

Stimulated regeneration of the crushed adult rat optic nerve correlates with attenuated expression of the protein tyrosine phosphatases RPTP α , STEP, and LAR

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We have evaluated the spatial and temporal expression patterns of three protein tyrosine phosphatases (PTPs), receptor PTP α (RPTP α), striatal enriched phosphatase (STEP), and leucocyte common antigen-related phosphatase (LAR), in the retina and optic nerve (ON) of adult rats in which the crushed ON was either regenerating after retinal ganglion cell (RGC) stimulation with intravitreal peripheral nerve (PN) grafting or lens injury (LI), or not regenerating (no treatment). In intact adult rats, all three PTPs were expressed by RGCs and ON glia. In both the regenerating and non-regenerating models, a postlesion rise in RPTP α , STEP, and LAR expression occurred in the RGC somata and in the ON. However, for RPTP α and LAR in the RGCs, and for RPTP α , STEP, and LAR in the ON, this postlesion increase was attenuated in the regenerating versus the non-regenerating models. ON PTP expression changes were localized to glia in the proximal and distal stumps, and to macrophages and extracellular matrix of the glial scar at the lesion site. Interestingly, neither RPTP α , STEP, nor LAR localized to intact or regenerating axons. One explanation of these findings is that RPTP α and LAR may modulate RGC survival, and that RPTP α , STEP, and LAR may modulate axon growth.

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Abbreviations: CAII, carbonic anhydrase-II; CNS, central nervous system; CSPG, chondroitin sulphate proteoglycans; dpi, days postinjury; ERK1/2, extracellular signal regulated kinases 1 and 2; GFAP, glial fibrillary acidic protein; GAP-43, growth associated protein-43; LI, lens injury; LAR, leucocyte common antigen-related phosphatase; MAG, myelin associated glycoprotein; OMgp, oligodendrocyte myelin glycoprotein; ON, optic nerve; ONC, optic nerve crush; ONC + PN, optic nerve crush and intravitreal peripheral nerve graft; PN, peripheral nerve; PTP(s), protein tyrosine phosphatase(s); R, receptor; RPTP α , receptor protein tyrosine phosphatase α ; RGC(s), retinal ganglion cell(s); STEP, striatal enriched phosphatase.

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Introduction

Protein tyrosine phosphatases (PTPs) comprise a large family of around 100 members divided into two groups: receptor (R) and non-receptor (cytosolic) PTPs. The antagonistic activities of protein tyrosine kinases and PTPs regulate cellular protein phosphotyrosine levels, phosphorylating proteins on tyrosine residues, and dephosphorylating phosphotyrosine residues, respectively (Tonks and Neel, 2001). Ligands have only been identified for few receptor PTPs (RPTPs). For example, heparan sulphate proteoglycans act as ligands for RPTP σ (Aricescu et al., 2002), and pleiotrophin is the RPTP ligand for phosphacan (Maeda et al., 1996). Ligand identification for leucocyte common antigen-related phosphatase (LAR) is still underway, although a certain LAR isoform was found to bind to the extracellular matrix laminin–nidogen complex (O’Grady et al., 1998). Furthermore, LAR homophilic ligand interactions occur, where a small ectodomain isoform, designated LARFN5C, functions as a ligand (Yang et al., 2003).

This study focuses on the role of three PTPs, receptor protein tyrosine phosphatase α (RPTP α), striatal enriched phosphatase (STEP), and LAR, in the regeneration of injured adult mammalian central nervous system (CNS) axons. LAR and RPTP α are RPTPs, whereas transmembrane and cytosolic isoforms of STEP exist. Several studies have implicated LAR in axon growth. For example, in LAR-deficient mice, decreased cholinergic fiber innervation of the dentate gyrus occurs (Van Lieshout et al., 2001; Yeo et al., 1997), neurite outgrowth following sciatic nerve crush is impaired (Van der Zee et al., 2003; Xie et al., 2001), and the collateral sprouting response following entorhinal cortex lesion is delayed (Van der Zee et al., 2003). By contrast, in other situations, LAR may have an inhibitory role in neurite outgrowth, because LAR-deficient PC12 cells have augmented nerve growth factor-induced neurite outgrowth (Tisi et al., 2000). Hence, LAR may inhibit or promote

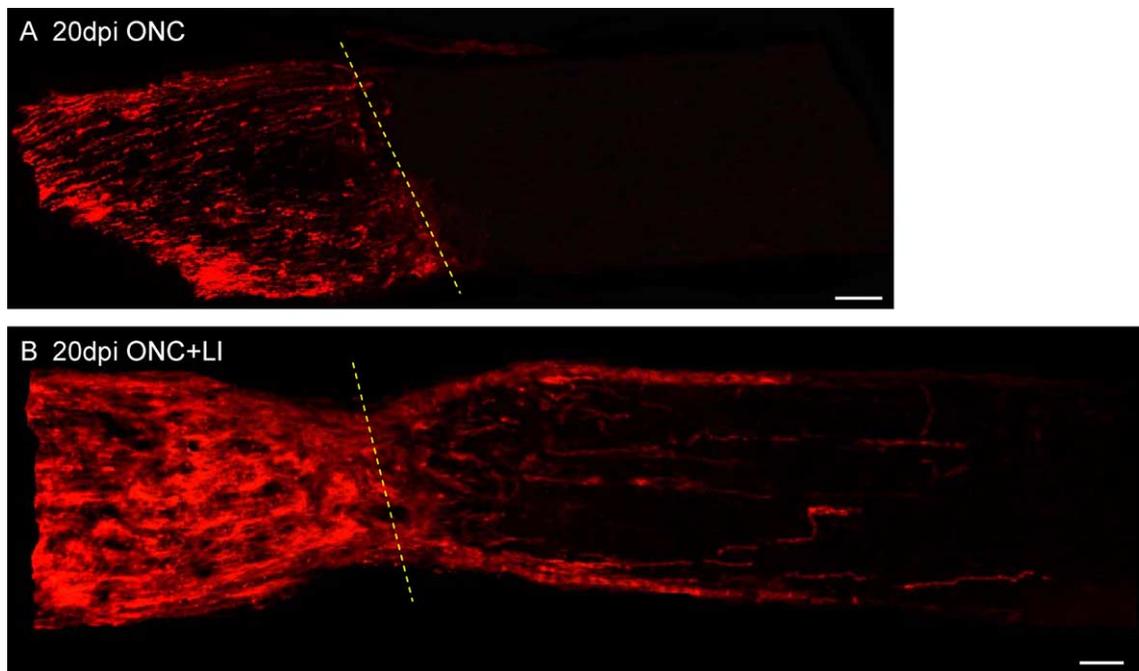


Fig. 1. DiI-labeled RGC axons in the adult rat ON 20 dpi after (A) ONC alone and (B) ONC combined with LI. The lesion site is marked by a yellow interrupted line (scale bars: 100 μ m).

axon regeneration depending on context. Indeed, PTPs can differentially affect neurite outgrowth of vertebrate CNS neurons depending on the area in which they are located (Garwood et al., 1999; Wang and Bixby, 1999). The involvement of RPTP α in adult mammalian CNS axon regeneration has not previously been investigated. Roles found for RPTP α so far include in vitro regulation of cell-substratum adhesion (Harder et al., 1998), involvement in neuronal differentiation in vitro (den Hertog et al., 1993), and a role in retinal ganglion cell (RGC) differentiation during zebrafish embryogenesis (Van der Sar et al., 2002). PC12 cells overexpressing STEP exhibit increased cAMP-induced neurite outgrowth (Okamura et al., 1999). STEP associates with extracellular signal regulated kinases 1 and 2 (ERK1/2) in rat fibroblasts and is thus a potential regulator of the ERK1/2 signaling pathway (Pulido et al., 1998). Furthermore, there are reported physical and functional associations of STEP with Fyn (a member of the Src family kinases), ERK, and the NMDA receptor in neurons (Nguyen et al., 2002; Paul et al., 2000, 2003; Pelkey et al., 2002).

