Selective Expression of the Immediate Early Gene *c-jun* in Axotomized Rat Medial Septal Neurons Is Not Related to Neuronal Degeneration

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In the present study, we use the anatomically well defined septohippocampal projection to study the molecular events involved in the reaction of neurons to axotomy. The expression of three immediate early genes (*c-fos*, *c-jun*, and *jun B*) was investigated in rat septohippocampal neurons after axotomy by bilateral fimbria–fornix transection (FFT). Moreover, the extent of retrograde degeneration in the septal complex was assessed by analyzing DNA fragmentation. In a postoperative time course analysis, a strong increase of *c-jun* immunoreactivity (IR) was observed in the nuclei of neurons in the medial septum/diagonal band complex (MSDB) 2 and 6 d postaxotomy, which was followed by a decline after 12 d and 3 weeks, respectively. Nine weeks after FFT, *c-jun* IR had disappeared. The *c-jun*-positive MS neurons were identified as former septohippocampal projection cells by double-labeling with the retrogradely transported tracer Fluoro-Gold injected into the hippocampus

before axotomy. In line with the immunocytochemical data, there was a massive induction of *c-jun* mRNA in the axotomized MS neurons as visualized by *in situ* hybridization histochemistry. *c-fos* mRNA and *c-fos* or *jun B* IR were not detectable in either unoperated or lesioned medial septal neurons. Experiments using the TdT-mediated deoxyuridine triphosphate nick-end-labeling technique, designed to detect nuclear DNA fragmentation in degenerating neurons, complemented this study. During the postoperative time range studied, MS neurons did not exhibit DNA fragmentation. We conclude that MSDB neurons survive axotomy by FFT and display characteristic changes in gene expression.

Key words: *septohippocampal projection; transcription factor; axotomy; retrograde degeneration; retrograde tracing; in situ hybridization*

The anatomically well defined fimbria–fornix system, which connects the septal complex with the hippocampal formation, has been used often as a paradigm to study lesion effects on CNS neurons, because this fiber tract can be transected completely without affecting vital functions of the animal. The septohippocampal projection neurons display either cholinergic (Lewis and Shute, 1967; Houser et al., 1983; Amaral and Kurz, 1985; Bialowas and Frotscher, 1987) or GABAergic phenotype (Köhler et al., 1984; Freund and Antal, 1988). Their cell bodies are located in the medial septum/diagonal band complex (MSDB) and undergo retrograde changes in response to axotomy by fimbria–fornix transection (FFT). Thus, there is a massive loss of immunocytochemically identified cholinergic and GABAergic medial septal neurons after FFT (Gage et al., 1986, 1988; Hefti, 1986; Williams et al., 1986; Armstrong et al., 1987; Kromer, 1987; Peterson et al., 1987; Frotscher, 1988; Naumann et al., 1994), which was interpreted as retrograde neuronal death of these cells (for review, see Gage et al., 1989). Recent studies, however, demonstrated that axotomized medial septal neurons may survive for long periods of time, as shown by prelabeling of these cells with the retrograde tracer Fluoro-Gold (FG) before axotomy and electron microscopy of the prelabeled and then axotomized neu-

rons after varying survival periods (Peterson et al., 1990, 1992; Naumann et al., 1992b). Moreover, the axotomized cholinergic neurons regain their IR for choline acetyltransferase (ChAT) after 6 months postlesion, again indicating survival rather than cell death (Naumann et al., 1994). In line with these studies after axotomy, Sofroniew et al. (1990, 1993) and Plaschke et al. (1994) demonstrated that removal of hippocampal target tissue by excitotoxic lesions did not result in a massive loss of ChAT-positive neurons in the MSDB, despite disconnection from neurotrophin-producing target cells.

