

CEP11004, a novel inhibitor of the mixed lineage kinases, suppresses apoptotic death in dopamine neurons of the substantia nigra induced by 6-hydroxydopamine

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Abstract

There is much evidence that the kinase cascade which leads to the phosphorylation of c-jun plays an important signaling role in the mediation of programmed cell death. We have previously shown that c-jun is phosphorylated in a model of induced apoptotic death in dopamine neurons of the substantia nigra *in vivo*. To determine the generality and functional significance of this response, we have examined c-jun phosphorylation and the effect on cell death of a novel mixed lineage kinase inhibitor, CEP11004, in the 6-hydroxydopamine model of induced apoptotic death in dopamine neurons. We found that expression of total c-jun and Ser73-phosphorylated c-jun is increased in this model and both colocalize with

apoptotic morphology. CEP11004 suppresses apoptotic death to levels of 44 and 58% of control values at doses of 1.0 and 3.0 mg/kg, respectively. It also suppresses, to approximately equal levels, the number of profiles positive for the activated form of caspase 9. CEP11004 markedly suppresses striatal dopaminergic fiber loss in these models, to only 22% of control levels. We conclude that c-jun phosphorylation is a general feature of apoptosis in living dopamine neurons and that the mixed lineage kinases play a functional role as up-stream mediators of cell death in these neurons.

Keywords: apoptosis, c-jun, c-jun kinase, Parkinson's disease, programmed cell death.

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Programmed cell death may play a role in the pathogenesis of the chronic neurodegenerative diseases and so may provide a target for therapeutic interventions. Such interventions may be more effective if they act up-stream in death pathways. In the intrinsic, or mitochondrial, pathway of programmed cell death, for example, it would seem desirable to intervene before the release of mitochondrial death mediators (Yuan and Yankner 2000).

One up-stream signaling cascade that mediates cell death is that of c-jun N-terminal kinase (JNK) phosphorylation of c-jun. Programmed cell death in neurons can be inhibited by neutralizing antibodies to c-jun (Estus *et al.* 1994) or by dominant negative mutants (Ham *et al.* 1995). Neurotrophic factor withdrawal-induced death increases activity of JNK (Xia *et al.* 1995; Park *et al.* 1996) and its activity is, in turn, regulated by a complex up-stream kinase cascade (Fig. 1), many components of which have also been implicated in the regulation of neuronal cell death *in vitro* (Xu *et al.* 2001; Bazenet *et al.* 1998; Gallo and Johnson 2002). This signaling

cascade is likely to be up-stream of mitochondrial release of death mediators because inhibition of death with a dominant negative c-jun blocks cytochrome *c* release *in vitro* (Whitfield *et al.* 2001).

Studies with an indolocarbazole derivative, CEP-1347, which inhibits the mixed lineage kinases (MLKs) (Maroney

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Abbreviations used: ABC, avidin–biotin horseradish peroxidase complexes; BSA, bovine serum albumin; DLK, dual leucine zipper kinase; JNK, c-jun N-terminal kinase; MLK, mixed lineage kinase; 6OHDA, 6-hydroxydopamine; PB, phosphate buffer; PBS, phosphate-buffered saline; PND, post-natal day; SN, substantia nigra; SNpc, SN pars compacta; TH, tyrosine hydroxylase.

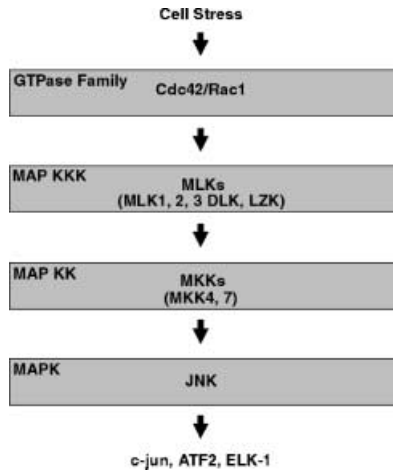


Fig. 1 The MAPK signaling pathways. A variety of cell stresses initially activate members of the Rho small GTPase family, Cdc42 and Rac1 (Bazenet *et al.* 1998). These kinases act up-stream of members of the mixed lineage kinase (MLK) family (Gallo and Johnson 2002) including MLKs 1, 2, 3, dual leucine zipper kinase (DLK) and leucine zipper kinase (LZK). These kinases are classified as MAPK kinase kinases (MAP KKKs). The MLKs phosphorylate and activate MAPK kinases (MAP KKS or MKKs). MKKs 4 and 7, in particular, have been shown to activate c-jun N-terminal kinase (JNK) isoforms (Gallo and Johnson 2002). JNK, in turn, phosphorylates and activates transcription factors including c-jun.

et al. 2001), have supported the importance of the JNK/c-jun signaling pathway in programmed cell death. *In vitro*, it suppresses death induced by neurotrophic factor withdrawal, oxidative stress and beta-amyloid treatment (Maroney *et al.* 1998, 1999; Bozyczko-Coyne *et al.* 2001). CEP-1347 also suppresses neuron death in living animals in studies of excitotoxicity (Saporito *et al.* 1998), natural cell death (Glicksman *et al.* 1998), axotomy (Glicksman *et al.* 1998) and noise-induced auditory hair cell loss (Pirvola *et al.* 2000).

We have had an interest in the role of the JNK/c-jun signaling pathway in programmed cell death of dopamine neurons of the substantia nigra (SN), which degenerate in Parkinson's disease. When these neurons are injured by the neurotoxin 6-hydroxydopamine (6OHDA) or axotomy, they show increased expression of c-jun and phosphorylated c-jun (Jenkins *et al.* 1993; Herdegen *et al.* 1998). We have shown that, when they are induced to die by apoptosis, following early target deprivation, they up-regulate c-jun, phosphorylated c-jun and JNK with the onset of death and these proteins colocalize with apoptotic morphology (Oo *et al.* 1999). The probable functional role of JNK/c-jun signaling in regulating the viability of these neurons is suggested by studies in the MPTP neurotoxin model, which have demonstrated an increased number of dopamine neurons following treatment with CEP-1347 (Saporito *et al.* 1999). However, these studies did not directly examine effects on cell death as opposed to restorative effects on phenotype. Furthermore, studies in the

MPTP model are somewhat difficult to interpret in relation to the canonical models of programmed cell death pathways because the morphology of cell death following MPTP is mixed. Acute treatment induces a necrotic form of cell death (Jackson-Lewis *et al.* 1995) and, while chronic low doses of MPTP induce apoptosis (Tatton and Kish 1997), they do not do so exclusively; a necrotic morphological pattern is also observed (Jackson-Lewis *et al.* 2000b). To explore the ability of MLK inhibition to suppress dopamine neuron death and to examine its role specifically in the intrinsic pathway we have here examined the effect of a novel MLK inhibitor, CEP11004 (Murakata *et al.* 2002), on apoptotic death in a developmental model induced by 6OHDA. We have previously shown that the morphology of death in this model is exclusively apoptotic (Marti *et al.* 1997) and we demonstrate herein that death is associated with activation of caspase 9, an up-stream caspase in the intrinsic pathway.

