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Delayed priming promotes CNS regeneration post-rhizotomy in Neurocan and Brevican-deficient mice

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A wealth of literature has provided evidence that reactive tissue at the site of CNS injury is rich in chondroitin sulfate proteoglycans which may contribute to the non-permissive nature of the CNS. We have recently demonstrated using a murine model of human brachial plexus injury that the chondroitin sulfate proteoglycans Neurocan and Brevican are differentially expressed by two subsets of astrocytes in the spinal cord dorsal root entry zone (DREZ) following dorsal root lesion (Beggah *et al.*, Neuroscience 133: 749–762, 2005). However, direct evidence for a growth-inhibitory role of these proteoglycans *in vivo* is still lacking. We therefore performed dorsal root lesion (rhizotomy) in mice deficient in both Neurocan and Brevican. Rhizotomy in these animals resulted in no significant increase in the number of sensory fibres regenerating through the DREZ compared to genetically matched controls. Likewise, a conditioning peripheral nerve lesion prior to rhizotomy, which increases the intrinsic growth capacity of sensory neurons, enhanced growth to the same extent in transgenic and control mice, indicating that absence of these proteoglycans alone is not sufficient to further promote entry into the spinal cord. In contrast, when priming of the median nerve was performed at a clinically relevant time, i.e. 7 weeks post-rhizotomy, the growth of a subpopulation of sensory axons across the DREZ was facilitated in Neurocan/Brevican-deficient, but not in control animals. This demonstrates for the first time that (i) Neurocan and/or Brevican contribute to the non-permissive environment of the DREZ several weeks after lesion and that (ii) delayed stimulation of the growth program of sensory neurons can facilitate regeneration across the DREZ provided its growth-inhibitory properties are attenuated. Post-injury enhancement of the intrinsic growth capacity of sensory neurons combined with removal of inhibitory chondroitin sulfate proteoglycans may therefore help to restore sensory function and thus attenuate the chronic pain resulting from human brachial plexus injury.

Keywords: nerve priming; CNS regeneration; proteoglycans; growth-inhibition

Abbreviations: CSPGs = chondroitin sulfate proteoglycans; CGRP = calcitonin gene-related peptide; DREZ = dorsal root entry zone; GFAP = glial fibrillary acidic protein; NF70 = neurofilament 70

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Introduction

Injury to the CNS leads to permanent loss of function due to the inability of severed nerve fibers to regenerate back to their targets. Lack of CNS repair has been attributed to several causes, including to extrinsic growth-inhibitory molecules associated with myelin, and to extracellular matrix chondroitin sulfate proteoglycans (CSPGs) produced by activated glial cells post-injury [reviewed in Filbin (2003), Silver and Miller (2004), Zurn and Bandtlow (2006)]. Treatment with antibodies against the myelin inhibitor Nogo (Schnell and Schwab, 1990), with a Nogo-66 receptor antagonist peptide (GrandPre *et al.*, 2002), or with enzymes degrading CSPGs (Bradbury *et al.*, 2002; Barritt *et al.*, 2006), has provided evidence in recent years for the growth-inhibitory function of these molecules. However, the limited ability to regenerate following injury

is also due to an intrinsic low capacity of adult axons to grow. Interestingly, the growth capacity of central branches of sensory neurons located in the spinal cord can be increased if their peripheral branches are previously cut (conditioning lesion), i.e. if their axonal growth program is activated before spinal cord lesion (Richardson and Issa, 1984; Neumann and Woolf, 1999). Yet the timing of this peripheral lesion is critical since peripheral nerve priming delayed by 2 weeks post-lesion fails to promote growth of central sensory axons (Neumann and Woolf, 1999). Only the combination of two priming lesions, one at the time of spinal cord injury, and one 1 week later, has so far been shown to facilitate growth in the same model (Neumann *et al.*, 2005).

Experimental model of human brachial plexus injury

Disconnection of primary sensory afferents via cervical dorsal root lesion in humans (brachial plexus injury) results in severe impairment of hand function and in intense intractable pain (Berman *et al.*, 1998). This may in part be due to the fact that sensory axons cannot re-enter the spinal cord at the dorsal root entry zone (DREZ) post-injury and project locally to the dorsal horn or to the brain stem. The DREZ is a non-permissive CNS region that undergoes Wallerian degeneration and gliosis, but no glial scar formation. However, despite the lack of scar formation, inhibition at the DREZ is particularly efficient (Ramer *et al.*, 2001*b*). For instance, peripheral nerve conditioning prior to spinal cord lesion in the rat stimulates growth within the spinal cord (Neumann and Woolf, 1999), but has little effect on the regeneration of sensory fibers through the DREZ following dorsal root injury (Chong *et al.*, 1999). We have recently shown that the reactive gliosis that occurs at the DREZ following dorsal root injury in mice (rhizotomy) coincides with increases in the expression of the chondroitin sulfate proteoglycans Neurocan, Brevican and Versican (Beggah *et al.*, 2005). This indicates that these proteoglycans may participate in growth-inhibition in this region. To evaluate whether Neurocan and Brevican are indeed inhibitory *in vivo*, dorsal root lesions were performed in mice deficient in both proteoglycans and compared to genetically matched control animals. In addition, since a peripheral conditioning lesion has been shown to have only modest effects on regeneration across the DREZ in the rat (Chong *et al.*, 1999), we wondered whether in the absence of Neurocan and Brevican such a priming lesion would result in improved enhancement of growth. Furthermore, to assess the possible effects of a priming lesion at a clinically relevant time, i.e. several weeks following injury, peripheral nerve lesion was performed 7 weeks post-rhizotomy in both knockout and control animals.

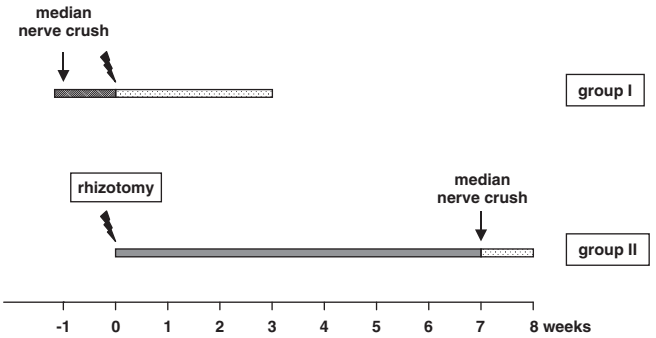

Fig. 1 Schematic illustration of the timing of the lesions. In group I, half of the mice received a median nerve crush 7 days prior to rhizotomy and all the animals were sacrificed 3 weeks later. In group II, all mice first underwent a rhizotomy, the median nerve of half of the animals was crushed 7 weeks later, and all animals were sacrificed 8 weeks following rhizotomy.

