Enhancement of axonal regeneration by in vitro conditioning and its inhibition by cyclopentenone prostaglandins

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Summary
Axonal regeneration is enhanced by the prior ‘conditioning’ of peripheral nerve lesions. Here we show that Xenopus dorsal root ganglia (DRG) with attached peripheral nerves (PN-DRG) can be conditioned in vitro, thereafter showing enhanced neurotrophin-induced axonal growth similar to preparations conditioned by axotomy in vivo. Actinomycin D inhibits axonal outgrowth from freshly dissected PN-DRG, but not from conditioned preparations. Synthesis of mRNAs that encode proteins necessary for axonal elongation might therefore occur during the conditioning period, a suggestion that was confirmed by oligonucleotide microarray analysis. Culturing PN-DRG in a compartmentalized system showed that inhibition of protein synthesis (but not RNA synthesis) in the distal nerve impaired the conditioning response, suggesting that changes in gene expression in cultured DRG depend on the synthesis and retrograde transport of protein(s) in peripheral nerves. The culture system was also used to demonstrate retrograde axonal transport of several proteins, including thioredoxin (Trx). Cyclopentenone prostaglandins, which react with Trx, blocked the in vitro conditioning effect, whereas inhibition of other signalling pathways thought to be involved in axonal regeneration did not. This suggests that Trx and/or other targets of these electrophilic prostaglandins regulate axonal regeneration. Consistent with this hypothesis, morpholino-induced suppression of Trx expression in dissociated DRG neurons was associated with reduced neurite outgrowth.

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Key words: Axonal regeneration, Axonal transport, Mass spectrometry, Microarray analysis, Neurotrophin, Prostaglandin

Introduction
Axotomy causes extensive changes in gene expression (Costigan et al., 2002; Xiao et al., 2002; Tanabe et al., 2003; Cameron et al., 2003; Boeshore et al., 2004; Nilsson and Kanje, 2005; del Signore et al., 2006) and it is believed that induction of these regeneration-associated genes (RAGs) is important for axonal regeneration because it is faster in vivo and in vitro after prior ‘conditioning’ peripheral nerve lesions (Oblinger and Lasek, 1984; Lankford et al., 1998; Ekström et al., 2003 and references therein). Consistent with this hypothesis, neurite outgrowth from dissociated dorsal root ganglion (DRG) neurons undergoes a spontaneous transcription-dependent change from short, highly branched to long, un-branched processes after 1-2 days in culture, whereas DRG neurons conditioned by prior axotomy in vivo are able to extend long and/or un-branched neurites in the first day in culture – even in the presence of transcriptional inhibitors (Smith and Skene, 1997). Although the significance of most changes in gene expression of axotomised neurons is unknown, several of the upregulated proteins can influence axonal regeneration, including growth-associated protein-43 (GAP43), cytoskeleton-associated protein-23 (Bomze et al., 2001), the Rho-family GTPase TC10 (Tanabe et al., 2000), fibroblast-growth-factor-inducible protein 14 (Tanabe et al., 2003), small proline-rich repeat protein 1A (Bonilla et al., 2002), integrin α7 (Werner et al., 2000; Ekström et al., 2003; Gardiner et al., 2005), Jun (Raivich et al., 2004) and activating transcription factor 3 (ATF3) (Seijffers et al., 2006; Seijffers et al., 2007).

The mechanisms that lead to changes in gene expression in axotomised neurons are poorly understood but probably involve retrograde axonal transport of activating signalling molecules (reviewed by Hanz and Fainzilber, 2006; Zhou and Snider, 2006; Raivich and Makwana, 2007); their identification is of considerable importance. Signal transducer and activator of transcription 3 (STAT3) might act as a retrograde activating signal because it is rapidly phosphorylated in lesioned nerves and, subsequently, appears in the nuclei of both sensory and motor neurons (Haas et al., 1999; Sheu et al., 2000; Schweiger et al., 2000; Lee et al., 2004). Inhibition of STAT3 phosphorylation with AG490 suppresses regenerative axonal growth of adult DRG neurons (Liu and Snider, 2001; Qiu et al., 2005). Jun is essential for the expression of certain RAGs and optimal axonal regeneration (Raivich et al., 2004), and retrograde axonal transport of its activator Jun terminal kinase (JNK) has been demonstrated (Lindwall and Kanje, 2005). In their phosphorylated form, the extracellular signal-regulated protein kinases 1 and 2 (Erk1/2) are also retrogradely transported and involved in initiation of axonal regeneration (Perlson et al., 2005).
In the present investigation, we demonstrate that *Xenopus* dorsal root ganglia (DRG) with attached short peripheral nerves (PN-DRG) that were incubated for 3 days in serum-free medium (in vitro conditioned) show enhanced axonal growth in response to neurotrophins, similar to PN-DRG axotomized by sciatic nerve lesions (in vivo conditioned), even in the presence of actinomycin D (ActD), indicating prior synthesis of mRNAs necessary to support axonal regeneration. Using the in vitro conditioning model to investigate signalling mechanisms involved in the control of axonal regeneration, we show that this might depend (in part) on synthesis and retrograde transport of proteins that are encoded by mRNA within the peripheral nerve. By 2D gel electrophoresis (2-DE) and liquid chromatography tandem mass spectrometry (LC-MS/MS), several retrogradely transported proteins were identified, including the oxidoreductase thioredoxin (Trx). Pharmacological inhibition of Trx function by using cyclohexenone prostaglandins blocked the in vitro conditioning effect, whereas inhibition of other signalling pathways thought to be involved in axonal regeneration did not, suggesting that Trx and/or other redox-sensitive factors regulate RAG expression and axonal regeneration. Moreover, morpholino oligonucleotide (MO)-induced suppression of Trx levels in dissociated DRG neurons was associated with reduced neurite outgrowth.

**Results**

De novo transcription is required for axonal growth following conditioning in vitro

Since neurite elongation rates of dissociated rat DRG neurons increase spontaneously during culture (Smith and Skene, 1997), which suggests the ‘conditioning lesion effect’ is mimicked in vitro, we investigated this possibility using *Xenopus* PN-DRG, which show excellent survival in culture (Tonge et al., 2004). In the absence of trophic factors, freshly dissected PN-DRG in collagen gels showed limited axonal outgrowth (Fig. 1A), but outgrowth distance was significantly increased \((P<0.01)\) compared with in vitro conditioned preparations (Fig. 1B). Brain-derived neurotrophic factor (BDNF) markedly increased the lengths (by 91%; \(P<0.001\)) and apparent numbers of outgrowing axons from freshly dissected PN-DRG (Fig. 1C) but even more so from in vitro conditioned preparations (Fig. 1D), with a significant \((P<0.001)\) increase (by 157%) in axon length compared with freshly dissected preparations.

