

Expression of c-fos, c-jun, and c-jun N-terminal kinase (JNK) in a Developmental Model of Induced Apoptotic Death in Neurons of the Substantia Nigra

Tinmarla F. Oo, Claire Henscheliffe, Daylon James, and Robert E. Burke

Department of Neurology, Columbia University College of Physicians and Surgeons, New York, New York, U.S.A.

Abstract: The transcription factors c-fos and c-jun have been proposed to play a role in the initiation of programmed cell death in neurons. We have shown that programmed cell death, with the morphology of apoptosis, occurs in dopamine neurons of the substantia nigra (SN) during normal postnatal development and that this death event can be induced by early striatal target injury. We have investigated the relationship between c-fos and c-jun protein expression and induced death in neurons of the SN. Although c-fos is induced, it is unlikely to play a role in cell death, because its expression is not well correlated with apoptotic death either temporally or at a cellular level. Expression of c-jun, however, is both temporally and regionally correlated with induction of death, and, at a cellular level, it colocalizes with apoptotic morphology. The increased expression of c-jun is likely to be functionally significant, because it is associated with increased c-jun N-terminal kinase (JNK) and phosphorylated c-jun expression. JNK expression also colocalizes with apoptotic morphology. We conclude that c-jun is likely to play a role in the initiation of apoptotic cell death in these neurons. **Key Words:** Programmed cell death—Apoptosis—Dopamine neuron—Substantia nigra—Parkinson's disease.

J. Neurochem. **72**, 557–564 (1999).

There is substantial evidence from both *in vivo* and *in vitro* studies that the transcription factors c-fos and c-jun may play a role in regulating programmed cell death in neurons. Expression of c-fos, for example, has been shown to precede normal developmental programmed cell death in several tissues and augmented apoptotic neuron death in cerebellum in the mouse mutant *weaver* (Smeyne et al., 1993). In an *in vitro* model of apoptotic death in sympathetic neurons induced by nerve growth factor withdrawal, *c-jun* mRNA expression was increased, and neurons were protected from death by intracellular microinjection of neutralizing antibodies to c-jun (Estus et al., 1994). In a similar model, other investigators have shown that nerve growth factor withdrawal induced c-jun phosphorylation and increased levels of c-jun protein (Ham et al., 1995). In addition, it was

shown that transfection with an expression vector for a dominant negative mutant of *c-jun* protected neurons from apoptotic death (Ham et al., 1995). Virdee et al. (1997) have shown that nerve growth factor deprivation causes an increase in c-jun N-terminal kinase (JNK) activity in primary rat sympathetic neurons and that suppression of this activity, at early postdeprivation times, rescues neurons. However, they also showed that the onset of c-jun expression and the timing of its phosphorylation did not correlate with death commitment and that c-jun expression was not confined to apoptotic nuclei, so they concluded that c-jun expression was perhaps necessary, but not sufficient, for cell death.

In an *in vivo* model of hypoxic–ischemic injury, Dragunow and colleagues demonstrated c-jun expression in neurons undergoing delayed death, probably due to programmed cell death (Dragunow et al., 1993, 1994; Dragunow and Preston, 1995). Expression of c-jun has also been observed by Ferrer et al. (1996a) *in vivo* in cerebellar neurons undergoing apoptotic death following exposure to ionizing radiation. These investigators have also shown that c-jun is expressed in apoptotic profiles during natural developmental cell death in brain (Ferrer et al., 1996b).

We have previously shown that natural cell death occurs in dopamine neurons of the substantia nigra (SN) pars compacta (SNpc), with the morphology of apoptosis (Janec and Burke, 1993; Oo and Burke, 1997). As for many other developing neural systems (Clarke, 1985), this natural cell death event can be augmented by a lesion to the target of these neurons (Macaya et al., 1994). We have shown that an axon-sparing lesion to the striatum

Received June 29, 1998; revised manuscript September 16, 1998; accepted September 17, 1998.

Address correspondence and reprint requests to Dr. R. E. Burke at Box 67, Department of Neurology, Columbia University College of Physicians and Surgeons, 710 West 168th Street, New York, NY 10032, U.S.A.

Abbreviations used: BSA, bovine serum albumin; JNK, c-jun N-terminal kinase; PB, phosphate buffer; PBS, phosphate-buffered saline; PND, postnatal day; QA, quinolinic acid; SN, substantia nigra; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata.

made with the excitotoxin quinolinic acid (QA) results in an eightfold increase in the number of apoptotic profiles in the SNpc, peaking at 24 h (Macaya et al., 1994). The induced cell death is identical in its apoptotic morphologic features at the light microscope level to that observed during natural cell death and is confirmed to be apoptotic by ultrastructural analysis and *in situ* DNA 3' end-labeling (Macaya et al., 1994). A suppressed silver staining technique demonstrates that induced cell death in this model is exclusively apoptotic. We have suggested that the SNpc shows augmented cell death in this model due to diminished developmental support provided in retrograde fashion by the striatal target, as envisioned by classic neurotrophic theory (Barde, 1989). The possibility that developing SNpc dopamine neurons depend on interaction with their target, the striatum, for viability is supported by the observation that selective lesion of their terminals within the striatum with the neurotoxin 6-hydroxydopamine, which spares intrinsic striatal neurons, also leads to an induction of apoptotic death (Marti et al., 1997).

Little is known of the molecular basis for induced apoptotic death in SN neurons in these models. The molecular events underlying programmed cell death can be thought of as occurring in three phases: an initial signaling phase, an effector phase, and a late phase in which cells undergo the structural changes characteristic of apoptosis (Goldstein, 1997). We have investigated whether c-fos or c-jun plays a role in the initial signaling phase of induced apoptotic death among SN neurons. To this end, we have studied the regional and temporal correlations between expression of these transcription factors and the occurrence of apoptotic death. In addition, we have examined at a cellular level the relationship between expression of these factors and the presence of apoptotic morphology. In relation to c-jun, we have also examined the possible functional significance of increased protein expression by examining its phosphorylation status and the expression of JNK.