Materials and Methods

Animals and surgery

All animal protocols were approved by and performed in accordance with the regulations of the local veterinary commission in Lausanne, Switzerland. Dorsal root lesions (rhizotomies) were performed in Neurocan and Brevican double-knockout mice bred on a C57Bl/6 genetic background and in genetically matched control animals (Zhou *et al.*, 2001; Brakebusch *et al.*, 2002). These mice are viable, have a normal life-span, and have no obvious functional deficits. Dorsal cervical spinal roots of 3–4 months-old mice were unilaterally transected as previously described (Beggah *et al.*, 2005). Briefly, animals were anesthetized with an intraperitoneal injection of a mix of xylazine (10 mg/kg) and ketamine (100 mg/kg). Hemilaminectomy of the right side of the dorsolateral cervical spine was performed from C5 to T1 to expose the dorsal roots. Following a longitudinal slit in the dura mater, dorsal roots C6 to C8 were completely transected midway between the DRG and the entry zone. Care was taken to avoid minimal damage to the spinal cord. The wound was sutured and the animals were allowed to recover. For peripheral nerve lesions, the median nerve of the right front paw was exposed, crushed 10 times for 1 s and once for 10 s with flat tweezers. Median nerve crush was performed either 7 days prior, or 7 weeks following rhizotomy. Two groups of experiments were performed. In the first group (group I), median nerve crush was performed 7 days prior to rhizotomy in half of the animals and all animals were sacrificed 3 weeks post-rhizotomy ($n = 56$, 14 mice for each condition: wild-type no crush; wild-type plus peripheral nerve crush; Neurocan/Brevican knockout no crush; Neurocan/Brevican knockout plus crush). In the second group (group II), median nerve crush was performed 7 weeks following rhizotomy in half of the animals and the animals were all sacrificed at 8 weeks ($n = 92$, 23 mice for each condition) (Fig. 1). Animals with partial lesions or with small contusion injuries in or close to the DREZ were excluded from the analysis. Thus 8, 11, 8 and 10 mice (group I), and 14, 16, 13 and 15 mice (group II) were included for quantification of regeneration.

Tissue processing and immunohistochemistry

At 3 or 8 weeks post-lesion, the animals were anesthetized with a lethal dose of pentobarbital and transcardially perfused with NaCl 0.9%, followed by perfusion fixation with 50 ml 4% paraformaldehyde in H_2O. The cervical part of the spinal cord together with dorsal root ganglia was carefully removed, post-fixed overnight at 4°C, cryoprotected in 30% sucrose in PBS, and rapidly frozen in OCT compound (Tissue-Teck, Netherlands) over liquid nitrogen. Ten micrometers thick serial transverse sections were cut on a cryostat (CM3000, Leica, Germany) starting at the level of root C6 up to root T1, collected on Superfrost Plus slides (Menzel-Glaser, Braunschweig, Germany), and processed for immunofluorescence labelling. To evaluate regeneration of sensory fibers through the dorsal root and into the spinal cord, sections were exposed overnight at 4°C to a mixture of rabbit anti-calcitonin gene related peptide (CGRP, 1:10 000; Peninsula Laboratories, San Carlos, CA, USA) for small and medium-sized unmyelinated axons, or mouse anti-neurofilament (neurofilament-70, 1:100, clone 2F11, DAKO) for large diameter myelinated axons, and rat anti-laminin α2 chain antibodies (1:150, clone 4H8-2, Alexis Corporation, Lausen, Switzerland) to outline the PNS/CNS border. To examine the possible growth of sensory axons along blood vessels, sections were labelled with goat anti-human glucose transporter 1 (Glut-1, 1:100, Santa Cruz Biotechnology, Inc.) (Choeiri *et al.*, 2002). In addition, glial activation in the DREZ was assessed using antibodies against nestin (1:1000, Chemicon, Temecula, CA, USA), CD11b (MAC-1) (1:100, SEROTEC, Oxford, UK), or NG2 proteoglycan (1:400, Chemicon, Temecula, CA, USA). The absence of Neurocan and Brevican in Neurocan and Brevican-deficient mice was visualized using anti-Neurocan antibodies (1:1000, raised in guinea pigs against a C-portion of the glycosaminoglycan (GAG)-binding region of mouse Neurocan; generous gift of Dr D. Zimmermann, University of Zurich, Switzerland) and anti-Brevican antibodies (1:5000; raised in rabbits against the entire core protein of the recombinant secreted form of rat Brevican; kind gift of Dr R. Timpl, Max-Planck-Institute for Biochemistry, Martinsried, Germany). Primary antibodies were visualized with Alexa Fluor 594 goat anti-rabbit, goat anti-rat or goat anti-guinea-pig secondary antibodies, or Alexa Fluor 488 goat anti-mouse or goat anti-rat antibodies (Molecular Probes, Invitrogen). The slides were cover-slipped using Gel Mount™ (Sigma, Switzerland). Digital images were captured with an Olympus BX40 epifluorescence microscope (Olympus, Switzerland) equipped with a Q-imaging Fast 139/4 digital camera (Canada), and processed using Adobe Photoshop software (Adobe systems, USA).

Quantification of regeneration and statistical analysis

Regeneration was quantified on digital images of serial transverse sections (8–15 sections/animal) taken at 30 µm intervals with a 20 × objective. Only fibres regenerating throughout cervical dorsal root C7 were assessed to minimize possible interference of fibres from adjacent non-lesioned roots. The number of CGRP- or neurofilament-positive sensory fibres growing into degenerating white matter of the DREZ outlined with anti-laminin staining of the peripheral root, was calculated as a regeneration index: number of fibres extending >10 µm beyond the PNS/CNS border divided by the number of fibres regenerating in the dorsal root in each animal. Only fibres present in the cone of the DREZ protruding into the dorsal root were counted (Fig. 1, supplementary material; numbered lines). Fibres visible more distally (asterisks) were considered to be extensions of the same fibres. To evaluate the length of the CGRP-positive fibres extending into the DREZ, regenerating axons were traced using a Morpho Expert Imaging Software (Explora Nova, La Rochelle, France) (Fig. 1, supplementary material; coloured lines). Only the segments visible in the cone of the DREZ and in the plane of the section were traced. The imaging system measures lengths in pixels which are then transformed into micrometers (1 pixel = 2.38 µm). The sum of the fibre lengths was divided by the number of fibres that had regenerated through cervical dorsal root C7 to give a mean fibre length per animal. The analyst was blind to the treatments and genotypes. Statistical differences in the regeneration indices and in the fibre lengths between various conditions were evaluated with a non-parametric test (Mann–Whitney) using the GraphPad PRISM® statistics program (GraphPad Software Inc., USA).