To investigate whether changes in axonal growth following in vitro conditioning were transcription dependent, effects of ActD on BDNF-induced axonal outgrowth from freshly dissected and in vitro conditioned PN-DRG were investigated. In the absence of neurotrophins, ActD significantly reduced axonal outgrowth distances from both freshly dissected and in vitro conditioned PN-DRG (by 64% and 68%; \(P<0.01\) and \(P<0.001\), respectively), compared with outgrowth from preparations cultured without ActD (Fig. 2). However, although ActD caused a marked \((P<0.001)\) reduction in axonal outgrowth distance from freshly dissected preparations (by 76%; Fig. 1E) in the presence of BDNF, it had little effect on in vitro conditioned preparations, which showed abundant axonal outgrowth for several days (Fig. 1F). Similar increases in axonal outgrowth from in vitro conditioned PN-DRG occurred in response to nerve growth factor (NGF), neurotrophin 3 (NT3) and neurotrophin 4 (NT4), also persisting in the presence of ActD (data not shown). These results (summarized in Fig. 2A) suggest that, during the initial conditioning period in vitro, neurons synthesize mRNAs that are necessary and sufficient to sustain subsequent axonal growth in response to neurotrophins for several days. To test this hypothesis, PN-DRG were incubated free-floating for 1 day with \(\alpha\)-amanitin (\(\alpha\)-AM), an irreversible RNA polymerase II inhibitor, followed by 3 days without the drug. When these preparations were subsequently cultured on collagen gels, (with or without ActD), BDNF did not increase axonal outgrowth (Fig. 2B). By contrast, PN-DRG that had been in vitro conditioned for 3 days before \(\alpha\)-AM exposure on day 4 showed vigorous BDNF-induced axonal outgrowth that was not significantly different from preparations incubated for 4 days without \(\alpha\)-AM treatment or significantly reduced by ActD (Fig. 2B), indicating that mRNA required for axonal growth is synthesized during the first 3 days in culture.

Transcriptional dependence of axonal growth following axotomy in vivo

To determine whether in vivo conditioned DRG neurons also show transcription-independent axonal regeneration, PN-DRG were cultured in collagen gels 3 or 5 days after unilateral sciatic nerve section. The mean axonal outgrowth distances from prior axotomized (in vivo conditioned) and contralateral (control) PN-DRG are summarized in Fig. 3. In the absence of BDNF, control PN-DRG showed sparse axonal outgrowth after 3 days in culture (similar to those shown in Fig. 1A) but axonal outgrowth distance...
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was slightly increased from 3-day in vivo conditioned PN-DRG and significantly so ($P<0.02$) from 5-day conditioned preparations (82% and 171%, respectively). In the presence of BDNF, dense axonal outgrowth occurred from control preparations (similar to Fig. 1C), with significant increases in axonal outgrowth distance ($P<0.02$) compared to preparations cultured without BDNF. Incubation with $\alpha$-AM on day 1 strongly inhibits subsequent BDNF-induced axonal outgrowth in collagen gels, but incubation with $\alpha$-AM on day 4 does not.

Changes in gene expression in cultured DRG

In view of the above observations, we carried out an oligonucleotide microarray analysis to compare gene expression in freshly dissected and 3-day in vitro conditioned DRG. Results of this experiment showed extensive increase and decreases in gene expression (Table S1 in supplementary material) with 58 identified genes that were upregulated fivefold or more in 3-day in vitro conditioned compared with freshly dissected DRG (Table 1). To verify changes in gene expression, quantitative PCR (qPCR) was carried out using the cDNA samples from control and conditioned DRG that had been used in the array analysis, with primers recognizing six genes that had been selected for their relevance in axonal regeneration. Arginase and galectin expression increase during axonal regeneration (Lange et al., 2004; Horie et al., 2005). Transportin mediates nuclear import of proteins lacking conventional nuclear localization signals (Carson et al., 2006) and might therefore be involved in the transport of signalling molecules that regulate expression of RAGs – similar to the role of importins in rodents, (Hanz et al., 2003). Vg1 RNA binding protein (Vg1RBP) mediates the translocation of $\beta$-actin and cofilin mRNAs to growth cones where it is involved in local protein synthesis during growth cone turning (Leung et al., 2006; Piper et al., 2006). Secreted frizzled-related protein sequence protein 1 (SFRP) is involved in axonal growth of retinal ganglion cells (Rodriguez et al., 2005) and Xenopus Hen1 is a transcription factor involved in neural development (Bao et al., 2000). Therefore, both might control expression of genes required for axon elongation. The results, normalized to those of 18S RNA, show all six genes were upregulated at least twofold (Fig. 4) consistent with extensive
changes in gene expression detected by microarray analysis in conditioned DRG.

Investigation of signalling pathways that regulate RAG expression

To investigate the involvement of signaling pathways thought to be involved in the initiation of axonal regeneration, we incubated PN-DRG for 3 days in medium that contained different kinase inhibitors, followed by culture for 3 days with BDNF in collagen gels, with and without ActD. The kinase inhibitors included AG490 (at 2 μM, 10 μM and 50 μM), which inhibits the Janus kinases 2 and 3 (JAK2 and JAK3, respectively) (Meydan et al., 1996; Lin et al., 2005) that are required for STAT3 phosphorylation; PD98059 (at 50 μM) and U0126 (at 10 μM), inhibitors of mitogen activated protein (MAP) kinases including Erks (Dudley et al., 1995; Favata et al., 1998); and SP600125 (at 200 μM), a JNK inhibitor (Bennett et al., 2001). We also incubated preparations with LY294002, an inhibitor of phosphoinositide 3-kinase (PI3K) (Vlahos et al., 1994) because of PI3K’s importance in axonal growth (reviewed by Zhou and Snider, 2006); k252a, which inhibits high-affinity neurotrophin receptors (Trks) and other kinases, including Ca2+/calmodulin-dependent protein kinase II (CAM kinase II), myosin light chain kinase, protein kinase A (PKA), protein kinase C (PKC) and protein kinase G (PKG) (Ruegg et al., 1989). We also used Ca2+-free RPMI medium because Ca2+ entry into lesioned nerves is thought to be important in initiating axonal regeneration (Perlson et al., 2005).

Results of these experiments (Fig. 5) showed that pre-incubation of PN-DRG with 50 μM AG490 inhibited subsequent BDNF-induced axonal outgrowth with and without ActD (by 80% and 57%, respectively; P<0.001 and P<0.005, respectively). However, at concentrations of AG490 as low as 2 μM also strongly inhibited BDNF-induced axonal outgrowth from both freshly dissected and in vitro conditioned preparations, similar to findings by Liu and Snider (2001). These results indicate that JAK/STAT signalling is necessary for axonal regeneration even after the induction of RAGs, and/or that AG490 also affects cells in other ways. For example, in addition to inhibiting JAK2, AG490 has antioxidant properties (Gorina et al., 2007); so, results of experiments involving its use should be treated with caution. In the presence of the other inhibitors and in Ca2+-free RPMI medium, BDNF-induced axonal outgrowth was generally reduced as expected from other studies (reviewed by Zhou and Snider, 2006). However, prior incubation of the preparations with these inhibitors or culturing in Ca2+-free medium did not impair subsequent BDNF-induced axonal outgrowth, even in the presence of ActD (data not shown), suggesting the expression of RAGs was not inhibited.

