c-Jun mediates axotomy-induced dopamine neuron death *in vivo*

S. J. Crocker*, W. R. Lamba*, P. D. Smith*, S. M. Callaghan*, R. S. Slack*, H. Anisman†, and D. S. Park*‡

*Neuroscience Research Institute, University of Ottawa, Ottawa, ON K1H 8M5, Canada; and †Institute of Neuroscience, Carleton University, Ottawa, ON K1S 5B6, Canada

Edited by P. S. Goldman-Rakic, Yale University School of Medicine, New Haven, CT, and approved September 6, 2001 (received for review April 10, 2001)

Expression of the transcription factor c-Jun is induced in neurons of the central nervous system (CNS) in response to injury. Mechanical transection of the nigrostriatal pathway at the medial forebrain bundle (MFB) results in the delayed retrograde degeneration of the dopamine neurons in the substantia nigra pars compacta (SNc) and induces protracted expression and phosphorylation of c-Jun. However, the role of c-Jun after axotomy of CNS neurons is unclear. Here, we show that adenovirus-mediated expression of a dominant negative form of c-Jun (Ad.c-JunDN) inhibited axotomy-induced dopamine neuron death and attenuated phosphorylation of c-Jun in nigral neurons. Ad.c-JunDN also delayed the degeneration of dopaminergic nigral axons in the striatum after MFB axotomy. Taken together, these findings suggest that activation of c-Jun mediates the loss of dopamine neurons after axotomy injury.

In the central nervous system (CNS), axon transection proximal to the neuron soma results in neuronal death and a failure of the injured axons to regenerate. Whereas the mechanisms regulating cell death or inhibition of neurite regeneration after axotomy remain unclear, increasing evidence suggests that these are regulated processes, controlled by multiple, discrete biochemical signals (1–3). Of the signaling pathways associated with neuronal death, a role for the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signaling pathway has been implicated in nerve injury (4, 5). In this regard, correlative studies have described increased expression of c-Jun and JNK activity after axotomy (6–8). However, the precise role of c-Jun expression in the response of CNS neurons to axotomy injury is incongruous, because expression/activation of c-Jun has been correlated with both the regeneration of nerve fibers and the mediation of neuronal death (refs. 9–11, and reviewed by Herdegen *et al.* in ref.12).

Here, we have addressed the role of c-Jun expression after axotomy injury in the central nervous system by using the medial forebrain bundle (MFB) axotomy model of dopamine neuron death *in vivo*. In this model, mechanical transection of the MFB results in the protracted loss of dopamine neurons in the substantia nigra pars compacta (SNc) and the commensurate depletion of dopaminergic innervation of the striatum (13, 14). This injury model has been well characterized for both the progress of neuronal death, and the activation/expression of the c-Jun/JNK pathway (6, 8, 13, 14). Accordingly, we engineered an adenovirus expressing a dominant negative c-Jun (15), and examined the functional consequences of impairing the c-Jun/JNK signaling pathway in nigrostriatal dopamine pathway after MFB axotomy. We provide evidence to support a preeminent role for the c-Jun/JNK pathway in axotomy-induced death of dopamine neurons *in vivo*.

Materials and Methods

Animals. Male Wistar rats (150–175 g; Charles River Breeding Laboratories) were used for all experiments in this study. All animals and procedures were approved by the University of Ottawa Animal Care Committee, and were maintained in strict accordance with the Guidelines for the Use and Treatment of Animals set out by the Animal Care Council of Canada.

Intrastriatal Administration of Adenoviruses and Fluorogold. Recombinant adenoviral vectors were constructed by using the Cre-*lox* system (16). The FLAG-tagged c-Jun dominant negative (c-JunDN) were generously provided by J. Whitfield and J. Ham (15). Adenoviruses (3 μl; 1×10^7 particles per μl per construct) containing either c-JunDN (Ad.c-JunDN) or lacZ (Ad.lacZ) were stereotaxically injected into the right dorsolateral striatum 0.7 mm rostral to bregma, 2.5 mm to the right of midline, and −5.2 mm relative to the skull surface, at an infusion rate of 0.5 μl/min by using a syringe pump (PHD2000, Harvard Apparatus; ref. 17). Injections of the fluorescent retrograde tracer, Fluorogold (FG; 0.2 μl of 2.0% in 0.9% saline; Fluorochrome, Engleweed, CO) were also administered bilaterally into the striata at the same coordinates as the adenovirus and the equivalent site of the contralateral hemisphere (18).

Medial Forebrain Bundle Axotomy. Unilateral axotomy of the MFB was performed one week after intrastriatal adenovirus administration by using a Scouten wire knife (Kopf Instruments, Tujunga, CA). The MFB was transected at the level −3.8 mm caudal to bregma: the knife was moved 2.4 mm to the right of the midline, and lowered 8.0 mm below the skull surface, and the blade was extended and raised 2.5 mm, then lowered and retracted, as described previously (refs. 17 and 19–22; Fig. 1*A*). Postmortem validation of the lesion site was determined in every animal by hematoxylin and eosin staining (Fig. 1*B*). To examine the effects of c-JunDN expression on axotomy-induced neuronal death, groups of rats received intrastriatal injections of either adenoviruses and FG (n = 6 per group) or adenovirus alone (n = 12 per group), followed by MFB axotomy 1 week later. Fourteen days after MFB axotomy, animals were perfused or subjected to HPLC analysis (see below). Additional groups of animals were used to examine changes in axotomy-induced gene expression and TH 3 (n = 3 per virus) or 7 days (n = 3–4 per virus) postaxotomy. A final group of adenovirus-axotomized rats (n = 6 per virus) were killed 50 days postaxotomy to assess the long-term effects of adenoviral gene expression on nigral neuron survival.

Immunohistochemistry. Animals were perfused transcardially with saline (0.9%; 200 ml) followed by an equivalent volume of paraformaldehyde (4.0% in 0.1 M phosphate buffer). Tissues were postfixed overnight and then cryoprotected by using 10% sucrose in 0.1 M phosphate buffer. Serial coronal sections were collected from the forebrain and midbrain levels of all animals and developed

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: c-JunDN, c-Jun dominant negative; Ad, adenovirus; DN, dominant negative; TH, tyrosine hydroxylase; MFB, medial forebrain bundle; SNc, substantia nigra pars compacta; MGN, medial geniculate nucleus; VTA, ventral tegmental area; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; CNS, central nervous system; FG, Fluorogold; CREB, cAMP-responsive element binding protein; MKK4, mitogen-activated protein kinase kinase 4; MTN, medial terminal nucleus.

