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ATF3 upregulation in glia during Wallerian degeneration: differential expression in peripheral nerves and CNS white matter

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Abstract

Background: Many changes in gene expression occur in distal stumps of injured nerves but the transcriptional control of these events is poorly understood. We have examined the expression of the transcription factors ATF3 and c-Jun by non-neuronal cells during Wallerian degeneration following injury to sciatic nerves, dorsal roots and optic nerves of rats and mice, using immunohistochemistry and *in situ* hybridization.

Results: Following sciatic nerve injury – transection or transection and reanastomosis – ATF3 was strongly upregulated by endoneurial, but not perineurial cells, of the distal stumps of the nerves by 1 day post operation (dpo) and remained strongly expressed in the endoneurium at 30 dpo when axonal regeneration was prevented. Most ATF3+ cells were immunoreactive for the Schwann cell marker, S100. When the nerve was transected and reanastomosed, allowing regeneration of axons, most ATF3 expression had been downregulated by 30 dpo. ATF3 expression was weaker in the proximal stumps of the injured nerves than in the distal stumps and present in fewer cells at all times after injury. ATF3 was upregulated by endoneurial cells in the distal stumps of injured neonatal rat sciatic nerves, but more weakly than in adult animals. ATF3 expression in transected sciatic nerves of mice was similar to that in rats. Following dorsal root injury in adult rats, ATF3 was upregulated in the part of the root between the lesion and the spinal cord (containing Schwann cells), beginning at 1 dpo, but not in the dorsal root entry zone or in the degenerating dorsal column of the spinal cord. Following optic nerve crush in adult rats, ATF3 was found in some cells at the injury site and small numbers of cells within the optic nerve displayed weak immunoreactivity. The pattern of expression of c-Jun in all types of nerve injury was similar to that of ATF3.

Conclusion: These findings raise the possibility that ATF3/c-Jun heterodimers may play a role in regulating changes in gene expression necessary for preparing the distal segments of injured peripheral nerves for axonal regeneration. The absence of the ATF3 and c-Jun from CNS glia during Wallerian degeneration may limit their ability to support regeneration.

Background

Following injury to peripheral nerve trunks and the initiation of Wallerian degeneration, the resident cells in the distal stump undergo proliferation and many changes in gene expression, and are joined by hematogenous monocytes. These events enable debris to be cleared, the extracellular matrix to be remodelled and the bands of Büngner to be made ready to receive the regenerating axons. Non-neuronal cells in the proximal stump are less affected than those in the distal stump. Transcription factors control the changes in gene expression that occur in the distal stump, and it has been recognised for some years that an upregulation of c-jun and c-fos in the non-neuronal cells occurs soon after injury and that Krox-20 and SCIP are also upregulated [1]. Wallerian degeneration also occurs in the CNS distal to a site of axotomy. The best known features of responses of CNS glia to axotomy are the generation of reactive astrocytes and microglia [2]. The transcriptional control of these cellular events is also poorly understood, but there is little upregulation of c-jun in the degenerating optic nerve following crush injury [3]. ATF3 is one member of a large family of bZip leucine zipper transcription factors that bind to promoters responsive to cAMP and phorbol esters at the related cAMP (CRE) and phorbol ester response elements and AP-1 sites [4]. ATF3 is particularly interesting in the context of axonal regeneration because it can form heterodimers with c-Jun [4] and its regulation in axotomised neurons closely mimics that of c-Jun [5]. However, there have been no previous studies of ATF3 expression in the non-neuronal cells of injured peripheral nerves or CNS glia during Wallerian degeneration. We show that ATF3 is upregulated by Schwann cells in degenerating segments of peripheral nerves and downregulated again following axonal regeneration. In contrast, CNS glia do not upregulate ATF3 during Wallerian degeneration.

Results

ATF3 expression in peripheral glia

In the sciatic nerve contralateral to injury and in unoperated control rats and mice, no expression of ATF3 was observed. In all nerve injury experiments the extent of axonal regeneration was assessed by immunofluorescence for neurofilament protein.

Sciatic nerve resection

Sciatic nerve resection in adult rats created a gap between the proximal and distal stumps. Outgrowths from the proximal and distal stumps into the gap were noticeable by 8 days after operation (dpo), and at 16 dpo or later, a thin strand of regenerated nerve connected the two stumps in some animals. The numerical density of cells in the endoneurium of the distal stump, as demonstrated by bisbenzimidazole staining of cell nuclei, increased 2 or 3 fold between 4 dpo and 16 dpo and remained high at 30 dpo.

By 1 dpo some cells in the endoneurium of the distal stump were ATF3+ but by 4 dpo large numbers of strongly ATF3+ nuclei were present throughout the endoneurium of the distal stump of the injured nerves (Fig. 1B). ATF3+ cells were of different sizes and shapes. They were rarely found in the perineurium but a few were present in the epineurium of the nerve, often associated with the tunica adventitia of blood vessels. Approximately 50% of the cell nuclei in the endoneurium were ATF3+ at 4 dpo (Fig. 2). In contrast, there were very few ATF3+ cell nuclei in the proximal stumps of the injured nerves at this stage (Fig. 1A). At 8 dpo, the number of ATF3+ cells in the distal stump had increased (Fig. 1D); approximately 70% of the cell nuclei in the distal stumps and their outgrowths were ATF3+. A few cells in the proximal stumps or their outgrowths were ATF3+ at 8 dpo (Fig. 1C), but most were only weakly immunoreactive. The situation remained similar at 16 dpo (Figs. 1E,1F). At 30 dpo the number of ATF3+ cells in the endoneurium of the distal stumps depended upon the extent of axonal regeneration that had occurred (Fig. 1H; c.f. proximal stump Fig. 1G). The only experiments in which significant numbers of axons did not regenerate into the distal stump were those in which the proximal stump was ligated. In such experiments the percentage of ATF3+ nuclei in the distal stump remained high (Fig. 2), similar to the counts at 16 dpo. However, in experiments in which the nerve was transected without ligation, the proportion of ATF3+ endoneurial cells varied from 10% to 56%. ATF3+ nuclei were found in cells apparently in contact with regenerating axons (Figs. 3A,3B), indicating that ATF3 is not immediately downregulated by Schwann cells on contact with axons. However, there was a tendency for ATF3+ endoneurial cells to be less abundant when regeneration was extensive (Fig. 3C). A few more cells in the endoneurium of the proximal stump had become ATF3+ by 16–30 dpo. There was no fluorescence in sections of nerve reacted without primary antibody, except for some autofluorescent cells found near the cut ends.

