine hydroxylase (TH) and p-JNK. **(A, D)** Representative photomicrographs of cells immunostained for p-JNK and TH after treatment with paraquat **(A)** or rotenone **(D)**. Arrows point to dopaminergic neurons with JNK phosphorylation. Scale bars = 20 μm. **(B, E)** Quantification of JNK phosphorylation in TH-positive dopaminergic neurons. **(C, F)** After 8 hours of paraquat **(C)** or rotenone **(F)** treatment, cells were lysed and total protein was analyzed by immunoblotting for p-JNK. Actin was used as a loading control. Veh, vehicle; Par, paraquat; Rot, rotenone. **p < 0.01; ***p < 0.005.

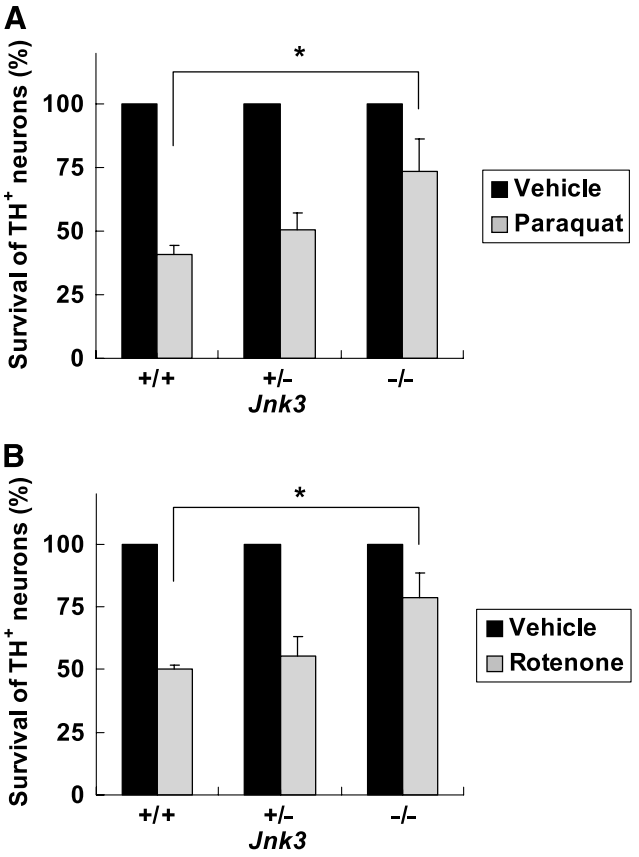

FIGURE 5. Tyrosine hydroxylase (TH)–positive dopaminergic neurons cultured from c-*Jun*-N-terminal kinase 3 (Jnk3)$^{-/-}$ mice are resistant to paraquat and rotenone toxicity. Cell cultures were prepared as in Figure 4 and treated on day 6 in vitro with 40 μmol/L paraquat **(A)**, 5 nmol/L rotenone **(B)**, or vehicle control. Numbers of surviving TH-positive neurons were scored 24 hours later and normalized to the control group. *$p < 0.05$.

neurons associated with PD (41, 42); both rotenone and paraquat generate reactive oxygen species (ROS), which mediate their toxicity for dopaminergic neurons (data not shown and [43, 44]). Consequently, we also investigated whether JNK3 plays a general role in oxidative stress–induced dopaminergic neuron death using *Jnk3*-null mice. Sodium arsenite is known to induce cell death through JNK activation and oxidative stress in both neurons and non-neuronal cells (45–48), and H_2O_2 is often used as a direct and general tool to cause oxidative damage to cells. Primary cultured dopaminergic neurons prepared from *Jnk3*$^{+/+}$ embryos were much more sensitive to sodium arsenite (Fig. 6A) and H_2O_2 toxicity (Fig. 6B) than those from *Jnk3*$^{-/-}$ embryos, suggesting that JNK3 contributes to the cell death of dopaminergic neurons caused by ROS.

Jnk3 Deletion Protects Dopaminergic Neurons Against Paraquat In Vivo

To evaluate the importance of JNK for dopaminergic neuron degeneration in vivo, mice were treated with paraquat. Motor function was examined 1 day after the last injection. Paraquat treatment of *Jnk3*$^{+/+}$ mice impaired performance on the rotarod and reduced the total distance traveled in the open-field test (Fig. 7; Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A111), indicating a deficit in motor function. In contrast, paraquat did not affect open-field activity or rotarod performance of *Jnk3*$^{-/-}$ mice.

