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Expression of the repulsive guidance molecule RGM and it receptor Neogenin after spinal cord injury in sea lamprey

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Abstract

The sea lamprey recovers normal-appearing locomotion after spinal cord transection and its spinal axons regenerate selectively in their correct paths. However, among identified reticulospinal neurons some are consistently bad regenerators and only about 50% of severed reticulospinal axons regenerate through the site of injury. We previously suggested (Shifman and Selzer, 2000) that selective chemorepulsion might explain why some neurons are bad regenerators and others not. To explore the role of additional chemorepulsive axonal guidance molecules during regeneration, we examined the expression of the repulsive guidance molecule (RGM) and its receptor neogenin by *in situ* hybridization and quantitative PCR. RGM mRNA was expressed in the spinal cord, primarily in neurons of the lateral gray matter and in dorsal cells. Following spinal cord transection, RGM message was downregulated in neurons close (within 10 mm) to the transection at 2 and 4 weeks, although it was upregulated in reactive microglia at 2 weeks post-transection. Neogenin mRNA expression was unchanged in the brainstem after spinal cord transection, and among the identified reticulospinal neurons, was detected only in “bad regenerators, Neurons that are known to regenerate well never expressed neogenin. The downregulation of RGM expression in neurons near the transection may increase the probability that regenerating axons will regenerate through the site of injury and entered caudal spinal cord.

Keywords

axonal guidance; spinal cord regeneration; *in situ* hybridization; RGM; neogenin; lamprey; microglia

Introduction

Unlike mammals, lampreys show regeneration of axons and functional recovery after complete spinal cord transection (Rovainen, 1976, Selzer, 1978, Wood and Cohen, 1979). However,

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lamprey reticulospinal neurons differ in their regenerative abilities. While some are consistently “good regenerators”(regenerating 75% of the time or more), other neurons “bad regenerators” (regenerating less than 10–20% of the time)(Davis and McClellan, 1994, Jacobs, et al., 1997, Swain, 1989). In general, among identified reticulospinal neurons only about 50% regenerate through the site of injury.

The failure of axonal regeneration in the CNS of mammals has been ascribed primarily to the presence of inhibitory factors in the extracellular environment of the injured axon (Aguayo, et al., 1991, Caroni and Schwab, 1988, David and Aguayo, 1981) and glial scar prevention of axonal growth (Galtrey and Fawcett, 2007, Qiu, et al., 2000). Several inhibitory molecules are associated with myelin (Brittis and Flanagan, 2001, Huber and Schwab, 2000, McKerracher, et al., 1994) and in matrix molecules secreted by reactive astrocytes and oligodendrocytes (Fawcett and Asher, 1999, Fitch and Silver, 1997, Snow, et al., 1990). However, because the lamprey CNS lacks myelin (Bullock, et al., 1984), failure of many reticulospinal axons to regenerate cannot be attributed to myelin-derived growth inhibitors (*e.g.*, MAG, MOG and Nogo). Moreover, lamprey spinal axons grow preferentially through a hemisection scar rather than around it (Lurie and Selzer, 1991), suggesting that reactive glial cells are supportive of axon regeneration. Therefore, other factors may be responsible for the heterogeneity in the regenerative abilities of lamprey reticulospinal neurons. Based on our previous data that showed upregulation of the netrin receptor UNC5 in “bad regenerating” reticulospinal neurons after spinal cord transection (Shifman and Selzer, 2000), we hypothesized that post-injury expression of some repulsive guidance molecules in the spinal cord and coordinated upregulation of their receptors in reticulospinal neurons could be responsible, at least in partial, for limiting the regenerative abilities of those neurons.

Research conducted during the last decade has identified several discrete classes of diffusible and transmembrane proteins that act as repellent cues in guiding axon growth during development and possibly during axon regeneration. Among them are the netrins, semaphorins, ephrins, slits and the newly described “repulsive guidance molecule” (RGM; reviewed in (Matsunaga and Chedotal, 2004, Moore and Kennedy, 2006)). RGM actually represents a new family of GPI membrane-bound proteins that play a critical role in axon guidance and other processes of neuronal development. RGMs contain a signal peptide, an RGD site, a von Willebrand factor (vWF) domain, a hydrophobic region, and a GPI (glycosylphosphatidylinositol) anchor (Monnier, 2002) and are divided into 3 classes – RGM A, RGM B and RGM C. Recent experimental data suggested that neogenin binds RGMs and act as their receptor, mediating intracellular signaling (Matsunaga and Chedotal, 2004, Wilson and Key, 2006, Yamashita, et al., 2007).

While involvement of semaphorins and netrins in restricting the ability of spinal cord axons to regenerate after injury (De Winter and Oudega M, 2002, Manitt, et al., 2006, Moreau-Fauvarque, et al., 2003, Pasterkamp and Verhaagen, 2001, Shifman and Selzer, 2000, Shifman and Selzer, 2007, Wehrle, et al., 2005) is well documented, the role of RGMs and their receptor neogenin in spinal cord regeneration has not received comparable attention. However, several recent articles described RGM expression after axonal injury (Doya, et al., 2006, Hata, et al., 2006, Schwab, et al., 2005, Schwab, et al., 2005).

To gain new insights into the function of RGM in axon regeneration, we used the highly accessible nervous system of larval sea lampreys (*Petromyzon marinus*), as a model system in which post-axotomy expression of RGM and its receptor neogenin could be studied *in vivo*at the single cell level. The lamprey brainstem contains several uniquely identifiable reticulospinal neurons, including the Mauthner neurons with crossed descending axons and several pairs of Müller cells whose axons descend ipsilaterally. Because their axons project almost the entire length of the spinal cord (Swain, et al., 1993), all these large reticulospinal

neurons are axotomized by a high spinal cord transection. Moreover, because the regenerative abilities of these neurons have been quantified previously (Jacobs et al., 1997), post-axotomy changes in RGM and neogenin expression could be related to the known regenerative abilities of identified neurons.

MATERIALS AND METHODS

Animals

Wild-type larval lampreys (*Petromyzon marinus*), 12–14 cm in length (4–5 years old) and in a stable stage of neurological development, were obtained from streams feeding lake Michigan and maintained in fresh water tanks at 16°C until the day of surgery.

Spinal cord transection

Animals were anesthetized by immersion in 0.1% tricaine methanesulfonate, and the spinal cords exposed from the dorsal midline at the level of the fifth gill. Transection of the spinal cord was performed with Castroviejo scissors, after which the wound was allowed to air dry over ice for one hour. Each transected animal was examined 24 hours after surgery to confirm that there was no movement caudal to lesion site. A transection was tentatively considered complete if on stimulation of the head, an animal could move only its head and body rostral to the lesion. Animals were allowed to recover in fresh water tanks at room temperature. At the specified recovery times, animals were re-anesthetized, and the spinal cords and brains were removed for *in situ* hybridization or RNA extraction for real-time PCR analysis. Experiments were carried out on 100 lampreys that were either untransected (n = 20) or permitted to recover 2 weeks (n = 40) or 4 weeks (n = 40). Experiments were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Riboprobe synthesis

Previously cloned cDNAs for lamprey neogenin and lamprey RGM were used as templates for the generation of digoxigenin-(DIG-) labeled sense and antisense riboprobes for *in situ* hybridization on spinal cord and brain wholemount preparations of control and spinal-transected animals. The neogenin (GenBank accession number **AY744917**) cRNA probe was transcribed from a 648-bp sequence spanning the intracellular domain (nucleotides 4052–4700) and the RGM probe was transcribed from a cloned sequence (nucleotides 1–581; GenBank accession number **EU449948**). Lamprey RGM and neogenin cDNAs cloned in pGEM-T Easy vector (Promega) were linearized with restriction enzymes and gel-purified. The DIG incorporation into probes was controlled by dot blots. The length and integrity of the probes was examined by gel electrophoresis. Sense RNA probes were used as controls.