Results

Sensory fibre regeneration following rhizotomy in Neurocan/Brevican-deficient and genetically matched control animals

The growth-inhibitory CSPGs Neurocan and Brevican have been shown to be expressed in subsets of astrocytes in the DREZ for several weeks following dorsal root lesion (Beggah *et al.*, 2005). To evaluate whether they participate in the non-permissive environment of the DREZ *in vivo*, regeneration of sensory fibres was analysed in Neurocan/Brevican double-knockout mice (Fig. 2C), 4 weeks following rhizotomy and compared to genetically matched control animals. The degree of regeneration into degenerating white matter of the DREZ was assessed by calculating a regeneration index = ratio of the number of fibres crossing the PNS/CNS border, divided by the number of fibres that have regenerated in the peripheral dorsal root (see Fig. 1, supplementary material). Regeneration of CGRP-positive sensory fibres was not significantly different in knockout and in control animals (Fig. 2A), corresponding to an average number of 1.25 ± 0.31 and 1.45 ± 0.25 fibres in the DREZ and 7.65 ± 1.0 and 8.4 ± 0.85 fibres in the dorsal root of knockout and wild-type animals, respectively. Similarly, the mean length of the CGRP-positive fibres growing across the DREZ (Fig. 2B) and the regeneration index of neurofilament-positive fibres was not different (0.61 ± 0.15 and 0.76 ± 0.15 fibres in the DREZ and 6.55 ± 0.63 and 6.86 ± 0.38 fibres in the dorsal root of knockout and wild-type animals, respectively; not shown). Absence of these two proteoglycans is therefore not sufficient to promote regeneration into the spinal cord.

Sensory fibre regeneration post-rhizotomy in Neurocan/Brevican-deficient and control mice following a pre-conditioning lesion of the median nerve

Since a peripheral conditioning lesion has been shown to promote the growth of lesioned sensory fibres within

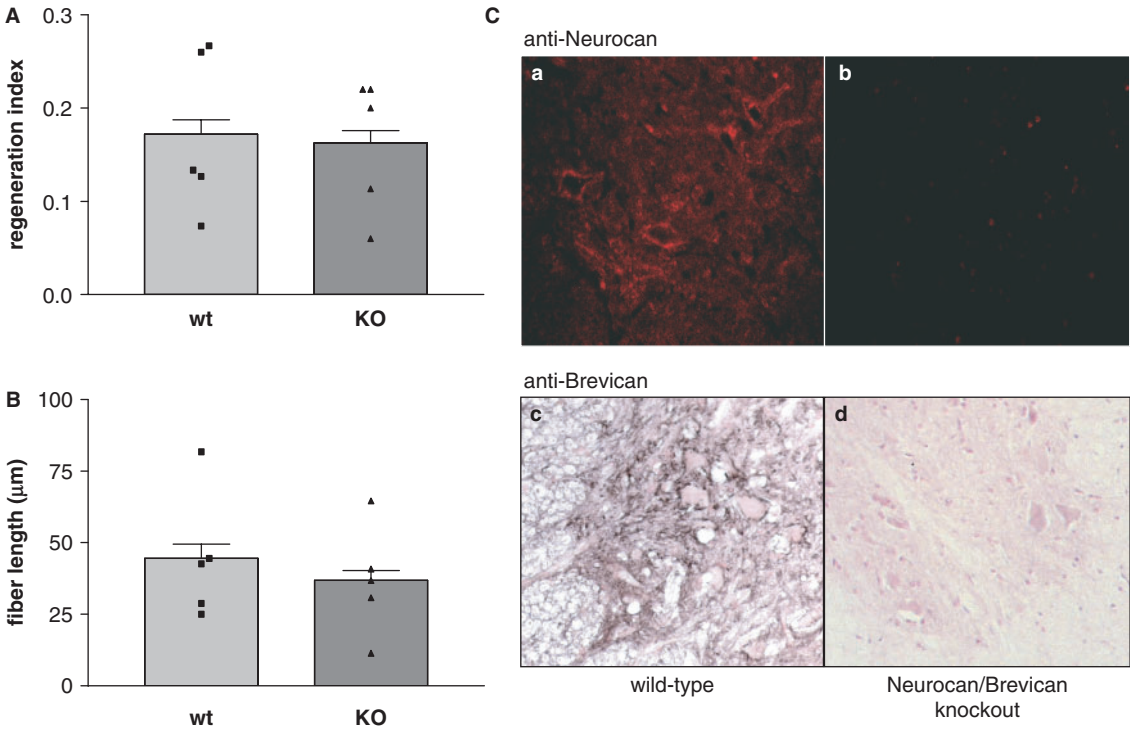

Fig. 2 Sensory fibre regeneration following rhizotomy in Neurocan/Brevican-deficient and genetically matched control mice. (**A**) Quantitative evaluation of the number of CGRP-positive fibres that have regenerated beyond the PNS/CNS border 4 weeks post-lesion calculated as a regeneration index (number of fibres present in the DREZ divided by the number of fibres regenerating in the dorsal root). No significant difference was observed in double-knockout animals (KO) compared to controls (wt). (**B**) Quantification of the mean fibre length in the DREZ (sum of the total fibre length divided by the number of fibres regenerating through the dorsal root) did not reveal any statistically significant differences between knockout and control animals. The data are expressed as means ± SEM; $n = 5$ in each group. (**C**) Absence of Neurocan and Brevican immunolabelling in perineuronal nets of motoneurons of the ventral spinal cord of Neurocan/Brevican-deficient mice (b, d) and their presence in wild-type controls (a, c).