Paraquat reduced the number of TH-positive dopaminergic neurons in the SNpc of *Jnk3*$^{+/+}$ mice but not *Jnk3*$^{-/-}$ mice (Fig. 8). The number of dopamine transporter–positive neurons was similarly decreased in *Jnk3*$^{+/+}$ mice but not in *Jnk3*$^{-/-}$ mice (percentage survival of dopamine transporter–positive neurons in paraquat-treated *Jnk3*$^{+/+}$ or *Jnk3*$^{-/-}$ mouse brains relative to the respective vehicle control–treated brains: 77.5% ± 2.0% and 99.2% ± 3.2%, respectively). These data demonstrate a critical role for JNK3 in the paraquat model of PD in vivo in mice.

DISCUSSION

We tested the hypothesis that the neural-specific JNK3 is a critical and common mediator of dopaminergic neuron death caused by paraquat and rotenone. Using RNA silencing and targeted gene deletion to block JNK3 expression, we found that JNK3 is the principal JNK isoform activated by

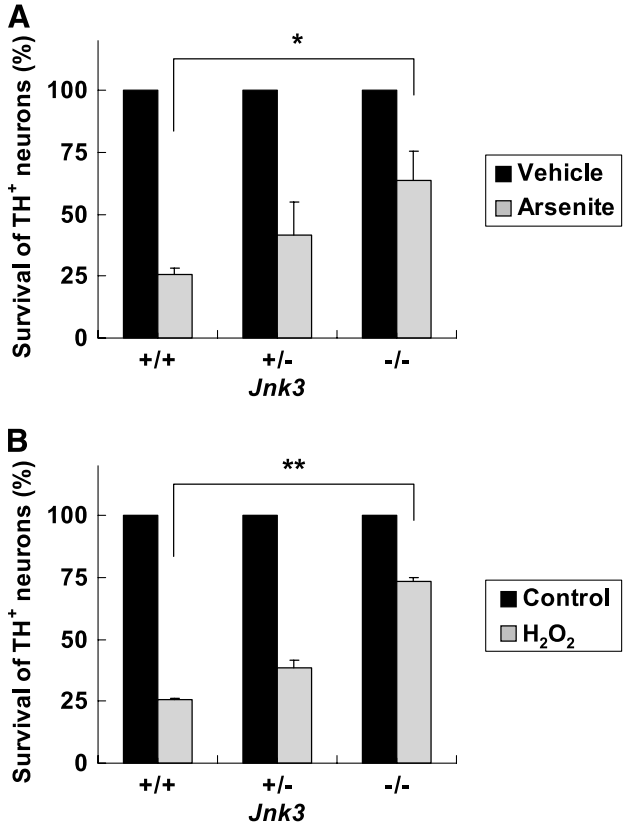

FIGURE 6. c-*Jun*-N-terminal kinase 3 (*Jnk3*) deletion prevents oxidative stress–induced dopaminergic neuron death. Cell cultures were prepared as in Figure 4 and treated with 10 μmol/L arsenite **(A)**, 0.5 μmol/L H_2O_2 **(B)**, or vehicle control on day 6 in vitro. Numbers of surviving tyrosine hydroxylase (TH)–positive neurons was scored 28 hours later and normalized to the vehicle control group. *$p < 0.05$; **$p < 0.01$.

rotenone and paraquat in primary dopaminergic neurons. Furthermore, blocking JNK3 expression protected primary dopaminergic neurons against paraquat and rotenone toxicity. Finally, *Jnk3*-null mice were protected from paraquat-induced motor deficits and dopaminergic neuron death in the SNpc. These data suggest a major role for JNK3 in dopaminergic neuron death in the pesticide models for PD.

There was still a slight increase in JNK phosphorylation in paraquat- or rotenone-treated JNK3$^{-/-}$ cultures (Fig. 4) or in cultures transfected with siRNA to JNK3 (Fig. 2). Furthermore, siRNA to JNK1 or JNK2 partially suppressed JNK phosphorylation, although to a much lesser degree than siRNA to JNK3 did (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A110). Thus, although paraquat and rotenone induce JNK activation primarily through JNK3, the JNK1 and JNK2 isoforms may also contribute to this process to a much smaller degree.