Sciatic nerve transection and reanastomosis

This procedure in adult rats allowed the regeneration of axons from the proximal stump into the distal stump of the injured nerve. Even at 4 dpo regenerating axons, identifiable as thin, continuous neurofilament-positive fibres, were present in parts of the distal stump within a few millimetres of the anastomosis, although a large amount of more particulate neurofilament immunoreactivity remained in the degenerating nerve fibres of the distal stump. By 16 dpo there was extensive regeneration of axons into the distal stump. Large numbers of ATF3+ cell nuclei were present in all parts of the endoneurium of the distal stump by 4 dpo and constituted 35 % of the total population of nuclei (Fig. 2C). By 16 dpo the number of ATF3+ endoneurial cells remained similar but the

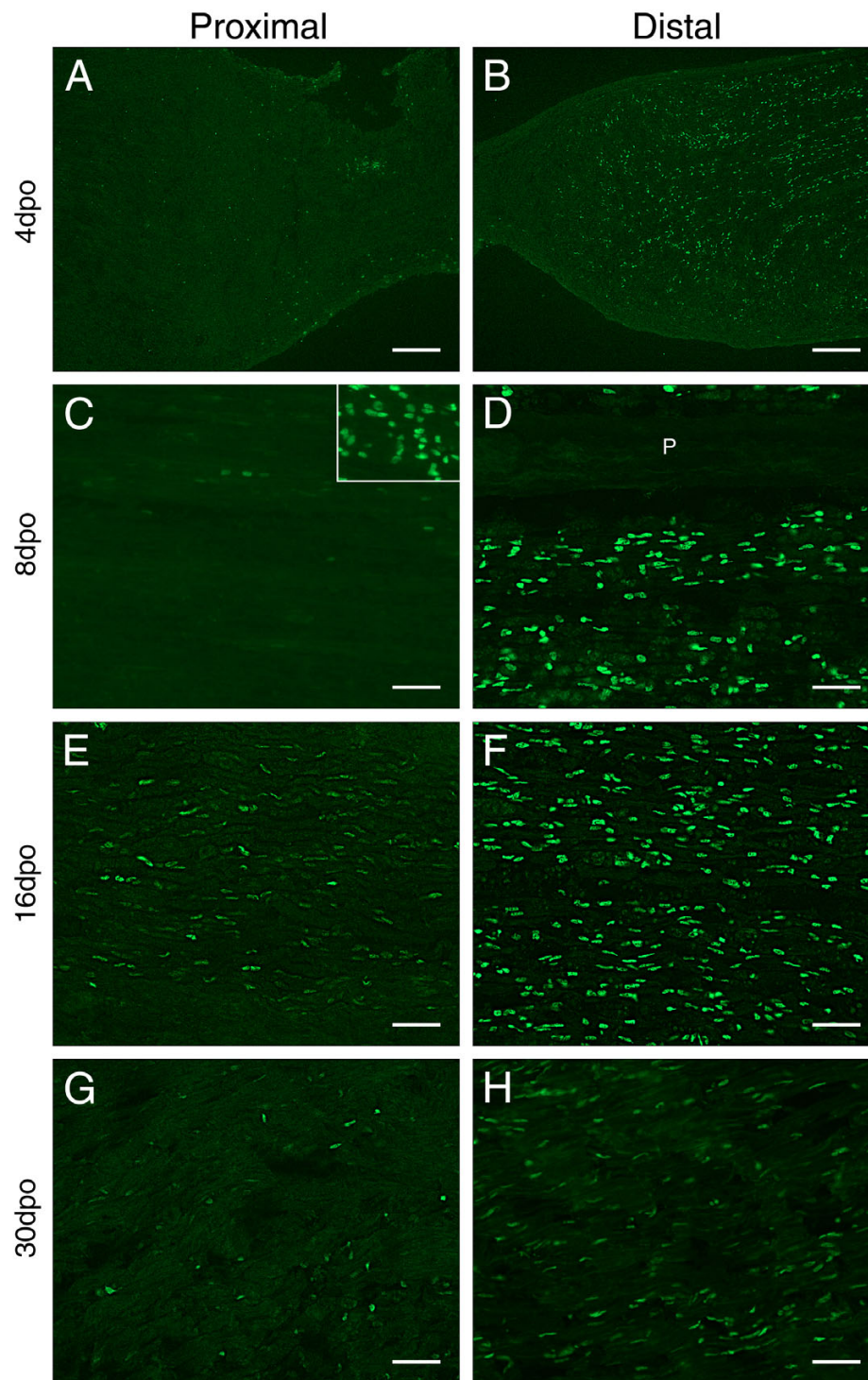


Figure 1

ATF3 immunoreactivity in the proximal and distal stumps of severed sciatic nerves in adult rats at 4, 8, 16 and 30 dpi. Figs. 1A, 1C, 1E and 1G show proximal stumps and Figs. 1B, 1D, 1F and 1H show distal stumps at corresponding survival times. ATF3 is strongly expressed by many cells in the distal stump at all times after injury but is more weakly expressed in the proximal stump, and by fewer cells. The inset in Fig. 1C shows part of a distal stump photographed at the same settings as the main panel. Note that there is no ATF3 immunoreactivity in the perineurium (P in Fig. 1D) or in intact sciatic nerve (not shown). Magnification bars = 200 μ m in A and B; 50 μ m in C and H.