Materials and methods

6-hydroxydopamine lesion and CEP11004 treatment regimen

All animal procedures were performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance with protocols approved by the Institutional Animal Care and Use Committee of Columbia University and the principles outlined in the National Institute of Health *Guide for the Care and Use of Laboratory Animals*. The 6OHDA lesions were performed as previously described (Marti *et al.* 1997). Rat pups at post-natal day (PND) 7 were pre-treated with 25 mg/kg desmethylimipramine, anesthetized by hypothermia and placed prone on an ice pack. The skull was exposed by a midline incision and a burr hole was placed 3.0 mm lateral to the left of bregma on the coronal suture. A 28-gauge cannula was then inserted vertically into the striatum to a depth of 4.0 mm from the top of the skull. 6-Hydroxydopamine hydrobromide (Regis, Morton Grove, IL, USA) was prepared at 15 µg (total weight)/1.0 µL in 0.9% NaCl/0.02% ascorbic acid and infused by pump (Harvard Apparatus, Holliston, MA, USA) at a rate of 0.25 µL/min. The cannula was slowly withdrawn 2 min after the end of the infusion. After recovery from anesthesia, pups were returned to the dams. A dose-response analysis was performed with doses ranging from 1.0 to 15 µg and it was determined that 2.5 µg was the minimally effective dose. Therefore, this was the dose given in the CEP11004 experiments. CEP11004 was initially dissolved in warm Solutol and then diluted in phosphate-buffered saline (PBS) to a final Solutol concentration of 10%. CEP11004 was administered subcutaneously the day before the 6OHDA lesion and given once per day thereafter up to and including post-lesion day 6.

Tissue processing and immunohistochemistry for c-jun, phospho-c-jun, tyrosine hydroxylase and activated caspase 9

C-jun immunostaining

c-jun immunostaining was performed as previously described (Oo *et al.* 1999). Pups were anesthetized by Halothane inhalation and then perfused intracardially with 0.9% saline for 5 min by gravity, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB)

for 10 min at room temperature. The brain was removed and the SN was blocked. The SN was post-fixed in 4% paraformaldehyde in 0.1 M PB for 3 h at 4°C and then placed in 20% sucrose in 0.1 M PB for 24 h prior to sectioning. Each SN was rapidly frozen by immersion in isopentane on dry ice and then sectioned in a cryostat at 30 µm. Sections were processed free-floating. They were initially washed with PBS, followed by PBS/0.5% bovine serum albumin (BSA) and then by PBS/0.5% BSA with Triton 0.1% for 15 min at 4°C. After an additional wash in PBS, sections were incubated with antiserum to c-jun (Ab-1; Oncogene Science, Cambridge, MA, USA) at 1 : 20 for 48 h at 4°C. Sections were then washed in PBS/0.5% BSA and then incubated with biotinylated Protein A, prepared in our laboratory, at 1 : 100 for 60 min at room temperature. After a wash in PBS/0.5% BSA, sections were incubated in avidin–biotin horseradish peroxidase complexes (ABC; Vector Laboratories, Burlingame, CA, USA) at 1 : 600 for 60 min at room temperature, followed by incubation in diaminobenzidine in the presence of hydrogen peroxide to generate a brown chromogen product. Sections were mounted on slides subbed in gelatin and then thionin counterstained to identify cellular morphology and intranuclear apoptotic chromatin clumps (Janec and Burke 1993). The Ab-1 (Oncogene Science) to c-jun is an affinity-purified rabbit polyclonal antibody raised against amino acids 209–225 in the DNA-binding domain in the C-terminal region of v-jun. Previous investigators have shown that Ab-1 identifies a single band of the appropriate molecular weight on western analysis of rat brain homogenates and its regional distribution of immunostaining correlates with expression of c-jun mRNA (Harlan and Garcia 1995).

Ser-73-phospho-c-jun immunostaining

Rat pups were anesthetized by hypothermia on wet ice in an oxygenated chamber. They were perfused intracardially with 4% paraformaldehyde/0.1 M PB/0.2 mM sodium orthovanadate at a rate of 5.0 mL/min by peristaltic pump for 10 min. The brain was then removed, the SN blocked and post-fixed in the same fixative overnight. The SN block was then cryoprotected for 24 h in 20% sucrose/0.1 M PB. The tissue was frozen as described and sectioned at 30 µm on a cryostat. Sections were processed free-floating, as described for c-jun. Sections were incubated with a rabbit anti-Ser73 phospho-c-jun polyclonal antibody (9164S; Cell Signaling Technology, Beverly, MA, USA) at 1 : 50 for 48 h. Thereafter, sections were treated with biotinylated Protein A and ABC, as described for c-jun. As for c-jun, sections were thionin counterstained to identify intranuclear apoptotic chromatin clumps. Use of this antibody to detect N-terminal phosphorylated c-jun has previously been reported (Vaudano *et al.* 2001).

Tyrosine hydroxylase immunostaining

Animals were anesthetized and perfused as described for c-jun. Brains were post-fixed for 1 week at 4°C and then cryoprotected. Representative coronal sections (30 µm) were taken from each of the major planes encompassing the SN (Paxinos–Watson planes 2.7, 3.2, 3.7 and 4.2) (Paxinos and Watson 1982). After washes with PBS (2× for 15 min each), sections were incubated in primary antibody (MAB5280; Chemicon, Temecula, CA, USA) mouse monoclonal anti-tyrosine hydroxylase (TH) at 1 : 40 in PBS/10% normal horse serum for 24 h at 4°C. Sections were then washed with PBS (2× 15 min each) and incubated with biotinylated horse anti-mouse IgG (Vector Laboratories) at 1 : 50 in PBS/10% normal horse serum

at 4°C. Sections were washed in PBS/BSA (2× for 15 min each) at room temperature and then incubated with ABC (Vector Laboratories) at 1 : 600 for 1 h at room temperature. Following washes in PBS (2× for 5 min each), sections were incubated in a solution of diaminobenzidine (50 mg in 100 mL Tris buffer, pH 7.6) containing glucose oxidase, ammonium chloride and D(+) glucose to generate H₂O₂. Following three 15-min washes in Tris buffer, pH 7.6, sections were then mounted on gelatin-coated slides, left to dry overnight at room temperature and thionin counterstained.

