The Differential Control of c-Jun Expression in Regenerating Sensory Neurons and Their Associated Glial Cells

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Damage to the axons of adult sensory neurons results in massively increased expression of the protooncogene c-jun both in neurons and in the associated Schwann cells. The role of growth factors and axon contact in mediating this expression was investigated in dissociated cultures of adult sensory neurons and glial cells that expressed c-jun within 24 hr of plating. Trk, trkB, and trkC growth factor receptor genes were expressed in discrete subpopulations of sensory neurons but addition of NGF or brain-derived neurotrophic factor (BDNF) did not inhibit the expression of c-jun. Similarly, axon contact was not sufficient to decrease c-jun expression in glial cells. However, c-jun expression could be downregulated in glial cells, but not neurons, by treatment with cAMP or forskolin and increased by raising intracellular calcium levels. The results suggest that c-jun levels are differentially regulated in neurons and glial cells but that NGF or BDNF do not regulate c-jun expression in damaged neurons.

[Key words: c-jun, regeneration, sensory neurons, glial cells, neurotrophins, second messengers]

A number of recent studies have implicated the c-jun protooncogene in the control of growth and differentiation in a variety of cell types including neurons and glial cells (Bos et al., 1990; de Groot et al., 1990; Yamaguchi-Iwai et al., 1990; Castellazzi et al., 1991; Jenkins and Hunt, 1991; Leah et al., 1991; De Felipe et al., 1992; Wong et al., 1992; Jenkins et al., 1993a–c). Following axon damage or block of axonal transport in peripheral sensory or motor neurons in the rat, there is a delayed expression of c-jun protein and mRNA that is maintained until the nerve has fully regenerated (Jenkins and Hunt, 1991; Leah et al., 1991; Herdegen et al., 1993). Similar modifications in c-jun expression occur following axotomy of rubrospinal (Jenkins et al., 1993a) or nigrostriatal (Jenkins et al., 1993b) neurons but these changes largely reversed within 14 d in parallel with the failure of these central neurons to regenerate. These observations suggested that the expression of c-jun was in some way important, if not essential, for the regeneration of adult neurons and implied that the mechanisms by which c-jun expression could be regulated in neurons and glial cells could be crucial for

our understanding of the regenerative process in adult mammals.

c-jun is the cellular homolog of v-jun the transforming oncogene of the avian sarcoma virus 17 (Maki et al., 1987; Sakai et al., 1989) and encodes for a 39 kDa nuclear phosphoprotein that is the major component of the AP-1 complex of transcriptional regulators (Bohmann et al., 1987; Halzonetis et al., 1988; Abate et al., 1990; Kerppola and Curren, 1991). c-jun is one of a family of closely related proteins (including Jun B and Jun D) that possess a leucine zipper through which they form homo- or heterodimers that will bind to DNA (Chiu et al., 1988) and act as transcriptional regulators (see Lamb and McKnight, 1991). However, the control of c-jun gene expression and protein activity is complex and may involve transcriptional, combinatorial, temporal, and posttranslational mechanisms that vary between different cell types (for reviews, see Karin and Smeal, 1992; Jackson, 1992).

c-jun has been shown to heterodimerize with c-fos, other fos-related proteins, and members of the CREB/ATF (cAMP response element-binding protein/activating transcription factor) family of binding proteins or to form Jun–Jun homodimers (Karin and Smeal, 1992). These different combinations of proteins can possess different DNA binding specificities, affinities, and transcriptional activities. Jun–Fos heterodimers possess both an increased DNA binding activity and trans-activating activity when compared with Jun–Jun homodimers although c-jun itself retains the highest transactivating potential (Smeal et al., 1989, 1991; Angel and Karin, 1991).

Following stimulation of neurons, neuronal cell lines, or glial cells with phorbol esters, polypeptide hormones, growth factors, cytokines or neurotransmitters, the expression of c-jun mRNA may follow “immediate early” gene kinetics (Bartel et al., 1989; Shen and Greenberg, 1990; Sheng et al., 1990; Wisden et al., 1990; McNaughton and Hunt, 1992). This is characterized by a rapid but transient expression of c-jun mRNA following stimulation and is not dependent upon new protein synthesis. However, in certain situations the induction of c-jun, but not c-fos, expression can be both delayed and prolonged (Brenner et al., 1989), for example, during the differentiation of F9 carcinoma cells with retinoic acid or in the neuronal response to axonal damage (Yang-Yen et al., 1990b; Jenkins and Hunt, 1991; Leah et al., 1991; Sleight, 1992).

The observation that in peripheral nerve, axoplasmic block was as effective as axon section or ligation in causing an increased c-jun expression in neurons, led us to suggest that the c-jun response may have resulted in part from deprivation of axonally transported target factors (Jenkins et al., 1993a–c). The most obvious candidates are the neurotrophins, NGF and brain-

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derived neurotrophic factor (BDNF), which are known to be retrogradely transported in sensory neurons (Hendry et al., 1974; DiStefano et al., 1992). Indeed, exogenously applied NGF has been shown to reverse the changes in peptide and growth factor receptor expression seen following peripheral nerve section in rat (Fitzgerald et al., 1985; Verge et al., 1989a,b, 1992). To look at these possibilities more closely we have established primary cultures of adult dorsal root ganglion (DRG) cells and their supporting satellite and Schwann cells and investigated the signals and intracellular events that lead to and modify c-jun expression. Our results demonstrate that c-jun expression in neurons and glial cells is differentially regulated and not obviously related to neurotrophin starvation.