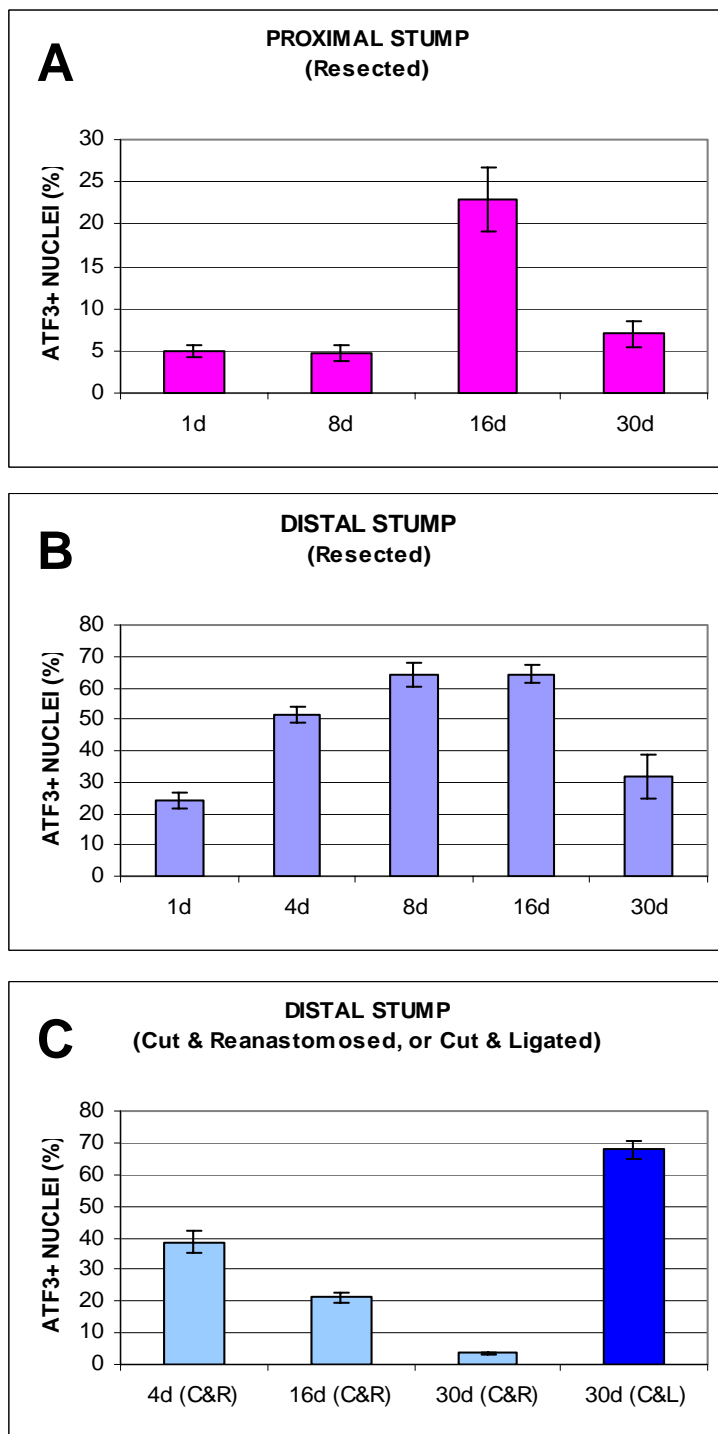
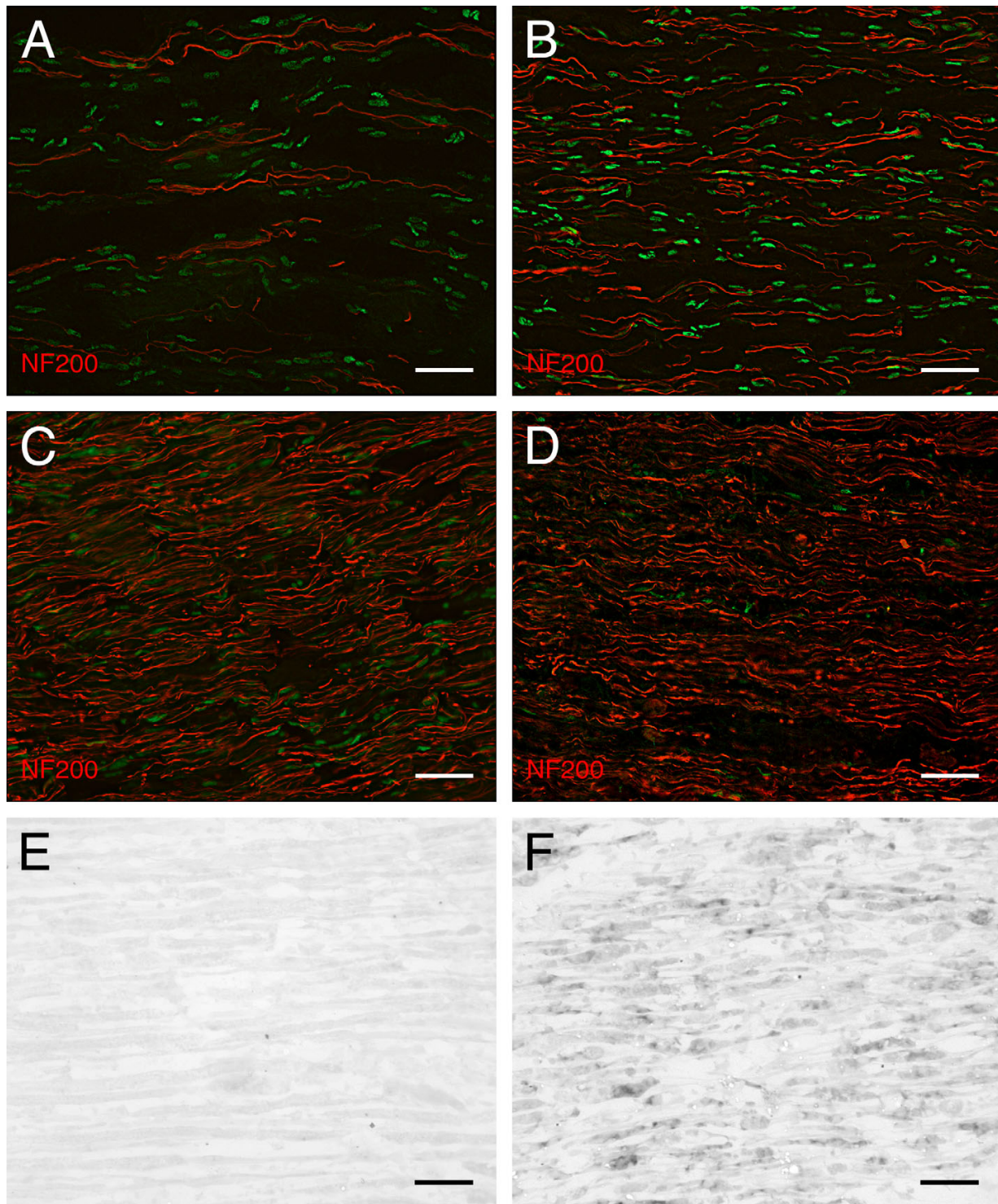