The pattern of gene expression of CNS neurons after axotomy is poorly understood; however, immediate early genes such as *c-jun*, *c-fos*, and *jun B* seem to be involved in lesion-induced neuronal events. The products of these genes function as transcription factors (Sheng and Greenberg, 1990; Morgan and Curran, 1991), which are differentially expressed after lesions of the CNS or peripheral nervous system (Jenkins and Hunt, 1991; Leah et al., 1991; Dragunow, 1992; Herdegen et al., 1992, 1993; Haas et al., 1993; Jenkins et al., 1993b; Koistinaho et al., 1993; Hüll and Bähr, 1994a,b; Brecht et al., 1995). Recent evidence suggests that *c-jun* expression in injured neurons is not merely a response to damage but rather is associated with the neuronal growth response (Jenkins et al., 1993a).

On the basis of the concept that medial septal neurons potentially survive axotomy and may regenerate axonal processes, we have investigated the expression of immediate early genes (*c-jun*, *c-fos*, and *jun B*) in the medial septal nucleus after FFT. In addition, we have studied these central axotomized neurons for DNA fragmentation, which is indicative of degenerating neurons

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in other lesion models (Berkelaar et al., 1994; Rabacchi et al., 1994; Nitatori et al., 1995).

MATERIALS AND METHODS

Animal surgery. Bilateral FFTs were performed as described by Naumann et al. (1992b). Briefly, adult male Sprague–Dawley rats (weighing 250–300 gm) were deeply anesthetized with a mixture of ketamin, rompun, and ketavet (2.5 ml/kg body weight, i.m.) and placed in a stereotaxic apparatus. The skull was opened bilaterally 1 mm posterior to bregma, extending 5 mm on either side of the midline. Under visual control, the overlying cortical tissue was removed by aspiration to access the fimbria–fornix, which was aspirated bilaterally. All surgery was performed in accordance with institutional guidelines for animal welfare. The animals were allowed to survive for 1 d ($n = 4$), 2 d ($n = 4$), 6 d ($n = 4$), 8 d ($n = 4$), 12 d ($n = 2$), 21 d ($n = 5$), and 9 weeks ($n = 3$). Unoperated animals ($n = 4$) served as controls.

Retrograde prelabeling of medial septal neurons. The retrograde tracer Fluoro-Gold (FG; Fluorochrome, Englewood, CO; 2.5% in H₂O) was stereotactically injected into the right hippocampus of five adult male anesthetized (see above) rats, as described previously (five injections of 30 nl FG each into different septotemporal portions of the hippocampus; coordinates from bregma: AP 3.4, ML 2.2, DV –3.38; AP 3.8, ML 3.0, DV –3.6; AP 5.5, ML 3.8, DV –4.1; AP 6.1, ML 4.1, DV –4.74; AP 5.8, ML 4.7, DV –6.6) (Naumann et al., 1992a). The animals were returned to their home cages for 5 d. Four of the FG-injected animals then received a unilateral FFT and were allowed to survive for another 6 d.

Immunohistochemistry. Animals were anesthetized as described above and transcardially perfused for 3 min with 0.1 M PBS, pH 7.2, followed by 4% paraformaldehyde buffered with PBS for 20 min. The brains were removed from the skull and immersed in the same fixative for 2 d. Tissue sections (50 μ m, coronal plane of the septal region and horizontal plane of the hippocampus) were cut on a vibratome and collected in PBS. For immunohistochemistry, a floating procedure was applied. Tissue sections were rinsed three times for 5 min in PBS and then pretreated for 30 min with a solution containing 10% normal goat serum (NGS), 0.25% Triton X-100, and PBS. Sections were rinsed again with PBS two times for 5 min followed by incubation with the primary antibody diluted in 1% NGS, 0.1% Triton X-100, and 0.01% sodium azide (normal horse serum was used for mouse monoclonal antibodies). The following antibodies were used in this study: rabbit polyclonal anti-c-jun/AP1, PC06 (Oncogene Science, Uniondale, NY), 100 μ g/ml, dilution 1:100; rabbit polyclonal anti-c-jun (636/3; gift from R. Bravo, Bristol-Meyers Squibb Pharmaceutical Research Institute, Princeton, NJ), dilution 1:25,000; rabbit polyclonal anti-c-fos, PC05 (Oncogene Science), 100 μ g/ml, dilution 1:100; rabbit polyclonal anti-jun B (gift from R. Bravo), dilution 1:4000; rabbit polyclonal anti-jun B (Biotechnology, Santa Cruz, CA), dilution 1:1000; and rat–mouse hybridoma anti-ChAT, type I (Boehringer Mannheim, Mannheim, Germany), dilution 1:9.

