Published in final edited form as:

Mol Cell Neurosci. 2008 February ; 37(2): 222–235.

Modulating Sema3A signal with a L1 mimetic peptide is not sufficient to promote motor recovery and axon regeneration after spinal cord injury

Erik Mire¹, Nicole Thomasset², Lyn B. Jakeman^{3,*}, and Geneviève Rougon^{1,*}

¹ Institut de Biologie du Développement de Marseille-Luminy, Université de la Méditerranée CNRS UMR 6216. Case 907, Parc Scientifique de Luminy, 13288 Marseilles Cedex 9 France

² INSERM U433 faculté Laennec Rue Guillaume Paradin 69372 Lyon Cedex 08 France

³ Department Physiology and Cell Biology, The Ohio State University, Columbus, Ohio USA

Abstract

We examined whether Sema3A which is upregulated at the site of spinal cord injury exerts a direct effect on axons. We used ASNKL peptide that prevents specifically the inhibitory effect of Sema3A on L1/Neuropilin1 (Nrp1) expressing axons. In the naïve mouse spinal cord, L1 is located on a subset of corticospinal axons, whereas Nrp1 is barely detectable. After contusion injury, Nrp1 is found on L1-negative immune cells whereas its expression does not increase on severed axons. L1 expressing axons sprout extensively into the lesion site but no difference in axon density could be detected in the lesion area of mice treated with ASNKL. In agreement, these mice did not recover a better motor function than controls. Similarly, culture of neurons sensitive to ASNKL on cryosections of lesioned spinal cords revealed no effect of Sema3A. Our data indicate a limited direct effect of Sema3A on axonal growth at the site of a contusion injury, and suggest that alternative mechanisms underlie positive effects of Sema3A inhibition on motor recovery.

Keywords

mouse; spinal cord injury; Semaphorin 3A; L1CAM; motor function recovery

INTRODUCTION

Both intrinsic growth capacities and extrinsic factors contribute to limit adult central nervous system (CNS) axon regeneration. The inhospitable milieu of the injured CNS results, in part, from the presence of myelin-specific inhibitory factors (Schwab and Bartholdi, 1996). In addition, CNS injury induces a glial scarring response that leads to increased expression of many molecules thought to inhibit regeneration (Silver and Miller, 2004). Furthermore, the recruitment of inflammatory cells and cytokines has a more complicated effect on CNS regeneration (Yiu and He, 2006). Most of the guidance molecules playing a crucial role in

Corresponding author: Dr Geneviève Rougon, Institut de Biologie du Développement de Marseille-Luminy, CNRS UMR 6216. Case 907, Parc Scientifique de Luminy, 13288 Marseille Cedex 9 France, mail: rougon@ibdm.univ-mrs.fr, tel: 33-(0)4 9126 9348, Fax: 33-(0)4 9126 9748.

*These authors contributed equally to this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

development and guidance of neuronal projections, such as Ephrins, Slits, Netrins and class 3 Semaphorins persist in the spinal cord after development is completed (Wehrle et al., 2005; Fabes et al., 2006; Harel and Strittmatter, 2006; Manitt et al., 2006). Their distribution, especially after injury, provides regenerating axons with a drastically altered signaling environment.

Semaphorins are a large family of secreted and membrane bound proteins involved in axon guidance, cell migration and cell death (Kruger et al., 2005; Mann et al., 2007). While Semaphorins are known to be expressed at sites of CNS injury, until recently, evidence for a significant contribution of guidance cues of the Semaphorin family in inhibition of axon regeneration were lacking. Using xantofulvin, a pharmacological inhibitor of Sema3A, Kaneko et al showed enhanced functional recovery after total spinal cord transection in rats. Pleiotropic effects including enhanced regeneration and/or preservation of injured axons, robust Schwann cell migration, decrease in apoptotic cell number and marked enhancement of angiogenesis are thought to be responsible for the observed recovery (Kaneko et al., 2006).

Sema3A, initially identified on the basis of its growth cone collapse-inducing activity (Luo et al., 1993) repels axons by binding to receptors composed of a binding subunit Neuropilin 1 (Nrp1) and a signal transducer of the PlexinA family, thus activating intracellular pathways that control actin dynamics (Castellani and Rougon, 2002; Tran et al., 2007). We have previously shown that Nrp1 and the Immunoglobulin superfamily cell adhesion molecule (Ig CAM) L1, associate through their extracellular domains, uncovering a link between L1 and Nrp1/Sema3A signaling. This is further supported by the finding that wild-type, but not L1-deficient axons, when exposed to soluble L1 or a 5 amino acid peptide (ASNKL) derived from L1 Ig1 domain and mimicking L1/Nrp1 binding site, switched their response to Sema3A from repulsion to attraction (Castellani et al., 2000; Castellani et al., 2002). This peptide thus represents a pharmacological tool to directly and specifically manipulate Sema3A responses from cells co-expressing L1 and Nrp1.

Here, we used this tool with the view to better decipher the multiple cellular effects of Sema3A after spinal cord contusive injury in mice. We show that *in vitro*, both Sema3A mediated inhibition of axon growth and growth cone collapse are blocked by ASNKL. We show that Sema3A is expressed in the lesion scar in mice, and that some spinal axons express Nrp1/L1, therefore making it possible to modulate their response to Sema3A with ASNKL peptide. However, delivery of the peptide *in vivo* does not improve motor recovery, nor does it increase axonal density in the lesion site in this model. Results from cryoculture assays using adult spinal cord sections showed that the complex environment of the injured spinal cord prevents embryonic neurons from responding to a modulation of Sema3A signal by ASNKL. Together our results suggest that modulation of Sema3A signaling on neurons is not sufficient to promote recovery and regeneration after spinal cord injury.

RESULTS

ASNKL peptide prevents inhibition of axon growth mediated by Sema3A

We previously showed that a ASNKL peptide interferes with Sema3A signaling resulting in conversion of the repulsive response of Nrp1/L1 expressing axons into an attraction (Castellani et al., 2002). To determine if this peptide also interferes with Sema3A signal in axon growth, we examined its effect in a neurite outgrowth assay. Dissociated embryonic cortical neurons were cultured on a polyLysine/laminin substrate in the presence (fig. 1B, D) or absence (fig. 1A,C) of recombinant Sema3A, with (fig. 1C, D) or without (fig. 1A, B) ASNKL peptide, and the longest neurite was measured. After two days *in vitro*, Sema3A reduced axonal length by 30% compared to control conditions (69.3 ± 8.2 %, student t test $p < 0.0001$). As predicted, the ASNKL peptide ($5 \cdot 10^{-6}$ M) protects axons from the Sema3A inhibitory effect (101.4 ± 3.5

student t test $p=0.17$) (fig. 1E). Similarly, ASNKL blocked Sema3A mediated collapse of growth cones (fig. 1F–I). These results confirm ASNKL bioactivity and reinforce the potential of L1 mimetic peptide as a Sema3A signal modulator in the context of guidance and growth of L1/Nrp1 expressing cortical axons.