‡To whom reprint requests should be addressed at: Neuroscience Research Institute, 451 Smyth Road, University of Ottawa, Ottawa, ON K1H 8M5, Canada. E-mail: dpark@uottawa.ca.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

NEUROBIOLOGY

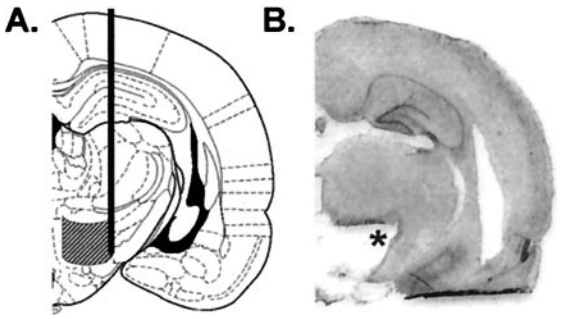

Fig. 1. (*A*) Schematic line drawing of a coronal section from rat brain indicating the site and extent of mechanical injury made by the Scouten wire knife to transect the MFB. Verification of anatomical MFB transection was accomplished by hematoxylin and eosin staining of sections from every animal (−3.6 to −4.16 mm caudal to bregma; ref. 24), as demonstrated by representative section shown (*B*). Line drawing schematic of coronal brain section adapted from atlas of Paxinos and Watson (24).

for immunohistochemical analysis of protein expression in striatal and nigral regions, respectively, as described previously (23). Sections from each subject and treatment group were stained in parallel by using these antibodies: FLAG (1:500, Chemicon), β-galactosidase (1:1000; Promega), phosphoserine-63 or 73 c-Jun (both 1:500; Santa Cruz Biotechnology), c-Jun (1:1500: no. 9162, NEB, Beverly, MA), JunD and cAMP-responsive element binding protein (CREB; both 1:2000; R. Bravo, Bristol-Myers Squibb, Princeton, NJ), FosB (1:5000; Santa Cruz Biotechnology), and tyrosine hydroxylase (TH, 1:10,000; Incstar, Stillwater, MN). Digital photomicrographs were captured by using a computer-based image analysis system, and striatal densitometry measurements (five sections per subject; +1.0 mm to −0.26mm from bregma) were made by using a subroutine program (Northern Eclipse, Empix Imaging, Mississauga, ON, Canada), as described (17).

Assessment of Dopamine Neuron Survival. SNc neuron survival after MFB axotomy was determined by immunohistochemical detection of the dopamine phenotype marker, TH, and then confirmed by the presence of the fluorescent tracer FG. Because of the topographic nature of the dopaminergic projections from the SNc to the striatum, retrograde transport of adenoviruses after intrastriatal administration only labeled a subpopulation of the SNc (ref. 17; −4.8 to −5.6 mm caudal from bregma, ref. 24), which included the medial terminal nucleus (MTN) of the accessory optic tract. Hence, the MTN was used as an anatomical land mark to ensure that the same level of the medial SNc was analyzed between hemispheres, animals, and treatment groups (17, 25, 26). Thus, every fifth section was collected through the medial SNc (six sections per animal). The number of nucleated TH- or FG-positive neurons was counted in both lesioned and contralateral SNc, and the proportion of nigral neuron survival was expressed as a percentage of the unlesioned hemisphere (17, 27).

High Performance Liquid Chromatography. Dopamine levels were determined in the caudate tissue extracts from MFB axotomized rats ($n = 6$ per group, adenovirus alone) 3 h after administration of D-amphetamine (2.0 mg/kg, s.c.). Briefly, tissues were placed in 0.5 ml homogenizing solution (5 ml methanol with 500 ml water, which contained 14.17 g monocholoracetic acid and 0.018 g EDTA) and stored at −80°C until analysis. Striatal concentrations of the dopamine metabolite 3,4-dihydroxyphenylacetic acid were measured by using HPLC with electrochemical detection, as described elsewhere (28, 29).

Statistical Analyses. All data were analyzed by using two-tailed, unpaired Student *t* tests (unless stated otherwise). Differences were considered significant when $P < 0.05$.

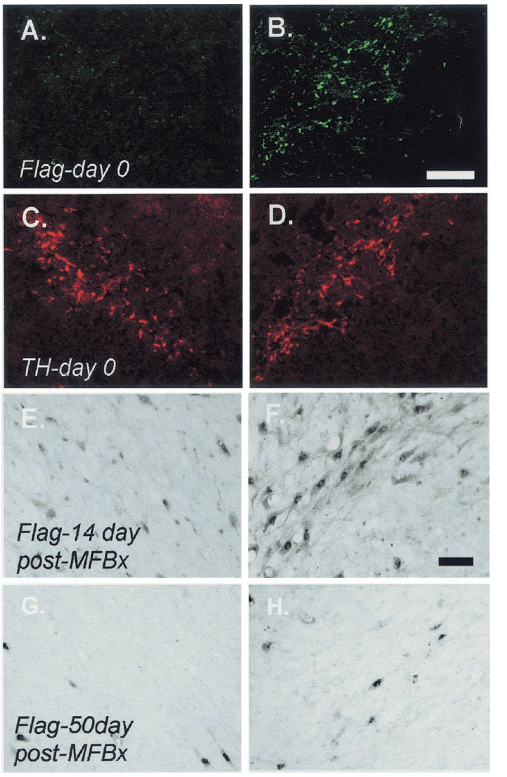

Fig. 2. Intrastriatal adenovirus administration results in retrograde labeling of dopamine neurons in the SNc. Expression of c-JunDN was detected 1 week later in the SNc ipsilateral to the adenovirus injection (*B*), but not in the contralateral hemisphere (*A*). Immunohistochemical detection of FLAG (Ad.c-JunDN) was predominantly in SNc neurons expressing TH (*C* and *D*). Fourteen days after MFB axotomy, FLAG immunoreactivity was still detectable in SNc neurons ipsilateral to injection of Ad.c-JunDN (*F*), yet by 50 days postinjury, adenovirus expression was not detectable in the SNc (*H*). FLAG expression was not detected in the contralateral SNc at these time points (*E* and *G, respectively*). Scale bars = 160 μm (*A–D*) and 40 μm (*E–H*).