Some of these results have been briefly reported elsewhere (De Felipe et al., 1993).

Materials and Methods

Cell cultures. Cultures of adult dorsal root ganglion cells (DRGs) were prepared as described previously (Lindsay, 1988) with minor modifications. Briefly, cleaned ganglia from adult Sprague-Dawley rats were collected in Dulbecco's minimal Eagle's medium (DMEM) plus 10% horse serum and treated twice for 1.5 hr with 0.25% collagenase. A single-cell suspension was then obtained by trituration (10–12 passages) through a fire-polished Pasteur pipette. Cells were collected, rinsed, and plated (2000 cells/ml) on to polyornithine (100 mg/ml) and laminin (5 mg/ml) coated coverslips. After 15–20 hr cells cultures were maintained in the same serum-containing medium or more usually transferred to DMEM defined medium (Bottenstein and Sato, 1979). Cells were maintained in culture at 37°C, 7.5% CO₂ for up to 30 d. Experiments were routinely done on 7–10-d-old cultures. Treatments were always carried out without replacing the culture medium to avoid the possibility that changing the medium may induce changes in protooncogene expression. Aliquots of 100 times concentrated stock substances were added directly to the culture medium (1 ml).

Peripheral nerve lesions. Adult male Sprague-Dawley rats (200–300 gm) were deeply anesthetized by intraperitoneal injection of 4 ml/kg Equithesin. Sciatic nerve ligation and section was performed through a 1 cm incision over the sciatic notch. In sham controls the nerve was exposed but not damaged. The animals survived for 1 d ($n = 3$), 4 d ($n = 2$), and 7 d ($n = 2$). Animals were deeply anesthetized with chloral hydrate and perfused transcardially with 4% paraformaldehyde in sodium phosphate buffer (PB; 0.1 M, pH 7.4); nerves and ganglia were removed, postfixed for 2 hr, and cryoprotected overnight in PB plus 30% sucrose. Nerves were sectioned at 40 μ m and incubated in antibodies, free floating, for 18–48 hr.

Immunohistochemistry. Cells were fixed in methanol (–20°C) for 5 min and freshly depolymerized paraformaldehyde (4% in 0.05% sodium phosphate buffer, pH 7.4, for 5 min) and incubated overnight in antibodies. Antiserum to c-jun was generated by immunizing rabbits with synthetic peptide conjugated to the carriers keyhole limpet hemoglobin or thyroglobulin. Subsequent characterization of this serum was by enzyme-linked immunoabsorbance assay (ELISA), Western blotting, immunoprecipitation, and immunocytochemistry. This indicated reactivity with a protein of the predicted molecular weight, and subcellular localization in response to various stimuli. A DRG cell culture protein extract analyzed by Western blotting revealed two bands in the region of 40–44 kDa that reacted with the c-jun antibody as predicted from previous studies (data not shown). The c-jun antiserum was raised against the sequence CQLMLTQQLQTF from the C-terminus of the c-jun peptide, which did not recognize jun-B or jun-D (H. Hurst, and G. I. Evan, unpublished observations) (diluted 1:2000). Sections were then processed with either an avidin-biotin complex horseradish peroxidase kit (Williams et al., 1990; Wisden et al., 1990) or with an avidin-fluorescein isothiocyanate (FITC) tertiary step (all from Vector). Sections were rinsed after all incubations in 0.1 M PB for 30 min and finally either reacted with 0.1% diaminobenzidine (DAB; Sigma) and 0.012% hydrogen peroxide to reveal a brown reaction product or mounted and viewed with conventional epifluorescence or with a Bio-Rad MRC-600 confocal microscope. Occasionally, a double labeling procedure was used. The first antibody, usually c-jun was used first and binding revealed with the HRP-linked method described above. The second antibody was then applied overnight (usually 3A10 and/or RT97) and the

procedure repeated except that 5-chloro-1-naphthol, which gives a blue reaction product, was used as the second chromogen. Alternatively, a double fluorescence procedure was used, the first antibody detected with an avidin-FITC probe and the second with an avidin-Texas red probe (both from Vector).

The c-jun antiserum has been used extensively in other studies (Wisden et al., 1990; Jenkins and Hunt, 1991; Jenkins et al., 1993a–c). Controls, which were all negative, included selective adsorption of antisera with 10 mg of native peptide and omission of the primary antibody. Because of the potential problems of cross-reactivity, immediate early gene immunoreactivity should be regarded as being “-like-activity.” Similarly, changes in the expression of c-jun may reflect increased synthesis of message or other mechanisms, such as increased stability of mRNA or protein. The methods used here are unable to distinguish between these possibilities.

The rabbit antisera to c-jun does not recognize either jun B or jun D. Other antibodies used included sensory neuron-specific monoclonal antibody 3A10 (diluted 1:2), which recognizes a neurofilament-related antigen (gift of J. Dodd and T. Jessell); RT97, which recognizes the 200 kDa phosphorylated neurofilament antigen (gift of B. Anderton); a mouse monoclonal antibody to GAP43 (1:25,000; Boehringer); rabbit antisera to c-fos (1:3000; Hunt et al., 1987) and NGFI-A (1:2000; Waters et al., 1990; Wisden et al., 1990); rabbit antisera to calcitonin gene-related peptide (CGRP; gift of Dr. K. Sternini; diluted 1:3000). Glial cells were identified with antibodies to glial acidic fibrillary protein (GFAP) and S100 (Dako).

