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Long-lasting Expression of JUN and KROX Transcription Factors and Nitric Oxide Synthase in Intrinsic Neurons of the Rat Brain following Axotomy

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In adult rats, the medial forebrain bundle (MFB) and mammillothalamic tract (MT) were unilaterally transected, resulting in axotomy of neurons in numerous areas such as the substantia nigra (SN), ventral tegmental area (VTA), nucleus (ncl.) mammillaris (MnM), and ncl. parafascicularis of the thalamus (PF). In these areas, expression of the transcription factor proteins c-JUN, JUN B, JUN D, c-FOS, FOS B, KROX-20, KROX-24, and CREB was investigated by immunocytochemistry up to 150 d. In parallel, the expression of nitric oxide synthase (NOS) was investigated both immunocytochemically and by the NADPH-diaphorase reaction (NDP), and the antibody against NOS was further characterized. The colocalization of c-JUN with NDP or NOS was also studied in the axotomized neurons.

c-JUN and JUN D became visible in nuclei of many neurons of the ipsilateral MnM, PF, VTA, and SN (predominantly in the pars compacta and those double labeled by tyrosine hydroxylase, TH) after 36 hr, not after 24 hr, following transection of MFB and MT. In MnM, c-JUN and JUN D persisted at a nearly maximal level for up to 150 d. In PF, these proteins returned to control levels after 75 d. Expression of c-JUN and JUN D declined in the VTA after 30 d, but in the SN, it already declined after only 10 d. KROX-24 had a later onset of expression, being visible after 3 d in all investigated areas, and its pattern was similar to that of JUN proteins, although labeling was visible in fewer nuclei and declined earlier. JUN B, c-FOS, FOS B, and KROX-20 were not expressed in these areas, and substantial alterations of CREB immunoreactivity (CREB-IR) could not be detected.

A subset of SN neurons (predominantly in the pars reticularis and negative for TH) presented an early and transient expression of all studied JUN, FOS, and KROX-24 proteins within 3 hr of transection that declined between 24 hr and 48 hr to basal levels. This expression pattern is typical of that caused by transynaptic stimulation (probably due to excitation of descending striatal neurons running within the MFB) and was clearly distinct from that evoked by c-JUN, JUN D, and KROX-24 IRs after 36 hr (predominantly in the pars compacta).

An ipsilateral increase in NOS and NDP became visible in many neurons of the MnM after 10 d, but not after 5 d, and this persisted up to 150 d. The temporospatial pattern of NDP was similar to the pattern of NOS-IR. In the MnM, 78–92% of neurons with NDP and 67–81% of neurons with NOS-IR were also labeled for c-JUN. NOS-IR and NDP did not change in VTA and SN and remained absent in PF.

These results indicate that axotomized intrinsic neurons of CNS show either individual transient or long-lasting changes of transcription factors that may underlie and mediate their different sprouting potencies. The particular persistence and lasting coexpression of c-JUN and NOS in MnM neurons indicate a protective role of NOS and c-JUN partnership for damaged neurons in the rat CNS.

[Key words: axotomy, transcription factors, JUN proteins, FOS proteins, KROX proteins, nitric oxide, nitric oxide synthase, central intrinsic neurons, regeneration, regenerative potency, NADPH-diaphorase reaction]

Neurons of mammalian CNS respond to lesions of their axons with an intense cell body response, and with selective changes in gene expression (Mikucki and Oblinger, 1991; Tetzlaff et al., 1991). The extracellular matrix and glial environment have been shown to be crucial for the control of axonal sprouting of the damaged neurons (David and Aguayo, 1981; Villegaz-Perez et al., 1988; Schnell and Schwab, 1990; Wictorin et al., 1990; Bastmeyer et al., 1991; David et al., 1991). In addition to these extraneuronal components, intrinsic properties of neurons contribute to the potency of the cell body response and axonal sprouting (Richardson et al., 1984; Carpenter et al., 1986; Lyon and Stelzner, 1987; Davies, 1989; Fawcett, 1992). These intrinsic properties could depend on a differential control of those genes that precede and underlie the cell body response. However, at present nothing is known about the nuclear molecular genetic mechanisms of damaged neurons that initiate and maintain axonal sprouting. We have investigated the expression of JUN proteins (c-JUN, JUN B, and JUN D) in axotomized neurons because these transcriptionally operating proteins are supposed to play a crucial role in cellular growth, differentiation, and restoration (Nakabeppu et al., 1988; Ryder and Nathans,

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1988; Ryseck et al., 1988; Hirai et al., 1989; Hsu et al., 1992; Kovary and Bravo, 1992). Recently, we have shown that selective changes in the expression of c-JUN, JUN D, and CREB transcription factors precede the onset of the cell body response, and persist during the sprouting efforts of axotomized neurons following transection of peripheral and optic nerve fibers (Herdegen et al., 1990, 1991c, 1992, 1993a,b; Leah et al., 1991).

In this study we have investigated the expression of eight JUN, FOS, KROX, and CREB transcription factors in axotomized intrinsic central neurons following transection of the medial forebrain bundle (MFB) and mammillothalamic tract (MT). The expression of these transcription factors was studied in thalamic and subthalamic neurons that project ascending axons into the MFB and MT such as the substantia nigra (SN) pars compacta (SNC), nucleus (n.) mammillaris (MnM), ventral tegmental area (VTA), and n. parafascicularis of thalamus (PF) (Nieuwenhuys et al., 1982; Veening et al., 1982). In addition to transcription factors, we have studied the expression of nitric oxide synthase (NOS; EC1.14.23) by immunocytochemistry and by the NADPH-diaphorase reaction (NDP). NOS catalyzes the transformation of arginine to nitric oxide (NO) (Moncada et al., 1991). NOS and/or NO, the previously identified endothelium-derived relaxing factor, are thought to be involved in neuron–glia interaction (Morris et al., 1992) and in the resistance of central neurons to ischemic and degenerative destruction (Ferrante et al., 1985; Beal et al., 1986; Koh et al., 1986; Uemura et al., 1990). Therefore, we wanted to know whether changes in NOS expression and NDP could be related to the initiation and/or maintenance of the cell body response of axotomized neurons, and to the expression of c-JUN transcription factor following transection of MFB and MT.

Materials and Methods

Animal experiments. Adult Sprague–Dawley rats 2 months old (250 gm body weight) were anesthetized (60 mg/kg pentobarbital, i.p.) and placed in a stereotaxic frame. After skin incision, a craniotomy (2 mm in diameter) was performed by an electric drill on the left frontal bone (bregma −2.30 frontal and 1.50 lateral, according to Paxinos and Watson, 1989). The dura was opened and a blade 1.5 mm wide was introduced to 8 mm and immediately withdrawn by a micromanipulator. The wound was closed by Gelfoam and the overlying skin was sutured. The rats were allowed to survive for 3 hr, 8 hr, 12 hr, 24 hr, and 36 hr ($n = 2$), 3, 5, 10, 20, 30, 45, 60, 75, 100, and 150 d ($n = 3$). The identical experimental procedure was performed in 1-year-old rats that were killed after 3 and 60 d ($n = 3$). In order to evaluate the effect of surgery on protein expression, the blade was introduced for 4 mm, the dorsal border of MT; or for 6 mm, the dorsal border of MFB. These sham controls survived each for 3 hr, 24 hr, and 10 d ($n = 2$). In all animals, the efficiency of operation for transection of MFB and MT was histologically investigated post-mortem and animals have been excluded when MFB and MT were only partially transected.

Immunocytochemistry. All rats were reanesthetized (100 mg/kg pentobarbital, i.p.) and killed by transcardial perfusion (4% paraformaldehyde in phosphate buffer). The brains were postfixed in the same fixative and cryoprotected by 30% sucrose for 72 hr. Cryostat cut coronal sections 35 μm thick were processed for immunocytochemistry as free-floating sections using the conventional avidin–biotin complex method as described in detail previously (Herdegen et al., 1991a). Immunoreactivities (IRs) were visualized by diaminobenzidine and intensified by nickel-cobalt.

All antibodies were polyclonal and generated in rabbits. They were diluted as follows: anti–c-JUN, 1:1000 (607/8) and 1:40,000 (636/3); anti–JUN B, 1:4000; anti–JUN D, 1:8000; anti–c-FOS, 1:40,000; anti–FOS B, 1:2000; anti–KROX-20, 1:10,000; and anti–KROX-24, 1:8000. The two antibodies against c-JUN showed *in vitro* either a specific reaction with c-JUN (code 607/8) or a cross-reaction with JUN B and JUN D (code 636/3). *In vivo*, these antibodies gave an identical pattern of immunoreactivity, and the antibody 636/3 was thus used because it produced less background labeling and an intense immunoreactivity (IR). The antibody against CREB (a generous gift from Dr. W. Schmid, German Cancer Research Center, Heidelberg, Germany) was diluted 1:3000. The specificity of all antibodies was demonstrated *in vitro* (Boshart et al., 1991; Kovary and Bravo, 1991) and *in vivo* by preabsorption experiments (Herdegen et al., 1991a, 1992). The monoclonal antibody against tyrosine hydroxylase (TH) (Boehringer, Mannheim, Germany) was diluted 1:4000.