The distribution of Sema3A and its binding partners has not been described in adult mice after spinal cord injury. In order to determine the expression of the three necessary partners for activity of the L1 mimetic peptide, namely Sema3A, Nrp1 and IgCAM L1, we performed immunohistofluorescence and *in situ* hybridization on naïve and injured spinal cords. We chose to use a closed contusion injury model (Material & Methods), as it seems presently to be the best available mimic of human injury (Rosenzweig and McDonald, 2004).

Sema3A is expressed in the lesion scar after contusive injury of the spinal cord

Previous studies showed expression of Sema3A in the adult rat spinal cord (De Winter et al., 2002; Pasterkamp and Verhaagen, 2001). We found that in naïve mouse spinal cord, *Sema3A* mRNA is located in the gray matter, in large cells of the ventral horn. The size and distribution of these cells is closely similar to that of motoneurons expressing *Choline Acetyltransferase (ChAT)* mRNA. Cells positive for the Sema3A protein also stained with neurofilament M, confirming the neuronal expression of Sema3A (supplementary data, fig. S1). After contusive injury, we performed double labeling for GFAP and fibronectin (fig. 2C, E, G) to localize the lesion scar and for Sema3A (fig. 2D, F, H) on parasagittal serial sections (fig. 2A, B). Sema3A is located on round cells in the lesion area as early as 4 days post injury (dpi) (fig. 2D). From 10 dpi to 35 dpi, Sema3A is mainly observed at the GFAP/fibronectin positive border of the injury site (arrows in fig. 2E–H and data not shown), with greatest expression associated with the astrocytic border. Some signal can also be detected in isolated cells located in the lesion core (arrowheads in fig. 2F and H). Thus, in agreement with previous studies of injured rat spinal cord, Sema3A is expressed in a subpopulation of spinal neurons and in the lesion scar at the injury site. Moreover, this expression at the lesion site is long lasting, as intense Sema3A protein expression can be detected at the latest time point studied (35 dpi).

Expression of L1 and Nrp1 in the naïve spinal cord

Co-expression of L1 and Nrp1 on axons is necessary to the activity of the ASNKL peptide. Therefore, we examined expression of the two proteins in the spinal cord (fig. 3A). In the naïve spinal cord, L1 is located in the superficial laminae of the dorsal horn (fig. 3B) and within the white matter, in axon tracts of the dorsal (*df*), dorsolateral (*dlf*) and ventral (*vf*) funiculi (fig. 3C, D and supplementary data, fig. S2). This expression is in agreement with that reported by previous studies in rats (Akopians et al., 2003; Runyan et al., 2005). A subset of these axons is also positive for a marker of the corticospinal tract (CST), PKC γ (supplementary data, fig. S2).

In our hands, available antibodies for detection of Nrp1 in adult mouse CNS tissue are not very sensitive. We used both *in situ* hybridization and immunodetection to determine Nrp1 localization in the naïve adult brain and spinal cord. *In situ hybridization* (supplementary data, fig. S3) revealed *Nrp1* mRNA expression at low level in cortical layer Vb, in agreement with published data for rat (De Winter et al., 2002; Hashimoto et al., 2004). Low levels of Nrp1 protein were also detected in the CST only with an antibody directed against the intracellular domain of Nrp1 (fig. 3F), whereas it is detected at higher levels in the superficial laminae of the dorsal horn with two different antibodies (fig. 3E and H). As for rats (Agudo et al., 2005), blood vessels were also detected with both antibodies (fig. 3F and I). Double staining with L1 showed prominent coexpression of the proteins in the *dlf* (fig. 3K–M).

Expression of L1 and Nrp1 in the injured spinal cord

After contusion, L1 positive structures detected in the lesion site are positive for SMI -31, indicating they are axons. L1 positive axons are interrupted at 4dpi (fig. 4F–H) illustrating section of fibers in the white matter compared to naïve spinal cord (fig. 4C–E). At this time, the injury site is completely devoid of L1 fibers. From 10 dpi, L1 positive axons are detected in the peripheral fibroglial scar whereas some of them can be seen in the lesion epicenter (fig 4I–K and data not shown). L1 positive axons in the injury site also express GAP-43, an axon growth marker, indicating that these axons spontaneously sprout into the lesion site (fig. 4L–N). This sprouting is maintained until the latest time post injury studied (35 dpi).

After injury, low levels of Nrp1 are detected throughout the spinal cord. Strikingly, high expression of Nrp1 is located in the leukocyte infiltrate at 4 dpi in the lesion epicenter (fig. 5C). Later, Nrp1 is detected in cells both rostral and caudal to the injury site, with distance increasing with time post injury. These cells present some process-like extensions (fig. 5D, E). To assess their nature we performed a double immunostaining of Nrp1 with CD11c, a marker of mouse dendritic cells. CD11c positive cell aggregates are located mainly in the white matter outside the lesion core. Nrp1 positive cells are detected into these aggregates (fig. 5D–F). These cells do not express L1CAM (fig. 5I–K).

ASNKL peptide fails to promote locomotor function after spinal cord contusion

We considered that the weak expression of Nrp1 detected on spinal cord axons might be due to the limited sensitivity of the anti-Nrp1 antibodies since results previously published (De Winter et al., 2002; Hashimoto et al., 2004; Kaneko et al., 2006) suggested expression after injury. Therefore we decided to test the effect of ASNKL peptide *in vivo*, after contusion of the spinal cord.

In order to ensure a localized delivery of the peptide to the site of injury, it was administered by repeated intrathecal bolus infusions (Jakeman et al., 1998), every 3rd day until 6th week post injury at the concentration of 1mM, to account for the dilution occurring in tissues after delivery. Locomotor recovery was assessed using the BMS score, developed for use in mice with spinal cord injury (Basso et al., 2006). At 1 dpi, all animals showed a BMS score close to 0 indicating complete paralysis of hind paw (fig. 6A). Control groups (no treatment, or PBS) exhibited spontaneous recovery of motor function and reached a plateau between 14 and 21 dpi, with no significant differences over the period studied although animals of the PBS group recovered slightly better (respectively 5.0+/-0.3 and 4.3+/-0.3). Strikingly, the treated group did not show any difference from controls in the maximum motor score (respectively 5.4+/-0.5; 5.4+/-0.5; 5.8+/-0.5 for no treatment, PBS and treated groups respectively), nor in the kinetics of the motor recovery to reach this plateau (fig. 6A). Moreover, using the BMS subscore to expand the components of the motor function, we did not find any significant improvement for the ASNKL peptide treated group compared to controls (fig. 6B) although a slight enhancement of BMS subscore was seen for treated mice at 35 and 42 dpi. Dissection of animals and dye infusion at the end of the testing period confirmed that the infusion cannulas were still partially patent at six weeks post-injury, despite connective tissue growth along their length (not shown). Analysis of spared white matter at the lesion epicenter revealed no difference in lesion length or white matter sparing measures between the groups (data not shown). Therefore, in these conditions, L1 mimetic peptide failed to promote motor recovery or promote tissue sparing after spinal cord injury in mouse.