MATERIALS AND METHODS

Striatal QA lesion

Timed pregnant female rats were obtained from Charles River Laboratories (Wilmington, MA, U.S.A.). On postnatal day (PND) 12 or 13, rat pups were anesthetized with methoxyflurane (Metofane) by inhalation and hypothermia. A burr hole was placed 3.0 mm lateral to bregma along the coronal suture, and a 28-gauge cannula was inserted into the brain to a depth of 4.0 mm below the skull. QA (Sigma), at a concentration of 480 nmol/ μ l [in 0.1 M phosphate-buffered saline (PBS), pH 7.2] was infused by pump at a rate of 0.5 μ l/min. At the end of the infusion, the cannula was slowly withdrawn after an interval of 2.0 min. Pups were maintained in a group under a heating lamp until recovery from anesthesia and were then returned to the dam. This procedure has been approved by the Institutional Animal Care and Use Committee of Columbia University. We have previously shown in this model that there is no difference in number of apoptotic profiles between the SN on the side contralateral to the QA injection and either the ipsilateral or the contralateral side of vehicle-injected animals (Macaya et al.,

1994; Kelly and Burke, 1996). For that reason, in these studies we have used the SN contralateral to the side of the QA injection as a control. The contralateral control is precisely matched to the experimental side in all variables related to tissue processing, including degree of fixation, section thickness, tissue permeabilization, and chromogen reaction.

Tissue preparation and immunohistochemistry

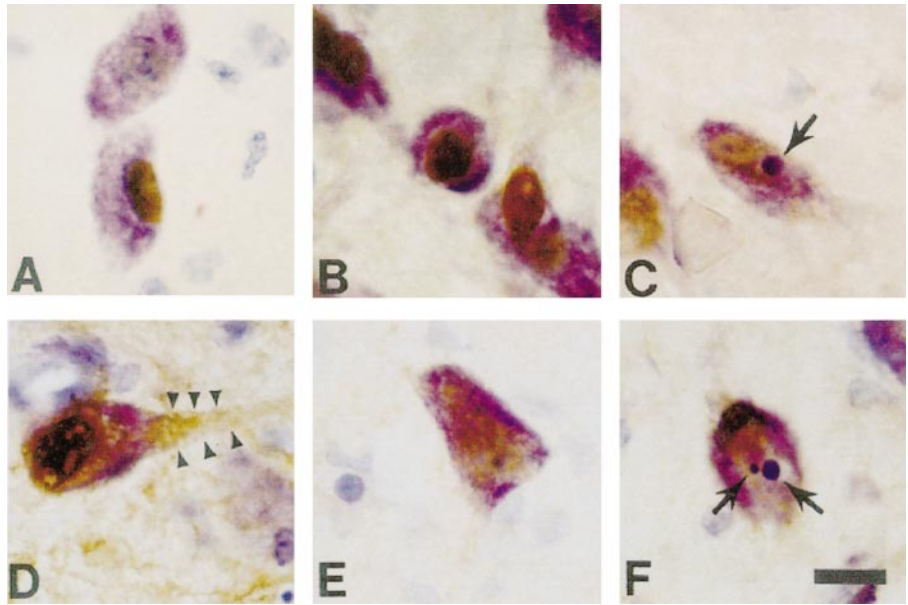
At varying postlesion times, pups were anesthetized by Metofane inhalation and then perfused intracardially with 0.9% NaCl for 5 min by gravity, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 10 min at room temperature. The brain was removed, and the SN and striatum were blocked. The SN was postfixed 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 before sectioning. Each SN was rapidly frozen by immersion in isopentane on dry ice and then sectioned in a cryostat. For the c-fos and c-jun staining, alternate 30- μ m sections were saved for each and collected in PBS. The striatal block was postfixed in 4% paraformaldehyde in 0.1 M PB for 1 week and then serially sectioned and thionin-stained to confirm lesion placement.

For c-fos and c-jun immunostaining, all sections were processed free-floating. Sections were initially washed with PBS, followed by PBS containing 0.5% bovine serum albumin (BSA) and then by PBS containing 0.5% BSA with 0.1% Triton for 15 min at 4°C. After an additional wash in PBS, sections were incubated with antiserum to either c-fos (Ab-5; Oncogene Science) at 1:1,000 in PBS containing 0.5% BSA or c-jun (Ab-1; Oncogene Science) at 1:20 for 48 h at 4°C. Sections were then washed in PBS containing 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 containing 0.5% BSA, sections were incubated in avidin-biotin horseradish peroxidase complexes (ABC; Vector Laboratories) 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 to slides subbed in gelatin and then thionin-counterstained to identify cellular morphology and intranuclear apoptotic chromatin clumps (Janec and Burke, 1993; Macaya et al., 1994). The Oncogene Science Ab-5 rabbit polyclonal serum was raised against residues 4–17 of human fos; it does not cross-react with the 39,000-kDa jun protein. Ab-5 has previously been demonstrated to be specific for c-fos protein in immunohistochemical studies of rat brain (Elmqvist et al., 1996). The Oncogene Science Ab-1 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. Prior 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).

For JNK immunostaining, primary antibody was diluted at 1:50, and a procedure identical to that outlined above was followed, with the exception that sections were cut at 20 μ m. The antibody used was an affinity-purified rabbit polyclonal antibody (JNK FL; catalogue no. 571; Santa Cruz) raised against recombinant full-length (amino acids 1–384) human JNK1 produced in *Escherichia coli*. This antibody recognizes a single band at 46 kDa in rat brain homogenates (Ferrer et al., 1997).