the spinal cord (Richardson and Issa, 1984; Neumann and Woolf, 1999), but to have only minor effects on regeneration into the DREZ following rhizotomy in the rat (Chong *et al.*, 1999), we hypothesized that if Neurocan and Brevican are indeed inhibitory *in vivo*, such a conditioning lesion may facilitate regeneration across the DREZ in Neurocan/Brevican-deficient mice, but not in controls. Representative examples of the growth of CGRP- and neurofilament-positive fibres in double-knockout and wild-type animals with or without a conditioning lesion 7 days prior to rhizotomy (Fig. 1, group I) are illustrated in Fig. 3. Rostral to caudal serial sections over the entire cervical dorsal root C7 from a Neurocan/Brevican-deficient mouse with a conditioning lesion are illustrated in supplementary Fig. 2 (supplementary material). Quantitative evaluation of regeneration revealed that a significantly larger number of CGRP- and neurofilament-positive fibres entered the DREZ after a peripheral nerve lesion in double-knockout, but also in wild-type mice (Fig. 4A and B). This corresponded to an average number of 0.93 ± 0.06 and 1.34 ± 0.19 CGRP-positive fibres in the DREZ and 6.51 ± 0.55 and 8.6 ± 0.52 fibres in the dorsal root of knockout and wild-type animals without conditioning, respectively and to 1.97 ± 0.23 and 2.45 ± 0.3 fibres in the DREZ and 8.59 ± 0.45 and 8.88 ± 0.29 fibres in the dorsal root of knockout and wild-type animals following conditioning lesion, respectively. Similarly, the mean CGRP fibre length within the DREZ was higher in both knockout and wild-type animals (Fig. 4C). Thus, a conditioning peripheral nerve lesion facilitates growth across the DREZ in mice, but absence of Neurocan and Brevican is not sufficient to enhance regeneration further.

Vascularization of the DREZ following rhizotomy

The small number of sensory axons regenerating into the spinal cord following rhizotomy and a conditioning lesion have previously been described to avoid the DREZ and grow along blood vessels (Chong *et al.*, 1999). Consequently, we double-labelled spinal cord sections with antibodies against the glucose transporter Glut-1, a marker for blood vessels, and laminin to delineate the PNS/CNS border in order to assess the presence of growth-permissive blood vessels in the DREZ (Fig. 5). Transversely cut blood vessels were observed in the peripheral dorsal root and reactive fibrous tissue (arrowheads), in the spinal cord (arrow), but never in the DREZ itself (asterisks) (Fig. 5A). The CGRP-positive fibres

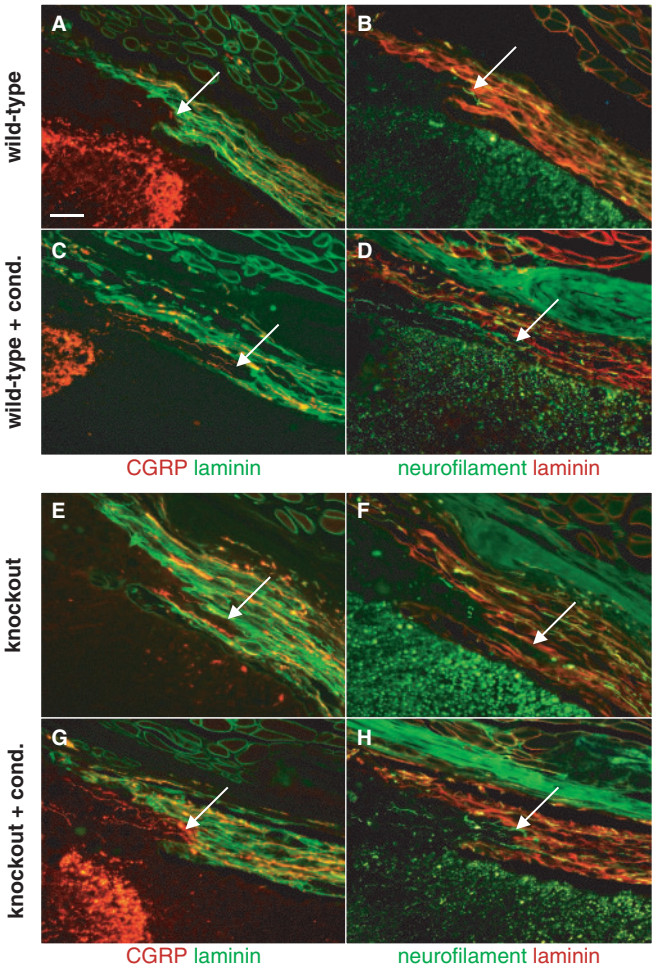

Fig. 3 Sensory fibre regeneration following rhizotomy in Neurocan/Brevican-deficient mice and in controls in the absence or in the presence of a pre-conditioning lesion of the median nerve (group I). (**A**, **C**) representative images illustrating CGRP-positive fibre regeneration (red) in wild-type mice 3 weeks following rhizotomy in the absence (A), or in the presence (C) of a conditioning lesion of the median nerve 7 days prior to rhizotomy (wild-type + cond.). Anti-laminin labelling (green) allows to outline the PNS/CNS border (arrows). (**B**, **D**) adjacent sections immunolabeled with antibodies against neurofilament protein (green) and laminin (red). (**E**, **G**) CGRP-positive fibre regeneration in Neurocan/Brevican-deficient mice (knockout) 3 weeks following rhizotomy in the absence (E), or in the presence (G) of a conditioning lesion of the median nerve 7 days prior to rhizotomy. (**F, H**) Adjacent sections immunolabeled with antibodies against neurofilament protein (green) and laminin (red). Scale bar = 50 μm.

