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Expression Profile and Role of EphrinA1 Ligand After Spinal Cord Injury

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Abstract

Spinal cord injury (SCI) triggers the re-expression of inhibitory molecules present in early stages of development, contributing to prevention of axonal regeneration. Upregulation of EphA receptor tyrosine kinases after injury suggest their involvement in the nervous system's response to damage. However, the expression profile of their ephrinA ligands after SCI is unclear. In this study, we determined the expression of ephrinA ligands after contusive SCI. Adult Sprague-Dawley female rats were injured using the MASCIS impactor device at the T10 vertebrae, and levels of ephrinA mRNA and protein determined at different time points. Identification of the cell phenotype expressing the ephrin ligand and colocalization with Eph receptors was performed with immunohistochemistry and confocal microscopy. Behavioral studies were made, after blocking ephrinA1 expression with antisense (AS) oligonucleotides, to assess hindlimb locomotor activity. Real-time PCR demonstrated basal mRNA levels of ephrin (A1, A2, A3, and A5) in the adult spinal cord. Interestingly, ephrinA1 was the only ligand whose mRNA levels were significantly altered after SCI. Although ephrinA1 mRNA levels increased after 2 weeks and remain elevated, we did not observe this pattern at the protein level as revealed by western blot analysis. Immunohistochemical studies showed ephrinA1 expression in reactive astrocytes, axons, and neurons and also their colocalization with EphA4 and A7 receptors. Behavioral studies revealed

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worsening of locomotor activity when ephrinA1 expression was reduced. This study suggests that ephrinA1 ligands play a role in the pathophysiology of SCI.

Keywords

Axonal outgrowth; EphA receptors; Inhibitory proteins; Trauma; Regeneration; Reactive astrocytes

Introduction

Spinal cord injury (SCI) triggers a complex cascade of events that include the infiltration of macrophages, inflammation, development of a glial scar, loss of myelin, cell death, and upregulation of nonpermissive molecules for axonal outgrowth (Fitch and Silver 1997; Fawcett and Asher 1999; Domeniconi et al. 2002; Niclou et al. 2006). Altogether, these events may contribute to the limited regeneration observed after trauma to the central nervous system (CNS). Several factors have been involved in the lack of axonal elongation observed after SCI. Among these are the limited amount of trophic factors, the loss of myelin, and the increase of inhibitory factors. Growth factors such as neurotrophins and glial cell line-derived neurotrophic factor (GDNF) have shown an increase in mRNA levels after an injury, but not significant when compared to levels seen after injury in the peripheral nervous system (Widenfalk et al. 2001). The loss of myelin is associated with most well-known inhibitory molecules such as Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) (McKerracher et al. 1994; Chen et al. 2000; Grandpre and Strittmatter 2001; Wang et al. 2002). Interestingly, these proteins bind to the Nogo receptor (NgR) (Domeniconi et al. 2002; Liu et al. 2002; Wang et al. 2002), which produces growth inhibition via a membrane complex that includes the neurotrophic receptor $p75^{NTR}$ (Wang et al. 2002). Unfortunately, deletion and blockade of these inhibitory targets results in only modest recovery of locomotor behavior and axonal regeneration (Teng and Tang 2005; Zheng et al. 2005). Moreover, targeted deletion of glial scar molecules such as NG2, did not demonstrate a complete axonal regeneration or infiltration of serotonergic efferent fibers into the scar tissue (de Castro et al. 2005). These findings suggest the involvement of additional inhibitory factors that could hinder axonal regeneration after trauma to the CNS.

Additional repulsive molecules that are expressed during CNS developmental stages include the semaphorins, netrins, and Eph receptors, all of which are highly conserved molecules among species. This last group of proteins is known to be upregulated after SCI (Irizarry-Ramirez et al. 2005; Cruz-Orengo et al. 2006; Figueroa et al. 2006). The Eph receptors are the largest identified subfamily of receptor tyrosine kinases and are classified into two groups: EphA and EphB, depending on which ephrin ligand activates them (Eph Nomenclature Committee 1997). In general, EphA receptors bind ephrinA molecules whereas the EphB receptors bind ephrinB molecules; however, one member of each class, EphA4 and EphB2, can be activated by both classes of ephrins (Himanen and Nikolov 2003) and within each group, although affinities vary, there is promiscuity. The ephrins are separated into two groups based on how they are attached to the cell membrane (Flanagan and Vanderhaeghen 1998). The ephrinA ligands (A1–A6) have a glycosyl-phosphatidylinositol (GPI)-anchor at their carboxyl terminal, and the ephrinB ligands (B1–B3) have a single transmembrane domain and a highly conserved carboxy-terminal tail.

EphA–ephrinA signaling mediates contact repulsion resulting in growth cone collapse. Interaction between Eph receptors and ephrin ligands elicit a cascade of events known as forward (a cascade of intracellular events in the cell with the Eph receptors) and reverse

signaling (a cascade of intracellular events initiated in the cell with the ephrin ligand), respectively. These events have been implicated in cell migration, fasciculation, and axonal outgrowth mainly by a repellent mechanism (Cowan and Henkemeyer 2002). In the developing CNS, Eph–ephrin interaction is associated primarily with a contact-dependent repulsive effect on axonal outgrowth.

Diversified roles for ephrin ligands have been documented through their involvement in mechanisms such as angiogenesis (Pandey et al. 1995), neurotrophic effects on sympathetic neurons (Gao et al. 2000), and neuronal outgrowth support (Zhou et al. 2001), among others. These data portray ephrin ligands as permissive molecules, therefore, the possibility that the expression of these molecules after SCI may not result in an inhibitory environment should not be discarded.

Since EphA receptors are upregulated after trauma (Willson et al. 2002; Du et al. 2007) and some of them are implicated in the pathophysiology of SCI (Goldshmit et al. 2004, 2006; Cruz-Orengo et al. 2006; Figueroa et al. 2006; Fabes et al. 2007), we evaluated the expression profile of ephrinA ligands after spinal cord lesion in adult rats at the mRNA and protein levels. Here, we determined the role of ephrinA1 ligand after SCI using antisense (AS) technology and monitoring functional locomotor recovery. The results obtained suggest that ephrinA1 expressed after SCI may play a different role than previously described during developmental stages. Contrary to repellent activities, the data of this project propose that ephrinA1 may affect neurite outgrowth, synapse formation or cell survival.

Methods

General Procedures

Experiments were performed using female Sprague-Daw-ley rats (Hilltop Lab. Scottdale, PA) (180–200 g). After being anesthetized with an intraperitoneal cocktail of ketamine (Fort Dodge Animal Health, Fort Dodge, IA, USA; 87.7 mg/kg), xylazine (Boehinger Ingelheim, Ridgefield, CT, USA; 4.2 mg/kg), and acepromazine (0.85 mg/kg), the animals received a laminectomy to remove the dorsal lamina of the T10 vertebrae. One group of rats received a moderate contusion by dropping a 10 g weight from a height of 12.5 mm over the exposed spinal cord, using the MASCIS Impactor device (Gruner 1992; Miranda et al. 1999). Sham rats received only the laminectomy. Post operative treatments given to the rats include bladder expression up to three times daily for the next 7 days post operation (DPO) or until micturition reflex was recovered. Antibiotic (Cefazolin; Bristol Myers Squibb, NY, USA; 25 mg/kg) and analgesic (Buprenex®; Reckett & Colman Pharmaceuticals, Inc. Richmond, VA, USA; 0.05 mg/kg) was administered twice daily for the next 7 DPO and the next 3 DPO, respectively (Santiago et al. 2009). Rats were killed at 2, 4, 7, 14, and 28 days post injury (DPI). All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at University of Puerto Rico School of Medicine, and adhere to the guidelines established by the National Institutes of Health.