Activated caspase 9 immunostaining

Brains were processed initially as described for c-jun and then incubated with a rabbit polyclonal antibody to the activated form of caspase 9 (anti-m9D368) (Fujita *et al.* 2000) at 1 : 200 for 48 h at 4°C. The sections were then washed in PBS/BSA and treated with biotinylated Protein A and ABC, as described for c-jun, except that the ABC step was performed twice for increased sensitivity. The sections were then incubated with diaminobenzidine and H₂O₂, as described, and thionin counterstained. The antiactivated caspase 9 antibody has previously been characterized (Fujita *et al.* 2000). Briefly, it was raised to the LDQLD³⁶⁸ C-terminal processing site of caspase 9. This antiserum reacts specifically with the processed form of expressed caspase 9 and not with the zymogen form. It detects only a band corresponding to the processed form of caspase 9 in staurosporine-treated wild type mouse embryonic fibroblasts and detects no bands in caspase 9 null mouse embryonic fibroblasts. Immunoreactivity detected by anti-m9D368 is augmented in the brains of homozygous null bcl-2 mice and observed in conjunction with positive TUNEL labeling.

Quantitative morphological analysis of c-jun, Ser73-phospho-c-jun-positive and activated-caspase 9-positive profiles

For each brain, immunostained sections (with thionin counterstain) were classified according to location within the SN pars compacta (SNpc), based on planes comparable to planes 4.2, 3.7 or 3.2 in the Paxinos–Watson atlas of adult rat brain (Paxinos and Watson 1982). At least two sections within each of these planes were selected and the SN in its entirety was scanned visually on both the non-injected (Control) and the 6OHDA-injected (Experimental) sides at 600×. Thus, ≥ 6 sections per animal were scanned. A profile was counted as positive if it contained brown chromogen nuclear staining well above background levels (as shown in Figs 2 and 3). The values for number of profiles were averaged among all of the individual sections scanned within a specific plane to obtain an overall value for that plane; these values were then added to obtain a measure of the number of profiles for each region on each side of each brain. No attempt was made to correct for double-counting error or to determine the absolute number of positive neurons per brain. For this reason, the counts are referred to as positive ‘profiles’ (Coggeshall and Lekan 1996). It should be noted, however, that double-counting error would not be expected to be large, given the small size of the nucleus in comparison to the section thickness (Saper 1996).

Qualitative morphological analysis of apoptosis in dopamine neurons

In the TH immunoperoxidase-stained sections, apoptosis was identified at the light microscope level by performing a thionin counterstain and visualizing intranuclear chromatin clumps as one or

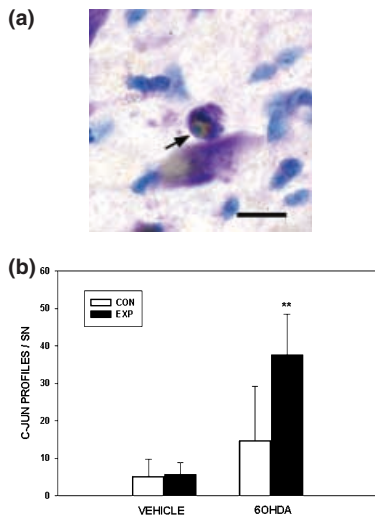


Fig. 2 The expression of c-jun is induced in substantia nigra pars compacta (SNpc) following intrastriatal injection of 6-hydroxydopamine (6OHDA). (a) Brown peroxidase staining for c-jun was observed within cell nuclei. In a number of instances, as in this profile, c-jun immunostaining colocalized with basophilic intranuclear chromatin clumps (arrow), characteristic of apoptosis. Bar, 10 μ m. (b) Quantitative analysis of the number of c-jun-positive profiles in SNpc demonstrates an about eightfold induction following intrastriatal 6OHDA (** $p < 0.02$, ANOVA; $n = 7$ vehicle injected; $n = 10$ 6OHDA injected). CON, Control, non-injected side; EXP, 6OHDA- or vehicle-injected side. SN, substantia nigra.

more intensely basophilic, homogeneously stained, round and distinctly bounded structures. We have previously shown for natural cell death in dopamine neurons, and for induction of this death event by either striatal lesion or the injection of 6OHDA, that apoptotic profiles so identified are confirmed to be apoptotic by electron microscopy (Macaya *et al.* 1994; Jackson-Lewis *et al.* 2000a). Profiles so identified can also be confirmed as apoptotic by TUNEL labeling, suppressed silver staining and immunostaining for activated caspase 3 and caspase cleavage products (Macaya *et al.* 1994; Marti *et al.* 1997; Jeon *et al.* 1999; Jackson-Lewis *et al.* 2000a; Oo *et al.* 2002).

Quantitative morphological analysis of apoptosis in neurons of the substantia nigra pars compacta

Apoptotic profiles in each SN were quantified by selecting two to three sections from each of Paxinos–Watson (Paxinos and Watson 1982) SN planes 4.2, 3.7 and 3.2 and scanning the entire SN at 600 \times . Apoptotic profiles were identified as cellular profiles containing one or more distinct, rounded basophilic chromatin clumps. Free extracellular chromatin clumps, lying outside an identifiable cellular profile, were not counted. The number of profiles in the two sections in a given plane were averaged to provide a measure for that plane and the averages for the three planes were then added to provide an index of the number of profiles for each SN. We have previously shown, using a physical disector technique (Gundersen 1986), that apoptotic profiles, as defined, are rarely split by the microtome blade (Oo and Burke 1997). Clarke and Oppenheim (1995) have

demonstrated a similar result. Thus, apoptotic profiles, identified by focusing through the section, represent unique and unbiased counts. To assess the number of apoptotic profiles derived from dopaminergic neurons of the SNpc, we have previously used two sets of criteria: cellular and regional. An apoptotic profile was considered to be dopaminergic by the cellular criterion if its cytoplasm was TH positive. It was considered to be derived from a dopamine neuron by the regional criterion if it was within 15 μ m of two TH-positive neurons. We used this regional criterion because dopamine neurons lose their cytoplasm as they undergo apoptosis, making strict phenotypic identification on the basis of cytoplasmic markers impossible (Macaya *et al.* 1994). We have shown for natural cell death in SNpc that there is a precise temporal and numerical correlation between numbers of profiles counted using these two criteria ($r = 0.939$) (Oo and Burke 1997), suggesting that they are likely to be derived from the same population.