For phosphorylated c-jun, primary antibody was diluted 1:200, and 20- μ m sections were also used. The antibody was a

FIG. 1. Immunoperoxidase stain, with thionin counterstain, for c-fos, c-jun, phosphorylated c-jun, and JNK in the SN. **A:** Immunostaining for c-fos in SNpr at 4 h post-striatal lesion. The c-fos staining is demonstrated as a brown reaction product; thionin counterstain demonstrates neuronal morphology. In this neuron, immunoreactivity is restricted to the nucleus. Similar observations were made in SNpc. **B:** Staining for c-jun in SNpc at 24 h postlesion. Brown peroxidase reaction was most distinct within the nucleus. Thionin counterstain demonstrated that cells with c-jun-positive nuclei were neurons. Before thionin counterstain, faint c-jun positivity could be observed in the cytoplasm and processes, but this staining has now been obscured by the counterstain. **C:** A c-jun-positive neuronal nucleus is demonstrated in SNpr at 24 h postlesion. In this particular profile, thionin counterstain identified a single, rounded, distinct chromatin clump within the nucleus (arrow). Such rounded, intensely basophilic chromatin clumps are characteristic of apoptosis (Janec and Burke, 1993; Clarke and Oppenheim, 1995). **D:** Brown peroxidase reaction product demonstrates phosphorylated c-jun immunoreactivity in this neuron in SNpr at 24 h postlesion. This profile demonstrates intense brown reaction product deposition within the nucleus and a faint brown product within the proximal portion of a neuronal process (small black triangles). **E:** JNK immunoreactivity in a neuron in the SNpr at 24 h postlesion. As for c-jun and phosphorylated c-jun, reactivity is most intense in the nucleus, but before thionin counterstain, faint reactivity was observed in the cytoplasm and processes. **F:** In this JNK-positive profile in SNpr at 24 h, thionin counterstain demonstrates two distinct, basophilic chromatin clumps (arrows), characteristic of apoptosis. Bar = 10 μ m.



mouse monoclonal antibody (catalogue no. 822; Santa Cruz) raised against a peptide corresponding to amino acids 56–69 of c-jun of human origin. This antibody reacts with c-jun phosphorylated on Ser⁶³ but does not react with c-jun that is not phosphorylated at Ser⁶³. For immunostaining with this mouse monoclonal antibody, sections were washed in PBS and then incubated with the primary antibody in PBS containing 10% horse serum for 24 h at 4°C. Following a wash in PBS, sections were incubated with biotinylated horse anti-mouse antibody at 1:50 (Vector Laboratories) for 24 h at 4°C. Sections were then incubated in avidin–biotin horseradish peroxidase complexes (ABC; Vector Laboratories) at 1:600 for 60 min at room temperature, followed by incubation in diaminobenzidine as described above. Sections stained for either JNK or phosphorylated c-jun were mounted onto gelatin-subbed sections and thionin-counterstained to identify cellular morphology and apoptotic chromatin clumps.

Quantitative morphological analysis

For each brain, immunostained sections (with thionin counterstain) were classified according to location within the SNpc, based on planes comparable to plane 4.2, 3.7, or 3.2 in the adult rat brain atlas of Paxinos and Watson (1982). One or two sections within each of these planes were selected, and the SN in its entirety was scanned visually on both the noninjected (control) side and on the QA-injected (experimental) sides at $\times 600$. Thus, three to six sections per animal were scanned. Both the SNpc and the SN pars reticulata (SNpr) were scanned, and positive profiles within each region were counted separately. A profile was counted as positive if it contained brown chromogen nuclear staining well above background levels (as shown in Fig. 1). 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 pointed out that, in general, there were very few positive profiles for any of these proteins on the control side, so the main purpose of the profile counts was to delineate the time course of expression on the experimental side. For that purpose, relative profile counts serve as well as absolute counts.

Each selected section was also scanned for the presence of apoptotic profiles. A profile was counted if it contained one or more intensely basophilic, distinct, and rounded chromatin clumps contained within a nucleus, as previously described (Janec and Burke, 1993; Macaya et al., 1994). We have previously shown in this model that profiles identified in this way at the light microscope level are confirmed to be apoptotic at the ultrastructural level and by 3' end-labeling. Chromatin clumps not enclosed within a nucleus were not counted.

RESULTS

Expression of c-fos

Expression of c-fos protein was induced in the SN following developmental striatal excitotoxic injury. In both the SNpc and SNpr, positive immunostaining was demonstrated exclusively in neurons, and it was restricted to the nucleus (Fig. 1A). In SNpc, c-fos-positive