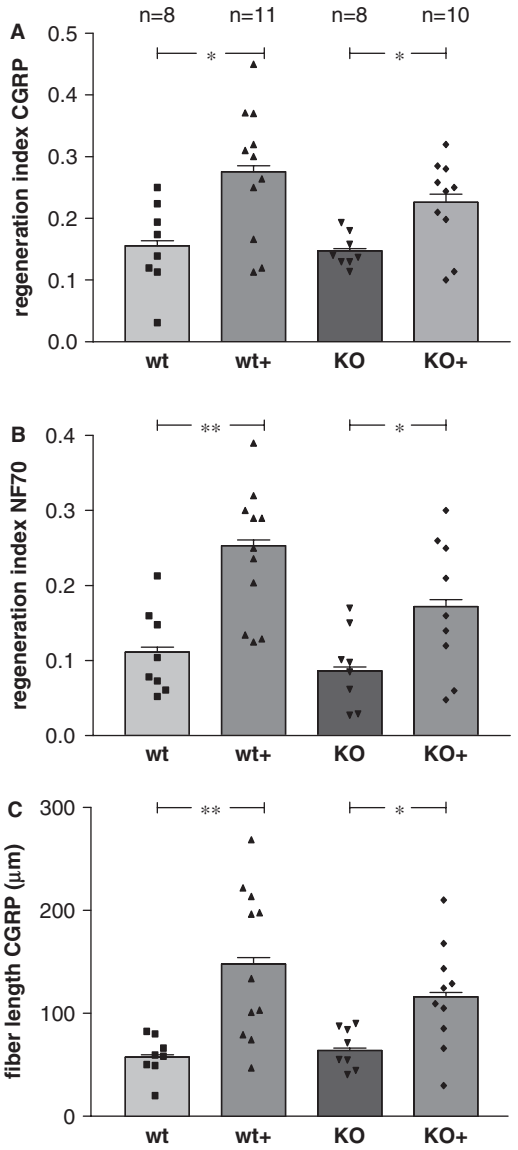

Fig. 4 Quantification of sensory fibre regeneration across the DREZ in double-knockout and control mice in the presence or the absence of a pre-conditioning lesion of the median nerve (group I). (**A**) The regeneration indices of CGRP-positive sensory fibres are significantly larger in Neurocan/Brevican-deficient (KO+) and control mice (wt+) that have received a conditioning lesion 7 days prior to rhizotomy compared to those without a peripheral nerve lesion (KO, wt): KO+/KO, $^{*}P < 0.05$; wt+/wt, $^{*}P < 0.05$. A similar difference was observed when the regeneration indices of neurofilament-positive sensory fibres (**B**) and the mean fibre lengths of CGRP-positive fibres (**C**) were evaluated. Neurofilament-positive fibres: KO+/KO, $^{*}P<0.05$; wt+/wt, $^{**}P < 0.01$. CGRP fibre length: KO+/KO, $^{*}P < 0.05$; wt+/wt, $^{**}P < 0.01$. The data are expressed as means ± SEM and analysed using the Mann–Whitney test.

that have crossed the PNS/CNS border in the adjacent section (Fig. 5B, asterisks) therefore do not grow along blood vessels but among activated glial cells present in the DREZ.

Effect of delayed priming of the median nerve on sensory fibre regeneration in Neurocan/Brevican-deficient and genetically matched control mice

To evaluate whether stimulating the growth capacity of sensory neurons at a clinically relevant time, i.e several weeks following rhizotomy, could facilitate growth, the median nerve of half of the animals of the Neurocan/Brevican-deficient group, and half of the genetically matched controls was crushed 7 weeks after rhizotomy and the animals were sacrificed 1 week later (Fig. 1, group II). The regeneration index of CGRP-positive fibres was

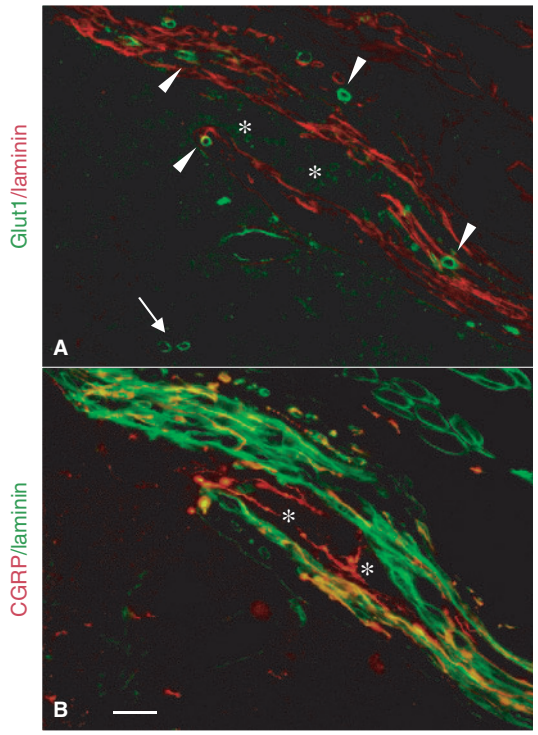

Fig. 5 Vascularization of the DREZ following rhizotomy and a conditioning lesion. (**A**, **B**) Double-immunolabelling with antibodies against the glucose transporter Glut-1 (green) to reveal blood vessels, and laminin (red) to reveal the PNS/CNS border (A) of a section adjacent to a spinal cord section double-labeled with anti-CGRP (red) and laminin (green) (B) from a mouse that had received a conditioning lesion 7 days prior to rhizotomy and was sacrificed 3 weeks later. Transversely cut blood vessels are visible in the peripheral dorsal root and in reactive fibrous tissue (A, arrowheads), spinal cord gray matter (arrow), but not in the DREZ (asterisks). Sensory fibres regenerating through the DREZ (B, asterisks) are thus not growing along blood vessels. Scale bar = 20 μm.

significantly higher with delayed priming in the Neurocan/Brevican knockout group ($P<0.001$), but not in wild-type controls, corresponding to an average number of 1.04 ± 0.1 and 1.58 ± 0.25 fibres in the DREZ and 6.84 ± 0.4 and 8.4 ± 0.27 fibres in the dorsal root of knockout and wild-type animals without priming, respectively and to 2.32 ± 0.26 and 2.03 ± 0.22 fibres in the DREZ and 7.19 ± 0.43 and 8.68 ± 0.51 fibres in the dorsal root of knockout and wild-type animals following priming lesion, respectively (Fig. 6A). The difference between knockout and wild-type animals with a priming lesion was also statistically significant ($P<0.05$). Delayed priming also significantly increased the length of CGRP-positive fibres ($P<0.01$, Fig. 6C) and the regeneration index of neurofilament-positive fibres ($P<0.05$, Fig. 6B) in the Neurocan/Brevican knockout group, but not in the wild-type controls. Delayed peripheral nerve priming thus promotes the growth of sensory axons in mice that are deficient in both Neurocan and Brevican, but not in controls. This is the first direct *in vivo* evidence that these proteoglycans participate in the non-permissive nature