Immunofluorescence double labeling for tyrosine hydroxylase and Ser73-phospho-c-jun

Animals were anesthetized, perfused and their brains were removed and frozen as described for phospho-c-jun. Sections were then rinsed in Tris-buffered saline (pH 7.4)/0.2% Triton/2% normal goat serum/2% normal base serum. They were then incubated with anti-TH at 1 : 40 and anti-Ser73-phospho-c-jun at 1 : 50 for 48 h at 4°C. Following rinsing in Tris-buffered saline, they were then incubated with horse anti-mouse-Texas Red (Vector Laboratories) at 1 : 75 and biotinylated goat anti-rabbit (Vector Laboratories) at 1 : 75 for 1 h at room temperature. Following a Tris-buffered saline rinse, they were incubated with fluorescein-conjugated avidin (Vector Laboratories) at 1 : 100 for 1 h. The sections were then mounted on gelatin-coated slides, dried, treated with Hoechst 33342 (0.0004%) (Molecular Probes, Eugene, OR, USA) (to demonstrate apoptotic chromatin clumps), rinsed and coverslipped with antifade medium (Dako, Carpinteria, CA, USA). The sections were then viewed under epifluorescence with an Eclipse 800 microscope (Nikon, Melville, NY, USA) or with an LSM 510 Multiphoton Confocal Microscope (Zeiss, Thornwood, NY, USA).

Striatal tyrosine hydroxylase-positive fiber staining and quantification

Animals were anesthetized and perfused as described for TH neuron staining. One week post-fixation, brains were frozen (without cryoprotection) on fine powdered dry ice. Striatal sections were cut at 30 μ m from Paxinos–Watson plane 8.7–10.2. Sections were rinsed in PBS and then treated with PBS/BSA and PBS/BSA/0.1% Triton. Sections were then incubated with a rabbit anti-TH polyclonal antibody (Calbiochem, San Diego, CA, USA) for 48 h at 4°C, treated with biotinylated Protein A and ABC as described and incubated with diaminobenzidine and H₂O₂. The optical density of the TH immunoperoxidase staining was determined as previously described (Burke *et al.* 1990) using an Analytical Imaging Station (Imaging Research, Inc., St Catharines, Canada).

Northern analysis

The SN was microdissected from PND14 animals as previously described (El-Khodori *et al.* 2001). RNA was isolated from each SN individually using an RNAeasy mini kit (Qiagen, Valencia, CA, USA). Each piece of tissue was homogenized in 350 μ L of buffer

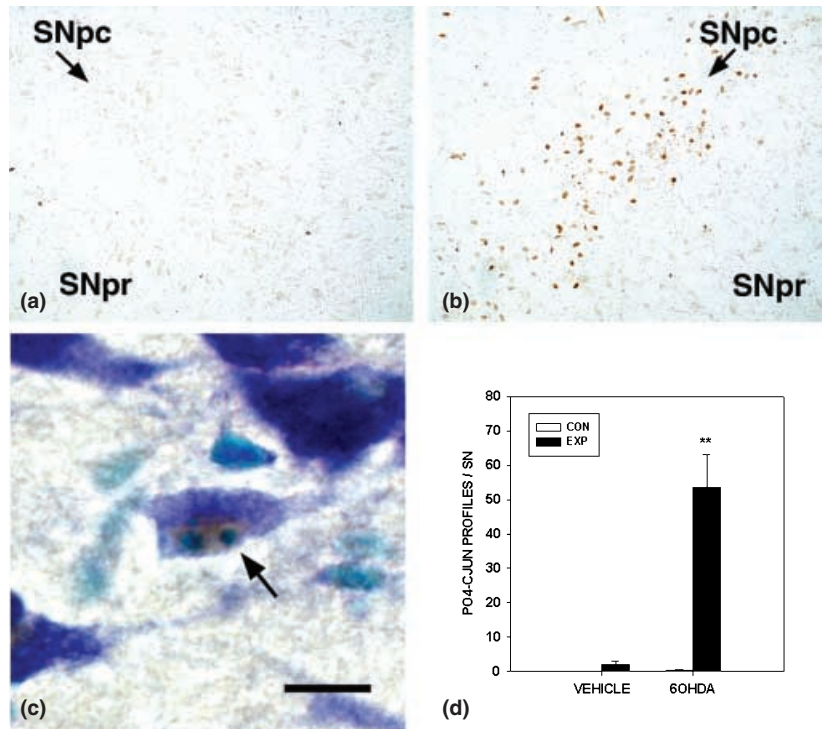


Fig. 3 Expression of Ser73-phospho-c-jun is induced in substantia nigra pars compacta (SNpc) following intrastriatal injection of 6-hydroxydopamine (6OHDA). (a and b) Low power photomicrographs of Ser73-phospho-c-jun immunostaining following intrastriatal 6OHDA. These micrographs represent the non-injected contralateral side (a) and the ipsilateral, injected side (b) of the same section. No staining is observed on the non-injected side. Robust staining is observed throughout the SNpc on the injected side. (c) At a cellular level, Ser73-phospho-c-jun staining was observed in the nuclei of neurons with

normal morphology. Occasionally apoptotic chromatin clumps were observed, as shown here (arrow), but, even in these instances, neuronal morphology appeared normal. Bar, 10 μ m. (d) Quantitative analysis of the number of phospho-c-jun-positive profiles demonstrates a marked induction following intrastriatal 6OHDA (** $p < 0.001$, ANOVA; $n = 4$ vehicle-injected; $n = 4$ 6OHDA-injected). CON, Control, non-injected side; EXP, 6OHDA- or vehicle-injected side; SN, substantia nigra; SNpr: SN pars reticulata; P04-c-jun: phosphorylated c-jun.

provided by passing it through a 27-gauge needle with a 1-mL syringe 10 times. Further steps of the RNA isolation were carried out according to the manufacturer's instructions. RNA concentration was then determined by measuring absorption at 260 nm on a GenQuant spectrophotometer (Pharmacia, Cambridge, UK); 10 μ g of each RNA sample per lane were electrophoresed in 1.4% agarose-formaldehyde gels and equal loading of lanes was confirmed by ethidium bromide staining. RNA was transferred onto an Immobilon-Ny+ membrane (Millipore Corporation, Bedford, MA, USA) using a capillary system (Sambrook *et al.* 1989) overnight in 2 \times saline sodium citrate. After transfer, RNA was cross-linked to the membrane by UV exposure in a FUNA-UV-Linker (Spectroliner, Westbury, NY, USA). Hybridization was performed overnight at 67°C in ULTRAhyb (Ambion, Austin, TX, USA). Washing was carried out according to the manufacturer's recommendations. The membrane was then dried in air, wrapped and exposed to a phosphorimager cassette. Radioactivity of bands was determined using a Phosphor Imager 445 SI and ImageQuant software.

Molecular probes for northern analysis

To create molecular probes for rat members of the MLK family we utilized known human and mouse sequences to identify candidates

for rat homologues in rat expressed sequence tag databases. Based on these sequences, primers were designed and RT-PCR was performed on mRNA derived from rat brain. Each candidate sequence was cloned and sequenced and, in each case, an open reading frame analysis revealed a highly homologous amino acid sequence to the human or mouse homologue of each corresponding MLK family member. Our sequences for these rat homologues have been entered in GenBank as the following Accession numbers: MLK1, AY240866; MLK2, AY240867; MLK3, AY240868; dual leucine zipper kinase (DLK), AY240864 and leucine zipper kinase, AY240865.