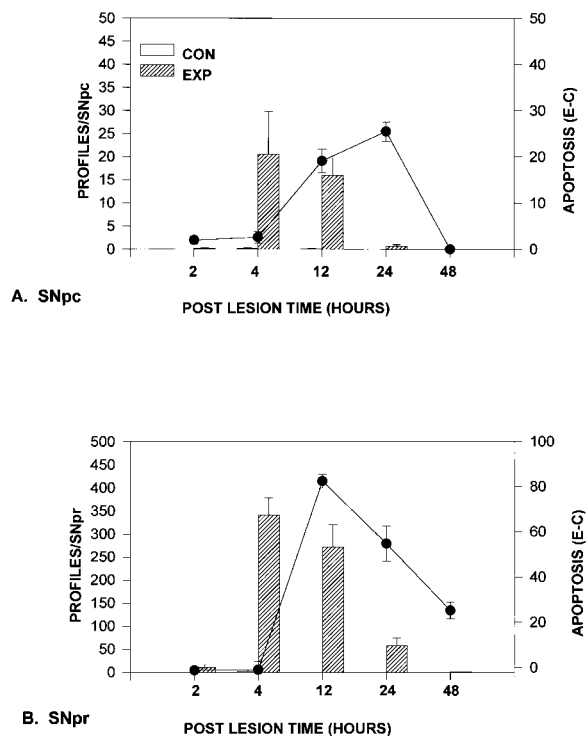


FIG. 2. Time course of c-fos expression in SN following striatal excitotoxic lesion at PND 12. **A:** In SNpc. PND 12 animals underwent striatal QA lesion as described in Materials and Methods and were processed for c-fos immunostaining. A total of 27 animals was studied ($n = 4-6$ at each time point). As described in Materials and Methods, one or two sections representative of each of the planes 4.2, 3.7, and 3.2 in the atlas of Paxinos and Watson (1982) were examined, and profiles were counted at $\times 600$. Counts within each plane were averaged, and the averages of each plane were added to provide an index of the number of profiles per region. Each section was thionin-counterstained to identify apoptotic chromatin clumps, and the number of apoptotic profiles was also determined. Because the number of apoptotic profiles due to natural cell death varies during this interval (Janec and Burke, 1993), we have expressed the number of induced profiles as the number on the experimental (E or EXP) side minus the number on the control (C or CON) side. The number of c-fos-positive profiles peaked at 4 h, before an induction of apoptotic profiles. By 24 h, the c-fos response had abated. **B:** In SNpr. As in SNpc, c-fos expression peaked at 4 h, before an induction of apoptotic death, and largely abated by 24 h.

profiles first appeared at 4 h postlesion (Fig. 2A). At that time, few apoptotic profiles were observed. Levels were comparable to those identified on the nonlesioned side (Fig. 2A) and as previously observed in normal animals at this postnatal age (Janec and Burke, 1993). By 12 h, there was a clear induction of the number of apoptotic profiles on the side of striatal injury (Fig. 2A) as previously reported (Macaya et al., 1994). At that time, the number of c-fos-positive profiles was comparable to the number observed at 4 h. At a cellular level, at 12 h, we did not observe colocalization of c-fos positivity with apoptotic chromatin clumps, defined by thionin counterstain. By 24 h, when induction of apoptotic profiles

peaked, the c-fos response had largely abated, and by 48 h no c-fos profiles were observed. At 4 h, when c-fos expression peaked, $14 \pm 2.2\%$ of neuronal profiles in SNpc were positive, based on an assessment of 1,290 neuronal profiles in five animals.

Expression of c-fos in SNpr followed a temporal pattern similar to that observed in SNpc. Expression was first induced at 4 h postlesion, before induction of apoptotic profiles (Fig. 2B). As in SNpc, induction persisted at 12 h, when, in SNpr, the number of apoptotic profiles peaked. At 12 h, we did not observe colocalization of c-fos staining and apoptotic chromatin clumps. In absolute terms, there were ~ 10 -fold more c-fos-positive profiles in SNpr than SNpc. At 4 h, $54.6 \pm 4.4\%$ of neuronal profiles in SNpr were c-fos-positive, based on an assessment of 465 profiles.

Expression of c-jun

Expression of c-jun protein was induced in both SNpc and SNpr following striatal injury (Fig. 1B). As for c-fos, staining was exclusively neuronal. At a cellular level, staining was predominantly nuclear, but faint homogeneous staining was observed in the cytoplasm of the cell soma and in processes. In SNpc, the first significant induction of c-jun-positive profiles occurred at 24 h (ANOVA, $p < 0.001$), at the peak of apoptotic cell death (Fig. 3A). Although there was a trend for an increase in number of profiles at 12 h, this did not achieve significance. At 24 h, some c-jun-positive profiles did colocalize with apoptotic chromatin clumps (Fig. 1C), but they were $< 5\%$ of all c-jun-positive profiles. In absolute terms, at the time of maximal expression, there were about eightfold more c-jun-positive profiles in SNpc than there were c-fos-positive profiles at its maximum at 4 h. These comparisons were made in the same animals, with alternate sections stained for c-fos and c-jun. At 24 h, $42 \pm 5.0\%$ profiles in the SNpc were c-jun-positive (in 1,677 profiles among six animals). By 48 h after striatal injury, the c-jun response in SNpc had abated, as had the apoptotic response.

Expression of c-jun in SNpr peaked at 12 h, at a time when the apoptotic response also peaked. Thus, in both SNpc and SNpr, there was a correspondence in time between maximal c-jun expression and the apoptotic response. In SNpr, the number of c-jun-positive profiles continued to be increased at 24 h. In SNpr, as in SNpc, there was occasional colocalization of c-jun expression and chromatin clumps, in $< 5\%$ of the c-jun-positive profiles. In absolute terms, the number of c-jun-positive profiles at maximum (12 h) in SNpr (187.9 ± 78) was comparable to their peak number in SNpc at maximum (165 ± 59 at 24 h). At 24 h, the percentage of c-jun-positive profiles was $41.3 \pm 3.7\%$ (in 484 profiles), quite similar to the number observed in the SNpc at that time.