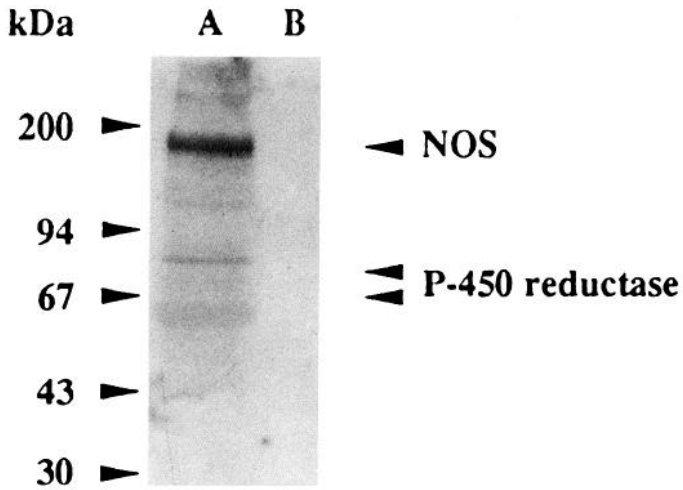

Figure 1. Immunoblotting of rat brain NOS. The anti-NOS antiserum against the N-terminal part of the NOS protein (in a dilution of 1:5000) was directed against rat brain synaptosol cytosol corresponding to about 10 ng of NOS (*NOS, lane A*) and against 2 μg of purified rat liver CPR-450 (*lane B*) applied onto 10% polyacrylamide SDS gels. The subunits of CPR-450 were localized by reversibly staining the nitrocellulose membrane with Ponceau-S.

To demonstrate the expression of nitric oxide synthase (NOS), two polyclonal rabbit antibodies were used. One was directed against the complete purified NOS protein (Mayer et al., 1990; Klatt et al., 1992) and was diluted 1:10,000 for immunocytochemistry. A second antibody was raised against the N-terminal peptide of NOS that does not share a homology with cytochrome P-450 reductase (CPR-450). This anti-NOS peptide antibody strongly reacted with the NOS peptide but did not cross-react at all with the CPR-450 protein (Fig. 1). Preabsorption of 1 nM NOS peptide with the corresponding anti-NOS antibody (diluted 1:5000) for 24 hr abolished the NOS-IR.

Activity of NOS was demonstrated by conventional NADPH-diaphorase histochemistry (NDP) using a medium containing 1 mM NADPH, 0.2 mM nitroblue tetrazolium, 0.2% Triton X-100 in 0.1 Tris-HCl at pH 7.2 for 30–45 min (Scherer-Singler et al., 1983; Fiallos-Estrada et al., 1993). For double-labeling, either sections first underwent NDP and were subsequently processed with the anti-c-JUN antibody, or sections first underwent c-JUN staining (visualization with nickel-cobalt) and were subsequently processed with the anti-NOS antibodies. All brains from every second time point were processed for anti-NOS antibodies.

Immunoblotting of rat brain NOS and purified rat liver cytochrome P-450 reductase. Rat brain synaptosomal cytosols with specific NOS activities of about 0.2 nmol L-citrulline × mg^{-1} × min^{-1} were prepared as previously described (Mayer et al., 1992). Assuming a specific activity of purified rat brain NOS of 1 μmol × mg^{-1} × min^{-1} (Bredt and Snyder, 1990), these preparations contained about 0.2 μg of NOS per milligram of total protein. CPR-450 purified from rat liver microsomes was a generous gift of Dr. M. Kastner (Institute of Toxicology and Embryopharmacology, University of Berlin). Rat brain synaptosomal cytosol (50 μm of total protein corresponding to about 10 ng of NOS) (Fig. 1, lane A) and 2 μg of purified CPR-450 (Fig. 1, lane B) were diluted with sample buffer and applied onto 10% polyacrylamide SDS gel. Electrophoresis and immunoblotting were carried out as previously described (Klatt et al., 1992), and the anti-NOS antiserum against the N-terminal part was used in a final dilution of 1:5000.

Effect of the operation on the animal behavior. Within the first postoperative week, the body weight either was not affected or was reduced by up to 15%. Thereafter, the increase of body weight paralleled that

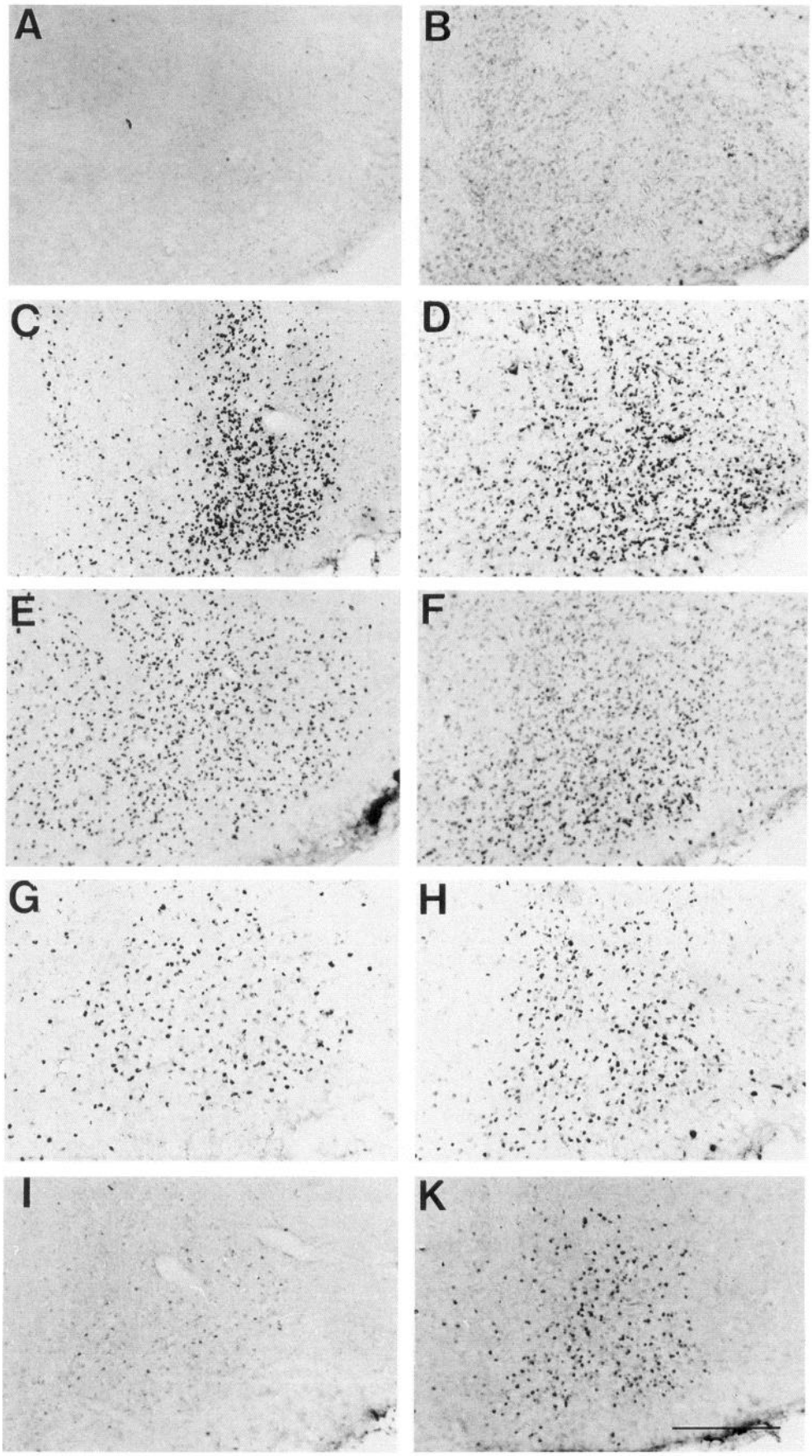

Figure 2. c-JUN, JUN D, and KROX-24 in MnM: c-JUN (*A, C, E, G*), JUN D (*B, D, F, H*), and KROX-24 (*I, K*) in MnM of untreated rats (*A, B, I*), and 10 d (*C, D, K*), 60 d (*E, F*), and 100 d (*G, H*) following transection of MFB and MT. The ventrolateral border is on the right-hand side. Scale bar, 300 μm.