ASNKL peptide does not attract regrowing axons toward the lesion site

As axon regeneration does not always correlate with functional improvement, we also examined the density of axonal growth in the lesioned spinal cord by quantifying the area of Tuj1 labeling within the lesion epicenter. Dense axonal staining is evident in the periphery of

the lesion, with few axons present in the center of the lesion (fig. 6C, D and E). An analysis of axonal density through the entire lesion in sections sampled at 200 micron intervals showed that positive staining within the lesion was inversely related to the distance from the epicenter (fig. 6F). Axon density did not differ between the three groups (1 way ANOVA; $f=2.064$; $p=0.162$; $df=2,15$; $n=6/\text{group}$). Thus, we find that treatment with ASNKL peptide does not enhance sprouting/regeneration of axons into the lesion site after spinal cord contusion.

ASNKL peptide does not promote axon growth on spinal cord cryosections

The lack of effect of the ASNKL modulating peptide *in vivo* could be due to different parameters. One could be related to the lack of response of adult axons themselves because of their weak coexpression of L1/Nrp1. Alternatively, it is possible that relieving inhibitory signaling of Semaphorin 3A is not sufficient to allow axons to overcome the complex environment of the injured adult spinal cord. To decide between these two non-exclusive hypotheses, we took advantage of an *in vitro* model where embryonic cortical neurons, known to respond to Semaphorin 3A inhibition, were cultured on naive or injured adult spinal cord cryosections (fig. 7A, B). We first evaluated the neurite outgrowth capacity in this model (fig. 7C). More neurons initiated neurites on naive spinal cord ($61.5\% \pm 5.1$) compared to the injured spinal cord sections taken at different times after injury ($43.0\% \pm 2.9$ at 7dpi, $51.5\% \pm 3.0$ at 14dpi and $37.9\% \pm 2.2$ at 28dpi, 1 way ANOVA $p < 0.0001$). Second, we examined the length of the longest neurite (fig. 7E). Tissue substrate from injured spinal cord was able to reduce neurite length compared to naive spinal cord by approximately 30% at all time points post injury studied ($100\% \pm 5.7$ for non injured (NI); $68.9\% \pm 2.3$ for 7dpi; $73.8\% \pm 5.9$ for 14dpi and $68.2\% \pm 2.6$ for 28dpi, 1 way ANOVA, $p < 0.0001$). Altogether these data indicate that injured spinal cord cryosections are less permissive to neurite growth than sections from naive spinal cord. Therefore, this model is suitable to detect potential effects on neurite growth of the ASNKL peptide.

As no differences were detected between growth on tissues from the three time points post injury examined, the data shown (fig 7D, F) correspond to pooled data obtained from these time points. When compared to naive spinal cord, treatment with ASNKL did not increase the percentage of cells with neurites on injured sections (fig. 7D, 2way ANOVA, lesion $p < 0.0001$, treatment $p = 0.308$, interaction $p = 0.794$). Strikingly, neurons still showed significantly shorter processes when treated with ASNKL peptide on injured sections (fig. 7E, 2way ANOVA, lesion $p < 0.0001$, treatment $p = 0.594$, interaction $p = 0.731$). No significant differences were observed when cell density was analyzed, indicating that the peptide did not affect adhesion or survival of dissociated neurons (data not shown). Together, these *in vitro* data indicate that although embryonic cortical neurons express Semaphorin 3A receptors, Np1 and L1 (Castellani et al., 2000), ASNKL peptide did not have any significant effect on their growth capacities when confronted with the environment of the injured adult spinal cord.

To exclude that the previous results are due to low amounts of Semaphorin 3A in the sections, in some experiments we added recombinant Semaphorin 3A to the cryosection assay. Strikingly, addition of exogenous Semaphorin 3A was able to reduce neurite length when neurons grew on a polyLysine/laminin substrate (fig. 8A, B and E, 73.03% of control, student t test $p < 0.001$), but had no detectable effect on the same neurons when cultured on spinal cord sections (fig. 8B, D, E) ($107.6\% \pm 4.5$ of neurite length in control conditions, student t test $p = 0.44$). These results show that inhibitory environment of the spinal cord prevents further inhibition of axon growth mediated by Semaphorin 3A.

DISCUSSION

One of the approaches to understand and treat CNS injury is to postulate that post-lesional events recapitulate, at least in part, the events that occur during development. Involvement of

Semaphorins in inhibition of axonal regeneration has long been suggested (De Winter et al., 2002; Pasterkamp and Verhaagen, 2001) but until recently there was no direct evidence for their implication in such phenomenon. The study from Kaneko and colleagues (Kaneko et al., 2006) is the first to provide a formal proof for a role of Sema3A in limiting regenerative responses and recovery of function after injury. It remains unclear however, whether axon regeneration is directly attributable to Sema3A-mediated inhibition of axon growth or if these effects are secondary to the modification of other processes.

In this study, we tried an original strategy to more directly assess the role of Sema3A in axonal growth in a closed contusion injury. Our *in vitro* studies confirm ASNKL peptide as a potent modulator of Sema3A signaling for guidance and growth of axonal projections. Indeed, ASNKL is able to convert repulsion into attraction (Castellani et al., 2002) and to block both growth cone collapse ((Castellani et al., 2004), this study) and reduction of neurite length (this study) induced by Sema3A. However, this conversion needs the presence of three partners precisely localized: L1 and Nrp1 on the axonal growth cone, and Sema3A in the environment. Our results and previous data in literature describes these partners in the appropriate locations: Sema3A in the scar and L1 and Nrp1 in cortical and sensory neurons (De Winter et al., 2002; Akopians et al., 2003; de Wit and Verhaagen, 2003; Hashimoto et al., 2004; Agudo et al., 2005).