To investigate the functional significance of the induction of c-jun protein expression at 24 h postlesion, we examined its phosphorylation status. Phosphorylation of Ser⁶³ induces increased transcriptional activity (Davis, 1994). We performed immunohistochemistry with a

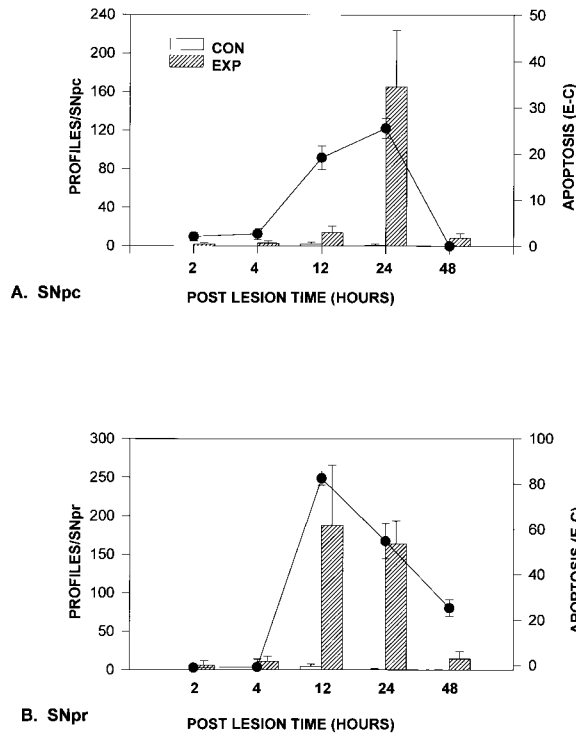


FIG. 3. Time course of *c-jun* expression in SN following striatal excitotoxic lesion at PND 12. **A:** In SNpc. The same animals analyzed for *c-fos* expression, shown in Fig. 2, had alternate sections processed for *c-jun* immunostaining. Expression of *c-jun* peaked at 24 h, coincident with the peak of apoptotic cell death. The response had abated by 48 h. **B:** In SNpr. Maximal expression of *c-jun* in SNpr occurred earlier than in SNpc, at 12 h, but like in SNpc, there was a correspondence between this peak of *c-jun* expression and the time of maximal induction of apoptotic death. E or EXP, experimental; C or CON, control.

mouse monoclonal antibody that reacts specifically with *c-jun* p39 phosphorylated on Ser⁶³ (catalogue no. SC-822; Santa Cruz). This reagent identified positive phosphorylated *jun* staining in both SNpc and SNpr (Fig. 1D). As with *c-jun*, staining was strictly neuronal and predominantly nuclear, but some faint staining was also observed in the cytoplasm of the cell soma and in processes. In both SNpc and SNpr, there was an induction of expression on the experimental side in comparison with values on the control side (Fig. 4). We did not attempt to make a direct, absolute comparison between the numbers of *c-jun*-positive and phosphorylated *jun*-positive profiles, because separate animals and immunostaining protocols were used. However, we noted that although numbers of *c-jun* profiles were comparable between SNpc and SNpr, there were approximately fourfold more phosphorylated profiles in SNpr. This may suggest that an active, phosphorylated status is more likely to be achieved in SNpr.

Expression of JNK

Phosphorylation of *c-jun* at Ser⁶³ is mediated by JNK (Davis, 1994). We examined whether increased protein expression of this kinase occurs in association with the

FIG. 4. Quantitative morphologic analysis of phosphorylated *c-jun* expression in SN at 24 h following developmental striatal excitotoxic lesion. A total of five animals underwent PND 12 striatal lesion and were processed for phosphorylated *c-jun* immunostaining at 24 h postlesion. One section from each of the planes 4.2, 3.7, and 3.2 in the atlas of Paxinos and Watson (1982) was examined, as described in Materials and Methods. At 24 h, both SNpc and SNpr show an induction of *c-jun* immunoreactivity (Fig. 3). At this time, both regions show an increase in the number of phosphorylated *c-jun*-reactive profiles. CON, control; EXP, experimental.

demonstrated increase in the number of neurons expressing phosphorylated *c-jun*. JNK expression was observed in SNpc and SNpr following striatal lesion. As for *c-jun* and phosphorylated *c-jun*, expression was strictly in neurons and predominantly in the nucleus (Fig. 1E). In both SNpc and SNpr, the time course of expression paralleled that observed for *c-jun* and the number of apoptotic profiles. Levels were minimal at 4 h, maximal at 24 h, and diminished by 48 h (Fig. 5). Although it is difficult to make direct absolute comparisons between the number

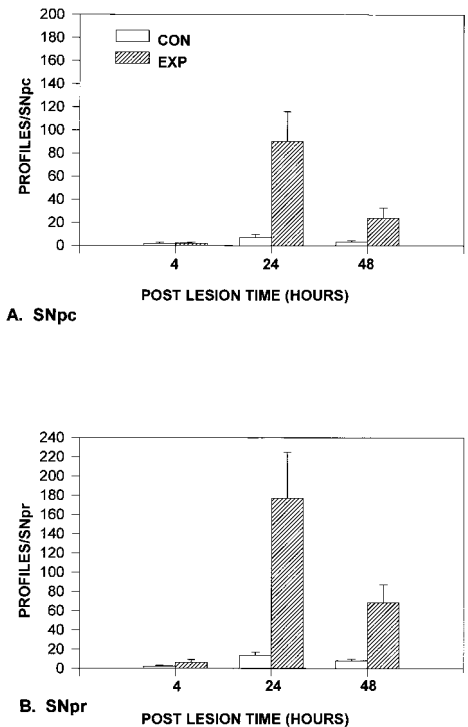


FIG. 5. Time course of JNK expression in SN following striatal excitotoxic lesion. A total of 17 animals underwent striatal lesion and were processed for JNK immunostaining at the postlesion times indicated (n = 5–7 at each time point). **A:** In SNpc. Similar to observations made for *c-jun* (Fig. 3), the expression of JNK was minimal at 4 h and peaked at 24 h. **B:** In SNpr. JNK expression in SNpr paralleled that in SNpc. In absolute terms, there were approximately twofold more JNK profiles than were observed in SNpc. CON, control; EXP, experimental.

of profiles observed for c-jun and JNK, because of differences across experiments and reagents, the percentage of JNK-positive profiles in SNpc ($21.8 \pm 4.9\%$ in 1,273 profiles among five animals) was approximately one-half that for c-jun-positive profiles (42%), suggesting that not all cells in SNpc with increased c-jun expression also express JNK. At a cellular level, $<5\%$ of JNK-positive profiles contained apoptotic chromatin clumps (Fig. 1F).