Non-radioactive *in situ* hybridization

Brains from PND14 animals were rapidly removed, frozen on dry ice and sectioned through the SN at 14 μ m. Sections were thaw mounted onto slides and stored at -80°C until use. On the day of hybridization, the sections were warmed, fixed by immersion in 4% paraformaldehyde/PBS, washed and then acetylated in triethanolamine/acetic anhydride solution. Following a wash in PBS, sections were pre-hybridized as previously described (Burke *et al.* 1994). Digoxigenin-11-UTP riboprobes were prepared as per the manufacturer's instructions (Roche Diagnostics GmbH, Penzberg, Germany). The size and integrity of labeled probe were confirmed by gel

electrophoresis. Hybridization was performed overnight at 68°C in a humidified chamber. Sections were washed in 0.2× saline sodium citrate at 68°C and then incubated overnight with an anti-digoxigenin antibody (Roche Diagnostics, Mannheim, Germany). Following a wash in 0.1 M Tris/0.15 M NaCl, sections were incubated with a solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate in a darkened humidified chamber overnight. Sections were then washed in Tris/EDTA (pH 8.0) and coverslipped with aqueous mounting medium (Dako).

Results

Expression of c-jun and Ser73-phospho-c-jun are induced in substantia nigra pars compacta following 6-hydroxydopamine

Following intrastriatal injection of 6OHDA at PND7, the expression of c-jun in SNpc was examined at post-lesion day 6, the time of peak cell death (Marti *et al.* 1997) in this model. A clear induction was observed, with the mean number of profiles increased about eightfold in comparison to vehicle-injected animals (Fig. 2). The immunostaining for c-jun at a cellular level was localized to the nucleus in all profiles (Fig. 2a). The large majority ($97.2 \pm 1.0\%$ for $n = 10$ animals) of cellular profiles with c-jun-positive nuclei had normal-appearing neuronal morphology on thionin counterstain. The remaining profiles ($2.8 \pm 1.1\%$) demonstrated intensely basophilic, round chromatin clumps, characteristic of apoptosis (Fig. 2a). There was a trend for the number of c-jun-positive profiles to be increased in the contralateral SNpc of 6OHDA-injected animals in comparison to vehicle-injected animals (Fig. 2b) suggesting a modest toxin effect on the non-injected side, but this did not achieve significance.

Following intrastriatal injection of 6OHDA, there was also a clear induction of phosphorylation of c-jun (Fig. 3). Interestingly, this induction was more robust, and more specific for injury, than the induction of total c-jun protein. c-jun protein was observed in a small but consistent number of cells even on the contralateral side of vehicle-injected animals. However, no Ser73-phospho-c-jun profiles were observed in that condition (Fig. 3d). While a small number of profiles were observed in SNpc following vehicle injection (1.9 ± 1.0 ; $n = 4$), there was a 28-fold increase in their number following 6OHDA injection (Fig. 3d). As for c-jun, the immunostaining for Ser73-phospho-c-jun was always nuclear and the large majority of stained cells showed normal neuronal morphology. Those few which showed colocalization of Ser73-phospho-c-jun staining with intranuclear apoptotic chromatin clumps showed normal neuronal morphology, suggesting that this staining is an early event in the course of apoptosis (Fig. 3c).

In order to confirm the expression of Ser73-phospho-c-jun in dopaminergic neurons of the SNpc, fluorescent double labeling was performed with immunostaining for TH. This

staining demonstrated phospho-c-jun staining exclusively within TH-positive neurons of the SNpc ipsilateral to the 6OHDA injection (Fig. 4). As observed on thionin stain, the neurons with phospho-c-jun-positive nuclei had normal neuronal morphology (Fig. 4) even when apoptotic chromatin was observed in the nucleus (Figs 4c–f).

Mixed lineage kinases are expressed in substantia nigra during development

Having shown that c-jun protein and its phosphorylation are induced in dopamine neurons of the SNpc following 6OHDA injection, we next sought to determine whether critical up-stream signaling components, particularly members of the MLK family, are expressed. We, therefore, assayed for the presence and relative abundance of mRNAs for the MLK family members by northern analysis in microdissected PND14 ventral mesencephalic tissues. As shown in Fig. 5, mRNAs for MLK 1, 2, 3, DLK and leucine zipper kinase were detectable in these tissues. The most abundant mRNA was that of DLK, which was almost four times more abundant than that of the next most abundant, MLK1. In order to confirm this higher level of expression of DLK anatomically, we performed *in situ* hybridization for DLK in comparison to another representative member of the MLKs, MLK3. This analysis demonstrated a much higher level of expression of DLK in the SN (Figs 5c and d). At a cellular level, this analysis revealed that high levels of expression of DLK are observed in neurons of the SNpc.

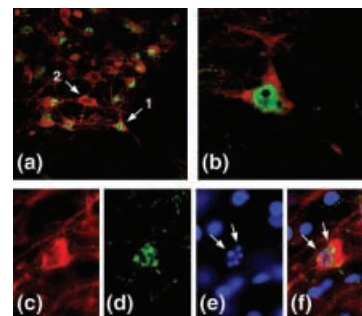


Fig. 4 Ser73-phospho-c-jun immunostaining is observed exclusively within tyrosine hydroxylase (TH)-positive neurons of the substantia nigra pars compacta (SNpc) following intrastriatal 6-hydroxydopamine injection. (a) An optical plane through the SNpc visualized at low power by confocal microscopy demonstrates that intranuclear Ser73-phospho-c-jun staining (green) is observed only in neurons with TH-positive cytoplasm (red). Profile 1, positive for both labels, is shown at higher power in (b). It has a normal polygonal shape with normal-appearing, extended neural processes. Profile 2 in (a) is TH positive but negative for phospho-c-jun. Occasional TH-positive neurons (c) which were positive for intranuclear phospho-c-jun staining (d) also contained apoptotic nuclear chromatin clumps, revealed by Hoechst 33342 dye (arrows in e and f).