Real-Time PCR (RT-PCR)

The mRNA levels of ephrinA ligands were determined with real-time RT-PCR. Animals were deeply anesthetized with pentobarbital (40–50 mg/kg) and perfused intracardially with 300 ml of 0.01 M phosphate buffered saline (PBS: Dulbecco's phosphate buffered saline, Sigma-Aldrich, St. Louis, MO, USA; pH 7.4) at 4°C. The 5 mm lesion epicenter was removed, followed by RNA extraction using Trizol (Invitrogen Co., Carlsbad, CA, USA) and RNA cleanup with DNAse I (Ambion Inc., Austin, TX, USA) according to the manufacturer instructions. The concentration of RNA was determined by absorbance at 260

nm and the integrity verified by electrophoresis using ethidium bromide staining. Reverse transcription reaction was performed using random hexamers (Promega Co., Madison, WI, USA), dNTP mix (Invitrogen Co.), 5× First Strand Buffer, 0.1 M DTT, and SuperScript II (Invitrogen Co.). RT-PCR amplification was carried out on the 96-well plate loaded with the cDNA and a cocktail of reverse and forward specific primers (Table 1) for ephrinA1, A2, A3, and A5 (Integrated DNA Technologies Inc., Coralville, Iowa, USA), Nuclease free water (Ambion Inc.) and SYBR Green (P/N 4309155; Applied Biosystems, Foster City, CA, USA) to monitor double stranded DNA as previously published (Figueroa et al. 2006). The amplification reaction was carried out in the Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA, USA) with a hot start of 3 min, and then each cycle consisted of 30 s at 94°C for denaturation, 30 s at the annealing temperature (T_m: Table 1), and 60 s at 72°C for extension, with an additional extension period of 5 min. Each gene was analyzed in triplicates ($n = 3$) and each sample was run in duplicates. The product of the PCR reaction was analyzed by electrophoresis in a 2% agarose gel, and fold change analysis standardized to the levels of GAPDH as reported previously (Figueroa et al. 2006). PCR products were purified using QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA, USA) and the identities of the amplified DNA fragments were verified by sequencing (ABI Prism 310 Applied Biosystems), confirming the specificity of the primers used.

Immunohistochemistry (IHC)

The spatial localization of ephrinA1 ligand was performed with IHC assays. Rats were deeply anesthetized and perfused at 2, 4, 7, 14, and 28 DPI ($n = 3$) with 300 ml of PBS at 4°C, followed by ice-cold paraformaldehyde (PFA, Fluka Chemika, Buchs, Switzerland) solution (4% in 0.1 MPBS). The spinal cord was removed and postfixed in 4% PFA/PBS at 4°C for 3 additional hours and cryoprotected by immersion in 30% sucrose, 0.1 M PBS at 4°C. Segments of the spinal cord (approximately 1.5 cm in length) were embedded in Tissue-Tek O.C.T. (Miles Inc., Ekhart, IN, USA), sectioned with a cryostat (Leica Cryostat CM1800; Nussloch, Germany) at 20 μm and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburg, PA, USA).

Double-labeling studies were performed as previously published (Cruz-Orengo et al. 2006; Figueroa et al. 2006). Briefly, the sections were post fixed, washed, and blocked with 3% Bovine Serum Albumin (BSA: Sigma-Aldrich). Then the sections were incubated with mouse anti-GFAP (1:100, Chemicon International Inc, Temecula, CA, USA), mouse anti-NeuN (1:200, Chemicon International Inc), mouse anti-ED1 (1:500, Serotec, Raleigh, NC, USA), mouse anti-NF-H (1:1000, Chemicon International Inc.), mouse anti-MAG (1:250, Chemicon International Inc, Temecula, CA, USA), and rabbit anti-ephrinA1 antibody (1:200 [sc-911], Santa Cruz Biotechnology, Santa Cruz, CA, USA) to identify reactive astrocytes, motorneurons, macrophages, axons or myelin, respectively. For the double-labeling assay related to Eph receptors, anti m-EphA4 (3 μg/μl), and anti m-EphA7 (5 μg/μl) (R&D Systems, Minneapolis, MN, USA) were used as standardized by Rosas et al. (2010). After a 24 h incubation at 4°C with the primary antibodies and three washes with PBS, donkey anti-rabbit Rhodamine (1:200, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), donkey anti-mouse Alexa (1:250), and donkey anti-goat Alexa (1:200, Invitrogen Detection Technologies, Eugene, OR, USA) were applied to the sections for 2 h at room temperature. The sections were washed and coverslipped with Slowfade Antifade Kit (Invitrogen Detection Technologies). Qualitative analysis was performed with Zeiss LSM5 Pascal confocal microscope systems (Carl Zeiss Microimaging, Peabody, MA, USA).

Western Blot

The temporal protein expression profile after SCI was determined through the use of western blots. Tissue from spinal cord (T10) segments (5 mm) of sham or injured rats were

homogenized in cold Tris lysis buffer (20 mM Tris, 150 mM NaCl, 5 mM NaF, 1 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin, 2 μg/ml antipain, 5 mM benzamidine, 1 mM DTT, 10 μg/ml leupeptin, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml trypsin inhibitors; pH 8) to prepare protein lysates, as reported previously (Cruz-Orengo et al. 2007). The homogenate was centrifuged for 90 min at 20,817×*g;* the obtained supernatant was collected and resuspended in ice-cold Tris lysis buffer with 1% of Nonident P-40 (NP-40) detergent for 45 min at 4°C. Afterwards, the mixture was centrifuged for 10 min (20,817×*g*) at 4°C and the resulting supernatant was transferred to a new cold (4°C) microtube. Analysis of protein concentration was performed using the Bio-Rad Protein Assay (Bio-Rad Laboratories) protocol, as suggested by the manufacturer's instructions. Membrane extracted proteins (30 μg) were analyzed in a 7.5% polyacrylamide-SDS gel, and electroblotted to a nitrocellulose membrane for 90 min following these parameters: 50 V, 300 mA, and 10 W. The nitrocellulose membrane was blocked with Blotto (3% milk, 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 2 h at room temperature. The membrane was probed with ephrinA1 antibody (1:400 [sc-20719], Santa Cruz Biotechnology) for detection in blocking solution and incubated overnight at 4°C. HRP-conjugated anti-rabbit IgG (1:10,000; Sigma) was used as secondary antibody and enhanced with SuperSignal West Dura extended version for 1 min (Pierce, Rockford, IL, USA) before exposure following manufacturer's instructions. Development and densitometric analysis methods of the nitrocellulose membranes were performed in the Versadoc™ Imaging System and Quantity One Software (Bio-Rad Laboratories). Levels of ephrinA1 protein were standardized to the amount of immunodetected GAPDH (1:5000; SIGMA-Aldrich) to monitor equal amount of cell extract at each time point studied. Specificity of the antibody was demonstrated by pre-absorbing it with 4 μg of the peptide used to generate the antibody.

