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JNK Inhibition Protects Dopamine Neurons and Provides Behavioral Improvement in a Rat 6-Hydroxydopamine Model of Parkinson's Disease

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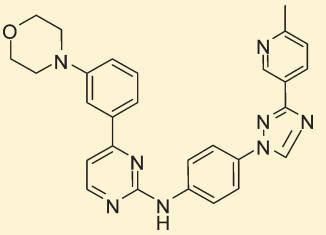
ABSTRACT: Parkinson's disease (PD) results from the loss of dopamine neurons located in the substantia nigra pars compacta (SNpc) that project to the striatum. A therapeutic has yet to be identified that halts this neurodegenerative process, and as such, development of a brain penetrant small molecule neuroprotective agent would represent a significant advancement in the treatment of the disease. To fill this void, we developed an aminopyrimidine JNK inhibitor (SR-3306) that reduced the loss of dopaminergic cell bodies in the SNpc and their terminals in the striatum produced by unilateral injection of 6-hydroxydopamine (6-OHDA) into the nigrostriatal pathway. Administration of SR-3306 [10 mg/kg/day (s.c.) for 14 days] increased the number of tyrosine hydroxylase immunoreactive (TH^+) neurons in the SNpc by 6-fold and reduced the loss of the TH^+ terminals in the striatum relative to the corresponding side of 6-OHDA-lesioned rats that received only vehicle ($p < 0.05$). In addition, SR-3306 [10 mg/kg/day (s.c.) for 14 days] decreased d-amphetamine-induced circling by 87% compared to 6-OHDA-lesioned animals given vehicle. Steady-state brain levels of SR-3306 at day 14 were 347 nM, which was approximately 2-fold higher than the cell-based IC_{50} for this compound. Finally, immunohistochemical staining for phospho-c-jun (p-c-jun) revealed that SR-3306 [10 mg/kg/day (s.c.) for 14 days] produced a 2.3-fold reduction of the number of immunoreactive neurons in the SNpc relative to vehicle treated rats. Collectively, these data suggest that orally bioavailable JNK inhibitors may be useful neuroprotective agents for the treatment of Parkinson's disease.



KEYWORDS: JNK, 6-OHDA, neuroprotection, Parkinson's disease

Since the 1960s, the mainstay for the treatment of Parkinson's disease has been levodopa (L-DOPA). Although initial response rates approach 90%,[1,2] L-DOPA loses efficacy over time and has numerous side effects that limit the overall effectiveness of this drug. Importantly, L-DOPA has never been shown to provide any neuroprotection in patients. Indeed, no drugs have shown neuroprotection, including rasagiline where recent double blind, delayed-start phase III studies in 1176 patients receiving rasagiline (1 or 2 mg per day) failed to demonstrate convincing neuroprotective disease-modifying effects.[3] Given that Parkinson's disease (PD) is pathologically characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and loss of projecting nerve fibers in the striatum, with concurrent motor deficits, any disease-modifying, neuroprotective agents would represent a significant advancement for the treatment of the disease.

One potential target for neuroprotection is c-jun-N-terminal kinase (JNK),[4−7] a member of the mitogen-activated protein (MAP) kinase family. JNK inhibition has been shown to be effective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model utilizing either JNK 2/3 knockout mice (KO),[8] an adenovirally delivered 154 amino acid JNK-interacting protein (JIP) construct,[9] or in the accompanying paper (Chambers et al.[10]) by SR-3306, a brain penetrant small molecule JNK inhibitor from the aminopyrimidine class.[11] In addition to the MPTP model, JNK has also been associated with dopaminergic neuronal death induced by 6-OHDA. In 2001, Björklund and colleagues showed that dopaminergic neuron cell death induced by intrastriatal injection of 6-OHDA led to increased levels of phospho-c-jun in the SNpc of rats.[12] These findings are supported by the work of Pan et al. where they too showed that 6-OHDA-induced dopaminergic neuron cell death was JNK dependent.[13] These reports, coupled with the finding that CEP11004, a mixed lineage kinase inhibitor upstream from JNK suppressed apoptotic cell death in dopaminergic neurons in the SNpc,[14] firmly plants JNK as a key target for neuroprotection. While a JNK pathway inhibitor has been shown to be efficacious in the rat 6-OHDA model, no direct JNK inhibitors have been tried in this model. One benefit of the 6-OHDA model over the mouse MPTP model is that it permits a well characterized and convenient behavioral assay by which to assess midbrain dopamine neuron loss via d-amphetamine-induced circling.[15,16]