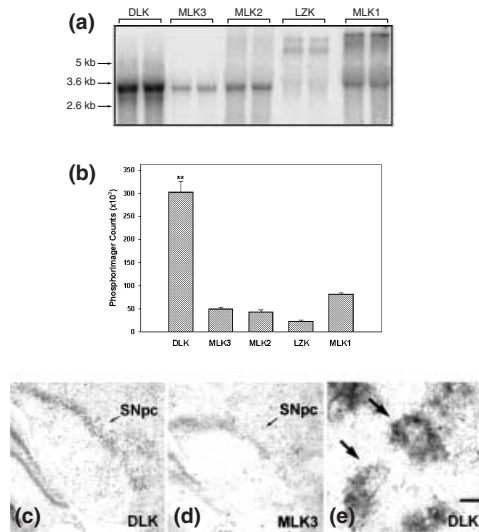


Fig. 5 Northern and *in situ* hybridization analysis of mRNA expression for members of the mixed lineage kinase (MLK) family in ventral mesencephalon. (a) A representative northern analysis demonstrates that mRNAs for dual leucine zipper kinase (DLK), MLK3, MLK2, leucine zipper kinase (LZK) and MLK1 are detectable in ventral mesencephalic tissue at post-natal day 14. For each mRNA species, samples from two animals are electrophoresed in adjacent lanes. (b) Quantification of radioactivity within bands by phosphorimager demonstrates that the most abundant mRNA species is that of DLK (each column represents the mean \pm SEM for $n = 6$ determinations). (** $p < 0.001$, ANOVA) (c) Non-radioactive *in situ* hybridization demonstrates robust expression of DLK in substantia nigra (SN) particularly the SN pars compacta (SNpc). An adjacent section, processed in parallel, probed for MLK3 (d) shows a much lower level of signal intensity. (e) A high power photomicrograph of the section shown in (c) demonstrates neuronal staining in the SNpc (arrows). Bar, 10 μ m.

CEP11004 suppresses 6-hydroxydopamine-induced apoptosis in substantia nigra dopamine neurons

In order to test the ability of CEP11004 to suppress apoptosis induced in dopamine neurons by 6OHDA, it was administered on the day before lesion and then each day until killing on the sixth post-lesion day. At a dose of 1.0 mg/kg/day, CEP11004 suppressed apoptosis to levels only 44% of those observed in vehicle-treated animals (Fig. 6a). To determine whether further suppression could be achieved with a higher dose, a second experiment was performed using 3.0 mg/kg/day. While this regimen also achieved suppression of apoptosis, it was somewhat less than that observed with 1.0 mg/kg/day; apoptosis was suppressed to levels 58% of those observed in vehicle-treated animals (Fig. 6b). This difference between the two doses, however, did not achieve significance ($p = 0.3$).

To confirm that apoptosis was suppressed in phenotypically defined dopamine neurons, we analyzed TH-positive apoptotic profiles (Fig. 6c). There was a suppression to 48% of vehicle controls, similar to that observed when both cellular and regional counting criteria were used.

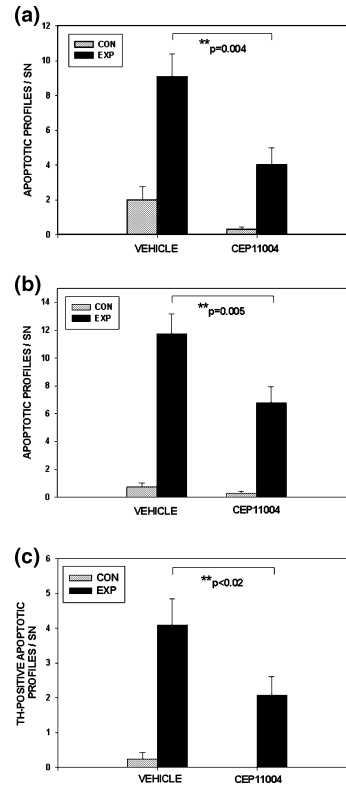


Fig. 6 CEP11004 suppresses apoptosis in dopamine neurons of the substantia nigra pars compacta (SNpc) induced by 6-hydroxydopamine (6OHDA). (a) CEP11004 administered at a dose of 1.0 mg/kg/day for 8 days, starting the day before lesion and continuing until the sixth post-lesion day, suppressed apoptosis in substantia nigra (SN) to 44% of vehicle treatment levels (** $p = 0.004$, ANOVA; Tukey all pairwise comparisons post-hoc; $n = 8$, vehicle; $n = 7$, 6OHDA). For this analysis all apoptotic profiles within the SNpc of tyrosine hydroxylase (TH)-stained sections, meeting cellular or regional criteria as defined in Materials and methods, were counted. CON, Control, non-injected side; EXP, 6OHDA-injected side. (b) CEP11004 3.0 mg/kg/day also suppressed apoptosis to 58% that of vehicle treatment (** $p = 0.005$, ANOVA; Tukey post-hoc; $n = 6$, vehicle; $n = 7$, 6OHDA). (c) The sections analyzed in (b) were also analyzed for only those profiles which met cellular criteria for being dopaminergic, i.e. they had a TH-positive cytoplasm. These TH-positive profiles also showed a significant suppression by CEP11004 (** $p < 0.02$, ANOVA).

Caspase 9 is activated following 6-hydroxydopamine injection and its activation is suppressed by CEP11004

To confirm that caspase 9 is activated when apoptosis is induced by 6OHDA, immunohistochemistry was performed with an antibody which exclusively recognizes the activated form (Fujita *et al.* 2000). Immunostaining was positive strictly in cellular profiles which also demonstrated classic basophilic intranuclear chromatin clumps by thionin counterstain (Fig. 7a). Staining was observed in some profiles exclusively within the cytoplasm (as shown in Fig. 7a) but, in others, it was either strictly perinuclear or in both cellular compartments. Given that 40–50% of apoptosis appears to be

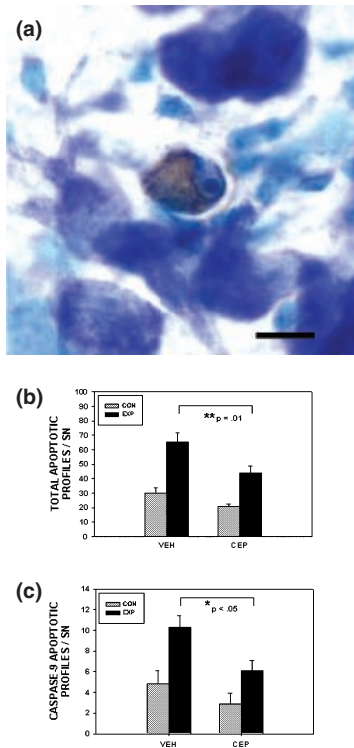


Fig. 7 CEP11004 (CEP) suppresses activation of caspase 9 upon induction of apoptosis by intrastriatal 6-hydroxydopamine (6OHDA). (a) Immunoperoxidase staining of activated caspase 9 within the cytoplasm of an apoptotic profile within the substantia nigra pars compacta (SNpc). A single, basophilic apoptotic chromatin clump is observed within the nucleus of this profile. Bar, 10 μm . (b) CEP11004 decreases the total number of apoptotic profiles identified by thionin stain within SNpc [$n = 9$ for both vehicle (VEH) and CEP11004 treatment groups; ANOVA, $p < 0.001$ for all groups; post-hoc Tukey, $**p = 0.01$ between vehicle and CEP11004 6OHDA (EXP) conditions]. CON, Control, non-injected side; EXP, 6OHDA-injected side. (c) In the same sections analyzed in (b) CEP11004 decreases the number of activated caspase 9 profiles in the SNpc following intrastriatal 6OHDA (ANOVA, $p < 0.001$ for all groups; post-hoc Tukey, $*p = 0.05$ between vehicle and CEP11004). SN, substantia nigra.