In situ hybridization. For *in situ* hybridization, cells grown on 19 mm coverslips were fixed in 4% paraformaldehyde for 5 min, dehydrated in alcohol, and stored in 100% alcohol until used. A 60-mer oligonucleotide probe that recognized c-jun mRNA or 45-mer oligonucleotide probes antisense to c-fos, Jun B, Jun D, or NGFI-A, or to trk, trkB, or trkC mRNA were 3' end labeled using ³²S-dATP as previously described (Wisden et al., 1990). The antisense sequences for the immediate early genes were described in Wisden et al. (1990) and on Northern blots were specific for the relevant transcripts. The trk oligonucleotide probe sequences were taken from Merlio et al. (1992).

Coverslips were exposed to Kodak XAR-5 film at room temperature for 3–4 d before dipping in Ilford K5 emulsion and exposing at 4°C for 2–8 weeks. Incubations of the cells with labeled probe plus 20-fold excess of cold oligonucleotides, or hybridization with sense probes resulted in no detectable signal. Cultured cells were analyzed by either qualitative densitometry of autoradiographs or silver grain counting of individual cells. All the experiments were repeated three times, and the number of cells analyzed was always greater than 20 per coverslip.

Quantification of protein immunofluorescence. Immunofluorescent intensity was measured using a confocal imaging system (Bio-Rad MRC-600). A scale of arbitrary units of fluorescent intensity was set up between 0 and 250 and the fluorescent intensity of control cells was adjusted to the middle of the this scale using the available software. This ensured that the average pixel luminosity measurement was proportional to the emitted fluorescence. Images of labeled cells were captured and analyzed with the laser off to ensure minimal photobleaching of preparations. Both control and treated cells were immunohistochemically processed at the same time and background levels were subtracted for each coverslip.

Neurons were scored as positive or negative using either c-jun immunofluorescence or presence of DAB reaction product. Generally at least three coverslips per treatment from three different experiments were used for quantification. Immunofluorescence was exclusively used to assess the concentration of reaction product. Data were analyzed using Dunn's test for multiple comparisons.

Results

DRG cultures

In the absence of mitotic inhibitors, a mixture of neurons and glial (satellite/Schwann) cells, and occasional fibroblasts formed a confluent monolayer within 7 d. These were morphologically identified with Hoffman optics, with phase contrast microscopy, or with monoclonal antibodies 3A10, RT97, or GAP43 (Fig. 1). Approximately 5% of the neuronal population in defined medium showed positive CGRP immunoreactivity (Fig. 1C). 3A10 and RT97 appeared to stain the arborizations of the same subpopulation of cultured neurons while all neurons stained

Figure 1. Immunohistochemical characterization of DRG neurons in culture. In DRG cultures all neurons stained positively with antibodies to GAP43 (*A*, 2 d *in vitro*) while only subsets of neurons were labeled with neurofilament antibody RT97 (*B*, 7 d *in vitro*) or CGRP antibody (*C*, 7 d *in vitro*). Scale bar, 40 μ m.

Figure 2. Nerve section results in an increased expression of c-jun protein in Schwann cells distal to the lesion. c-jun immunoreactivity appears in Schwann cells surrounding the degenerating portion of sectioned sciatic nerve (*A*) (4 d postlesion) but not in the proximal portion of the nerve (*B*) or contralateral nerve (not shown). Immunoperoxidase technique. Scale bar, 150 μ m.

positively with GAP43 antibody (Fig. 1*A,B*). However, in many neurons, 3A10 stained only perikaryal cytoplasm (44%) (see Fig. 3). Glial cells stained positively with GFAP and S100 and a very small number (<1%) with GAP43. Unless otherwise stated, all experiments were carried out in N2 defined culture medium.

Expression of c-jun protein and mRNA

In tissue sections taken from normal DRG and sciatic nerve *in vivo*, levels of c-jun mRNA or protein are low (Jenkins and Hunt, 1991; Fig. 2). c-Jun protein was substantially increased within the Schwann cell nuclei of the distal segment, but not the proximal segment, of the cut sciatic nerve at all time points following nerve section (Fig. 2*A,B*). The reaction in the DRG cell bodies has been described previously. Within 24 hr following nerve section, crush, or block of axoplasmic transport there was a massive expression of c-jun mRNA and protein in the damaged neurons that was shown to be maintained for the period of nerve regeneration (Jenkins and Hunt, 1992; Leah et al., 1992).

High levels of c-jun protein and mRNA were also found in both neurons and Schwann cells from 24 hr until 30 d (the longest period assayed), when cultured in either defined medium (Fig. 3; see Fig. 8*A,C,E*) or in the presence of serum (data not shown), suggesting that the results of axotomy *in vivo* on both neurons and glial cells could be maintained *in vitro*. Other members of the Jun family, Jun D and Jun B mRNA were also present both in neurons and in glial cells although levels of Jun B were extremely low. No expression of c-fos or NGFI-A mRNA or their proteins was detected in cultures (data not shown). In thionin-counterstained preparations, neurons could be easily identified by their intense staining and eccentrically placed nuclei. All neurons had high levels of mRNA for c-jun. However, not all neurons had nuclei that were positive labeled with the c-jun antibody; 20% of neurons were c-jun negative (Table 1) and measurement of cell body area revealed that these were predominantly large-diameter neurons (>30 μ m). Double labeling studies with 3A10 or RT97 revealed that many of these

large c-jun-negative neurons had extensive arborizations (Fig. 3*B*).

c-Jun protein and mRNA were also highly expressed in pure cultures of Schwann cells derived from sciatic nerve as well as in satellite cells plated with neurons, indicating that the presence of neurons is not required for c-jun expression in peripheral glial cells (not shown). No c-jun expression was seen in fibroblasts. Glial c-jun immunoreactivity was unaffected by contact with overlying axons (Fig. 4).