Following optic nerve (ON) crush (ONC) in mammals, adult RGCs do not regenerate beyond the lesion site into the distal ON stump. However, long distance regeneration is stimulated by lens and lens capsule injury (LI) (Fischer et al., 2000; 2001; Leon et al., 2000), and by intravitreal peripheral nerve (PN) grafting (Berry et al., 1996, 1999). Neurotrophic factors released by the PN implant (Berry et al., 1996) and a proteinaceous factor released by macrophages infiltrating the eye following LI (Leon et al., 2000; Lorber et al., 2002; Yin et al., 2003), and present in the PN implant (Berry et al., 1996), appear responsible, at least in part, for axon regeneration. Although several PTPs have been implicated in neuronal responses in the developing and mature CNS, their role in RGC axon regeneration in mature mammals remains to be defined. In this study, we chose to look at two PTPs, namely RPTP α and STEP, that had not been previously investigated in the context of CNS axon regeneration, and one PTP, LAR, that previous studies

had implicated as a modulator of both CNS and PNS axon regeneration.

As a first step in investigating a role for RPTP α , STEP, and LAR in adult rat RGC axon regeneration, we chose to evaluate their expression in the retina and ON following either ONC alone (non-regenerating ON model) and ONC combined with LI/intravitreal PN grafting (regenerating ON models). We hypothesize that differential PTP expression in the regenerating versus non-regenerating ON models would highlight a role for PTPs in RGC axon regeneration.

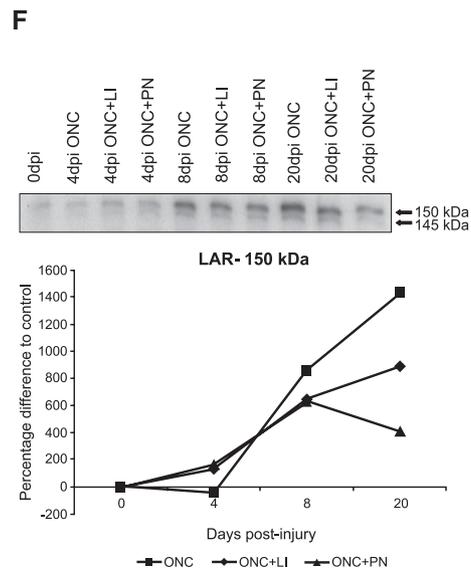
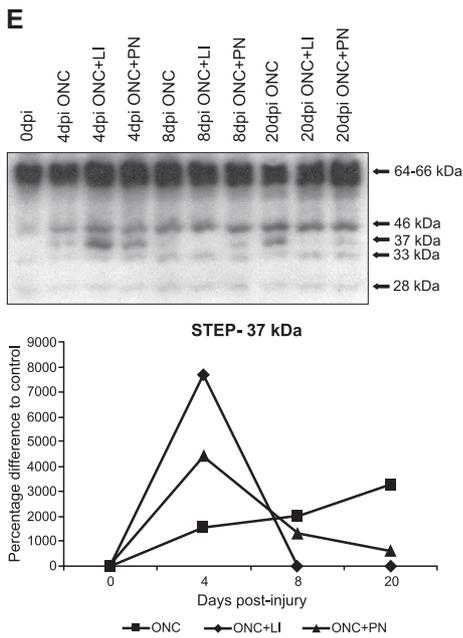
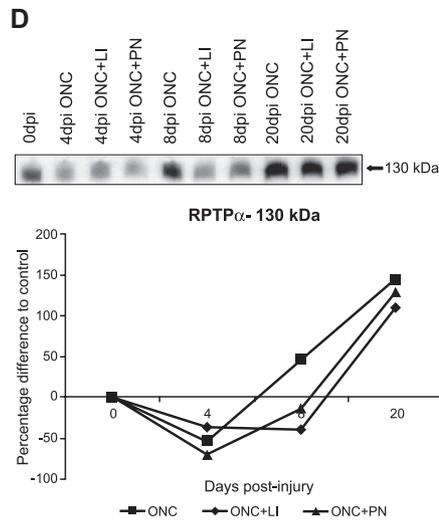
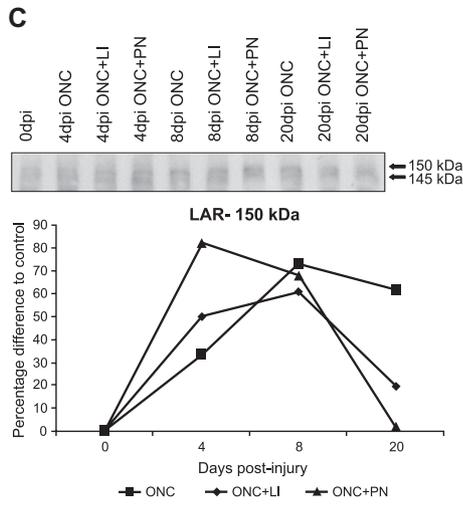
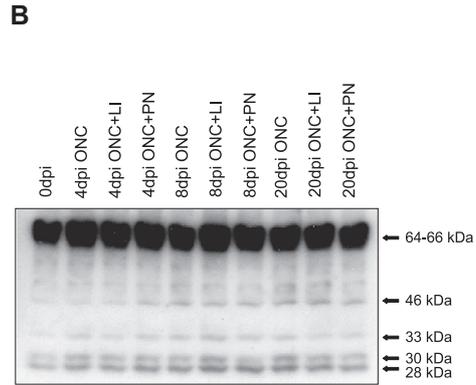
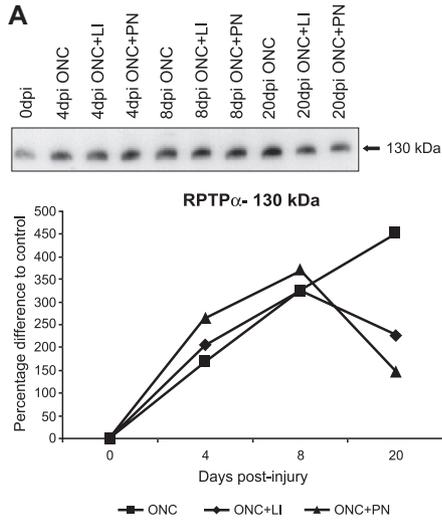
Results

Following ONC, the axons of RGCs do not regenerate past the lesion site into the distal ON stump (Fig. 1A), but do so after either intravitreal PN grafting (Berry et al., 1996, 1999) or LI (Fig. 1B). We studied the spatiotemporal expression of RPTP α , STEP, and LAR in non-regenerating and regenerating (induced via either intravitreal PN grafting or LI) adult rat retina and ON by immunohistochemistry and Western blotting. Immunohistochemistry revealed cellular PTP localization, while Western blotting provided quantification of changes in protein expression. The cellular PTP localization was studied at 0, 2, 4, 6, 8, 10, 12, and 20 days postinjury (dpi) both in the retina and ON of regenerating and non-regenerating models, allowing selection of three (4, 8, 20 dpi) key time points for Western blot analysis.

