Experimental Neurology 217 (2009) 242–251

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

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

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ARTICLE INFO

Article history:
Received 13 November 2008
Revised 10 February 2009
Accepted 12 February 2009
Available online 5 March 2009

Keywords:
Axonal guidance
Spinal cord regeneration
In situ hybridization
RGM
Neogenin
Lamprey
Microglia

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, M. I., and Selzer, M. E., 2000a. Expression of netrin receptor UNC-5 in lamprey brain; modulation by spinal cord transection. Neurorehabilitation and Neural Repair 14, 49–58; Shifman, M. I., and Selzer, M. E., 2000b. In situ hybridization in wholemounted lamprey spinal cord: localization of netrin mRNA expression. Journal of Neuroscience Methods 104, 19–25) 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.

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, lamprey reticulospinal neurons differ in their regenerative abilities. While some are consistently "good regenerators" (regenerating 75% of the time or more), other neurons are "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 with 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, MAG and Nogo). Moreover, lamprey spinal axons grow preferentially through a hemisection scar rather than around it (Lurie and Selzer, 1991a,b,c), 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, 2000a,b), 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 partially, 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

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0014-4886/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2009.02.011

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 et al., 2002, Manitt et al., 2006, Moreau-Fauvarque et al., 2003, Pasterkamp and Verhaagen, 2001, Shifman and Selzer, 2000a,b, 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., 2005a,b).

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 transaction

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 1 h. Each transected animal was examined 24 h 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 wholemount *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, 2000a,b). 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 transaction

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