DISCUSSION

This analysis demonstrates that c-fos expression at the protein level is increased in the SN following developmental striatal excitotoxic injury. Expression occurs exclusively in the nucleus of neurons, in both SNpc and SNpr, where we have previously (Macaya et al., 1994), and in the current study, shown that an induced apoptotic cell death event takes place. However, we have found that there was not a close correspondence in time between induction of c-fos expression and induction of apoptotic death. In addition, we did not observe at a cellular level any colocalization of c-fos staining and apoptotic morphology. There are two principal interpretations of these results. The first is that c-fos may be an early initiator of apoptotic cell death in SNpc and SNpr, but by the time individual cells develop an apoptotic morphology, they no longer express c-fos. Alternatively, c-fos expression may relate to another cellular response and not to cell death. For example, intra-striatal injection of QA would be expected to result initially in augmented firing rates in striatal efferent neurons. The SNpc and especially the SNpr receive important afferent projections from the striatum (Gerfen et al., 1987); their c-fos expression may relate to this augmentation in afferent input (Dragunow and Faull, 1989).

We favor the interpretation that c-fos expression in this model is not likely to play a role in the apoptotic cell death response. The time required for an individual cell to undergo apoptotic death is brief, generally a few hours (Oppenheim, 1991; Pittman et al., 1993), whereas the induction of cell death in this model extends over a 12–48-h period. Therefore, at the time of maximal death in this model, e.g., 24 h in SNpc, it would be expected that there would be a spectrum of cells in the different phases of apoptosis: some at the early initiation phase and some at later stages characterized by apoptotic morphology. However, at 24 h in SNpc there was virtually no induction of c-fos, and at no time did we observe c-fos immunoreactivity at a cellular level in association with apoptotic morphology. Another reason for doubting a relationship between c-fos expression and cell death in this model is that there were 10-fold more c-fos-positive profiles in SNpr than in SNpc, and yet the levels of induced death in SNpr were only twofold greater. An additional reason for questioning a role for c-fos expression in the death process is that we observed expression consistently confined to the nucleus. Smeyne et al. (1993) noted that within a few hours after kainate injection, c-fos immunoreactivity was confined to neuronal

nuclei. However, several days later, as a prelude to impending cell death, c-fos expression was distributed throughout the cell. We did not observe this pattern of immunostaining. We therefore conclude that the early expression of c-fos in neuronal nuclei within the SN after striatal QA injection is more likely to be related to the acute effects of excitotoxin injection than to the later apoptotic cell death process.

In contrast to c-fos, c-jun expression showed a close temporal relationship to the occurrence of induced apoptotic cell death in both SNpc and SNpr. In both structures, at a regional level, maximal expression of c-jun correlated with maximal levels of induced apoptotic death. In addition, at a cellular level, c-jun expression was identified in some apoptotic profiles, demonstrated by the presence of intensely basophilic, distinct, rounded chromatin clumps. The presence of rounded chromatin clumps, revealed by basophilic dyes, has been confirmed at an ultrastructural level by many investigators to be associated with apoptotic morphology (Cunningham et al., 1982; Ferrer et al., 1990; Ferrer, 1992; Sloviter et al., 1993; Clarke and Oppenheim, 1995), and we have confirmed that such is the case in this model (Macaya et al., 1994). Therefore, the presence of such clumps in these c-jun-positive profiles can be interpreted as specific morphologic evidence of apoptotic cell death. Another feature of the immunostaining that suggests a role for c-jun in cell death is that its expression was not confined to the nucleus, but was observed in the cytoplasm as well. As previously mentioned, this distribution of c-fos was observed before cell death (Smeyne et al., 1993). Similarly, Ferrer et al. (1996b) have reported c-jun expression in the cytoplasm in the setting of apoptotic death in brain.

However, unlike some other *in vivo* models in which c-jun expression has been associated with apoptotic morphology at a cellular level, this model induced this association only rarely. At the time of maximal c-jun expression, $<5\%$ of positive profiles exhibited apoptotic morphology. In contrast, Ferrer et al. (1996b) noted that during natural developmental cell death in rat brain, the majority of cells with strong c-jun immunoreactivity appeared apoptotic. When cell death was induced by irradiation of the brain, c-jun expression was restricted to apoptotic cells (Ferrer et al., 1996b). This difference in the prevalence of apoptotic morphology in c-jun-positive profiles is likely to be due to either the differences in the paradigms used to examine apoptotic death or the age of the animals studied. Ferrer et al. (1996b) confined their studies to less mature animals. This difference suggests that, in the model we have studied, c-jun expression is not exclusively related to an induced death response. In the SNpc, for example, at 24 h postlesion, 42% of neuronal profiles were c-jun-positive. It is unlikely that this number of neurons will go on to die in this model. We have previously analyzed in detail quantitative aspects of SNpc dopamine neuron loss in this model and have found that on average $\sim 20\%$ of these neurons are lost (Burke et al., 1992; Macaya and Burke, 1992). It seems likely that at least some of the c-jun expression in this

model either is related to another cellular response or does not inevitably lead to cell death. One possibility is that a sprouting response may occur in neurons destined to survive, and c-jun has been implicated as playing a role in initiating such regenerative responses (Haas et al., 1996; Herdegen et al., 1997). Thus, we conclude for this model, as did Virdee et al. (1997) for sympathetic neurons, that c-jun expression may be necessary, but is not sufficient, for cell death to occur.