Wholemount brain and spinal cord *in situ* hybridization

Wholemount preparations preserve three-dimensional information, which allows for the rapid and accurate identification of labeled cells. The lamprey spinal cord can be studied by whole-mount *in situ* hybridization, in part because of its flat shape and in part because it lacks myelin (Bullock, et al., 1984), making the entire CNS translucent. Hybridizations of DIG-labeled riboprobes to wholemounted lamprey brain and spinal cord, respectively, were performed using methods optimized for the lamprey as previously described (Shifman and Selzer, 2000). DIG-labeled sense RNA probes were used as internal controls and did not produce hybridization signals. Images were captured digitally using an Zeiss AxioCam CCD Video Camera attached to a Zeiss Axioskop microscope with AxioVision software, and scale bars were added. Images were imported into Adobe Photoshop CS2 (Adobe Systems, Inc., San Jose, CA). The images were cropped and adjusted for brightness and contrast, and labels were added.

Quantitative real-time RT-PCR assessment of RGM mRNA expression in the spinal cord after transection

Total RNA was extracted at 2 weeks or at 1 month after injury from rostral segments of spinal cord (including the injury epicenter), from segments 10 mm caudal to the transection, from the caudal remainder of the cord, and from sham-operated cords, using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was suspended in diethylpyrocarbonate (DEPC)-treated water, followed by treatment with DNAse1 using the DNA-Free Kit (Ambion, TX, USA) to remove any traces of contaminating genomic DNA. The yield and purity of RNA were checked by spectrophotometric determination at 260 and 280 nm. Integrity of RNA was determined by the presence of 28S and 18S ribosomal RNA by electrophoresis of samples through 1.0% agarose gels. The first strand cDNA synthesis reaction from total RNA was catalyzed by Superscript II Reverse Transcriptase (Invitrogen) and random primers according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

Primer design

The DNA sequence of lamprey RGM (see above) and of lamprey Hypoxanthine-guanine phosphoribosyltransferase (HPRT 1; Genbank accession number **FJ155927**) were found using MegaBLAST programs to search a lamprey Trace Archive database that has been developed to store the raw genomic data underlying all of the sequences generated by genome projects. Cloning and sequencing of lamprey glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Genbank accession number **AAT70328**) and 18S rRNA sequence (Genbank accession number **M97575**) was reported by others (Pancer, et al., 2004, Stock and Whitt, 1992). Sequences sharing homology with RGM, GAPDH, 18S rRNA and HPRT were amplified by RT-PCR. Only the open reading frame, or CDS coding for proteins, was chosen from these sequences. The primers were designed using Primer Express 3 software (Applied Biosystems CA, USA) according to the user's manual. The specificity of primers was confirmed by homology search against the GenBank database. The sequences of primers and probes are shown in Table 1. All the primers were synthesized by IDT, Inc (Coralville, IA, USA). To test the specificity of primers for lamprey-specific genes, nonquantitative PCRs were performed using 2 μl of first-strand synthesis cDNA from lamprey spinal cord RNA. The 50-μl PCR reaction additionally contained the following components: 0.4 μM of each primer, 10 μl 5X Green GoTaq® Flexi Buffer (Promega, WI) and 1.25 U GoTaq® DNA polymerase (Promega, WI). The PCR reactions were run in a programmable themocycler GeneAmp® PCR System 9700 (Applied Biosystems) using an initial denature temperature of 95°C for 4 min, 35 cycles of 95°C for 45 s, 52°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min. A total of 20 μl of final PCR product was separated in a 1.5% agarose gel, stained with ethidium bromide, and photographed.

For all gene expression studies using quantitative PCR it is necessary to compensate for differences between samples due to material losses, differences in RT yields and PCR inhibition. Normalization ideally should include an endogenous control gene that has constant expression in all the samples compared. There is no universal control gene, expressed at a constant level under all conditions and in all tissues. The best way to choose the proper reference gene is by running a panel of potential genes on a number of representative test samples. The gene(s) most appropriate for normalization are chosen in each case. We used the well-known housekeeping genes GAPDH, HPRT 1 and 18S rRNA in our preliminary experiments and found that GAPDH had the most constant expression in all the samples, and was therefore the best endogenous control gene.

Quantitative RT-PCR was performed at the Quantitative PCR Facility of Virus and Molecular Core Services of the University of Pennsylvania using an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems) under 9600 Emulation Mode using the following

conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min and 1 cycle 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, 60°C for 15 s. The PCR reagents, except primers, were from the Power SYBR® Green PCR Master Mix (Applied Biosystems). The PCR for the target (RGM) and endogenous control (GAPDH) were performed in separate tubes in triplicate on cDNA samples in a MicroAmp Optical 96-well reaction plate (Applied Biosystems) according to manufacturer's instructions. A Power SYBR® Green PCR Master Mix was mixed with 200 nM forward and reverse primer for RGM (300nM for GAPDH) and 10 ng cDNA. From this mixture, an aliquot of 20 μl was placed in each of three wells. Each plate contained no template control (NTC) in which DEPC water replaced the cDNA as well as 5 serial concentrations of cDNA standard to allow calculation of a standard curve.

Standard curve construction

Standard curves were prepared for both the target and the endogenous reference. Standard curves were based on RT-PCR of known quantities of cDNA synthesized from spinal cord RNA containing the sequences of interest. Each standard curve was generated based on five 10-fold serial dilutions of a cDNA (100, 10, 1, 0.1, 0.01ng), quantified by a spectrophotometer. The reaction kinetics was represented by an amplification curve, in which a region where the fluorescence increases exponentially was observed. The PCR cycle number at which the fluorescence crosses a threshold (C_T value) can be related to the amount of starting templates. A standard curve plotting C_T values against the logarithm of input DNA copy quantity (log attmoles) was constructed for each gene. The standard curves for all the genes analyzed were linear over a range of at least 0.00096–29.29.4 attmoles with a linear correlation coefficient > 0.987.

