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THE EXPRESSION OF CHEMOREPULSIVE GUIDANCE RECEPTORS AND THE REGENERATIVE ABILITIES OF SPINAL-PROJECTING NEURONS AFTER SPINAL CORD INJURY

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Abstract—Spinal cord injury (SCI) in mammals leads to permanent loss of function because axons do not regenerate in the central nervous system (CNS). To date, treatments based on neutralizing inhibitory environmental cues, such as the myelin-associated growth inhibitors and chondroitin sulfate proteoglycans, or on adding neurotrophic factors, have had limited success in enhancing regeneration. Published studies suggested that multiple axon guidance cues (repulsive guidance molecule (RGM) family, semaphorins, ephrins, and netrins) persist in adult animals, and that their expression is upregulated after CNS injury. Moreover, many adult CNS neurons continue to express axon guidance receptors. We used the advantages of the lamprey CNS to test the hypotheses that the regenerative abilities of spinal-projecting neurons depend upon their expression of chemorepulsive guidance receptors. After complete spinal transection, lampreys recover behaviorally, and injured axons grow selectively in their correct paths. However, the large identified reticulospinal (RS) neurons in the lamprey brain are heterogeneous in their regenerative abilities – some are high regeneration capacity neurons (probability of axon regeneration >50%), others are low regeneration capacity neurons (<30%). Here we report that the RGM receptor Neogenin is expressed preferentially in the low regeneration capacity RS neurons that regenerate poorly, and that downregulation of Neogenin by morpholino antisense oligonucleotides enhances regeneration of RS axons after SCI. Moreover, lamprey CNS neurons co-express multiple guidance receptors (Neogenin, UNC5 and PlexinA), suggesting that the regenerative abilities of spinal-projecting neurons might reflect the summed influences of the chemorepulsive guidance receptors that they express.

Key words: axon guidance, mRNA expression, identifiable neurons, lamprey, Mauthner cell, spinal cord regeneration.

INTRODUCTION

Complete spinal cord injury (SCI) in mammals, including humans, leads to permanent paralysis due to absence of axonal regeneration in the central nervous system (CNS). To elucidate this problem, during the past several decades, great importance has been placed on inhibitory factors present in the extracellular environment within the injured CNS (David and Aguayo, 1981; Caroni and Schwab, 1988; Aguayo et al., 1991). A number of these inhibitory factors are associated with myelin (Caroni and Schwab, 1988; McKerracher et al., 1994; Spillmann et al., 1998; Huber and Schwab, 2000; Brittis and Flanagan, 2001), or with matrix molecules secreted by reactive astrocytes (Snow et al., 1990; Fitch and Silver, 1997). However, treatments based on neutralizing inhibitory environmental cues, such as the myelin-associated growth inhibitors and chondroitin sulfate proteoglycans, or adding neurotrophic factors, have had limited success in enhancing regeneration and functional recovery, so additional factors must exist to explain this inability of cut axons to re-grow in the mature CNS. One possibility is that the same chemorepulsive molecules that guided axon development persist in the mature CNS in order to prevent important established connections from being displaced haphazardly by new ones. While this would be adaptive in the uninjured CNS, it might complicate recovery from injury. Several families of axon guidance molecules – semaphorins, ephrins, netrins, RGM (repulsive guidance molecule) and slits – guide axons to their targets during development by providing attractive or repulsive cues. Many axon guidance molecules continue to be expressed in the adult CNS (Manitt et al., 2001; de Wit and Verhaagen, 2003), and their expression is upregulated following injury (Ara et al., 2004; Schwab et al., 2005a; Shifman et al., 2009). Moreover, many CNS neurons continue to express receptors for these guidance molecules (Giger et al., 2010), suggesting that even in the adult CNS, neurons may continue to respond to the guidance cues. Many studies have focused on the

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Abbreviations: ANOVA, analysis of variance; CNS, central nervous system; MOs, morpholino oligonucleotides; Mth, Mauthner; mth′, auxiliary Mauthner; RGM, repulsive guidance molecule; RS, reticulospinal; SCI, spinal cord injury; TdT, terminal deoxynucleotidyl transferase.

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roles of netrins, semaphorins and ephrins in the pathophysiology of SCI (Bolsover et al., 2008; Giger et al., 2010). By comparison, the involvement of RGM and its receptor Neogenin in axon regeneration after SCI has received little consideration, although a few reports have described induction of RGM immunoreactivity after CNS injury in mammals (Schwab et al., 2005a, 2005b; Doya et al., 2006; Hata et al., 2006), suggesting a possible role for RGM in blocking axon regeneration.