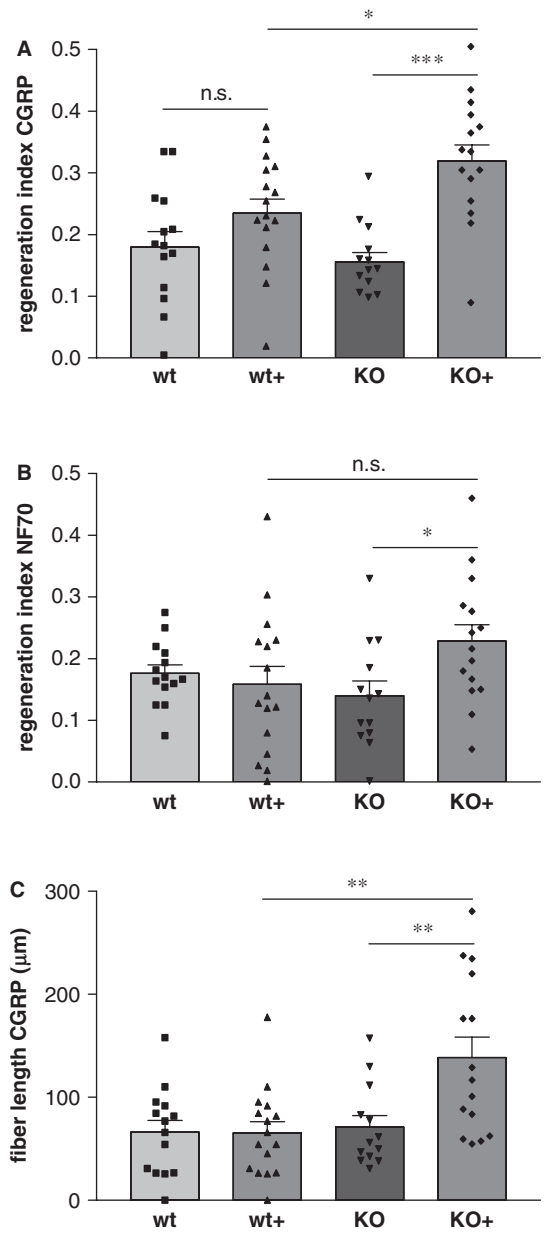

Fig. 6 Quantification of sensory fibre regeneration across the DREZ in the presence or the absence of a post-lesion priming of the median nerve (group II). (**A**) The regeneration indices of CGRP-positive fibres are significantly larger in Neurocan/Brevican-deficient mice that have received a post-priming lesion 7 weeks post-rhizotomy (KO+, $n=15$) compared to those without a peripheral nerve lesion (KO, $n=13$) ($^{***}P<0.001$). There is also a significant difference between KO+ and wt+ animals ($^{*}P<0.05$) and KO+ and wt mice ($^{**}P<0.01$). In contrast, there is no statistical difference in the regeneration index between wild-type controls with (wt+, $n=16$), or without (wt, $n=14$) a post-priming lesion (n.s., $P>0.05$). (**B**) The regeneration indices for neurofilament-positive fibres are significantly different between KO+ and KO mice ($^{*}P<0.05$), but not between KO+ and wt+ animals ($P>0.05$). **(C)** The mean differences in CGRP-positive fibre lengths between KO+ and KO, as well as KO+ and wt+ mice are also statistically different ($^{**}P<0.01$ for both). The data are expressed as mean values ± SEM and analysed using the Mann–Whitney test.