Expression of growth factor receptors in DRG cultures

Before monitoring the effects of growth factors on c-jun levels in our cultures, we looked for the presence of trk receptor mRNAs that mediate the effects of the neurotrophins. Growth factor receptor mRNA was differentially expressed by sensory neurons in our cultures (Fig. 5). Of 120 labeled sensory neurons, trkA mRNA was present on 40%, trkB mRNA on 25%, and trkC mRNA on 30% of neurons. No attempt was made to assess whether these were expressed in the same or overlapping populations of neurons or whether different ganglia expressed different proportions of the three trk receptor mRNAs. However, larger-diameter neurons appeared to preferentially label by trkC (Fig. 5*C*). There was also evidence for the presence of trkB and trkC on glial cells.

Effect of growth factors and cytokines on c-jun levels in DRG cultures

Cultures maintained for 7 d in defined medium were treated for 30 min, 2 hr, or 24 hr before fixation with a number of neurotrophins and cytokines. In addition, some cultures were maintained in defined medium with NGF or BDNF or both for the whole 7 d period.

c-jun mRNA levels were not changed by addition of growth factors at any time point in either neurons or Schwann cells. Similarly, the total number of c-jun-positive neurons (and neuronal survival) was unaffected by growth factor treatment and

Figure 3. The majority of neurons were c-jun immunopositive *in vitro*. The majority of neuronal and glial nuclei were c-jun positive (*A*, immunoperoxidase technique). However, a small number of neuronal nuclei were not c-jun positive (*B*, solid arrow) although a second antibody (3A10) revealed the extensive arborizations of both c-jun-positive (*A*) and -negative (*B*) neurons. In this 7 d culture, 3A10 staining was also seen to be restricted to the perikaryal cytoplasm in a subset of neurons (arrowheads, *B*). A background of positive glial cell staining (open arrows) can also be seen. Scale bar, 40 μ m.

no changes in c-jun protein immunofluorescence levels were found in neurons after treatment with any of these compounds. However, in Schwann cells 2 hr treatment with all the factors, except transforming growth factor- β , produced a significant increase in the c-jun protein immunofluorescence levels (30%) (see Table 2). This change in glial cell c-jun protein-like immunoreactivity was not maintained at the 24 hr time point except in the case of BDNF, and this change had disappeared by 4 d *in vitro* (Table 1).

Finally, the addition of the tyrosine kinase inhibitor laven- dustin for 24 hr at 1 μ M (O'Dell et al., 1991) was found to be without effect on either c-jun mRNA or protein levels in neurons or glial cells, suggesting that this group of receptors was not involved in the maintenance of c-jun expression.

Effects of second messenger stimulation on c-jun protein and mRNA

To determine the influence of second messenger candidates on the expression of c-jun in DRG cells in culture, levels of c-jun mRNA and protein were studied after phorbol 12-myristate 13-acetate (PMA) treatment, which stimulates protein kinase C, or stimulation of protein kinase A (PKA) with forskolin, which elevates cAMP through adenylate cyclase activation, or by addition of the membrane-permeable analog of cAMP, dibutyl- cyclohexyl adenosine monophosphate (dBcAMP). The effects of artificially raising or lowering internal calcium levels and of de- polarization were also studied.

Calcium levels and depolarization. Glial cell but not neuronal

Figure 4. Axon contact *in vitro* does not downregulate c-jun expression in glial cells. In a 7 d DRG culture, an axon labeled with the antibody RT97 grows over strongly c-jun-immunopositive glial cells (arrowheads) without reducing levels of expression of the proto-oncogene. Scale bar, 40 μ m.

Figure 5. Trk receptor mRNAs were expressed in subpopulations of DRG neurons *in vitro*. Cultures (7 d *in vitro*) were hybridized for trk (*A*), trkB (*B*), and trkC (*C*) using ^{35}S antisense oligonucleotide probes. Subpopulations of DRGs were labeled with each probe. Arrowhead indicates glial cell. Exposure time, 8 weeks. Scale bar, 40 μm .

Table 1. Long-term effect of neurotrophins on c-jun expression in neurons

	Control	NGF	BDNF
Total number of neurons counted	478 ± 46	450 ± 40	496 ± 65
% Neurons not expressing c-jun protein	19.6 ± 1.1	21.1 ± 0.2	16.6 ± 2.0
c-Jun protein levels	134.6 ± 2.0	136.5 ± 6.5	125.3 ± 4.3

Cultures were continuously exposed to NGF or BDNF from plating to fixation (5 d) in defined medium. Neurons from three coverslips in three separate experiments were analyzed by tracking across the maximum diameter of each 19 mm coverslip and scoring each neuron and measuring c-jun immunofluorescence. The results indicate that there was no effect of growth factors on either cell survival or the percentage of c-jun-positive neurons.

levels of c-jun protein and mRNA were influenced by changing intracellular calcium levels.