Incubation with the primary antibodies was performed at room temperature (RT) for 5 hr and then for 12 hr at 4°C on a rotating shaker. Thereafter, the sections were washed carefully in PBS and exposed to the secondary biotinylated goat anti-rabbit and horse anti-mouse antibodies (Vector Laboratories, Burlingame, CA), respectively, for 2 hr at RT (diluted in PBS, 1:250). After washes in PBS, the tissue-bound antibodies were visualized using the Vectastain ABC Kit (Vector Laboratories) and reacted with 3,3'-diaminobenzidine (DAB) [(Sigma, Deisenhofen, Germany) 0.05% in PBS with 0.002% H₂O₂]. The development of the reaction product was monitored visually. As the last step, tissue sections were mounted on gelatin-coated slides, air-dried, dehydrated in graded ethanol, and coverslipped with permount. As a negative control, incubation with the primary antibody was omitted. No immunostaining occurred under these conditions.

Double-labeling c-jun/FG. The FG-injected and fimbria–fornix-transected animals were transcardially perfused, vibratome sections were prepared, and c-jun immunostaining was performed as described in detail above. Visualization was achieved by a DAB reaction with nickel intensification, which resulted in a dark brown nuclear reaction product. Thereafter, the sections were washed again in PBS and preincubated in 10% NGS for 30 min followed by incubation with a polyclonal antibody solution to FG (Biogenesis, Bournemouth, UK), diluted 1:4000, for 2 d at 4°C. Next, after exposure to a biotinylated anti-rabbit IgG (1:250; 2 hr) and the ABC reagents (ABC-Elite, Vector Laboratories), the tissue-bound peroxidase was visualized with DAB for 5 min, which resulted in a brown cytoplasmic precipitate.

Acetylcholinesterase histochemistry. To assess the completeness of the

FFT, hippocampal sections of all experimental animals were processed for acetylcholinesterase (AChE) histochemistry according to Mesulam et al. (1987). Only those brains in which the typical AChE pattern in the fascia dentata and hippocampus proper was lost as a consequence of the FFT were included in the analysis.

TdT-mediated deoxyuridine triphosphate nick-end-labeling. The procedure of Gavrieli et al. (1992) was modified and adapted for vibratome sections. Animal perfusion and tissue fixation was performed as described for immunohistochemistry. Vibratome sections of 50 μ m were cut from septal and hippocampal tissue as described above and mounted on gelatin-coated slides for the TdT-mediated deoxyuridine triphosphate nick-end-labeling (TUNEL) procedure. Sections were rinsed with 0.1 M PBS, pH 7.2, and treated for 15 min with 0.3% Triton X-100 followed by a 5 min wash in PBS. Nuclei stripping was performed by incubation with 20 μ g/ml proteinase K (Boehringer Mannheim) in 10 mM Tris/HCl, pH 8.0, for 30 min at RT, and then the sections were rinsed thoroughly four times in distilled H₂O for 2 min. Endogenous peroxidase was inactivated by covering the sections with 3% H₂O₂ for 10 min at RT. Again, sections were rinsed in water, two times for 5 min, and equilibrated for 10 min in terminal transferase buffer composed of 5 mM cobalt chloride, 25 mM Tris/HCl, pH 6.6, 0.2 M potassium cacodylate, and 0.25 mg/ml BSA before the actual incubation with 0.3 U/ μ l terminal deoxynucleotidyl transferase (Boehringer Mannheim) and biotinylated deoxyuridine triphosphate (dUTP), 40 pmol/ μ l. The tailing reaction was performed in a small volume (10 μ l/section) under parafilm at 37°C for 60 min in a humid chamber. The reaction was stopped by transferring the tissue into a solution containing 300 mM sodium chloride and 30 mM sodium citrate for 15 min at RT. After two rinses in PBS, 5 min each, the sections were treated with 2% BSA in PBS followed by 0.3% Triton X-100 in PBS and subsequently processed with standard avidin–biotin–peroxidase (Vectastain ABC Kit, Vector Laboratories) to visualize the incorporated biotinylated dUTP.