resistant to CEP11004 in this model, we sought to determine whether this resistance may be due to other death pathways acting independently of caspase 9. Such a possibility would predict that CEP11004 would suppress a greater percentage of activated caspase 9 profiles than total profiles in SNpc. We observed, however, that while CEP11004 suppressed caspase 9 profiles, to a level of 58% of vehicle-treated controls (Fig. 7c), this level was not significantly different from that observed for all thionin-stained profiles (67%) (Fig. 7b) or for profiles in SNpc in TH-immunostained material meeting either cellular or regional criteria (44%; Fig. 6a). We, therefore, conclude that the cell death in this model which is not suppressible by CEP11004 utilizes caspase 9 to a similar degree as that which is suppressible.

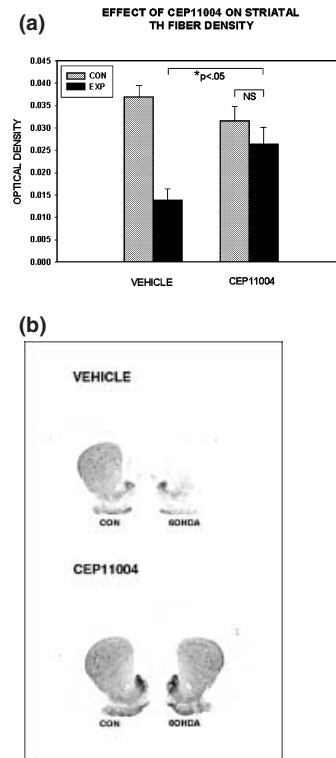


Fig. 8 CEP11004 protects striatal dopaminergic fibers in the intrastriatal 6-hydroxydopamine (6OHDA) model. (a) Optical density measurements of striatal tyrosine hydroxylase (TH) immunoperoxidase reaction product reveal a 0.023 unit (62%) reduction by 6OHDA in vehicle-treated animals. In the CEP11004-treated animals, there is only a 0.005 unit (17%) reduction. CON, Control, non-injected side; EXP, 6OHDA-injected side ($*p < 0.05$, post-hoc Tukey). (b) Representative TH-stained coronal sections of the striatum in comparable planes (Paxinos–Watson 9.7) from vehicle- and CEP11004-treated animals. There is preservation of staining on the 6OHDA-treated side in the CEP11004-treated animals. NS, not significant.

CEP11004 suppresses degeneration of tyrosine hydroxylase-positive striatal dopaminergic fibers following 6-hydroxydopamine injection

As cell death mechanisms involved in the destruction of axons may be different from those which are operative at the level of the cell body (Raff *et al.* 2002), we investigated whether CEP11004 also protects striatal dopaminergic fibers in the 6OHDA model. Administration of CEP11004 led to a clear preservation of striatal TH-positive fibers, quantified by measurement of optical density of peroxidase reaction product over the striatum (Fig. 8). Optical density values were reduced by a mean of 0.023 units in vehicle-treated animals, whereas the reduction was only 0.005 units in CEP11004-treated animals. Thus, CEP-treated animals showed a reduction only 22% of that observed in vehicle-treated animals. In the ANOVA of the data presented in Fig. 8(a), there was no significant difference between optical

densities in the 6OHDA-treated striatum and in the contralateral control striatum in CEP11004-treated animals.

Discussion

The present study confirms previous observations, made at a regional level (Vaudano *et al.* 2001), that increased expression of c-jun and phospho-c-jun accompanies the death of SNpc dopamine neurons induced by intrastriatal injection of 6OHDA and demonstrate, at a cellular level, colocalization with apoptotic morphology. Using a suppressed silver stain technique, which is capable of detecting necrosis (Nadler and Evenson 1983; Jackson-Lewis *et al.* 1995), and variant morphologies of programmed cell death (Oo *et al.* 1996) we have previously shown that the morphology of cell death in this intrastriatal 6OHDA model is exclusively apoptotic throughout its time course (Marti *et al.* 1997). We have previously shown that increased expression of c-jun and phospho-c-jun also accompanies the exclusive induction of apoptosis in a developmental target injury model (Macaya *et al.* 1994; Oo *et al.* 1999) and an axotomy model (El-Khodori and Burke 2002) (and unpublished observations). We, therefore, conclude that increased expression of c-jun and phospho-c-jun is a universal correlate of apoptotic death in dopamine neurons during development. This is the case in spite of the fact that, while these models share the classic light microscope features of neuronal apoptosis, they differ in terms of the cellular distribution of activated caspase 3 (Jeon *et al.* 1999) and caspase cleavage products (Oo *et al.* 2002), indicating that there are likely to be different underlying cellular mechanisms.

Increased expression of c-jun or phospho-c-jun also accompanies induction of apoptosis in dopamine neurons in the adult setting. Intrastriatal injection of 6OHDA in adults induces apoptosis (Marti *et al.* 2002) and this lesion has been associated with increased expression of c-jun (Jenkins *et al.* 1993; Vaudano *et al.* 2001) and phospho-c-jun (Vaudano *et al.* 2001). Likewise, low, chronic doses of MPTP induce apoptosis (Tatton and Kish 1997) and this regimen induces phosphorylation of MKK4, JNK and c-jun (Saporito *et al.* 2000; Xia *et al.* 2001). However, the precise relationship between c-jun signaling and any specific pathway of cell death in these adult models is somewhat difficult to identify because mixed morphologies of cell death occur in both the adult 6OHDA (Marti *et al.* 1997) and the low dose MPTP model (Jackson-Lewis *et al.* 2000b). In these studies, expression of c-jun-related signaling molecules was not identified in conjunction with either apoptotic morphology or caspase activation at a cellular level. In fact, one possible, but equally interesting, interpretation of these studies may be that c-jun signaling is associated with necrosis or variant morphologies of programmed cell death either instead of, or in addition to, apoptosis. This may also be the case in an adult medial forebrain bundle axotomy model in which

apoptotic morphology and *in situ* end labeling are not observed (Crocker *et al.* 2001) and yet increased expression of c-jun and phospho-c-jun have been reported (Herdegen *et al.* 1998). In any case, we can conclude, on the basis of the present work, that c-jun signaling is a common feature of apoptosis in dopamine neurons during development and, based on studies by others, that it is also a common feature of cell death in adult models, where the relationship to apoptosis, specifically, remains to be defined.

