Research Report

Induction of c-Jun phosphorylation in spinal motoneurons in neonatal and adult rats following axonal injury

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ABSTRACT

This study aims to address if phosphorylation of the transcription factor c-Jun is associated with lesion-induced death of spinal motoneurons, and if this cellular response is modulated by glial-cell-line-derived neurotrophic factor (GDNF). We found that after both distal axotomy and root avulsion, spinal motoneurons in neonatal rats expressed phosphorylated c-Jun (p-c-Jun) and almost all injured motoneurons in these animals died. Similarly, root avulsion in adult rats also induced p-c-Jun expression that preceded the loss of motoneurons. In contrast, neither motoneuron death nor p-c-Jun induction was found after distal axotomy of spinal nerves in adult rats. Application of GDNF after distal axotomy in the neonatal model prevented motoneuron death but did not alter the expression of p-c-Jun in the surviving motoneurons. We conclude that c-Jun phosphorylation correlates with the cellular events leading to motoneuron death and that its expression cannot be modulated by GDNF. We further showed that expression of p-c-Jun was not correlated with the expression of growth-associated protein-43 (GAP-43), whose expression was closely correlated both temporally and spatially with periods of axonal outgrowth, suggesting that p-c-Jun may not be related with axonal regeneration of injured motoneurons.

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1. Introduction

Neuronal responses to axonal interruption are often mediated by inducible transcription factors, which in turn serve to regulate the expression of downstream genes to carry out an adaptive alteration in cell behavior. C-Jun is a component of the heterodimeric AP-1 transcription factor (Jochum et al., 2001). As a component of the AP-1 complex, c-Jun can dimerize with Jun, Fos, activating transcription factor (ATF) and other transcription factors to control numerous target genes.
Axonal damage induces rapid expression of c-Jun in the mammalian peripheral nervous system (PNS) (Wu et al., 1994) and central nervous system (CNS) (Herdegen et al., 1993; Leah et al., 1991). The transcriptional activity of c-Jun can be enhanced by phosphorylating its serine (Ser) 63 and Ser 73 residues (Derijard et al., 1994; Smeal et al., 1994). The dichotomous role of c-Jun N-terminal kinase (JNK)-mediated c-Jun activation in neurons following different stimuli is well documented (Herdegen et al., 1997). This may actually reflect the different neuronal lineages or developmental...
stages in the animals studied. For example, activation of c-Jun was suggested to be associated with cell death in neonatal sympathetic neurons (Bruckner et al., 2001; Ellers et al., 2001) and hippocampal neurons (Schlingensiepen et al., 1993) due to a deprivation of neurotrophic factor. In contrast, many studies indicated that JNK-mediated c-Jun activation was required for the axonal outgrowth of dorsal root ganglion (DRG) sensory neurons (Kenney and Kocsis, 1998; Lindwall et al., 2004; Lindwall and Kanje, 2005) and retinal ganglion cells (Robinson, 1995). The role of JNK-mediated c-Jun activation in injured motoneurons remains unclear.

Spinal motoneuron death due to peripheral nerve injury is dependent on their states of maturation and the extent of damage. During embryonic and early postnatal development, both distal axotomy and spinal root avulsion result in massive motoneuron cell loss (Kashiwhara et al., 1987; Li et al., 1994; Pollin et al., 1991; Snider et al., 1992; Yuan et al., 2000, 2006). Similarly, a significant motoneuron loss is reported following root avulsion lesion in adult rats (Koliatsos et al., 1994; Wu et al., 2003; Yuan et al., 2006). However, no neuronal death occurs in adult rats when peripheral nerve is transected at more distal sites to the cell bodies (Gu et al., 1997; Kashiwhara et al., 1987; Snider et al., 1992; Yuan et al., 2006).

GDNF is one of the most effective motoneuron survival factors (Airaksinen and Saarma, 2002; Bohn, 2004) and numerous studies have demonstrated that GDNF prevents motoneuron death following nerve injuries (Li et al., 1994, 1995; Wu et al., 2003; Yuan et al., 2000).

GAP-43 is a small acidic membrane protein associated with successful axonal growth in the nervous system (Skene and Willard, 1981). It was found to be re-expressed in almost all known cases of successful axon regeneration (Benowitz and Lewis, 1983). Later studies have shown that this protein is regulated directly by c-Jun [reviewed by Herdegen et al. (1997)].