In positive controls, a DNase I treatment was performed before incubation with terminal transferase. Tissue sections were digested with 1 μ g/ml DNase I (Sigma) for 15 min in a buffer containing 50 mM Tris/HCl, pH 7.5, 10 mM MgSO₄, 0.1 mM dithiothreitol, and 50 μ g/ml BSA. After careful washes in distilled H₂O (4 times for 2 min), the tissue was processed further for TUNEL.

As an endogenous control for the detection of apoptotic cells, whole-mounts of newborn rat retina were carried through the TUNEL procedure.

In situ hybridization histochemistry. *In situ* hybridization histochemistry with synthetic oligonucleotide probes was performed as described by Haas et al. (1993). In short, the oligonucleotide sequence was as follows: c-jun (45-mer) (antisense 5'-GCA ACT GCT GCG TTA GCA TGA GTT GGC ACC CAC TGT TAA CGT GGT-3') homologous to the last 15 amino acids of the predicted c-jun protein (Angel et al., 1988). The sense oligonucleotide to this sequence was used as negative control.

The oligonucleotides were labeled by 3'-end tailing using a 30:1 molar ratio of [α -³²P]dATP to oligonucleotide; 0.3 pmol of oligonucleotide was tailed in the presence of 30 μ Ci of [α -³²P]dATP (3000 Ci/mmol; DuPont NEN, Bad Homburg, Germany) with terminal deoxynucleotidyl transferase (Boehringer Mannheim) in a buffer containing 25 mM Tris/HCl, pH 6.5, 0.2 M potassium cacodylate, 0.25 mg/ml BSA, and 1.5 mM CoCl₂ for 15 min at 37°C and purified by ethanol precipitation. The tail length was 10–15 nucleotides as determined by PAGE.

For *in situ* hybridization histochemistry, the tissue was fixed by transcardial perfusion with 4% paraformaldehyde (4 animals with bilateral FFT and two unoperated controls). Cryostat sections of 20 μ m (coronal plane of the septal region and horizontal plane of the hippocampus) were prepared and mounted on silanized glass slides. Permeabilization of the tissue included HCl and proteinase K treatment. Brain sections were then prehybridized with hybridization buffer at 42°C for 2–3 hr. The hybridization solution contained 20% formamide, 4 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 50 mM Tris/HCl, pH 7.0, 5 mM EDTA, 1% SDS, 5 $\times$ Denhardt's solution, 0.1 mg/ml sonicated denatured salmon sperm DNA, 0.1 mg/ml tRNA, and 10% dextran sulfate. This solution was drained and replaced with the hybridization mixture, which was the same but included 10,000 cpm/ μ l radiolabeled oligonucleotide probe. Sections were covered with parafilm and incubated in a moist chamber at 42°C overnight. The sections were washed three times for 15 min in a buffer containing 1 $\times$ SSC, 0.1% SDS at 42°C. Thereafter, a more stringent wash with 0.2 $\times$ SSC, 0.1% SDS was performed twice for 15 min at RT. Slices were dehydrated through graded ethanols containing 0.3 M ammonium acetate, coated with Kodak NTB-2 photo emulsion, and

exposed at 4°C for 3 weeks. They were developed in Kodak D19 developer and fixed in 24% sodium thiosulfate. As a last step, the sections were counterstained with cresyl violet, dehydrated, and mounted in DePeX (Gurr, BDH Chemicals, Poole, UK).