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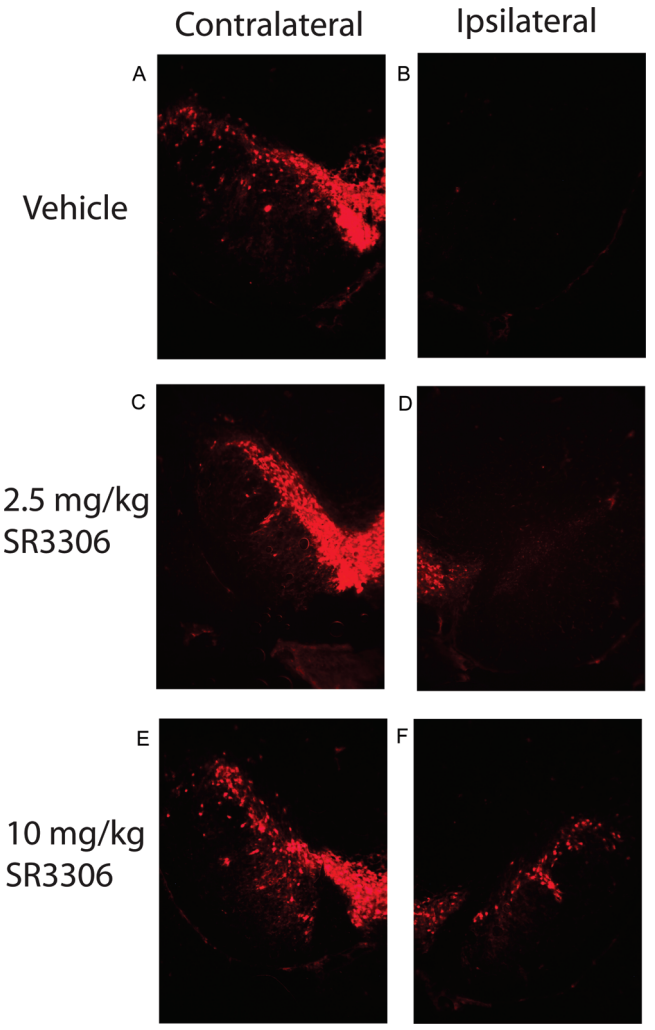

Figure 1. JNK inhibition protects dopaminergic neurons in the SNpc after 6-OHDA lesion. Tyrosine hydroxylase immunohistochemistry for TH on midbrain sections from (A) contralateral 6-OHDA/vehicle, (B) ipsilateral 6-OHDA/vehicle, (C) contralateral 6-OHDA/2.5 mg/kg SR-3306, (D) ipsilateral 6-OHDA/2.5 mg/kg SR-3306, (E) contralateral 6-OHDA/10 mg/kg SR-3306, and (F) ipsilateral 6-OHDA/10 mg/kg SR-3306-treated rats.

Our results represent the first demonstration that a brain penetrant JNK inhibitor reduced the ability of unilateral injections of 6-OHDA into the nigrostriatal pathway to promote the loss of cell bodies in the SNpc and terminals in the striatum. Importantly, this neuroprotection was manifested in protection against behavioral deficits induced by d-amphetamine, indicating that surviving dopamine neurons were functional. These observations, coupled with concordant neuroprotective effects of SR-3306 in a mouse MPTP model (accompanying paper by Chambers et al.[10], indicate that JNK inhibitors may represent a viable therapeutic approach for the treatment of Parkinson's disease.