Intrathecal Infusion

In order to infuse the AS oligonucleotides, procedure was done as described by Figueroa et al. (2006). We performed another laminectomy at the T12 level after the contusion lesion at the T10. A small incision at the dura mater was made using a 32-gauge needle allowing the catheter to be inserted and extended rostrally to the lesion epicenter. AS oligonucleotides were infused with Alzet® mini-osmotic pumps Model 2004, Alzet® Osmotic Pumps, Cupertino, CA, USA) connected to a 28 g rat intrathecal catheter (internal diameter = 0.18 mm; outside diameter = 0.36 mm; Alzet® Osmotic Pumps). A subcutaneous pocket was made towards the caudal region to place the mini-osmotic pump with the catheter towards the incision. Pumps were primed overnight at 37°C to dispense 2.5 or 5 nanomoles (nmol) of the ephrinA1 AS or scrambled control oligonucleotide sequence (SCR). Oligonucleotides of either sequence were infused at a constant rate of 0.25 μl/h for 28 days after the surgery.

Oligonucleotide Design

EphrinA1 AS oligonucleotide (5′-AAAGGAACUCCAUAGCGCGGGGCCG-3′) was designed in accordance to the sequence reported for ephrinA1 (NM_053599) in the National Center for Biotechnology Information (NCBI) database. This 25 nucleotides (nt's) long AS oligonucleotide was designed to be complementary to the ephrinA1 template from position 51–75, in such a way that the sequence of the AS that is complementary to the ATG initiation codon (position 63–65) was positioned in the middle of AS oligonucleotide. The rest of the nt's comprehending the whole AS sequence were complementary to upstream and downstream areas to the initiation codon with 12 and 10 nt's assigned for each area, respectively. On the other hand, the SCR control sequence (5′-GGCCGCGGUCCCGUCGACUUCUAUA-3′) was designed so that it had the same G+C content as the ephrinA1 AS but, taking into account that as analyzed by BLAST the sequence did not was even partially complementary to any other mRNA sequences related to the spinal cord of the rat. In order to enhance the half-life of both oligonucleotides (AS and

SCR), specific 2′-O-methylations on both ends of the oligonucleotides (5 nt's on each end) and phosphorothioate modifications to the remaining nt's located in the center region (15 nt's total) were requested upon designing (Integrated DNA Technologies Inc., Coralville, IA, USA). Reduction in ephrinA1 protein expression, due to intrathecal infusion of AS oligonucleotides, was monitored by western blot and densitometric analysis, as detailed before.

Behavioral Assessment

Hindlimb locomotion was evaluated using Basso–Beattie–Bresnahan (BBB) Open Field Locomotor scale and grid-walking tests (Behrmann et al. 1992; Basso et al. 1995; Merkler et al. 2001). Previously trained rats infused intrathecally with either a control (scrambled sequence) or ephrinA1 AS oligonucleotides, were evaluated in an open field at 7, 14, 21, and 28 days after surgery. Treatment for the different groups was unknown to the two evaluators who observed the animals during a period of 4 min (movement of the hindlimbs, weight support, coordination, etc.). Animals with an optimal locomotor behavior were given a score of 21, while lower scores were given to those animals that presented any level of locomotor dysfunction. Both hindlimbs were scored separately and the average was reported as the animal's score for each of the specific time points. In the grid-walking test (Merkler et al. 2001; Cruz-Orengo et al. 2006; Figueroa et al. 2006; Santiago et al. 2009), the animals walked over a ladder horizontally placed (15 inches from the ground) with bars randomly distanced. Blinded evaluators counted the number of errors (hindlimb slide between the bars) for each leg and the average was reported as the animal's score for each specific time point.

Data Analysis

The data were expressed as the mean ± standard error of the mean (SEM). To compare differences among samples, statistical analyses were performed by ANOVA followed by post hoc tests. In Stat version 3.0 software (GraphPad Software Inc., San Diego, CA, USA) was used to analyze the data and the differences were considered significant if $P < 0.05$.

Results

Ephrin mRNA Studies in the Adult Spinal Cord and After SCI

RT-PCR experiments were performed to determine the presence of ephrinA1, A2, A3, and A5 ligands in the adult spinal cord. Figure 1 (top panel) showed that several ephrinA ligands are expressed basally in the spinal cord of adult rats. PCR products were analyzed using a 2% agarose gel electrophoresis in order to evaluate amplicon specificity for ephrinA1 (149 bp), ephrinA2 (123 bp), ephrinA3 (118 bp), and ephrinA5 (117 bp). Sequencing of the purified PCR products confirmed the identity of these ephrin transcripts in the adult spinal cord. We could not detect ephrinA4 mRNA in the adult spinal cord.

The expression profile of ephrinA1 mRNAs after SCI was analyzed using RT-PCR. The PCR amplification curve showed the exponential increase of the product and the cycle number at which the amplification of the cDNA segment began, and the melting curve demonstrated the absence of primer dimer, uncontaminated samples and the specificity of the primer (data not shown). Quantitative analysis demonstrates a differential pattern of expression for the studied ephrinA ligands (Fig. 1; lower panels). The mRNA levels of ephrinA1 transcripts decreased by 5.49-folds and 4.65-folds at 2 and 4 DPI, respectively. By 7 DPI, the mRNA levels of ephrinA1 returned to basal levels. However, the mRNA increased at 14 and 28 DPI by 4.60-folds and 5.74-folds, respectively (ANOVA, followed by Tukey–Kramer post hoc test; $*P < 0.05$, $n = 3$, duplicates per group). Although the

mRNA profile of the other ephrinA ligands (A2, A3, and A5) showed a similar pattern when compared to ephrinA1, the changes were found not significant (Fig. 1; lower panels).

Cell Phenotypes Expressing EphrinA1 in the Injured Spinal Cord

Since ephrinA1 ligand was modulated by injury, the cell phenotype expressing this ligand was determined in the spinal cord. IHC studies and confocal microscopy analyses showed the localization of ephrinA1 molecules in several cells of the spinal cord ($n = 3$) as early as 2 DPI that remained until 28 DPI. Double-labeling assays revealed the colocalization of this protein with reactive astrocytes in the white matter. Figure 2 shows the presence of ephrinA1 (Fig. 2a) in GFAP-positive astrocytes (Fig. 2b) in the rostral region of the contused spinal cord as demonstrated by colocalization analysis of fluorochrome signals (Fig. 2c), as early as 2 DPI. The immunoreactive cells were also observed at the lesion epicenter and in regions caudal to it, that could be observed until 28 DPI (data not shown). Localization of ephrinA1 immunoreactivity (Fig. 2d, g) was also seen in axons (neurofilaments) (Fig. 2e, f) and in gray matter neurons (NeuN) (Fig. 2h, i) located in the spinal cord segments, approximately 1.0–1.5 cm regions rostral to the lesion epicenter (and caudal to it; data not shown). The expression of ephrinA1 was observed as early as 2 DPI that remained until 28 DPI, but representative pictures of spinal cords from 2 DPI were used for panels (a–f) and 14 DPI (g–i). Double-labeling analysis of injured sections demonstrated that ephrinA1 protein expression (Fig. 2j) was not apparent in oligodendrocytes (MAG) (Fig. 2k) and macrophages (ED1) (data not shown) when sections were scanned sequentially and merged (Fig. 2l), at any of the time points studied (2, 4, 7, 14, and 28 DPI).

