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Upregulation of activating transcription factor 3 (ATF3) by intrinsic CNS neurons regenerating axons into peripheral nerve grafts

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Abstract

The expression of the transcription factor ATF3 in the brain was examined by immunohistochemistry during axonal regeneration induced by the implantation of pieces of peripheral nerve into the thalamus of adult rats. After 3 days, ATF3 immunoreactivity was present in many cells within approximately 500 μm of the graft. In addition, ATF3-positive cell nuclei were found in the thalamic reticular nucleus (TRN) and medial geniculate nuclear complex (MGN), from which most regenerating axons originate. CNS cells with ATF3-positive nuclei were predominantly neurons and did not show signs of apoptosis. The number of ATF3-positive cells had declined by 7 days and further by 1 month after grafting when most ATF3-positive cells were found in the TRN and MGN. 14 days or more after grafting, some ATF3-positive nuclei were distorted and may have been apoptotic. In some experiments of 1 month duration, neurons which had regenerated axons to the distal ends of grafts were retrogradely labeled with DiAsp. ATF3-positive neurons in these animals were located in regions of the TRN and MGN containing retrogradely labeled neurons and the great majority were also labeled with DiAsp. SCG10 and c-Jun were found in neurons in the same regions as retrogradely labeled and ATF3-positive cells. Thus, ATF3 is transiently upregulated by injured CNS neurons, but prolonged expression is part of the pattern of gene expression associated with axonal regeneration. The co-expression of ATF3 with c-*jun* suggests that interactions between these transcription factors may be important for controlling the program of gene expression necessary for regeneration.
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Introduction

Axons in injured peripheral nerves regenerate vigorously following injury, but most axons in the CNS show only an initial sprouting response, which is soon aborted. The failure of axonal regeneration in the CNS is believed to result, in part, from an inadequate cell body response to axotomy. When motor or primary sensory (DRG) neurons are axotomized, they undergo dramatic changes in morphology and gene expression (e.g., Chong et al., 1992; Lieberman, 1971; Mason et al., 2002) but some CNS neurons show virtually no response to axotomy (Kloss et al., 1999; Mason et al., 2003a,b; Miller et al., 1989). Some populations of CNS neurons can regenerate axons over long distances through nerve grafts implanted into the brain or spinal cord (Morrow et al., 1993; Richardson et al., 1980), and this ability can be correlated with the extent of the cell body response to axotomy (Anderson et al., 1998). The two thalamic nuclei with the greatest capacity for axonal regeneration are the thalamic reticular nucleus (TRN) and subregions of the medial geniculate nucleus (MGN) (Benfey et al., 1985; Morrow et al., 1993). Following nerve graft implantation, neurons in these nuclei show similar changes in growth-related gene expression to those seen in regenerating peripheral neurons (Mason et al., 2003a,b). The key molecules for orchestrating the programs of gene expression necessary for regenerating an axon are transcription factors.

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The transcription factor whose expression has been most closely associated with axonal regeneration is c-*jun*, which is upregulated by DRG and motor neurons following peripheral nerve injury (Jenkins and Hunt, 1991) and by intrinsic CNS neurons when regenerating axons into peripheral nerve grafts (Vaudano et al., 1998). More recently, it has been recognized that another transcription factor, ATF3, is also upregulated strongly and selectively by injured sensory and motor neurons and following peripheral nerve injury (Tsujino et al., 2000; Tsuzuki et al., 2001, 2002). Its expression is correlated with the duration of axonal regeneration (Tsujino et al., 2000). ATF-3 is one member of a large family of bZip leucine zipper transcription factors that bind to promoters responsive to cAMP and phorbol ester at the related cAMP (CRE) and phorbol ester response elements and AP-1 sites (reviewed in Hai and Hartman, 2001). ATF3 is particularly interesting in the context of axonal regeneration because it can form heterodimers with c-Jun (see review by Hai and Hartman, 2001) and, in contrast to c-Jun, is undetectable in the great majority of undamaged neurons. Heterodimers of ATF3 and c-Jun bind to different sites and presumably have different effects on transcription than homodimers. ATF3 is a stress-induced factor and is also upregulated by injured CNS neurons (Takeda et al., 2000) but whether such neurons express ATF3 when regenerating axons is not clear. We have therefore examined the pattern of ATF3 expression in intrinsic CNS neurons when regenerating axons into peripheral nerve grafts in the thalamus. We have also correlated ATF3 expression with that of c-*jun* and SCG10, a protein which is strongly upregulated by most regenerating neurons (Mason et al., 2002) and therefore acts as a good marker for such cells.