In the present study we induced spinal nerve injuries, either by root avulsion or distal axotomy of the spinal nerve, both alone or in combination with GDNF treatment, to investigate: (i) the possible relationship between c-Jun phosphorylation and the death of spinal motoneurons, (ii) the modulation of this cellular response after GDNF treatment and, (iii) the relationship of p-c-Jun and GAP-43 expression in neonatal and adult rats following distal axotomy.

### Table 1 – Phosphorylated c-jun (ser63) immunohistochemical positivity in axotomized and avulsed motoneurons in neonatal or adult rats at different survival times. The staining intensity (-, absent; +, moderate; ++, intense; ++++, very intense) was assessed as compared to the non-operated side.

<table>
<thead>
<tr>
<th>Time post-lesion</th>
<th>1 day</th>
<th>3 days</th>
<th>1 week</th>
<th>2 weeks</th>
<th>4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distal axotomy in the neonatal</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Avulsion in the neonatal</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Distal axotomy in the adult</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Avulsion in the adult</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
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### 2. Results

#### 2.1. Age- and injury type-dependent induction of phosphorylated c-Jun following axonal injury

In neonatal rats, a massive motoneuron loss was observed in both distal axotomized (Fig. 1B) and root avulsed (Fig. 1C) spinal cords compared with normal control (Fig. 1A). Motoneuron loss was evident at 3 days post-avulsion and 7 days post-axotomy. Almost all motoneurons died within 2 weeks following either distal axotomy or root avulsion (Fig. 1D).

Neonatal motoneurons in the normal contralateral side of spinal cord did not express p-c-Jun (Fig. 1E). In contrast, intensive p-c-jun-immunopositive motoneuron nuclei in the lesioned side of spinal cord could be observed from 1 day following both distal axotomy (Fig. 1G) and root avulsion (Fig. 1I) before declining with time in parallel to neuronal survival (Table 1 and Fig. 1D respectively).

Counterstaining with neutral red showed that spinal motoneurons in neonatal rats expressed p-c-Jun following both distal axotomy (Figs. 1G and H) and root avulsion (Figs. 1I and J). More than 95% of surviving motoneurons were p-c-Jun positive at all time points examined post-injury from days 3 to 14 (Fig. 1K).
In adult rats, the induction of motoneuron death was injury type-dependent. No motoneuron death was found following distal axotomy (Figs. 2B and D) compared with normal control (Fig. 2A). In contrast, significant motoneuron loss was found following root avulsion at 4 weeks post-injury (Figs. 2C and D). Induction of c-Jun activation was also injury type-dependent. No induction of p-c-Jun was observed in lesioned motoneurons following distal axotomy (Figs. 2G and H and Table 1), compared with normal control (Figs. 2E and F). In contrast, p-c-Jun immunopositive nuclei were observed in root avulsed motoneurons (Figs. 2I and J and Table 1) with about 70% positive for p-c-Jun (Fig. 2K).
2.2. **GDNF did not inhibit c-Jun activation in neonatal motoneurons following axotomy**

To investigate the effect of GDNF on c-Jun activation, GDNF was given topically in the lesioned sites following distal axotomy in neonatal rats and following root avulsion in both neonatal and adult rats.

In the sham-treated control animals, a remarkable motoneuron loss was observed in axotomized neonatal rats and root avulsed neonatal and adult rats (Fig. 3A). In the GDNF-treated rats, survival of motoneurons significantly increased in both neonatal and adult rats (P<0.01) (Fig. 3A). GDNF treatment (Figs. 3C, E, and G) did not change the number of p-c-Jun-positive motoneurons compared with vehicle treatment (Figs. 3B, D and F) at 3 days post-injury following axotomy in neonatal rats (Figs. 3B and C) or root avulsion in neonatal (Figs. 3D and E) and adult rats (Figs. 3F and G). At this time point, the percentage of p-c-Jun positive motoneurons in both vehicle and GDNF-treated groups was similar (Fig. 3H, P>0.05).

2.3. **Age-dependent induction of GAP-43 following distal axotomy**

Previous studies have shown that c-Jun participates directly in regulating the GAP-43 protein [reviewed by Herdegen et al. (1997)]. Hence, we investigated the pattern of GAP-43 expression in the remaining nerves following distal axotomy in neonatal and adult rats. Weak GAP-43-immunopositive fibers were found in the proximal stump following distal axotomy in neonatal rats (Fig. 4B), which was comparable to age-matched normal control (Fig. 4A). However, following distal axotomy in adult animals, the proximal stump showed intense GAP-43-immunopositive fibers at 3 days post-injury (Fig. 4D) compared with the contralateral normal control (Fig. 4C), indicating a robust regenerative effort of adult axotomized motoneurons.