Interactions of EphrinA1 Ligands with Cells Containing Eph Receptors

As previously established, ephrinA1 molecules act as ligands of Eph receptors thus we decided to determine colocalization after an injury to the spinal cord through the use of immunohistochemical assays and confocal microscopy. Figure 3 shows the presence of ephrinA1 (Fig. 3a, d) in the white matter of an injured spinal cord. Colocalization was observed with both, EphA4 (Fig. 3b, c) and with EphA7 (Fig. 3e, f) receptors.

Expression and Spatial Profile of EphrinA1 Ligand in the Spinal Cord

EphrinA1 protein expression in the spinal cord was validated using western blot analysis. The specificity of the antibody and the presence of ephrinA1 in the spinal cord of injured rats were confirmed through preabsorption of the antibody with the antigen used to generate it (Fig. 4). EphrinA1 immunoreactivity after SDS-PAGE and immunoblotting was observed between 60 and 75 kDa. This was validated using an additional commercially available antibody against ephrinA1 (sc-911; Fig. 4a). We also determined the spatial profile for ephrinA1 ligand in the spinal cord of adult control rats (Fig. 4b). After protein extraction from cervical, thoracic, lumbar and sacral segments, western blot analysis was made revealing greater amount of ephrinA1 in the thoracic and cervical segments, followed by lower expression in lumbar and sacral regions.

Temporal Profile of EphrinA1 Ligand After Injury

Preliminary experiments were performed to determine the amount of protein extract necessary to evaluate expression changes within the linear range of detection (data not shown). Thirty-micrograms (30 μg) of solubilized spinal cord extracts were used in the ephrinA1 analysis at different time points after injury (days post injury, DPI). Samples from injured spinal cords at 2, 4, 7, 14, and 28 DPI showed downregulation of ephrinA1 when compared to the sham group (Fig. 5). The levels of ephrinA1 ligand decreased at 2 DPI by 82% and remained diminished until 28 DPI by 88%, contrary to what was observed with the mRNA (ANOVA, followed by Bonferonni post hoc test; $*P < 0.05$, $n = 3$ per group).

Behavioral Assessment After Silencing EphrinA1 Gene Expression

To investigate the role of ephrinA1 ligand present after SCI, we proceeded to administer AS oligonucleotides to block its expression and monitor functional locomotor recovery using the BBB (Fig. 6a) and grid-walking (Fig. 6b) tests. After 28 days, blockade of ephrinA1 expression was determined through western blot analysis. A dose-dependent effect was seen when comparing ephrinA1 levels from injured animals treated with concentrations of 2.5 nmol and 5 nmol of ephrinA1 AS, versus injured animals treated with the SCR 5 nmol oligonucleotide sequence (data not shown). Similar studies has been performed in our laboratory, showing AS oligonucleotide diffusion over the lesion epicenter, that reached the interior of the cells and blocked gene expression (Cruz-Orengo et al. 2006; Figueroa et al. 2006). Locomotor evaluation of the hindlimbs demonstrated that animals (n = 8) treated with the highest concentration of AS oligonucleotides (5 nmol) had a diminished recovery when compared to those animals (n = 4) with the SCR 5 nmol oligonucleotide sequence. Significant results for the BBB assay were observed at 7 DPI when comparing the injured group receiving 5 nmol of ephrinA1 AS oligonucleotides (3.06 ± 1.90) and the injured group treated with SCR 5 nmol oligonucleotide sequence (8.31 ± 0.94). Significant results were also seen between the injured group treated with ephrinA1 AS 5 nmol at 14 DPI (7.19 ± 2.36), 21 DPI (9.5 ± 1.48), and 28 DPI (11 ± 1.75) when compared to injured animals treated with the SCR sequence at 14 DPI (14.13 ± 2.02), 21 DPI (14.0 ± 1.16), and 28 DPI (16.88 ± 2.59) (Fig. 6a). Statistical analysis with ANOVA followed by Bonferroni post hoc test showed a significant difference between ephrinA1 AS and SCR control treated groups (F = (3, 87) = 90, $^{**}P < 0.01$, Fig. 6a).

As with the BBB open field test, the grid-walking assay showed a reduction in hindlimb activity of animals (n = 8) treated with ephrinA1 AS oligonucleotide as determined by the number of errors in paw placement. Significant results were observed between the injured group receiving 5 nmol of ephrinA1 AS oligonucleotides at 14 DPI (21.69 ± 4.14), 21 DPI (17.88 ± 3.73), and 28 DPI (13.06 ± 4.53) when compared to the injured animals (n = 4) treated with SCR 5 nmol oligonucleotide sequence at 14 DPI (9.5 ± 3.16), 21 DPI (8 ± 1.87), and 28 DPI (4.13 ± 1.80). No significant results were observed at 7 DPI (Fig. 6b). Both studies, BBB open field test and grid-walking, showed an apparent dose-dependent reduction in locomotor activity when animals (n = 8) treated with 2.5 and 5 nmol ephrinA1 AS oligonucleotides were compared with injured animals treated with the SCR oligonucleotides. Analysis of variance and Bonferonni post hoc test showed a significant effect of ephrinA1 AS oligonucleotide infusion compared to the performance of rats treated with SCR oligos on locomotor performance in the grid-walking test (F = (3, 77) = 49.6, $^{**}P < 0.01$, Fig. 6b).

Discussion

During development, axonal outgrowth is dependent on a precise balance between repulsive and attractive cues, which include Eph receptors and ephrin ligands. The expression of these molecules decreases from the early developmental stages towards adulthood. Interestingly, evidence suggests that after SCI, adult cells revert to a developmental phenotype and re-express a wide array of molecules that prevent growth (Emery et al. 2003). For instance, our own published data supports the expression of the EphA repellent molecules after SCI (Willson et al. 2002; Irizarry-Ramirez et al. 2005; Cruz-Orengo et al. 2006; Figueroa et al. 2006). The activation and signaling of these upregulated receptors depends on their ligand binding. However, these ephrin ligands could be involved in other cellular processes not related to growth cone collapse. An example of this mechanism are the experiments done by Marquardt et al. (2005) which show that ephrinA ligands have a role in growth cone and axonal spreading after Eph/ephrin interaction. Thus, the idea has been put forward that the

expression and activation of ephrinA ligands after injury might play a role in the modulation of tissue repair, regeneration, and plasticity.