RESULTS

Dopaminergic Neurons in Rats Treated with SR-3306 Are Protected Against 6-OHDA Intoxication. To test the hypothesis that small-molecule-mediated JNK inhibition is neuroprotective in a rat model of PD, we examined the ability of SR-3306 to prevent the loss of nigrostriatal neurons in the 6-OHDA model. Dopaminergic neurons were labeled with an antibody against tyrosine hydroxylase (TH) in brain sections from rats treated with 6-OHDA that received either vehicle (Figure 1A, B), or 2.5 mg/kg (Figure 1C, D) or 10 mg/kg SR-3306 (Figure 1E, F). Vehicle or SR-3306 was delivered subcutaneously daily for 14 days via constant infusion using osmotic minipumps. TH immunoreactivity in the SNpc contralateral (Figure 1 A, C, E) or ipsilateral (Figure 1 B, D, F) to the 6-OHDA lesion was examined in all animals from the three groups. Relative to the contralateral side (Figure 1A), rats treated with 6-OHDA showed a near complete loss of TH-positive neurons in the ipsilateral SNpc (Figure 1B). By comparison to the contralateral side (Figure 1C), 6-OHDA-lesioned rats treated with 2.5 mg/kg SR-3306 showed a slight increase in TH-positive neurons in the ipsilateral side (Figure 1D). In contrast, 10 mg/kg SR-3306 was clearly protective against 6-OHDA-induced neurodegeneration when comparing the contralateral side to the ipsilateral side (Figure 1E, F). To quantify the neuroprotective effects of SR-3306, unbiased stereological counts of the TH-positive cells in the SNpc at 14 days after 6-OHDA intoxication was carried out for each animal in all of the groups. Figure 2 presents the number of TH-positive cells for the three treatment groups (6-OHDA/vehicle, 6-OHDA/2.5 mg/kg SR-3306, and 6-OHDA/10 mg/kg SR-3306). Unbiased stereological counts revealed that the 6-OHDA-lesion decreased the number of TH-positive cells by >90% in the ipsilateral side compared to the contralateral side ($p < 0.01$). Administration of 2.5 mg/kg SR-3306 to 6-OHDA-treated animals did not enhance the survival of TH^+ neurons in the ipsilateral side (Figure 2). In contrast, treatment with 10 mg/kg SR-3306 increased the number of TH-positive cells in the ipsilateral side by approximately 6-fold compared to the 6-OHDA-lesioned animals that received vehicle ($p < 0.01$) (Figure 2).

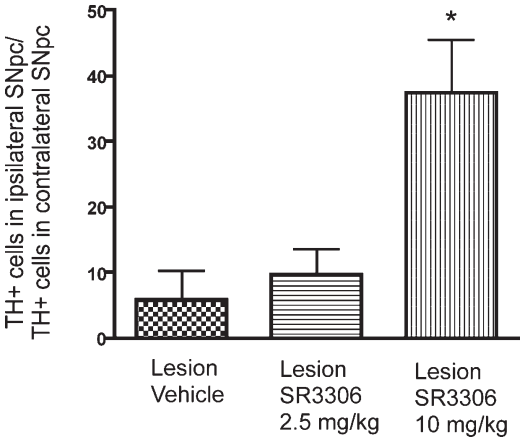

Figure 2. Unbiased stereological counts of TH-positive cells in the SNpc at 7 days after MPTP intoxication. Beginning at bregma −4.0, brains were sectioned at 40 μm for 24 sections, discarding every other section until bregma −7.04. Inhibition of JNK by 10 mg/kg SR-3306 dosed subcutaneously by constant infusion showed sparing of TH-positive neurons. Three groups were analyzed: 6-OHDA/vehicle (n = 11/group), 6-OHDA/2.5 mg/kg SR-3306 (n=11/group), and 6-OHDA/10 mg/kg SR-3306 (n=11/group). 3.6 μg of 6-OHDA was dosed by two injections into the medial forebrain bundle. For SR-3306 treated groups, SR-3306 was dosed subcutaneously by constant infusion 30 min prior to the 6-OHDA dose. Data are expressed as the number of TH^+ neurons (±SEM) surviving 14 days after the 6-OHDA treatment as detected by TH immunohistochemistry (*$p < 0.01$; established by two-way ANOVA also followed by Tukey's post hoc test).