Materials and methods

Surgery

All surgical procedures were approved by the UCL ethical committee and licensed by the Home Office. Twenty-three adult female Sprague–Dawley rats, weighing 180–250 g at the time of implantation, received peripheral (tibial) nerve grafts to the thalamus. The rats were anesthetized with Halothane, nitrous oxide, and oxygen mixture. Segments approximately 1.5 cm long of the left tibial nerve were removed and one end implanted through a craniotomy into the left thalamus, using coordinates taken from the atlas of Paxinos and Watson (1986). The graft was fixed to the skull surrounding the craniotomy with cyanoacrylate glue (Histoacryl, B. Braun, Germany). The distal end of the graft was left lying on the surface of the skull.

Fixation and immunohistochemistry

Four rats had a survival time of 3 days, eight had a survival time of 7 days, four survived for 14 days, and seven had a survival time of 1 month, following peripheral nerve graft implantation. At the relevant time point, the rats were anesthetized with halothane and overdosed with Sagatal (0.8 ml per rat; ip). They were then perfused through the left cardiac ventricle with phosphate buffer (50–100 ml, 0.1 M, pH 7.4) followed by paraformaldehyde (500 ml, 4%) in phosphate buffer. Brains were removed, taking care not to disturb the graft, and cryoprotected for 2–3 days at 4°C in 30% buffered sucrose (pH 7.4).

Brains were sectioned on a freezing microtome at a thickness of 40 μm. Serial sections were taken in triplets, so that the first in every three could be reacted for ATF3, the second for SCG10, and the third for c-Jun. This allowed a direct comparison of expression patterns to be made for all three molecules.

All the sections were incubated for 15 min in hydrogen peroxide made up in Tris buffered saline (TBS −0.03%). They were then washed in TBS, incubated in blocking serum and primary antibody was then added. Polyclonal anti ATF3 (Santa Cruz) was used at a dilution of at 1:1000 in TBS (0.05 M), incubated for 2 days at 4°C.; anti SCG10 (made in the laboratory of Dr Grenningloh; Pellier-Monnin et al., 2001; Riederer et al., 1997) at 1:8000 in TBS (0.05 M), incubated overnight at 4°C; and anti c-Jun (phosphorylated at Ser 73—New England Biolabs Inc.) at 1:2000 in TBS (0.05 M), incubated overnight at 4°C. The sections were then washed in TBS, and the primary antibodies localized using biotinylated anti-rabbit IgG (Vector) at 1:200 in 2% normal rat serum. The sections were then washed again in TBS, and reacted using the ABC process (Vector).

Retrograde labeling

In 3 rats surviving 1 month after grafting, the retrograde tracer, DiAsp (Di-4-10-Asp; Molecular Probes) was used to identify neurons that had regenerated axons into the grafts. A few crystals of DiAsp were placed on the freshly exposed distal end of the graft 2 weeks before perfusion with fixative and cryoprotection as described above. Sections were cut at a nominal thickness of 40 μm on a freezing microtome and mounted in PBS on glass slides. Photographs of DiAsp labeled neurons were taken with a Hamamatsu Orca digital camera and OpenLab software on a G4 Apple Macintosh computer. The sections were then removed from the slides and processed for ATF3 immunocytochemistry using the ABC-DAB method as described above.

Analysis of results

The sections were analyzed using a Leica DM R microscope under dark-field, bright-field, and phase-contrast illumination. Camera lucida drawings were produced of the graft site and any labeled cell bodies or axons, and estimates of their sizes were made using a graticule. The

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