Figure 2

Histograms showing the percentage of ATF3+ nuclei in the proximal (A) and distal (B, C) stumps of injured sciatic nerves in adult rats. The data in Figs. 2A and 2B are from animals in which the sciatic nerve was cut and a segment resected, resulting in restricted regeneration. The ATF3+ nuclei in the proximal stumps were more weakly stained as well as less numerous than those in the distal stump. There is no bar for 4 dpo in Fig. 2A because so few nuclei were ATF3 immunoreactive (<1%). Fig. 2C allows a comparison between the number of ATF3+ nuclei in the distal stumps of nerves that were cut and reanastomosed, to allow axonal regeneration, and nerves which were cut and ligated, to prevent regeneration. It is apparent that axonal regeneration into distal stumps is associated with a downregulation of ATF3.

**Figure 3**

Sciatic nerves from adult rats. Figs. 3A,3B and 3C, show the variation in ATF3 staining (green) and axonal regeneration (neurofilament immunoreactivity-red) in the distal stumps of resected sciatic nerves at 30 dpo. Some nuclei are closely related to regenerating axons; axonal regeneration does not result in immediate downregulation of ATF3. Fig. 3D shows the distal stump from a sciatic nerve which was cut and reanastomosed 30 days previously. Axonal regeneration is extensive and ATF3 expression has declined. Figs. 3E and 3F are in situ hybridization preparations showing the absence of ATF3 mRNA in intact nerve (E) and its upregulation in the distal stump of a resected nerve at 8 dpo (F). Magnification bars = 50 μ m.

frequency of ATF3+ cell nuclei had fallen to approximately 20%. Some ATF3+ cells in the distal stump were found in close proximity to regenerating axons. By 30 dpo most ATF3 immunoreactivity had disappeared from the distal stump (Fig. 3D) and most of the cells which remained ATF3+ (<5% of endoneurial cells) were much less strongly immunoreactive than at earlier times. The proximal stumps contained only a few ATF3+ cells, located very close to the site of reanastomosis.

Expression of c-jun following sciatic nerve injury

Strong c-jun immunoreactivity was found in the endoneurium of distal segments of injured sciatic nerves at times when ATF3 immunoreactivity was also expressed.

ATF3 mRNA

In situ hybridization demonstrated that ATF3 mRNA was absent from intact sciatic nerves but present in distal stumps at 8 dpo (Figs. 3E,3F).

Nerve injury in mice

To confirm that the pattern of ATF3 expression in damaged nerves is similar in other species, the left sciatic nerves of adult mice were transected or had a 3 mm segment resected. Sciatic nerve resection produced results similar to those obtained with rats. ATF3 was present throughout the distal stump but very little expression was observed in the proximal stump at 5, 8, 17 and 30 dpo. Simple transection of nerves allowed considerable axonal regeneration to occur and resulted in endoneurial expression of ATF3 in the distal stump at 4 dpo and 8 dpo (Fig. 4) but not at 20 dpo.

Sciatic nerve transection in neonatal rats

Four and seven days after the sciatic nerve was transected in P1 rat pups, the distal stumps contained numerous ATF3+ nuclei (Fig. 5), which were, however, slightly less intensely immunoreactive than those in injured adult nerve.

Identity of ATF3+ cells

In the endoneurium of the distal stump, most ATF3+ nuclei were found in S100+ and p75+ cells (Figs. 6A,6B), indicating that Schwann cells comprised the major ATF3+ population. Using phase contrast optics, ATF3+ nuclei could be identified within bands of Büngner between macrophages containing myelin debris (Fig. 6C). Cells surrounding endoneurial blood vessels (i.e. endothelial cells and pericytes) in the distal stump were consistently ATF3-negative, except in the distal stump of severed nerves at 1 dpo, where a minority of nuclei around vessels were weakly immunoreactive. The ATF3+ cells at the periphery of epineurial blood vessels in injured nerves were S100+ and presumed to be Schwann cells of injured nervi vasorum.

ATF3 expression in CNS glia

Dorsal root transection

Each dorsal rootlet contains a cone of CNS tissue, termed the dorsal root entry zone (DREZ). Following dorsal root injury, astrocyte processes extend outward from the DREZ into the rootlets. Axons regenerate along the rootlets towards the DREZ but only a few grow back into the spinal cord [6,7]. At 1, 4 and 8 dpo ATF3+ cell nuclei were present in the dorsal roots and rootlets, between the site of transection and the DREZ (Figs. 6D,6E). At 1 dpo, ATF3+ nuclei were more numerous near to the injury site than near to the DREZ, but at later stages they were present throughout the segment of the root between the lesion and the spinal cord. No strongly ATF3+ cells were found in the DREZ or in the dorsal column rostral to the DREZ, which was undergoing Wallerian degeneration. However, some ATF3+ nuclei were found at the CNS/PNS interface.

Expression of c-Jun during Wallerian degeneration

Cell nuclei immunoreactive for c-Jun were found in the dorsal roots between the lesion site and the spinal cord (Fig. 6F). Nuclei clearly immunoreactive for c-Jun were not found in the DREZ or degenerating dorsal columns of the spinal cord.