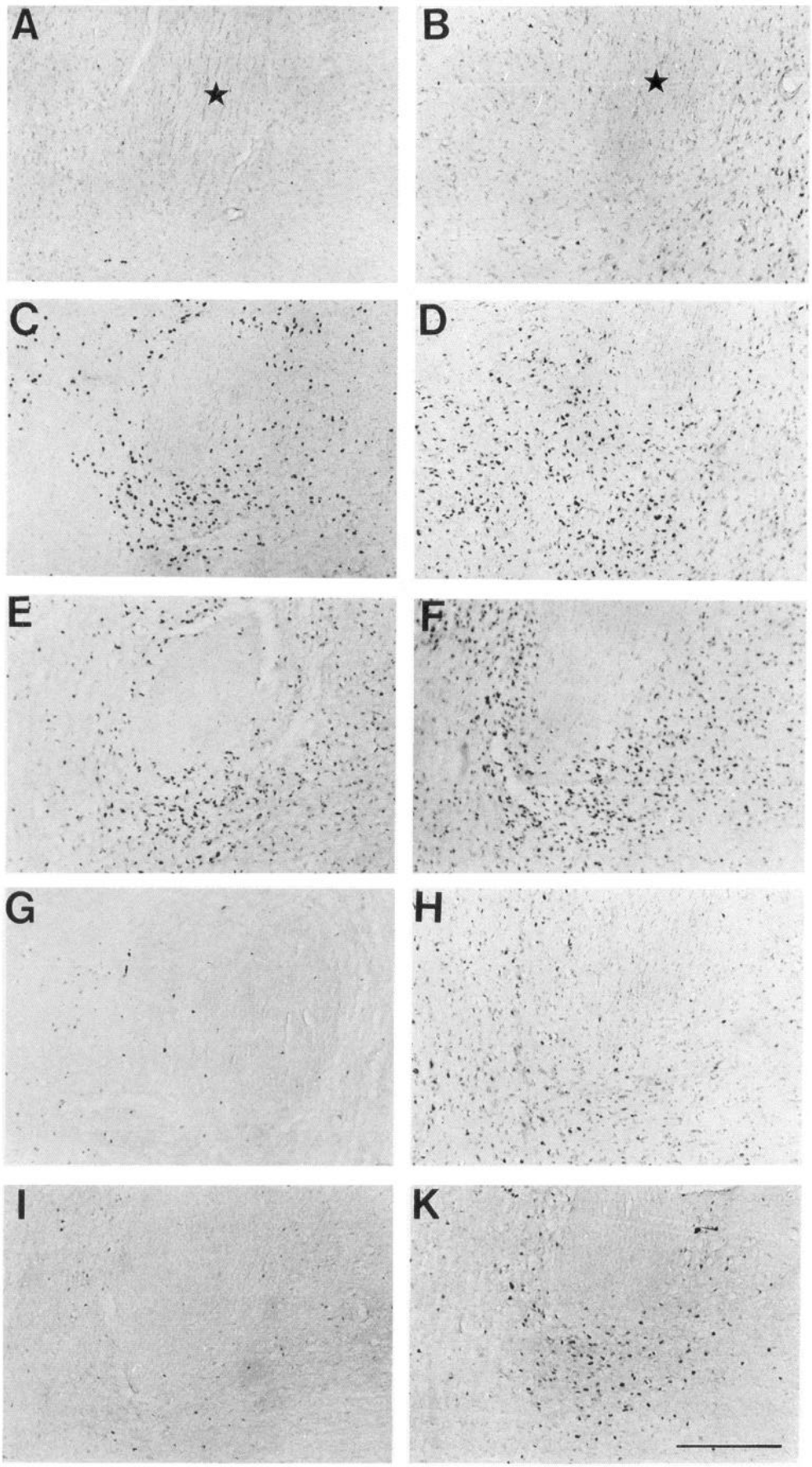

Figure 3. c-JUN, JUN D, and KROX-24 in PF: c-JUN (*A, C, E, G*), JUN D (*B, D, F, H*), and KROX-24 (*I, K*) in PF of untreated rats (*A, B, I*), and 10 d (*C, D, K*), 30 d (*E, F*), and 75 d (*G, H*) following transection of MFB and MT. The *stars* mark the fasciculus retroflexus. Dorsal is up and lateral to the right. Scale bar, 300 μm.

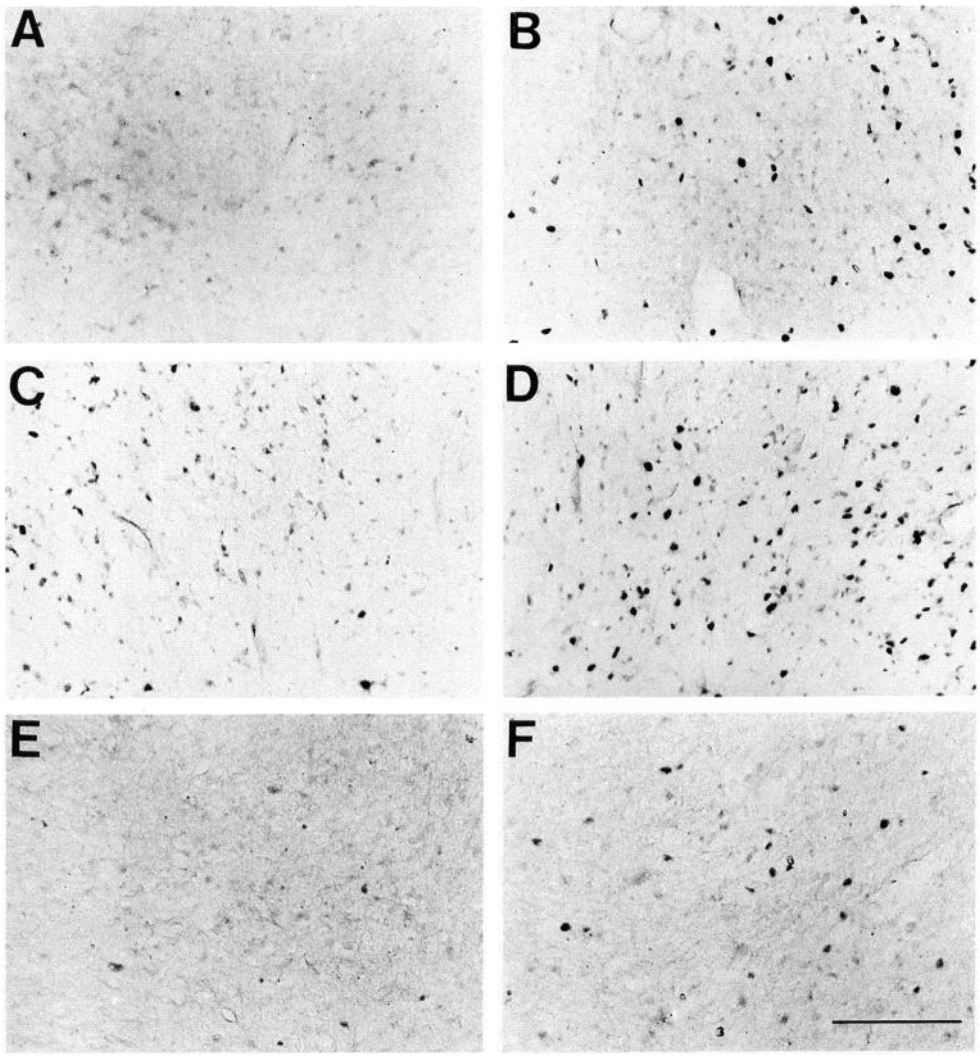

Figure 4. c-JUN, JUN D, and KROX-24 in VTA: c-JUN (*A, B*), JUN D (*C, D*), and KROX-24 (*E, F*) in VTA of untreated rats (*A, C, E*), and 10 d following transection of MFB and MT (*B, D, F*). Dorsal is up and lateral is to the right. Scale bar, 150 μm.

of untreated rats. Their social behavior and self-care were not affected. Six rats were excluded from the experiments and were killed within 24 hr following the surgery because of postsurgery stress. These rats are not included in the pool of investigated animals.

Statistics. Immunoreactive cell nuclei of MnM, PF, VTA, and SN were counted ipsilaterally to MFB and MT transection. For each area, two sections with the highest number of labeled nuclei were chosen in each rat and all counted numbers were averaged (mean ± SD) for each time point of survival. We counted only those nuclei whose intensity of immunoreactivity was above that of the contralateral area.

Results

Expression of transcription factor proteins

Ncl. mammillaris (MnM), ncl. parafascicularis (PF), and ventral tegmental area (VTA). Thirty-six hours, but not 24 hr, after MFB and MT transection, the expression of c-JUN and JUN D was induced in many nuclei of the ipsilateral MnM, PF, and VTA. After 5 d these proteins reached their maxima in both the number of labeled nuclei and the intensity of labeling (Figs. 2*A–D*, 3*A–D*, 4*A–D*, 5). For c-JUN and JUN D we counted a maximum number in MnM of 131 ± 31 and 145 ± 28 labeled cells per section (c/s), in PF of 103 ± 17 and 92 ± 23 c/s, and in VTA of 35 ± 13 and 46 ± 14 c/s, respectively. In MnM, the number of labeled nuclei remained at a submaximal level up to 150 d (Figs. 2*E–H*, 5). c-JUN and JUN D distinctly declined in PF after 60 d, and were at basal levels after 100 d (Figs. 3*E–H*, 5). In the VTA, the maximal expressions declined after 30 d and returned to basal levels after 45 d (Fig. 5). c-JUN and JUN D showed a close temporal and spatial pattern. The expression of KROX-24 was slightly different from that of the JUN proteins; its delayed onset occurred between 36 hr and 3 d, the number of labeled neurons was lower, and the IR decreased earlier (Figs. 2*I–K*; 3*I–K*; *E, F*; 5). The maximal numbers of KROX-24 were 88 ± 22 c/s in MnM, 65 ± 16 c/s in PF, and 24 ± 8 c/s in VTA. During the whole observation period the IRs did not change in the contralateral areas. In MnM, PF, and VTA, we could not detect IRs of JUN B, c-FOS, FOS B, and KROX-20 (Fig. 6).

Substantia nigra (SN). In the SN, two distinctly separated temporospatial patterns of JUN, FOS, and KROX-24 were observed. Within 3 hr, c-JUN, JUN B, JUN D, c-FOS, FOS B, and KROX-24 proteins became visible in the ipsilateral SN, predominantly in the medioventral area of the reticulated part (SNR) (Figs. 7*A–D*; 8*A,C,E*; 9). The patterns of these early IR were similar for all proteins. Between 8 hr and 12 hr, the maximal numbers of labeled neurons were counted for c-FOS (45 ± 12), JUN D (42 ± 13), JUN B (39 ± 10), c-JUN (36 ± 9), KROX-24 (29 ± 12), and FOS B (21 ± 7). These IRs declined between 24 hr and 36 hr (Figs. 8*B,D*; 9), apart from JUN D,