L1 and axon regeneration

We observed a restricted expression pattern of L1 in spinal cord white matter, on distinct tracts in the *df*, *dlf* and *vf*. Moreover, we showed that many of the axons surrounding or growing into the lesion site were both GAP-43 and L1 positive, further supporting L1 expression as a characteristic of regenerating axons. L1 homophilic interaction is known to promote neurite growth *in vivo* and *in vitro* (Kobayashi et al., 1995; Mohajeri et al., 1996). However, the role of this interaction in the development of CNS fiber tracts has been refined by data obtained in a mouse line expressing L1 deleted for its homophilic and integrin binding functions, which does not exhibit the severe fiber tract defects present in the L1 knockout mouse (Itoh et al., 2004). In our mouse model of contusion injury, L1 expression was found on axons, thus suggesting that axon-cell homophilic contacts are not the basis of the sprouting of L1 positive fibers into the lesion site. A recent study of recovery and axonal sprouting in L1 knock out mice after spinal cord injury may provide additional insight in L1 function in regeneration. After injury, L1 knockout mice exhibit higher amounts of sprouting of CST fibers in the spinal cord at several segments rostral to the injury site (Jakeman et al., 2006), but no difference in sprouting into the lesion epicenter. These results suggest that L1 contributes significantly to restrict CST sprouting at sites distant from the lesion. In contrast, it is likely that the balance of L1 interactions occurring at the scar and in its vicinity contributes to the high amount of sprouting fibers we observed into and around the lesion site. In support of such complex mechanisms is the observation that perturbing the interactions of L1 interactions via the treatment with L1-Fc of contused rats, results in limited, although significant, recovery compared to control (Roonprapunt et al., 2003) while its overexpression by adeno-associated virus resulted in strikingly better motor function after spinal cord injury (Chen et al., 2007).

Sema3A inhibits regenerative responses

By combining primary neuronal cultures and spinal cord contusion experiments, our study demonstrates that modulation of Sema3A signaling by the L1 mimetic peptide is not sufficient to promote functional recovery and axon regeneration after spinal cord injury. How can our results match with those obtained by Kaneko and colleagues? Efficiency of the two compounds concerning inhibition of Sema3A effects on neurites is not an issue, at least *in vitro*. Importantly, xantofulvin, as it prevents binding of Sema3A to Nrp1, is able to impede a diversity of mechanisms mediated by Sema3A such as Schwann cell migration and

neuroprotection. Schwann cells are known to constitute a permissive substrate for regeneration of axons in the injured spinal cord (Bamber et al., 2001; Bunge and Pearce, 2003). Therefore, enhanced migration of Schwann cells in the lesion site after inhibition of Sema3A as shown by Kaneko et al, can likely account for enhanced axonal density at the lesion epicenter. This idea is further supported by the absence of regeneration beyond the lesion site, similar to previous studies of regenerating axons after Schwann cells grafts (Xu et al., 1997; Grimpe et al., 2005). Another target of xantofulvin could be angiogenesis and the inflammatory process. We found Nrpl protein on blood vessels and on immune cells first infiltrating the lesion and then migrating outside the injured area. As these cells do not express L1, they were unlikely to be responsive to an effect of the L1 mimetic peptide even if sensitive to Sema3A.

Sema3A may not directly inhibit axon regeneration

If Sema3A directly inhibits regeneration of axons, one would expect, at least an enhancement of axonal density at the lesion site after administration of ASNKL peptide to contused spinal cord. This was not observed and correlated with the lack of improvement of the locomotor function. Although we cannot rule out a misexpression of L1 and Nrpl on axons *in vivo*, our *ex vivo* experiments demonstrate that ASNKL peptide addition is not sufficient to drive L1/Nrpl positive axons to overcome inhibitory factors in the injured spinal cord. In addition, we show that recombinant Sema3A is unable to reduce the length of neurites growing on spinal cord sections while it does when neurons are cultured on a permissive substrate. This indicates that the inhibitory adult spinal cord environment does not allow detection of additional inhibition mediated by Sema3A. Finally, it is essential to appreciate other interactions of Sema3A that may contribute to its complex activity *in vivo*. Sema3A although a secreted protein, is associated with extracellular matrix components such as chondroitin-sulfate proteoglycans (CSPGs) and heparin-sulfate proteoglycans (HSPGs) (De Wit et al., 2005), and treatment with Heparinase III potentiates its effect on growth cone collapse. Thus, it is possible that especially in the context of the injured adult spinal cord, a number of extracellular matrix components block the effect of Sema3A on axonal growth. Alternatively, lack of effect of the Sema3A blocking peptide could be due to additional factors modulating the intrinsic signaling state of the neurons, such as the intracellular levels of cyclic nucleotides. Indeed, levels of cAMP and cGMP are known to modulate the response of growth cones to extracellular cues such as Sema3A and MAG (Song et al., 1997). In the same way, neurotrophins such as nerve growth factor (NGF) have the ability to enhance regenerative capacities of axons (Chaudhry et al., 2006) and in this context, Sema3A is able to restrict NGF-induced axon growth (Tang et al., 2007). This suggests that in combination with growth promoting strategies, ASNKL treatment might be able to promote axon regeneration. Whatever the mechanisms at play, the injured spinal cord environment is able to modulate the ability of neurons to interpret extracellular cues. Although we have shown that both adult and embryonic neurons do not respond to ASNKL peptide within this environmental setting, the mechanisms of the environmental influence remain unknown.

We propose that pharmacological blockade of Sema3A alone promotes a regenerative response after spinal cord injury by regulating cell migration, neuroprotection and/or inflammation rather than by direct inhibition of axon regeneration, as preventing only its actions on neurons with a L1 mimetic peptide is not sufficient to stimulate regeneration or promote functional recovery.

MATERIALS and METHODS

Animals and spinal cord injury

For this study, we used a total of 42 female C57/B16J mice aged approximately 10 weeks. The mice were anesthetized with a cocktail of ketamine (10mg/kg) and xylazine (80mg/kg) and

then received a spinal contusion at the vertebral level of T₉ after laminectomy as described previously (Jakeman et al., 2000; Ma et al., 2001). Using an electro-magnetic device (OSU device), the closed dura on the dorsal surface of the spinal cord was rapidly displaced 0.5 mm and the impactor retracted in a rapid manner, the entire epoch occurring in 20 msec. Postoperative care included supplemental saline and prophylactic antibiotic to prevent bladder infection. Manual bladder expression was performed twice daily. No animals were lost prior to the end of the study.

Three groups of 6 mice received no treatment, phosphate buffer saline (PBS) at 3, 6, 9 and 12 day post injury (dpi), or L1 mimetic peptide in PBS at 3, 6, 9 and 12 dpi then twice per week until the time of perfusion at 6th week post-injury. For delivery, an intrathecal cannula inserted at the T13-L1 vertebral junction with the tip placed at the level of T10 one week before injury to allow the complete healing of the dura prior the injury. On the indicated dpi, the mice were anesthetized with isoflurane for infusion of 4µl of ASNKL peptide at 1mM. Mice were tested for locomotor function of the hindlimbs using Basso Mouse Scale (BMS), as described in (Basso et al., 2006), one day after cannula placement and one day prior to injury as a control. Then BMS testing was performed in a blind fashion at 1, 7, 14, 21, 28, 35, and 42 dpi. Results are presented as score $\pm$ SEM and analyzed using Anova test.