3. **Discussion**

3.1. **C-Jun phosphorylation in dying spinal motoneurons**

The results of the present study show a striking difference in the expression of p-c-Jun in injured spinal motoneurons dependent on animal age and type of injury. P-c-Jun was markedly induced 1 day after both distal axotomy and root avulsion in neonatal rats. However, induction of p-c-Jun was injury type-dependent in adult rats. Expression of p-c-Jun was found in adult motoneurons following root avulsion but not distal axotomy. It has been proposed that retrogradely transported chemical signals activated by nerve injury can induce nerve cell body responses including a variety of anatomical changes and modifications in gene expression (Zhang and Ambron, 2000). Thus, a more rapid onset of response may be expected for injuries involving shorter distances between sites of lesion and cell bodies. Several studies have demonstrated a more rapid onset of c-Jun induction in neonatal sciotic nerve transection compared with adult transection presumably due to the shorter distance between cell bodies and the site of lesion in neonatal animals (Herdegen et al., 1992). In our study, the absence of p-c-Jun in the adult axotomized motoneurons cannot be explained by the longer distance to cell bodies from the lesion site since c-Jun could not be detected at time points up to 28 days post-injury, which is sufficient time for the transport of putative signals from the lesion site to cell bodies at a velocity of 90–120 mm/day (Herdegen et al., 1992).

In all lesions leading to a loss of spinal motoneurons (i.e. root avulsion in the adult and neonatal rat, and distal axotomy in the neonatal rat), a rapid induction of p-c-Jun was found in injured motoneurons. Conversely, there was a lack of p-c-Jun-immunoreactivity after distal axotomy in the adult rat, which was coincident with robust regenerative efforts and a lack of cell death in axotomized spinal motoneurons. Taken together, this indicates that the induction of c-Jun phosphorylation may be involved in the process leading to cell death in spinal motoneurons.

GAP-43 is a prominent growth-cone component widely correlated with competence for axon regeneration (Herdegen et al., 1997). The GAP-43 gene contains a highly conserved AP-1 site, which can strongly modulate its transcription (Eggen et al., 1994). GAP-43 is co-expressed with c-Jun in retinal ganglion cells after axon injury (Schaden et al., 1994), and positive relations between the expression of c-Jun and GAP-43 are also observed in DRG cells, and cortical neurons following axotomy (Chong et al., 1994; Tetzlaff et al., 1994).

Our result showed that axotomized adult but not neonatal motoneurons display vigorous regenerative efforts indicated by intensive GAP-43 expression. However, no induction of p-c-Jun could be observed in the adult axotomized motoneurons. These results suggest that GAP-43 induction in axotomized
motoneurons is not accompanied by p-c-Jun. Thus, c-Jun activation may not be required for axonal regeneration of spinal motoneurons following axotomy. This observation is different from a previous finding, where phosphorylation of c-Jun was found to be important for axonal regeneration of sensory neurons (Lindwall and Kanje, 2005). Our results further reaffirm the notion that c-Jun activation plays distinctly different roles in different neuronal types.
3.2. GDNF does not modulate p-c-Jun in spinal motoneurons

The mechanism by which c-Jun activation is regulated remains unclear. It has been suggested that c-Jun phosphorylation occurs in neurons that fail to obtain sufficient trophic factor support in both in vitro and in vivo investigations (Sun et al., 2005; Virdee et al., 1997). P-c-Jun has been identified as one of the earliest biochemical signals of neuronal death after nerve growth factor (NGF) deprivation in sympathetic neurons (Deckwerth et al., 1998). Addition of NGF results in a complete suppression of JNK-mediated c-Jun phosphorylation and an arrest of apoptosis (Virdee et al., 1997). Previous evidence suggests that motoneuron degeneration following axonal injury may be due to a deprivation of neurotrophic factors from their targets or from Schwan cells of peripheral nerves, as application of neurotrophic factors can arrest death following axotomy or root avulsion (Wu et al., 2003; Yuan et al., 2000). Thus, we speculated that c-Jun activation induced in dying motoneurons may be due to a failure to obtain sufficient trophic support. If so, application with neurotrophic factors, e.g., GDNF, to dying motoneurons, should prevent c-Jun activation and enhance motoneuron survival. As expected, GDNF rescued almost all motoneurons from death. However, GDNF did not inhibit c-Jun activation in injured motoneurons. This finding contrasts with previous findings where GDNF treatment inhibited injury induced c-Jun activation in substantia nigra dopaminergic neurons (Vaudano et al., 2001) and cerebellar Purkinje neurons (McAlhany et al., 2000). It is therefore likely that mechanisms by which c-Jun activation is regulated may be different in different neuronal types. In support of this suggestion, Lindwall and Kanje (2005) have shown that NGF deprivation induces c-Jun activation in sympathetic neurons and re-addition of NGF inhibits c-Jun activation while these responses do not occur in sensory neurons although the survival of both types of neurons is dependent on NGF.