Northern blot analysis. Immunostaining for c-fos was supplemented by a Northern blot analysis for c-fos mRNA, which was carried out as described by Haas et al. (1993). In brief, unoperated ($n = 2$) and fimbria-fornix-transected (2 and 7 d, $n = 2$ each) rats were killed by decapitation, the brains were frozen on dry ice, and the medial septal nuclei were microdissected with a sterile razor blade. Cytoplasmic RNA of the septal area was prepared from each animal individually and size-fractionated by agarose gel electrophoresis as described. As a positive control for c-fos mRNA expression, RNA extracted from PC12 cells [treated with nerve growth factor (NGF) for 30 min] was used. Northern transfer was performed by vacuum blotting on Hybond N nylon membrane with 20× SSC. A c-fos cDNA probe was radiolabeled by random priming with [α - 32 P]dCTP (3000 Ci/mmol; DuPont NEN). Hybridization and washing conditions were the same as described previously (Haas et al., 1993).

RESULTS

Control of FFT by AChE histochemistry and ChAT immunostaining

To assess the completeness of the FFT, sections of hippocampus were processed for AChE histochemistry. The loss of AChE-positive fiber staining in the hippocampus has been used widely as a control for the complete transection of septohippocampal fibers (Naumann et al., 1994). AChE staining was performed at all studied time points from 6 d onward except at early stages (1 and 2 d postlesion), because the degeneration of AChE-positive fibers in the hippocampus requires ~1 week. In the deafferented hippocampi, there was a dramatic loss of AChE-positive fibers when compared with unoperated controls, which show a dense fiber network stained by AChE histochemistry. ChAT-IR was examined in unoperated control animals and at time points from 1 d up to 9 weeks in axotomized neurons of the MS. In line with previous reports (references cited in the introductory remarks), there was a dramatic loss of ChAT-IR neurons as early as 6 d postlesion (Fig. 1*a,b*).

Analysis of DNA fragmentation

The TUNEL technique designed to detect DNA fragmentation was applied to sections of the medial septal nucleus at the following postoperative time intervals: 1, 2, 6, 7, 8, 12, and 21 d. No nuclear labeling was observed in the septal region, indicating the absence of DNA fragmentation at the time points studied (Fig. 1*c*). In DNase I-treated control sections of the MS, however, a clear nuclear staining was observed (Fig. 1*d*).

To monitor the sensitivity of the TUNEL procedure applied in this study, another control experiment was performed. Whole-mounts of newborn rat retinae were examined for apoptotic retinal ganglion cells (RGCs), which undergo naturally occurring cell death at this stage of development (Cunningham, 1982). Numerous nuclei of RGCs were labeled in this experiment, indicating the high sensitivity of our procedure (data not shown).

Expression of immediate early genes in response to FFT

In unoperated control animals, no IR was observed for c-jun (Fig. 2*a*), jun B, and c-fos. After FFT we noticed a selective and long-lasting increase in immunostaining for c-jun in MSDB neurons (Fig. 2). No immunolabeling for c-fos and jun B was found in lesioned rats. In line with the immunocytochemical findings, no c-fos mRNA was detected in the MSDB by Northern blot analysis of unoperated or axotomized animals (Fig. 3).

Many c-jun-IR neuronal nuclei were observed in the MS as

early as 2 d postaxotomy. In our time course analysis, the number of c-jun-positive neurons was highest at 6 d postlesion (Fig. 2*b*), followed by a decline around postlesional day 12 (Fig. 2*c*). After 3 weeks, the population of c-jun-positive MS neurons dropped remarkably. No c-jun-positive neurons were found after a survival period of 9 weeks (Fig. 2*d*).

To confirm that an increase in c-jun-IR in axotomized medial septal neurons was accompanied by enhanced mRNA synthesis, *in situ* hybridization histochemistry was used. 33 P-labeled oligonucleotide probes specific for c-jun mRNA applied to tissue sections of the MS revealed a strong increase of c-jun mRNA in this brain region (Fig. 4*a,b*). Dense clusters of silver grains were concentrated over the MS and diagonal band of Broca. An accumulation of silver grains over Nissl-counterstained MS neurons indicated enhanced c-jun gene expression compared with that in unoperated controls (Fig. 4*c*). As in our immunocytochemical preparations with c-jun antibodies, the c-jun mRNA increase was restricted to the MSDB. The lateral septum that does not project to the hippocampus was spared. No hybridization was observed with sense controls.