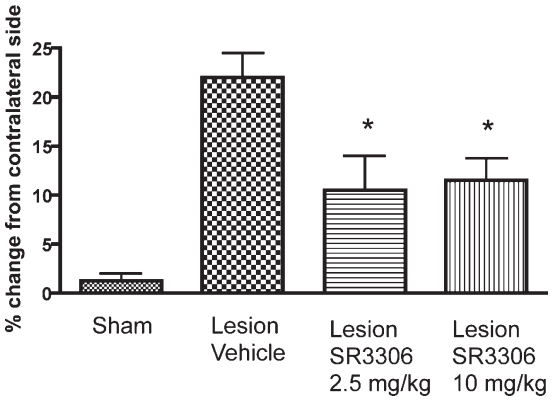

Figure 3. JNK inhibition protects striatal fiber density after 6-OHDA lesion. Striatal fibers were stained for TH, and the percent difference from the contralateral side was calculated. The three treatment groups were 6-OHDA/vehicle (n = 6), 6-OHDA/2.5 mg/kg SR-3306 (n = 3), and 6-OHDA/10 mg/kg SR-3306 (n = 6). *p < 0.05; established by one-way ANOVA.

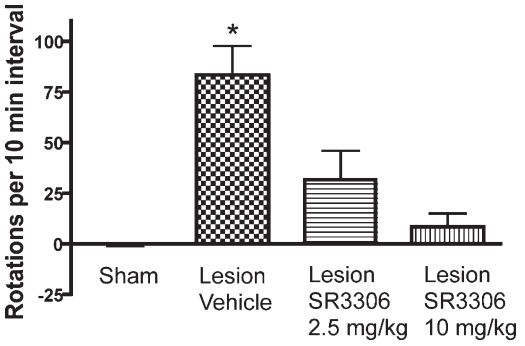

Figure 4. JNK inhibition reduces unilateral rotational behavior after amphetamine challenge in 6-OHDA lesioned rats. The lesioned rats were given a d-amphetamine challenge (5 mg/kg, intraperitoneal) 5 min prior to measurement of rotation behavior commencing. The rotation scores were collected in seven 10 min intervals with a computerized video activity monitoring system.

We extended these findings in the SNpc by assessing the effects of SR-3306 on the loss of TH^+ fibers in the striatum produced by 6-OHDA. Optical density measurements were performed to quantify striatal TH positive fibers ipsilateral and contralateral to the lesion. The density of TH immunoreactive fibers remaining in the ipsilateral striatum were expressed as a percentage of those detected in the striatum contralateral to the 6-OHDA lesion. Figure 3 showed that on average 6-OHDA produced a 22% loss of TH fiber density in the ipsilateral striatum. Both 2.5 and 10 mg/kg treatment with SR-3306 showed a 2-fold reduction in the loss of TH+ fibers in the striatum (p < 0.05).

To establish whether SR-3306 protected nigrostriatal neurons were functional, we measured the rotational behavior of rats challenged with 5 mg/kg (i.p.) of d-amphetamine 14 days after 6-OHDA lesion (Figure 4). In rats that had received unilateral injections of 6-OHDA lesion, d-amphetamine produced approximately 80 unilateral rotations in a 10 min interval whereas sham-treated animals did not show a rotational bias. 6-OHDA-lesioned animals treated with 2.5 mg/kg SR-3306 showed a decrease in the number of unilateral rotations that was not statistically

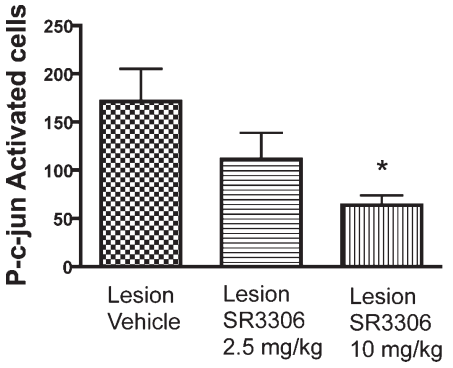

Figure 5. SR-3306 inhibits 6-OHDA-induced phosphorylation of c-jun in the substantia nigra pars compacta. Number of p-c-jun positive cells as determined by immunohistochemistry for three treatment groups (6-OHDA/vehicle (n=6), 6-OHDA/2.5 mg/kg SR-3306 (n=3), 6-OHDA/10 mg/kg SR-3306 (n = 6)). *p < 0.05; established by one-way ANOVA.

significant, whereas 6-OHDA-lesioned animals treated with 10 mg/kg SR-3306 showed an approximate 8-fold decrease (near complete reduction) in unilateral rotational behavior (p < 0.01) (Figure 4).