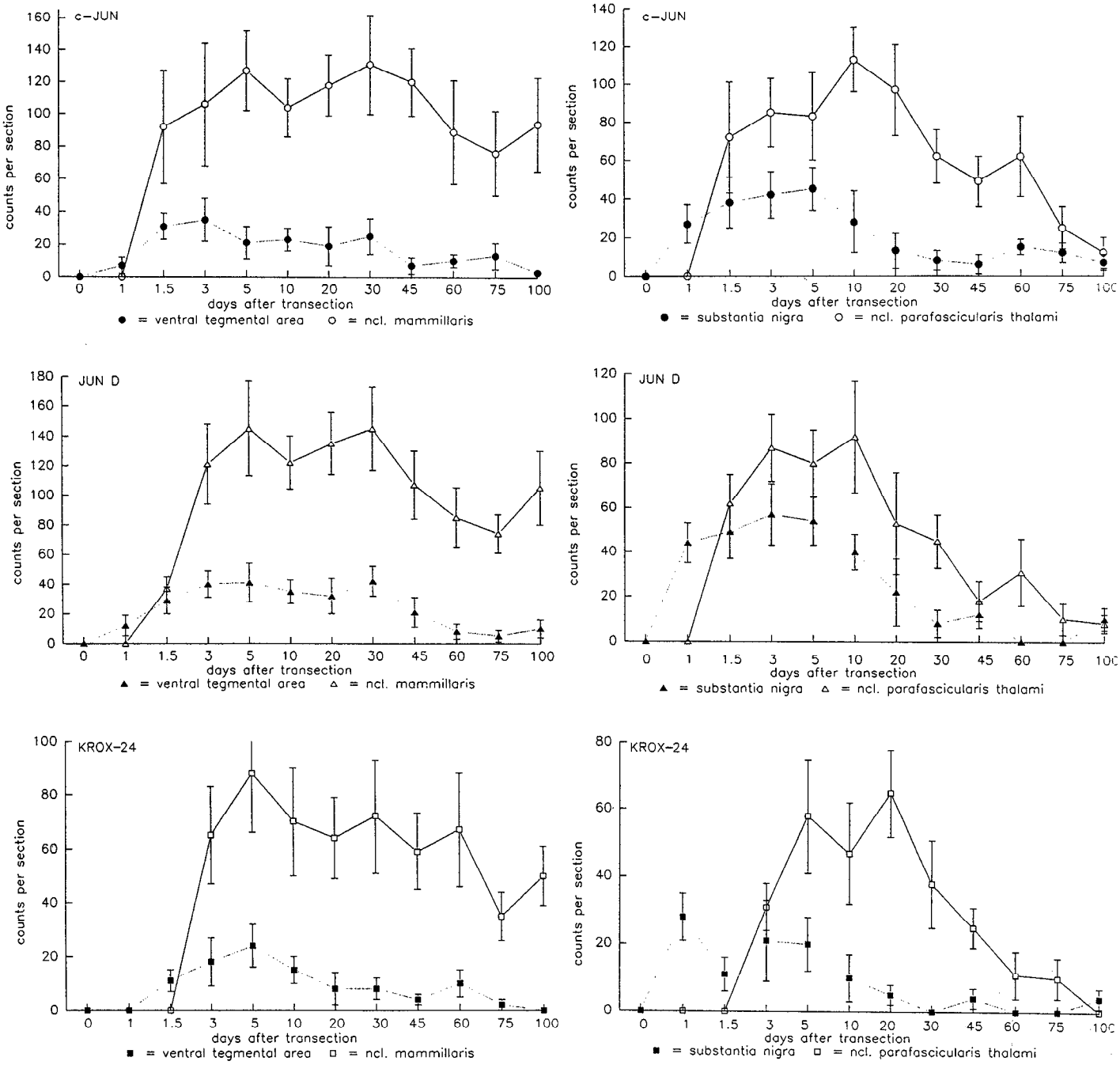

Figure 5. Time course of c-JUN, JUN D, and KROX-24 expression: the number of nuclei labeled by c-JUN, JUN D, and KROX-24 in MnM, VTA (*left column*), PF, and SN (*right column*). The counts (per 35 μm section) give the number of nuclei (mean ± SD) that show a suprabasal intensity of IR.

which persisted in the SNR for up to 10 d (Fig. 7*F*). Double labeling of c-FOS or c-JUN with TH, which marks the major population of SNC neurons, revealed that TH and c-FOS or c-JUN proteins are almost exclusively expressed in different neurons between 3 hr and 36 hr (Fig. 8*A*).

After 36 hr, a new pattern of immediate-early gene (IEG) expression appeared in the ipsilateral SN. c-JUN, JUN D, and KROX-24 proteins were predominantly expressed in the SNC (Figs. 7*E–H*, 9), and double labeling of TH with c-JUN showed a high colocalization in SNC neurons (Fig. 8*E*). The maximal expression of c-JUN, JUN D, and KROX-24 in the SN was visible after 5 d. In clear contrast to MnM, PF, and VTA, however, the IRs had already declined after 10 d (Fig. 7*G,H*), and the KROX-24–IR was only weak in the SN during the entire observation period. After 30 d, only a few labeled neurons were visible in SN. Suprabasal expression was also visible in a few neurons of the contralateral SNC that project within the ipsilateral MFB (Niewenhuys et al., 1982).

Expression of CREB. The CREB protein was constitutively expressed in apparently every neuronal and glial cell as described previously (Gonzalez et al., 1989; Herdegen et al., 1992). Because of its intense and ubiquitous expression, it was difficult

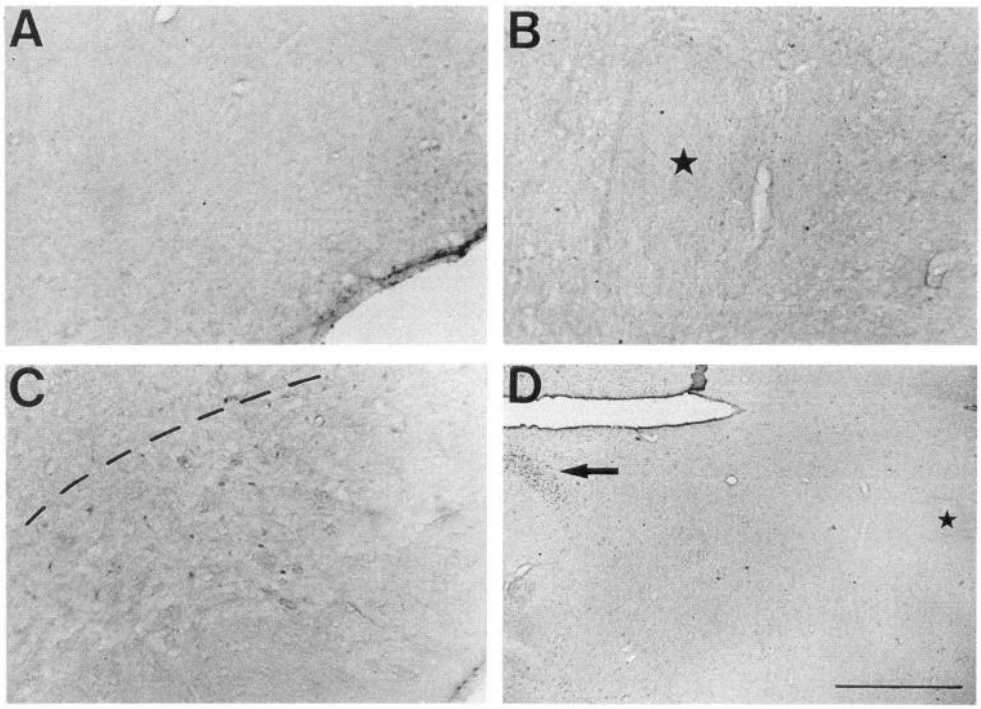

Figure 6. Absence of c-FOS, JUN B, and KROX-20 in axotomized neurons: lack of expression of c-FOS in MnM (*A*) and PF (*B*), of JUN B in MnM (*C*), and of KROX-20 in PF (*D*) 20 d following transection of MFB and MT. In *C* the *dashed line* marks the border between SNC and SNR. In *B* and *D*, the *stars* mark the fasciculus retroflexus, and in *D* the *arrow* marks the basal KROX-20–IR in the dorsomedial hypothalamus. In *A–C*, dorsal is up and lateral is to the right; in *D* dorsal is to the right and the third ventricle is on the left. Scale bars: *A–C*, 300 μm; *D*, 850 μm.

to assess significant changes in CREB-IR. In some animals we found a nonreproducible increase in labeled glial cells and a decrease of CREB-IR in neuronal nuclei ipsilateral to the transection.

NADPH-diaphorase reaction (NDP) and nitric oxide synthase (NOS)-IR

The activity of NOS was demonstrated by the NDP. The blue product of this substrate reaction showed a distribution in the cytoplasm, dendrites, axons of neurons, and blood vessels. In order to determine whether NDP was virtually related to the expression of NOS, brain sections were incubated with the anti-NOS antibodies. The pattern of NDP was congruent to the patterns of NOS-IR evoked by both anti-NOS antibodies, that is, in the neocortex and the retrorubral field (Fig. 10*A–D*).

In the MnM of untreated rats a distinct NDP was visible only in a few neurons, and a weak NOS-IR could be discriminated from background in about 20–40 neurons per section (Fig. 12*A,C*). In the SN and VTA, NOS-IR and NDP were present in a few neurons, but were completely absent in neurons of PF (Fig. 12*I*). The number of neurons labeled by NDP and NOS-IR selectively increased in MnM neurons 10 d following transection of MFB and MT, reached their maxima between 20 and 30 d (Figs. 11*A*; 12*B,D,E*; 13*A,B*), and persisted at submaximal levels for up to 150 d (Figs. 11*B*, 12*F*, 13*A,B*). In PF, neuronal NOS-IR and NDP remained absent during the complete observation period (Fig. 12*G,H*). In the VTA and SN, NOS-IR and NDP, which were present in a few neurons in rats, did not change following transection of MFB and MT (Fig. 12*I–K*).

Interestingly, NDP was also enhanced in the neuropil around axotomized neurons, indicating an increased vascularization by capillary blood vessels (Fig. 12*H*). This increase of NDP was not visible in adjacent areas, thus demonstrating a local effect on vascularization following axotomy.

Double labeling of NDP and NOS with c-JUN

Sections labeled by NDP and NOS were processed for c-JUN immunocytochemistry. In MnM, 78–91% of NDP-positive neurons also expressed c-JUN up to 150 d postaxotomy (Figs. 12*F*, 13*A*). Double labeling of NOS and c-JUN yielded colocalization values between 67% and 81% (Figs. 11*A,B*; 13*B*). These lower values of NOS colocalization compared to those of NDP colocalization could be explained by the finding that the small cytoplasm of many mammillary neurons gives only a weak NOS-IR that could not always be discriminated from nuclear c-JUN–IR (Fig. 11). Moreover, Figure 13*B* includes only those NOS-positive neurons whose intensity of IR surpassed that of contralateral MnM neurons. In VTA few double-labeled neurons were seen irrespective of the time of survival.