Although it is well established that most septohippocampal neurons project to the hippocampus via the fimbria-fornix and therefore are axotomized by FFT (Jacab and Leranth, 1995), an attempt was made in the present study to identify c-jun-positive septohippocampal projection neurons by retrograde labeling with FG before axotomy. This tracer was shown in previous studies to be a reliable marker for septohippocampal projection neurons that stays in the cell bodies for extended periods of time after tracer injection into the hippocampus (Naumann et al., 1992*a*).

In these double-labeling studies, unilateral fimbrial lesions were performed. We observed a heavy c-jun immunolabeling of medial septal neurons on the lesioned side, which suggests that c-jun immunostaining is causally related to the lesion. The few c-jun-positive cells on the contralateral side most likely belong to the crossed septohippocampal projection (Peterson, 1989), which was also transected in these experiments. Many double-labeled neurons were observed in the MS, suggesting that immunostaining for c-jun was induced by axotomy (Fig. 5).

In the double-stained sections (Fig. 5*a–c*), the heavily c-jun-labeled nuclei of septohippocampal neurons stood out against the less intensely FG-labeled cytoplasm of those cells. In contrast, solely FG-stained neurons displayed a pale nucleus surrounded by a halo of FG immunolabeling (Fig. 5*c*). The c-jun-positive neuronal population was always FG-labeled, but not all FG-stained cells were c-jun-positive (Fig. 5*b*). In control animals that were only retrogradely labeled with FG, intense nuclear staining was absent (Fig. 5*d*).

DISCUSSION

In the present study, we have shown that injured septohippocampal neurons selectively express c-jun mRNA and protein in a long-term manner, but not c-fos or jun B. In addition, we have demonstrated that these axotomized central neurons do not exhibit DNA fragmentation.

Previous studies have suggested that medial septal neurons degenerate and die after axotomy by transection of the fimbria-fornix. Thus, Daitz and Powell (1954) and McLardy (1955*a,b*) observed a loss of large neurons in the MS in Nissl stain and concluded that these cells degenerate as a result of axotomy. In line with these early observations, many investigators found a dramatic loss of ChAT-IR neurons in the MS after FFT (Gage et al., 1986, 1988; Hefti, 1986; Williams et al., 1986; Armstrong et al.,

Figure 1. ChAT immunostaining and *in situ* labeling of DNA fragmentation (TUNEL) in sections of the MSDB. *a, b*, ChAT immunostaining in the MSDB. *a*, Unoperated control animal. Many ChAT-IR neurons are present in the MS and diagonal band of Broca. *b*, Six days after FFT. Loss of ChAT immunostaining in the MSDB. Scale bar, 200 μ m. *c, d*, TUNEL analysis in the MSDB. *c*, Three weeks after FFT. No DNA fragmentation is detected in the MSDB after this as well as after other survival periods. *d*, DNase I treatment resulted in the labeling of many MSDB nuclei. MS, Medial septum; DB, diagonal band of Broca. Scale bar, 100 μ m.

Figure 2. c-jun immunostaining in the MS after various survival times following FFT. *a*, In an unoperated control animal, only background staining is seen. *b*, Six days after lesion, many c-jun-positive nuclei are seen in the MSDB. *c*, Twelve days after FFT, the number of c-jun-positive nuclei has decreased when compared with 6 d postlesion. *d*, Nine weeks after FFT, c-jun-positive nuclei have disappeared. MS, Medial septum; DB, diagonal band of Broca. Scale bars, 200 μ m.

Figure 3. Northern blot analysis of *c-fos* mRNA expression in the septal area after FFT. The autoradiogram of one representative experiment is shown. Only the positive control (NGF-treated PC12 cells) gives a hybridization signal. *c-fos* mRNA is not found in septal RNA from either unoperated or operated animals. *Lane 1*, PC12 cells treated with NGF for 30 min. *Lane 2*, Septal nucleus of unoperated animal. *Lane 3*, Septal nucleus 2 d after FFT. *Lane 4*, Septal nucleus 7 d after FFT. RNA sizes are given in kilobases.