Given the encouraging levels of protection produced by SR-3306 as demonstrated by quantification of dopamine neurons in the SNpc and striatal fiber density as well as decreased d-amphetamine-induced circling, we next determined if these effects were correlated with the inhibition of JNK activity in vivo. To do this, we utilized immunohistochemical detection of phospho-c-jun (p-c-jun) and counted the number of p-c-jun positive cells present in the SNpc 14 days after the 6-OHDA lesion in animals treated with vehicle, or two doses of SR-3306 (Figure 5). Rats that received 6-OHDA lesions showed approximately 170 phospho-c-jun containing cells in the ipsilateral SNpc (Figure 5). The number of phospho-c-jun containing cells was reduced in the presence of 2.5 mg/kg SR-3306 to approximately 120, whereas the number of phospho-c-jun containing cells in the 10 mg/kg SR-3306-treated group was reduced approximately 2.3-fold to 75 (p < 0.05) (Figure 5).

Finally, to measure the concentration of SR-3306 in the plasma and brain during the 14 day delivery of the compound, we took plasma samples at days 1−14 and brain samples at day 14 and used LC-MS/MS to determine SR-3306 levels (Figure 6). The results showed that on all days there were on average 4.3-fold higher levels of SR-3306 in the plasma at the 10 mg/kg dose than at the 2.5 mg/kg dose. Moreover, on day 14, there was 5.4-fold higher concentration in the brain for the 10 mg/kg dose than the 2.5 mg/kg dose. This result was very similar to that seen for the plasma difference between the doses on day 14 which was 6.1-fold. In addition, the plasma/brain ratio on day 14 for the 10 mg/kg dose of SR-3306 was 1.5 and the plasma/brain ratio on day 14 for the 2.5 mg/kg dose of SR-3306 was 1.3 (Figure 6). Finally, it should be noted that the plasma protein binding in rat for SR-3306 was 97%.

DISCUSSION

In the accompanying paper by Chambers et al.,[10] we demonstrated that SR-3306 was a highly selective (over 340 kinases and 80 receptors and ion channels), brain penetrant, potent inhibitor of JNK with cell-based IC_{50} = 216 nM, and showed that it

SR-3306 Plasma levels (nM)

Day	1	2	3	4	6	7	8	9	10	13	14	Brain 14
2.5 mg/kg/day	347	104	187	130	190	138	116	131	127	109	85	64
10 mg/kg/day	1007	459	446	357	783	624	613	554	489	588	522	357

Figure 6. Time course for plasma and brain levels of SR-3306 after 14 day subcutaneous infusion. SR-3306 was formulated in 30% β-cyclodextran containing 0.07% HCl, and four Sprague−Dawley rats were used for each dose. SR-3306 was dosed at 2.5 mg/kg or 10 mg/kg in subcutaneous minipumps at a rate of 5 μL/h, and after 24 h on days 1, 2, 3, 4, 6, 7, 8, 9, 10, 13, and 14 blood, and day 14 brain were collected. The plasma and brain samples were analyzed for compound by LC-MS/MS.

protected dopaminergic neurons against MPTP neurotoxicity in vitro and in vivo. One of the virtues of the MPTP mouse model is that MPTP has been shown to produce Parkinson's like symptoms in man[17] and thus is considered a relevant model for PD. One of the limitations of this mouse model is that some researchers have suggested that it does not provide any reliable behavioral motor disorder measures.[16] The 6-OHDA model on the other hand does permit comparison of cellular and behavioral neuroprotection enabling determination of the functionality of surviving nigrostriatal neurons. Additionally, since the lesion is unilateral, within animal comparisons between the contralateral and ipsilateral side can be made. Another virtue of using the rat model is that it enabled us to implant minipumps of sufficient capacity to subcutaneously deliver a constant level of compound. This prevented the peaks and troughs in plasma drug concentrations associated with an oral dosing regimen.