The correlation in time between expression of c-jun in this model and the expression of JNK and phosphorylated c-jun suggests that the induction of c-jun is likely to be functionally significant. Like c-jun, expression of phosphorylated c-jun was observed in the cytoplasm of neurons (Fig. 1D) as well as in the nucleus. As previously mentioned, this pattern has been reported for c-fos and c-jun in the setting of apoptotic death. Increased expression of JNK could, in both SNpc and SNpr, be correlated at the cellular level with apoptotic morphology. The possibility that JNK expression in these cells may play a role in mediating cell death is consistent with evidence in vitro that JNK does mediate apoptotic cell death in both nonneuronal (Chen et al., 1996) and PC12 (Xia et al., 1995) cells.

In spite of the widespread expression of c-jun in this model and the implication that it may therefore play a role in regenerative responses to injury, it is nevertheless clear that its expression does closely correlate with induction of death temporally at a regional level and in some instances at a cellular level. We therefore conclude that c-jun is likely to play some role in the initiation of apoptotic death in this model, as proposed for apoptotic death induced by hypoxia-ischemia (Dragunow et al., 1993, 1994; Dragunow and Preston, 1995), brain irradiation, and natural cell death (Ferrer et al., 1996b). This conclusion is compatible with substantial evidence obtained in vitro that c-jun plays a critical role in mediating apoptotic death in neurons (McBride and Feringa, 1991; Estus et al., 1994). In relation to apoptotic death in dopamine neurons, it would be of interest to determine in future studies whether c-jun expression plays a role in apoptotic death induced in models of parkinsonism induced either by 6-hydroxydopamine (Marti et al., 1997) or by *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Tatton and Kish, 1997). Expression of c-jun has been described in SNpc following striatal 6-hydroxydopamine injection (Jenkins et al., 1993), but it remains unclear whether it is playing a role in the cell death process or is a regenerative response. Given the preliminary evidence that apoptotic neuron death may occur in the parkinsonian brain (Tompkins et al., 1997), it would also be of interest to know whether c-jun is expressed in the parkinsonian brain, particularly in relation to apoptotic morphology. In another neurodegenerative disorder, Alzheimer's disease, there is some evidence for expression of c-jun in association with DNA 3' end-labeling, indicative of programmed cell death (Cotman and Anderson, 1995).

Acknowledgment: We are grateful to Ms. Pat White for excellent secretarial assistance. This work was supported by grant NS 26836 from the National Institutes of Health, the Parkinson's Disease Foundation, the Smart Family Foundation, and the Lowenstein Foundation.

REFERENCES

- Barde Y. A. (1989) Trophic factors and neuronal survival. *Neuron* **2**, 1525–1534.
- Burke R. E., Macaya A., DeVivo D., Kenyon N., and Janec E. M. (1992) Neonatal hypoxic-ischemic or excitotoxic striatal injury results in a decreased adult number of substantia nigra neurons. *Neuroscience* **50**, 559–569.
- Chen Y., Wang X., Templeton D., Davis R. J., and Tan T. (1996) The role of c-jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. *J. Biol. Chem.* **50**, 31929–31936.
- Clarke P. G. H. (1985) Neuronal death in the development of the vertebrate nervous system. *Trends Neurosci.* **8**, 345–349.
- Clarke P. G. H. and Oppenheim R. W. (1995) Neuron death in vertebrate development: *in vivo* methods, in *Methods in Cell Biology: Cell Death* (Schwartz L. M. and Osborne B. A., eds), pp. 277–321. Academic Press, San Diego.
- Coggeshall R. E. and Lekan H. A. (1996) Methods for determining numbers of cells and synapses: a case for more uniform standards of review. *J. Comp. Neurol.* **364**, 6–15.
- Cotman C. W. and Anderson A. J. (1995) A potential role for apoptosis in neurodegeneration and Alzheimer's disease. *Mol. Neurobiol.* **10**, 19–45.
- Cunningham T. J., Mohler I. M., and Giordano D. L. (1982) Naturally occurring neuron death in the ganglion cell layer of the neonatal rat: morphology and evidence for regional correspondence with neuron death in superior colliculus. *Dev. Brain Res.* **2**, 203–215.
- Davis R. J. (1994) MAPKs: new JNK expands the group. *Trends Biol. Sci.* **19**, 470–473.
- Dragunow M. and Faull R. (1989) The use of c-fos as a metabolic marker in neuronal pathway tracing. *J. Neurosci. Methods* **29**, 261–265.
- Dragunow M. and Preston K. (1995) The role of inducible transcription factors in apoptotic nerve cell death. *Brain Res. Rev.* **21**, 1–28.
- Dragunow M., Young D., Hughes P., MacGibbon G., Lawlor P., Singleton K., Sirimanne E., Beilharz E., and Gluckman P. (1993) Is c-jun involved in nerve cell death following status epilepticus and hypoxic-ischaemic brain injury? *Mol. Brain Res.* **18**, 347–352.
- Dragunow M., Beilharz E., Sirimanne E., Lawlor P., Williams C., Bravo R., and Gluckman P. (1994) Immediate-early gene protein expression in neurons undergoing delayed death, but not necrosis, following hypoxic-ischaemic injury to the young rat brain. *Mol. Brain Res.* **25**, 19–33.
- Elmqvist J. K., Scammell T. E., Jacobson C. D., and Saper C. B. (1996) Distribution of Fos-like immunoreactivity in the rat brain following intravenous lipopolysaccharide administration. *J. Comp. Neurol.* **371**, 85–103.
- Estus S., Zaks W. J., Freeman R. S., Gruda M., Bravo R., and Johnson E. M. (1994) Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. *J. Cell Biol.* **127**, 1717–1727.
- Ferrer I. (1992) The effect of cycloheximide on natural and x-ray-induced cell death in the developing cerebral cortex. *Brain Res.* **588**, 351–357.
- Ferrer I., Bernet E., Soriano E., del Rio T., and Fonseca M. (1990) Naturally occurring cell death in the cerebral cortex of the rat and removal of dead cells by transitory phagocytes. *Neuroscience* **39**, 451–458.
- Ferrer I., Olive M., Blanco R., Cinos C., and Planas A. M. (1996a) Selective c-Jun overexpression is associated with ionizing radiation-induced apoptosis in the developing cerebellum of the rat. *Mol. Brain Res.* **38**, 91–100.