Intracellular levels of calcium were raised using the calcium ionophore A-23178, or lowered by buffering the medium with ethylene glycol-bis(β -aminoethyl ether) *N,N,N',N'*-tetra-acetic acid (EGTA). After 24 hr of treatment with A-23178 (10 and 100 nM), levels of c-jun protein in glial cells, but not neurons, were significantly increased (Fig. 6) and mRNA levels increased twofold (Fig. 7). Higher doses of the ionophore (1 μ M) led to massive cell death in both cell populations. Treatment for 30 min or 2 hr with low-dose ionophore had no effect on c-jun protein levels.

When extracellular Ca^{2+} levels were buffered to 0.8 mM (but not 1.7 or 1.3 mM) with EGTA for 24 hr, c-jun protein levels were reduced in glial cells but not in neurons (Table 3). Lower Ca^{2+} levels led to cell death after 24 hr treatment. mRNA levels responded in a similar fashion.

High K^+ concentrations (54 mM), veratridine (10 μ M), or TTX (1 μ M) were added to 7-d-old DRG cultures in order to ask whether depolarization or Na^+ channel activity could induce changes in c-jun gene expression. No differences in c-jun protein or mRNA were found 24 hr after these treatments.

PMA and cAMP. PMA treatment (200 nM) for 2 hr or 24 hr had no effect on either c-jun protein or mRNA in either of the

cell populations studied (Fig. 7, Table 4). However, forskolin (10 μ M) and, to a greater extent, dBcAMP (0.5–1.0 mM) induced a time-dependant decrease in c-jun mRNA (Fig. 7, Table 4) and c-jun protein immunoreactivity in satellite and Schwann cells. This downregulation of c-jun expression was specific for glial cells, having no effect on c-jun levels in neurons. In Figure 8, *B*, *D*, and *F* show the virtually complete disappearance of c-jun protein and mRNA (85–90% decrease) after 24 hr of dBcAMP treatment in glial cells but not in neurons. Lower doses of dBcAMP (10 μ M) did not downregulate the expression of c-jun (Table 5). We were able to antagonize partially the forskolin effect with the specific PKA blocker H8 (*N*-[2-methylamino]ethyl)-5-isoquinoline sulfonamide dihydrochloride; 30 μ M) given 2 hr prior and during 24 hr forskolin treatment (Fig. 9). cAMP treatment did not change jun B or jun D gene expression in either of the cell types (data not shown).

Mitogenic effects of cAMP. Because of the known mitogenic effect of cAMP on Schwann cells (Wood and Bunge, 1975), we examined the relationship between the reduction of c-jun expression and cell division in DRG cultures maintained in serum-free medium. The effects of dBcAMP on c-jun levels and cell division in Schwann cells or satellite cells were dissociable. dBcAMP had a significant mitogenic effect at concentrations of

Table 2. Effect of growth factors on c-jun protein levels in glial cells from DRG cultures

Treatment	2 hr	24 hr
Control	140.3 ± 4.6	150.0 ± 9.4
TGF β	156.7 ± 4.9	145.7 ± 11.5
IGF I	200.8 ± 8.0*	133.0 ± 10.7
EGF	187.4 ± 8.0*	126.0 ± 8.1
PDGF	181.1 ± 9.5*	165.0 ± 10.6
NGF	218.8 ± 7.5*	142.8 ± 9.2
IGF II	189.6 ± 7.2*	154.6 ± 17.4
FGF	182.3 ± 5.3*	145.7 ± 10.7
BDNF	206.0 ± 7.6*	190.0 ± 10.8*

Cultures were maintained for 7 d in defined medium and treated for 30 min (not shown), 2 hr, or 24 hr before fixation with either nerve growth factor (NGF, 50 ng/ml, Sigma), epidermal growth factor (EGF, Bachem, 100 ng/ml), insulin-like growth factor I (IGF I, Bachem, 200 ng/ml), IGF II (Bachem, 200 ng/ml), platelet-derived growth factor (PDGF, Calbiochem, 100 ng/ml), basic or acidic fibroblast growth factor (FGF) (Bachem, 100 ng/ml), transforming growth factor- β (TGF β) (Sigma, 2 ng/ml), or brain-derived neurotrophic factor (BDNF, gift of Drs. J. Wood and H. Luebbert, Sandoz). BDNF was supplied in supernatant from a recombinant cell line containing a functional BDNF gene. By bioassay supernatant was found to be effective at a dilution of 1:40. In this study it was used at a concentration of 1:20. The immunofluorescence from at least 50 glial cells taken from each of three separate experiments was analyzed.

* $p < 0.05$, Dunn's test.

Figure 6. c-Jun immunofluorescence is enhanced in glial cells but not neurons by raising intracellular calcium levels. Treatment of DRG cultures with A-23178 for 24 hr caused a dose-dependent increase in c-jun immunofluorescence in satellite glial cells.

Figure 7. Differential effect of second messengers on c-jun mRNA expression in glial cells. DRG cultures were treated for various times with dBcAMP (0.5 mM), forskolin (10 μ M), PMA (200 nM), and the calcium ionophore A-23178 (100 nM) and hybridized with a c-jun antisense oligonucleotide probe. The exposure time was 3 weeks. Grain counts on individual cells were performed with a 60 $\times$ objective. *, $p < 0.05$, Dunn's test.