Results

Adenoviral Mediated Expression of a Dominant Negative (DN) c-Jun in Nigral Neurons. We first determined the retrograde transportation and expression of the c-JunDN-FLAG and lacZ containing adenoviruses in the dopamine neurons of the substantia nigra pars compacta after intrastriatal administration. By using immunofluorescence histochemistry, expression of c-JunDN was detected in dopamine neurons of the SNc 1 week after intrastriatal adenovirus injection. FLAG-like immunoreactivity, which detected the tag epitope added to the c-JunDN (Fig. 2*B*), revealed that adenoviral-mediated gene expression was found in neurons colabeled by TH (Fig. 2*D*). Similar results were obtained for expression of β-galactosidase (Ad.lacZ; data not shown). Adenovirally delivered proteins were not detected in the contralateral hemisphere of either adenovirus-treated group (Fig. 2 *A* and *E*). In the ventral midbrain, expression of adenoviral delivered proteins was largely exclusive to dopamine neurons. Expression of c-JunDN in SNc neurons persisted after MFB axotomy and revealed predominantly nuclear localization of the c-JunDN protein, although some cytoplasmic staining was also observed (Fig. 2*F*). However, by 50 days after MFB axotomy, adenoviral-mediated expression was no longer detectable in the ipsilateral SNc (Fig. 2*H*).

c-JunDN Prevents Axotomy-Induced Dopamine Neuron Death. To determine whether c-JunDN expression could prevent the loss of nigral dopamine neurons after MFB axotomy, groups of rats were injected with adenovirus and received axotomy 1 week later (Fig. 3).

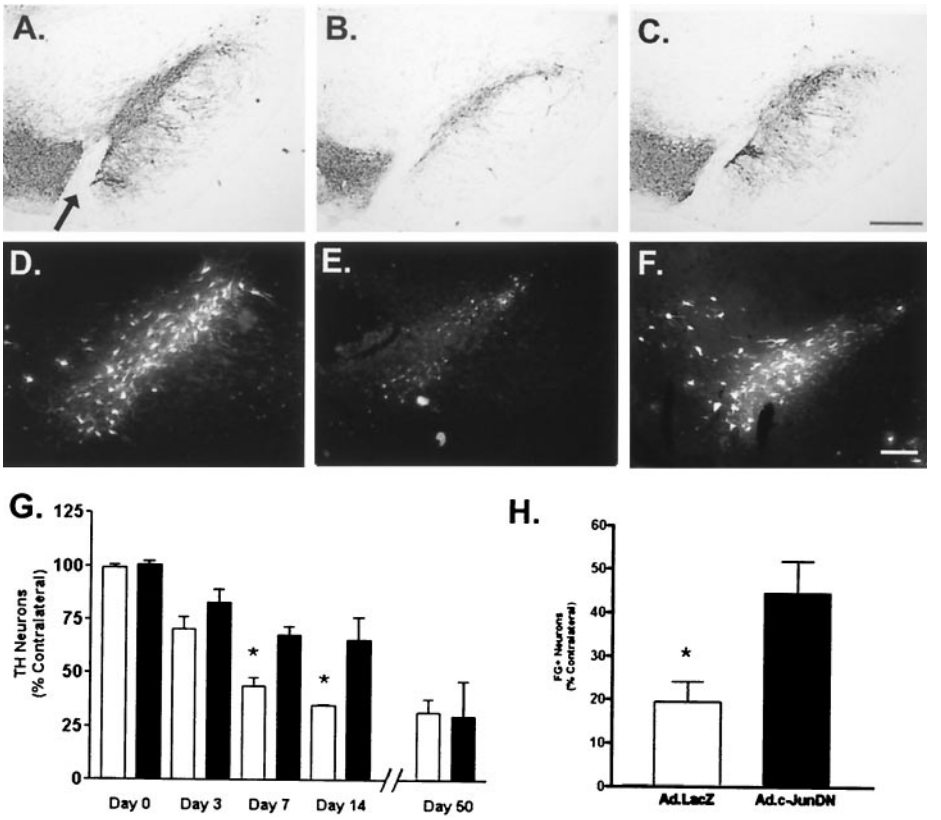

Fig. 3. Ad.c-JunDN attenuates loss of nigral dopamine neurons after MFB axotomy. Representative TH immunoreactivity in the SNc from unlesioned (*A*), and rats injected with either Ad.lacZ (*B*), or Ad.c-JunDN (*C*), 14 days after MFB axotomy. Arrow indicates the medial terminal nucleus (MTN), an anatomical landmark used to ensure for comparisons between sections (see *Materials and Methods*). Retrograde labeling of neurons in the SNc after intrastriatal injection of FluoroGold (FG) from unlesioned (*D*) Ad.lacZ or Ad.c-JunDN 14 days after MFB axotomy (*E* and *F*, respectively). (*G*) Quantification of TH neurons in the SNc at varying times after MFB axotomy: Ad.lacZ (white bars), Ad.c-JunDN (black bars) ($P < 0.0001$, ANOVA; *, $P < 0.001$, Newman-Keuls Multiple Comparison test; $n = 3$–4 per group, days 0–7, 50, $n = 6$ day 14). (*H*) Quantification of FG-labeled neurons in the SNc confirmed increased survival of SNc neurons after MFB axotomy in Ad.c-JunDN-treated rats, when compared with lesioned Ad.lacZ-treated animals ($n = 6$ per group; *, $P < 0.02$ *t* test). Scale bar in *C* = 350 μm (*A*-*C*) and 100 μm in *F* (*D*–*F*).