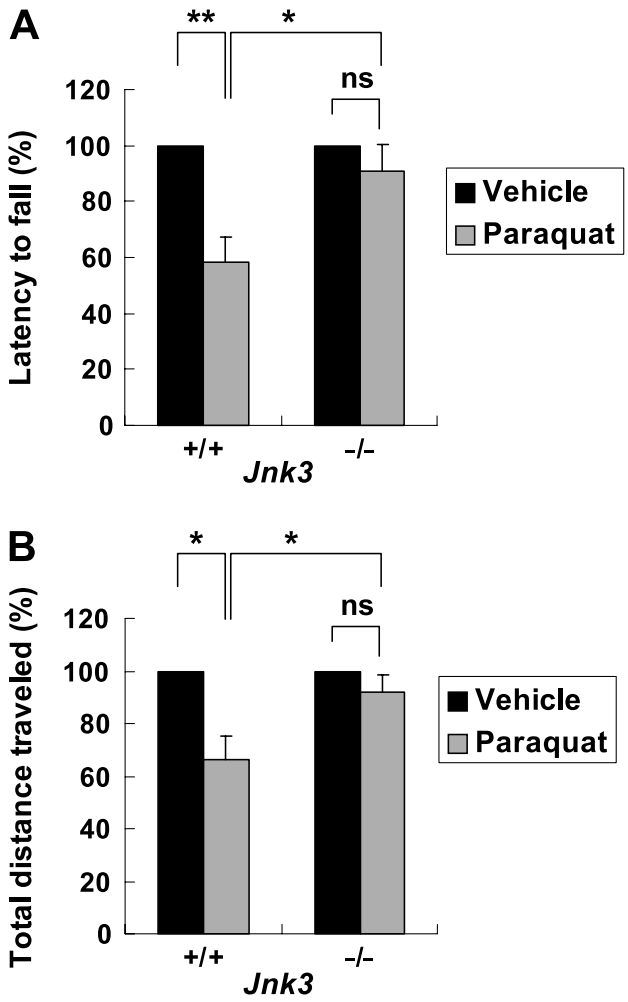

FIGURE 7. Paraquat-induced motor deficits in mice are attenuated by c-*Jun*-N-terminal kinase 3 (*Jnk3*) deletion. c-*Jun*-N-terminal kinase 3 (Jnk3)$^{+/+}$ and Jnk3$^{-/-}$ littermates were treated with paraquat (10 mg/kg) or vehicle control saline twice per week for 6 weeks and assessed for motor functions. **(A)** Latency to fall in accelerated rotarod test. **(B)** Locomotor activity in an open-field test, quantified as total distance traveled. *p < 0.05; **p < 0.01; ns, not significant.