10 μ M but which had no effect on c-jun expression. dBcAMP concentrations of 0.5 mM were not mitogenic (Raff et al., 1978) but had a maximal effect in reducing c-jun levels (Table 5).

Effects of dexamethasone and retinoic acid

It has been shown that in some cell systems addition of dexamethasone or retinoic acid blocks positive feedback of AP1 onto the c-jun promoter (see Discussion). However, no effect of these compounds on c-jun protein or mRNA levels in neurons or glial cells was seen, suggesting that autoregulation of c-jun expression as a means of raising levels of expression may not be operative in this system (data not shown).

Discussion

The results of these experiments are complex but demonstrate that the increased expression of c-jun by neurons and glial cells following axotomy can be reproduced and maintained in vitro and that the levels of c-jun protein and mRNA in neurons and glial cells can be differentially regulated by intracellular second messengers.

We previously suggested that the effectiveness of axoplasmic block in initiating the increased expression of c-jun in DRG cells may have been due to loss of a target-derived factor such as NGF (Jenkins and Hunt, 1991; Leah et al., 1991; Jenkins et al., 1993c). In culture, sensory neurons expressed the tyrosine kinase receptor mRNAs *trk*, *trkB*, and *trkC*, which code for

Table 3. Buffering extracellular calcium levels reduces the expression of c-jun immunofluorescence in glial cells but not in neurons

Extra-cellular Ca^{2+} (mM)	c-Jun immunofluorescence (relative intensity)	
	Schwann cells	Neurons
1.8	112 $\pm$ 4.2	171 $\pm$ 11
1.7	100 $\pm$ 4.9	184 $\pm$ 15
1.3	115 $\pm$ 5.3	201 $\pm$ 10
0.8	84 $\pm$ 5.3*	167 $\pm$ 10

Seven day DRG cultures were treated with different concentrations of the calcium chelator EGTA in defined medium, to buffer extracellular Ca^{2+} levels. The concentrations of EGTA were determined with an EQCAL program (Biosoft); 1.8 mM represents the normal concentration of Ca^{2+} in the medium used.

* $p < 0.05$, Dunn's test.

high-affinity receptors for the neurotrophins NGF, BDNF (and NT4), and NT3, respectively, and mediate their functions *in vivo* (see review by Chao, 1992). NGF high-affinity binding sites have been identified on approximately half of lumbar DRG cells including those containing substance P and CGRP and some of the larger-diameter neurons (Verge et al., 1989; Ernfors et al., 1990, 1993). Furthermore, the loss of *trk* from sensory neurons following axotomy can be partially reversed by NGF infusion (Verge et al., 1992), as can the loss of substance P immunoreactivity both *in vivo* and *in vitro* (Fitzgerald et al., 1985; Lindsay and Harmar, 1989; Lindsay et al., 1989). BDNF, but not NGF, is expressed by some DRG cells *in vivo* (Ernfors and Persson, 1991), and in cultured adult sensory neurons BDNF enhances neurite outgrowth but is not required for survival (Lindsay, 1988). It has been shown that NGF, BDNF, and NT3 display distinct patterns of retrograde axonal transport in peripheral neurons (DiStefano et al., 1992), which suggests but does not prove a functional role for these neurotrophins and their receptors. Our results also suggest that neurotrophin receptors are expressed on sensory neurons even when maintained in serum-free medium but, importantly, that the addition of NGF or BDNF to these cultures does not affect the expression of c-jun mRNA in neurons or glial cells. These results strongly suggest that the expression of c-jun is not related to growth factor starvation but to loss of unknown target-derived factors that act to repress c-jun expression in the intact nerve. This is not a particularly surprising result if it is accepted that the stimulus for regeneration is the loss of transported factors that follows axon damage. However, while NGF and probably BDNF are able to sustain and encourage cell growth and differentiation and to reverse many of the neurochemical events associated with axon damage, it seems unlikely that absence of these factors could account for the changes in c-jun expression seen in axotomized neurons. A change in c-jun protein but not mRNA levels was seen in glial cells 2 hr following treatment with a variety of growth factors. These changes may reflect a change in mRNA stability (Sherman et al., 1990) or be associated with an increased stability of the protein or a specific inhibition of the degradative machinery. The long-lasting effect of BDNF is of interest as there is a marked increase in the synthesis of this neurotrophin mRNA in peripheral nerve following nerve section and in isolated Schwann cells and at levels 10 times greater than those previously reported for NGF mRNA (Meyer et al., 1992). Taken together with the observation that Schwann cells and satellite cells express *trkB* and *trkC* mRNA (Carroll et al.,

Figure 8. cAMP treatment reduces levels of c-jun expression in glial cells but not neurons. At 7 d in culture c-jun immunofluorescence (*A*) and mRNA (*C*, *E*) were seen in both glial satellite cells (arrowheads) and neurons (arrows). Twenty-four hours following treatment with dBcAMP the levels of c-jun were substantially reduced in glial cells but not neurons. This was true for both protein (*B*) and mRNA (*D*, *F*). *E* and *F* are dark-field photomicrographs. Scale bars, 30 μ m.

