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ACTIVATING TRANSCRIPTION FACTOR 3 INDUCTION IN SYMPATHETIC NEURONS AFTER AXOTOMY: RESPONSE TO DECREASED NEUROTROPHIN AVAILABILITY

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Abstract—Activating transcription factor 3 (ATF3) is induced in a high proportion of axotomized sensory and motor neurons after sciatic nerve transection. In the present study, we looked at the expression of this factor in the superior cervical ganglion (SCG) after axotomy and after other manipulations that induce certain aspects of the cell body response to axotomy. Sympathetic ganglia from intact rats and mice exhibit only a very occasional neuronal nucleus with activating transcription factor 3–like immunoreactivity (ATF3-IR); however, as early as 6 h and as late as 3 weeks postaxotomy, many of the neurons showed intense ATF3-IR. A second population of cells had smaller and generally less intensely stained nuclei, and at least some of these cells were satellite cells. Lesions distal to the SCG induced by administration of 6-hydroxydopamine or unilateral removal of the salivary glands produced increases in ATF3-IR similar to those seen after proximal axotomy, indicating that this response is not strictly dependent on the distance of the lesion from the cell body. Two proposed signals for triggering ATF3 expression were examined: reduction in nerve growth factor (NGF) availability and induction of the cytokine leukemia inhibitory factor (LIF). While administration of an antiserum raised against NGF to intact animals induced ATF3-IR, induction of ATF3-IR after axotomy was not reduced in LIF null mutant mice. Since axotomy, 6-hydroxydopamine, and sialectomy are known to decrease the concentration of NGF in the SCG, our data suggest that these decreases in NGF lead to increases in ATF3-IR. Furthermore, since the number of neurons in the SCG expressing ATF3-IR was greater after axotomy than after antiserum against NGF treatment, this raises the possibility that decreased NGF is not the only process regulating ATF3 expression after axotomy.

Key words: cell body response, leukemia inhibitory factor, nerve injury, nerve growth factor, sialectomy, superior cervical ganglion.

Following axotomy, neurons in the peripheral nervous system can regenerate their axons and reinnervate target tissues. It has long been recognized that such regeneration is dependent on changes in neuronal gene transcription and translation, changes which are part of the "cell body response" to axotomy (Brattgard et al., 1957; Lieberman, 1971; Watson, 1974; Grafstein, 1975). Genes whose expression increases after axotomy, such as growth-associated protein-43 (GAP-43) and tubulin, have been referred to as regeneration-associated genes (RAGs; e.g. Theriault et al., 1992). While for many years only a handful of RAGs had been identified, our knowledge has greatly expanded recently through the use of global molecular biological techniques. For example, a microarray analysis of the rat superior cervical ganglion (SCG) 2 days following postganglionic nerve transection revealed 187 genes whose expression changed by a factor of 2 or more, 103 of which increased their expression (Boeshore et al., 2004). A long-term goal of such research is to delineate the molecular signals that trigger these axotomy-induced changes in gene expression. For example, expression of the neuropeptide galanin is upregulated in sympathetic and sensory neurons after axotomy, and this response is triggered by a reduction in the availability of target-derived nerve growth factor (NGF) and an induction of the cytokine leukemia inhibitory factor (LIF) (Rao et al., 1993; Sun and Zigmond, 1996; Shadiack et al., 1998, 2001).

One of the genes whose expression increased in axotomized sympathetic neurons is activating transcription factor 3 (ATF3) (Boeshore et al., 2004), a member of the ATF/cyclic AMP responsive element binding protein family of transcription factors (Hai and Hartman, 2001). By 6 h after surgery, ATF3 mRNA is already fivefold higher in axotomized than in sham-operated SCG, and it is elevated by eightfold at 48 h (Boeshore et al., 2004). Lindwall and Kanje (2005) recently reported neuronal nuclei expressing activating transcription factor 3–like immunoreactivity (ATF3-IR) in neonatal SCG after 24 h in organ culture. This transcription factor has received attention as a possible indicator of nerve injury, particularly in the case of sensory neurons (Tsujino et al., 2000; Tsuzuki et al., 2001; Dussor et al., 2003; Mizusawa et al., 2003; Obata et al., 2003; Takahashi et al., 2003; Wang et al., 2003; Averill et al., 2004).

ATF3 was first identified in a screen of mRNAs induced in the liver after partial hepatectomy and was originally named liver regeneration factor-1 (Hsu et al., 1991). It was subsequently found to increase in a number of non-neural tissues in response to a variety of physiological stressors

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Abbreviations: anti-NGF, antiserum against nerve growth factor; ATF3, activating transcription factor 3; ATF3-IR, activating transcription factor 3–like immunoreactivity; DRG, dorsal root ganglion; GAP-43, growth-associated protein-43; LIF, leukemia inhibitory factor; NGF, nerve growth factor; RAG, regeneration-associated gene; SCG, superior cervical ganglion; TH, tyrosine hydroxylase; 6-OHDA, 6-hydroxydopamine.

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and was implicated in apoptosis (Chen et al., 1996; Hai et al., 1999; Hartman et al., 2004). The induction of ATF3 in the nervous system was first reported by Chen et al. (1996) in the dentate gyrus in response to administration of the convulsant drug pentylenetetrazol, an effect the authors attributed to neural damage. ATF3 was also identified in a search for genes whose expression increased following NGF withdrawal from a subclone of PC12 cells (PC6-3 cells) and from embryonic SCG neurons, genes that the authors proposed were involved in apoptosis (Mayumi-Matsuda et al., 1999). Finally, ATF3 is induced in retinal ganglion cells after intraorbital crushing of the optic nerve, a procedure which leads to extensive neuronal cell death (Takeda et al., 2000). In contrast to this association with apoptosis, ATF3 is also induced in adult sensory and motor neurons after axotomy (Tsujino et al., 2000), a situation in which the majority of the neurons do not die.