Many interesting findings were associated with this study. First, the results showed that a constant infusion of 10 mg/kg SR-3306 provided brain levels at day 14 that were at 347 nM (Figure 6), a concentration that was only 1.6-fold higher than the cell-based IC_{50} for this compound. Despite having brain concentrations only 1.6-fold above the cell-based IC_{50}, and free brain concentrations near 10 nM based on the 97% plasma protein binding, SR-3306 inhibition of JNK provided a protection of approximately 6-fold of the dopaminergic neurons in the SNpc to a level that was 30% of the total population of SNpc neurons detected in the contralateral side. These results suggest that a brain concentration of SR-3306 that was near the cell-based IC_{50} is still sufficient to achieve dopaminergic neuronal survival in the 6-OHDA model. The on target mechanism demonstrated in Figure 5 through inhibition of c-jun phosphorylation and the highly selective nature of SR-3306 collectively suggest that JNK inhibition is an attractive neuroprotective strategy in PD. Indeed, these results are consistent with the resistance of JNK KO mice to MPTP-induced dopamine neuron loss[8] and our own pharmacological findings demonstrating the neuroprotective effects of small molecule mediated JNK inhibition in the MPTP model.

Second, the protective effects of SR-3306 on dopaminergic neuronal survival seen in the SNpc were extended to the striatum. These results are consistent with both Hunot et al.[8] and Xia et al.[9] who utilized JNK KO mice and the adenovirally expressed JIP protein, respectively, and showed increases in dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA). In the accompanying paper by Chambers et al.,[10] we demonstrated that SR-3306, when dosed orally in mice, produced no protection of dopamine terminal in the striatum. By contrast, SR-3306 achieved a 50% protection for dopamine terminals in the striatum. The difference between the MPTP and 6-OHDA studies utilizing SR-3306 may be due to numerous factors including the constant infusion dosing in the 6-OHDA study, species differences, or greater protection of striatal projections due to the longer (14 day) dosing regimen in the 6-OHDA study. Moreover, MPTP is known to compromise the striatal terminals to a much greater extent than the cell bodies. It is interesting to speculate that if a higher dose of SR-3306 was given in the 6-OHDA study, such that the steady-state brain concentration was 3−5-fold higher than the cell-based IC_{50}, that the neuroprotective effect as measured by TH staining in the SNpc or in striatal density fiber would have been higher. It is unclear if >99% JNK inhibition is required to achieve full protection of dopaminergic neurons in the SNpc and striatal projections, or if something less than that would be sufficient for full protection.

A third interesting finding was that protection of 30% of the dopaminergic neurons in the SNpc was sufficient to completely inhibit d-amphetamine-induced circling. If considered in the context of what is generally regarded as the scenario for a patient initially presenting to the clinic with PD symptoms (i.e., 60−70% dopaminergic cell loss with modest motor deficits), these results are quite encouraging. For example, it may be that JNK inhibition capable of maintaining approximately 30% of the total pool of dopaminergic neurons is enough to prevent a further loss of motor function in patients. While these interpretations are largely speculative, it is conceivable that less than 100% JNK inhibition is enough to prevent disease progression.

Given the good dose linear pharmacokinetics in both the plasma and brain for SR-3306, it would be relatively straightforward to design an experimental dosing regimen that could provide brain levels of SR-3306 which would presumably inhibit >99% JNK activity. With those results, one could then determine the maximal amount of dopamine neuron protection that could be afforded by JNK small molecule inhibition, and relate this to the maximum behavioral benefit. As a corollary to this, one may be able to also determine the minimal amount of JNK inhibition that is needed to maintain maximal neuronal survival and prevent motor deterioration.

In summary, we have shown that SR-3306 is a selective, potent, highly brain penetrant JNK inhibitor that displays efficacy in protecting dopaminergic neurons in the SNpc and their projections to the striatum of rat brains challenged with 6-OHDA. Moreover, protection of approximately 30% of the dopaminergic neurons in the SNpc correlated with a nearly 90% reduction in behavioral deficits as assessed by d-amphetamine-induced circling. These results suggest that SR-3306 should be considered for further evaluation as the first oral JNK inhibitor with adequate bioavailability and brain penetration necessary to be an effective neuroprotective agent for the treatment of Parkinson's disease.

METHODS

Experimental Animals. Male Sprague−Dawley rats (Charles River Laboratories) of 225−250 g were used in this study. The animals were house in a controlled environment under a 12 h light/dark cycle and were allowed food and water ad libitum until 48 h postsurgery.

Immediately postsurgery, the animals were fed mash supplemented with fresh fruit. Animals were food restricted on standard rat chow beginning 48 h after surgery to give a slow and steady weight gain.