1987; Kromer, 1987; Frotscher, 1988; Naumann et al., 1992b); however, prelabeling of septohippocampal neurons with retrograde tracer before axotomy and intracellular staining of the prelabeled and then axotomized neurons revealed survival of many septohippocampal projection cells after fimbria–fornix lesion (Frotscher and Naumann, 1992; Naumann et al., 1992b; Peterson et al., 1992).

Intracellular staining of prelabeled and axotomized cells proved the survival of individual neurons, but may not be the adequate method for monitoring the extent of degeneration or cell death occurring in the entire population of axotomized neurons. Recent studies have provided evidence that the TUNEL technique labels not only apoptotic cells but also degenerating neurons (Berkelaar et al., 1994; Rabacchi et al., 1994; Nitatori et al., 1995). In line with our previous reports on axotomized and then intracellularly stained neurons, our present results with the TUNEL technique do not provide evidence for a massive degeneration of septohippocampal projection neurons after axotomy. Nuclear DNA fragmentation could not be detected in any of the postlesional stages analyzed. We have reason to assume that the applied procedure is highly sensitive, because we were able to monitor apoptotic cell death in the developing retina as well as DNA fragmentation by DNase treatment. Nevertheless, we cannot ignore the fact that some degeneration has occurred in the present experiments, which may have escaped the analysis of the present postlesional stages. In fact, previous studies have indicated that DNA fragmentation is a rapid process (Johnson and Deckwerth, 1993). It should be pointed out, however, that our electron microscopic studies analyzing the time course of retrograde neuronal changes after axotomy did not reveal clumping of nuclei in axotomized septohippocampal neurons (Frotscher and Naumann, 1992; Naumann et al., 1992b). In summary, we conclude that there is no massive retrograde cell death in the MS after FFT.

In agreement with the lack of degeneration in the TUNEL experiments, we found an increased IR for the immediate early gene *c-jun* in the axotomized cells. This induction of *c-jun*-IR was selective, because no similar immunostaining was observed with antibodies against *c-fos* and jun B protein. Moreover, no *c-fos* mRNA was detected by Northern blot analysis in the septal complex after FFT. This lack of *c-fos* expression is in agreement with other studies of axotomized neurons (Jones and Evinger, 1991; Dragunow, 1992; Herdegen et al., 1992; Haas et al., 1993). The increased immunostaining for *c-jun* was

accompanied by an enhanced *c-jun* mRNA expression. We have reason to assume that the expression of *c-jun* in septal neurons is causally related to the axotomy of these cells, because IR was found only in identified (FG-prelabeled) septohippocampal projection neurons and was restricted to the MSDB. Also, in the double-labeling experiments, which were performed only unilaterally, the *c-jun* immunolabeling was found almost exclusively on the lesioned side. The completeness of the FFT was proven by AChE staining of the hippocampus. Only rats devoid of AChE-positive fibers in the hippocampus and fascia dentata were included in the present study.

The selective immunostaining for *c-jun* in axotomized medial septal neurons raises the question of the functional significance of this immediate early gene in the present experimental paradigm. Recent *in vitro* studies have shown that *c-jun* is necessary for neuronal apoptosis in NGF-dependent sympathetic neurons during development (Estus et al., 1994; Ham et al., 1995). However, *c-jun* is also expressed in regenerating and sprouting neurons of adult animals after peripheral nerve transection (Jenkins and Hunt, 1991; Leah et al., 1991; Herdegen et al., 1992; Haas et al., 1993; Jenkins et al., 1993a). It is tempting to speculate that the increased IR for *c-jun* observed in the present study is related to neuronal survival rather than cell death, because no obvious signs of degeneration were found (see above). In fact, the septohippocampal neurons not only may survive axotomy but may have the capacity to regenerate and reinnervate their appropriate target tissue (Cadelli and Schwab, 1991; Linke et al., 1995).