Data analysis

A relative standard curve method was used to quantify RGM mRNA at different times post-transection. NTC and DNA standards were run in the same plate with cDNA from injured spinal cord of each of the different sample regions (rostral, 10 mm caudal and caudal) at different times after injury. For each sample, the amount of target and endogenous reference was determined from the appropriate standard curve. The data were analyzed as described in "Relative Quantification Getting Started Guide" (Applied Biosystems). Results were analyzed by two-way ANOVA with Bonferroni post-hoc tests using Prism, version 5 (GraphPad, USA). Experiments were performed four times with each assay ran in triplicate per experiment and the results are shown as the fold-difference in the level of normalized RGM mRNA expression in control spinal cord and after transection.

Lectin histochemistry

To identify microglial cells, wholemounts were labeled with GSA isolectin I-B_4 after RGM-labeled cells were revealed colorimetrically by *in situ* hybridization. The spinal cord wholemounts were washed in PBS and incubated with Fluorescein-labeled GSL I–isolectin B_4 (5μg/ml Griffonia (Bandeiraea) simplicifolia lectin I GSL I-B_4, Vector), which is a specific marker for microglia and labels D-galactose residues that are expressed by both resting and activated microglial cells (Boya, et al., 1991, Streit, 1990, Streit and Kreutzberg, 1987). Each step was followed by washing three times for 10 min with PBS. Wholemounts were coverslipped in VECTASHIELD® Mounting Medium (Vector, USA), and observed under a fluorescence microscope. Control for lectin staining consisted of (a) treating tissue prior to staining with α-galactosidase from coffee beans (Sigma) at 1 u/ml in phosphate-citrate buffer (pH 5.1) for 2 hours at 37°C; and (b) incubating with GSA I-B_4 in the presence of 0.1 M melibiose (6-0-α-D-galactopyranosyl-D-glucose) to saturate lectin binding sites and prevent interaction with sugars of tissue components.

Cell countingand determination of microglia numbers

The numbers of GSA I-B_4 and RGM-positive GSA I-B_4-labeled cells were counted in the transected lamprey spinal cord and in unmanipulated control lamprey at different time points and locations. This was achieved by capturing five (approximately 210 μm × 180 μm) adjacent fields of view in the area of the highest microglia density at a magnification of × 400 beginning approximately 0.5 mm caudal (or rostral) to the center of the site of transection of the spinal cord, and continuing counting at 1.0 mm and 5 mm. Five fields of view were captured from each distance from the transection site (0.5; 1 and 5 mm) at 2 or 4 weeks post-transection and evaluated in the spinal cord of each animal. Our preliminary observation indicated that observed numbers of GSA I-B_4-labeled microglial cells and RGM–labeled microglial cells were similar in caudal and rostral parts of transected spinal cord (up to 20 mm from the transection site). Therefore, we averaged the cell counts from rostral and caudal parts and presented them as one total cell count.

Statistics

The statistics were performed using Prism, version 5 (GraphPad, USA) applying a one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test to analyze changes in the number of GSA I-B_4-labeled cells after 2 weeks and 4 weeks post-lesion. A two way ANOVA followed by Bonferroni post-test was applied to analyze the number of RGM-positive GSA I-B_4-labeled cells at 0.5 mm, 1mm and 5mm from the transection site after 2 and 4 weeks post-lesion and to compare numbers of RGM-positive GSA I-B_4-labeled cells at each time point. Results are presented in bar graphs as means ± standard deviation.

RESULTS

RGM detection by PCR

PCR using the primers for RGM and the lamprey versions of standard housekeeping genes yielded single bands of the expected sizes for all (Fig. 1). Of the housekeeping genes, GAPDH had the most constant expression in preliminary experiments (data not shown) and was selected as the control gene for quantitative RT-PCR of RGM.

Cellular localization of RGM mRNA expression in control spinal cord

RGM mRNA was expressed throughout the spinal cord of control animals. Label was found in dorsal cells, edge cells and in medium sized neurons in the lateral grey matter (Fig. 2A).

RGM mRNA expression rostral and caudal to the transection

The spatiotemporal expression of RGM mRNA after complete spinal cord transection was analyzed by wholemount *in situ* hybridization at two and four weeks post-lesion. At 14 days, RGM mRNA expression was strongly downregulated in neurons of the spinal gray matter close to the transection site, both caudally and rostrally (Figs. 2B and C). Only a few dorsal cells continued to express RGM mRNA 0.5 mm caudal to the transection (Fig. 2C). Expression increased with distance from the transection, but the intensity of RGM-specific *in situ* hybridization signal and the number of RGM-expressing neurons were still noticeably reduced at 10 mm (Fig. 2E compared with Fig. 2A). The appearance of RGM expression patterns in the spinal cord region more than 20 mm from the lesion resembled those in uninjured controls (Fig. 2F). This labeling was found primarily in dorsal cells, edge cells and neurons of the spinal gray matter. In addition, many small cells (presumably microglia) throughout the spinal cord expressed RGM (Fig. 2D).

Four weeks after spinal cord injury, the transection site was easily identified in wholemount spinal cord preparations by the narrowing of the spinal cord and widening of the central canal

(Fig. 2G). The transection zone was approximately 500 μm long, as described previously (Yin and Selzer, 1983). RGM mRNA expression was not detected at the lesion site (Fig. 2G) but at distances greater than 20 mm from transection, was present at reduced levels in medium sized neurons of the spinal gray matter, in dorsal cells and in lateral interneurons (Fig. 2H).

Identification of the non-neuronal RGM-expressing cell types in lamprey spinal cord after injury

During our examination of RGM expression in the transected spinal cord, we detected populations of small, seemingly non-neuronal, cells that expressed RGM (Fig. 2D and Figs. 3C, G and H). By their sizes and shapes, they may be microglial cells (Boya, et al., 1991,Streit, 1990,Streit and Kreutzberg, 1987). They were located on the surface of the spinal cord (Fig. 3G), although some of them were located just below the cord surface.

Microglial cell activation after spinal cord transection

Fluorescein-labeled GSL I–isolectin B_4, which is commonly used to identify macrophages/microglial cells in spinal cord and brain of mammals (Boya, et al., 1991, Streit, 1990, Streit and Kreutzberg, 1987), was used here to analyze the microglial response in the spinal cord 0.5–5 mm caudal and rostral to the lesion site at 14 days and 30 days after transection. To determine whether the RGM-labeled cells in the spinal cord are microglial cells, lectin histochemistry was performed after RGM *in situ* hybridization (Fig. 3A–H). In normal cord, few of the small, rounded RGM-expressing cells were detected on the surface of control spinal cord (Fig. 3A). These cells displayed morphological hallmarks of resting microglia (spindle shaped cell bodies and an elongated nucleus). Because these RGM-expressing cells were also labeled with IB_4 lectin, (Fig. 3B), we identified these RGM-expressing cells as resting microglia. Moreover, several lectin-positive but RGM-negative cells were seen in control spinal cord, indicating that microglial cells might be present in control, untransected spinal cord (Fig. 3B). The specificity of the lectin histochemical staining was confirmed by the complete elimination of staining if the fluorescein-labeled GSL I–isolectin B_4 was pre-incubated in the presence of 0.1 M melibiose, or if the tissue was treated with α-galactosidase from coffee beans (Sigma) prior to staining (data not shown).