Optic nerve crush

Following optic nerve crush in adult rats there is considerable axonal sprouting in the proximal stump [8] but only very limited axonal regeneration [9]. At 4, 8 and 30 dpo there were a few strongly ATF3+ cell nuclei in the meninges and in the lesion site (Fig. 6G). A few cells in the proximal and distal stumps exhibited a signal slightly above background levels; this was not present in controls in which the primary antibody was omitted, but we believe that such staining may represent non-specific binding of the rabbit antiserum. Many (but not all) large nuclei in the ganglion cell layer of the retina on the operated side were strongly ATF3+ (Fig. 6H) at all survival times examined. No ATF3 was present in ganglion cell layer nuclei on the unoperated side. This confirms previous findings [10] that ATF3 is upregulated by axotomised retinal ganglion cells.

Discussion

We have shown that ATF3 expression is initiated by endoneurial cells, including many Schwann cells, in the distal stump of injured nerves within 24 hours of axotomy, that it is eventually downregulated following axonal regeneration into the distal stump, but that its expression remains high if regeneration is reduced by nerve resection. Following dorsal root injury the cells around degenerating axons in the dorsal root upregulate ATF3 but cells in the CNS tissue of the DREZ and dorsal columns, where the same axons are degenerating, do not upregulate ATF3.