Our observations that motoneuron survival is increased while c-Jun activation is not suppressed after GDNF treatment following axonal injury further indicate that motoneuron protection is not necessarily accompanied by suppression of c-Jun activation. A recent study has also demonstrated that neuroprotection is not correlated with the suppression of c-Jun activation (Lomb et al., 2007). In their study, they found that both ethyl 3,4-dihydroxybenzoic acid (DHB) and dimethylloxalylglycine (DMOG) can inhibit apoptosis of sympathetic neurons caused by trophic factor deprivation. However, only DHB blocked c-Jun activation. Thus, c-Jun activation in injured motoneurons may not respond to GDNF deprivation induced by axonal injury and the neuroprotection of GDNF does not work via suppression of c-Jun activation.

4. Experimental procedures

4.1. Animals

Postnatal day 1 (PN1) and adult female Sprague–Dawley rats were used. PN1 animals were anesthetized under deep hypothermia and adults with ketamine (80 mg/kg) and xylazine (8 mg/kg). All surgical interventions and subsequent care and treatment were approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong.
4.2. Lesion model and surgical procedures

For ventral root avulsion, neonatal and adult rats were placed on the surgical table and a dorsal laminectomy was carried out. The seventh cervical (C7) spinal roots were exposed under a surgical microscope and both the dorsal and ventral roots were avulsed following procedures described previously (Wu, 1996). The avulsed dorsal and ventral roots were cut away from the peripheral nerve and examined under the microscope to confirm the success of the lesion.

For distal axotomy, the C7 spinal nerve was cut about 10 mm away from the spinal cord. Spinal peripheral nerve is a mixed nerve of sensory and motor fibers. To isolate motor axons following PN nerve injury, the DRG was removed to survive for 1, 3, 7, 14 and 28 days, with 5 rats in each postoperative time period.

4.3. GDNF administration in neonatal rats

GDNF was applied as previously described (Chai et al., 1999; Wu et al., 2003; Yuan et al., 2000). Briefly, immediately following distal axotomy or root avulsion, a small piece of Gelfoam soaked in 1 μg/μl of GDNF (n=5) (Pharmacia and Upjohn, Peapack, NJ) was placed in contact with the proximal stump of the C7 spinal nerve or spinal cord. Sham-treated control rats (n=5) received Gelfoam soaked in normal saline.

4.4. Perfusion and tissue processing

At the end of the postoperative survival period, rats were deeply anesthetized with ketamine and xylazine and perfused intracardially with normal saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). The C7 spinal segment and proximal stump of the spinal nerve from rats following distal axotomy were removed and immersion-fixed in the same fixative for 6 h, and then placed into 30% sucrose in 0.1 M PBS overnight. Transverse serial sections were cut at 40 μm and collected in wells containing PBS.

4.5. Immunohistochemistry

After rinsing with PBS, spinal sections were incubated with a rabbit IgG polyclonal antibody against p-c-Jun (1:1000, ser63, cell signaling technology, Beverly, MA) and the remaining peripheral nerve stump sections with anti-GAP-43 (1:800, Chemicon, CA) overnight at room temperature. The primary antibody was diluted in PBS containing 0.2% Triton X-100 and 3% normal goat serum. Following incubation in the primary antibody, sections were visualized by using Alexa 488 or 568-conjugated secondary antibodies (1:400 Molecular probes) under a fluorescent microscope (Zeiss).

4.6. Motoneuron counts and morphometric analysis

Sections immunostained with antibody against p-c-Jun were counterstained with neutral red. The number of surviving motoneurons was counted on both the intact and the lesioned sides as described previously (Wu et al., 2003; Yuan et al., 2006). The total number of surviving motoneurons on the lesioned side was expressed as a percentage of the number of motoneurons on the contralateral side. The number of motoneurons was compared among different treatment groups. Data were analyzed with a one way ANOVA followed by Turkey Kramer multiple comparison tests.

Acknowledgments

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References