Quantification of TH-positive neurons in the lesioned SNc, when compared with the unlesioned (contralateral) hemisphere, revealed that, 3 days postaxotomy, Ad.lacZ-treated animals had 70.67 ± 5.2% (mean ± SEM) of nigral neurons, whereas Ad.c-JunDN had 83.09 ± 5.9% survival ($P > 0.15$). However, by 7 days after MFB axotomy, Ad.lacZ-treated animals exhibited significant neuronal loss whereas Ad.c-JunDN-treated axotomized rats revealed significantly more nigral TH^+ neurons (43.95 ± 3.8% vs. 67.76 ± 4.1%, respectively; $P < 0.0008$). Increased neuronal survival was observed in Ad.c-JunDN-treated rats 14 days after axotomy (65.52 ± 10.7%), whereas Ad.lacZ-treated animals exhibited increased loss of TH^+ nigral neurons at this same time point (34.97 ± 0.6%; $P < 0.01$). Because axotomy injury may elicit a loss of TH expression rather than cell death (30), we also assessed SNc neuron survival by using FG as an independent marker of cell survival. Animals subject to axotomy and expressing c-JunDN had more FG-labeled SNc neurons (44.49 ± 7.3%) than lacZ-treated controls (19.40 ± 4.7%; $P < 0.02$; Fig. 3*H*). Although the numbers of FG^+ cells were slightly lower in both treatment groups than that assessed by TH staining, this is likely due to the fact that ≈23% of SNc neurons project to the contralateral nigra, hence these neurons would not be FG labeled (14). Taken together, these findings are consistent with the notion that c-JunDN is neuroprotective in this model of neuronal injury.

Previous work by others has determined that expression and activation of c-Jun in nigral neurons is maintained for up to 50 days after MFB axotomy (8). Because expression of c-JunDN significantly reduced axotomy-induced neuronal loss, we also determined whether protection by c-JunDN depended on sustained expression adenovirus-mediated protein. Quantitative assessment of SNc neurons survival 50 days after MFB axotomy revealed that the degree of neuronal loss did not differ between Ad.c-JunDN and Ad.lacZ treatment groups (36.75 ± 9.38% vs. 31.47 ± 4.37%, respectively) (Fig. 3*G*). Hence, neuroprotection in Ad.c-JunDN-treated animals was lost by 50 days postaxotomy (57 days post adenovirus administration)—and consistent with the observed loss of adenovirus-mediated gene expression (Fig. 2).

c-JunDN Attenuates Striatal Dopaminergic Fiber Degeneration After Axotomy. Mechanical transection of the MFB separates the dopaminergic nerve fibers in the striatum from the nigral cell bodies. Because we had observed protection of nigral neurons, we also investigated whether expression of c-JunDN effected the loss of the nigral axons distal to the site of transection. Striatal sections from Ad.lacZ- and Ad.c-JunDN-treated animals 14 days after axotomy were assessed for immunohistochemical detection of TH, and the density of fibrous staining was quantified. In lacZ-treated animals, axotomy induced a profound depletion of dopaminergic fibers in the striatum ipsilateral to the MFB transection (Fig. 4*B*). When compared with the contralateral hemisphere (Fig. 4*A*), axotomy induced an 87% depletion of striatal TH immunoreactive fiber labeling (Fig. 4*E*). In contrast, MFB axotomy reduced the density of fibrous labeling in the ipsilateral striatum of Ad.c-JunDN-treated rats by only 49.2% (Fig. 4 *C* and *D*). The difference in dopaminergic afferent staining in the striatum of the lesioned sides was significantly different ($P < 0.02$), whereas striatal dopaminergic fibrous

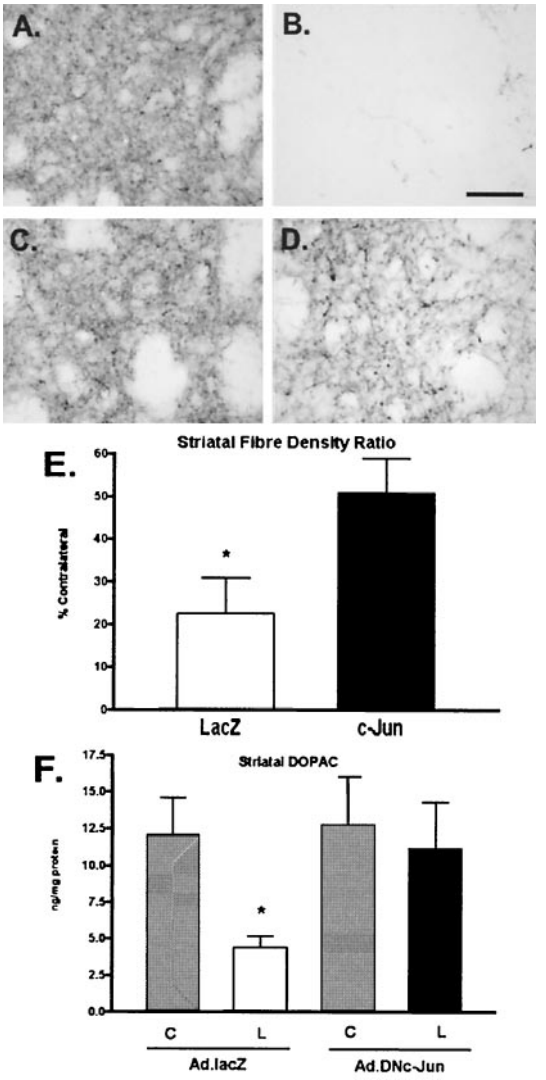

Fig. 4. Degeneration of dopaminergic axon fibers in the striatum is attenuated by c-JunDN. Immunohistochemical detection of dopamine fibers in the striatum of adenovirus-treated rats was examined 2 weeks after MFB axotomy. Immunostaining for TH in the unlesioned hemisphere of Ad.lacZ and Ad.c-JunDN axotomized rats revealed dense afferent fibrous staining in the striatum (*A* and *C*; respectively). A profound reduction in striatal TH fiber staining in Ad.lacZ was observed (*B*), whereas Ad.c-JunDN-treated animals exhibited a marked attenuation of this loss (*D*). Quantification of striatal densities is shown in *E* (*, $P < 0.02$). Axotomized striatal afferents in Ad.c-JunDN-treated animals released dopamine in response to administration of amphetamine (2.0 mg/kg, s.c.), whereas Ad.lacZ-treated animals exhibited a marked deficit in responsiveness (*F*, $P < 0.02$). Scale bar = 60 μm.

labeling in the contralateral hemisphere of both treatment groups did not differ ($P > 0.86$).