The present study also demonstrates that the association between c-jun and phospho-c-jun both in time and spatially at a cellular level with apoptosis is likely to indicate a functional role because inhibition of the MLKs, which act as up-stream mediators of c-jun phosphorylation, by administration of CEP11004 suppresses apoptotic cell death. This conclusion is supported by observations in the aforementioned adult models of dopamine neuron degeneration. Saporito *et al.* (1999) have demonstrated that treatment with CEP1347 results in an increased number of dopamine neurons following MPTP. This investigation, however, did not directly assess effects on the magnitude of cell death so it is possible that the increase in dopamine neuron number could have been due to restorative effects on phenotype, conversion of phenotype or recruitment of new neurons. However, our finding that CEP11004 inhibits cell death directly is supported by observations of Xia *et al.* (2001) who demonstrated that viral transfection of dopamine neurons with the JNK-binding domain of JNK-interacting protein-1 suppresses activated caspase 3 induction by low dose MPTP. It is further supported by the observations of Crocker *et al.* (2001) who demonstrated, in the adult axotomy model, that viral transfection of dopamine neurons with a dominant negative form of c-jun prevents the loss of Fluorogold retrogradely labeled neurons. Our observations extend these results by demonstrating *in vivo* that it is possible to suppress apoptotic cell death by intervening at the level of the MLKs, up-stream of JNK and c-jun. It is important to note that CEP11004 was not likely to be protective in the 6OHDA model on the basis of inhibition of 6OHDA uptake into terminals by the dopamine transporter. Saporito *et al.* (1999) have shown that the closely related analog, CEP1347, does not inhibit the dopamine transporter.

The present demonstration of an increased abundance of apoptotic profiles immunopositive for the activated form of caspase 9 in the SNpc following intrastriatal 6OHDA suggests that there is utilization of the canonical intrinsic, or mitochondrial, pathway of programmed cell death (Budihardjo *et al.* 1999). The ability of CEP11004 to suppress the number of these profiles suggests that the MLKs are up-stream of caspase 9. This conclusion is supported by the observations of Harris *et al.* (2002) who demonstrated, for sympathetic ganglia in culture, that another MLK inhibitor, CEP1347, blocks mitochondrial cytochrome *c* release.

We have found that 3.0 mg/kg/day of CEP11004 does not suppress death to any greater degree than 1.0 mg/kg/day, suggesting that suppression to levels of 44–58% is maximal in this model. It is unlikely that this apparent resistance is due to the presence of DLK as the dominant MLK in SN. While CEP11004 is more potent at MLKs 1, 2 and 3, it also inhibits DLK (D. Bozycko-Coyne, personal communication). It is also unlikely that the resistant population represents a different phenotype of dying cells. When the analysis of the effect of CEP11004 is restricted to TH-positive apoptotic profiles in the SNpc, the level of death suppression at 3.0 mg/kg remains 48%, similar to the level of suppression for all apoptotic profiles in SNpc at 1.0 mg/kg. We interpret the existence of this resistant cell death to mean that there are alternative pathways of death in this model which do not utilize the MLKs. These alternative pathways, however, utilize the activation of caspase 9 in the intrinsic pathway to the same degree as the MLK pathway because suppression of caspase 9 profiles was similar to the overall suppression of cell death.

The ability of CEP11004 to inhibit apoptotic death in dopamine neurons at the level of the MLKs, up-stream of other components of the JNK/c-jun kinase cascade and the activation of caspase 9, would suggest that it has the potential to forestall apoptosis early in its course. Our observations support this concept. The large majority of phospho-c-jun-positive neurons showed normal neuronal morphology and TH-positive phenotype, suggesting an early stage in the biochemical and morphological process of cell death. An alternative interpretation, that phospho-c-jun positivity in some instances serves as a marker for a process unrelated to cell death, such as a regenerative response, is unlikely for several reasons. First, the large majority of TH-positive neurons in this model die, so the markers expressed are more likely to be playing a role in cell death. Second, if we only consider those cellular instances in which apoptotic nuclear chromatin clumps were observed, indicating a commitment to die, even these profiles showed preserved neuronal morphology and TH phenotype, indicating an early stage in the death process. Third, while we did not conduct a time course study in this model, in another model of induced apoptosis, that of early target injury, the induction of c-jun occurred in phase with the induction of death (Oo *et al.* 1999). Finally, the ability of CEP11004 to directly suppress cell death, demonstrated herein, indicates that a major role for this signaling pathway is to mediate cell death rather than a regenerative response.

Our observation that CEP11004 treatment preserved striatal dopaminergic fiber staining suggests that the MLKs, in the c-jun signaling pathway, play a role not only in the destruction of the cell soma but also in the destruction of axons and terminal arborizations. It is important to recognize this as a distinct role for the MLKs because it is now clear that the molecular pathways which mediate axonal destruction are separate and distinct from those which mediate soma destruction (Raff *et al.* 2002). The preservation of

TH-positive fibers in our study is unlikely to be a secondary consequence of preservation of dopamine neurons because the 6OHDA injection is directly into the striatum so destruction of fibers is likely to occur initially. A role for the c-jun signaling pathway in dopamine axonal degeneration is supported by observations of other investigators in other models. Saporito *et al.* (1999) demonstrated preservation of striatal dopaminergic terminal markers by CEP1347 treatment in the MPTP model as did Xia *et al.* (2001) following viral transfection with the JNK-binding domain of JNK-interacting protein-1. Similarly, Crocker *et al.* (2001) demonstrated preservation of striatal dopaminergic fibers by viral transfection with dominant negative c-jun following adult axotomy. Thus, the c-jun signaling pathway may be an important therapeutic target not only for the preservation of dopamine neurons but also for their terminal axonal structures. This is a potentially important therapeutic goal because striatal dopaminergic terminal markers are reduced out of proportion to cell body loss in Parkinson's disease, suggesting that terminal structures may suffer the brunt of pathology early in the course of the disease (Kish *et al.* 1988).

In conclusion, we have shown that phosphorylation of c-jun is likely to be a universal feature of apoptosis of dopamine neurons in a developmental setting. It is also widely observed in adult models of induced death of these neurons in which a variety of death morphologies, including apoptosis, occur. We have shown that the kinase pathway leading to c-jun phosphorylation is likely to be playing a functional role in the apoptotic death process because inhibition of the MLKs with CEP11004 directly suppresses apoptosis. The c-jun phosphorylation kinase pathway is likely to be acting, at least in part, through the intrinsic pathway of programmed cell death by activating caspase 9. Our cellular analysis of phospho-c-jun-immunopositive profiles suggests that MLK inhibition may intervene early in the cell death process prior to irreversible morphological alteration. Mixed lineage kinase inhibition may, therefore, provide a therapeutic strategy for the treatment of neurodegenerative diseases affecting dopamine neurons, such as Parkinson's disease, in which programmed cell death has been postulated to occur.

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