Since our experiments did not confirm the involvement of hitherto-postulated signalling pathways that control the expression of genes required for axonal regeneration, we used the PN-DRG preparations to investigate its dependence on axonal protein synthesis because mRNA translation of β-importin and vimentin in lesioned axons is important for the retrograde transport of signalling proteins.
molecules that are involved in the initiation of axonal regeneration (Hanz et al., 2003; Perlson et al., 2005). Translation of many proteins is known to occur in axons (Giuditta et al., 2002; Piper and Holt, 2004; Willis et al., 2005) and may be essential for efficient axonal regeneration (Verma et al., 2005). To investigate the involvement of axonal protein synthesis for the induction of genes required for axonal regeneration, PN-DRG preparations were incubated in two-compartment culture dishes (Edbladh et al., 1994) allowing localized application of drugs to either DRG or the cut end of the attached peripheral nerve. After 3 days, the DRG and the proximal ~1-2 mm part of the peripheral nerve (which had not been exposed to CHX) in the inner compartment (with or without CHX), prior to culturing the DRG and proximal part of the peripheral nerve (which had not been exposed to CHX) in collagen gels with BDNF (with or without ActD). Protein synthesis inhibition in the peripheral nerve significantly reduced subsequent BDNF-induced axonal outgrowth in the presence of ActD.

In three separate experiments, 2-DE gels of DRG proteins stained with silver showed several hundred spots (Fig. 7) of which only ~35 were radioactive on each gel. 2-DE gels of the control DRG (from the outer compartments) showed similar patterns of (non-radioactive) silver-stained spots (data not shown) and addition to the inner compartment of vinblastine (100 μM), which blocks axonal transport in amphibian nerves (Hanson and Edstrom, 1977), also prevented appearance of radio-labelled proteins in the DRG, indicating that this depends on retrograde axonal transport rather than isotope leakage. This conclusion is also reinforced by the fact that on 2D-gel autoradiographs of DRG containing [35S]methionine-[35S]cysteine, virtually all silver stained spots were radioactive (data not shown), a pattern completely different to the radioactive spots from a silver-stained gel were excised and subjected to trypsin digestion, LC-MS/MS and database searching, resulting in the identification of 32 proteins (Table 2). However, 13 spots contained more than one protein and in those cases it was uncertain which protein was radioactively labelled and therefore retrogradely transported. Several proteins in the radioactive spots, including cofilin, profilin, vimentin, actin, tubulin, UchL-1, enolase,
glyceraldehyde-3-phosphate dehydrogenase and aldolase, are translated from mRNAs in regenerating axons (Willis et al., 2005) and retrograde axonal transport of vimentin has been demonstrated (Perlson et al., 2005). To investigate the presence of mRNAs encoding other proteins than those identified in the spots, we harvested axons that had been grown in matrigel through 8-μm pores in a membrane barrier and were therefore free from contaminating migratory cells (Fig. 8). To confirm the absence of cellular contamination, others (see also Willis et al., 2005) have used the absence of γ-actin as a marker of axonal RNA purity as this is restricted to neuronal cell bodies (Bassell et al., 1998). However, several bands were consistently detected in the present samples by using primers for Xenopus γ-actin, making this approach unsuitable here. As a consequence, both axonal and cell-body samples were assayed for both genomic DNA as well as Trx mRNA because we identified the latter as a candidate axonal mRNA (see below). Assaying the genomic DNA samples by PCR for a 5’ flanking sequence of the zinc-finger transcription factor GATA2 (Brewer et al., 1995) revealed a strong signal in the cell body samples, whereas levels were insignificant in the axonal preparation (Fig. 9). By contrast, reverse transcriptase (RT)-PCR revealed that Trx cDNA were abundant in both axonal and cell-body samples, confirming that any cellular contamination was either absent or negligible.

RT-PCR of cDNAs generated from mRNAs of the outgrowing axons in matrigel by using primers that recognize seven cDNAs that correspond to proteins identified in the radioactive spots (and, therefore, presumably retrogradely transported) showed that mRNAs for all seven proteins were present in the axons (Fig. 10). This suggests that most proteins in the radioactive spots on the 2D gels can be translated in axons and subsequently be transported to the soma.

The retrogradely transported proteins comprised several different classes, including enzymes and cytoskeletal molecules. However, apart from dynein and vimentin, which are known to be involved in retrograde axonal transport (Vallee et al., 2004; Perlson et al., 2005), none of the signalling molecules thought to be involved in initiating axonal regeneration – such as STAT3, JNK or Erk1/2 (reviewed by Hanz and Fainzilber, 2006) – was detected, although the possibility that such factors are first synthesized in the soma and then anterogradely transported along axons before returning by retrograde transport cannot be excluded. However, our finding that Trx might be retrogradely transported could be relevant because in addition to functioning as an anti-oxidant, it also regulates activity of various signalling molecules including apoptosis signalling kinase, JNK and PKC. Furthermore, it can translocate to the nucleus where it regulates activities of several transcription

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Fig. 7. Retrograde axonal transport of proteins in vitro. (A,B) Representative 2-DE separation of proteins from DRG after culture in compartmentalized culture dishes with the end of the peripheral nerve incubated with [35S]metionine-[35S]cysteine for 2 days. In the autoradiograph of the gel shown in A, up to 100 radioactive spots are visible, of which ~35 (arrows) were also seen in autoradiographs of gels from other experiments. The silver-stained gel in B shows many more spots, of which only a small subset correspond to those on the autoradiograph (arrows).

Fig. 8. Isolated axon preparations. (A-D) BDNF-stimulated axonal growth, visualized by Calcein Orange-Red fluorescence (A) is associated with migrating cells whose nuclei are labeled by DAPI (B). Axons that have extended through a Nuclepore membrane (C) are devoid of migrating cells (D). The bright foci that are visible in C represent localized thickenings of axons, not cells, as shown by the absence of DAPI labeling in D. Scale bar, 100 μm.
factors, including NF-κB, activator protein 1 (AP1) and p53 (reviewed by Yoshioka et al., 2006), that are involved in axonal regeneration (Di Giovanni et al., 2006; Raivich et al., 2004; Gallagher et al., 2007).