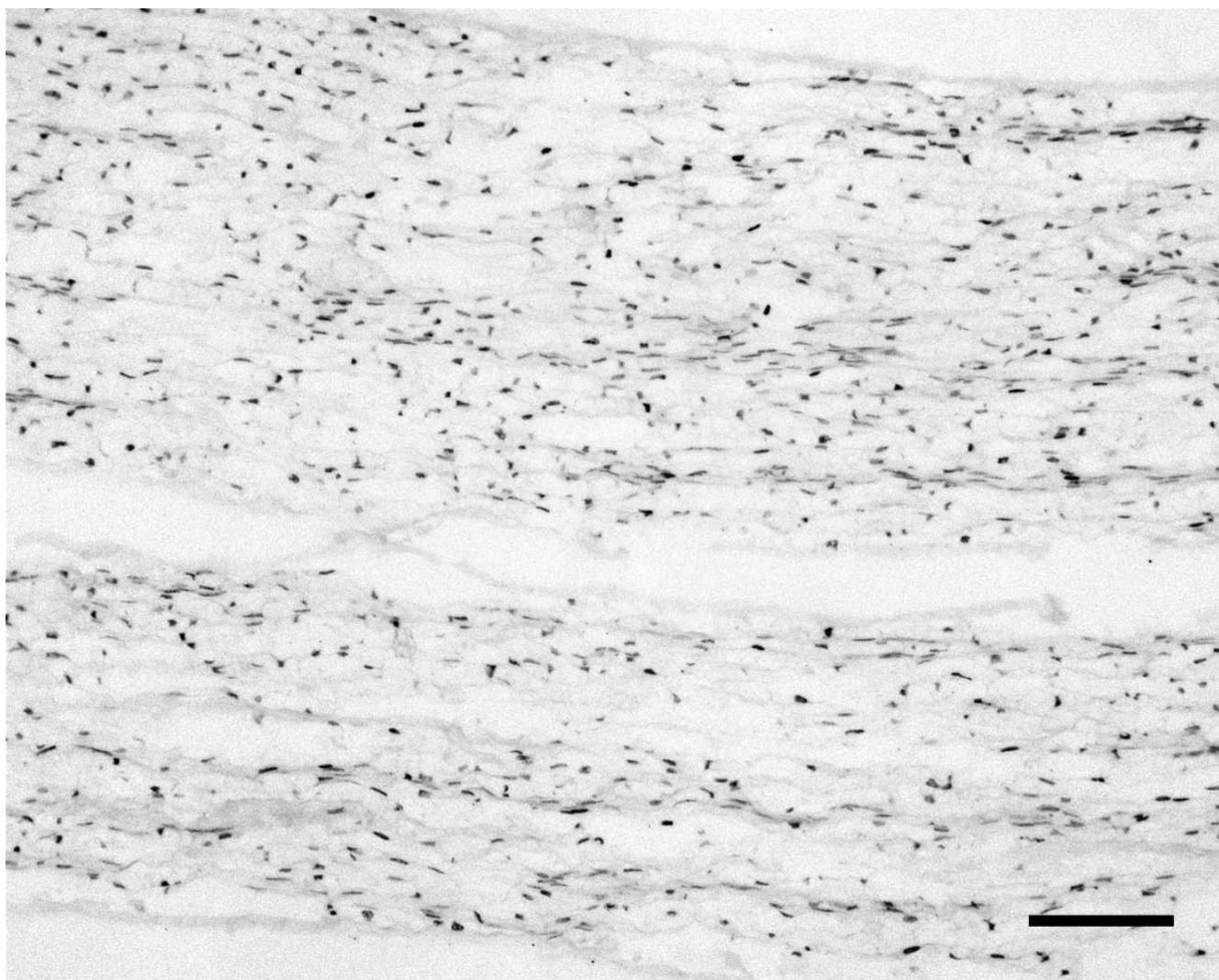


Figure 4

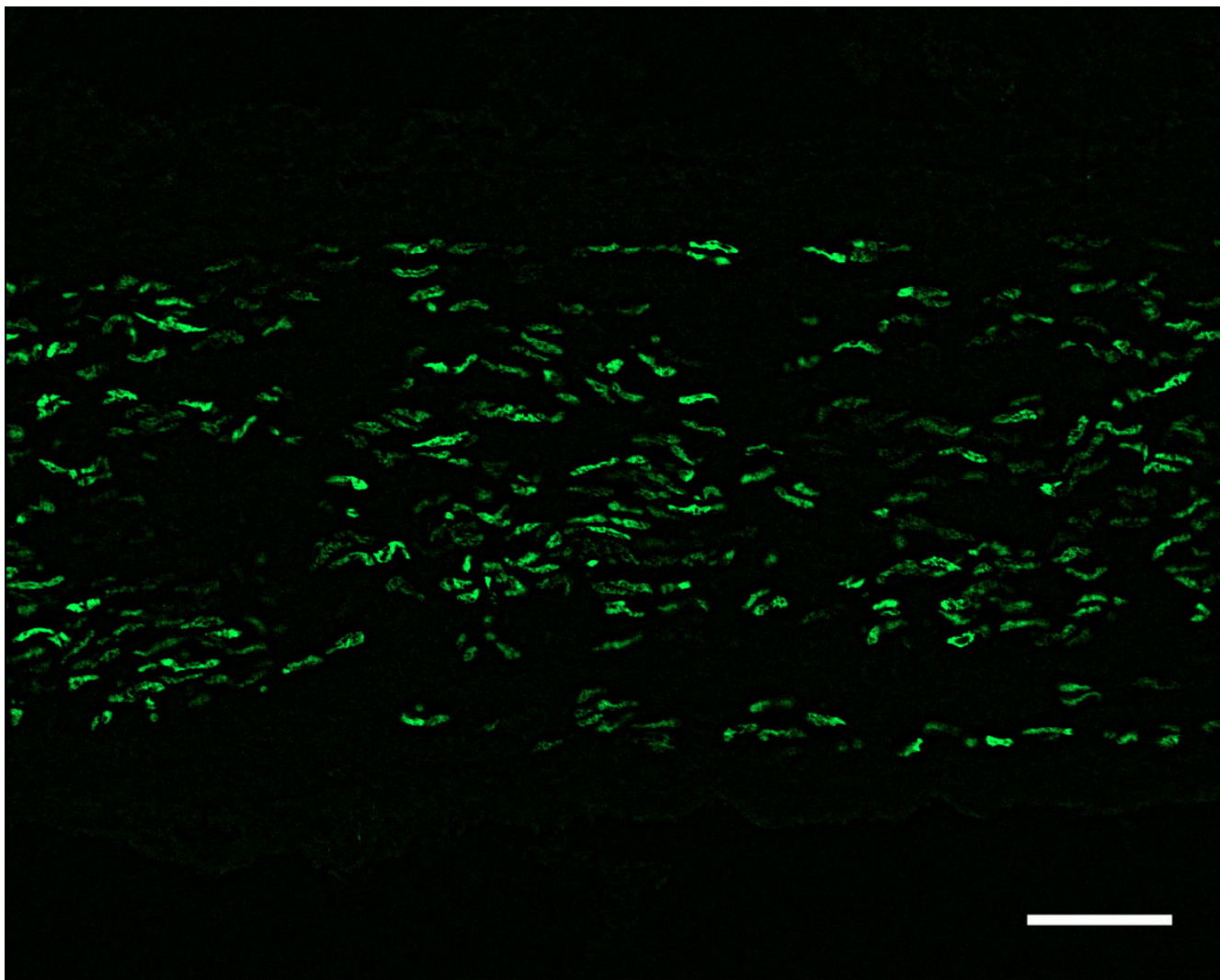
The distal stump of a transected sciatic nerve from an adult mouse at 8 dpo, immunoreacted for ATF3 using the ABC technique. The endoneurium contains many ATF3+ nuclei. Magnification bar = 50 μ m.

Direct injury to optic nerves resulted in strong expression of ATF3 by some cells at the lesion site and weak immunoreactivity in the proximal and distal stumps.

The expression of ATF3 by endoneurial cells appears to be negatively regulated by signals from axons. Nerve injury causes death of the axons in the distal stump, resulting in upregulation of ATF3 in the endoneurium. This does not appear to be a general stress response since ATF3 was not upregulated by perineurial cells or by most endothelial cells in the distal stump, both of which undergo considerable changes after nerve injury, or by most cells at the injured end of the proximal stump. The ATF3 expressing

cells in the distal stump include S100+, p75+ Schwann cells but may also include other cell types, because of the variety of size and shape of the ATF3+ nuclei. The pattern of expression of ATF3 in damaged nerves strongly resembles that of c-Jun [11,12].

CNS glia in the DREZ and dorsal columns do not upregulate ATF3 during Wallerian degeneration. Following dorsal root section, ATF3 was upregulated around the degenerating axons in the part of the root containing peripheral glia but not by glia around the same degenerating axons within CNS tissue. In crushed optic nerves the pattern of immunoreactivity was somewhat different, in

**Figure 5**

The distal stump of a sciatic nerve from a neonatal rat. The nerve was resected at P1 and the animal killed at P5. Many endoneurial cells express ATF3. Magnification bar = 50 μ m.

that ATF3 was clearly expressed by some cells in the lesion sites, and there was weak immunoreactivity in the proximal and distal stumps. The main difference between optic nerve and dorsal root injuries is that only the former involve direct damage to the CNS. Lesion sites on optic nerves are invaded by a number of different types of cell including macrophages, meningeal cells and Schwann cells [8]. It has been shown previously that c-Jun is not upregulated in damaged optic nerves [3]. We conclude that CNS glia do not upregulate ATF3 in the course of Wallerian degeneration, although they may weakly upregulate ATF3 in response to a direct injury. It appears that in CNS glia, ATF3 expression, like that of c-Jun, is not nega-

tively regulated by signals from axons, and that the transcriptional regulation of Wallerian degeneration in the CNS is very different from that in peripheral nerves. It has long been assumed that reactive astrocytes are an impediment to axonal regeneration in the CNS but it is now clear that one reason for the failure of axonal regeneration in the CNS may be that the reaction of CNS glia to axotomy is inadequate to support the regenerative process.