Expression patterns in 1-year-old rats

Expression of transcription factors as well as NOS and NDP was also investigated in 1-year-old rats 3 and 60 d following MFB and MT transection. The patterns of all IRs and NDP did not differ from those in 3-month-old rats. Only the numbers and the intensity of KROX-24 labeled nuclei were slightly increased in ipsilateral nuclei of MnM, PF, VTA, and SNC (not shown).

Control experiments

Numerous control experiments demonstrated that the reported expression of the transcription factors was the consequence of transection of the fiber tract. The effects of surgery on JUN, FOS, and KROX expression in MnM, PF, VTA, and SN were assessed by graded introduction of the blade. Introduction down to 4 mm, the dorsal border of MT, did not change IR; introduction down to 6 mm, the dorsal border of MFB, transected the MT without affecting the MFB and induced c-JUN, JUN D, and KROX-24 selectively in MnM; only the introduction

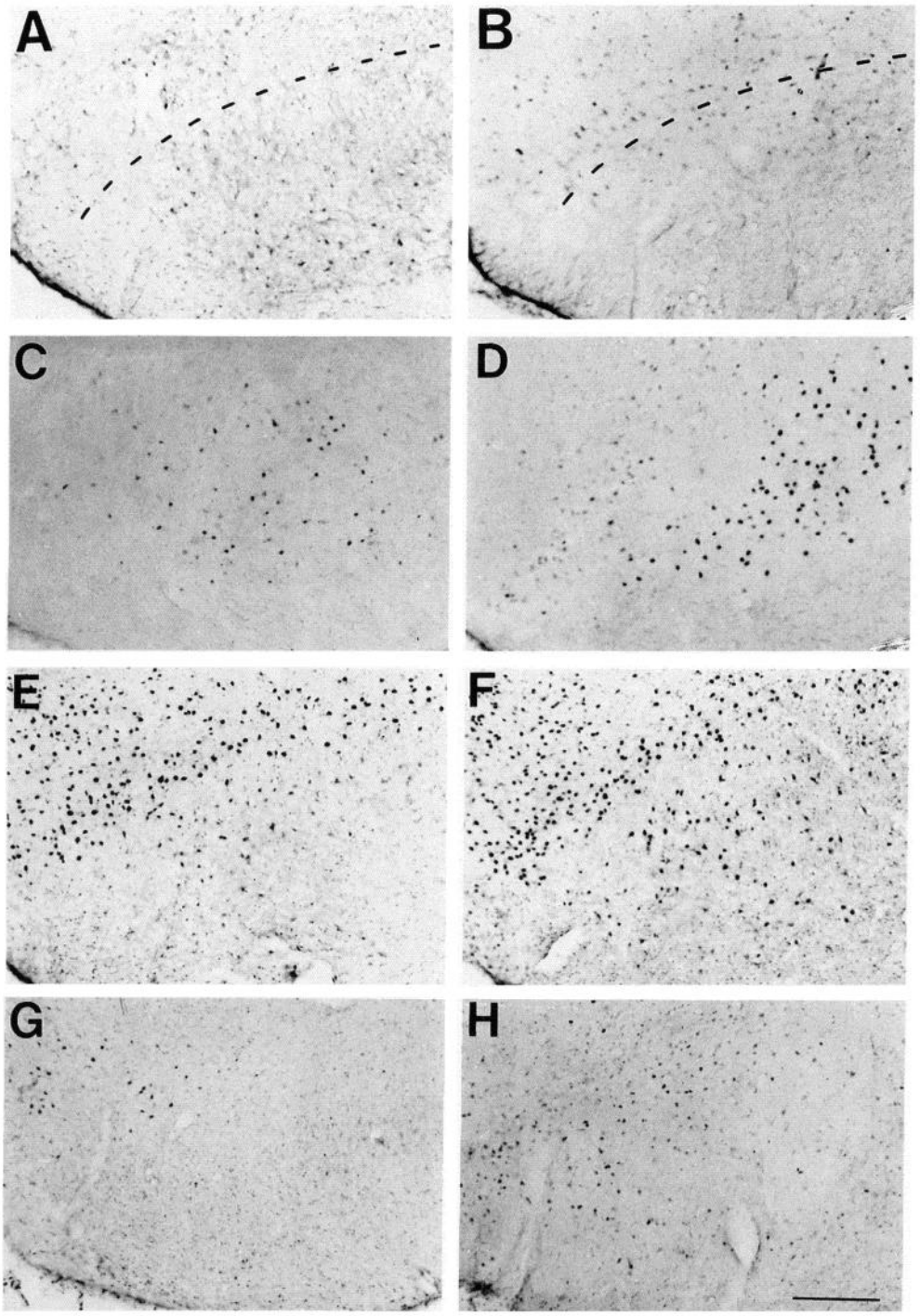

Figure 7. c-JUN, JUN D, and KROX-24 in SN: c-JUN (*A, C, E*), JUN D (*B, D, F*), and KROX-24 (*G, H*) in SN of untreated rats (*A, B, G*), and 8 hr (*C, D*) and 5 d (*E, F, H*) following transection of MFB and MT. The *dashed lines* mark the border between SNR and SNC. Scale bar, 300 μm.

up to 9 mm with transection of MFB induced the described patterns of IR in PF, VTA, and SN (SNC and SNR).

Discussion

The present study describes the expression of transcription factor proteins, NOS and NDP histochemistry in axotomized intrinsic neurons of the adult rat brain. The expression of c-JUN, JUN B, JUN D, c-FOS, FOS B, KROX-20 (also termed Egr-2), KROX-24 (also termed NGFI-A, Egr-1, Zif268), and CREB [also termed CRE-BP1 (Gonzalez et al., 1989; Macgregor et al., 1990)] was investigated in the MnM, PF, VTA, and the SN following transection of the MFB and MT. Our findings can be summarized as follows. (1) Axotomy of intrinsic neurons of CNS induces the expression of c-JUN, JUN D, and KROX-24, but not of JUN B, c-FOS, FOS B, or KROX-20 proteins. (2) The onset of expression started between 24 and 36 hr after axotomy. (3) The expression of JUN and KROX-24 proteins persisted differentially in each group of axotomized neurons, ranging from 10 d in SN to at least 150 d in MnM. (4) NDP and NOS-IR were selectively increased in MnM where a high proportion of NDP- and NOS-labeled neurons also expressed c-JUN. The expression of NOS was demonstrated by a specific antibody

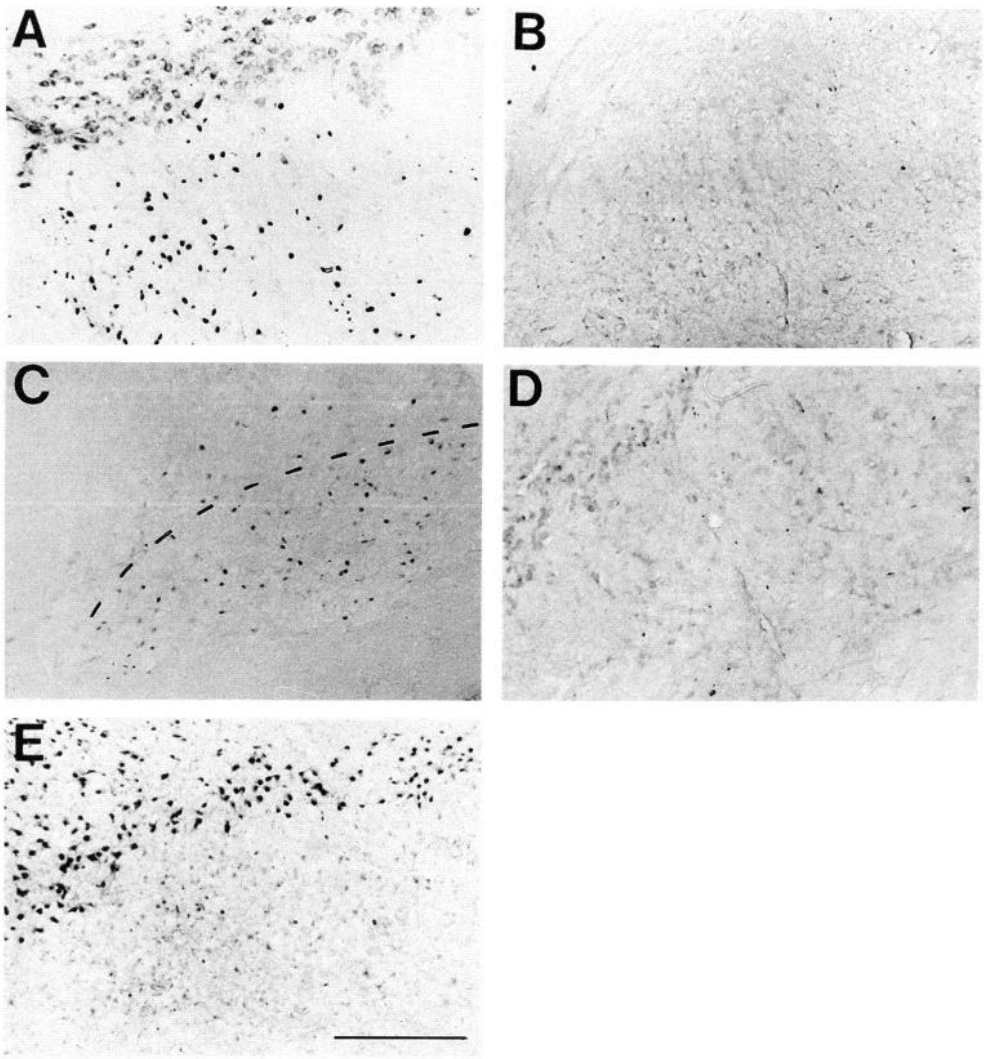

Figure 8. c-JUN, JUN B, and c-FOS in SN. c-FOS (*A, B*), JUN B (*C, D*), and c-JUN (*E*) in the SN 8 hr (*A, C*), 36 hr (*B, D*), and 10 d (*E*) following transection of MFB. The *dashed line* marks the border between SNR and SNC. c-FOS (*A*) and c-JUN (*E*) are colabeled with TH, which is only present to SNC: c-FOS–IR in SNR and TH-IR in SNC are exclusive (*A*) whereas c-JUN is mostly expressed in TH-labeled neurons. TH-IR had already strongly declined after 10 d. Scale bar, 300 μm.