Cyclopentenone prostaglandins block the in vitro conditioning effect on axonal regeneration

To investigate possible involvement of Trx in axonal regeneration, we incubated PN-DRG for 1 day with 50 μM prostaglandin A1 (PGA1), or with 5 μM 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) that covalently binds Trx (Moos et al., 2003; Shibata et al., 2003), and observed significant inhibition of subsequent BDNF-induced axonal outgrowth in the presence (P<0.001 and P<0.01, respectively) or absence (P>0.001 and P>0.05, respectively) of ActD. By contrast, incubation of PN-DRG with these prostaglandins for 1 day after 3-day in vitro conditioning did not inhibit subsequent axonal outgrowth, even in the presence of ActD (Fig. 11). Moreover, in three separate experiments using the two-

Table 2. Retrogradely transported proteins identified by 2-DE and mass spectrometry

| Spot number | Identified protein | GenBank accession number | Mascot score | Peptides matched | Sequence coverage | Molecular size (kDa) | pI  |
|-------------|-------------------|--------------------------|--------------|-----------------|------------------|---------------------|-----|
| 1           | Not identified    |                          |              |                 |                  |                     |     |
| 2           | SH3 domain binding glutamic acid-rich protein-like3          | 46249884          | 171          | 2               | 46%              | 10685               | 4.85|
| 3           | Calpactin-1       | 51873774          | 207          | 4               | 33%              | 8334                | 4.91|
| 4           | Profilin          | 76775311          | 646          | 11              | 84%              | 14962               | 7.49|
| 5           | Trx               | 49119156          | 488          | 8               | 69%              | 11565               | 4.87|
| 6           | Nuclear transport factor 2                                | 6093543           | 105          | 3               | 44%              | 14468               | 4.89|
| 7           | Haematopoietic stem progenitor cell 300                   | 3851232          | 84           | 2               | 26%              | 8741                | 5.33|
| 8           | Not identified    |                          |              |                 |                  |                     |     |
| 9           | Cofilin-1-A       | 1168993           | 1111         | 21              | 79%              | 19054               | 7.6 |
| 10          | Cofilin-1-B       | 1168995           | 1025         | 10              | 77%              | 19108               | 7.6 |
| 11          | Dic1              | 34785679          | 231          | 4               | 63%              | 10335               | 6.82|
| 12          | Calpactin-1       | 51873774          | 42           | 5               | 52%              | 11322               | 6.82|

Proteins were excised from radioactive spots on 2D gels of DRGs following metabolic labelling in the distal part of the peripheral nerve in a 2-chamber culture system. Note that the retrogradely transported proteins (with the exception of vimentin) differ from those up-regulated in the cultured DRGs (listed in Table 1).
compartment culture system, application of PGA1 or 15d-PGJ2 to the distal part of the peripheral nerve of PN-DRG for 3 days reduced subsequent BDNF-induced axonal outgrowth in the presence of ActD from 594±47 μm (controls) to 312±17 μm and 406±31 μm (P<0.01 and P<0.05; respectively). These results are consistent with the hypothesis that Trx is involved in regulating expression of RAGs and axonal regeneration, although involvement of other protein(s) bound by these cyclopentenone prostaglandins – including the redox-sensitive transcription factors Jun, p53 and NF-κB (reviewed by Kim and Surh, 2006) that are known to be involved in axonal growth (Raivich et al., 2004; Di Giovanni et al., 2006; Gutierrez et al., 2005; Gallagher et al., 2007) – should also be considered.

To determine whether Trx synthesis is required for axonal regeneration, we electroporated FITC-labelled MOs recognizing

Xenopus Trx and zebrafish embryonic β-globin (as control) into dissociated DRG neurons, and observed fluorescence – often localized to nuclei – in most neurons (Fig. 12). DRG neurons showed Trx-like immunoreactivity over both soma and neurites (Fig. 12) which in three separate experiments and after 2 days in vitro, was 44±4% lower in the cultures treated with Trx MOs than in those treated with β-globin MOs. In cultures from freshly dissected DRG, the mean neurite outgrowth distance from neurons electroporated with Trx MOs (148±28 μm) was significantly less (P<0.05) than from those electroporated with β-globin MOs (250±40 μm). By contrast, there was no significant difference between the mean distances of neurite outgrowth from neurons dissociated from 3-day in vitro conditioned DRG following electroporation with MOs using Trx or β-globin (501±28 μm or 543±10 μm, respectively).

Discussion

Changes in gene expression after axotomy or culture

The present findings indicate that changes in gene expression following axotomy are essential for axonal regeneration because axonal growth in response to BDNF and other neurotrophins in
freshly dissected PN-DRG was almost abolished by the inhibition of mRNA synthesis, but not from in vivo or in vitro conditioned preparations. These observations suggest that mRNAs that encode proteins that are required for axonal growth are synthesized in response to axotomy or culture in vitro within 3 days, and are sufficiently stable to sustain regeneration thereafter for several days. Transcription-independent neurite outgrowth from in vivo and in vitro conditioned mammalian DRG neurons, and also NGF-primed PC12 cells has been reported previously (Smith and Skene, 1997; Twiss and Shooter, 1995).

To investigate changes in gene expression, we used oligonucleotide microarray analysis on RNA extracted from fresh and cultured DRG, and found extensive changes in gene expression. Of the 58 genes that show increases in expression of more than fivefold in cultured DRG (Table 1), at least seven, including arginase, galectin, cyclin B1, metallothionein and glutathione S-transferase are upregulated in mammalian sensory or sympathetic ganglia following axotomy (Costigan et al., 2002; Tanabe et al., 2003; Cameron et al., 2003; Boeshore et al., 2004; Nilsson and Kanje, 2005), which suggests their involvement in evolutionarily conserved programs of axonal regeneration and/or neuronal survival. Arginase has neuroprotective properties and its forced expression promotes neurite outgrowth on myelin, normally inhibitory to axonal regeneration (Cai et al., 2002). Expression of galectin is correlated with axonal regeneration, which it might influence by stimulating macrophages to release factor(s) that promote axonal growth and migration of Schwann cells (Horie et al., 2005). Both glutathione-S-transferase and metallothionein have neuroprotective functions (Townsend and Tew, 2003; Penkowa et al., 2005). In cultured DRG, upregulation of other genes that might be important for axonal regeneration include vimentin, which is involved in retrograde axonal transport of activating signals for initiation of axonal growth (Hanz et al., 2003; Perlson et al., 2005), and the cytoskeletal proteins paullin and calponin (Huang et al., 2004; Plantier et al., 1999). Vg1RBP is also of interest because, in developing neurons, it is involved in the transllocation of mRNAs to growth cones (Leung et al., 2006; Piper et al., 2006) but might also be important during axonal regeneration.

It is unclear which signalling pathways are involved in the control of gene expression following axotomy. Extensive studies of GAP43, the best known RAG, have not identified any factor(s) that are directly responsible for its observed upregulation during regeneration. However, nerve-injury-induced expression of GAP43 and Trx1 tubulin (also known as Tuba1a) is absent in CAAT-enhancer-binding protein β (C/EBPβ)-knockout mice and moreover, Trx1 tubulin is a direct C/EBPβ target (Nadeau et al., 2005). Other RAGs whose expression might be regulated by C/EBPβ include arginase (Gray et al., 2005) and SPRR1A (Preverand et al., 2004). C/EBPβ is transcriptionally regulated by STAT3 (Niehof et al., 2001) and can be activated by phosphorylation through different pathways involving MAPK, PKC, CAM kinase and Erk1/2 (Ramji and Foka, 2002; Park et al., 2004). Since phosphorylated Erk1/2 and STAT3 are retrogradely transported in injured snail and mammalian nerves (Sung et al., 2001; Perlson et al., 2005; Lee et al., 2004) these could promote C/EBPβ activity in lesioned nerves, leading to expression of specific RAGs. However, in the present study, pharmacological inhibition of MAPK, PKC, CAM kinase and Erk1/2 pathways failed to prevent the in vitro conditioning effect on axonal regeneration and although this was blocked by inhibition of JAK/STAT signalling using AG490, its specificity is uncertain (Gorina et al., 2007).