Microglia expressing RGM mRNA increase after injury

Following injury, cellular elements of noticeably different shape were labeled in the spinal cord. A detailed analysis of changes in tissue adjacent to the injury is provided in Figure 3 and its legend. In the first two weeks post-transection, activated microglia were observed in the region rostral and caudal to the lesion center. These cells had very small rounded cell bodies and no cells larger than 5 μm or possessing obvious neuronal morphology were labeled with RGM (Fig. 3C, G and H). During this time, the number of RGM-expressing cells increased significantly 0.5–5 mm rostral and caudal to the injury site (Fig. 3C and Fig. 4B), accompanied by rapid accumulation of reactive microglial cells, as evidenced by increasing IB_4 lectin reactivity (Fig. 3D and Fig. 4A). Most of the lectin-labeled cells also expressed RGM (Fig. 3D). Microglial RGM-expression was further examined 30 days after spinal cord transection. Although the localization, shapes and numbers of IB_4 lectin-labeled microglial cells resembled those at 14 days (Figs. 3E, F and 4A), activated microglia that were also RGM-positive were largely absent from the spinal cord (Fig. 3E). RGM-expressing cells resembled the small spindle-shaped cells in control spinal cord (Fig. 3A and E) and because they were labeled by IB_4 lectin they are likely to be resting microglial cells (Fig. 3F).

Time course of increases in the numbers of RGM–expressing microglia/macrophages in the spinal cord post-transection

In lesioned spinal cords a significant accumulation of activated microglia was observed between 0.5 and 5 mm from the transection site at 14 days ($p<0.05$, mean=188.7/mm^2, SEM= 46.3) and 30 days after injury ($p<0.05$, mean=146.5/mm^2, SEM= 7.2) as compared to control tissue (mean=16.3/mm^2, SEM=3.9; Fig. 4A). At 14 days post-transection, the proliferation of activated microglia contributed to an almost 12-fold increase ($P<0.05$) in total numbers of IB_4 lectin-labeled cells, compared to control cords. At one month after injury, the numbers of IB_4 lectin – stained microglia were still 9-fold greater than in controls cords.

In parallel with morphological signs of activation, reactive microglia appeared to upregulate RGM expression (Figs. 3C–F and 4B). A significant accumulation of RGM-expressing cells was seen two weeks after transection (Fig. 4B). This increase extended up to 10 mm caudal and rostral to the transection site but was not evident 20 mm from the transection. However, after 4 weeks of survival, the numbers of RGM-expressing cells decreased dramatically (Fig. 4B). The greatest numbers of these cells were detected close to transection site (0.5 mm), whereas at 1 mm and 5 mm from the transection cell counts decreased by almost 42% and 75% respectively (Fig. 4B). This uneven cell distribution was observed at 2 weeks but not at 4 weeks post-transection (Fig. 4B). However, the total numbers of activated microglia did not change significantly between 14 and 30 days after spinal cord transection (Fig**. 4A**).

RT-PCR determination of temporal and spatial changes in RGM mRNA expression after spinal cord transection

Our *in situ* expression data were confirmed by quantitative PCR, which showed downregulation of RGM expression 2 and 4 weeks after spinal transection. RGM mRNA was quantified at 2 weeks and 1 month after injury in segments of spinal cord representing the rostral part (including the injury epicenter), the first 10 mm caudal to the transection, and remainder of the cord caudal to it. Figure 5 shows the temporal changes in mRNA expression of RGM in these samples after transection. Two-way ANOVA indicated significant effects of both time and location after injury and of the interaction between these factors (location $F=38.74$; time $F=225.68$; interaction $F=12.56$. Significant effect with $p < 0.001$). In tissue rostral to the injury site, RGM mRNA was decreased by 47% at 2 weeks and 26% at 30 days. In tissue within 10 mm caudal to the transection, RGM mRNA decrease by 40% 2 weeks after injury and 24% at 30 days after injury. A different pattern was seen for the expression of RGM after transection in spinal cord more then 10 mm caudal to transection site. RGM expression was decreased by only 20% at 2 weeks post-transection and then was increased by 16% by 30 days.

Changes in neogenin expression in control animals and after spinal cord transection

Neogenin is a member of the immunoglobulin superfamily of transmembrane receptors and a homologue of DCC, a netrin receptor that performs highly-conserved *in vivo* roles in cell migration and axon guidance. Like DCC, neogenin is a netrin receptor that is prominently expressed by differentiating neurons in the central nervous system. Recent experimental data suggested that neogenin bind RGM and act as it receptor mediating RGM signaling(Matsunaga and Chedotal, 2004, Yamashita, et al., 2007).

We used nonradioactive *in situ* hybridization to study neuronal expression of neogenin in lamprey brain neurons projecting to spinal cord. Neogenin mRNA was present most often in the cytoplasm of a several reticulospinal neurons, most prominently in the Mauthner neurons, B neurons of the bulbar region B_1, B_3 and B_4 cells and in neurons of the isthmic region – I_1 and I_2 (Fig. 6A). Also two of mesencephalic neurons - M_1 and M_2 were labeled with neogenin probe (data not shown). Expression for any given neuron was variable from animal to animal

and it was common to observe expression for neogenin in only one of a pair of neurons in an animal. There were virtually no other labeled cells. No labeling was observed when the sense RNA probe was used instead of the antisense probe.

Changes in neogenin expression after spinal cord transection

In order to examine whether neogenin might be involved in the regenerative response of spinal projecting neurons, *in situ* hybridization for neogenin was performed at one, two and four weeks post-transection, consistent with the times when axons are dying back (first two weeks) and then regenerating through the proximal stump, respectively. The numbers of neogenin–expressing neurons did not change significantly at one, two and four weeks post-transection (Chi-square = 6.238, P = 0.1006, p> 0.05). As with expression in control animals, the level of receptor expression post-transection was variable, *i.e.* among cell types there was some degree of variability in expression levels at different times. However, beginning at four weeks post-transection, there was an apparent reduction in the intensity of staining of neogenin-expressing cells **(**Fig. 6D). Neurons were pale and appeared to be shrinking, which may indicate some degenerative processes or incipient neuronal death.

The expression of neogenin appeared to be limited to the Mauthner cell and a few other neurons that are known to regenerate poorly, specifically I_1, B_1 B_3 and B_4 (Jacobs, et al., 1997). On the other hand, some neurons that are known to regenerate well, such as the I_3–I_5, the auxiliary Mauthner, B_2, B_5 and B_6 (Jacobs, et al., 1997) never showed neogenin message expression.

DISCUSSION

The present study demonstrates that the spinal cord of the large larval (4–5 years old) lamprey contains many cells that produce RGM, the expression of which is downregulated near the lesion after spinal cord transection. By contrast, RGM was up-regulated at the lesion site after spinal cord injury in rats (Hata, et al., 2006, Kyoto, et al., 2007, Schwab, et al., 2005). Since spinal-projecting axons regenerate in lampreys but not in mammals, RGM might play a role in inhibiting axonal regeneration after CNS injury, and thus posttraumatic downregulation of RGM expression in the lamprey spinal cord could be one reason why some of its axons regenerate. Interestingly, while there was little effect of transection on RGM expression remote (more than 20 mm) from the transection site, expression was decreased dramatically in neurons close to the lesion at 2 and 4 weeks post-transection, the period of most intense regeneration, when axons are growing back toward the transection site after initial retraction (Lurie and Selzer, 1991, Rovainen, 1976, Yin and Selzer, 1983).