ATF3 is likely to be involved in the transcriptional regulation of the changes in gene expression by Schwann cells by which bands of Büngner are prepared for their role in supporting axonal regeneration. A large number of

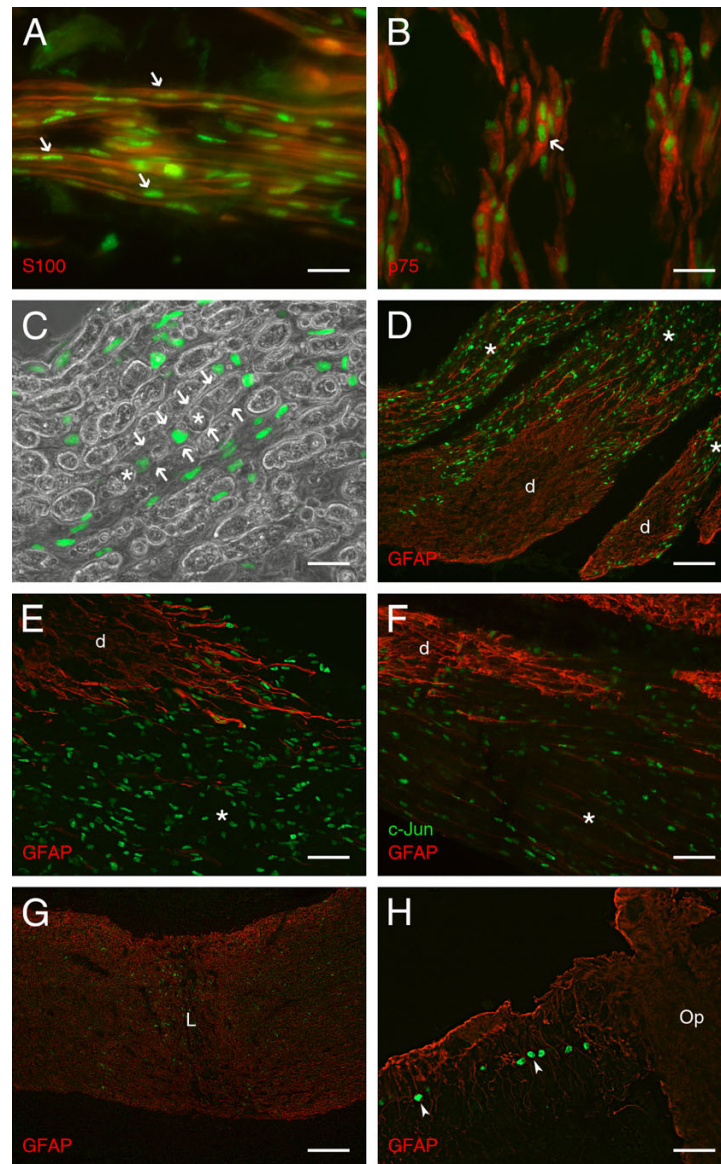


Figure 6

Figs. 6A and 6B show the distal stumps of sciatic nerves 30 days (A) and 8 days (B) after resection of a piece of the nerve. ATF3+ nuclei (green) are found in cells that co-express the Schwann cell markers S100 (red in A) and p75 (red in B). The arrows indicate some of the nuclei clearly situated in cytoplasm expressing the Schwann cell markers. Fig. 6C is a double exposure showing a phase contrast image and ATF3 immunofluorescence (green) of a distal stump of a nerve 4 days after resection. ATF3+ nuclei are found between debris-laden macrophages (*) inside bands of Büngner (one of which is outlined by arrows). Figs. 6D and 6E show sciatic nerve dorsal rootlets immunoreacted for ATF3 and GFAP, 8 days after dorsal rhizotomy. The dorsal root entry zone of the spinal cord (d) can be identified by its content of GFAP+ astrocytes (red) which extend processes into the peripheral part of the rootlets (*). ATF3 expression is largely confined to the peripheral rootlet where Schwann cells are found. Fig. 6F shows a dorsal rootlet immunoreacted for c-Jun (green) and GFAP (red) 24 hours after dorsal rhizotomy. The pattern of c-Jun expression precisely follows that of ATF3 (compare with Fig. 6E), being confined to the peripheral, Schwann cell-containing part of the rootlet (*) and being absent from the dorsal root entry zone (d). Figs. 6G and 6H are taken from a rat in which the left optic nerve was crushed 30 days previously, and are immunoreacted for ATF3 (green) and GFAP (red). In Fig. 6G the injury site (L) is marked by reduced GFAP staining. Some nuclei in the lesion are clearly ATF3+ and some cells within the optic nerve tissue proximal and distal to the lesion are very weakly ATF3+ but the expression is much weaker than in damaged peripheral nerves. Fig. 6H is taken from the retina of the same animal, and shows ATF3+ nuclei in the ganglion cell layer (arrowheads). Op = optic nerve head. Magnification bars = 25 μ m in A-C; 100 μ m in D and G; and 50 μ m in E, F and H.

changes in gene expression occur in the distal stump of injured nerves [13]. The denervated Schwann cells are known to upregulate expression of a number of cell surface molecules which are presumed to aid axonal regeneration, including NCAM, L1, CHL1 and p75, while markers of the myelinated state including MAG and P0 are downregulated [14,15]. Expression of neurotrophic molecules including NGF and BDNF [16], and extracellular matrix molecules including collagens, laminin and tenascin-C [17-19] are increased in the endoneurium. The expression of several cytokines is dramatically upregulated in the endoneurium of damaged nerves, e.g. [20,21]. It is not yet known, however, whether any of these genes is controlled by ATF3. Since ATF3 and c-Jun are both expressed by Schwann cells in the distal stumps of damaged peripheral nerves and they are known to be capable of interacting to form heterodimers, it is tempting to speculate that such heterodimers are a key modulator of gene expression during Wallerian degeneration. ATF3 and c-Jun in extracts from neuronal cell lines co-precipitate [22]. ATF3/c-Jun heterodimers are known to bind to different sites and have different effects on gene expression to ATF3 homodimers (in particular they activate rather than repress gene expression [4]), but their target genes in peripheral glia remain to be identified. Finally, since ATF3 and c-Jun are co-expressed both by regenerating neurons and the glial cells through which they regenerate, the possibility that they are controlling the same genes in both types of cell must be raised. The cell adhesion molecule CHL1 and GAP-43 are upregulated by motor and sensory neurons and by Schwann cells in injured nerves during axonal regeneration [23-26] and many other molecules, such as p75, are upregulated by Schwann cells and some axotomised neurons; it will be interesting to see if they are regulated by ATF3/c-Jun heterodimers. The GAP-43 gene has an AP-1 site [27] but there is little evidence that it is regulated primarily by c-Jun [28]. Recently, it has been reported that ATF3 is anti-apoptotic and a neurite growth-promoting factor in cultured neurons, apparently acting through HSP27 [29,22]. Such properties would also be valuable if displayed by Schwann cells in the distal stumps of injured nerves.