Spatiotemporal expression of RPTP α , STEP, and LAR

Western blots

Retina. Western blotting detected the 130-kDa band of RPTP α , as previously described (Den Hertog et al., 1994). RPTP α was



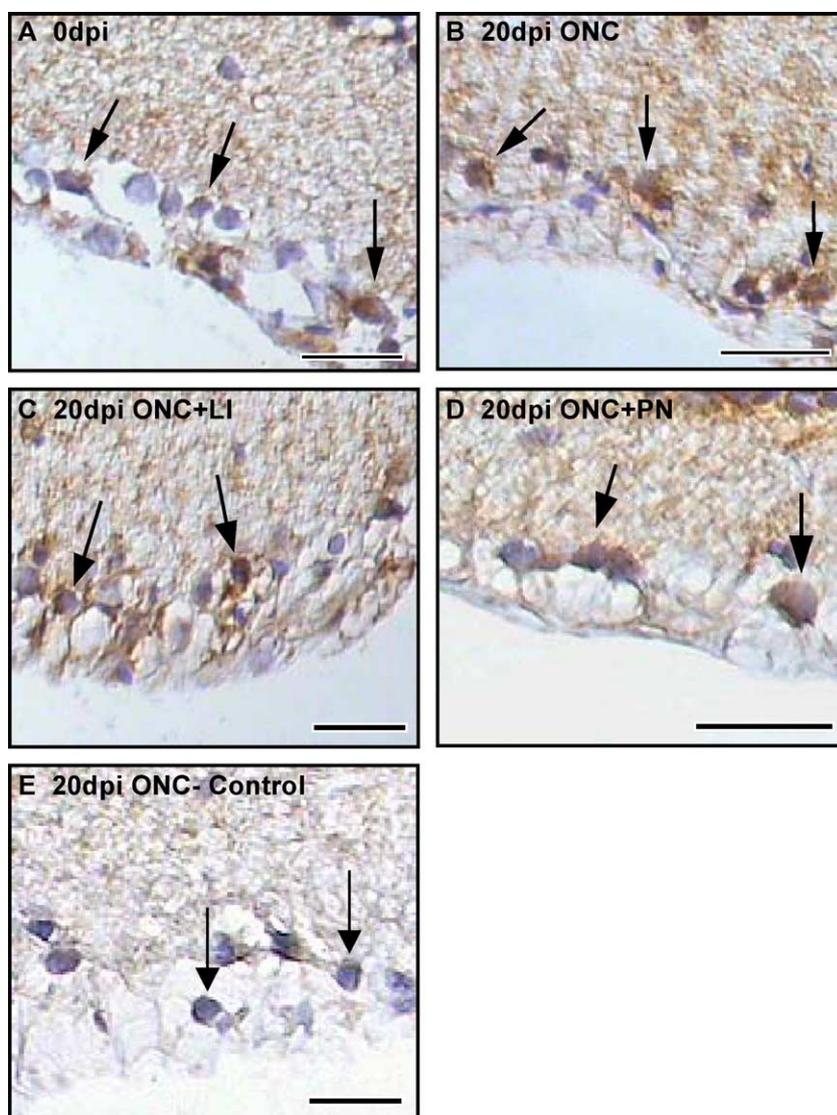


Fig. 3. RPTP α expression in adult rat RGCs (arrows) in the retina (A) of an intact animal, (B) 20 dpi after ONC alone, (C) 20 dpi after ONC and LI, (D) 20 dpi after ONC, and intravitreal PN grafting; (E) preabsorbed RPTP α antibody control on a retinal section at 20 dpi following ONC alone, to show specificity of the RPTP α antibody (scale bars: 50 μ m). The postinjury increase in expression is attenuated in regenerating (ONC + LI, ONC + PN) versus non-regenerating RGCs (ONC). PTP labeling is in brown, whereas nuclei are counterstained in blue.

expressed at a low level in the retina of control adult rats, with expression rising acutely after injury both in regenerating and non-regenerating models (Fig. 2A). RPTP α expression was most prominent at 20 dpi in the retina of the non-regenerating model, with attenuated levels of expression seen at this time point when ONC was combined with either LI or intravitreal PN grafting to stimulate RGC axon regeneration (Fig. 2A).

Five previously reported STEP isoforms, namely STEP₂₈, STEP₃₀, STEP₃₃, STEP₄₆, and STEP_{64–66} (Boulanger et al., 1995; Bult et al., 1997; Lombroso et al., 1993; Raghunathan et al., 1996), were expressed in the normal adult rat retina (Fig. 2B).

Expression of all five isoforms rose after injury, with no major differences in expression seen between regenerating and non-regenerating models up to 20 dpi (Fig. 2B).

Western blotting detected 145- and 150-kDa splice forms of the LAR extracellular subunit (Streuli et al., 1992). As the LAR antibody used is specific for the N-terminal of the LAR 150-kDa ectodomain, we concentrated on quantification of the 150-kDa band. Expression of LAR 150 kDa was low in the retina of controls, but rose modestly after injury in both the regenerating and non-regenerating models (Fig. 2C). At 4 dpi, expression was increased in regenerating versus non-regenerating retina but this

Fig. 2. Western blots for (A) RPTP α , (B) STEP, and (C) LAR from intact adult rat retina and from retina of rats in regenerating and non-regenerating models at 4, 8, and 20 dpi. Western blots for (D) RPTP α , (E) STEP, and (F) LAR from intact adult rat ON and from ON in regenerating and non-regenerating models at 4, 8, and 20 dpi. Protein size estimates (in kDa) are indicated on the right. For A, C, D, E, and F, the postinjury changes in RPTP α (A, D-130 kDa band), STEP (E-37 kDa), and LAR (C, F-150 kDa band) have been quantified. Quantification is only relative as Western blots consist of six pooled samples per time point, which does not allow statistical analysis.

was subsequently reversed. At 20 dpi, expression of LAR 150 kDa was reduced in the retina of regenerating versus non-regenerating models (Fig. 2C).

Optic nerve. As found for the retina, ON Western blots detected the 130-kDa band of RPTP α . RPTP α was expressed at significant levels in the intact adult rat ON, but by 4 dpi, expression levels fell in the ON of both the regenerating and non-regenerating models (Fig. 2D). However, by 8 dpi, expression levels had markedly increased in the ON of the non-regenerating model and were higher compared to regenerating models (Fig. 2D). At 20 dpi, expression levels in the ON of both the non-regenerating and regenerating models were increased to almost equivalent levels (Fig. 2D).

Four different STEP isoforms, STEP₂₈, STEP₃₃, STEP₄₆, and STEP_{64–66}, were expressed in the ON of intact adult rats (Fig. 2E). Expression of STEP₂₈, STEP₃₃, STEP₄₆, and STEP_{64–66} increased postinjury, with no major reproducible differences in expression occurring between the regenerating and non-regenerating models. Of note, expression of a further isoform, STEP₃₇, was induced at 4 dpi both in regenerating and non-regenerating ON models. However, the temporal response of STEP₃₇ expression was different between the two models. Thus, in the absence of sustained axon regeneration, levels of STEP₃₇ peaked late, at around 20 dpi. By contrast, levels of STEP₃₇ peaked earlier, at 4 dpi, in both regenerating ON models. Interestingly, the induction of STEP₃₇ expression at 4 dpi was higher after LI than after intravitreal PN grafting (Fig. 2E).

As found for the retina, Western blotting of the ON for LAR detected 145- and 150-kDa splice forms of the LAR extracellular

subunit. LAR 150 kDa was expressed at a low level in the ON of control adult rats (Fig. 2F). Expression remained low at 4 dpi, but was strongly increased by 8 dpi in the ON of both regenerating and non-regenerating models, with lower levels found in regenerating models. At 20 dpi, LAR 150 kDa expression levels continued to increase in the ON of the non-regenerating model, with attenuated expression further apparent in regenerating models (Fig. 2F).

Immunohistochemistry

We have illustrated the Western blots for RPTP α , STEP, and LAR in the retina and ON samples of regenerating and non-regenerating models, since the three PTPs exhibited differences in the patterns of expression between regenerating and non-regenerating models at different dpi. However, photomicrographs of immunoreactivity in the retina and ON of only one of the PTPs, namely RPTP α , are illustrated because the results obtained were similar in terms of cell localization and distribution to those of STEP and LAR. Equivalent data for STEP and LAR are available on request from the corresponding author. We have chosen to show the RPTP α immunohistochemistry photomicrographs of the retina at 20 dpi and of the ON at 8 dpi, where the differences in expression between regenerating and non-regenerating models were most prominent.