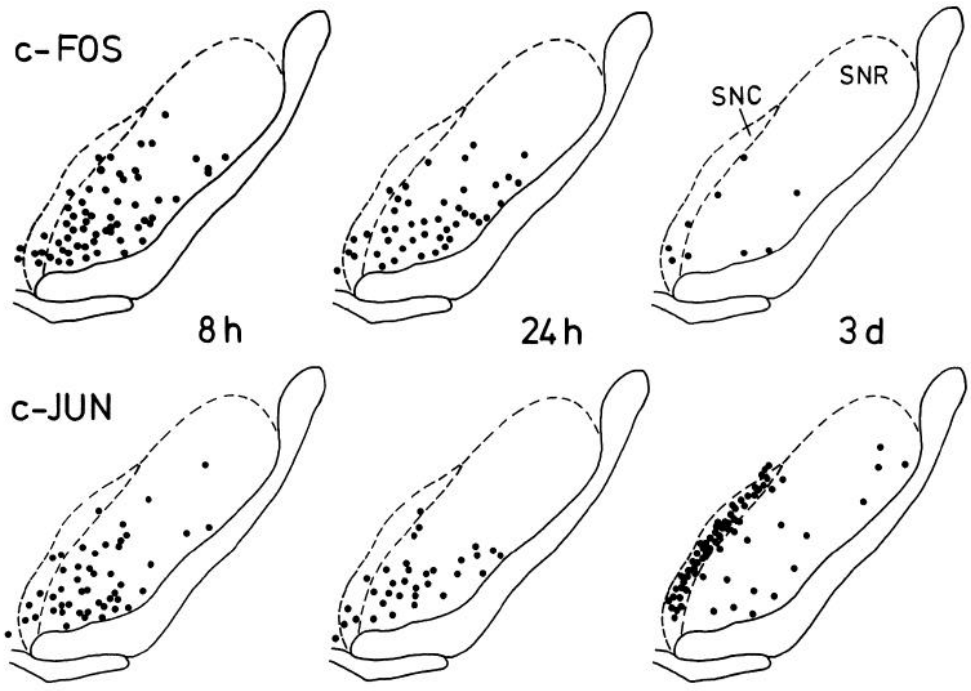

Figure 9. c-FOS and c-JUN in SN: neurons labeled by c-FOS (*upper row*) and c-JUN (*lower row*) 8 hr, 24 hr, and 3 d following transection of MFB and MT in SNC and SNR. Each *dot* represents one labeled nucleus.

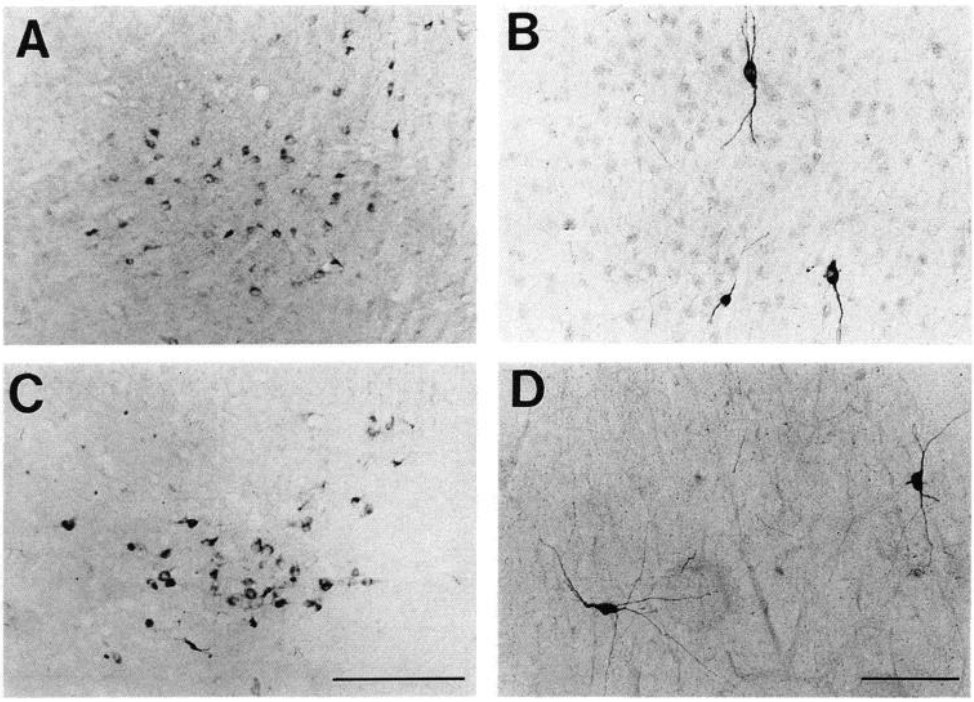

Figure 10. Comparison of NOS-IR and NDP: NOS-IR (*A, B*) and NDP (*C, D*) in neurons of layers II–IV of parietal cortex (*A, C*) and the retrorubral field (*B, D*). Scale bars: *A* and *C*, 100 μm; *B* and *D*, 50 μm.

against the N-terminus of NOS. (5) The expression patterns of 1-year-old rats did not differ compared to those of 3-month-old rats.

Expression of JUN and KROX-24 proteins is related to the transection of MFB and MT

The ascending and descending fiber tracts that project through the MFB and MT are well described (reviewed by Nieuwenhuys et al., 1982; Veening et al., 1982). According to these investigations, transection of the MFB and MT results in axotomy of the ipsilateral ascending projections of MnM, PF, VTA, and SN. In sham-operated controls, IRs of all proteins did not differ from those in untreated rats. In the experimental and sham-operated groups we found an induction of all the JUN, FOS, and KROX-24 proteins but not of KROX-20 in glial and/or neuronal cells around the damaged tissue and in the ipsilateral cortical hemisphere. This pattern of expression is characteristic for mechanical local lesions of the brain and is probably propagated by local inflammatory factors and by cortical spreading depression (Dragunow et al., 1991; Gass et al., 1992a,b; Herdegen et al., 1993c).

After transection of MFB, all the JUN, FOS, and KROX-24 proteins appeared within 3 hr in the SNR, but not in TH-labeled neurons that mark most neurons of the SNC. This is the pattern of an early and transient JUN, FOS, and KROX-24 expression resembling the well-known pattern evoked by *transynaptic* stimulation of neurons (Herdegen et al., 1991a, 1992, 1993c; Gass et al., 1992a). This might be due to transection of strial axons that project into the MFB to the SN, predominantly to SNR (Fallon and Loughlin, 1985), and recently we have shown that transection of peripheral nerve fibers evokes a transient expression of JUN, FOS, and KROX-24 proteins in the deafferented spinal neurons, probably by spontaneous impulse discharge arising from transected nerve fibers (Leah et al., 1991; Herdegen et al., 1992).

In contrast, the selective expression of c-JUN, JUN D, and KROX-24 became visible in MnM, VTA, PF, and SN neurons between 24 hr and 36 hr. The IRs were mainly restricted to the ipsilateral side and persisted between 10 and 150 d. Apart from axotomy, transynaptic stimulation and deafferentation could also induce IEG expression. As mentioned above, transynaptic mechanisms (e.g., via neuronal circuits) should also induce JUN

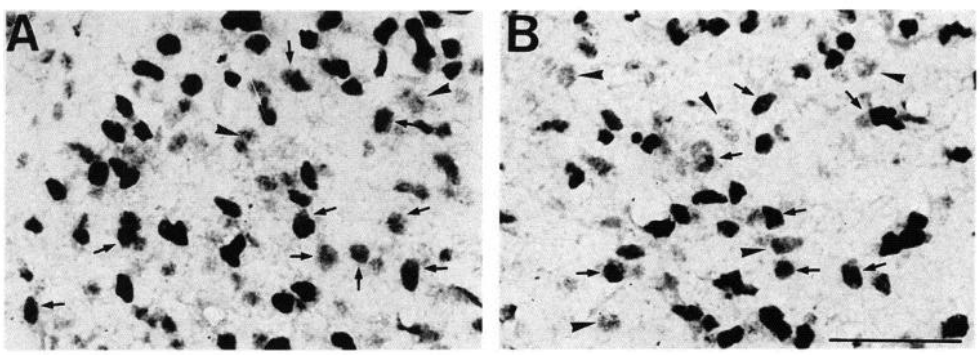

Figure 11. NOS-IR and colocalization with c-JUN in MnM neurons: cytoplasmic NOS-IR and nuclear c-JUN–IR in MnM neurons 30 d (*A*) and 150 d (*B*) following transection of MT. *Arrows* mark colocalization of c-JUN and NOS; *arrowheads* mark neurons labeled by NOS but not by c-JUN. Scale bar, 100 μm.