Axotomy downregulates RGM expression in neurons

Some effects of spinal cord transection on expression of RGM could be explained as direct consequences of axotomy, *e.g.*, the almost complete loss of RGM mRNA expression from the dorsal cells close to the transection site. These cells strongly expressed RGM in control animals. Dorsal cells are primary sensory neurons arrayed in two distinct rows on either side of the midline (Rovainen, 1967, Rovainen, 1979). Most project at least as far as the rostral spinal cord (Tang and Selzer, 1979) and are thus axotomized by the high-level spinal cord transection in the present study. Corresponding morphological and electrophysiological changes described previously in axotomized dorsal cells were maximal at 3 weeks and, like the loss of RGM expression, the intensities of those changes were also inversely related to the distance of the cell from the transection (Yin, et al., 1981).

The roles of RGM in embryonic development have been well described (Matsunaga and Chedotal, 2004) but their functions in the postnatal CNS are less clear. In mammals, the RGM receptor, neogenin displays sustained expression in adulthood (Keeling, et al., 1997,

Meyerhardt, et al., 1997), when it might function in the maintenance of established connections by restricting spontaneous or aberrant axon sprouting. Several recent reports have described modulation of RGM expression by injury. Up-regulation of RGM at the lesion site after spinal cord injury was detected in rats neurons, neurites, blood-borne cells infiltrating the lesion, activated microglia/macrophages and some reactive astrocytes in areas of ongoing scar and oedema formation (Hata, et al., 2006, Kyoto, et al., 2007, Schwab, et al., 2005). Inhibition of RGM by a blocking antibody facilitated axon re-growth and improved locomotor behavior after spinal hemisection in adult rats (Hata, et al., 2006).

Reactive microglia upregulate RGM

The present findings suggest that in lamprey spinal cord, microglia are activated and increase in numbers for several weeks post-transection. In the normal adult CNS, microglia are distributed throughout the neural parenchyma and constitute a population of cells that proliferate and turn over slowly (Lawson, et al., 1992). Microglia are activated swiftly after spinal cord injury (Popovich, et al., 1997, Sroga, et al., 2003). Reactive microgliosis induced by acute neural injury includes massive, but usually transient expansion of the microglial cell population, induction of a wide range of myeloid markers, trophic factors, cytokines, free radicals and nitric oxide, and the acquisition of a phagocytic phenotype (Ladeby, et al., 2005, Streit, 1994, Streit, 2002). Macrophages/microglial cells may have important functions in axonal regeneration by secreting growth-inhibiting and/or -promoting molecules (Sandvig, et al., 2004, Streit, 1994, Streit, 1996, Streit, 2002, Streit, 2002).

In the present study, a marked increase in isolectin-B_4 labeled cells was detected 14 day after a lesion and continued for at least another two weeks. Concurrently, expression of RGM mRNA in reactive microglial cells reached a maximum at 14 days post-injury and returned to pre-transection levels by 30 days. Our findings are in agreement with previous reports describing increased numbers of RGM-expressing macrophages/microglia after spinal cord injury in rodents and man (Hata, et al., 2006, Schwab, et al., 2005). However, in the present study, the reversal of RGM upregulation long before regeneration is complete, and the limitation of the reactive microgliosis to the surface of the cord, raise the question of how microglial expression of this GPI membrane-bound protein could be affecting regeneration axons located far from spinal cord surface. The lamprey glial scar differs from that in mammals. It consists of enlarged processes of glial cells whose cell bodies are located mainly in the proximal and distal stumps. There are no neuronal perikarya and relatively few glial cell bodies within the scar, except for the glial/ependymal cells reconstituting the enlarged central canal (Lurie and Selzer, 1991, Lurie and Selzer, 1991). Unlike the scar in lesioned mammalian spinal cord, which contains RGM-, Sema3- and/or netrin-expressing cells (Hata, et al., 2006, Pasterkamp, et al., 1999, Schwab, et al., 2005, Wehrle, et al., 2005), the transection scar in the lamprey spinal cord contained no cells that are positive for any of these chemorepulsive guidance molecules. This may partially explain previous observations that lamprey spinal axons grow preferentially through a hemisection scar rather than around it (Lurie and Selzer, 1991).

The *in situ* hybridization and quantitative real-time PCR experiments showed an overall reduction of RGM expression 2 and 4 weeks after injury in spinal cord areas close to the transection. Thus RGM expression in microglial cells was not enough to compensate for almost total disappearance of RGM–producing spinal cord neurons.

Possible role of RGM in regeneration

Axon regeneration in the lamprey spinal cord is incomplete (Lurie and Selzer, 1991, Selzer, 1978, Yin and Selzer, 1983). Fewer than 50% of severed axons grow into the distal stump within 10–12 weeks post-transection, and most of those grow less than 1 cm beyond the transection site (Lurie and Selzer, 1991, Lurie and Selzer, 1991, Rovainen, 1976, Yin and

Selzer, 1983). The presence of chemorepulsive axonal guidance molecules RGM (this article) and Sema3 and Sema4 in lamprey spinal cord (Shifman and Selzer, 2006, Shifman and Selzer, 2007) and the down-regulation of netrin (Shifman and Selzer, 2007), which in some cases can act as a chemoattractant, could contribute to the regenerative failure of some lamprey axons after spinal cord transection however, further experiments will be needed to elucidate RGM role in spinal cord regeneration.

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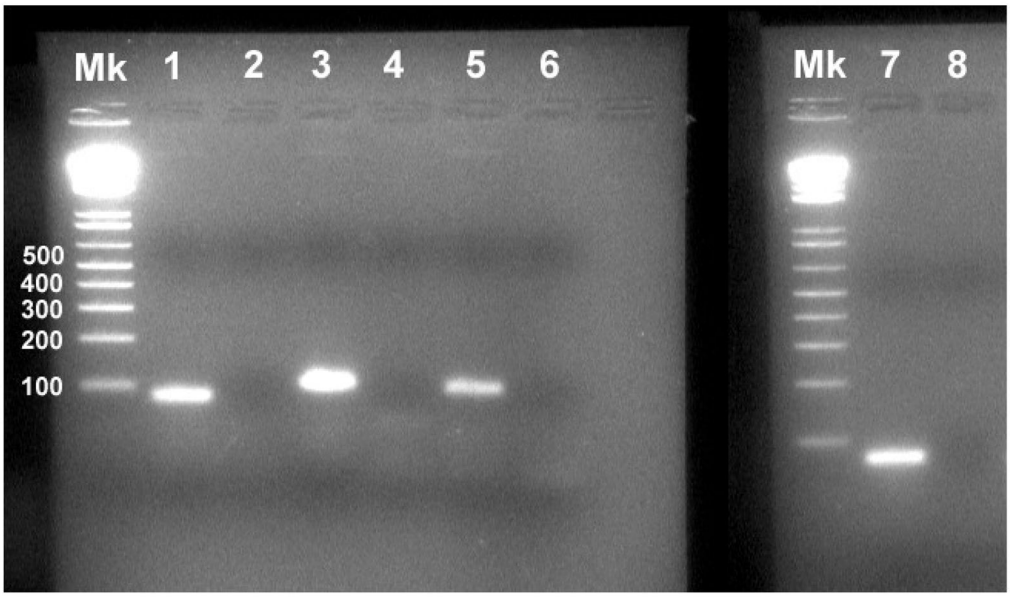