Retrograde signalling
The mechanisms by which peripheral nerve lesions cause changes in gene expression in neurons might involve retrograde activating signals that are generated at the site of the axon lesion (reviewed
by Hanz and Fainzilber, 2006). To investigate whether effects of in vitro conditioning on subsequent axonal growth depend on such signals, preparations were incubated in two-compartment culture dishes, enabling the application of drugs to either the DRG or the distal end of the peripheral nerve. Here we show that blockade of protein synthesis in the distal part of the nerve reduces subsequent BDNF-induced axonal growth in the presence of ActD. This suggests that the retrograde signal is generated, at least in part, by local translation from existing mRNAs because local application of ActD to this part of the nerve did not block the conditioning effect. These observations are consistent with previous findings (Hanz et al., 2003; Perlson et al., 2005) that demonstrate the importance of intra-axonal synthesis of β-importin and vimentin in retrograde signalling, and initiation of axonal regeneration. However, other proteins that might be carried by importin-dynein complexes were not identified in those investigations. Retrograde transport of ~30 proteins was demonstrated in cultured frog nerves by Edbladh et al. (Edbladh et al., 1994), and of >100 proteins in lesioned nerves of the mollusc Lymnia (Perlson et al., 2004), although the identities and possible roles of those proteins in axonal regeneration are largely unknown.

In this study, we have demonstrated the retrograde axonal transport of ~35 proteins, 32 of which were identified – including nine that are known to be synthesized within regenerating mammalian axons (Willis et al., 2005). However, we did not detect any of the signalling molecules that are thought to mediate the expression of RAGs following axotomy in vivo (reviewed by Hanz and Feinzilber, 2006). Nevertheless, the possible retrograde transport of Trx is of interest because it influences the activity of various transcription factors (reviewed by Yoshioka et al., 2006), including NF-κB, which is important for axonal growth in developing neurons (Gutierrez et al., 2005; Gallagher et al., 2007), and also p53 and the AP1 transcription factors Jun and ATF3, which are known to be involved in axonal regeneration within mature neurons (Di Giovanni et al., 2006; Raivich et al., 2004; Seijffers et al., 2006; Seijffers et al., 2007). Trx might thus have a pivotal role in regulating the activities of several transcription factors that are important for axonal regeneration. It is interesting to note that, in PC12 cells, Trx is induced by NGF and is essential for neurite-like outgrowth (Bai et al., 2003).

To investigate the involvement of Trx, we used PGA1 and 15d-PGJ2, which both react with Trx (Moos et al., 2003; Shibata et al., 2003), and found that incubation of freshly dissected PN-DRG with these prostaglandins on day 1 of a 4-day period of in vitro conditioning resulted in the inhibition of subsequent BDNF-induced axonal growth. Incubation on day 4, however, did not, which suggests that these electrophilic prostaglandins had blocked – during the first days in culture – synthesis of mRNA that is necessary for axonal growth. Incubation of freshly dissected PN-DRG with PGJ2, which both react with Trx (Moos et al., 2003; Shibata et al., 2003), and other redox-sensitive factors are involved in axonal regeneration, probably by regulating the expression of RAGs, although the possibility that the cyclopentenone prostaglandins inhibit axonal regeneration by other mechanisms is not excluded. However, whether Trx acts as a retrograde signal within lesioned axons during initiation of axonal regeneration or whether it is involved globally remains to be determined.

Materials and Methods

Reagents were purchased from Sigma (Poole, UK) unless otherwise stated. *Xenopus laevis* were supplied by Blades Biologics Ltd (Edenbridge, UK), Aquatic Environment Services (Tonbridge, UK), Neil Hardy Aquatica Ltd (Carshalton, UK) or bred at King’s College London and the University of Bristol (UK). Surgical procedures were licensed by the British Home Office and approved by the Animal care and Use Committee of King’s College London. Post-metamorphic animals (~1.5-2 cm body length) were anaesthetized by immersion in an aqueous solution (0.1% w/v) of tricaine methanesulphonate (MS 222) and killed by destruction of the brain. Lumbar and thoracic PN-DRG were removed and either incubated free floating for 3 days in vitro conditioning or cultured in batches of four to five in alginate (Worthington) or matrigel (Beckton Dickenson, Oxford, UK) as described previously (Tonge et al., 1998). In vivo conditioning, animals were anaesthetized with MS 222 and the sciatic nerve in one leg was exposed and sectioned, followed by wound closure with medical adhesive (Histoacyrl, Melsungen, Germany) 3 or 5 days prior to removal of the PN-DRG for culture. Neurotrophic factors: NGF (PeproTech EC, London, UK), BDNF (R&D Systems Europe Ltd, Abingdon, UK), NT3 and NT4 (Alomone Laboratories, Jerusalem, Israel) were used at 50 ng/ml. Actinomycin D (AcID), cycloheximide (CHX) and α-amanitin (α-AM; Merck Chemicals, Nottingham, UK) were used at concentrations of 5 μg/ml, 10 μg/ml and 20 μg/ml, respectively. ActD inhibits RNA polymerases I, II and III, whereas α-AM inhibits RNA polymerase II and, at the concentrations used in this study, reduces RNA synthesis in *Xenopus* skeletal muscle by ~90% and 70%, respectively (Brehm et al., 1987). PGA1 or 15d-PGJ2 (Axoxa Ltd, Nottingham, UK), AG490, PD98059, UO126, SP600125, LY294002, k252a (Merek Chemicals, UK) and vinblastine were used as described in individual experiments. In some experiments, the effects of drug application to either DRG or the distal 2-3 mm end of the peripheral nerve were studied using a two-compartment culture system (Edbladh et al., 1994), comprising a 35-mm plastic dish and inner cylindrical compartment with a small notch for the nerve, coated with silicone grease to prevent leakage between compartments. After 3 days, PN-DRG were removed, the peripheral nerve trimmed followed by culture in collagen gels. To obtain isolated axons for RT-PCR, PN-DRG were cultured in matrigel on membranes with 8-μm pores (Nucleopore track-etched membranes; Thermo Fisher Scientific Inc, Loughborough, UK) glued to the bottom of 4-well culture dishes with a drop of matrigel and folded in half to provide a vertical barrier facing the cut ends of the peripheral nerves. In response to BDNF, axons extended through the pores (unaccompanied by cells) and into matrigel beyond the barrier, and were harvested after 10 days by removing the folded membrane (together with the PN-DRG), leaving the outgrown axons in the matrigel on the bottom of the culture dish.