- Ferrer I., Olive M., Ribera J., and Planas A. M. (1996b) Naturally occurring (programmed) and radiation-induced apoptosis are associated with selective c-jun expression in the developing rat brain. *Eur. J. Neurosci.* **8**, 1286–1298.
- Ferrer I., Planas A. M., and Pozas E. (1997) Radiation-induced apoptosis in developing rats and kainic acid-induced excitotoxicity in adult rats are associated with distinctive morphological and biochemical c-Jun/AP-1 (N) expression. *Neuroscience* **80**, 449–458.
- Gerfen C. R., Herkenham M., and Thibault J. (1987) The neostriatal mosaic: II. Patch- and matrix-directed mesostriatal dopaminergic and non-dopaminergic systems. *J. Neurosci.* **7**, 3915–3934.
- Goldstein P. (1997) Controlling cell death. *Science* **275**, 1081–1082.
- Haas C. A., Deller T., Naumann T., and Frotscher M. (1996) Selective expression of the immediate early gene c-jun in axotomized rat medial septal neurons is not related to neuronal degeneration. *J. Neurosci.* **16**, 1894–1903.
- Ham J., Babij C., Whitfield J., Pfarr C. M., Lallemand D., Yaniv M., and Rubin L. L. (1995) A c-jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron* **14**, 927–939.
- Harlan R. E. and Garcia M. M. (1995) Charting of Jun family member proteins in the rat forebrain and midbrain: immunocytochemical evidence for a new Jun-related antigen. *Brain Res.* **692**, 1–22.
- Herdegen T., Skene P., and Bahr M. (1997) The c-Jun transcription factor—bipotential mediator of neuronal death, survival and regeneration. *Trends Neurosci.* **20**, 227–231.
- Janec E. and Burke R. E. (1993) Naturally occurring cell death during postnatal development of the substantia nigra of the rat. *Mol. Cell. Neurosci.* **4**, 30–35.
- Jenkins R., O'Shea R., Thomas K. L., and Hunt S. P. (1993) c-Jun expression in substantia nigra neurons following striatal 6-hydroxydopamine lesions in the rat. *Neuroscience* **53**, 447–455.
- Kelly W. J. and Burke R. E. (1996) Apoptotic neuron death in rat substantia nigra induced by striatal excitotoxic injury is developmentally dependent. *Neurosci. Lett.* **220**, 85–88.
- Macaya A. and Burke R. E. (1992) Effect of striatal lesion with quinolinate on the development of substantia nigra dopaminergic neurons: a quantitative morphological analysis. *Dev. Neurosci.* **14**, 362–368.
- Macaya A., Munell F., Gubits R. M., and Burke R. E. (1994) Apoptosis in substantia nigra following developmental striatal excitotoxic injury. *Proc. Natl. Acad. Sci. USA* **91**, 8117–8121.
- Marti M. J., James C. J., Oo T. F., Kelly W. J., and Burke R. E. (1997) Early developmental destruction of terminals in the striatal target induces apoptosis in dopamine neurons of the substantia nigra. *J. Neurosci.* **17**, 2030–2039.
- McBride R. L. and Feringa E. R. (1991) Ventral horn motoneurons 10, 20 and 52 weeks after T-9 spinal cord transection. *Brain Res. Bull.* **28**, 57–60.
- Oo T. F. and Burke R. E. (1997) The time course of developmental cell death in phenotypically defined dopaminergic neurons of the substantia nigra. *Dev. Brain Res.* **98**, 191–196.
- Oppenheim R. W. (1991) Cell death during development of the nervous system. *Annu. Rev. Neurosci.* **14**, 453–501.
- Paxinos G. and Watson C. (1982) *The Rat Brain in Stereotaxic Coordinates*. Academic Press, San Diego.
- Pittman R. N., Wang S. L., DiBenedetto A. J., and Mills J. C. (1993) A system for characterizing cellular and molecular events in programmed neuronal cell death. *J. Neurosci.* **13**, 3669–3680.
- Sloviter R. S., Dean E., and Neubort S. (1993) Electron microscopic analysis of adrenalectomy induced hippocampal granule cell degeneration in the rat: apoptosis in the adult central nervous system. *J. Comp. Neurol.* **330**, 337–351.
- Smeyne R. J., Vendrell M., Hayward M., Baker S. J., Miao G. G., Schilling K., Robertson L. M., Curran T., and Morgan J. I. (1993) Continuous c-fos expression precedes programmed cell death *in vivo*. *Nature* **363**, 166–169.
- Tatton N. A. and Kish S. J. (1997) In situ detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange. *Neuroscience* **77**, 1037–1048.
- Tompkins M. M., Basgall E. J., Zamrini E., and Hill W. D. (1997) Apoptotic-like changes in Lewy-body-associated disorders and normal aging in substantia nigral neurons. *Am. J. Pathol.* **150**, 119–131.
- Virdee K., Bannister A. J., Hunt S. P., and Tolkovsky A. M. (1997) Comparison between the timing of JNK activation, c-Jun phosphorylation, and onset of death commitment in sympathetic neurons. *J. Neurochem.* **69**, 550–561.
- Xia Z., Dickens M., Raingeaud J., Davis R. J., and Greenberg M. E. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270**, 1326–1331.