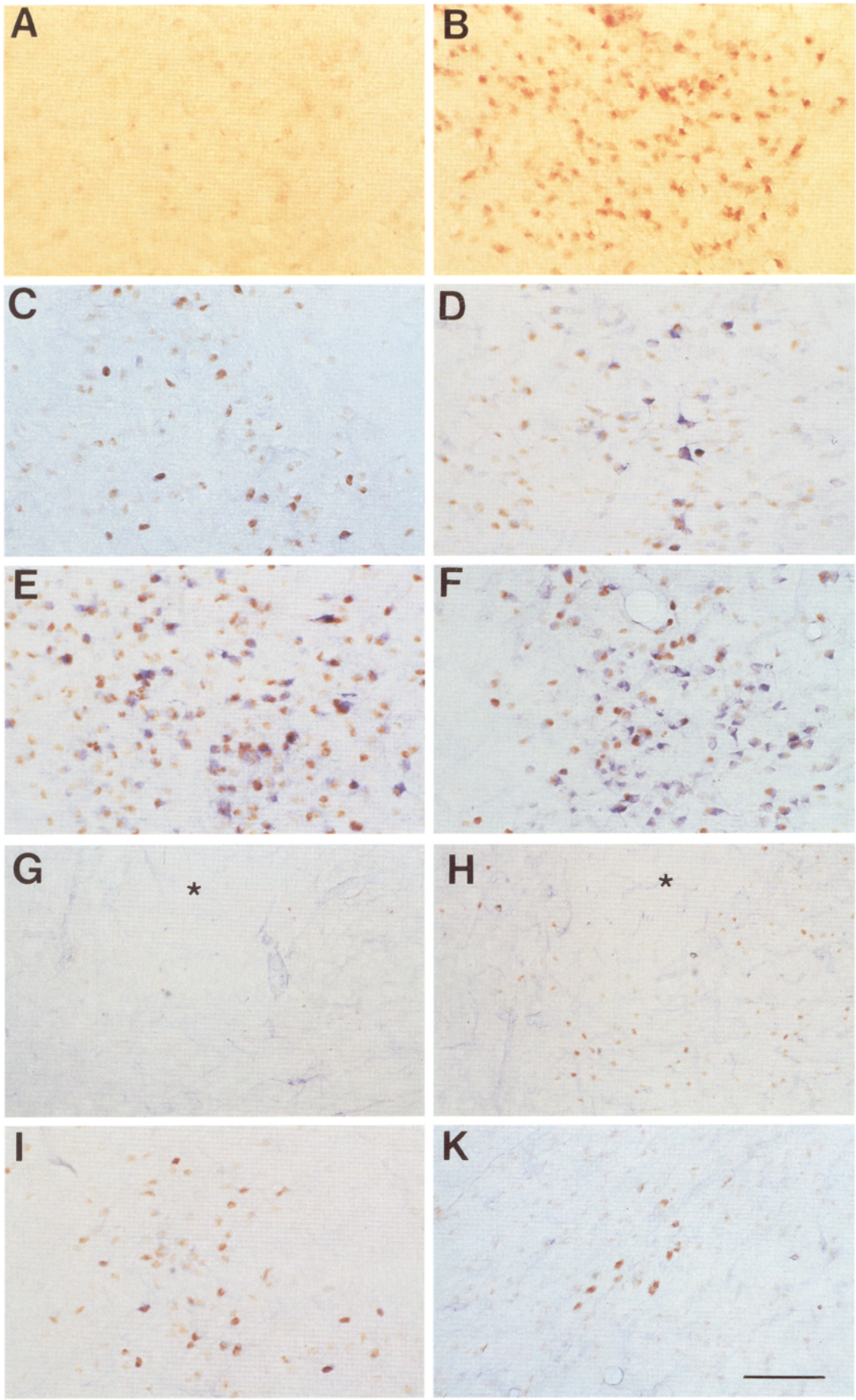

Figure 12. NOS-IR and NDP in MnM, VTA, PF, and SN: NOS-IR in MnM of untreated rats (*A*) and 30 d following transection of MFB and MT (*B*), NDP and c-JUN–IR in MnM after 3 d (*C*), 10 d (*D*), 30 d (*E*), and 150 d (*F*), and NDP and c-JUN–IR in PF of untreated rats (*G*) and after 30 d (*H*), and in VTA (*I*) and SN (*K*) after 10 d. The *asterisks* (*G, H*) mark the fasciculus retroflexus. Scale bar, 150 μm.

B, c-FOS, and FOS B. However, to our knowledge, transynaptic induction has never been reported to evoke a selective expression of c-JUN, JUN D, and KROX-24 proteins. Deafferentation per se of second-order neurons does not induce IEG expression in spinal neurons (Leah et al., 1991; Herdegen et al., 1992). Concordant to our data, dissociated patterns of c-Jun- and c-Fos-IR in SNC and SNR have been recently observed following mechanically and chemically induced axotomy of SN neurons (Jenkins et al., 1993; Weiser et al., 1993).

The signals for induction of IEGs following axotomy remain to be elucidated. Transection of MFB and MT produces a proximal axon stump of 1–3 mm length, and in spite of this short distance, the JUN and KROX expression only started between 24 hr and 36 hr. In contrast, JUN expression is induced within 10 hr following peripheral nerve transection in axotomized primary afferent neurons displaying a peripheral axon stump of 6–7 mm length (Herdegen et al., 1991c, 1992). The comparatively late onset of axotomy-associated protein synthesis in central intrinsic neurons, which does not start before 24 hr, is a general finding (Miller et al., 1989; Mikucki and Oblinger, 1991; Tetzlaff et al., 1991), and our data indicate that this might be related to the delayed expression of transcription factors.

The selective temporospatial pattern of transcription factor expression in axotomized neurons demonstrates that, as early as at the level of transcriptional operations, cell body responses can show differential reactions due to an identical lesion. The temporal onset of c-JUN, JUN D, and KROX-24 expression is fairly similar in all axotomized neurons, whereas the decrease was differentially regulated. Thus, SN neurons express IEG-encoded proteins only for up to 20 d whereas MnM neurons do so at least up to 150 d. This difference could be related to the different potencies for neuronal survival and axonal sprouting of central neurons (Carpenter et al., 1986; Lyon and Stelzner, 1987) depending on the neuronal genotype. For instance, axotomized GABAergic rat thalamic neurons preferentially grow axons into peripheral nerve grafts whereas GABA-negative neurons fail to do so (Benfey et al., 1985). Furthermore, the regenerative potency of adrenergic neurons *in vitro* is stronger than that of serotonergic and dopaminergic neurons (Björklund et al., 1971, 1973; Seiger and Olson, 1977). The dopaminergic neurons of the SN have a particularly low potency for axonal sprouting in cell culture assays (Seiger and Olson, 1977) and their survival depend on trophic supply from striatal neurons (Burke et al., 1992). Besides SN neurons, VTA neurons also show a restricted expression of transcription factors. Interestingly, SN and VTA neurons are ontogenically derived from a common cell complex (Seiger and Olsen, 1973). It is a relevant question whether the *in vitro* reduced potency might be related to the rather transient expression of transcriptionally acting proteins such as JUN and KROX-24 in axotomized neurons of SN *in vivo*.

The differential expression of transcriptional factors could be the result of selective neuronal responsiveness to neurotrophic factors such as NGF, brain-derived neurotrophic factor, or ciliary neurotrophic factor that support survival of central neurons such as dopaminergic neurons of SN *in vitro* (Hyman et al., 1991; Knüsel et al., 1991). The neurotrophic effects of these factors depend on the binding to their appropriate (high affinity) receptors. Recently, expression of *trk*B and *trk*C mRNAs was reported in MnM and SN of untreated rats, with a particularly intense labeling of lateral MnM (Merlio et al., 1992), that area with the most sustained c-JUN and NOS expression. Upregulation of *trk*B mRNA was observed in injured spinal neurons (Frisen et al., 1992), and the *trk*B protein constitutes a receptor for the brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 (Klein et al., 1991, 1992).

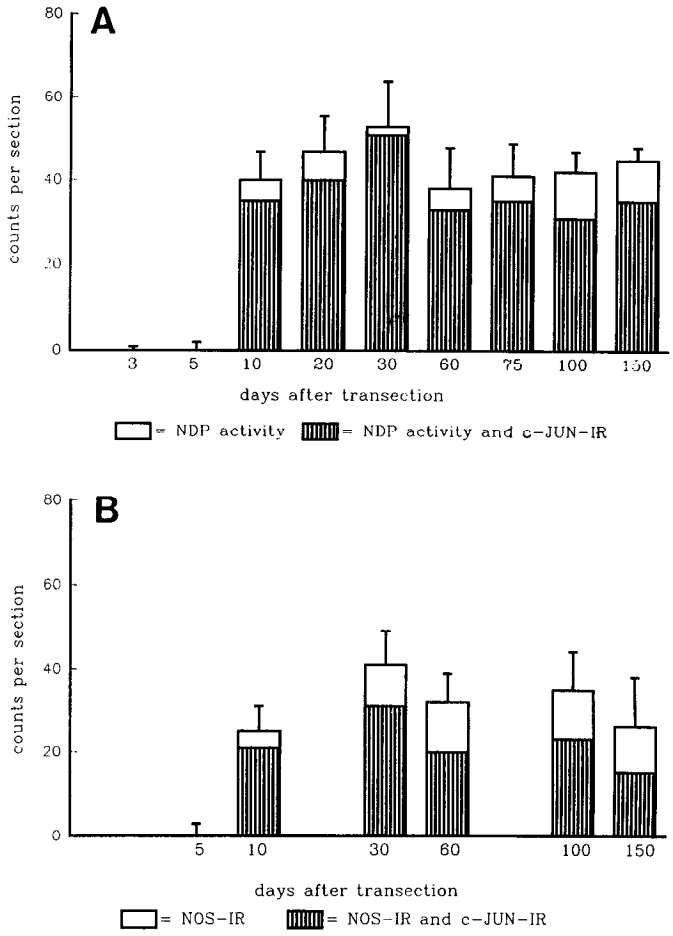

Figure 13. Time courses of NDP and NOS and their colocalization with c-JUN in MnM neurons. The *bars* represent the numbers (mean ± SD) of MnM neurons labeled by NDP (*A*) and NOS (*B*), and the *hatched* parts represent the number of those neurons colabeled for c-JUN. For NOS (*B*), only every second time point was investigated.