In this study, we demonstrate for the first time the basal expression of several key members of the ephrinA family in the adult rat spinal cord. RT-PCR results showed gene amplification for ephrinA1, A2, A3, and A5 in the spinal cord of adult rats, but no ephrinA4 was detected with this approach in the adult vertebrate spinal cord. Similar reports about the absence or low levels of ephrinA4 in the adult CNS have been published by others (Carpenter et al. 1995; Moss et al. 2005). The presence of these molecules suggests a functional role for EphA–ephrinA signaling in the adult CNS, since our work and that of others demonstrates that EphA receptors are also expressed in basal levels in the spinal cord (Xu et al. 2003; Goldshmit et al. 2004; Cruz-Orengo et al. 2006; Figueroa et al. 2006). Currently their role in the adult CNS is unknown, however, it is logical to speculate that these ligands might be responsible for supporting and stabilizing spinal cord circuitry as has been shown in the hippocampus, thalamus, and retinal axons (Brownlee et al. 2000; Symonds et al. 2007).

EphrinA1 expression varied among the different regions of the spinal cord, being the thoracic–cervical areas the regions with the highest levels of this protein with a reduced amount in the sacral–lumbar segments [thoracic ≥ cervical > lumbar ≥ sacral]. The differences in ephrinA1 expression could be correlated to the varied populations of cells (interneurons, motor neurons, astrocytes, oligodendrocytes, and microglia) and tracts (sensory and motor) along the spinal cord segments and their roles in each region. The differential ephrinA1 expression throughout the spinal cord is similar to the pattern of EphA4 receptor that has been shown during topographical mapping of the corticospinal tract (Canty et al. 2006), suggesting a possible implication of regionally localized Eph/ephrin ligands during cell migration, synapse formation or during regeneration.

The expression of ephrinA ligands was determined after injury since traumatic events to the spinal cord lead to the upregulation of inhibitory molecules. RT-PCR data showed a biphasic modulation of the ephrinA1 mRNA levels after injury to the spinal cord without a significant change in the mRNA levels of ephrinA2, A3, and A5. We observed an acute tendency to reduced mRNA levels followed by a significant increase during the more chronic stages of the studied endpoints (e.g., 14 and 28 DPI). Trauma to the spinal cord results in an acute and robust inflammatory reaction that leads to necrosis, cell death, formation of a glial scar, axonal retraction, and collapse (Popovich et al. 1999). Macrophages are responsible for removing the debris generated after the contusion, which might contribute to the observed reduction in ephrin ligand expression at 2 and 4 days after injury (Popovich et al. 1999). The contusion injury also catalyzes events such as necrosis and apoptosis that might contribute to the initial decrease in the levels of ephrinA1 ligands.

The gradual upregulation of ephrinA1 could be related to gliosis and the proliferation of reactive astrocytes at the lesion epicenter. Interestingly, the ephrinA1 mRNA levels were significantly upregulated at 14 DPI and were maintained until 28 DPI. This suggests that ephrinA1 signaling could potentially be involved in more chronic events, such as axonal guidance and the reorganization or stabilization of CNS fibers. Our findings correlate with an increase in RNA levels of Eph receptors seen after excitotoxicity elicited in the adult rat hippocampus (Moreno-Flores and Wandosell 1999) and after SCI (Willson et al. 2002). The upregulation maintained by the expression of this ligand during different time points might be due to the fact that the CNS environment surrounding the trauma forms a glial scar which provides a chemical and physical barrier, thus limiting further entrance or exit of other molecules. Altogether, these results validated our EphA receptor studies and suggested that

the activation of ephrinA1 ligand may be one of multiple mechanisms activated and involved in axonal guidance and synapse formation after trauma in the adult CNS.

The Eph receptors mediate contact repulsion by causing growth cone collapse and/or retraction of lamellipodia and filopodia after ligand binding, mainly through RhoA related mechanisms (Shamah et al. 2001; Yamazaki et al. 2009). The expression of these receptors has also been confirmed in neurons and astrocytes of the adult CNS, and after CNS trauma (Biervert et al. 2001) including SCI (Bundesen et al. 2003; Irizarry-Ramirez et al. 2005). As with Eph receptors, these ligands are found in areas of the developing and adult CNS but their expression after SCI is unknown. The presence of ephrinA1 ligand after SCI was confirmed by immunohistochemical assays and the cell phenotype established by double-labeling studies. As with their conjugate receptors, ephrin ligands were colocalized with astrocytes in gray (Fig. 2c) and white matter at 7 DPI (data not shown). The microenvironment generated after SCI includes the proliferation of reactive astrocytes which hinder the possibility of making functional connections in the CNS. To some extent we might attribute the upregulation of this ligand to the increase in reactive astrocytes after SCI, as was previously reported for the Eph receptors (Willson et al. 2002), or as observed during diseases like multiple sclerosis (Sobel 2005). The expression of ephrinA1 in GFAP-positive cells, as well as EphA receptors, suggests that this EphA–ephrinA interaction could regulate certain aspects of astrocyte–astrocyte interaction during the glial scar formation. We cannot discard the possibility that ephrinA–EphA signaling could be involved in the formation and stabilization of synapses (Aoto and Chen 2007) in the injured cord.

The presence of ephrinA1 in reactive astrocytes, motor neurons, and axons after SCI suggest that expression of this ligand may be involved in the nonpermissive environment for neurite outgrowth. However, since protein levels decreased when the mRNA levels of ephrinA1 increased, this suggested that SCI might trigger a decrease in ligand translation, an increase in ligand degradation after synthesis or the activation of metalloproteases that cleaves the ligand extracellularly (de Castro et al. 2000; Wells et al. 2003; Hsu et al. 2008). Originally, we considered the possibility that these metalloproteases could cleave ephrinA1 from the plasma membrane reducing the levels of this ligand in our western blots, as previously published for ephrinA2 (Hattori et al. 2000) and ephrinA5 (Janes et al. 2005). The soluble ephrinA1 may interact with EphA receptors in the surrounding cells of the injured cord, as previously shown in embryonic neuronal growth cone collapse assays (Wykosky et al. 2008), inducing the cascade of events that block neurite outgrowth. However, analysis of ephrinA1 ligand in soluble fractions from injured samples were reduced and not elevated as expected (data not shown). This allows us to discard the possibility of an increase in metalloprotease activity, as the mechanism responsible of reducing the levels of this ligand from the plasma membrane. Therefore, we could establish posttranscriptional (mRNA stability), translational (protein synthesis) or post-translational regulation (protein stability, trafficking or sorting) for ephrinA1 ligand after trauma to explain its downregulation.

Colocalization of ephrinA1 protein and EphA4 and A7 receptors lead us to suggest that ephrinA–EphA signaling could activate molecular pathways involved in growth cone collapse, glia–glia interactions and/or cell death. In this case the abundance of Eph receptors does not correlate with the expression levels of ephrinA1 ligand proteins; however, these basal levels of the ligand might be achieving their role as inhibitory molecules or as activators of inhibitory mechanisms through the Eph receptors expressed at the injury site. This response could be accountable for preventing axonal outgrowth or necessary cell-to-cell interactions after SCI. Moreover, ephrinA1 expression could promote axonal demyelination by inducing apoptosis in EphA7-expressing oligodendrocytes. This idea was supported by our own findings that suggest that EphA7 can mediate apoptotic cell death in a subpopulation of spinal cord oligodendrocytes after SCI (Figueroa et al. 2006). Another

possible function for this ligand in CNS injury could have been the modulation of spinal circuitry and pain after SCI. Our previous studies suggest that upregulation of EphA4 receptor after trauma may prevent the development of abnormal pain syndromes and could potentially be exploited as a preventive analgesic mediator to chronic neuropathic pain (Cruz-Orengo et al. 2006). However, we could not discard the possibility that expression of ephrinB ligands may correlate with the abundance of Eph receptors producing a nonpermissive environment for axonal regeneration and this will need further investigation.