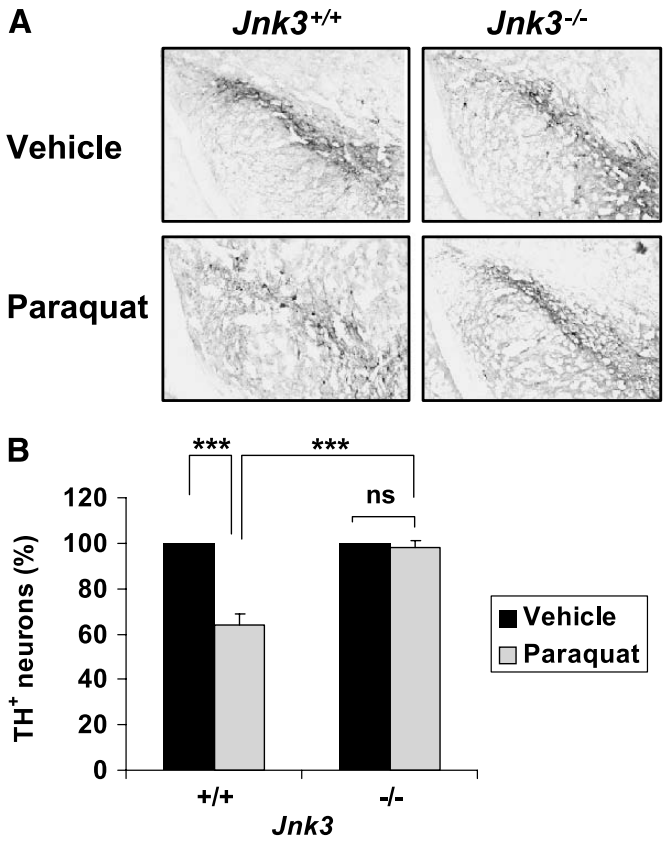

FIGURE 8. c-*Jun*-N-terminal kinase 3 (*Jnk3*) deletion protects tyrosine hydroxylase (TH)–positive dopaminergic neurons in the substantia nigra pars compacta (SNpc) from paraquat toxicity in vivo. The Jnk3$^{+/+}$ and Jnk3$^{-/-}$ littermates were treated with paraquat as in Figure 7. After motor function tests, mice were killed for TH immunostaining. **(A)** Representative photomicrographs of SNpc sections stained for TH. **(B)** Stereological quantification of the number of TH-positive neurons in the SNpc, normalized to vehicle control–treated groups. ***p < 0.005; ns, not significant.

The *JNK3* gene deletion or siRNA suppression of JNK3 expression also did not completely protect dopaminergic neurons in culture against paraquat, and there was still a small loss of TH-positive neurons after paraquat treatment (Figs. 3, 5). On the other hand, TH-positive neurons in the brains of mice treated with paraquat were almost completely spared in JNK3-null mice (Fig. 8). This slight discrepancy may be the result of differences in experimental paradigms used for in vitro versus in vivo studies. For example, 40 μmol/L paraquat was used in the cell culture study, which killed approximately 60% of the TH-positive neurons (in Fig. 5). This concentration is likely higher than that reached in the mouse brain after intraperitoneal injection of paraquat in vivo, which killed about 38% of TH-positive neurons. Paraquat at higher concentrations (e.g. 40 μmol/L in culture) may kill TH-positive neurons through JNK3-independent mechanisms.

The rotenone in vivo PD model was initially established in rats (8). Interestingly, Inden et al (16) recently reported the

rotenone mouse model after oral administration. It will be interesting in the future to determine whether *JNK3* gene deletion protects dopaminergic neurons against rotenone in vivo in the mouse brain.

Oxidative damage has been implicated as a major convergence point, whereby genetic, dietary, and environmental factors may cause dopaminergic neuron degeneration (41, 42). These include treatments with rotenone, paraquat, MPTP, and 6-hydroxydopamine (43, 44, 49, 50). Our data demonstrate that *Jnk3* deletion also protects primary dopaminergic neurons against H_2O_2, a direct ROS, and against sodium arsenite, which induces cell death in multiple cell types through oxidative stress (45–48). Thus, JNK3 may be generally required for ROS-mediated dopaminergic neuron degeneration.

c-*Jun*-N-terminal kinase activation has been reported in α-synuclein transgenic mouse brains and human PD brains (50, 51), suggesting a role for JNK activation in PD pathogenesis. The critical role for JNK in dopaminergic neuron death in the MPTP, 6-hydroxydopamine, rotenone, and paraquat PD models suggests that pharmacological inhibitors of JNK signaling may be useful for PD treatment.

Disappointingly, a clinical trial using CEP1347 for PD treatment was terminated because it failed to offer significant improvement (52). However, among the 3 JNK isoforms, JNK1 and JNK2 are ubiquitously expressed in adult tissues, and CNS neurons have extremely high basal JNK1/2 activities (46, 47, 53). c-*Jun*-N-terminal kinase 1 and JNK2 undoubtedly have important physiological functions in the adult, and side effects associated with inhibition of these enzymes would limit tolerable doses of JNK inhibitors. Consequently, general inhibition of all JNK isoforms, such as those achieved by CEP1347, may offer limited benefit to dopaminergic neurons at tolerable doses. By contrast, JNK3 is neural specific (31, 54–56) and does not exhibit high basal activity in the brain (46, 47). Furthermore, JNK3 is expressed in striatum (55) and in SN neurons (57). Our data suggest that selective inhibition of the neural-specific JNK3 isoform may be more specific for slowing PD progression. Furthermore, individual polymorphisms in *Jnk3* gene may contribute to individual susceptibility to PD.