Figure 9. The forskolin-mediated reduction of c-jun immunofluorescence in glial cells can be partially blocked with a PKA antagonist. The PKA antagonist H8 (30 μ M) was given 2 hr before and during 24 hr forskolin treatment. The effect of forskolin treatment on reducing c-jun levels in glial cells was partially antagonized. *, forskolin versus controls, and **, H8 + forskolin versus forskolin, $p < 0.05$, Dunn's test.

1992), this suggests that BDNF might have an auto- or paracrine effect on c-jun expression in Schwann cells as well as being available to encourage the regeneration of those axotomized sensory neurons expressing trkB receptors. The rather widespread but transient effect of other factors on glial cell c-jun protein levels suggests that multiple pathways may converge upon the c-jun protein as a key mediator in the cellular response to denervation.

A previous report had indicated that c-jun expression in neonatal Schwann cells *in vitro* could be downregulated by forskolin (Monuki et al., 1989). Here we show that the effects of increased intracellular calcium levels or stimulation with second messenger candidates such as cAMP on c-jun levels were *entirely* on the glial cell population even in mixed neuron–glial cultures. Stimulation with PMA had no effect at the time points used. However, addition of stable cAMP analog or forskolin to the cultures resulted in a massive and rapid loss of c-jun mRNA from glial cells but with no effect on neuronal levels. These results are similar to those described for the regulation of BDNF expression in Schwann cell cultures but the reverse of those described for NGF mRNA regulation (Meyer et al., 1992).

Denervation of Schwann cells following peripheral nerve section has been shown to result in a substantially changed pattern of gene expression that is thought to encourage the regeneration of the damaged neuron. Thus, there is an upregulation of low-affinity NGF receptor, an increased expression of BDNF and NGF as well as c-jun described here (Taniuchi et al., 1988; Meyer et al., 1992) and an increase in the production of matrix factors (Martini and Schachner, 1988). It has also been reported that denervated nerve provides a better substrate for growth of explanted adult DRG cells than intact nerve (Bedi et al., 1991) perhaps because of a changed complement of adhesion factor molecules on the surface of denervated Schwann cells (Corn-

Table 4. Effect of second messenger activation on c-jun protein immunofluorescence levels in rat DRG cultures

Treatment	Schwann cells	Neurons
Control	107.5 $\pm$ 4.0	183.5 $\pm$ 11.9
Forskolin 16 hr	40.8 $\pm$ 3.0*	190.0 $\pm$ 15.0
dBcAMP 4 hr	72.3 $\pm$ 4.2*	170.0 $\pm$ 15.1
dBcAMP 8 hr	26.9 $\pm$ 3.1*	185.2 $\pm$ 15.8
dBcAMP 24 hr	18.1 $\pm$ 2.1*	187.0 $\pm$ 18.0
PMA 2 hr	114.0 $\pm$ 9.2	162.5 $\pm$ 6.1
PMA 24 hr	112.1 $\pm$ 7.0	173.0 $\pm$ 8.5

Forskolin, dBcAMP, or PMA was added to cultures for various periods of time before fixation, immunofluorescence labeling for c-jun protein, and subsequent semiquantitative analysis.

* $p < 0.05$, Dunn's test.

brooks et al., 1983; Martini and Schachner, 1988; Martini et al., 1990). Finally, treatment of DRG cultures grown on Schwann cell beds with forskolin or dBcAMP substantially retards the growth of dissociated sensory neurons (Palmer et al., 1993). Much of this data would fit with the suggestion that axon–glial cell contact activates a cAMP second messenger system within the glial cell (see Lemke, 1990) that then inhibits the expression of numerous genes such as c-jun and BDNF and discourages further axon growth. However, in our cultures, while axons were mostly seen running over glial cells, they were without effect on c-jun expression in these glial cells. This suggests that other conditions beyond contact between axons and glial cells need to be met before downregulation of gene expression occurs or that the axon must secure a target before its influence on associated glial cells is registered. However, the close correlation between the pattern of gene expression seen in denervated Schwann cells, the coordinate expression of the transcription factor c-jun, and the coregulation of many of these genes by cAMP suggests that c-jun may serve a pivotal role in the regenerative process.

It was previously shown that within hours of axotomy there is a transient expression of c-fos in Schwann cells and a later increase in NGF expression (Hengerer et al., 1990). Further analysis suggested that c-fos could mediate this increased NGF expression (at least in fibroblasts) via one of the intronic AP1 sites present on the NGF gene, presumably after dimerizing with a second leucine zipper containing protein such as c-jun. However this expression of c-fos is transient and was not seen under the conditions described here. Moreover, *in vitro*, the expression of NGF mRNA is upregulated by dBcAMP whereas c-jun expression is, like BDNF, downregulated, again suggesting that rather different mechanisms are at work.

The molecular mechanisms that result in the long-term ex-

Table 5. The effects of cAMP stimulation on c-jun expression and cell division in glial cells are dissociable

Levels	Number of cells	c-Jun protein
Control	17.9 $\pm$ 0.6	154 $\pm$ 7
dBcAMP 10 μ M	32.4 $\pm$ 2.7*	169 $\pm$ 6
dBcAMP 500 μ M	16.5 $\pm$ 1.5	50 $\pm$ 3*

DRG cultures (24 hr after plating) were plated at the same density and stimulated with dBcAMP for 3 d. Two coverslips from each of three separate experiments were analyzed. The mean number of glial cells in four squares of 0.1 mm²/coverslip was counted and measured.