Generation of anti-Nrp1 antibodies

Site-directed anti-Nrp1 antibodies was raised using a synthetic 15 aminoacid peptide (NH₂-KKDKLNPQSNYSEA-COOH) from the Nrp1 C-terminal domain (Chen et al., 1998). A rabbit was immunized intradermally with 50ug of peptide in complete Freund's adjuvant and boosted twice with the same amount of peptide in incomplete Freund's adjuvant. Serum was collected at day 74 and antibodies were purified on an Nrp1 peptide-Sepharose column. Bound antibodies were eluted with 0.1 M glycine, pH2 and the eluant neutralized with 0.1 M Tris, pH 8. Preimmune serum served as control. We verified that these antibodies gave a positive signal on permeabilized COS7 cells transfected with a Nrp1 construct (not shown).

Tissue processing

After anesthesia, animals were perfused transcardially with PBS followed by 4% of paraformaldehyde in PBS. Spinal cords and brains were removed and postfixed 2 h, rinsed overnight in 0.2M Phosphate Buffer and transferred to 30% sucrose for cryoprotection. Sagittal or coronal sections of 16µm were taken from a block of approximately 0.5cm on a cryostat (Leica, Germany). For *in situ hybridization* in the cortex of naive mouse, 20 µm thick brain sections were cut in coronal plane.

Expression pattern study

Four mice per time point received 0.5mm displacement injury as described above and were sacrificed for each following time points: 4, 10, 14, 21, and 35dpi.

Immunohistochemistry—Sections selected from the epicenter region processed as described above were submitted to immunohistofluorescence with the following antibodies: monoclonal mouse anti Sema3A (kind gift from Dr. Barzilai, Tel Aviv, Israel (Shirvan et al., 2002)), polyclonal rabbit anti neurofilament M (Chemicon, USA), monoclonal rat anti L1 CAM (Rathjen and Schachner, 1984), monoclonal mouse anti SMI31 (Sternberger, USA), monoclonal mouse anti GFAP (Sigma, USA), polyclonal anti Fibronectin (Sigma, USA) polyclonal goat anti Nrp1 extracellular (R&D Systems, UK) and polyclonal rabbit anti Nrp1 intracellular prepared as described above (1/50), polyclonal anti GAP43 (Chemicon, USA), FITC conjugated anti CD11c, rabbit polyclonal anti PKC gamma (Santa Cruz, USA), and rabbit anti-beta tubulin III (Tuj1) (Genetex, Inc, St. Antonio TX, USA). Briefly, after permeabilisation with 0.1–0.3% Triton in PBS, tissue sections were blocked with 5% BSA in

PBS and incubated with the primary antibody overnight at 4°C. After rinsing in PBS, the sections were incubated with the appropriate conjugated (Cy3-, FITC-, TexasRed-, or biotinylated-) secondary antibody (Jackson ImmunoResearch, USA) for 1 h at room temperature. Sections were then rinsed and mounted with immunomount medium or (Tuj1) incubated in ABC *Elite* complex (Vector Labs, Burlingame, CA, USA) followed by diaminobenzidine in the presence of H₂O₂ and NiCl₂. In some cases, sections were processed for DAPI (0.5 mg/ml for 5 min) to stain nuclei. Control sections were processed as above except for the primary antibody that was omitted. Pictures were taken with a CCD camera adapted on an Axioplan 2 motorized microscope equipped with an Apotome function (Zeiss, Germany) and processed with the Adobe Photoshop software. Tuj1 measures were made on images collected with a 10x objective using a Dage 777 camera and MCID imaging program (Microcomputer Imaging Device, Imaging Research, Amersham, Inc.). Axon staining was calculated as the percentage of positive stained pixels within a defined region in the center of the lesion, as described previously (Ma et al., 2004).

In situ hybridization (ISH)—Sections from fixed brain and spinal cord were also subjected to ISH for *Sema3A*, *Nrp1* and *ChAT*. In brief, DIG (Roche, USA) labeled probes were obtained by *in vitro* reverse transcription with T3 polymerase (Promega, USA) using linearized cDNA templates (gift from Dr. Mann, Marseilles, France (Cohen et al., 2005)). After postfixation and acetylation, sections were prehybridized in hybridization solution for 2 h at 70°C and hybridized with 100 ng/ml of RNA probe at 70°C overnight. Stringent washes followed by a blocking step of 1 h were performed before overnight incubation with alkaline phosphatase conjugated anti DIG antibody 1/5000 (Roche, USA) at room temperature. Alkaline phosphatase was revealed using NBT/BCIP (Promega, USA).

Cell culture

Production of conditioned media—Alkaline phosphatase (AP) or full-length *Sema3A*-AP cDNA plasmids were transfected in HEK T293 cells with the lipofectamine technique (Invitrogen), supernatants were collected 48 h after transfection and centrifuged at 1500 rpm for 5min. Supernatants were dosed for AP activity and concentrated if necessary.

Neurite outgrowth assay—Embryonic cortical neurons (E13.5 to E17.5) were cultured on poly-D-Lysine/Laminin substrate for 48 h in Neurobasal Medium complemented with Glutamax and B27 (Invitrogen). *Sema3A* (10 to 20 nM) or OptiMEM (control) was added to the medium with or without ASNKL peptide at 10⁻⁶M. Neurons were fixed with 2% PFA diluted in PBS and stained for neurofilament SMI-31 1/1000 (Sternberger, USA). The longest neurite of each cell was measured with the Neuron J software (NIH). Results are presented as mean values of 4 independent experiments.

Cryoculture assay—Dissociated cortical neurons from E14.5 embryos were cultured on adult spinal cord cryosections as described previously (Golding et al., 1996). Briefly, fresh spinal cord from naive or injured mice taken at one, two and four weeks after injury were frozen in liquid nitrogen, then cut in 10 µm thick sections with a cryostat and mounted on poly-L-lysine coated coverslips. Sections were kept at -80°C until use. Cortical neurons were grown for 24 h on cryosections in Neurobasal Medium complemented with Glutamax and B27 (Invitrogen) in the presence or absence of ASNKL peptide at 10⁻⁶M and for some experiments *Sema3A*-AP at a final concentration of 20 nM. The longest neurite of each cell was measured and percentages of cells with neurites as well as the cell density were determined with the Image J software (NIH). Results are presented as mean values of at least 5 independent experiments with mean SEM and were analyzed using one way Anova.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work is sponsored by the CNRS and grants from AFM (Association Française contre les Myopathies), ARSEP (Association de Recherche contre la Sclérose en Plaque) and IRME (Institut de Recherche sur la Moelle Epinière) to GR and NINDS R0143246 and P30 NS-045758 (LJ). EM is supported by a MENRT fellowship. We acknowledge the use of the “RIO mouse exploratory function platform” CIML-IBDM and are grateful to Drs Barzilai and F. Mann for the gift of reagents. We would like to thank all the Rougon’s group and Pharmaxon members for their help and discussions and Qin Feng Yin, Zhen Guan and Patricia Walters for technical assistance.