Antisense oligonucleotides were infused to the injured spinal cord to block ephrinA1 expression in the plasma membrane and/or reduce the limited amount that could be in the extracellular milieu (probably due to metalloprotease activity). Therefore, the repulsive environment that limited amount of ephrinA1 may generate (either by its presence in the membrane or as a soluble ligand) will be reduced and some functional locomotor recovery should be observed. Behavioral assays were performed to injured animals infused with the ephrinA1 AS and SCR control sequence. Significant deterioration was observed beginning at 7 DPI (BBB open field test) and at 14 DPI in the grid-walking test in animals infused with ephrinA1 AS when compared with those animals infused with the SCR control sequence (Fig. 6a, b). Similar results were observed at 21 DPI and 28 DPI, revealing that ephrinA1 ligand do not play a role as a direct contributor to the inhibitory environment generated after a lesion. As seen previously, ephrin ligands have been associated with various mechanisms that support growth or elongation, such as neurite outgrowth (Zhou et al. 2001) and axon branching of sympathetic neurons (Gao et al. 2000), outgrowth of filopodia from astrocytes (Nestor et al. 2007), or retinal axon branching due to an interaction between ephrinA ligands and neurotrophin receptors (Marler et al. 2008). Moreover, soluble ephrinA ligands have been shown to induce angiogenesis (Pandey et al. 1995) and in cultures of rat spinal cord exhibit a neurotrophic factor activity (Magal et al. 1996). Besides SCI, we can account for certain tumors, such as glioblastoma multiforme, where there is an increase in EphA receptors and a decrease in ephrinA ligands. In this case ephrinA1 ligand acts as tumor suppressing molecule (Wykosky et al. 2008). Our data leads us to suggest that the basal levels of ephrinA1 ligand do not necessarily act as inhibitors or repulsive signals, but as molecules that might stabilize remaining axonal connections.

Various molecules such as netrins and their receptors, associated with the developmental processes of the CNS, have been found to possess attractive and repulsive effects (Hong et al. 1999). In agreement with this finding, we propose a bi-functional effect for ephrinA1 ligands after injury to the spinal cord. Although the exact mechanism by which ephrinA ligands can act as a repulsive and attractive molecule is not known, different mechanisms have been postulated. Evidence suggests that the concentration of ligand present (Hansen et al. 2004), a cis or trans interaction (Yin et al. 2004), the segregation of the ligand and receptor on the same cell (Marquardt et al. 2005), or the regulation by which these molecules are internalized might play a role in the mechanism that is activated. It has also been established that spatiotemporal patterns play a role in molecular responses to ephrin ligands.

According to our data, we may suggest that ephrinA1 ligand does not provide direct inhibition responsible for the limited axonal outgrowth seen after SCI, contrary to what is seen with other molecules such as Nogo, OMgp, MAG, chondroitin sulfate, and Eph receptors. Although, just as with Eph receptors, ephrinA ligands appear to function in the adult CNS to help regulate and organize axonal pathfinding after trauma, but in a way that opposes the traditional mechanism involved in developmental stages of the CNS.

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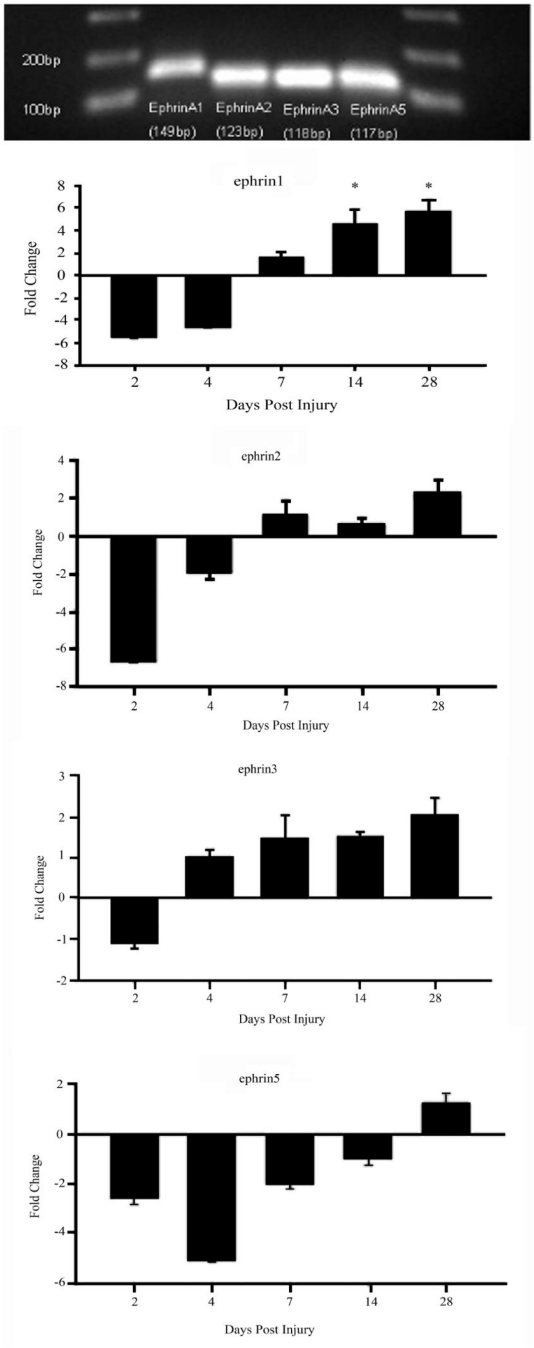

Fig. 1.