Deletion of the *Jnk3* gene alone also protects many types of neurons against several other forms of injury, including ischemia/hypoxia, excitotoxicity, and axotomy of facial motor neurons or dorsal root ganglion neurons (32, 58, 59). Cortical neurons cultured from *Jnk3*$^{-/-}$ mice were also protected from β-amyloid toxicity (60). Thus, specific inhibition of JNK3 may provide a novel therapy for various forms of neurodegeneration including PD.

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REFERENCES

1. Olanow CW, Tatton WG. Etiology and pathogenesis of Parkinson's disease. Annu Rev Neurosci 1999;22:123–44
2. Dauer W, Przedborski S. Parkinson's disease: Mechanisms and models. Neuron 2003;39:889–909
3. Brown TP, Rumsby PC, Capleton AC, et al. Pesticides and Parkinson's disease—is there a link? Environ Health Perspect 2006;114:156–64
4. Costello S, Cockburn M, Bronstein J, et al. Parkinson's disease and residential exposure to maneb and paraquat from agricultural applications in the central valley of California. Am J Epidemiol 2009;169:919–26
5. Ecobichon DJ. Toxic effects of pesticides. In: Klaassen CD, ed. *Casarett & Doull's Toxicology: The Basic Science of Poisons*. New York, NY: The McGraw-Hill Companies, Inc, 2001:763–810
6. Hsuan SL, Klintworth HM, Xia Z. Basic fibroblast growth factor protects against rotenone-induced dopaminergic cell death through activation of extracellular signal–regulated kinases 1/2 and phosphatidylinositol-3 kinase pathways. J Neurosci 2006;26:4481–91
7. Klintworth H, Newhouse K, Li T, et al. Activation of c-*Jun*-N-terminal protein kinase is a common mechanism underlying paraquat- and rotenone-induced dopaminergic cell apoptosis. Toxicol Sci 2007;97:149–62
8. Betarbet R, Sherer TB, MacKenzie G, et al. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 2000;3:1301–6
9. Sherer TB, Kim JH, Betarbet R, et al. Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. Exp Neurol 2003;179:9–16
10. Manning-Bog AB, McCormack AL, Li J, et al. The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: Paraquat and alpha-synuclein. J Biol Chem 2002;277:1641–44
11. McCormack AL, Di Monte DA. Effects of L-dopa and other amino acids against paraquat-induced nigrostriatal degeneration. J Neurochem 2003;85:82–86
12. Manning-Bog AB, McCormack AL, Purisai MG, et al. Alpha-synuclein overexpression protects against paraquat-induced neurodegeneration. J Neurosci 2003;23:3095–99
13. Brooks AI, Chadwick CA, Gelbard HA, et al. Paraquat elicited neurobehavioral syndrome caused by dopaminergic neuron loss. Brain Res 1999;823:1–10
14. Thiruchelvam M, McCormack A, Richfield EK, et al. Age-related irreversible progressive nigrostriatal dopaminergic neurotoxicity in the paraquat and maneb model of the Parkinson's disease phenotype. Eur J Neurosci 2003;18:589–600
15. Thiruchelvam M, Richfield EK, Baggs RB, et al. The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and maneb: Implications for Parkinson's disease. J Neurosci 2000;20:9207–14
16. Inden M, Kitamura Y, Takeuchi H, et al. Neurodegeneration of mouse nigrostriatal dopaminergic system induced by repeated oral administration of rotenone is prevented by 4-phenylbutyrate, a chemical chaperone. J Neurochem 2007;101:1491–504
17. Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell 2000;103:239–52
18. Weston CR, Davis RJ. The JNK signal transduction pathway. Curr Opin Genet Dev 2002;12:14–21
19. Abou-Sleiman PM, Muqit MM, Wood NW. Expanding insights of mitochondrial dysfunction in Parkinson's disease. Nat Rev Neurosci 2006;7:207–19
20. Hald A, Lotharius J. Oxidative stress and inflammation in Parkinson's disease: Is there a causal link? Exp Neurol 2005;193:279–90
21. Dawson TM, Dawson VL. Molecular pathways of neurodegeneration in Parkinson's disease. Science 2003;302:819–22
22. Moore DJ, West AB, Dawson VL, et al. Molecular pathophysiology of Parkinson's disease. Annu Rev Neurosci 2005;28:57–87
23. Peng J, Andersen JK. The role of c-*Jun*-N-terminal kinase (JNK) in Parkinson's disease. IUBMB Life 2003;55:267–71
24. Saporito MS, Thomas BA, Scott RW. MPTP activates c-Jun NH(2)-terminal kinase (JNK) and its upstream regulatory kinase MKK4 in nigrostriatal neurons in vivo. J Neurochem 2000;75:1200–8
25. Xia XG, Harding T, Weller M, et al. Gene transfer of the JNK interacting protein-1 protects dopaminergic neurons in the MPTP model of Parkinson's disease. Proc Natl Acad Sci U S A 2001;98:10433–38
26. Saporito MS, Brown EM, Miller MS, et al. CEP-1347/KT-7515, an inhibitor of c-*Jun*-N-terminal kinase activation, attenuates the 1-methyl-4-phenyl tetrahydropyridine-mediated loss of nigrostriatal dopaminergic neurons in vivo. J Pharmacol Exp Ther 1999;288:421–27
27. Choi WS, Yoon SY, Oh TH, et al. Two distinct mechanisms are