Retina. In the retina, we have focused on PTP expression in RGCs, where the observed postinjury changes were greatest. As found by Western blotting, RPTP α expression was relatively low in control RGCs (Fig. 3A). At 20 dpi following ONC alone, RGC RPTP α

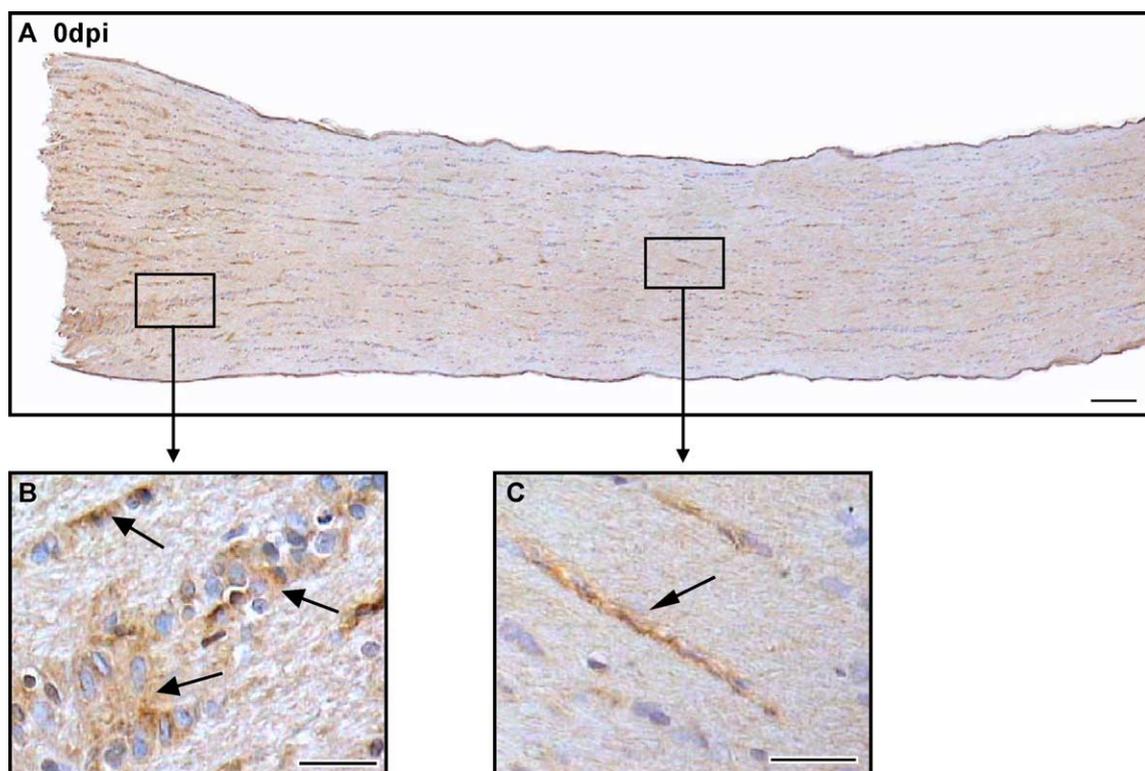


Fig. 4. RPTP α expression in the ON of an intact adult rat. Panel A shows a low-power overview of the ON (scale bar: 100 μ m). RPTP α expression (arrows) in the equivalent tissue of the (B) proximal and (C) distal stumps after ON crush (scale bar for B and C: 50 μ m). RPTP α expression is found throughout the ON. PTP labeling is in brown, whereas nuclei are counterstained in blue.

expression levels had increased (Fig. 3B), with attenuated levels of expression apparent when ONC was combined with either LI (Fig. 3C) or intravitreal PN grafting (Fig. 3D). To show specificity of the RPTP α antibody, we applied primary RPTP α antibody solution that was preincubated with excess glutathione-agarose linked GST-RPTP α to a retinal section in which RPTP α expression was highest (Fig. 3B), that is, that of a rat 20 dpi with ONC alone (Fig. 3E). This control showed no staining, establishing specificity of the antibody.

Optic nerve. We have chosen to show low power, overview pictures of the intact adult rat ON (Fig. 4A), 8 dpi following ONC alone (Fig. 5A), 8 dpi following ONC combined with LI (Fig. 6A), and 8 dpi following ONC combined with intravitreal PN grafting (Fig. 7A), to give an overview of RPTP α expression at key time points, when differences in expression between regenerating and non-regenerating models were most prominent.

RPTP α was expressed predominantly by glia in the ON of intact adult rats (Figs. 4A–C). At 8 dpi in the ON of the non-regenerating model, RPTP α staining was strongly increased in glia (Figs. 5A–D), with attenuated levels of immunoreactivity in the

ON of the regenerating models, induced either via LI (Figs. 6A–D), or intravitreal PN grafting (Figs. 7A–D). The attenuated protein levels in the ON of regenerating versus non-regenerating models were clearly visible in the proximal stump, lesion site, and distal stump of the ON (compare Figs. 5B–D, 6B–D, 7B–D).

To further define which cell types the PTPs colocalized within the various ON segments, we undertook colocalization studies, choosing for this analysis time points of 8 and 20 dpi in the non-regenerating model, when PTP expression was strongest. Again, RPTP α colocalization (Figs. 8A–H) is shown as a representative for STEP and LAR, because all three PTPs colocalized within the same ON cell types. In the proximal ON stump, RPTP α predominantly localized to GFAP + astrocytes (Fig. 8A), whereas, in the lesion, RPTP α was found within ED1 + macrophages/microglia (Fig. 8B). RPTP α also colocalized with collagen I (Fig. 8C), laminin (Fig. 8D), and astrocytes (not shown) in the ON scar. In the distal ON stump, RPTP α localized to CAII + oligodendrocytes (Fig. 8E) and NG2 + synantocytes (Berry et al., 2002; Butt et al., 2002; Sandvig et al., 2004) (Fig. 8F), but not to astrocytes (not shown). RPTP α localized to occasional oligodendrocytes in the proximal ON stump (not shown). Interestingly,