MO-induced inhibition of Trx expression

To obtain isolated neurons, DRG were removed from 2-3 animals, trimmed and incubated in 0.125% collagenase type III (Worthington) for 4 hours at 24°C, followed by trituration in RPMI medium containing 10% bovine serum. Following centrifugation, cells were re-suspended in 100 μl of Rat Neuron Nucleofector™ solution (Axama Biosystems, Germany), diluted to 80% with water, containing 2 μl of 1 mM 3′-labelled carboxyfluorescein MOs followed by electroporation using program O-05 of a Nucleofector™ (Axama Biosystems). After electroporation, cells were cultured for 2 days in 4-well dishes (Nunc) pre-coated with 10 μg/ml laminin in PBS and containing 10 ng/ml of BDNF in RPMI medium. The MO sequences were 5′-ATGTT-GTGAAACCATATGGCGTTT-3′ (*Xenopus* Trx1) and 5′-ATCCAGCACCACA-TCTGAGCAGCAAC-3′ (zebrafish embryonic β-globin). The β-globin MO was a kind gift of Adam Rodaway (King’s College, London, UK). Additionally, some dissociated DRG neurons were cultured for 1 day without electroporation.

Immunocytochemistry

PN-DRG were fixed after 3 days culture, for one hour using 3.5% formaldehyde in PBS, followed by incubation with mAb 6-11B-1 which recognizes acetylated α-
tubulin (LeDizet and Piperno, 1991) and axons visualized using peroxidase-conjugated avidin as described (Tonge et al., 1998). Stained preparations were mounted in Glycergel (DakoCytomation, High Wycombe, UK) and photographed using a microscope equipped with phase contrast (BH2; Olympus, Japan) and C5810 digital camera (Hamamatsu Photonics, Welwyn Garden City, UK). Dissociated DRG neurons were fixed after 1 or 2 days in culture with the same fixative for 30 minutes, blocked for 1 hour in Tris-buffered saline (TBS) containing 0.3% (v/v) Triton X-100 and 3% (w/v) non-fat dried milk (NFDm) in PBS, prior to incubation for 2 hours with a rabbit polyclonal antibody to human Trx (Abcam), diluted 1:500. Following washing with PBS, preparations were incubated with TRITC-conjugated goat anti rabbit IgG (1:300 dilution) for 1 hour, washed again with PBS, mounted in Vectashield containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, USA), viewed using an Eclipse TE2000 fluorescence microscope and images captured using a DXM1200F digital camera (both from Nikon, Tokyo, Japan). Cross-reactivity of the antibody for Xenopus Trx was verified in one experiment by western blotting, showing recognition of a 12 kDa band in protein extracts from both mouse and Xenopus heart (data not shown). Moreover, in a further experiment, prior incubation of the antibody with recombinant human Trx reduced subsequent mean fluorescence labelling of dissociated Xenopus DRG neurons by 65% (36 neurons). Relative expression levels of Trx were assessed from images (captured using the same exposure time) by comparing the averaged immunofluorescence intensity of labelled neuronal somas against background along orthogonal axes using a PC version of NIH Image (Scion Image).

Outgrowth measurements

After staining, PN-DRG preparations were viewed under a Nikon TMS inverted-phase-contrast microscope. Where large numbers of outgrowing axons were present (forming a halo), distances from the cut end of the peripheral nerve to the tips of the outgrowing axons were measured at five separate points, using an eye piece scale and graticule, and their mean calculated for each PN-DRG. Where axonal outgrowth was sparse, the lengths of the five longest axons from the cut end of the peripheral nerve of each preparation were measured and their mean calculated. In some experiments, axonal outgrowth was visualized by fluorescence microscopy following overnight incubation with 1 μg/ml Calcein-AM or Calcein Orange-Red (Invitrogen, Paisley, UK), membrane-permeable dyes which are hydrolysed by intracellular esterases in viable cells yielding green or orange fluorescent products, respectively. Some preparations were fixed, mounted in Vectashield containing DAPI, and viewed using the fluorescence microscope with the digital camera described above. In cultures of dissociated DRG neurons, neurite outgrowth was quantified from 15-20 digital images per well by measuring the longest neurite of neurons, labeled by Calcein, using Scion Image.

Oligonucleotide microarray analysis

Total RNA was extracted from two separate pools, each comprising about 60 freshly dissected (control) or 3-day in vitro conditioned DRG using Absolutely mini RNA kits (Stratagene, Cambridge, UK) according to the manufacturer’s instructions. RNA quality was assessed on an Agilent 2100 bioanalyzer using RNA LabChips according to the manufacturer’s instructions. RNA from control and in vitro conditioned DRG was used to generate biotinylated cRNA according to Affymetrix protocols and hybridized with separate Affymetrix Xenopus GeneChips, comprising >14,400 transcripts (Affymetrix UK High Wycombe, UK). The arrays were scanned and analysed at the Genomics Centre, King’s College London, using a Hewlett Packard GeneArray Scanner and Affymetrix MicroArray Analysis Suite 5.0 software. Standard software settings were used to establish the presence or absence of transcripts, relative mRNA abundance, and also to determine the fold differences in transcript abundance in the RNA samples.

Quantitative PCR

To validate the microarray data, Quantitative PCR (qPCR) was carried out on cDNA that had been prepared using random decamers from samples of RNA used in the microarray analysis, using avian myeloblastosis virus reverse transcriptase (AMV RT) according to the manufacturer’s protocol (Promega, Southampton, UK). Control reactions omitting AMV RT were also performed. Relative expression levels of different transcripts were calculated (in triplicate) by qPCR using fluorescent SYBR Green 1 technology on an ABI PRISM 7000 HT sequence detection system/LightCycler (Applied Biosystems) and normalized to those of 18S RNA. Primers (supplementary material Table S2) were designed using Primer Express software (Applied Biosystems, Warrington, UK) with default settings, applied to Xenopus cDNA sequences derived from the NCBi web site http://www.ncbi.nlm.nih.gov. PCR reactions were performed on a MicroAmp Optical 96-well reaction plate and the Ct method was used for relative quantification as described (Brewer et al., 2005).

Non-quantitative PCR and reverse-transcription PCR

For analysis of the axonal RNA panel, total RNA was also extracted from isolated axon preparations in matrigel and used to generate cDNA as described above. Control reactions omitting AMV RT were also performed. cDNAs were used as templates for PCR reactions using 40 cycles and primers designed using Primer Express software (Applied Biosystems, UK) applied to Xenopus sequences derived from the NCBi web site. Individual primers were divided into two pools for parallel RNA and DNA analyses. From one pool, total RNA was prepared from DRG and axon samples, using the Absolutely RNA T MicroPrep Kit (Stratagene). For conventional RT-PCR, cDNA preparation was performed as described by Weber et al. (Weber et al., 2000). To control for contamination with genomic DNA, parallel reactions were carried out, with and without AMV RT. PCR was carried out using GoTaq premix (Promega) according to the manufacturer’s instructions.