To determine whether the nigrostriatal fibers protected by Ad.c-JunDN could be stimulated to release dopamine, we challenged groups of adenovirus-treated MFB axotomized rats with amphetamine (2.0 mg/kg, s.c.), and analyzed striatal concentrations of released dopamine as measured by levels of 3,4-dihydroxyphenylacetic acid, the principal metabolite of dopamine in rodents. Depletion of striatal dopaminergic fibers in Ad.lacZ-treated animals was reflected by a 63% reduction in 3,4-dihydroxyphenylacetic acid levels after amphetamine administration in the denervated striatum ($P < 0.04$ paired *t* test, Fig. 4*F*). In contrast, and consistent with the observed neuroprotection of striatal TH fibers in Ad.c-JunDN-treated rats, amphetamine-induced dopamine release in the striatum ipsilateral to the MFB transection was normalized in Ad.c-JunDN-treated animals ($P > 0.15$ paired *t* test, Fig. 4*F*).

Axotomy-Induced Phosphorylation (Ser^{73}) of c-Jun Is Attenuated by c-JunDN. Transcriptional activation of c-Jun is facilitated by phosphorylation by members of the mitogen-activated protein (MAP) kinase superfamily (reviewed in ref. 31). Specifically, phosphorylation of Serine residues 63 and 73 (pSer^{63} and pSer^{73}) of c-Jun by JNK/SAPK enhances c-Jun transactivation (32–34). Likewise, JNK activity is positively regulated by the upstream kinase, MAP kinase kinase 4 (MKK4), which phosphorylates threonine and tyrosine residues in JNK. To determine whether expression of c-JunDN in the SNc influenced axotomy-induced changes in activation of c-Jun by JNK/SAPK, we examined changes in phosphorylation of residues Serine 63 (pSer^{63}) or Serine 73 (pSer^{73}) of c-Jun by using epitope-specific antibodies. No changes in pSer^{63} phosphorylation were detected at any time after axotomy in either adenovirus treatment group, consistent with previous reports (8). However, the possibility remains that Ser^{63} of c-Jun may be a labile site and the perfusion/fixation of tissues may negatively effect the ability to detect the weak phosphorylation of c-Jun at this residue (35). In contrast, axotomy induced an increase in phosphorylation of Ser^{73} in the ipsilateral SNc of Ad.lacZ-treated rats (Fig. 5*D*). Interestingly, pSer^{73} was also observed in the ventral tegmental area (VTA) and medial geniculate nucleus (MGN) adjacent to the SNc (Fig. 5 *A* and *G*). Because retrograde labeling from the striatum labeled only SNc neurons, the VTA and MGN were used as an internal control for quantification of pSer^{73} immunoreactivity. Quantification revealed that phosphorylation of c-Jun (pSer^{73}) was blocked in the SNc (Fig. 5 *D–F*), where c-JunDN was expressed. No differences in activation of c-Jun were detected in either the VTA (Fig. 5 *A–C*) or MGN (Fig. 5 *G–I*) of Ad.lacZ- and Ad.c-JunDN-treated animals 7 days after axotomy. Consistent with this observation, induction of endogenous c-Jun protein in the SNc after axotomy was also impaired only in animals expressing c-JunDN (Fig. 6*F*, which is published as supporting information on the PNAS web site, www.pnas.org). c-JunDN expression also did not appear to effect the expression pattern of other select immediate early genes in the SNc after axotomy. As previously reported by others, expression of JunD (6) is induced, CREB (36) reduced, and expression of FosB in the SNc is unchanged after axotomy (6). When compared with tissues from Ad.lacZ-treated animals, axotomy-induced expression of these three IEG proteins was not affected by expression of c-JunDN (Fig. 6). To also address the possibility that the observed impairment of Ser^{73} phosphorylation of c-Jun by c-JunDN in nigral neurons was due to an interruption of upstream c-Jun pathway kinases, we examined for axotomy-induced expression of pMKK4. By using an antibody specific for phosphorylated (activated) MKK4 (Thr^{261}), an increase in pMKK4 was evident in nigral neurons by 3 days after MFB axotomy, and diminished by 7 days postaxotomy, similar to what has been previously reported (37). More importantly, elevated pMKK4 in SNc neurons after axotomy was observed in both Ad.c-JunDN and Ad.lacZ treatment groups (data not shown). Hence, in this axotomy model, modulation of endogenous c-Jun expression rather than interruption of upstream signaling elements of the c-Jun/JNK pathway likely mediated the neuroprotective actions of c-JunDN.

Discussion

c-Jun and Axotomy-Induced Neuronal Death. Axotomy of the nigrostriatal dopamine pathway by mechanical transection of the medial forebrain bundle evokes the sustained induction of the c-Jun/JNK pathway in nigral neurons (6). However the role of c-Jun in neurons of the CNS after axotomy injury has been controversial (12) because expression of c-Jun may serve to function either as a survival response to stress or as a potential mediator of neuronal demise after injury (15, 38–43). In addition, a role of c-Jun expression in axonal regrowth after fimbria-fornix transection of