ATF3 is a particularly interesting RAG for several reasons. First, its change in expression is among the earliest reported after axotomy. Second, as ATF3 is a transcription factor, it is likely that it is involved in triggering some of the later occurring changes in gene expression. Third, at least for sensory and motor neurons, ATF3 protein has been reported to be undetectable in intact neurons but detectable in virtually all axotomized neurons (Tsujino et al., 2000). Fourth, overexpression of ATF3 has recently been shown to stimulate neurite outgrowth in SCG and dorsal root ganglion (DRG) neurons and PC12 cells and to decrease apoptosis in SCG neurons, PC12 cells and hippocampal neurons (Nakagomi et al., 2003; Pearson et al., 2003; Francis et al., 2004; Seijffers et al., 2006).

Tsujino et al. (2000) proposed ATF3 expression as a specific marker for injured neurons. Two more recent papers, however, suggest that this description needs to be qualified both with respect to the distance between the site of nerve injury and the neuronal cell body and in terms of the cellular specificity of the ATF3 response. Mason et al. (2003) found that whether or not ATF3 expression was observed in axotomized corticospinal neurons depended on how far away from the cell body the axons were severed, with expression only occurring after a proximal lesion. Hunt et al. (2004) observed that, in addition to increasing in neurons in the DRG and ventral spinal cord after sciatic nerve lesion, it also increases in Schwann cells in the sciatic nerve, predominantly in the distal portion of the severed nerve, where Wallerian degeneration takes place.

The present study had two primary goals. The first was to characterize the induction of ATF3 mRNA and protein in sympathetic ganglia after axonal damage, particularly with respect to the effect of the distance from the cell body that the injury takes place and the cell types expressing the transcription factor. The second goal was to characterize the molecular signals that trigger these inductions of ATF3 after nerve injury and in particular to investigate the role of two molecules known to be involved in other aspects of the response to injury, namely the neurotrophin NGF and the cytokine LIF.

EXPERIMENTAL PROCEDURES

Animals and surgery

Our initial characterization of ATF3 expression in sympathetic ganglia after postganglionic nerve transection was done with both rats and mice, while subsequent experiments characterizing the signals that trigger this induction were done with mice. Adult male Sprague–Dawley rats (200–250 g) were purchased from Zivic-Miller (Zelienople, PA, USA). Seven-week-old male C57Bl/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). LIF −/− mice (n=12) were obtained from Philip Brulet (Pasteur Institute, Paris, France; Escary et al., 1993). The animals were housed under controlled lighting and with *ad libitum* access to food and water for at least a week prior to use. For surgery, rats were anesthetized by i.p. injection of a mixture of ketamine (43 mg/kg), xylazine (8.6 mg/kg), and acepromazine (1.4 mg/kg). Mice were injected with ketamine (100 mg/kg) and xylazine (10 mg/kg). Neurons in the SCG were axotomized by transecting the two major postganglionic trunks of the ganglion, the internal and external carotid nerves, approximately 2 mm from where they exit the ganglion. Sham-operated animals had their postganglionic trunks exposed but not transected. Six hours or 2, 5, 7, or 21 days later (rats: n=7, 20, 4, and 3 respectively; mice at 6 h, n=4, at 2 days, n=6, and at 7 days, n=12) the animals were reanesthetized and perfused transcardially with 4% paraformaldehyde. Alternatively, the animals were killed by CO_2 inhalation, and the SCG was removed and fixed by immersion in 4% paraformaldehyde for 1–2 h at 4 °C. Similar results were obtained following fixation by perfusion and by immersion. In some experiments, ganglia were also examined from unoperated control animals (rats, n=2, and mice, n=2). In one group of mice (n=6), the submandibular, parotid and sublingual glands were removed unilaterally ("sialectomy"), and 7 days later the ipsilateral and contralateral SCG was examined.

6-Hydroxydopamine (6-OHDA) and antiserum against nerve growth factor (anti-NGF) treatment

Mice were injected with 6-OHDA (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 100 mg/kg dissolved in 0.9% saline with 0.1% ascorbic acid intraperitoneally (n=3) or with the vehicle alone (n=2). Seven days later, the mice were perfused and the ganglia processed for immunohistochemistry. anti-NGF (obtained from Dr. J. Diamond McMaster University, Ontario, Canada) was raised in sheep by injecting them with 2.5 S NGF purified from male mouse salivary glands (Diamond et al., 1992). Mice were injected daily intraperitoneally with 2.5 (n=5) or 7.5 (n=5) μl/g anti-NGF or normal sheep serum given at the higher dose (n=10; Sigma-Aldrich) for 7 days. Ganglia were dissected and immersion fixed 24 h after the last dose.

In situ hybridization

Adult male rats were killed by CO_2 inhalation 6 h (n=2), 2 days (n=3), or 5 days (n=3) after axotomy or after a sham-operation. The SCG were dissected, desheathed, and fixed by immersion in 4% paraformaldehyde for 1–2 h at 4 °C. The ganglia were cryoprotected in sucrose and embedded in O.C.T. embedding compound (Electron Microscopy Sciences, Hatfield, PA, USA). Ten micron frozen sections were mounted on Fisher ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA, USA), air dried for 2–3 h, and stored at −20 °C. Each slide contained sections of SCG from two different animals for each of three conditions (unoperated, sham operated, and axotomized). A pDrive plasmid containing a 146 bp PCR product for ATF3 was linearized with *Hind*III and *Bam*HI restriction enzymes (Roche Applied Sciences, Indianapolis, IN, USA). Sense and antisense probes were generated using a DIG RNA Labeling kit (SP6/T7) (Roche Applied Sciences) ac-