References

- Agudo M, Robinson M, Cafferty W, Bradbury EJ, Kilkenny C, Hunt SP, McMahon SB. Regulation of neuropilin 1 by spinal cord injury in adult rats. *Mol Cell Neurosci* 2005;28:475–484. [PubMed: 15737738]
- Akopians A, Runyan SA, Phelps PE. Expression of L1 decreases during postnatal development of rat spinal cord. *J Comp Neurol* 2003;467:375–388. [PubMed: 14608600]
- Bamber NI, Li H, Lu X, Oudega M, Aebischer P, Xu XM. Neurotrophins BDNF and NT-3 promote axonal re-entry into the distal host spinal cord through Schwann cell-seeded mini-channels. *Eur J Neurosci* 2001;13:257–268. [PubMed: 11168530]
- Basso DM, Fisher LC, Anderson AJ, Jakeman LB, McTigue DM, Popovich PG. Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. *J Neurotrauma* 2006;23:635–659. [PubMed: 16689667]
- Bunge MB, Pearse DD. Transplantation strategies to promote repair of the injured spinal cord. *J Rehabil Res Dev* 2003;40:55–62. [PubMed: 15077649]
- Castellani V, Rougon G. Control of semaphorin signaling. *Curr Opin Neurobiol* 2002;12:532–541. [PubMed: 12367632]
- Castellani V, Falk J, Rougon G. Semaphorin3A-induced receptor endocytosis during axon guidance responses is mediated by L1 CAM. *Mol Cell Neurosci* 2004;26:89–100. [PubMed: 15121181]
- Castellani V, De Angelis E, Kenwrick S, Rougon G. Cis and trans interactions of L1 with neuropilin-1 control axonal responses to semaphorin 3A. *Embo J* 2002;21:6348–6357. [PubMed: 12456642]
- Castellani V, Chedotal A, Schachner M, Faivre-Sarrailh C, Rougon G. Analysis of the L1-deficient mouse phenotype reveals cross-talk between Sema3A and L1 signaling pathways in axonal guidance. *Neuron* 2000;27:237–249. [PubMed: 10985345]
- Chaudhry N, de Silva U, Smith GM. Cell adhesion molecule L1 modulates nerve-growth-factor-induced CGRP-IR fiber sprouting. *Exp Neurol* 2006;202:238–249. [PubMed: 16860320]
- Chen H, He Z, Bagri A, Tessier-Lavigne M. Semaphorin-neuropilin interactions underlying sympathetic axon responses to class III semaphorins. *Neuron* 1998;21:1283–1290. [PubMed: 9883722]
- Chen J, Wu J, Apostolova I, Skup M, Irintchev A, Kugler S, Schachner M. Adeno-associated virus-mediated L1 expression promotes functional recovery after spinal cord injury. *Brain* 2007;130:954–969. [PubMed: 17438016]
- Cohen S, Funkelstein L, Livet J, Rougon G, Henderson CE, Castellani V, Mann F. A semaphorin code defines subpopulations of spinal motor neurons during mouse development. *Eur J Neurosci* 2005;21:1767–1776. [PubMed: 15869472]
- De Winter F, Oudega M, Lankhorst AJ, Hamers FP, Blits B, Ruitenberg MJ, Pasterkamp RJ, Gispens WH, Verhaagen J. Injury-induced class 3 semaphorin expression in the rat spinal cord. *Exp Neurol* 2002;175:61–75. [PubMed: 12009760]
- de Wit J, Verhaagen J. Role of semaphorins in the adult nervous system. *Prog Neurobiol* 2003;71:249–267. [PubMed: 14687984]
- De Wit J, De Winter F, Klooster J, Verhaagen J. Semaphorin 3A displays a punctate distribution on the surface of neuronal cells and interacts with proteoglycans in the extracellular matrix. *Mol Cell Neurosci* 2005;29:40–55. [PubMed: 15866045]