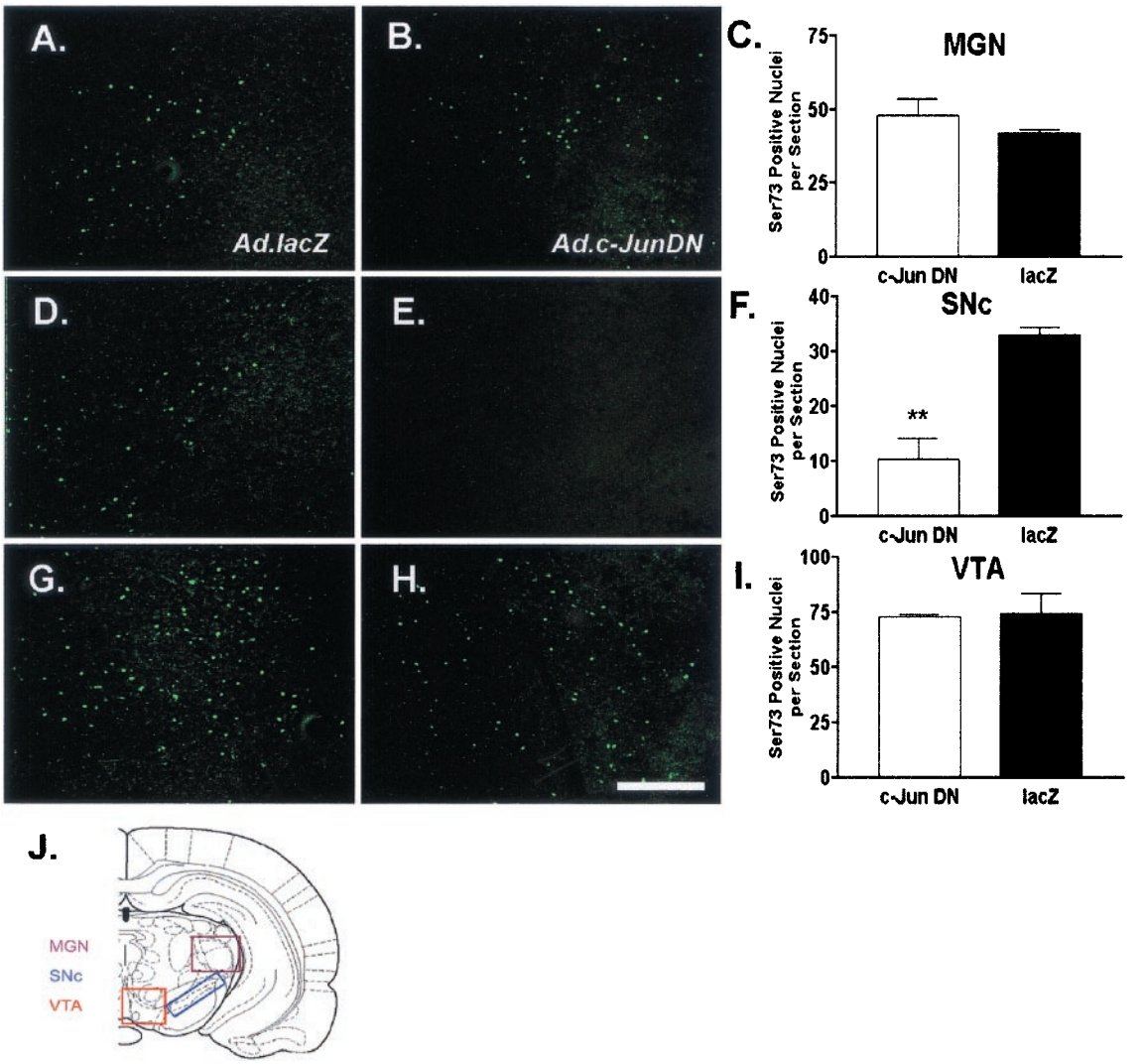

Fig. 5. MFB axotomy-induced phosphorylation of c-Jun Ser[73] is significantly attenuated by Ad.c-JunDN in the SNc. Increased c-Jun-Ser[73] was detected in the MGN (*A, B,* and *C*), the SNc (*D, E,* and *F*), and VTA (*G, H,* and *I*), of Ad.lacZ (*A, D,* and *G*), and Ad.c-JunDN (*B, E,* and *H*) animals after MFB axotomy. Quantification of Ser[73] immunoreactive cells (*C, F,* and *I*) in the MGN, SNc, and VTA is shown ($P < 0.001$ ANOVA; **, $P < 0.01$ Newman-Keuls test). (*J*) Schematic of coronal rat brain section indicating square area used for quantitative analyses of c-Jun phosphorylation (adapted from Paxinos and Watson, ref. 24). Scale bar = 180 μm.

cholinergic neurons (44) and retinal ganglion cells (45) has also been suggested. In contrast, c-Jun expression has been correlated with cell death of dopamine neurons after MFB transection (8). Interestingly, treatment with FK506, which attenuates neuronal loss in this model, also reduced expression of c-Jun (25). Given the uncertainty of the role of c-Jun, we directly tested the hypothesis that expression of c-Jun in dopamine neurons is a key determinant of whether a neuron will survive axotomy. Our results demonstrate that expression of c-JunDN is neuroprotective, supporting the notion that axotomy-induced neuronal death is signaled through a c-Jun/JNK dependent pathway.

Neuroprotection by c-Jun Dominant Negative. The question of how c-JunDN may interfere with neuronal death signals to evoke neuroprotection in this axotomy model has several possible answers. First, c-JunDN may interfere with stress-activated gene expression in nigral neurons. *In vitro* studies have previously shown that N-terminal deletions of c-Jun (Δ169 c-Jun) do not impair the bZIP-mediated binding of c-JunDN to endogenously expressed IEG proteins, suggesting that overexpression of c-JunDN may modulate cellular responses by "quenching" resident Fos and Jun transcriptional activities (46, 47). Therefore, the ability of c-JunDN to prevent axotomy-induced death of nigral dopamine neurons *in vivo* may reflect the ability of c-JunDN to bind IEG proteins and thereby alter axotomy-induced neuronal gene expression. However, it is important to note that expression of c-JunDN did not effect expression patterns of several IEGs, such as JunD, CREB, or FosB, indicating certain selectivity of c-JunDN action. Second, we have shown that axotomy-induced phosphorylation of c-Jun (Ser[73]) in the SNc was attenuated by expression of c-JunDN. This observation is likely accountable by active repression of endogenous c-Jun expression by c-JunDN, as previously described *in vitro* (48). Alternatively, c-JunDN may have directly interfered with JNK *in vivo*, although absence of the c-Jun delta (δ) domain, amino acids 31–60, has been reported to reduce the affinity of c-JunDN for JNK (49). Finally, the protracted expression of c-JunDN before the axotomy may have also altered basal activator protein-1 (AP-1)-regulated gene expression, and therein modified the subsequent genetic response of the neurons to injury.