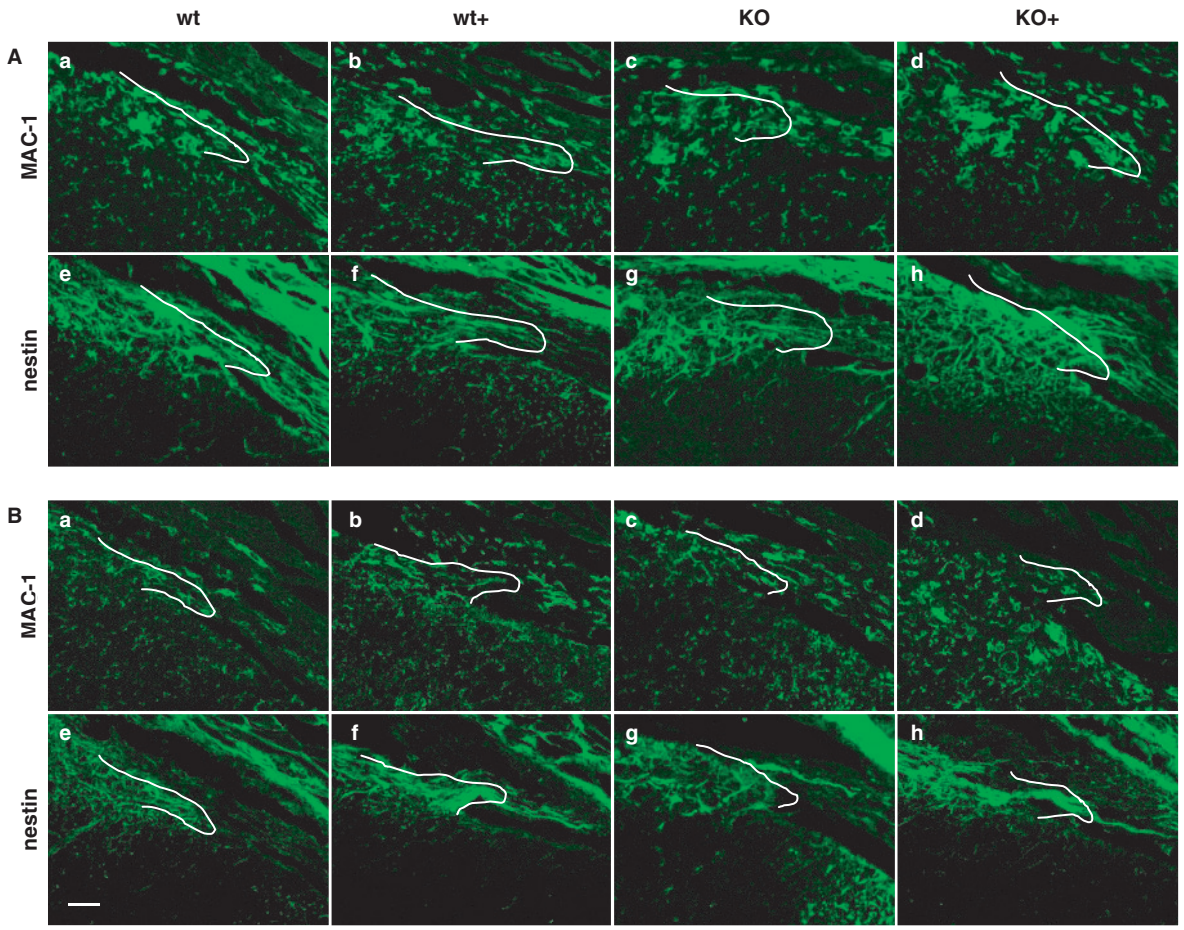

Fig. 7 Glial cellactivation in the DREZ following rhizotomy with or without a priming lesion. (**A**) Mice sacrificed 3 weeks post-rhizotomy in the absence or in the presence of a conditioning median nerve crush 7 days prior to rhizotomy (group I). Representative images of spinal cord sections co-labeled for the microglia/macrophage marker MAC-1 (a–d), and nestin, a marker for reactive astrocytes (e–h) (adjacent sections). Gliosis in wild-type (wt; a, e), wild-type plus conditioning (wt+; b, f), Neurocan/Brevican-deficient (KO; c, g) and Neurocan/Brevican-deficient mice plus conditioning (KO+; d, h). (**B**) Mice sacrificed 8 weeks post-rhizotomy in the absence or in the presence of median nerve priming 7 weeks post-rhizotomy (group II). Representative images of adjacent spinal cord sections co-labelled for MAC-1 (a–d) and nestin (e–h). Gliosis in wild-type (a, e), wild-type plus priming (b, f), Neurocan/Brevican-deficient (c, g) and Neurocan/Brevican-deficient mice plus priming (d, h). The dashed lines indicate the location of the PNS/CNS border as outlined with laminin antibody labelling on adjacent sections. Scale bar = 50μm.

of the DREZ and that sensory fibre regeneration can be facilitated when their growth capacity is stimulated post-rhizotomy.

Reactive gliosis in the DREZ following rhizotomy with or without a priming lesion

To correlate increased regeneration through the DREZ with possible variations in the intensity of gliosis in response to rhizotomy and/or median nerve crush in the two groups of animals, spinal cord sections of animals sacrificed 3 weeks after rhizotomy with or without a pre-conditioning lesion 7 days earlier (group I) and animals sacrificed 8 weeks after rhizotomy with or without a priming lesion at 7 weeks (group II) were processed for immunohistochemical staining with antibodies to visualize activated astrocytes (nestin) and microglia/macrophages (MAC-1) (Fig. 7A and B). Globally a similar intensity of gliosis was observed in all groups of animals and was maintained up to 8 weeks post-lesion.

Discussion

We have demonstrated recently using a murine model of brachial plexus injury in humans that the expression of the chondroitin sulfate proteoglycans Neurocan and Brevican is increased in the DREZ following rhizotomy (Beggah *et al.*, 2005). However, although both proteoglycans have previously been demonstrated to have growth-inhibitory properties *in vitro* (Friedlander *et al.*, 1994; Yamada *et al.*, 1997), direct evidence for their contribution to the non-permissive CNS environment *in vivo* is still lacking.

The present study demonstrates that delayed priming of the median nerve 7 weeks following rhizotomy promotes the growth of a subpopulation of sensory fibres through the DREZ in Neurocan/Brevican-deficient, but not in control mice. This is the first *in vivo* evidence that Neurocan and Brevican contribute to the non-permissive CNS environment several weeks after lesion. Moreover, this demonstrates that post-lesion enhancement of the growth capacity of sensory neurons can facilitate regeneration into the spinal cord, provided the amount of growth-inhibitory proteoglycans present in the DREZ is reduced. Simultaneous activation of the growth program of sensory neurons and enzymatic removal of inhibitory chondroitin sulfate proteoglycans post-injury may therefore have utility in the clinic to ameliorate sensory function and thus attenuate the severe pain caused by human brachial plexus injury (Anand and Birch, 2002).

Absence of Neurocan and Brevican does not facilitate the growth of sensory fibres beyond the PNS/CNS border

Comparison of sensory fibre regeneration in Neurocan/Brevican double-knockout and genetically matched control mice following rhizotomy did not reveal any differences in the regeneration indices or mean fibre lengths between these two groups of animals. This may in part be due to the presence of additional inhibitory molecules in the region such as the CSPGs Versican V1, Versican V2 and NG2 (Beggah *et al.*, 2005), as well as to inhibitory glycoproteins associated with myelin (Filbin, 2003). Treatment with chondroitinase ABC (ChABC), for instance, the enzyme which removes the glycosaminoglycan chains of all CSPGs, has previously been shown to promote regeneration and functional recovery following injury of the rat spinal cord (Bradbury *et al.*, 2002; Barritt *et al.*, 2006). Furthermore, regeneration of sensory axons through the DREZ following rhizotomy has recently been described to be facilitated in mice genetically engineered to express ChABC in reactive astrocytes (Cafferty *et al.*, 2007). Absence of Neurocan and Brevican alone may therefore not be sufficient to render the DREZ more permissive for growth.

Peripheral nerve conditioning 7 days prior to injury promotes regeneration of sensory fibres in both Neurocan/Brevican-deficient and control mice