Figure 1. PCR amplification of lamprey RGM cDNA

Gel electrophoresis of PCR products from lamprey spinal cord cDNA yielded single bands at the expected sizes in base pairs (bp) for each target gene. No band was observed in the PCR products of the no template control (NTC). The RGM product was 96 bp, HPRT 1 was 68 bp, 18S rRNA was 75 bp and GAPDH was 72 bp. Molecular weight marker (Mk) was 1 Kb Plus (Invitrogen). Lanes are labeled as follows: #1 - 18S rRNA, #2 – NTC, #3 – RGM, #4 – NTC, #5 – GAPDH, #6 – NTC, #7 - HPRT 1, #8 – NTC.

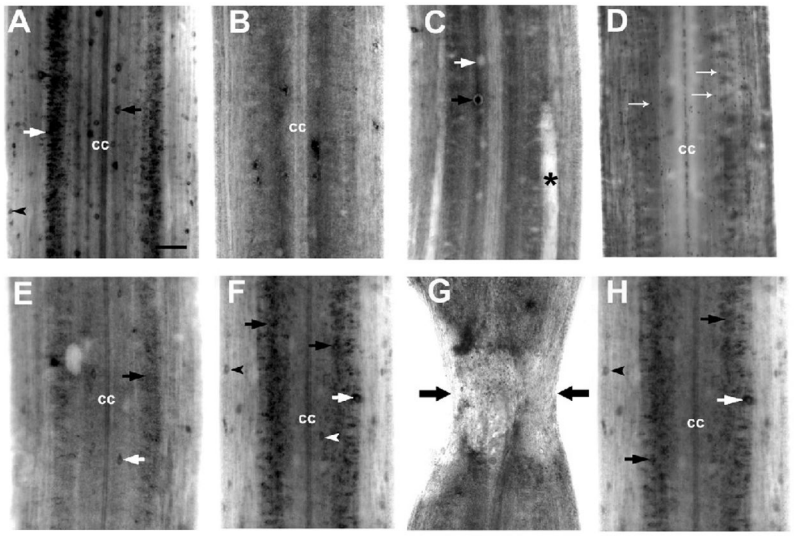

Figure 2. Expression of RGM mRNA in lamprey spinal cord after spinal cord transection

A, *In situ* hybridization in wholemount control spinal cord shows labeling for lamprey RGM in dorsal cells (black arrow), edge cells (black arrowhead) and in neurons of the spinal grey matter (white arrow). (cc) - central canal. **B–F,** RGM mRNA expression two weeks after spinal cord transection. Expression was strongly downregulated 500 μm rostral (**B**) and caudal (**C)** to the transection in neurons of the spinal gray matter. Only a few dorsal cells (black arrow) expressed RGM mRNA, while most dorsal cells stopped expressing it (the clear silhouette of a dorsal cell is indicated by the white arrow); asterisk – a swollen, degenerating Mauthner axon. **D,** Small cells (presumably microglia) throughout the spinal cord express RGM (white arrows). **E,** Expression increased with distance from the transection (10 mm caudal), but the intensity of RGM-specific *in situ* hybridization signal and the number of RGM-producing dorsal cells (white arrow) and neurons of the spinal grey matter (black arrow) were very small compared to control spinal cords. **F,** At distances more than 20 mm caudal to the lesion RGM mRNA expression patterns in neurons of the spinal grey matter (black arrows) resembled those in the uninjured control. Labeling was found primarily in dorsal cells (white arrowhead), edge cells (black arrowhead), lateral interneurons (white arrows) and neurons of the spinal gray matter (black arrows). **G and H,** RGM mRNA expression in spinal cord 1 month after transection. **G,** Absence of *in situ* hybridization signal for RGM in the transection site. **H,** Caudal to the transection (20 mm), RGM mRNA expression was present at reduced levels in medium sized neurons of the spinal grey matter (black arrows), in edge cells (black arrowhead) and lateral interneurons (white arrow). Scale bar, 100 μm (**A**).