Conclusions

ATF3 and c-Jun are expressed by Schwann cells, but not CNS glia, during Wallerian degeneration and may control the programme of gene expression by which Schwann cells support axonal regeneration.

Methods

Animal utilization and surgical procedures

All surgical procedures were approved by the UCL ethical committee and licenced by the Home Office. Adult female Sprague Dawley rats, rat pups on postnatal day 1 (P1) and adult C57bl mice of both sexes were anaesthetised with

Halothane, nitrous oxide and oxygen mixture. In 20 rats the left sciatic nerve was transected in the thigh, and 2–3 mm of the nerve resected to create a gap between the proximal and distal stumps. This allows slow regeneration of axons with few entering the distal stump before 16 days post operation (dpo) but variable and sometimes substantial numbers of axons present in the distal stump at 30 dpo. These animals were killed at 1 dpo (n = 4), 4 dpo (n = 3), 8 dpo (n = 3), 16 dpo (n = 3) and 30 dpo (n = 4). In three of the animals with resected nerves the proximal stump was ligated and turned aside to prevent axonal regeneration into the stump. These rats were killed at 30 dpo. In 9 rats the left sciatic nerve was transected and reanastomosed with 10/0 sutures to allow rapid axonal regeneration into the distal stump. These rats were killed at 4 dpo (n = 3), 16 dpo (n = 3) and 30 dpo (n = 3). In 8 rats the left L3-6 dorsal roots were cut using microsurgical scissors. This produces Wallerian degeneration in the segment of dorsal root between the lesion and the spinal cord, in the dorsal root entry zone (DREZ) and in the ascending dorsal column (fasciculus gracilis) of the spinal cord rostral to the lesion. The animals were killed at 1 dpo (n = 3), 8 dpo (n = 3) and 30 dpo (n = 2). In 6 rats the left optic nerve was crushed with watchmakers' forceps 2 mm from the eyeball. These rats were killed at 4 dpo (n = 2), 8 dpo (n = 2) and 30 dpo (n = 2). In 6 P1 rat pups the left sciatic nerve was transected. The animals were killed at 4 dpo (n = 3) and 7 dpo (n = 3). In 4 adult C57bl mice the left sciatic nerve was transected. This allows rapid axonal regeneration because the ends soon become reapposed. These mice were killed at 4 dpo (n = 1), 8 dpo (n = 1) and 20 dpo (n = 2). Approximately 3 mm was resected from the left sciatic nerve of 7 further mice which were killed at 5 dpo (n = 2), 8 dpo (n = 2), 17 dpo (n = 2) and 30 dpo (n = 1). All animals were killed by decapitation while deeply anaesthetised. Samples from operated and unoperated animals of the sciatic nerves, spinal cord, optic nerves and retina were fresh-frozen in Tissue Tek (Sakura Finetek, Netherlands), cooled by dry ice.

Antibodies and immunohistochemical techniques

Fresh-frozen sections were cut at 20 μ m, directly onto Superfrost Plus slides using a Bright cryostat and fixed in 4% paraformaldehyde (PFA). The sections were rinsed several times in phosphate buffered saline (PBS) prior to incubation for 30 minutes at room temperature (RT) in blocking solution (0.1 M PBS, 5% NGS, 0.5% Triton-X 100). The samples were then incubated with primary antibodies, diluted appropriately in blocking solution, at RT overnight, rinsed several times with PBS and incubated with fluorophore-conjugated secondary antibodies, diluted in PBS, for two hours at RT. The sections were then rinsed several times in PBS before being stained with bisbenzimidazole (2.5 μ g/ml) for 7 minutes. The samples were rinsed several more times in PBS before being

mounted in DABCO. Primary antibodies: rabbit polyclonal anti-ATF3 (Santa Cruz); rabbit polyclonal anti-neurofilament 200 kDa antibody (Sigma); rabbit polyclonal anti-c-Jun antibody (a kind gift of Dr A. Behrens, Cancer Research UK, London, UK); mouse monoclonal anti-bovine glial fibrillary acidic protein (Sigma); mouse monoclonal anti-S100 (Sigma); mouse monoclonal anti-p75 (Sigma). Secondary antibodies: monoclonal tetramethylrhodamine conjugated goat anti-mouse (Molecular Probes); monoclonal Alexa-Fluor-conjugated goat anti-rabbit (Molecular Probes). Both secondary antibodies were adsorbed against rat serum prior to use. Control sections, which were not incubated with primary antibodies, were used to ensure signal specificity.

In situ hybridisation

In situ hybridisation was carried out as previously described using digoxigenin-labelled antisense and sense riboprobes [30,31]. The specificity of the ATF3 hybridisation signal was verified by comparison with the sections processed with sense probe under identical conditions.

Microscopy

All microscopy was performed using a Zeiss Axioplan microscope equipped with Openlab image analysis software. All immunofluorescence micrographs are digital images and are deconvolved except Fig. 1C, Fig. 1C inset, and Fig. 6C.

Statistical analysis

All sections for statistical analysis were immunoreacted for ATF3, and stained with bisbenzimidazole (which labels all nuclei). The percentage of ATF3+ nuclei was calculated from comparable fields, photographed using a $\times 20$ objective, from each of three adjacent sections of injured sciatic nerve, per animal. These data were collated with those from at least two other animals, depending on the injury model and timepoint (see above). The group average and the standard error of the mean were subsequently calculated. Graphs were generated in Microsoft Excel.

Authors' contributions

DH carried out immunohistochemistry, performed the statistical analysis and prepared the figures. MRJM made the ATF3 probe and carried out the *in situ* hybridization. K H-I. performed all the experiments on mice and some rat sciatic nerve injury experiments. PNA performed the surgery on rats and the photomicroscopy. JW provided technical assistance with all parts of the study. DH, K H-I, RC, ARL and PNA drafted the manuscript. DH and K H-I made the initial observation of ATF3 upregulation in the distal stumps of injured nerves, separately and contemporaneously. All the authors contributed to planning the study and to interpreting the results. All authors read and approved the final manuscript.

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