The limited ability to regenerate following injury is not only due to the growth-inhibitory properties of the CNS environment, but also to the low capacity of adult axons to grow. Since a peripheral conditioning lesion was shown to increase the expression of growth-associated proteins (Schreyer and Skene, 1991), to promote axonal regeneration within the spinal cord (Richardson and Issa, 1984; Neumann and Woolf, 1999), but to have only minor effects on regeneration across the DREZ following rhizotomy in the rat (Chong *et al.*, 1999), we evaluated whether in Neurocan/Brevican-deficient mice, i.e. in animals in which the DREZ is potentially more permissive, such a lesion may facilitate re-growth into the spinal cord. Our results show that a significantly larger number of sensory fibres entered the DREZ after a conditioning lesion 7 days prior to rhizotomy in double-knockout mice. However, regeneration into the spinal cord was also facilitated in wild-type mice. This discrepancy with previous findings in the rat in which nerve conditioning had only minor effects on regeneration across the DREZ (Chong *et al.*, 1999) may be due to the different species used. Spinal cord injury, for instance, leads to the formation of a cystic cavity at the lesion site in the rat, whereas the injury site is filled with dense fibrous tissue in mice (Fitch *et al.*, 1999; Jakeman *et al.*, 2000). These differences may result from species-specific injury-induced inflammatory reactions (Sroga *et al.*, 2003), thus leading to reactive tissues with distinct inhibitory potencies. Second, the fact that the absence of Neurocan and Brevican did not lead to additional facilitation of growth following a conditioning lesion may indicate that these two proteoglycans only contribute to a minor proportion to the non-permissive properties of the DREZ. Support for this latter hypothesis is provided by an earlier study showing that pre-conditioning with zymosan combined with degradation of all CSPGs with ChABC prior to rhizotomy, but not single treatment alone, significantly increased regeneration of sensory axons across the DREZ in the rat (Steinmetz *et al.*, 2005).

Sensory fibres entering the DREZ do not grow along blood vessels

Previous results in the rat had lead the authors to conclude that the small number of sensory axons that entered the DREZ following rhizotomy grew along blood vessels or other laminin-rich structures present in the region (Kozlova *et al.*, 1997; Chong *et al.*, 1999). We did not find evidence supporting these findings, as illustrated in the sections double-labelled for CGRP and the laminin $\alpha 2$ chain, or neurofilament and laminin $\alpha 2$ (Fig. 3 and 5B). Furthermore, immunolabelling with antibodies against the glucose transporter Glut-1 did not reveal longitudinally extending blood vessels in transverse sections of the DREZ, as described earlier for the DREZ of the cat (Berthold and Carlstedt, 1977), as well as rat lumbar ventral rootlets (Kaar and Fraher, 1987). Regenerating sensory axons thus do not grow along blood vessels, but among activated glial cells. We have previously shown that CGRP-positive sensory fibres can grow along astrocytic processes extending several hundred microns into the peripheral dorsal root post-rhizotomy (Beggah *et al.*, 2005).

Delayed priming of the median nerve promotes regeneration of CGRP-positive sensory fibres in Neurocan/Brevican-deficient, but not in genetically matched controls

So far, peripheral nerve lesions have only been described to promote regeneration when they were performed either prior to spinal cord injury (Neumann and Woolf, 1999), or both at the time of injury and one week later (Neumann *et al.*, 2005). The present study demonstrates that one single median nerve lesion, delayed by 7 weeks post-rhizotomy, promotes the growth of sensory fibres across the DREZ in Neurocan/Brevican-deficient mice, but not in controls. This is the first direct *in vivo* evidence that Neurocan and Brevican may participate in the non-permissive properties of the DREZ post-injury. The only functional effects described in Neurocan- and Brevican-deficient mice so far were deficits in the maintenance of hippocampal long-term potentiation, probably due to the absence of these CSPGs in perineuronal nets (Zhou *et al.*, 2001; Brakebusch *et al.*, 2002). The modest magnitude of the enhanced growth following delayed priming in the double-knockout mice may be due to the presence of additional inhibitory molecules in the region such as the CSPGs Versican V1, Versican V2 and NG2 (Beggah *et al.*, 2005), inhibitory myelin glycoproteins (Filbin, 2003), and/or to the short interval between the priming lesion and sacrifice (1 week). This may also explain the lack of evidence of functional reconnection within laminae I and II of the spinal dorsal horn, as evaluated by immunolabelling with phosphorylated ERK1/2 antibodies following noxious stimulation (not shown). The lesser effect on neurofilament than CGRP-positive sensory axons may be the result of distinct binding sites for Neurocan and Brevican present on different subpopulations of sensory fibres, as previously described for receptors for neurotrophic factors and laminin (McMahon *et al.*, 1994; Gardiner *et al.*, 2005).

In addition to revealing an inhibitory role of Neurocan and Brevican in the DREZ *in vivo*, our results also provide the first evidence that delayed peripheral nerve priming can promote regeneration across the DREZ provided Neurocan and Brevican are absent. This contrasts with experiments in the rat in which priming delayed by 3 days post-lesion, combined with degradation of all CSPGs, did not promote growth post-rhizotomy (Steinmetz *et al.*, 2005). This may be due to two main reasons: (i) inflammatory reactions are distinct in the rat and mouse, leading to reactive tissue with diverse growth-inhibitory properties (Sroga *et al.*, 2003); (ii) priming was performed as long as 7 weeks, and not 3 days, post-injury, time at which inflammation and gliosis may have subsided. Preliminary attempts to visualize differences in intensity of the glial reaction over time, between knockout and control mice, as well as between groups with or without priming, did not reveal any major differences in the overall immunolabelling for reactive astrocytes and microglia/macrophages. However, a more detailed analysis of the molecular events underlying inflammation and gliosis at the DREZ over time, as well as between groups of animals, will be needed to fully understand why delayed priming promotes growth in Neurocan/Brevican-deficient mice and not in controls. Reduced facilitation of regeneration with neurotrophin-3 delivery postponed by 1 week following rhizotomy in the rat has previously been ascribed to the delayed invasion of the DREZ by ED-1-expressing phagocytes (Ramer *et al.*, 2001a).

In summary, our results demonstrate that regeneration across the DREZ post-rhizotomy can be promoted by delayed peripheral nerve priming, provided the growth-inhibitory properties of this CNS region are attenuated due to the absence of Neurocan and Brevican. This is additional evidence that combinatorial strategies are better than single strategies alone (Lu *et al.*, 2004; Fouad *et al.*, 2005; Steinmetz *et al.*, 2005). Moreover, since a conditioning lesion effect has previously been shown to be mimicked by dibutyril cAMP or rolipram (Neumann *et al.*, 2002; Qiu *et al.*, 2002; Nikulina *et al.*, 2004;), simultaneous enhancement of the growth capacity of sensory neurons with pharmacological agents, and enzymatic degradation of inhibitory chondroitin sulfate proteoglycans following injury, i.e. at a clinically relevant time, may offer a feasible therapeutic approach to promote repair and attenuate the severe chronic pain caused by human brachial plexus injury.

Supplementary material

Supplementary materials are available at *Brain* online.

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