Expression of ephrinA1, A2, A3, and A5 ligands in the adult spinal cord and after spinal cord injury. Real-time PCR demonstrated the expression of ephrinA ligands in the adult spinal cord. (*Top panel*) Specific PCR products of 149 bp (ephrinA1), 123 bp (ephrinA2), 118 bp (ephrinA3), and 117 bp (ephrinA5) migrated to the expected position as observed in a 2% agarose gel impregnated with ethidium bromide. (*Lower panels*) Real-time RT-PCR analysis for ephrinA1 showed an initial decrease in the levels of this gene at 2 and 4 DPI, relative to GAPDH, that returned to the basal levels by 7 DPI. Results demonstrate a significant upregulation at 14 and 28 DPI. EphrinA2, A3, and A5 did not show any

significant changes (ANOVA followed by a Tukey–Kramer post hoc test, $P < 0.05$, $n = 3$). *The bars* represent the mean ± standard error of the mean (SEM)

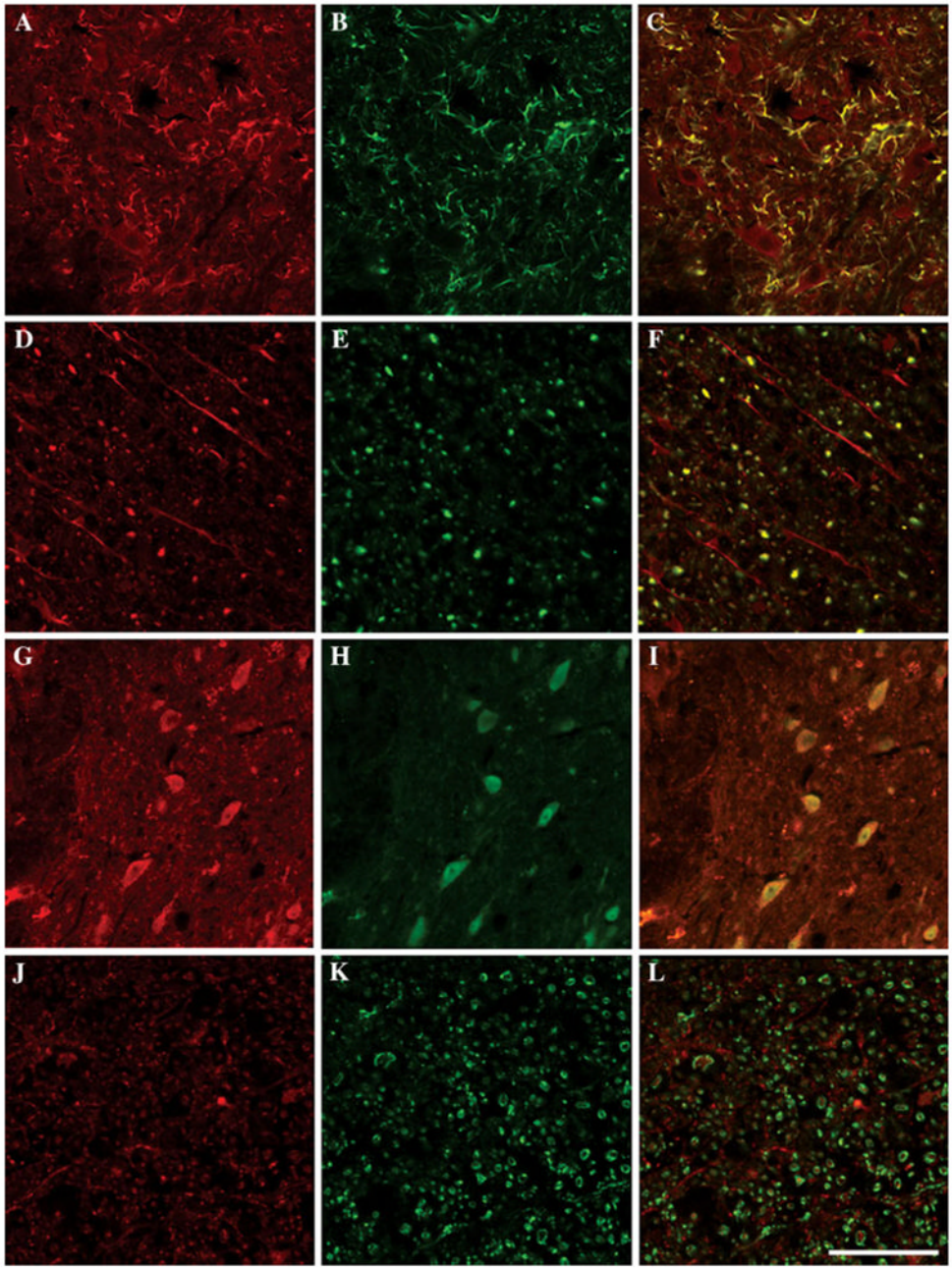

Fig. 2.

Cell phenotypes expressing ephrinA1 in the spinal cord. Immunocolocalization and confocal microscopy studies (n = 3) for ephrinA1 (**a, d, g, j**) demonstrated the presence of this molecule in reactive astrocytes/GFAP (**b**), as observed for axons/neurofilaments (**e**), and neurons/NeuN (**h**) in regions rostral to the lesion epicenter (**c, f, i**). The expression was observed as early as 2 DPI that remained until 28 DPI, but representative pictures of spinal cords from 2 DPI were used for panels (**a–f**) and 14 DPI (**g–i**). Double-labeling experiments demonstrate no colocalization between ephrinA1 (**j**) and oligodendrocytes/MAG (**k**) presented after the lesion to the spinal cord (**l**). *Scale bar* 10 μm

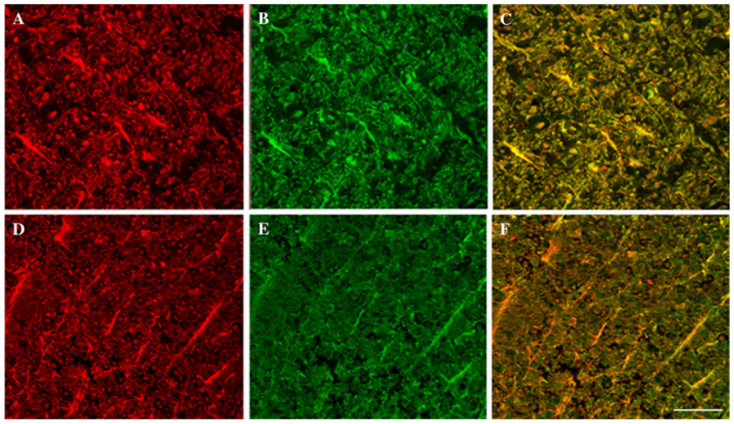

Fig. 3.

EphrinA1 and Eph receptors colocalized in the contused spinal cord. Immunohistochemical assays showed the colocalization of ephrinA1 (**a, d**) with receptors EphA4 (**b**) and EphA7 (**e**) after a lesion to the spinal cord of an adult rat (**c, f**). *Scale bar* 10 μm

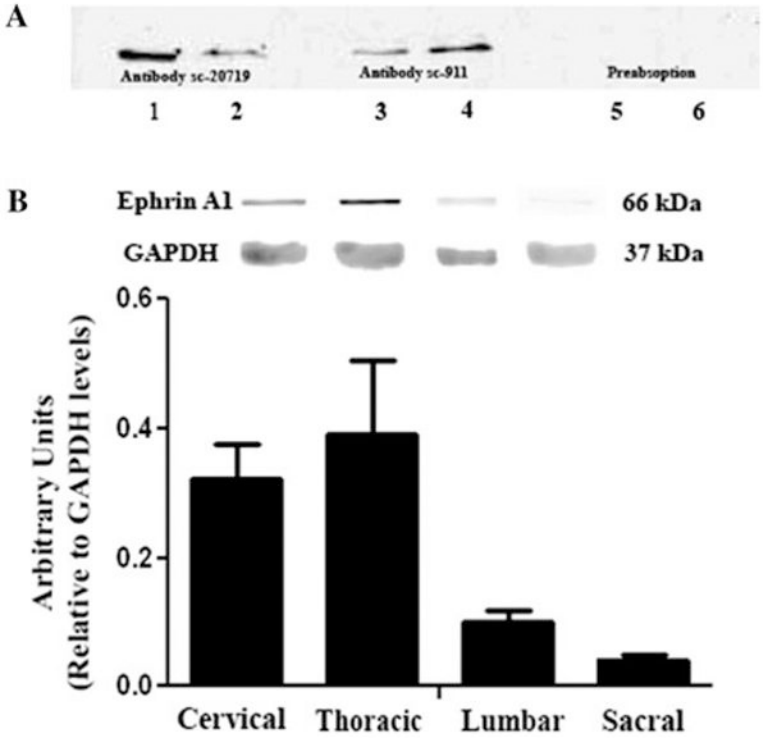

Fig. 4.