The results of this study support the notion that axotomy-related c-Jun expression in CNS neurons is associated with neuron death. This finding, however, is inconsistent with the role proposed for

c-Jun in medial septal neurons (MSN) after transection of the fimbria-fornix (44). Whereas SNc neurons degenerate within weeks after axotomy (14), MSN neurons may survive for an extended period before dying (10, 11, 30). The disparity in rates of survival in these two populations of CNS neurons may be related to the induction of IEG expression in the axotomized neurons. For instance, whereas axotomy of MSN elicits sustained expression of c-Jun, these neurons do not display prolonged expression of other numerous leucine zipper-containing proteins, which are clearly expressed after MFB axotomy (6). This induction of a variety of potential activator protein-1 binding partners in SNc neurons after MFB axotomy may lead to death signals not robustly activated in MSN neurons (11). Whereas the downstream effects of c-Jun activation, which mediate axotomy-induced injury, are unknown, several groups have identified c-Jun in the regulation of several target genes linked to cell death pathways, including the proapoptotic Bcl-2 member BIM (50, 51), the tumor suppressor p53 (52), and cyclin D1 (53, 54). The contribution of these and other c-Jun activated targets in the axotomy-induced death of SNc neurons remain to be elucidated.

c-Jun and Axonal Degeneration. One important observation of the present study was that overexpression of c-JunDN significantly attenuated the loss of afferent striatal dopaminergic fibers. It is becoming increasingly evident that the synapse can function and respond to stimuli or stress independent of the neuronal soma, as exemplified by the demonstration that death-related processes can occur in synaptosomal preparations and anucleated neurons (55, 56). These findings suggest that autonomous biochemical events within neurites, separate from the regulation of neural soma, may retain the machinery for delayed axonal death and thereby offer the potential for extended survival of axons by inhibition of these localized events (57).

Whereas the exact mechanism by which c-JunDN delayed neurite degeneration after MFB axotomy is presently unclear, there are several plausible explanations for this observation. First, because overexpression of c-JunDN preceded axotomy in the CNS injury model used in the present study, it could be proposed that the presence of c-JunDN modified the regulation of c-Jun responsive target genes before the injury. Alternatively, administration of Ad.c-JunDN into the axon field, the striatum, may have also affected the response of the postsynaptic cells to the axotomy of nigrostriatal dopaminergic fibers. For instance, the survival of transected *Aplysia* axons can be significantly prolonged independent of *de novo* protein synthesis (58), or when contact with neurons is retained (59). The survival of severed dopaminergic axons by expression of c-JunDN merits further study to elucidate the nature of this merotrophism (60). Interestingly, several pools of inducible JNK reside within the cytoplasm and nucleus of neurons (61). In this context, overexpression of c-JunDN may obstruct the major available pool of stress-inducible JNK in the axoplasm, and inhibit JNK phosphorylation of cytoplasmic target after injury (61).

c-Jun as a Therapeutic Target for Neuroprotection. Central to our understanding of the potential molecular regulation of neuronal fate after axotomy is the role of proteins that interact with, or are regulated by, c-Jun. Elucidation of axotomy-induced c-Jun regulated genes may further our understanding of the basic processes governing neuron fate after axotomy injury. Nevertheless, our findings suggest that strategies that inhibit the c-Jun/JNK signaling cascade, such as pharmacological JNK inhibitors or the application of c-Jun antisense, may offer the potential of clinical utility for situations of CNS nerve fiber injury.

We thank C. J. Lee, Z. Kulczycki, and P. Bradley for technical assistance. We also thank J. Ham of University College London, for helpful suggestions. This work was supported by grants (to D.S.P.) from the Ontario Neurotrauma Foundation (ONF) and the Canadian Institutes of Health Research (CIHR). S.J.C. is a recipient of an ONF postdoctoral fellowship. D.S.P. is a recipient of the Glaxo-Wellcome Chair for Stroke and is a CIHR Scholar.