- Fabes J, Anderson P, Yanez-Munoz RJ, Thrasher A, Brennan C, Bolsover S. Accumulation of the inhibitory receptor EphA4 may prevent regeneration of corticospinal tract axons following lesion. *Eur J Neurosci* 2006;23:1721–1730. [PubMed: 16623828]
- Golding JP, Shewan D, Berry M, Cohen J. An in vitro model of the rat dorsal root entry zone reveals developmental changes in the extent of sensory axon growth into the spinal cord. *Mol Cell Neurosci* 1996;7:191–203. [PubMed: 8726103]
- Grimpe B, Pressman Y, Lupa MD, Horn KP, Bunge MB, Silver J. The role of proteoglycans in Schwann cell/astrocyte interactions and in regeneration failure at PNS/CNS interfaces. *Mol Cell Neurosci* 2005;28:18–29. [PubMed: 15607938]
- Harel NY, Strittmatter SM. Can regenerating axons recapitulate developmental guidance during recovery from spinal cord injury? *Nat Rev Neurosci* 2006;7:603–616. [PubMed: 16858389]
- Hashimoto M, Ino H, Koda M, Murakami M, Yoshinaga K, Yamazaki M, Moriya H. Regulation of semaphorin 3A expression in neurons of the rat spinal cord and cerebral cortex after transection injury. *Acta Neuropathol (Berl)* 2004;107:250–256. [PubMed: 14727128]
- Itoh K, Cheng L, Kamei Y, Fushiki S, Kamiguchi H, Gutwein P, Stoeck A, Arnold B, Altevogt P, Lemmon V. Brain development in mice lacking L1-L1 homophilic adhesion. *J Cell Biol* 2004;165:145–154. [PubMed: 15067019]
- Jakeman LB, Wei P, Guan Z, Stokes BT. Brain-derived neurotrophic factor stimulates hindlimb stepping and sprouting of cholinergic fibers after spinal cord injury. *Exp Neurol* 1998;154:170–184. [PubMed: 9875278]
- Jakeman LB, Chen Y, Lucin KM, McTigue DM. Mice lacking L1 cell adhesion molecule have deficits in locomotion and exhibit enhanced corticospinal tract sprouting following mild contusion injury to the spinal cord. *Eur J Neurosci* 2006;23:1997–2011. [PubMed: 16630048]
- Jakeman LB, Guan Z, Wei P, Ponnappan R, Dzwonczyk R, Popovich PG, Stokes BT. Traumatic spinal cord injury produced by controlled contusion in mouse. *J Neurotrauma* 2000;17:299–319. [PubMed: 10776914]
- Kaneko S, Iwanami A, Nakamura M, Kishino A, Kikuchi K, Shibata S, Okano HJ, Ikegami T, Moriya A, Konishi O, Nakayama C, Kumagai K, Kimura T, Sato Y, Goshima Y, Taniguchi M, Ito M, He Z, Toyama Y, Okano H. A selective Sema3A inhibitor enhances regenerative responses and functional recovery of the injured spinal cord. *Nat Med* 2006;12:1380–1389. [PubMed: 17099709]
- Kobayashi S, Miura M, Asou H, Inoue HK, Ohye C, Uyemura K. Grafts of genetically modified fibroblasts expressing neural cell adhesion molecule L1 into transected spinal cord of adult rats. *Neurosci Lett* 1995;188:191–194. [PubMed: 7609906]
- Kruger RP, Aurandt J, Guan KL. Semaphorins command cells to move. *Nat Rev Mol Cell Biol* 2005;6:789–800. [PubMed: 16314868]
- Luo Y, Raible D, Raper JA. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* 1993;75:217–227. [PubMed: 8402908]
- Ma M, Basso DM, Walters P, Stokes BT, Jakeman LB. Behavioral and histological outcomes following graded spinal cord contusion injury in the C57Bl/6 mouse. *Exp Neurol* 2001;169:239–254. [PubMed: 11358439]
- Ma M, Wei P, Wei T, Ransohoff RM, Jakeman LB. Enhanced axonal growth into a spinal cord contusion injury site in a strain of mouse (129X1/SvJ) with a diminished inflammatory response. *J Comp Neurol* 2004;474:469–486. [PubMed: 15174067]
- Manitt C, Wang D, Kennedy TE, Howland DR. Positioned to inhibit: netrin-1 and netrin receptor expression after spinal cord injury. *J Neurosci Res* 2006;84:1808–1820. [PubMed: 16998900]
- Mann F, Chauvet S, Rougon G. Semaphorins in development and adult brain: Implication for neurological diseases. *Prog Neurobiol* 2007;82:57–79. [PubMed: 17537564]
- Mohajeri MH, Bartsch U, van der Putten H, Sansig G, Mucke L, Schachner M. Neurite outgrowth on non-permissive substrates in vitro is enhanced by ectopic expression of the neural adhesion molecule L1 by mouse astrocytes. *Eur J Neurosci* 1996;8:1085–1097. [PubMed: 8752578]
- Molyneaux BJ, Arlotta P, Hirata T, Hibi M, Macklis JD. Fezl is required for the birth and specification of corticospinal motor neurons. *Neuron* 2005;47:817–831. [PubMed: 16157277]
- Pasterkamp RJ, Verhaagen J. Emerging roles for semaphorins in neural regeneration. *Brain Res Brain Res Rev* 2001;35:36–54. [PubMed: 11245885]

- Rathjen FG, Schachner M. Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. *Embo J* 1984;3:1–10. [PubMed: 6368220]
- Roonprapunt C, Huang W, Grill R, Friedlander D, Grumet M, Chen S, Schachner M, Young W. Soluble cell adhesion molecule L1-Fc promotes locomotor recovery in rats after spinal cord injury. *J Neurotrauma* 2003;20:871–882. [PubMed: 14577865]
- Rosenzweig ES, McDonald JW. Rodent models for treatment of spinal cord injury: research trends and progress toward useful repair. *Curr Opin Neurol* 2004;17:121–131. [PubMed: 15021237]
- Runyan SA, Roy R, Zhong H, Phelps PE. L1 CAM expression in the superficial dorsal horn is derived from the dorsal root ganglion. *J Comp Neurol* 2005;485:267–279. [PubMed: 15803510]
- Schwab ME, Bartholdi D. Degeneration and regeneration of axons in the lesioned spinal cord. *Physiol Rev* 1996;76:319–370. [PubMed: 8618960]
- Shirvan A, Kimron M, Holdengreber V, Ziv I, Ben-Shaul Y, Melamed S, Melamed E, Barzilai A, Solomon AS. Anti-semaphorin 3A antibodies rescue retinal ganglion cells from cell death following optic nerve axotomy. *J Biol Chem* 2002;277:49799–49807. [PubMed: 12376549]
- Silver J, Miller JH. Regeneration beyond the glial scar. *Nat Rev Neurosci* 2004;5:146–156. [PubMed: 14735117]
- Song HJ, Ming GL, Poo MM. cAMP-induced switching in turning direction of nerve growth cones. *Nature* 1997;388:275–279. [PubMed: 9230436]
- Tang XQ, Heron P, Mashburn C, Smith GM. Targeting sensory axon regeneration in adult spinal cord. *J Neurosci* 2007;27:6068–6078. [PubMed: 17537979]
- Tran TS, Kolodkin AL, Bharadwaj R. Semaphorin Regulation of Cellular Morphology. *Annu Rev Cell Dev Biol*. 2007
- Wehrle R, Camand E, Chedotal A, Sotelo C, Dusart I. Expression of netrin-1, slit-1 and slit-3 but not of slit-2 after cerebellar and spinal cord lesions. *Eur J Neurosci* 2005;22:2134–2144. [PubMed: 16262652]
- Xu XM, Chen A, Guenard V, Kleitman N, Bunge MB. Bridging Schwann cell transplants promote axonal regeneration from both the rostral and caudal stumps of transected adult rat spinal cord. *J Neurocytol* 1997;26:1–16. [PubMed: 9154524]
- Yiu G, He Z. Glial inhibition of CNS axon regeneration. *Nat Rev Neurosci* 2006;7:617–627. [PubMed: 16858390]

Figure 1. ASNKL peptide blocks inhibition of axon growth and growth cone collapse mediated by Sema3A

Embryonic cortical neurons were cultured for 48 h in the presence of AP conditioned medium (A, C) or Sema3A-AP (B, D) from HEK transfected cells with (C, D) or without (A, B) ASNKL peptide at 10^{-6} M and stained with anti SMI-31. Cell bodies are at the bottom of the picture for each condition. Scale bar 40 μ m. (E) Quantification of Sema3A effects in the presence or absence of ASNKL peptide. Data are presented as mean $\pm$ SEM normalized to values obtained in AP conditions. Sema3A reduces by 30% neurite length in control conditions while it has no effect in the presence of ASNKL peptide. Photomicrographs of growth cones stained with Tuj1 in the presence of control medium (F), Sema3A (G) with ASNKL peptide (H) at 10–

6 M. Scale bar 5 μ m. (J) Quantification of complex growth cones for each condition. Results are presented as mean $\pm$ SEM from 3 independent experiments. Sema3A increases the percentage of collapsed growth cones and ASNKL peptide prevents this effect.