What could be the function of *c-jun* in the axotomized neurons? *c-jun* is one member of a family of closely related proteins (including jun B and *c-fos*) that possess a leucine zipper through which they form hetero- or homodimers that will bind to the AP-1 DNA consensus element in target genes (Chiu et al., 1988; Nakebeppu et al., 1988; Cohen et al., 1989). In fact, *c-jun* can also homodimerize with itself to activate transcription (Karin and Smol, 1992), and this is possibly the case in axotomized MSDB neurons, which do express *c-jun* but not *c-fos* and jun B. A possible target for *c-jun* action could be the ChAT gene that contains an AP-1 site in its promoter (Hahn et al., 1992) and is inducible by AP-1 proteins *in vitro* (Bausero et al., 1993). We hypothesize a downregulation of ChAT expression by *c-jun* in axotomized medial septal neurons. As mentioned, there is a massive loss of ChAT-IR cells after FFT paralleled by an increase in *c-jun*. Moreover, IR for *c-jun* ceases after 3 weeks postlesion, whereas ChAT-IR recovers in axotomized septohippocampal neurons with increasing survival time (Naumann et al., 1994).

The molecular mechanisms that lead to *c-jun* induction are still unknown. Block of axonal transport by colchicine mimics the effect of axotomy and causes *c-jun* induction (Leah et al., 1991, 1993). This indicates that disconnection from target-derived factors activates *c-jun* expression. In the septohippocampal system, it is known that the target cells in the hippocampus produce NGF and brain-derived neurotrophic factor (BDNF) and their mRNAs (Korsching et al., 1985; Ayer-LeLievre et al., 1988; Ernfors et al., 1990; Hofer et al., 1990; Phillips et al., 1990; Wetmore et al., 1991). NGF and BDNF are retrogradely transported from the hippocampus to the parent cell bodies in the MS (Schwab et al., 1979; Seiler and Schwab, 1984; DiStefano et al., 1992). One may speculate that FFT disconnects MSDB neurons from their growth factor supply, and in turn this could cause *c-jun* expression. Evidence against a growth factor-dependent regulation of *c-jun* in

Figure 4. Localization of *c-jun* mRNA in the MSDB by *in situ* hybridization histochemistry with ^{33}P -radiolabeled oligonucleotides. *a*, Dark-field photograph of unoperated animal. Only background levels of silver grains are detected over the tissue section. *b*, Dark-field photograph 6 d after FFT. Note the strong accumulation of silver grains over the MSDB, indicating increased *c-jun* mRNA levels in these areas. MS, Medial septum; DB, diagonal band of Broca; LS, lateral septum; LV, lateral ventricle. Scale bars, 1,000 μm . *c*, Bright-field photograph of medial septal neurons shown in *b* at higher magnification. Neurons that display a strong hybridization signal for *c-jun* mRNA are indicated by arrows. Tissue was counterstained with cresyl violet. Scale bar, 60 μm .

Figure 5. Colocalization of the retrograde tracer FG and c-jun IR in axotomized neurons of the MSDB. *a*, Medial septal nucleus (MS) after unilateral retrograde tracing with FG followed by ipsilateral FFT (survival time, 6 d). *Framed area* is shown at higher magnification in *b*. Scale bar, 200 μ m. *b*, Framed area in *a* at higher magnification. *Arrows* indicate double-labeled neurons with cytoplasmic FG IR and nuclear, Ni/Co-enhanced c-jun labeling. *Open arrow* points to a neuron that is only FG-labeled. Scale bar, 50 μ m. *c*, High magnification of c-jun/FG double-labeled neuron (*arrow*) and an FG-positive cell (*open arrow*). Scale bar, 40 μ m. *d*, Single immunostaining for FG. Note absence of intense nuclear staining. Scale bar, 50 μ m.

neurons comes from *in vitro* studies on dorsal root ganglion cells, however, where the addition of NGF or BDNF did not inhibit the expression of *c-jun* (DeFelipe and Hunt, 1994). In these sensory neurons, *c-jun* expression is obviously not related to neurotrophin deprivation; a suppressing effect of other growth factors, however, cannot be excluded. The results of the present study do not allow one to draw conclusions as to the mode of *c-jun* induction. They do point to a possible involvement of *c-jun* in neuronal survival and regeneration, however, whereas the two other transcription factors studied here, *jun B* and *c-fos*, do not seem to play a role.

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