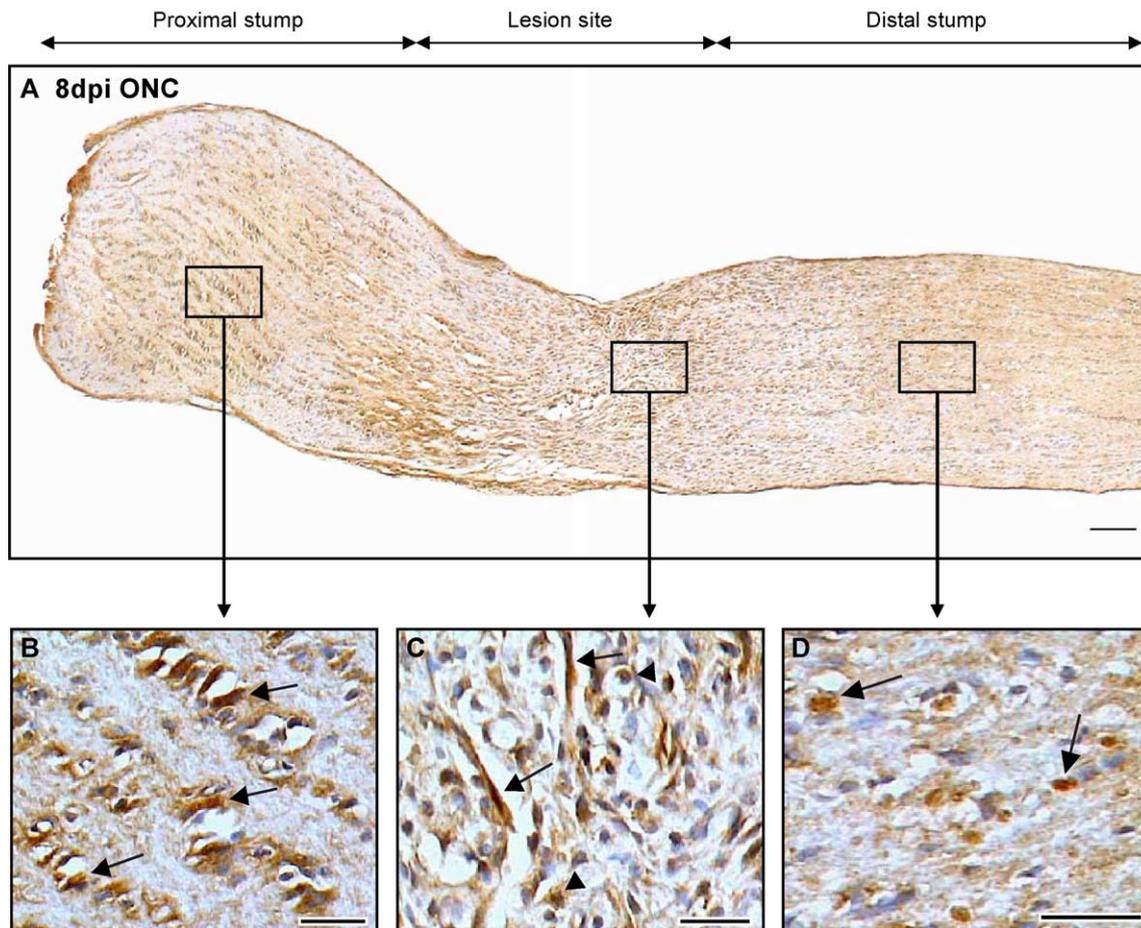


Fig. 5. RPTP α expression in the ON of an adult rat 8 dpi after ONC alone. (A) Low-power overview of the ON illustrating the three regions of the crushed ON, namely the proximal ON segment that extends from the eye to the lesion site, the lesion site itself, and the distal ON segment that extends from the lesion site to the optic chiasm (scale bar: 100 μ m). RPTP α expression (arrows) in (B) the proximal stump, (C) the lesion site (RPTP α expression in macrophages and microglia, arrowheads; RPTP α expression in extracellular scar matrix, arrows), (D) the distal stump (scale bar for B, C, and D: 50 μ m). RPTP α expression is strongly increased in the proximal stump, the lesion site, and the distal stump of the ON. PTP labeling is in brown, whereas nuclei are counterstained in blue.

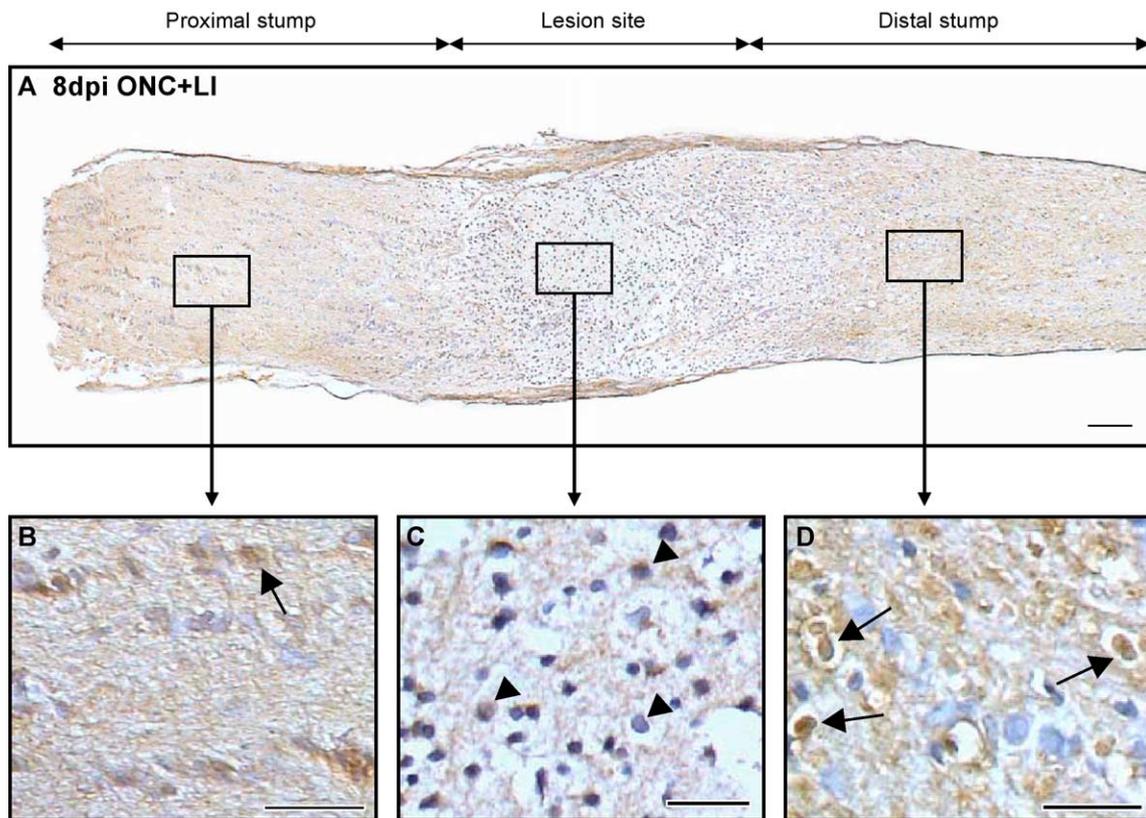


Fig. 6. RPTP α expression in the ON of an adult rat 8 dpi after ONC and LI. (A) Low-power overview of the ON (scale bar: 100 μ m). RPTP α expression (arrows) in (B) the proximal stump, (C) the lesion site (RPTP α expression in macrophages and microglia, arrowheads), (D) the distal stump (scale bar for B, C, and D: 50 μ m). RPTP α expression in the proximal stump, lesion site, and distal stump is lower compared to the non-regenerating ON. PTP labeling is in brown, whereas nuclei are counterstained in blue.

RPTP α did not localize to either intact (Figs. 9A–C) or regenerating RGC axons (Figs. 9D–F).

Discussion

Several factors limit the success of CNS axon regeneration after injury, including axon growth inhibitory molecules that are expressed along the projection pathway of growing axons, such as myelin-associated glycoprotein (MAG), NOGO (Bandtlow and Schwab, 2000; Hunt et al., 2003; McKerracher et al., 1994; Sandvig et al., 2004), oligodendrocyte myelin glycoprotein (OMgp), and chondroitin sulphate proteoglycans (CSPG) (Asher et al., 2000; Fidler et al., 1999; Levine, 1994; Niederost et al., 1999; Sandvig et al., 2004). Essential neurotrophic factors and their cognate receptors may also be limited in supply (Logan and Berry, 2002). As a consequence, after adult ONC, RGC axons do not regenerate beyond the lesion (Berry et al., 1996, 1999). However, it is possible to stimulate RGC axon regeneration across the lesion site and into the distal ON stump by either intravitreal PN grafting (Berry et al., 1996, 1999) or by LI (Fischer et al., 2000, 2001; Leon et al., 2000). We hypothesize that, if PTPs play a role in CNS axon regeneration, their expression will be differently modulated in regenerating versus non-regenerating ON models. In both models, axons sprout in the first week after injury in the proximal ON stump and grow within the lesion site. When further stimulated to grow by either LI or intravitreal PN grafting, significant numbers of axons cross the ON lesion site at 8 dpi and grow robustly into

the distal ON stump. By contrast, axons in the ON of the non-regenerating model fail to grow beyond 8 dpi (Berry et al., 1996, 1999).

The attenuated expression of LAR and RPTP α in the RGCs that becomes most apparent at 20 dpi, together with the coincident enhanced RGC survival observed in regenerating versus that of non-regenerating model (Berkelaar et al., 1994; Leon et al., 2000), suggests that LAR and RPTP α may promote late phases of neuronal apoptosis. A role for LAR in apoptosis has been suggested by Weng et al. (1998) who, by overexpressing LAR in cultured mammalian U2OS cells, activated the caspase pathway and induced apoptosis. Furthermore, LAR-deficient PC12 cells have decreased serum deprivation-induced apoptosis (Tisi et al., 2000). RPTP α is involved in the activation of the src pathway (den Hertog et al., 1993; Harder et al., 1998; Pallen, 2003), a signaling pathway that is reported to regulate cell survival both positively and negatively depending on context (Karni and Levitzki, 2000; Zhong et al., 2002).