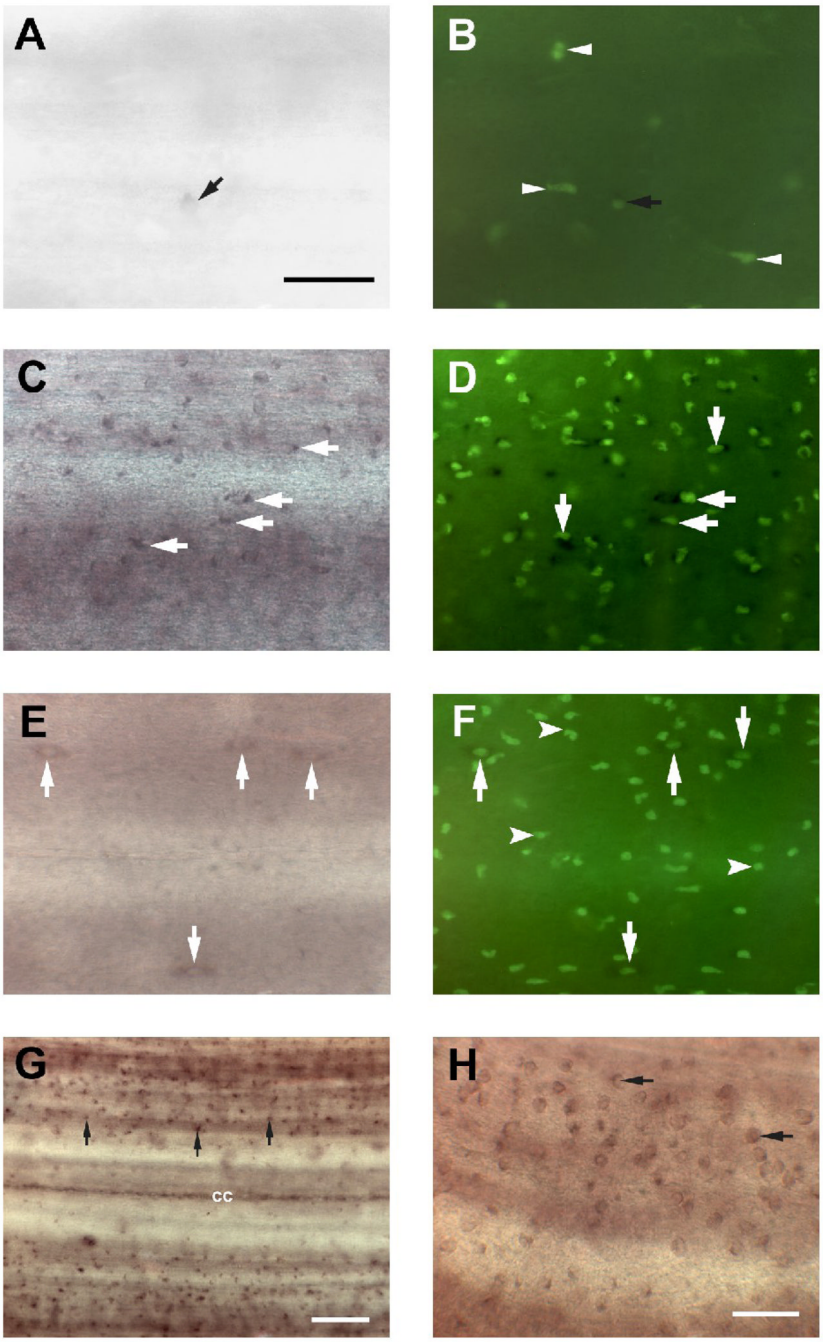

Figure 3. Microglial activation after spinal cord transection includes RGM upregulation

A, *In situ* hybridization in control spinal cord wholemount revealed the presence of small RGM-expressing cells (black arrow) on the surface of the spinal cord. Scale bar, 20 μm. **B,** Fluorescein-labeled GSL I–isolectin B_4 histochemistry revealed several lectin-labeled cells in control spinal cord (white arrowheads) including RGM–expressing cells (black arrow). In controls, few isolectin B_4-labeled profiles were RGM-positive. **C,** Two weeks after spinal cord transection, increased numbers of small, rounded cells (white arrows), reminiscent of activated microglia/macrophages, were labeled with the RGM probe, and a dense accumulation of reactive macrophages/microglial cells, as evidenced by increasing IB_4 lectin reactivity, was seen (**D**). Most of the lectin-labeled cells co-expressed RGM mRNA (white arrows). **E,** Only

a few RGM–expressing cells were detected in the spinal cord 1 month after injury. Some slender, elongated RGM-expressing cells (white arrows) resembled the macrophages/microglial cells seen in control spinal cord (Fig. 3A)**. F,** At this time, numerous small, round macrophages/microglial cells (white arrowheads) could be detected by labeling with IB_4 lectin in the spinal cord, but these were never labeled by the RGM probe. On the other hand, there were some larger, elongated RGM-expressing cells that also were labeled with IB_4 lectin (white arrows). **G and H,** RGM-expressing cells exhibited very small rounded cell bodies (no larger than 5 μm) and possessed obvious non-neuronal morphology (black arrows). Scale bar, 20 μm(**G, H**).

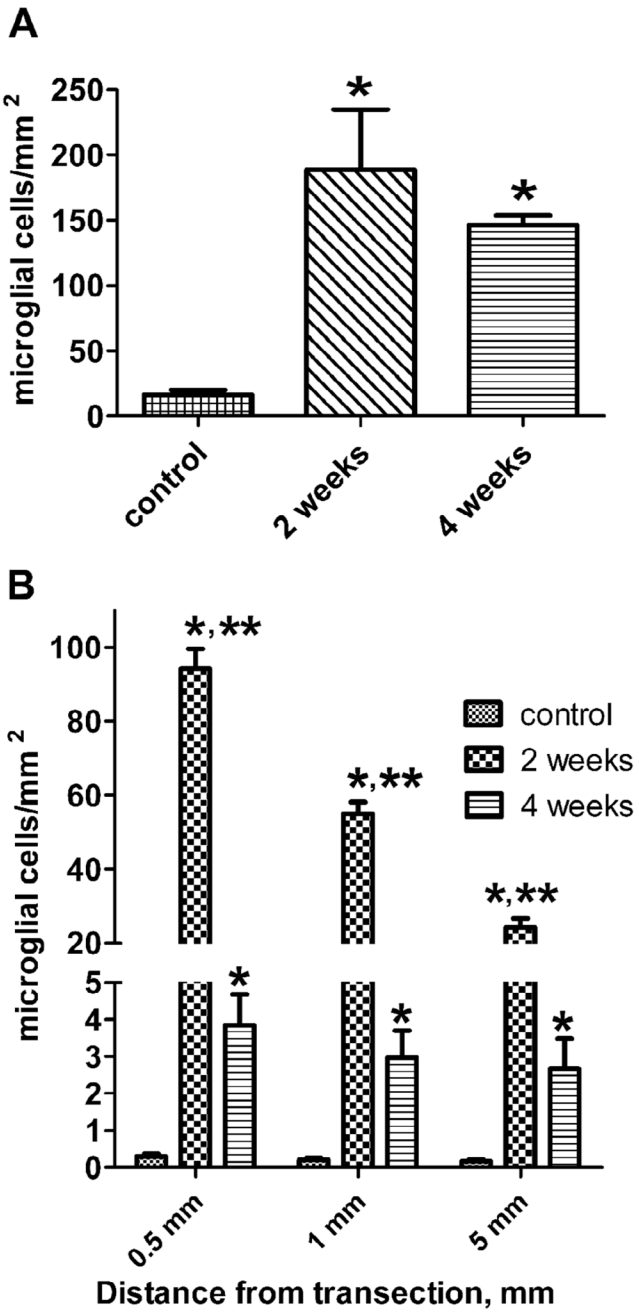

Figure 4. Microglial proliferation and RGM expression in response to spinal cord transection

A, Increase in the number of fluorescein-labeled GSL I–isolectin B_4 microglial cells in the spinal cord between 0.5 and 5 mm rostral and caudal to the lesion site at 14 and 30 days post-lesion. *P*-values are indicated as follows: * $P < 0.05$ compare to control. Differences between 14 days and 30 days numbers are not significant. Data are presented as mean with standard deviation and analyzed using a one way ANOVA followed by Tukey's multiple comparison test. **B,** Temporal and spatial dependence of RGM expression in microglial cells post-transection. The number of RGM-expressing microglial cells at 2 and 4 weeks post-transection are compared between control and lesioned spinal cord 0.5, 1 and 5 mm from the transection. Control *vs.* 2 weeks: * $P<0.001$; control *vs.* 4 weeks: $P>0.05$, ns, not significant; 2 weeks *vs.*

4 weeks: ** $P<0.001$. Data are presented as means and standard deviations and analyzed using a two way ANOVA followed by a Bonferroni post-test.