Increase of NDP and NOS-IR in the MnM

Axotomy induces a long-lasting increase of both the NOS-IR and the NDP substrate reaction (NDP) in MnM. There is strong evidence that NDP neurons reflect the distribution of NOS in the rat brain (Bredt et al., 1991a; Dawson et al., 1991; Hope et al., 1991). The two antibodies used in the present study were directed against the entire protein or the N-terminal part of the NOS protein (Mayer et al., 1990; Klatt et al., 1992). They displayed a congruent pattern between NOS-IR structures and NDP-labeled structures in both the CNS and PNS (Kummer et al., 1992; Fiallos-Estrada et al., 1993). Our finding of a basal NOS-IR in MnM is confirmed by the previous report of a basal NOS mRNA and protein production in the rat brain (Bredt et al., 1991a). We have to take into consideration that both the NDP and the NOS-IR could have been evoked by CPR-450, which shares a close homology with NOS (Bredt et al., 1991b). However, one anti-NOS antibody did not at all cross-react with the CPR-450 protein (Fig. 1). It is also unlikely that CPR-450 contributes to our described changes of NDP and NOS-IR because

the occurrence of CPR-450 is only restricted to a few areas in the CNS (Kapitulnik et al., 1987). Moreover, CPR-450 is differentially regulated because axotomy of SN neurons increases CPR-450 (Haglund et al., 1984) whereas we observed no changes of NOS-IR in axotomized SN neurons.

The close and lasting coexpression of c-JUN in NDP-labeled mammillary neurons suggests an advantageous role of NOS in the cell body response to axotomy. Several reports indicated a protective effect of the presence of NOS against ischemic, toxic, and degenerative insults in the CNS (Ferrante et al., 1985; Beal et al., 1986; Koh et al., 1986; Uemura et al., 1990). Therefore, it is an important observation that the most persisting presence of JUN and KROX-24 proteins occurs in those axotomized neurons that also show a long-lasting increase of NOS-IR and NDP. We suggest that the persistence of these transcription factors reflects a high endogenous regenerative capacity for axonal sprouting that could be supported by the action of NOS via NO (Bredt et al., 1990), for example, by lowering the intracellular levels of calcium (Garg and Hassid, 1991) or by a self-stimulation of the axotomized neurons via NO that might compensate for the axotomy-induced functional inactivation. It was also suggested that the release of NO from resistant NOS neurons could kill nearby neurons during the neurotoxicity (Bredt and Snyder, 1992). Our data indicate the viability of many neurons contacted by NDP fibers and perikarya for up to 150 d, arguing against NO-mediated cell death following axotomy. Finally, the late increase of NOS-IR and NDP between 5 and 10 d excludes a major role for the cell body response but ascribe to NOS a major function during the later postaxotomy period.

The increase of NOS-IR demonstrates the inducibility of the NOS gene at least in those areas with basal NOS expression. Very similar, NOS mRNA and protein increased following axotomy in primary afferent neurons that displayed basal NOS expression, and these axotomized neurons also showed a high colocalization of NOS and c-JUN (Verge et al., 1992; Fiallos-Estrada et al., 1993). Thus, axotomy presents an adequate stimulus for *de novo* expression of NOS in the rodent PNS and CNS.

Meaning of c-JUN, JUN D, and KROX-24 expression

It is a striking phenomenon that an intense and long-lasting expression of c-JUN and JUN D is not at all paralleled by expression of c-FOS and FOS B proteins. It was demonstrated that an increase of c-*jun* mRNA in axotomized neurons underlies the enhanced JUN-IR (Jenkins and Hunt, 1991; Rutherfurd et al., 1992; Hass et al., 1993; Jenkins et al., 1993). The mechanism of signal transduction is not known that induces JUN without FOS proteins; its investigation might also offer a novel key for uncovering the (selective) second messenger systems operating for the onset and maintenance of the cell body response.

It was generally accepted that JUN and FOS proteins have to form heterodimers for effective transcriptional control (reviewed by Curran and Franza, 1988). However, several reports have already described a dissociation of c-*jun* and c-*fos* expression *in vitro* (Bartel et al., 1989; Zwiller et al., 1991; Trejo et al., 1992). Axotomy presents the first *in vivo* pathophysiological condition for such a selective expression pattern. This dissociation was already visible by the basal constitutive expression of c-JUN and JUN D in the CNS in the absence of any c-FOS expression (Herdegen et al., 1991b, 1993c; Mellström et al., 1991).

Our data also demonstrate the dissociation of KROX-24 and c-FOS expression following axotomy. In contrast, previously the induction of *krox-20* and *krox-24* mRNA has been reported to be closely linked to that of c-*fos* mRNA *in vitro* and *in vivo* (Chavrier et al., 1988, 1989; Christy et al., 1988; Lemaire et al., 1988; Sukhatme et al., 1988; Gass et al., 1992a). Moreover, transynaptic stimulation of central neurons, for example, by noxious somatosensory stimulation, cortical spreading depression, and epileptic seizures, induces FOS, JUN, and KROX-24 proteins in a characteristic temporospatial order in CNS neurons. In these cases the numbers of KROX-24– and c-FOS–labeled neurons exceed those labeled by c-JUN and JUN D (Herdegen et al., 1991a, 1993c; Gass et al., 1992a,b).

The absence of FOS proteins following axotomy (Sharp et al., 1989; Leah et al., 1991; Herdegen et al., 1992) raises the question about effective transcriptional operations of JUN proteins. Apart from FOS proteins, c-JUN is able to form *in vitro* transcriptional complexes with CREB/ATF proteins (Benbrook and Jones, 1990; Macgregor et al., 1990), helix-loop-helix protein MyoD (Bengal et al., 1992), and liver regeneration factor-1 (Hsu et al., 1992). c-JUN can bind to various classes of DNA consensus sequences such as CRE and AP-1 (Sassone-Corsi et al., 1990; Ryseck and Bravo, 1991), and moreover, JUN proteins have the capability for a dynamic composition of transcription complexes (Ryseck and Bravo, 1991; Sonnenberg et al., 1989a). This functional variability predetermines transcription factors such as c-JUN for transcription control of effector genes during the regeneration process. Axotomy evokes complex changes of induction and suppression of genes, and by formation of variable complexes with changing partners, c-JUN could be involved in the intricate transcription control in damaged neurons.

The present and recent data demonstrate that the expression of c-JUN and JUN D is a constituent of the cell body response following complete axotomy of peripheral and central neurons in the rat (Herdegen et al., 1990, 1991c, 1992, 1993a,b; Jenkins and Hunt, 1991; Leah et al., 1991; Dragunow, 1992; Fiallos-Estrada et al., 1993; Jenkins et al., 1993). Moreover, appearance of JUN-IR in axotomized retinal ganglion cells of the goldfish indicates a conserved reaction during evolution (Herdegen et al., 1993a).

KROX-20 and JUN B were not induced by axotomy but both genes are expressed in the brain during development (Wilkinson et al., 1989a,b). These findings show that even at the transcriptional level the cell body response cannot be simply considered as replication of developmental processes. The absence of JUN B in axotomized neurons deserves particular interest for two reasons: first, conflicting data contribute to JUN B a function in both the growth arrest, that is, in myeloid cells (Lord et al., 1990), and the promotion of sprouting, that is, in primary hippocampal neurons (Schlingensiepen et al., 1992). JUN B represses the activation of promoters mediated by c-JUN and c-FOS (Schütte et al., 1989; Hsu et al., 1992; Deng and Karin, 1993), counteracting the transcriptional upregulation of target genes of c-JUN. Thus, the absence of JUN B could contribute to the long-lasting expression of c-JUN because c-JUN upregulates its own transcription. Second, the absence of JUN B protein suggests a block of its mRNA translation. The induction of *jun B* mRNA was found in axotomized facial motoneurons (Haas et al., 1993), whereas nuclear JUN B–IR could not be detected (C. A. Haas and T. Herdegen, unpublished observations). Our data clearly demonstrate that SN neurons can present

a distinct JUN B-IR following transynaptic stimulation (Fig. 8C) but not following axotomy.

Functions of JUN and FOS proteins have also to be discussed in regard to their modulation of further transcription factors. JUN and FOS proteins positively and negatively interact the DNA binding of steroid hormone receptors and of vitamin A and D derivatives, and vice versa (Schüle et al., 1990; Shemshedini et al., 1991; Touray et al., 1991; Zhang et al., 1991). Because regenerative efforts of axotomized neurons are also under the control of steroid hormones (Scheff et al., 1980; Yu and Yu, 1983; Gorski, 1985), these humoral factors could contribute to the differential expression patterns and variable functions of AP-1 proteins in the cell body response.

The IEG encoded c-JUN, JUN D, and KROX-24 proteins are nuclear transcription factors (Milbrandt, 1987; Almendral et al., 1988; Ryseck et al., 1988; Hirai et al., 1989; Bravo, 1990). What could be their target genes in axotomized central neurons? At present, the NFG, prodynorphin, and proenkephalin genes have been described as target genes of JUN and FOS proteins (Sonnenberg et al., 1989b; Hengerer et al., 1990; Naranjo et al., 1991). There is evidence that intrinsic central neurons can respond to axotomy with a long-lasting expression of regeneration-associated effector genes, for example, with expression of GAP-43 mRNA and T-α-1-tubulin mRNA up to 50 d (Kalil and Skene, 1986; Skene, 1989; Mikucki and Oblinger, 1991; Tetzlaff et al., 1991). The prolonged increase of JUN and KROX-24 proteins could be the transcriptional prerequisite for persisting expression of such genes. Our results suggest that the fate of damaged central neurons depends on their molecular genetic organization and capacity to control gene expression, in addition to the already documented inhibitory effects of the extracellular matrix (Schnell and Schwab, 1990; David et al., 1991). The present findings of differential transcriptional operations and their putative meaning for the potency of axonal sprouting could be relevant for new strategies to improve the recovery of central nerve fibers after injury.

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