involved in 6-hydroxydopamine- and MPP+-induced dopaminergic neuronal cell death: Role of caspases, ROS, and JNK. J Neurosci Res 1999;57:86–94
28. Newhouse K, Hsuan SL, Chang SH, et al. Rotenone-induced apoptosis is mediated by p38 and jnk map kinases in human dopaminergic SH-SY5Y cells. Toxicol Sci 2004;79:137–46
29. Peng J, Mao XO, Stevenson FF, et al. The herbicide paraquat induces dopaminergic nigral apoptosis through sustained activation of the JNK pathway. J Biol Chem 2004;279:32626–32
30. Ries V, Silva RM, Oo TF, et al. JNK2 and JNK3 combined are essential for apoptosis in dopamine neurons of the substantia nigra, but are not required for axon degeneration. J Neurochem 2008;107:1578–88
31. Gupta S, Barrett T, Whitmarsh AJ, et al. Selective interaction of JNK protein kinase isoforms with transcription factors. EMBO J 1996;15: 2760–70
32. Yang DD, Kuan CY, Whitmarsh AJ, et al. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the *Jnk3* gene. Nature 1997;389:865–70
33. Choi WS, Kruse SE, Palmiter RD, et al. Mitochondrial complex I inhibition is not required for dopaminergic neuron death induced by rotenone, MPP+, or paraquat. Proc Natl Acad Sci U S A 2008;105:15136–41
34. Wang Y, Luo W, Reiser G. Proteinase-activated receptor-1 and -2 induce the release of chemokine GRO/CINC-1 from rat astrocytes via differential activation of JNK isoforms, evoking multiple protective pathways in brain. Biochem J 2007;401:65–78
35. Reeves R, Thiruchelvam M, Baggs RB, et al. Interactions of paraquat and triadimefon: Behavioral and neurochemical effects. Neurotoxicology 2003;24:839–50
36. Cole TB, Martyanova A, Palmiter RD. Removing zinc from synaptic vesicles does not impair spatial learning, memory, or sensorimotor functions in the mouse. Brain Res 2001;891:253–65
37. Fernagut PO, Chalon S, Diguet E, et al. Motor behaviour deficits and their histopathological and functional correlates in the nigrostriatal system of dopamine transporter knockout mice. Neuroscience 2003;116:1123–30
38. Rozas G, Lopez-Martin E, Guerra MJ, et al. The overall rod performance test in the MPTP-treated-mouse model of Parkinsonism. J Neurosci Methods 1998;83:165–75
39. Mandir AS, Przedborski S, Jackson-Lewis V, et al. Poly(ADP-ribose) polymerase activation mediates 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP)–induced parkinsonism. Proc Natl Acad Sci U S A 1999;96: 5774–79
40. Hof PR, Young WG, Bloom FE, et al, eds. *Comparative Cytoarchitectonic Atlas of the C57BL/6 and 129/sv Mouse Brains With CD ROM*. The Netherlands: Elsevier, 2000
41. Fahn S, Cohen G. The oxidant stress hypothesis in Parkinson's disease: Evidence supporting it. Ann Neurol 1992;32:804–12
42. Danielson SR, Andersen JK. Oxidative and nitrative protein modifications in Parkinson's disease. Free Radic Biol Med 2008;44:1787–94
43. Sherer TB, Betarbet R, Stout AK, et al. An in vitro model of Parkinson's disease: Linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage. J Neurosci 2002; 22:7006–15