For genomic DNA analysis, samples from the second pool were diluted in 0.5 ml 50 mM Tris (pH 7.5), 1 mM EDTA, 0.5% Tween with 200 μg/ml proteinase K and digested overnight at 56°C. After extraction, the DNA samples were precipitated with isopropanol (RNA used as carrier), and resuspended in Tris-EDTA. PCR (as described for RT-PCR, except the annealing temp was 58°C) was performed on cDNA and PCR reactions are shown in supplementary material Table S2.

Detection of retrogradely transported proteins by metabolic labelling and 2-DE

PN-DRG preparations (eight to ten per experiment) were placed in the two-compartment culture system described above; to each inner compartment, containing 2-3 mm of the distal peripheral nerve, 0.32 Mβ [35]methionine-[15]S]cysteine (GE Healthcare 99%, Omnifit St Giles, UK) was added. After 2 days in culture the DRG were pooled and solubilized in DeStak Rehydration Solution with 0.5% Phospholipase (pH 3-10) (GE Healthcare, UK) and separated by iso-electric focusing (IEF) using pH 3-10 Immobiline DryStrips (GE Healthcare, UK) for the first dimension. After focusing, the strips were equilibrated in buffer containing 6 M urea (pH 8.8, Ampholines Tris-HCl (pH 3-10), 30% v/v glycerol, 2% SDS with 1% v/v DTT) for 15 minutes, followed by 2.5% IAA in the same buffer for a further 15 minutes. In the second dimension, the IEF strips were run on 12% SDS-PAGE. Gels were then fixed with 50% methanol and 10% acetic acid, followed by transfer to 30% methanol 3% glycerol overnight. The next day, gels were dried and exposed to X-ray films for up to 30 days. After the development of the films, gels were re-swelled and silver-stained using a protocol compatible with mass spectrometry (Yan et al., 2000). Autoradiographs and images of silver-stained gels were scanned at 360 pixels per inch resolution using an EPSON GT-7000 scanner and images compared manually.

Analysis of 2DE spots by LC-MS/MS

Following autoradiography and silver-staining, spots of interest were excised from gels and subjected to reduction, alkylation and digestion (with trypsin) prior to subsequent analysis by LC-MS/MS using published procedures (Hye et al., 2006; Gallio-Montbrun et al., 2007). Briefly, peptides were extracted from gel pieces by a series of acetonitrile and aqueous washes, lyophilized and re-suspended in 50 mM ammonium bicarbonate before analysis by LC-MS/MS. Chromatographic separations were performed using Ultimate LC or Ultimate 3000 systems (Dionex, Camberley, UK). Peptides were ionised by electrospray ionization using Z-spray sources fitted to Q-TOF micro (Waters Co, Milford, MA) or MicroQ (Applied Biosystems, UK). Both instruments were set to run in automated switching mode, selecting precursor ions based on their intensity, for sequencing by collision-induced fragmentation. MS/MS analyses were conducted using collision energy profiles that were chosen based on the m/z and the charge state of the peptide. Mass spectral data were searched against the Swiss Prot (Uniprot release 52.40, NCBI non-redundant database) using Mascot software (v2.2, Matrix Science, London, UK). Data searching was performed using specific amino-acid-modification parameters (e.g. variable cysteine carboxamidomethylation and methionine oxidation). Assumptions included peptide-mass tolerance of 1 Da, fragmentation tolerance 0.05 Da and a maximum of two missed cleavages. MS/MS-sequencing information was obtained for all peptides included in the results (MOWSE algorithm score of 95% confidence) based on matching evidence of MS/MS fragmentation to protein sequences in the databases. Where reported matches were below 95% confidence limits, each peptide was visually verified before inclusion. The percentages of sequences covered are given in Table 2.

Statistical analysis

Experiments were repeated at least three times unless otherwise stated. Differences between means were evaluated throughout using Student’s t-test and considered significant at P<0.05.

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References

Bai, J., Nakamura, H., Kwon, Y. W., Hattori, I., Yamaguchi, Y., Kim, Y. C., Kondo, N., Oka, S., Ueda, S., Masutani, H. et al. (2003). Critical roles of thioredoxin in nerve growth factor-mediated signal transduction and neurite outgrowth in PC12 cells. J. Neurosci. 23, 503-509.

Bao, J., Talagme, D. A., Role, L. W. and Gautier, J. (2000). Regulation of neurogenesis by interactions between HEN1 and neuronal LMO proteins. Development 127, 425-435.
Basell, G. J., Zhang, H., Byrd, A. L., Femino, A. M., Singer, R. H., Taneja, K. L., Lifshitz, J. C., Roman, J. M., and Kosik, K. S. (2001). Sorting of beta-actin mRNA and protein to neurites and growth cones in culture. *J. Neurosci.* 21, 251-265.

Bennett, B. L., Sasaki, D. T., Murray, B. W., O'Leary, E. C., Sakata, S. T., Xu, W., Leisten, J. C., Motwala, A., Pierce, S., Satoh, Y. et al. (2001). *SP600125, an inhibitor of JNK1 and JNK2, reduces axonal injury and death in the early phase of excitotoxic neuronal injury.* *Proc. Natl. Acad. Sci. U.S.A.* 98, 13681-13686.

Boeshore, K. L., Schreiber, R. C., Vaccariello, S. A., Sachs, H. H., Salazar, R., Lee, J., Ratan, R. R., Leahy, P. and Zigmond, R. E. (2004). Novel changes in gene expression following axotomy of a sympathetic ganglion: a microarray analysis. *J. Neurobiol.* 59, 216-235.

Bomze, H. M., Bulsara, K. R., Iskandar, B. J., Caroni, P. and Pate Skene, J. H. (2002). Axonal regeneration evoked by replacing two growth cone proteins in adult neurons. *Nat. Neurosci.* 5, 34-43.

Bonilla, I. E., Tanabe, K. and Strittmatter, S. M. (2001). Small, proline-rich repeat protein 2A is expressed by axotomized neurons and promotes axonal outgrowth. *J. Neurosci.* 21, 1305-1315.

Brehm, P., LeClair, R. M. and Moody-Corbett, F. (2001). Evidence for a sequential postlesional function of the cytokines. *J. Neurosci.* 21, 400-404.

Bolinna, I. E., Tanabe, K. and Strittmatter, S. M. (2002). Small, proline-rich repeat protein 1A is expressed by axotomized neurons and promotes axonal outgrowth. *J. Neurosci.* 22, 1305-1315.

Breitling, P., Sahlmann, V. M. and Moor-Keil, H. (2001). 15-deoxy-Delta12,14-prostaglandin J2 as a potential endogenous regulator of redox-sensitive transcription factors. *Biochem Pharmacol.* 72, 1612-1615.

Boeshore, K. L., Schreiber, R. C., Vaccariello, S. A., Sachs, H. H., Salazar, R., Lee, J., Ratan, R. R., Leahy, P. and Zigmond, R. E. (2004). Novel changes in gene expression following axotomy of a sympathetic ganglion: a microarray analysis. *J. Neurobiol.* 59, 216-235.

Bomze, H. M., Bulsara, K. R., Iskandar, B. J., Caroni, P. and Pate Skene, J. H. (2002). Axonal regeneration evoked by replacing two growth cone proteins in adult neurons. *Nat. Neurosci.* 5, 34-43.