Figure 2. Sema3A expression at the lesion site after spinal cord injury

Diagrams illustrating parasagittal section plane (A) and representing a sagittal section (B) of the spinal cord as illustrated in A. Box corresponds to the area where the photomicrographs were taken. Immunohistofluorescence of GFAP (red), fibronectin (green) (C, E, G) localizing the lesion scar and of Sema3A (red) (D, F, H), with nuclei in blue, in the injured spinal cord. Arrows point to examples of Sema3A positive cells profiles in the scar, which is most prominent at later time points at the edges of the lesion site. Arrowheads indicate isolated cells in the lesion core shown at higher magnification in insets. Asterisks indicate the lesion core. Scale bars 18 μ m.

Figure 3. Expression of L1 and Nrp1 in the naïve spinal cord

(A) Diagram illustrating a section through the spinal cord in the coronal plane. L1 protein (B, C, D) is expressed in the grey matter (dh) and white matter (dlf). Low levels are detected in the df. Nrp1 protein is detected in the dh, at lower levels in the dlf and the basal df (cst) with antibodies raised against the intracellular domain (E, F, G). Antibodies raised against the extracellular domain of Nrp1 allow detection only in the dh and at lower levels in the dlf (H, I, J). Signal is also evident in blood vessels (arrowheads). Double immunostaining for L1 (green) and Nrp1 (red) shows coexpression at in the dlf and the dh (K, L, M). Scale bar 100 μ m. cst, corticospinal tract, df, dorsal funiculus, dlf, dorsolateral funiculus, dh dorsal horn, vf, ventral funiculus, vh, ventral horn.

Figure 4. L1 positive fibers sprout into the lesion site

Diagrams illustrating parasagittal section plane (A) and representing a sagittal section (B) of the spinal cord as illustrated in A. Box corresponds to the area where the photomicrographs were taken. Double immunohistochemistry for L1 (green) and SMI31 (red) on sagittal sections of mouse spinal cord at 0 (C, D, E), 4 (F, G, H) and 14 (I, J, K) dpi. Arrows indicate L1 positive fibers around and into the lesion site. (L, M, N). Example of fibers double stained for L1 (green) and GAP-43 (red) in the lesion site at 14dpi. Arrows indicate L1 positive fibers into the lesion site. Asterisks indicates the lesion core. Scale bars 50 μ m.

Figure 5. Immune cells in the spinal cord express Nrp1 after lesion

Diagrams illustrating parasagittal section plane (A) and representing a sagittal section (B) of the spinal cord as illustrated in A. Box corresponds to the area where the photomicrographs were taken except for panel C which was taken in the lesion epicenter. Nrp1 is detected on immune cells (arrows) in the spinal cord from 4 to 35 dpi (C, D, E) (insets are higher magnifications of arrowed cells). Example of a double staining (F, G, H) with CD11c (green) illustrating Nrp1 (red) expression on dendritic cells. Double staining (I, J, K) of Nrp1 (red) and L1 (green) shows that these cells do not express L1 (arrows). Scale bars 18 μ m.

Figure 6. Mice treated with ASNKL L1 mimetic peptide do not show improvement of motor function and do not have enhanced axonal density at the lesion site

(A) Mice treated with ASNKL (●) do not show improved motor recovery compared to non treated (□) or PBS treated (▲) mice in the BMS for locomotion. Results are presented as mean values of 6 tested mice per group $\pm$ SEM. (B) analysis of BMS subscores does not reveal significant differences between the three tested groups. (C, D) Photomicrographs and analysis of axonal density within the lesion center at 6 weeks after injury. Immunostaining with Tuj1 antibodies in the section corresponding to the lesion epicenter. Dense axonal staining is evident in the periphery, with very few axons present in the center of the lesion. Scale bar 100 μ m. (E) Analysis of axonal density in the lesion epicenter section. Positive staining density within a 1 mm² sample box in the lesion center did not differ between the three treatment groups. Symbols represent mean $\pm$ SEM (1 way ANOVA; $f=2.064$; $p=0.162$; $df=2,15$; $n=6$ /group). (F) Analysis of axonal density through the entire lesion in sections sampled at 200

μm intervals centered on the epicenter. Positive staining within the lesion was inversely related to the distance from the epicenter, with no difference in axonal density between treatment groups. Symbols represent mean $\pm$ SEM (2 way ANOVA with repeated measures; Distance $p < 0.001$; $f, df = 18.59, 6$; Treatment $p = 0.470$; $f, df = 0.80, 2$).

Figure 7. ASNKL peptide fails to promote neurite growth on spinal cord cryosections

Examples of embryonic neurons stained for Tuj1 (red) grown on naïve (A) or injured (B) adult spinal cord sections with the lesion site localized by strong laminin staining (blue). Scale bars 30µm.

Quantification of % cells with neurites (C, D) and neurite length (E, F) on naïve (NI, n=6) and injured (7dpi, n=6; 14dpi, n=5; 28dpi, n=6) spinal cord sections. The two parameters were significantly lower on injured spinal cord sections whatever the time after injury compared to naïve spinal cord (1way ANOVA, $p < 0.0001$). ASNKL peptide treatment does not significantly increase the number of cells with neurites (D) or their length (F) (NI, n=3; NI+ASNKL, n=3; injured, n=5; injured+ASNKL, n=6). (2way ANOVA, lesion $p < 0.0001$, treatment $p = 0.308$,

interaction $p=0.794$ for percentage of cells with neurites, 2way ANOVA, lesion $p<0.0001$, treatment $p=0.594$, interaction $p=0.731$ for neurite length). Results are presented as mean $\pm$ SEM, normalized to control (NI) values for neurite length.

Figure 8. Sema3A-mediated inhibition of axon growth cannot be detected on adult spinal cord cryosections

(A–D) photomicrographs of neurons stained with Tuj1 antibodies growing on poly-d-lysine/laminin (A, C) or spinal cord sections (B, D) in the presence (C, D) or absence (A, B) of exogenously added Sema3A. Scale bar 30µm. (E) Sema3A reduces neurite length on PDL/laminin (student t test $p < 0.001$) while it has no effect on neurites growing on spinal cord sections (student t test $p = 0.44$). Results are presented as mean $\pm$ SEM of values normalized to control conditions.