44. McCormack AL, Atienza JG, Johnston LC, et al. Role of oxidative stress in paraquat-induced dopaminergic cell degeneration. J Neurochem 2005; 93:1030–37
45. Bernstam L, Nriagu J. Molecular aspects of arsenic stress. J Toxicol Environ Health, Part B Crit Rev 2000;3:293–322
46. Namgung U, Xia Z. Arsenite-induced apoptosis in cortical neurons is mediated by c-*Jun*-N-terminal protein kinase 3 and p38 mitogen-activated protein kinase. J Neurosci 2000;20:6442–51
47. Namgung U, Xia Z. Arsenic induces apoptosis in rat cerebellar neurons via activation of JNK3 and p38 MAP kinases. Toxicol Appl Pharmacol 2001;174:130–38
48. Cai B, Chang SH, Becker EB, et al. p38 MAP kinase mediates apoptosis through phosphorylation of BimEL at Ser-65. J Biol Chem 2006;281: 25215–22
49. Pan J, Wang G, Yang HQ, et al. K252a prevents nigral dopaminergic cell death induced by 6-hydroxydopamine through inhibition of both mixed-lineage kinase 3/c-Jun NH2-terminal kinase 3 (JNK3) and apoptosis-inducing kinase 1/JNK3 signaling pathways. Mol Pharmacol 2007;72:1607–18
50. Hunot S, Vila M, Teismann P, et al. JNK-mediated induction of cyclooxygenase 2 is required for neurodegeneration in a mouse model of Parkinson's disease. Proc Natl Acad Sci U S A 2004;101:665–70
51. Frasier M, Walzer M, McCarthy L, et al. Tau phosphorylation increases in symptomatic mice overexpressing A30P alpha-synuclein. Exp Neurol 2005;192:274–87
52. The Parkinson Study Group PRECEPT Investigators. Mixed lineage kinase inhibitor CEP-1347 fails to delay disability in early Parkinson disease. Neurology 2007;69:1480–90
53. Figueroa-Masot XA, Hetman M, Higgins MJ, et al. Taxol induces apoptosis in cortical neurons by a mechanism independent of Bcl-2 phosphorylation. J Neurosci 2001;21:4657–67
54. Martin JH, Mohit AA, Miller CA. Developmental expression in the mouse nervous system of the p49(3F12) SAP kinase. Mol Brain Res 1996;35:47–57
55. Mohit AA, Martin JH, Miller CA. p493F12 kinase: A novel MAP kinase expressed in a subset of neurons in the human nervous system. Neuron 1995;14:67–78
56. Kumagae Y, Zhang Y, Kim OJ, et al. Human c-*Jun*-N-terminal kinase expression and activation in the nervous system. Brain Res 1999;67:10–17
57. Lee JK, Park J, Lee YD, et al. Distinct localization of SAPK isoforms in neurons of adult mouse brain implies multiple signaling modes of SAPK pathway. Brain Res Mol Brain Res 1999;70:116–24
58. Kuan CY, Whitmarsh AJ, Yang DD, et al. A critical role of neural-specific JNK3 for ischemic apoptosis. Proc Natl Acad Sci U S A 2003;100: 15184–89
59. Keramaris E, Vanderluit JL, Bahadori M, et al. c-*Jun*-N-terminal kinase 3 deficiency protects neurons from axotomy-induced death in vivo through mechanisms independent of c-*Jun* phosphorylation. J Biol Chem 2005;280:1132–41
60. Morishima Y, Gotoh Y, Zieg J, et al. {beta}-Amyloid induces neuronal apoptosis via a mechanism that involves the c-*Jun*-N-terminal kinase pathway and the induction of fas ligand. J Neurosci 2001;21:7551–60