6-Hydroxydopamine Lesion Surgery and Mini-Pump Insertion. Sprague−Dawley rats were anesthetized by intraperitoneal injection of a ketamine hydrochloride, xylazine, and acepromazine maleate mixture (60, 1.2, and 1.6 mg/kg, respectively), and unilateral lesions of the right striatum and substantia nigra pars compacta were made by two injections of 6-hydroxydopamine hydrobromide (6-OHDA) (Sigma, St. Louis, MO) and 3.6 μg of 6-OHDA hydrogen bromide in 2.0 mg of L-ascorbate in sterile saline (0.9% sodium chloride). The animals were placed in a Kopf stereotaxic apparatus and a burr hole drilled to allow two injections of 6-OHDA into the medial forebrain bundle. The first injection of 2.5 μL was made at stereotaxic coordinates AP −4.4, ML −1.2, and DV −7.8 with the incisior bar set at 2.4 mm below the inter aural line (IA). The second injection was made with 3.0 μL of 6-OHDA injected at AP −4.0, ML −0.8, and DV −8.0 with the incisor bar set at 3.4 above the IA. The injections were made with a Kopf microinjection unit (Kopf Instruments, Tujunga, CA) with a 26 gauge Hamilton syringe set at 0.5 μL/min, and the needle was left in place for 5 min postinjection to prevent backfilling along the injection tract. Animals received Alzet 2ML2 osmotic pumps (Durect Corp.) containing vehicle (hydroxypropyl-β-cyclodextrin, 0.07% hydrochloric acid), 2.5 mg/kg/day SR-3306 dissolved in vehicle, or 10 mg/kg/day SR-3306 implanted subcutaneously. Two weeks after the lesion surgery, the efficacy of the lesion was determined by measuring rotational behavior following d-amphetamine administration.

Behavioral Testing. The lesioned rats were given an amphetamine challenge (5 mg/kg, intraperitoneal) (Sigma, St. Louis, MO) 5 min prior to measurement of rotation behavior commencing. The rotation scores were collected in seven 10 min intervals with a computerized video activity monitoring system (Videomex, Columbus Instruments).

Immunohistochemistry. One hour following rotation testing animals were sacrificed by an overdose of phenobarbital (euthansol, CDMV) followed by cardiac perfusion with 4% paraformaldehyde (PFA) in a 0.1 M sodium phosphate buffer (pH 7.4). The brains were removed and further postfixed in 4% PFA at 4 °C for 2 days, followed by cryoprotection in 30% sucrose for a minimum of 24 h. Symmetrical 30 μm thick sections were cut on a freezing microtome (Leica) and stored in a Millonig's solution. Every twelfth section was processed for immunohistochemistry. Free-floating sections were pretreated in 1% H_2O_2 in phosphate-buffered saline for 15 min, in blocking solution (5% goat or horse serum/phosphate buffered saline [PBS]) for 1 h at room temperature, followed by the appropriate antibody at 4 °C overnight. For proper identification of the SNpc, all sections were incubated with either polyclonal rabbit anti-tyrosine hydroxylase (TH) (1:2000; Chemicon, Temecula, CA) or monoclonal mouse anti-TH (1:2000; Chemicon, Temecula, CA). Sections were then washed with PBS containing 0.1% Triton X-100, incubated with biotinylated horse anti-mouse secondary antibody for the mouse monoclonal anti-TH or biotinylated goat anti-rabbit for the polyclonal anti-TH (both from Vector Laboratories, Burlington, Ontario) in 5% horse serum/PBS for 1 h at room temperature, placed in avidin−biotin complex (ABC Elite; Vector Laboratories, Burlington, Ontario) in PBS for 1 h, and, finally, placed for 20 min in 0.05% diaminobenzidine in PBS. In between steps, sections were washed for 3 × 20 min in PBS with 0.1% Triton X-100. Alternatively, sections were secondarily labeled with an Alexa fluor 594-conjugated goat anti-rabbit antibody (Invitrogen, Burlington, Ontario). Sections were mounted on unsubbed glass slides and coverslipped in glycerol in PBS.