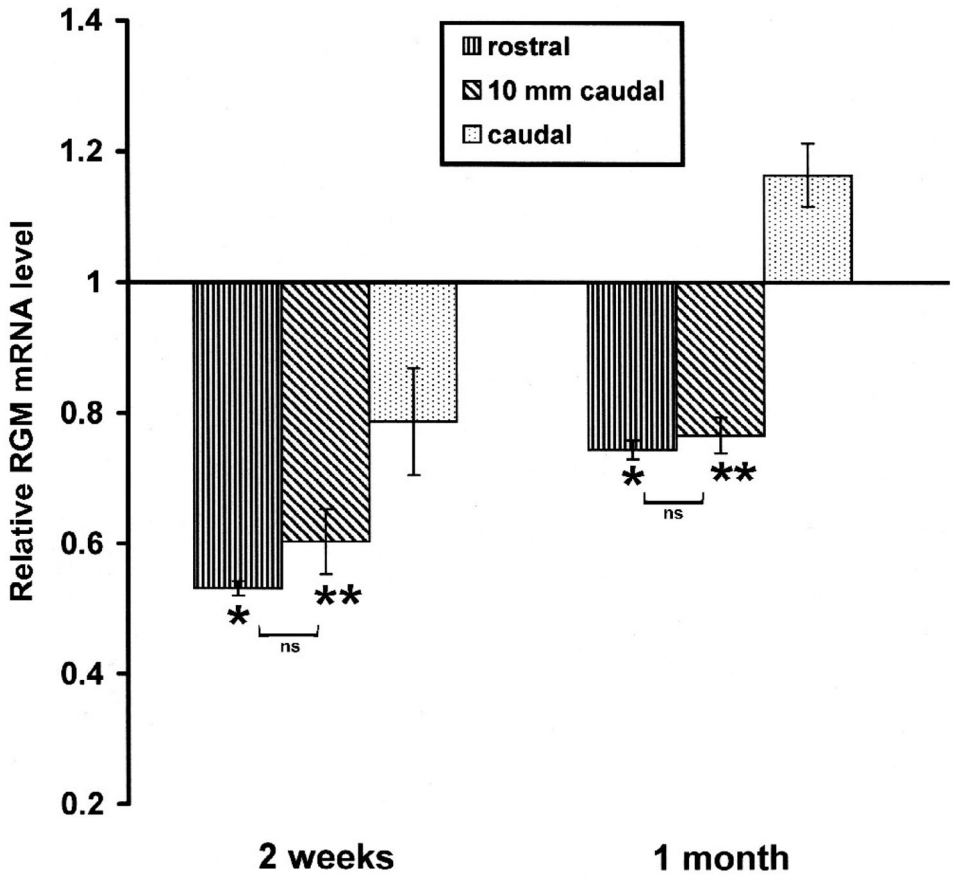

Figure 5. Relative changes of mRNA expression of RGM post-transection in spinal cord rostral to the lesion (including the transection site), the first 10 mm caudal to the transection, and further caudal tissue

The time course of relative changes of mRNA expression in spinal cord after injury was determined by quantitative real-time RT–PCR. Expression of RGM and GAPDH mRNA was measured in the presence of their respective primers and the PCR reaction was performed in triplicate. The level of RGM mRNA expression was calculated after normalizing against the GADPH mRNA level in each sample and is presented as the fold-difference in the level of RGM mRNA expression after transection compared with the untransected control sample, which was assigned a value of 1. Two-way ANOVA was used to compare experimental groups, with corrections for multiple post-hoc comparisons by the Bonferroni test. Non-significantly (ns) different: rostral vs 10 mm caudal - $P > 0.05$; significantly different: * rostral vs caudal - $P < 0.001$; ** 10 mm caudal vs caudal - $P < 0.01$.

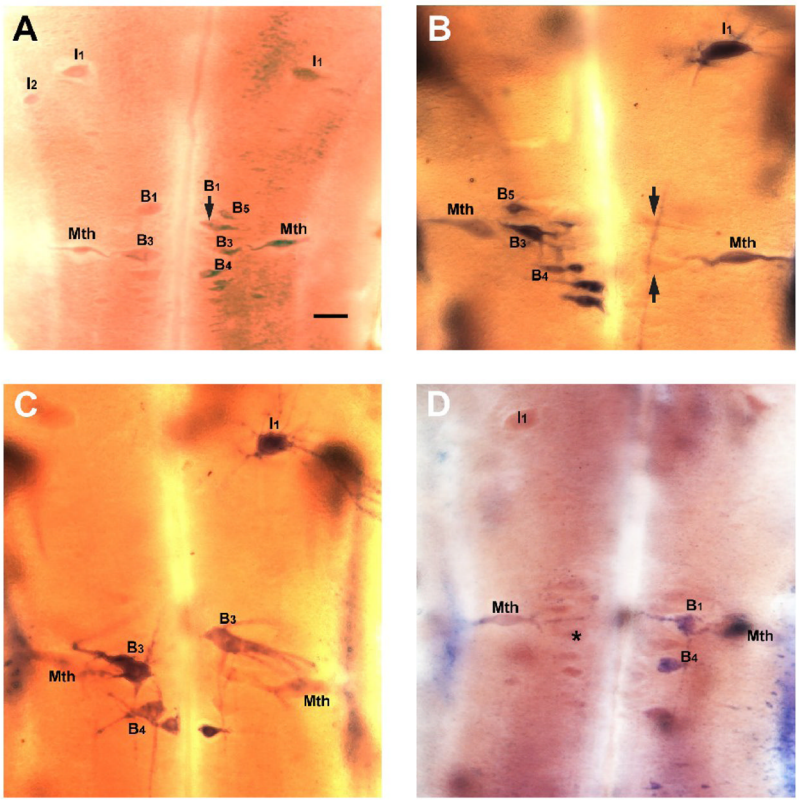

Figure 6. Expression of the RGM receptor neogenin in reticulospinal neurons after spinal cord transection

A, *In situ* hybridization for neogenin in control animals. The rhombencephalon shows asymmetric neogenin expression in the Mauthner neurons (Mth), in the I_1 and I_2 neurons and in B neurons of the bulbar region (B_1, B_3, B_4, B_5). Scale bar – 100 μm. **B**, One week after transection, neogenin is expressed in the Mauthner neurons (Mth), I_1 neuron and B neurons in the bulbar region. Note that neogenin expression was heterogeneous and was often stronger in one of a pair of cells or only one neuron was labeled. The silhouettes of several unlabeled B neurons are indicated by black arrows. **C,** At two weeks post-transection, neogenin is expressed in an I_1 neuron and B neurons and very faintly in Mauthner neurons (Mth). **D,** At four weeks after transection, neogenin is expressed only in the B1 and B4 neurons. Note the shrinking Mauthner neurons leaving empty outlines around labeled neurons. The silhouette of an unlabeled B neuron is indicated by asterisk.

Table 1

Sequence of primer pairs for the lamprey genes used for the real-time PCR

Gene (GenBank Accession #)	Sequence 5′–3′	Fragment size(bp)
RGM (**EU449948**)		96
Forward primer	CCTCGTCGACAACCCTTACC	
Reverse primer	AAGATGATGGTCAGCTTGTTGGT	
HPRT 1 (**U03700**)		68
Forward primer	GCGCTCAACCGCAACAC	
Reverse primer	CAGTAGCTCTTGAGTCGGATGAAG	
18S rRNA (**M97575**)		75
Forward primer	GTAGTTGGTGGAGCGATTTGTCT	
Reverse primer	GGCCGCGTAGCTAGTTAGCA	
GAPDH(**AAT70328**)		72
Forward primer	TGCAAAGCACGTCATCATCTC	
Reverse primer	TTCTCGTGGTTTACTCCCATCA	