By contrast, the lamprey shows robust axon regeneration after SCI (Rovainen, 1976; Selzer, 1978; Wood and Cohen, 1979; Davis and McClellan, 1994). Therefore, previously we examined expression of RGM and its receptor Neogenin by *in situ* hybridization and quantitative real-time PCR in this animal (Shifman et al., 2009). RGM mRNA was expressed in the spinal cord, primarily in neurons of the lateral gray matter and in dorsal cells, but unlike the immunoreactivity responses in mammals, lamprey RGM mRNA was down-regulated in neurons within 10 mm of the transection at 2 and 4 weeks and not changed further from the injury. Since this distance encompasses the range of most regeneration for these axons, it suggested a possible regeneration-promoting response to injury in the lamprey. However, proliferating reactive microglial cells in the same region of spinal cord upregulated their expression of RGM mRNA. Moreover, mRNA expression for the RGM receptor Neogenin was unchanged in the axotomized brain neurons. Nevertheless, among identified reticulospinal (RS) neurons, Neogenin mRNA was detected primarily in those with low regeneration capacity. Neurons that regenerate well rarely expressed Neogenin. These findings left the role of Neogenin and RGM in inhibiting regeneration unclear.

To clarify the role of Neogenin in CNS axon regeneration, we now have extended our previous observations on expression of Neogenin and two other chemorepulsive receptors, UNC5 and PlexinA, to longer post-transection times, and also have determined the effect of Neogenin knockdown with antisense morpholino oligonucleotides (MOs) on regeneration of RS axons. The lamprey CNS has exceptional advantages for such a study, including the presence of individually identified RS neurons with previously determined regenerative abilities, in which the expression of guidance receptors during regeneration can be studied on the single cell level *in vivo*.

The spinal-projecting axons in the lamprey originate in several nuclear groups that include 36 individually identifiable RS neurons, including giant Müller and Mauthner cells, and a variable number of unidentified neurons (Fig. 1A and B–E) (Swain et al., 1993). The identified RS neurons are symmetrically arranged on the left and right sides of the brainstem. All neurons project to the ipsilateral spinal cord, except for the Mauthner (Mth) and auxiliary Mauthner (mth′) cells, which project contralaterally. The identified RS neurons vary from one another in their regenerative capabilities (Swain, 1989; Davis and McClellan, 1994; Jacobs et al., 1997). Some regenerate their axon beyond the transection with high probability (*e.g.*, M_4, I_3, I_4, mth′, B_5, B_6; regeneration probabilities > 50%; high regeneration capacity), whereas others regenerate poorly (*e.g.*, Mth, I_1, M_2; regeneration probabilities < 30%; low regeneration capacity), and still others fall in between (*e.g.*, M_1, I_2, B_2; intermediate regeneration capacity) (Jacobs et al., 1997). The presence in the same brain regions of both "good-regenerating" and "bad-regenerating" neurons, which project in the same axon tracts, suggests that differences in their regenerating abilities depend upon factors intrinsic to the neurons. Therefore, the lamprey provides a unique opportunity to compare properties of neurons with high *vs.* low regenerating capacity in the same brain and animal. Only recently have studies on mouse retinal ganglion cells begun to approach this capability (Duan et al., 2015).

Here we report that the RGM receptor Neogenin is preferentially expressed in the low regeneration capacity RS neurons and that downregulation of Neogenin by MOs enhances the regeneration of RS neurons after SCI. Moreover, many neurons co-express multiple guidance receptors (Neogenin, UNC5 and PlexinA), which suggests that the regenerative abilities of spinal-projecting neurons are determined, in part, by the combination of chemorepulsive guidance receptors that they express.

EXPERIMENTAL PROCEDURES

Animals

Larval lampreys (*Petromyzon marinus* L.), 10–14 cm in length (4–7 years old), were obtained from commercial suppliers (Northwoods Custom Products and Services, Marquette, MI or Lamprey Services, Ludington, MI) and maintained in freshwater tanks at 16 °C with the appropriate ventilation conditions until the day of surgery. Animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1996). Animal experimental protocols were approved by the Institutional Animal Care and Use Committee at Temple University. All the methods and experiments followed the approved Temple University guidelines. All efforts were made to minimize the number of animals used and their suffering.

Spinal cord transections

Anesthesia was administered by immersion of lampreys in 0.1% tricaine methanesulfonate (Sciencelab, Houston, TX, USA). After anesthesia the spinal cord was uncovered *via* a dorsal incision and transected at the level of the 5th gill with Castroviejo scissors. The wound was air dried over ice for one hour. Twenty-four hours after surgery, each lamprey was examined to check that there was no movement caudal to the lesion site. Surgery was considered successful if the lamprey could move only its head and body rostral to the transection. Lampreys then were allowed to recuperate in an aquarium at room temperature. After specific recovery times, they were re-anesthetized, and their brains dissected for *in situ* hybridization. Experiments