Furthermore, the attenuated expression of LAR and RPTP α found in RGCs of regenerating versus those of non-regenerating models suggests that both PTPs may also inhibit axonal regeneration, perhaps by activating disassembly of microtubules, thereby disrupting the supply of structural elements necessary for axonal outgrowth. Interaction-trap assays show that Trio, a serine–threonine kinase, binds to LAR (Debant et al., 1996). Interestingly, one of the Trio guanine nucleotide exchange factor domains is a specific activator of RhoA, a GTPase that signals axon growth inhibition by inducing growth cone collapse

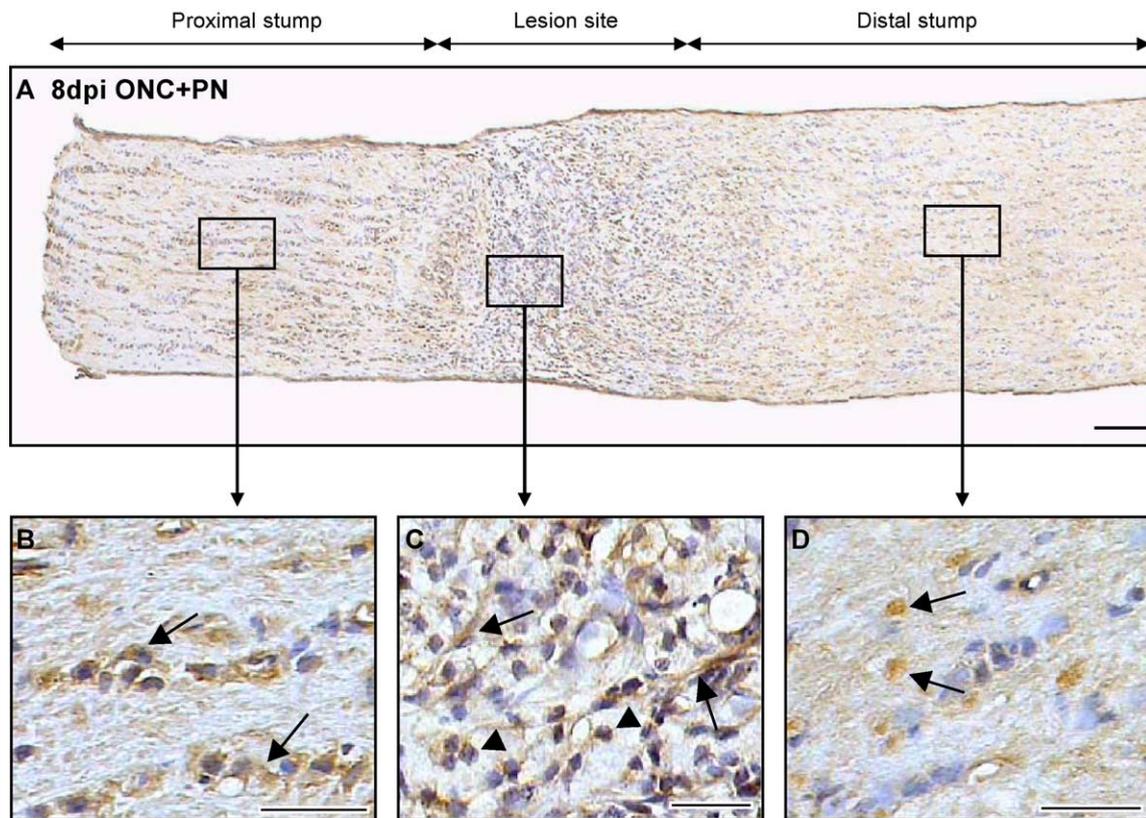


Fig. 7. RPTP α expression in the ON of an adult rat 8 dpi after ONC and intravitreal PN grafting. (A) Low-power overview of the ON (scale bar: 100 μ m). RPTP α expression (arrows) in (B) the proximal stump, (C) the lesion site (RPTP α expression in macrophages and microglia, arrowheads; RPTP α expression in extracellular scar matrix, arrows), (D) the distal stump (scale bar for B, C, and D: 50 μ m). RPTP α expression in the proximal stump, lesion site, and distal stump is lower compared to the non-regenerating ON. PTP labeling is in brown, whereas nuclei are counterstained in blue.

(Kaufmann et al., 1998; Sandvig et al., 2004). This suggests that LAR signals disruption of orderly actin formation, causing growth cone collapse, via the RhoA pathway. Ligand identification for the RPTPs is incomplete. It has been shown recently that a small ectodomain isoform, designated LARFN5C, can function as a homophilic ligand for LAR (Yang et al., 2003). Therefore, unknown ligands, present in the extracellular milieu of the retina, may bind to LAR and RPTP α present on RGC somata, thereby directly signaling cell death and axonal growth inhibition. Interestingly, expression levels of STEP isoforms are not different in regenerating and non-regenerating RGCs, arguing against a direct role for STEP in RGC survival and axonal regeneration.

The observed attenuated expression of LAR and RPTP α in ON glia in regenerating compared to non-regenerating models implies that local expression by glia in the ON may also exert inhibitory effects on axonal regeneration. However, neither LAR nor RPTP α localizes to RGC axons, so that a direct signaling effect on RGC axon regeneration would thus require that they act as ligands for as yet unidentified receptors on RGC axons. LAR and RPTP α may also signal axon growth inhibition indirectly by acting as glial receptors to which unknown ligands can bind, thereby initiating glial expression of inhibitory ligands that engage the axon growth inhibitory receptors on axon growth cones. The localization of LAR and RPTP α to astrocytes, oligodendrocytes, and synantocytes together with the known expression of inhibitory CSPG by astrocytes, oligodendrocytes (Asher et al., 2000; Fidler et al., 1999; Levine, 1994; Niederost et al., 1999), and synantocytes

(Berry et al., 2002; Butt et al., 2002; Sandvig et al., 2004), and MAG and NOGO by oligodendrocytes (Bandtlow and Schwab, 2000; McKerracher et al., 1994), prompts speculation that PTPs may upregulate postinjury expression of glial-derived axon growth inhibitory molecules. The time course of attenuated LAR and RPTP α ON expression suggests that these two PTPs act sequentially to inhibit axon growth. Interactions between RPTP α and LAR via their membrane-distal domains occur, indicating a specific mechanism of cross-talk between the two PTPs that may regulate their biological functions (Blanchetot and den Hertog, 2000).

Interestingly, whereas STEP₂₈, STEP₃₃, STEP₄₆, and STEP₆₄₋₆₆ are constitutively expressed in the intact adult ON, STEP₃₇ expression is only induced upon injury. Attenuated expression of the STEP₃₇ isoform was associated with sustained axon regeneration at later time points in the injury response. This suggests that STEP₃₇ may also negatively influence axonal regeneration. STEP₃₇, a cytosolic STEP isoform also present in the adult striatum and substantia nigra (Boulanger et al., 1995; Lombroso et al., 1993), may act as an intracellular signaling molecule, possibly involved in the upregulation of glial inhibitory ligands.

Whereas RPTP α and LAR may act sequentially to exert growth inhibition, the synchronous time course of attenuated STEP₃₇ and LAR expression in the ON suggests that these two PTPs act in concert. The differential time course of STEP₃₇ expression after ON lesions when regeneration is induced by LI or by intravitreal PN grafting suggests that the regeneration response in these two models may involve slightly different signaling pathways.