Bonilla, I. E., Tanabe, K. and Strittmatter, S. M. (2001). Small, proline-rich repeat protein 2A is expressed by axotomized neurons and promotes axonal outgrowth. *J. Neurosci.* 21, 400-404.
Plantier, M., Fattoum, A., Menn, B., Ben-Ari, Y., Der Terrissian, E. and Represa, A. (1999). Acidic calponin immunoreactivity in postnatal rat brain and cultures: subcellular localization in growth cones, under the plasma membrane and along actin and glial filaments. *Eur. J. Neurosci.* 11, 2801-2812.

Preserand, S., Yasukawa, H., Muller, O. G., Kjekshus, H., Nakamura, T., St Amant, T. R., Yajima, T., Matsumura, K., Duplair, H. and Iwatake, M. et al. (2004). Small proline-rich protein 1A is a gpl30 pathway- and stress-inducible cardioprotective protein. *EMBO J.* 23, 4517-4525.

Raihii, G. and Makwana, M. (2007). The making of successful axonal regeneration: genes, molecules and signal transduction pathways. *Brain Res. Rev.* 53, 287-311.

Raihii, G., Bohatschek, M., Da Costa, C., Iwata, O., Galiano, M., Hristova, M., Nateri, A. S., Makwana, M., Riera-Sans, L., Wolfer, D. P. et al. (2004). The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. *Neuron* 43, 57-67.

Ramji, D. P. and Foka, P. (2000). CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem. J.* 365, 561-575.

Rodriguez, J., Esteve, P., Weiln, C., Ruiz, J. M., Fermin, Y., Trousse, F., Dwivedy, A., Smith, D. S. and Skene, J. H. P. (1997). A transcription-dependent switch controls transcription (STAT).

Shibata, T., Yamada, T., Ishii, T., Kumazawa, S., Nakamura, H., Masutani, H., Yodoi, J. and Uchida, K. (2000). Differential patterns of ERK phosphorylation after sciatic nerve transection in the rat. *J. Neurosci.* 20, 7911-7920.

Shu, J. Y., Kulhanek, D. J. and Eckenstein, F. P. (2000). Differential patterns of ERK and STAT3 phosphorylation after sciatric nerve transection in the rat. *Exp. Neurol.* 166, 392-402.

Shibata, T., Yamada, T., Ishii, T., Kumazawa, S., Nakamura, H., Masutani, H., Yodoi, J. and Uchida, K. (2000). Thioredoxin as a molecular target of cyclopentenone prostaglandins. *J. Biol. Chem.* 278, 26046-26054.

Smith, D. S. and Skene, J. H. P. (1997). A transcription-dependent switch controls competence of adult neurons for distinct modes of axon growth. *J. Neurosci.* 17, 646-658.

Sung, Y. J., Walters, E. T. and Ambrose, R. T. (2004). A neuronal isoform of protein kinase G couples mitogen-activated protein kinase nuclear import to axotomy-induced long-term hyperexcitability in Aplysia sensory neurons. *J. Neurosci.* 24, 7583-7595.

Tanabe, K., Tachibana, T., Yamashita, T., Che, Y. H., Yoneda, Y., Ochi, T., Tohyama, M., Yoshikawa, H. and Kiyama, H. (2000). Small GTP-binding protein TC10 promotes nerve elongation in neuronal cells, and its expression is induced during nerve regeneration in rats. *J. Neurosci.* 20, 4138-4144.

Tanabe, K., Bonilla, L., Winkles, J. A. and Strittmatter, S. M. (2003). Fibroblast growth factor-inducible-14 is induced in axotomized neurons and promotes neurite outgrowth. *J. Neurosci.* 23, 9675-9686.

Tongs, D., Edstrom, A. and Ekstrom, P. (1998). Use of explant cultures of peripheral nerves of adult vertebrates to study axonal regeneration in vitro. *Prog. Neurobiol.* 54, 459-480.

Tonge, D. A., Pountney, D. J., Leclerc, P. G., Zhu, N. and Pizzey, J. A. (2004). Neurotrophin-independent attraction of growing sensory and motor axons towards developing Xenopus limb buds in vitro. *Dev. Biol.* 265, 169-180.

Townsend, D. M. and Tew, K. D. (2003). The role of glutathione-S-transferase in anti-cancer drug resistance. *Oncogene.* 22, 7369-7375.

Twiss, J. L. and Shooter, E. M. (1995). Nerve growth factor promotes neurite regeneration in PC12 cells by translational control. *J. Neurochem.* 64, 550-557.

Vallee, R. B., Williams, J. C., Varma, D. and Barnhart, L. E. (2004). Dynemin: An ancient motor protein involved in multiple modes of transport. *J. Neurobiol.* 58, 189-200.

Verma, P., Chierzi, S., Codd, A. M., Campbell, D. S., Meyer, R. L., Holt, C. E. and Fawcett, J. W. (2005). Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration. *J. Neurosci.* 25, 331-342.

Vahos, C. J., Matter, W. F., Hui, K. Y. and Brown, R. F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinoethyl)-8-phenyl-1H-benzopyran-4-one (LY294002). *J. Biol. Chem.* 269, 5241-5248.

Weber, H., Simyes, C. E., Walmsey, M. E., Rodaway, A. R. F. and Patient, R. K. (2000). A role for GATA5 in Xenopus endoderm specification. *Development* 127, 4345-4350.

Werner, A., Willem, M., Jones, I. L., Kreutzberg, G. W., Mayer, U. and Raivich, G. (2000). Impaired axonal regeneration in alpha7 integrin-deficient mice. *J. Neurosci.* 20, 1832-1830.

Willis, D., Li, K. W., Zheng, J. Q., Chang, J. H., Smit, A., Kelly, T., Merianda, T. T., Sylvester, J., van Minnen, J. and Twiss, J. L. (2005). Differential transport and local translation of cytoskeletal, injury-response, and neurodegeneration protein mRNAs in axons. *J. Neurosci.* 25, 778-791.

Xiao, H. S., Huang, Q. H., Zhang, F. X., Bao, L., Lu, Y. J., Guo, C., Yang, L., Huang, W. J., Fu, G., Xu, S. H. et al. (2002). Identification of gene expression profile of dorsal root ganglion in the rat peripheral axotomy model of neuropathic pain. *Proc. Natl. Acad. Sci. USA* 99, 8360-8365.

Yan, J. X., Wait, R., Berkelman, T., Harry, R. A., Westbrook, J. A., Wheeler, C. H. and Dunn, M. J. (2000). A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. *Electrophoresis* 21, 3666-3672.

Yoshikawa, J., Schreiter, E. R. and Lee, R. T. (2006). Role of thioredoxin in cell growth through interactions with signalling molecules. *Antioxid. Redox Signal.* 8, 2143-2151.

Zhou, F. Q. and Snider, W. D. (2006). Intracellular control of developmental and regenerative axon growth. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 361, 1575-1592.