* $p < 0.05$, Dunn's test.

pression of c-jun in neurons and glial cells are unknown but may well be cell type specific. Experimental investigation of the maintained expression of c-jun in non-neuronal cells has suggested that the positive autoregulation of c-jun expression through an AP1 site on the promoter of c-jun itself may play a key role in translating a transient stimulus into a long-term response (Angel et al., 1988; Schutte et al., 1989; Yang-Yen et al., 1990b). In fibroblasts, there is evidence to suggest that this response can be inhibited by the coexpression of a second member of the Jun family, Jun B (Chiu et al., 1989; Schutte et al., 1989; Yang-Yen et al., 1990b), while data from the study of collagenase expression have demonstrated that the interaction of AP1 with its DNA binding site can be inhibited by either dexamethasone (Jonat et al., 1990; Schule et al., 1990; Yang-Yen et al., 1990a) or retinoic acid (Yang-Yen et al., 1990b, 1991; Schule et al., 1991), thus blocking the positive feedback onto the c-jun promoter. However, in both neurons and glial cells we failed to find either an induction of Jun B expression following stimulation with cAMP or forskolin or an effect of either retinoic acid or dexamethasone, suggesting that such mechanisms may not be operative in these cells. Dexamethasone (5 mg/kg) likewise failed to antagonize the effects of axon section on c-jun expression *in vivo* (C. De Felipe and S. P. Hunt, unpublished observations).

The mechanism by which c-jun expression is downregulated in glial cells but not neurons is also particularly obscure in that most previous reports have suggested that cAMP operates through a CREB protein acting at the cAMP response element (CRE) on the c-jun promoter causing increased gene expression (Lamph et al., 1990; de Grost and Sassone-Corsi, 1992). PKA-mediated phosphorylation of CREB activates gene transcription, while dephosphorylation inhibits c-jun expression. However, recent studies have suggested that the presence of other members of the CREB family could act as transcriptional repressors (Benbrook and Jones, 1990) and genes encoding cAMP-responsive element modulator (CREM) proteins, with antagonist properties, have been described (Foulkes et al., 1991). The presence of IP1, a trans-activating inhibitor of AP1 binding to DNA, is likewise inactivated by phosphorylation following stimulation with cAMP (Auwerx and Sassone-Corsi, 1991) and unlikely to explain the present results although a novel inhibitory interaction may well be operative (Baichwal and Tjian, 1989; Baichwal et al., 1991) at least in Schwann cells.

The function of c-jun in neuronal regeneration is also unclear. Not all neurons in culture were clearly labeled with the c-jun antibody although all neurons were expressing c-jun mRNA. This suggests that either there is no direct correlation between neuronal growth and differentiation and c-jun expression, at least in this subpopulation, or that in larger-diameter neurons there is a more rapid turnover of c-jun protein. In the sensory ganglion *in vivo*, section of the dorsal root results only in a weak and variable expression of c-jun protein, yet the damaged axons will regenerate to the spinal cord (Jenkins et al., 1993c). However this regeneration is greatly facilitated by a conditioning crush to the peripheral branch of the neuron, which also results in a massive expression of c-jun protein in damaged neurons (Richardson and Issa, 1984; Jenkins et al., 1993). This suggests that c-jun expression can facilitate but is not an absolute requirement for growth in all nerve cells. The mechanisms by which c-jun can facilitate growth are unknown. We recently pointed out that the suggestion of a causal relationship between other changes in gene expression seen in sensory neurons following axotomy and c-jun expression may be an oversimplification.

The expression of a number of other genes is substantially altered in DRGs following peripheral nerve section. Neurofilament and the peptides substance P and CGRP are downregulated whereas increased levels of expression of actin, tubulin, GAP43, and the neuropeptides galanin, neuropeptide Y, and vasoactive intestinal polypeptide have been reported (Barbut et al., 1981; Gibson et al., 1984; Shehab and Atkinson, 1986; Hökfelt et al., 1987; McGregor et al., 1989; Nielsch and Keen, 1989; Van der Zee et al., 1989; Villar et al., 1989; Noguchi et al., 1990; Woolf et al., 1990; Xu et al., 1990; Dumoulin et al., 1991; Wakiska et al., 1991; Wong and Oblinger, 1991). However, most of these changes in gene expression were restricted to subsets of sensory neurons and occurred at different times after axotomy. For example, the upregulation of GAP43 expression occurs initially in small-diameter sensory neurons and involves larger-diameter DRGs only at longer survival times (Sommerville et al., 1991). In contrast, the change in c-jun expression was rapid and appeared simultaneously in both small and large sensory neurons within 24 hr, regardless of chemical phenotype (Jenkins et al., 1991c; Leah et al., 1991). Thus, it seems unlikely that c-jun expression leads directly to changes in the expression of other downstream genes simply by direct interaction with the relevant promoter sequences.

Recent studies suggest that genes such as c-fos and c-jun can act as selectors of cell responsiveness to external stimuli (Diamond et al., 1990). c-Jun-transformed fibroblasts, which express up to four times their normal levels of the gene product, grow in medium with reduced serum content, suggesting a greater capacity to respond to available growth-promoting factors (Castellazzi et al., 1991, 1993). We suggest that the activation of c-jun could prime neurons and glial cells to respond in a novel way to factors in their environment and so accelerate repair.

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