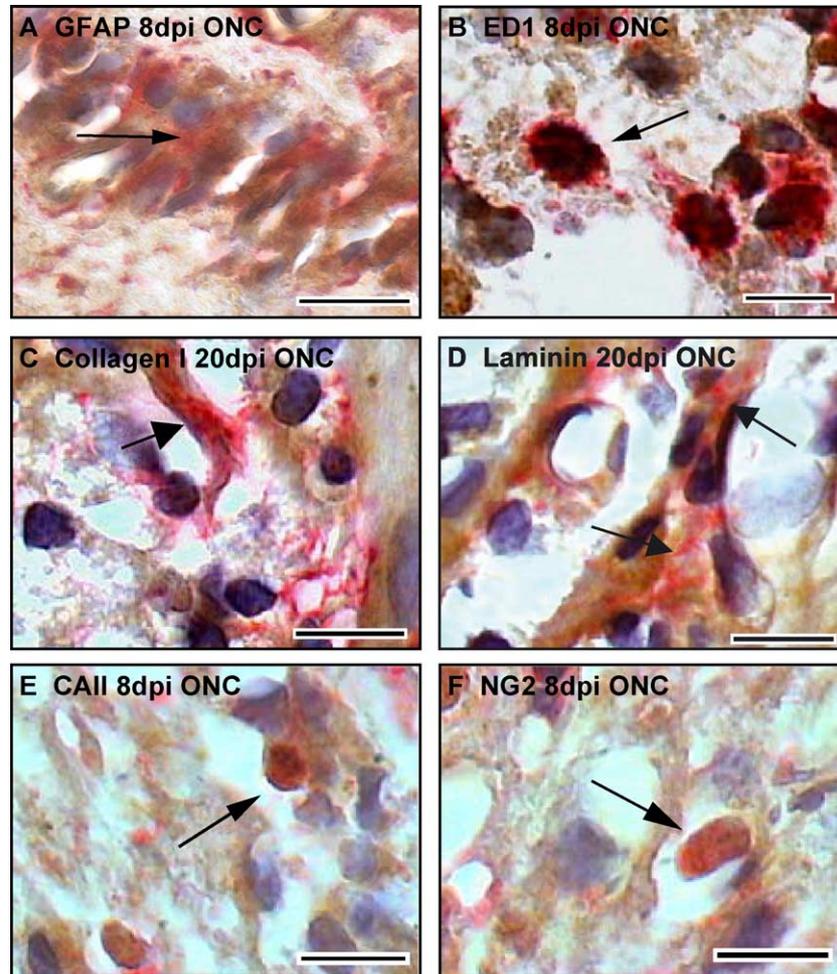


Fig. 8. Colocalization of RPTP α with (A) GFAP + astrocytes in the proximal ON stump at 8 dpi; (B) ED1 + macrophages/microglia in the ON lesion site at 8 dpi; (C) collagen I in the ON lesion site at 20 dpi; (D) laminin in the ON lesion site at 20 dpi; (E) CAII + oligodendrocytes in the distal ON stump at 8 dpi; (F) NG2 + synantocytes in the distal ON stump at 8 dpi, after adult rat ONC. Arrows indicate colocalization of RPTP α with the various cell types in the ON. Therefore, RPTP α colocalizes with glia, extracellular scar matrix, and macrophages/microglia in the ON. RPTP α expression is in brown, cell marker is in red and nuclei are counterstained in blue (scale bars: 20 μ m).

In summary, we have found attenuated expression of LAR and RPTP α in regenerating versus non-regenerating RGC somata, and attenuated expression of LAR, STEP₃₇, and RPTP α in glia of regenerating versus non-regenerating ON. There are two alternative interpretations of the significance of these observations. The first is that LAR and RPTP α contribute to postinjury RGC apoptosis, whereas LAR, RPTP α , and STEP₃₇ play a role in RGC axon regeneration. The high PTP levels in macrophages, in the extracellular matrix of the ON scar, and, importantly, in the glia of the distal non-regenerating ON stump imply that those molecules may contribute significantly to the observed failure of axon regeneration. In addition, the raised PTP levels seen in glia throughout the proximal non-regenerating ON stump may also negatively influence the collateral axon sprouting observed by Berry et al. (1996). A second interpretation of the observed reduction in PTP levels in ON glia in the regenerating models compared to those in the non-regenerating model might be that growth-stimulated axons directly modulate glial PTP expression either by down-regulation of PTP gene transcription or by posttranslational destruction of the PTPs by proteinases released from axon growth cones. Future experiments using transgenic animals will allow the rigorous testing of the hypotheses resulting from the current study.

Experimental methods

Surgical procedures

Surgical procedures were licensed by the British Home Office. Adult, female 200–250 g Fischer rats ($n = 80$) were kept in a pathogen-controlled environment in standard cages and allowed to feed and drink ad libitum. Rats were assigned to three treatment groups: A, B, and C, each consisting of five animals (two rats for immunohistochemical and three rats for Western blotting studies) killed at 4, 8, and 20 dpi. For anterograde RGC axon labeling studies, an additional three animals each for groups A and C were set up. All animals were anaesthetized by intraperitoneal injection of Hypnorm/Hypnovel (Janssen Pharmaceuticals, Oxford, UK). Animals of treatment groups A, B, and C received an intraorbital ONC. In addition, animals of group B also received a PN graft into the vitreous body of the eye at the time of the ONC. For detailed description of the ONC and intravitreal PN grafting see Berry et al. (1996, 1999). The lens was injured in animals of group C at the time of the ONC by inserting the tip of a 25-gauge needle into the eye 2 mm anterior to the nerve head, perpendicular to the sclera to puncture the lens to a depth of about 0.5 mm. LI was confirmed by

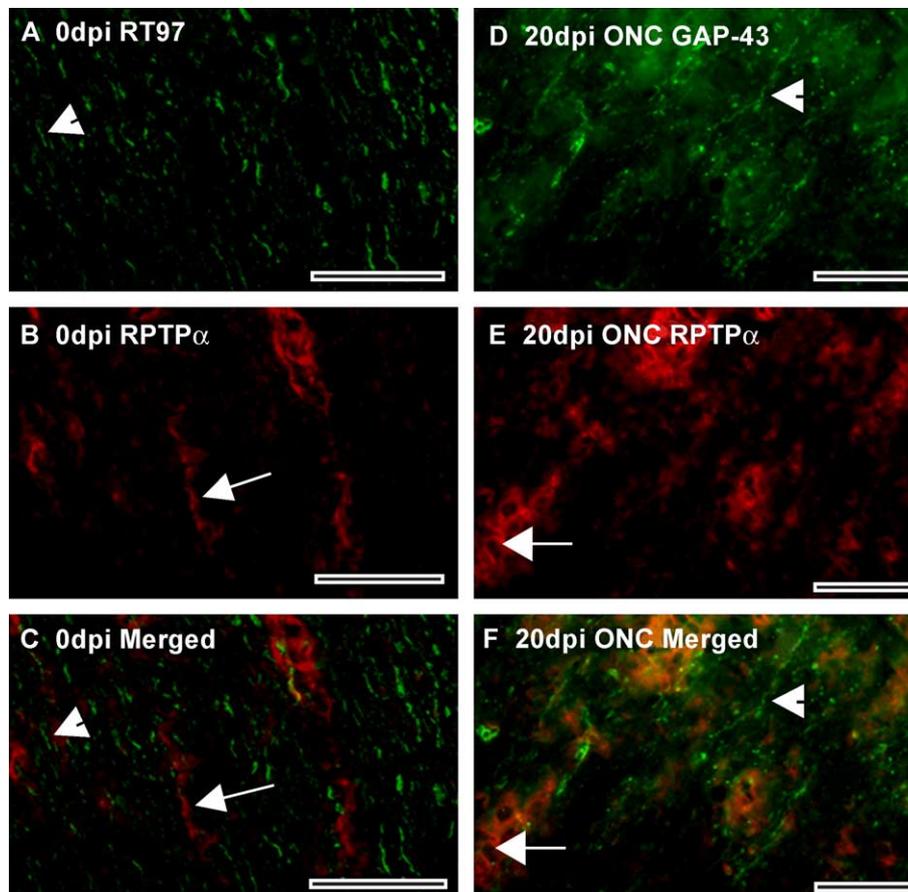


Fig. 9. Non-colocalization of RPTP α with RT97 + axons in the intact adult rat ON (A–C) and GAP-43 + axons (D–F) in the proximal stump of the adult rat ON at 20 dpi following ONC. A shows RT97 + axons, whereas D shows GAP-43 + axons; B and E show RPTP α labeling; C shows the merged picture of RT97 and RPTP α labeling (A + B); whereas F shows the merged picture of GAP-43 and RPTP α labeling (D + E). RPTP α does not colocalize with either intact or regenerating axons in the ON. RPTP α is in red (white arrow), whereas RT97/GAP-43 + axons are in green (white arrowhead) (scale bars: 50 μ m).

direct visualization through the cornea. Two additional animals each for groups A, B, and C were sacrificed at 2, 6, 10, and 12 dpi for immunohistochemical studies. Group D (two rats for immunohistochemical and three rats for Western blotting studies) comprised control rats that received no treatment.