For detection of striatal fibers, polyclonal rabbit anti-TH (1:2000; Chemicon, Temecula, CA) was used as the primary antibody. Sections were then washed with PBS containing 0.1% Triton X-100, incubated with biotinylated goat anti-rabbit for the polyclonal anti-TH (1:500, Vector Laboratories, Burlington, Ontario) in 5% horse serum/PBS for 1 h at room temperature, placed in avidin−biotin complex (ABC Elite; Vector Laboratories, Burlington, Ontario) in PBS for 1 h, and, finally, placed for 20 min in 0.05% diaminobenzidine in PBS. In between steps, sections were washed for 3 × 20 min in PBS with 0.1% Triton X-100. Sections were mounted on unsubbed glass slides and coverslipped in glycerol in PBS.

A similar procedure as described above was followed for phosphorylated c-jun detection. Sections were blocked with 5% goat serum/PBS for 1 h at room temperature and then incubated in rabbit polyclonal antibody against phospho-c-jun (Ser73) (1:100, Cell Signaling) followed by biotinylated goat anti-rabbit (1:500, Vector Laboratories, Burlington, Ontario). For proper identification of the SNpc, sections were incubated with monoclonal mouse anti-TH (1:2,000; Chemicon, Temecula, CA). The tissue was washed in a similar manner to the processing for the diaminobenzidine staining, but detection of the primary antibody was by goat anti-mouse Cy3 tagged secondary antibody (1:100, Jackson Immunoresearch, West Grove, PA).

Stereological Counting of TH^+ Dopaminergic Cells. The total number of surviving TH+ immunoreactive cells in the substantia nigra were estimated using unbiased stereology using the optical fractionator probe and stereological software (Stereo Investigator 6.01, Microbrightfield Biosciences, Williston, VT). The optical fractionator probe was used on every sixth immunostained section throughout the grafted areas and consisted of a 50 × 50 μm counting frame with a height of 11.5 μm. The section thickness was estimated every dissector measurement and then averaged for each section.

Density Measurements of Striatal Fiber Tracts. The extent of striatal fiber damage was assessed by optical density of TH stained sections using a densitometry software program (Image J 1.40, National Institutes of Health). The relative optical density of TH immunoreactivity in lesioned versus unlesioned striatum was compared. The TH-immunoreactive fibers remaining in the lesioned striatum were expressed as a percentage of the contralateral striatum.

Statistical Analysis. The rotational scores were assessed for within and between group differences at a significance level of $p < 0.05$ using two-way ANOVA and Tukey's post hoc testing. The statistical analysis for TH^+ cell counts and density of striatal fiber tracts were conducted using two-way ANOVA also followed by Tukey's post hoc test.

Rat Pharmacokinetics. SR-3306 was formulated in 30% β-cyclodextran containing 0.07% HCl, and four Sprague−Dawley rats were used for each dose. SR-3306 was dosed at 2.5 or 10 mg/kg in subcutaneous minipumps at a rate of 5 μL/h, and after 24 h on days 1, 2, 3, 4, 6, 7, 8, 9, 10, 13, and 14 blood, and day 14 brain were collected. Plasma was generated, and the samples were frozen at −80 °C. The plasma and brain were mixed with acetonitrile (1:5 v/v or 1:5 w/v, respectively). The brain sample was sonicated with a probe tip sonicator to break up the tissue, and samples were analyzed for compound levels by LC-MS/MS. Plasma compound levels were determined against standards made in plasma and brain levels against standards made in blank brain matrix. All procedures were approved by the Scripps Florida IACUC.

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Author Contributions

C.E.C., G.S.R., M.D.C., and P.L. conceived and designed the experiments; C.E.C. and S.K. performed the experiments; C.E.C., G.S.R., M.D.C., and P.L. analyzed the data; and G.S.R and P.L. wrote the manuscript.

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Notes
The authors declare that they have no competing financial interests.

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■ ABBREVIATIONS

DOPAC, 3,4-dihydroxyphenylacetic acid; 6-OHDA, 6-hydroxydopamine; HVA, homovanillic acid; JIP, JNK-interacting protein; JNK, c-jun-N-terminal kinase; KO, knockout; L-DOPA, levodopa; MAP, mitogen-activated protein; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; p-c-jun, phospho c-jun; SEM, standard error of the mean; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; t.i.d., three times daily.

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