1. Herdegen, T. & Waetzig, V. (2001) *Oncogene* **20,** 2424–2437.
2. Horner, P. J. & Gage, F. H. (2000) *Nature (London)* **407,** 963–970.
3. Park, D. S., Stefanis, L. & Greene, L. A. (1997) *Trends Cardiovasc. Med.* **7,** 294–301.
4. Dragunow, M., Young, D., Hughes, P., MacGibbon, G., Lawlor, P., Singleton, K., Sirimanne, E., Beilharz, E. & Gluckman, P. (1993) *Brain Res. Mol. Brain Res.* **18,** 347–352.
5. Vaudano, E., Rosenblad, C. & Bjorklund, A. (2001) *Eur. J. Neurosci.* **13,** 1–14.
6. Leah, J. D., Herdegen, T., Murashov, A., Dragunow, M. & Bravo, R. (1993) *Neurosci.* **57,** 53–66.
7. Kenney, A. M. & Kocsis, J. D. (1998) *J. Neurosci.* **18,** 1318–1328.
8. Herdegen, T., Claret, F. X., Kallunki, T., Martin-Villalba, A., Winter, C., Hunter, T. & Karin, M. (1998) *J. Neurosci.* **18,** 5124–5135.
9. Herdegen, T., Bastmeyer, M., Bahr, M., Stuermer, C., Bravo, R. & Zimmermann, M. (1993) *J. Neurobiol.* **24,** 528–543.
10. Dragunow, M. (1992) *Brain Res. Mol. Brain Res.* **15,** 141–144.
11. Butterworth, N. J. & Dragunow, M. (1996) *Brain Res. Mol. Brain Res.* **43,** 1–12.
12. Herdegen, T., Skene, P. & Bahr, M. (1997) *Trends Neurosci.* **20,** 227–231.
13. Weiser, M., Baker, H., Wessel, T. C. & Joh, T. H. (1993) *J. Neurosci.* **13,** 3472–3484.
14. Brecknell, J. E., Dunnett, S. B. & Fawcett, J. W. (1995) *Neurosci.* **64,** 219–227.
15. Ham, J., Babji, C., Whitfield, J., Pfarr, C. M., Lallemand, D., Yaniv, M. & Rubin, L. L. (1995) *Neuron* **14,** 927–939.
16. Hardy, S., Kitamura, M., Harris-Stansil, T., Dai, Y. & Phipps, M. L. (1997) *J. Virol.* **71,** 1842–1849.
17. Crocker, S. J., Wigle, N., Liston, P., Thomspon, C. S., Lee, C. J., Xu, D. G., Roy, S., Nicholson, D. W., Park, D. S., MacKenzie, A. E., *et al.* (2001) *Eur. J. Neurosci.* **14,** 391–400.
18. Yamada, M., Oligino, T., Mata, M., Goss, J. R., Glorioso, J. C. & Fink, D. J. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 4078–4083.
19. Knusel, B., Beck, K. D., Winslow, J. W., Rosenthal, A., Burton, L. E., Widmar, H. R., Nikolics, K. & Hefti, F. (1992) *J. Neurosci.* **12,** 4391–4402.
20. Beck, K. D., Valverde, J., Alexi, T., Poulson, K., Moffat, B., Vandlen, R. A., Rosenthal, A. & Hefti, F. (1995) *Nature (London)* **373,** 339–341.
21. Tseng, J. L., Baetge, E. E., Zurn, A. D. & Aebischer, P. (1997) *J. Neurosci.* **17,** 325–333.
22. Moon, L. D. F., Brecknell, J. E., Franklin, R. J. M., Dunnett, S. B. & Fawcett, J. W. (2000) *Exp. Neurol.* **161,** 49–66.
23. Crocker, S. J., Morelli, M., Wigle, N., Nakabeppu, Y. & Robertson, G. S. (1998) *Brain Res. Mol. Brain Res* **53,** 69–77.
24. Paxinos, G. & Watson, C. (1996) *The Rat Brain in Stereotaxic Coordinates: Compact Edition* (Academic, New York).
25. Winter, C., Schenkel, J., Burger, E., Eickmeier, C., Zimmermann, M. & Herdegen, T. (2000) *Neuroscience* **95,** 753–762.
26. Sauer, H., Rosenblad, C. & Bjorklund, A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8935–8939.
27. Bilang-Bleuel, A., Revah, F., Colin, P., Locquet, I., Robert, J. J., Mallet, J. & Horellou, P. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 8818–8823.
28. Hayley, S., Brebner, K., Lacosta, S., Merali, Z. & Anisman, H. (1999) *J. Neurosci.* **19,** 5654–5665.
29. Seegal, R. F., Brosch, K. O. & Bush, S. (1986) *J. Chromatogr.* **377,** 131–144.
30. Hagg, T., Fass-Holmes, B., Vahlsing, H. L., Manthorpe, M., Conner, J. M. & Varon, S. (1989) *Brain Res.* **505,** 29–38.
31. Davis, R. J. (2000) *Cell* **103,** 239–252.
32. Derijard, B., Hibi, M., Wu, I. W., Barrett, T., Su, B., Deng, T., Karin, M. & David, S. J. (1994) *Cell* **76,** 1025–1037.
33. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E., Ahmad, M. F., Avruch, J. & Woodgett, J. R. (1994) *Nature (London)* **369,** 156–160.
34. Binetruy, B., Smeal, T. & Karin, M. (1991) *Nature (London)* **351,** 122–127.
35. Smeal, T., Hibi, M. & Karin, M. (1994) *EMBO J.* **13,** 6006–6010.
36. Brecht, S., Gass, P., Anton, F., Zimmermann, M. & Herdegen, T. (1994) *Mol. Cell. Neurosci.* **5,** 431–441.
37. Saporito, M. S., Thomas, B. A. & Scott, R. W. (2000) *J. Neurochem.* **75,** 1200–1208.
38. Leah, J. D., Herdegen, T. & Bravo, R. (1991) *Brain Res.* **566,** 198–207.
39. Saporito, M. S., Brown, E. M., Miller, M. S. & Carswell, S. (1999) *J. Pharmacol. Exp. Ther.* **288,** 421–427.
40. Dragunow, M., Xu, R., Walton, M., Woodgate, A., Lawlor, P., MacGibbon, G. A., Young, D., Gibbons, H., Lipski, J., Muravlev, A., *et al.* (2000) *Mol. Brain Res.* **83,** 20–33.
41. Nishi, K. (1997) *Brain Res.* **771,** 133–141.
42. Hirata, Y., Adachi, K. & Kiuchi, K. (1998) *J. Neurochem.* **71,** 1607–1615.
43. Ward, L. & Hagg, T. (2000) *Eur. J. Neurosci.* **12,** 1867–1881.
44. Haas, C. A., Diller, T., Naumann, T. & Frotscher, M. (1996) *J. Neurosci.* **16,** 1894–1903.
45. Robinson, G. A. (1995) *Brain Res. Mol. Brain Res.* **30,** 61–69.
46. Brown, P. H., Alani, R., Preis, L. H., Szabo, E. & Birrer, M. J. (1993) *Oncogene* **8,** 877–886.
47. Brown, P. H., Chen, T. K. & Birrer, M. J. (1994) *Oncogene* **9,** 791–799.
48. Eilers, A., Whitfield, J., Babij, C., Rubin, L. L. & Ham, J. (1998) *J. Neurosci.* **18,** 1713–1724.
49. May, G. H. W., Allen, K. E., Clark, W., Funk, M. & Gillespie, D. A. F. (1998) *J. Biol. Chem.* **273,** 33429–33435.
50. Putcha, G. V., Moulder, K. L., Golden, J. P., Bouillet, P., Adams, J. A., Strasser, A. & Johnson, E. M. (2001) *Neuron* **29,** 615–628.
51. Whitfield, J., Neame, S. J., Paquet, L., Bernard, O. & Ham, J. (2001) *Neuron* **29,** 629–643.
52. Kirch, H.-C., Flaswinkel, S., Rumpf, H., Brockmann, D. & Esche, H. (1999) *Oncogene* **18,** 2728–2738.
53. Bakiri, L., Lallemand, D., Bossy-Wetzel, E. & Yaniv, M. (2000) *EMBO J.* **19,** 2056–2068.
54. Osuga, H., Osuga, S., Wang, F., Fetni, R., Hogan, M. J., Slack, R. S., Hakim, A. M., Ikeda, J. E. & Park, D. S. (2000) *Proc.Natl. Acad. Sci. USA* **97,** 10254–10259. (First Published August 15, 2000; 10.1073/pnas.170144197)
55. Mattson, M. P., Keller, J. N. & Begley, J. G. (1998) *Exp. Neurol.* **153,** 35–48.
56. Jacoson, M. D., Burne, J. F. & Raff, M. C. (1994) *EMBO J.* **13,** 1899–1910.
57. Perry, V. H., Brown, M. C. & Lunn, E. R. (1992) *Eur. J. Neurosci.* **4,** 1000–1002.
58. Benbassat, D. & Spira, M. E. (1993) *Exp. Neurol.* **122,** 295–310.
59. Benbassat, D. & Spira, M. E. (1994) *Eur. J. Neursci.* **6,** 1605–1614.
60. Alvarez, J., Giuditta, A. & Koenig, E. (2000) *Prog. Neurobiol.* **62,** 1–62.
61. Coffey, E. T., Hongisto, V., Dickens, M., Davis, R. J. & Courtney, M. J. (2000) *J. Neurosci.* **20,** 7602–7613.