Anterograde RGC axon labeling

Animals were killed at 20 dpi by perfusion with 4% paraformaldehyde at 4°C under anesthesia and, after removing the eyes and ON intact, the vitreous body and lens were dissected from each eye and a DiI crystal (Molecular Probes, Oregon, USA) placed against the optic disc. Eyes with their ON were kept for 1 month at 37°C in an incubator in 4% paraformaldehyde, and then embedded in OCT mounting media (Miles Inc, PA, USA). Serial sections, 10 μ m thick, were cut with a cryostat (Bright Instrument Co. Ltd., Huntington, Cambridgeshire, UK), set at –20°C, and DiI staining of ON axons was observed under a Zeiss fluorescence microscope (Zeiss, Hertfordshire, UK).

Immunohistochemistry

Animals were killed by perfusion with 4% paraformaldehyde at 4°C under anesthesia. Whole eyes and ON were removed and immersed overnight in cold 4% paraformaldehyde and washed

in cold phosphate-buffered saline (Sigma, Poole, UK). Tissue was dehydrated through a graded series of alcohols, infiltrated with polyester wax (Sigma) at 37°C (Steedman, 1957), and blocked and stored in wax at 4°C. Serial sections, 7 μ m thick, were cut with a cryostat (Bright Instrument Co. Ltd.) at 4°C, floated onto gelatin-coated slides (Surgipath, Cambridge, UK), and allowed to dry at room temperature overnight before storing at 4°C.

Rehydrated sections were incubated in blocking solution (50 mM Tris–HCl, pH 7.5; 150 mM NaCl, 0.1% Tween 20 (TBST); 4% serum) for 30 min at room temperature. Primary antibody was applied overnight at 4°C in a TBS (50 mM Tris–HCl, pH 7.5; 150 mM NaCl) solution containing 4% serum. Concentrations of the antibodies used were as follows: polyclonal rabbit anti-STEP 1–369 (Munoz et al., 2003), used at 1/1600 on ON and eye sections; polyclonal rabbit anti-LAR (Schaapveld, 1997), used at 1/2500 on ON, and at 1/3000 on eye sections; polyclonal rabbit anti-RPTP α 5478 (Den Hertog et al., 1994), used at 100 μ g/ml on ON and eye sections. In some cases, slides were double labeled with cell marker antibodies. In cases of double labeling, a biotin-blocking step (DAKO, Cambridge, UK) was performed in between both staining steps. The cell marker antibodies used were monoclonal mouse anti-ED1 macrophage antibody (Serotec, Oxford, UK) at 1/200 for hematogenous macrophages and microglia; monoclonal mouse anti-glial fibrillary acidic protein (GFAP) antibody (Sigma)

at 1/400 for astrocytes; polyclonal rabbit anti-carbonic anhydrase-II (CAII) antibody (prepared by Dr. N. Gregson, KCL, Guy's Campus, London) at 1/20,000 for oligodendrocytes; polyclonal rabbit anti-growth-associated protein-43 (GAP-43) antibody (prepared by Dr. R. Curtis and G. Wilkin, Imperial College, London) at 1/1000 for regenerating axons; monoclonal mouse anti-neurofilament antibody, RT97 (Serotec) at 1/100 for intact axons; polyclonal rabbit anti-NG2+ antibody (Chemicon, Harrow, UK) at 1/50 for synantocytes; polyclonal rabbit anti-laminin antibody (Sigma) at 1/100 as a marker for the basal laminae of the glia limitans externa and blood vessels; polyclonal rabbit anti-collagen I antibody (Monosan, Uden, The Netherlands) at 1/10.

The following day, slides were washed 4×5 min in TBST. Secondary antibody (either biotinylated goat anti-rabbit, or biotinylated horse anti-mouse antibody, 1/200, Vector Laboratories, Peterborough, UK) was applied for 1 h at 4°C in a TBS solution containing 4% serum. Following 4×5 min washes in TBS, enhancement was done using avidin/biotinylated horse radish peroxidase or phosphatase (Vector Laboratories) for 30 min at room temperature. Slides were then washed 4×5 min in TBS. Horseradish peroxidase or phosphatase staining was developed using either 3,3'-diaminobenzidine tetrahydrochloride or Vector Red, respectively (Vector Laboratories), followed by counterstaining with Haemalum (BDH, Poole, UK).

Additional heat antigen retrieval at 95°C for 10 min using 10 mM trisodium citrate (Sigma) (pH 6) was applied when staining for the rabbit anti-STEP antibody. Immunohistochemistry for the rabbit anti-RPTP α antibody varied in the following way (adapted from Den Hertog et al., 1996). Blocking was done for 1 h at room temperature in a TBST solution containing 4% swine serum. Sections were incubated in primary antibody (in blocking buffer) overnight at 4°C followed by 4×10 min washes with TBST. Secondary antibody (biotinylated swine anti-rabbit antibody 1/100, DAKO) was applied in TBS containing 1% bovine serum albumin (Sigma) for 1 h at room temperature. Steps for double labeling with RT97/GAP-43 included: (1), incubation of sections in a TBS solution containing 0.3% Triton and 1.5% serum for 30 min at room temperature after developing staining for LAR, STEP, or RPTP α with Vector Red; (2) application of primary anti-RT97 or anti-GAP-43 antibody overnight at 4°C in a TBS solution containing 1.5% serum; (3) application of secondary antibody (Alexa 488 goat anti-mouse, 1/100 or Alexa 488 goat anti-rabbit, 1/100, Molecular Probes) in a TBS solution for 1 h at room temperature.

As a control, immunohistochemistry was done without anti-LAR, STEP, and RPTP α antibodies. In addition, the procedure was performed with an antibody solution either incubated with excess glutathione-agarose linked GST-RPTP α (Den Hertog et al., 1994) or GST-STEP (Munoz et al., 2003) before use. In none of these control experiments was a specific signal obtained.

Western blotting

For each treatment group, a total of six ON and six retinae from three animals per time point were pooled. Tissues were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% NP-40; Protease inhibitor cocktail (Sigma)), kept in the same buffer for further lysis for 30 min at 4°C, and spun at 13,000 rpm for 25 min at 4°C. After removing the supernatant, the tissue was homogenized in lysis buffer and spun at 13,000 rpm for 25 min at 4°C. The supernatant was removed and added to the previous

supernatant. Protein content of the various samples was determined with the Folin-Lowry assay (BioRad, Hemel Hempstead, UK).

Equal amounts of protein (10 μ g) were loaded onto either 4–12% or 12% Novex precast gels (Invitrogen, Paisley, UK), followed by transfer to polyvinylidene difluoride membranes (Millipore, Bedford, USA). Gels were run in duplicate for each antibody or tissue and membranes were stained with Ponceau S Solution (Sigma) to ensure equal loading of protein. Membranes were incubated in 5% non-fat dry milk in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, containing 0.05% Tween 20, for 1 h at room temperature, followed by incubation with primary antibody overnight at 4°C. Concentrations of the primary antibodies were as follows: polyclonal rabbit anti-RPTP α 5478 (Den Hertog et al., 1994), used at 2 μ g/ml; monoclonal mouse anti-LAR (Transduction Labs, Lexington, KY), used at 1/250; polyclonal rabbit anti-STEP 1–369 (Munoz et al., 2003), used at 1/1000; secondary antibodies (peroxidase-conjugated donkey anti-rabbit IgG and peroxidase-conjugated sheep anti-mouse IgG; 1/10,000; Amersham, Buckingham, UK) were applied for 1 h at room temperature. Chemiluminescence was detected using the Amersham ECL kit, followed by exposure of the membranes to X-ray film. Autoradiographs in the linear range were densitometrically scanned and percentage differences to control were visualized. Quantification of Western blots was only relative as each time point consisted of six pooled samples, which did not allow statistical analysis.

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