EphrinA1 protein is differentially expressed in the adult spinal cord. **a** Western blot demonstrates the migration of ephrinA1 protein at the same position using antibodies sc-20719 and sc-911. A preabsorbtion was made using a peptide for sc-911 (*lanes 5 and 6*), indicating the specificity of the antibody. Incubation of the protein samples with laemmli loading buffer, containing 8 M urea (*lanes 2, 4, and 6*), did not affect the migration of the protein confirming that the immunoreactive band is the matured glycosylated ligand. **b** Spatial profiling was determined by western blot, revealing a tendency for increased levels of ephrinA1 protein in thoracic and cervical areas of spinal cord segments of an adult control rat. *The bars* represent the mean ± SEM

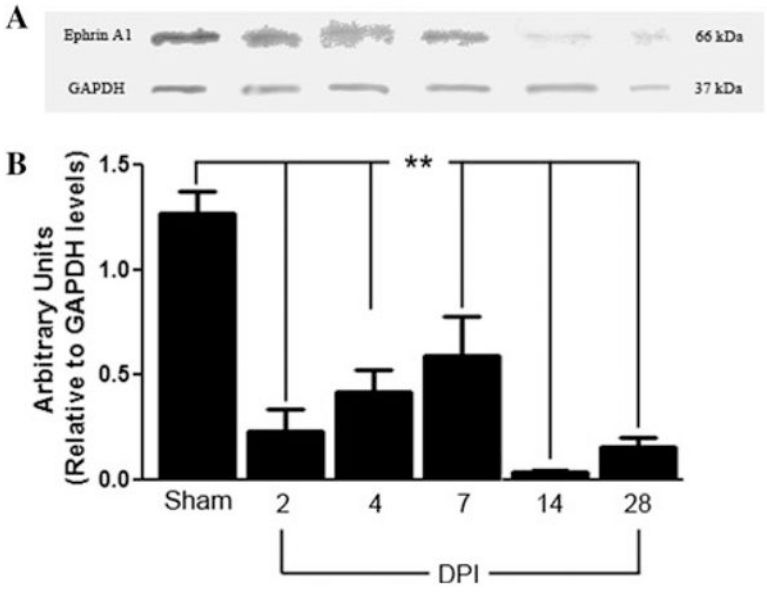

Fig. 5.

EphrinA1 temporal protein profile after SCI. **a** Western blot demonstrates ephrinA1 protein migration in a 7.5% polyacrylamide-SDS gel at 66 kDA. **b** Western blot analysis for ephrinA1 shows a constant downregulation as compared with the sham group. All indicated time points for ephrinA1 are established relative to GAPDH (ANOVA followed by a Bonferroni post hoc test, $P < 0.05$, $n = 3$). *The bars* represent the mean ± SEM)

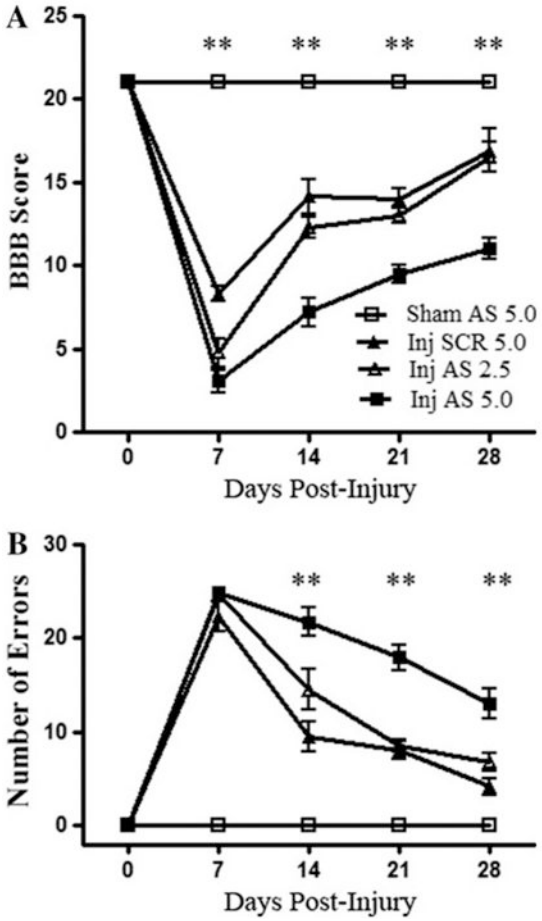

Fig. 6.

Effect of ephrinA1 expression blockade in locomotor behaviors. **a** Infusion of 5 nmol antisense (AS) oligonucleotide blocked basal ephrinA1 expression after SCI and significantly reduced BBB scores at all time points when compared to rats infused with scramble (SCR) control oligonucleotides. **b** A parallel result was observed as the number of errors increased in the grid-walking test when animals were intrathecally infused with 5 nmol of ephrinA1 AS oligonucleotides. Significant scores were observed 14, 21, and 28 DPI when compared to rats infused with SCR control oligonucleotides (ANOVA followed by a Bonferroni post hoc test, $P < 0.01$, $n = 8$). *The bars* represent the mean ± standard error of the mean

Table 1
Sequence of primers used to amplify specific ephrinA ligands

Primer	Sequence	Product size (bp)	T_m (°C)	Accession number
Forward ephrinA1	5′-GAAGAGACTCCAAGCAGATG-3′	149	52.6	NM_053599
Reverse ephrinA1	5′-CCAGGGCAAGCAAATACC-3′		52.4	
Forward ephrinA2	5′CTGCGGCTGAAAGTTTAC-3′	123	50.7	XM_234903
Reverse ephrinA2	5′-GACTGTAGTGAGGAAGAGG-3′		50.3	
Forward ephrinA3	5′-CATTGGAAGTGTCTGAGG-3′	118	48.7	XM_574979
Reverse ephrinA3	5′-CGTTGATCTTCACATTGG-3′		47.7	
Forward ephrinA5	5′-ATTAGAACCAGCAGATGACAC-3′	117	52.9	NM_053903
Reverse ephrinA5	5′-GCCAGGAGGAACAGTAGG-3′		52.7	
Forward GAPDH	5′-AACTTTGGCATCGTGGAA-3′	222	51.1	AF106860
Reverse GAPDH	5′-TACATTGGGGGTAGGAACAC-3′		53.4	

bp base pairs, T_m melting temperature (annealing temperature)