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Reduction of EphA4 receptor expression after spinal cord injury does not induce axonal regeneration or return of tcMMEP response

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Abstract

Spinal cord injury (SCI) causes an increase of inhibitory factors that may restrict axonal outgrowth after trauma. During the past decade, the Eph receptors and ephrin ligands have emerged as key repulsive cues known to be involved in neurite outgrowth, synapse formation, and axonal pathfinding during development. Given the non-permissive environment for axonal regeneration after SCI, we questioned whether enhanced-expression of the EphA4 receptor with repulsive activity for axonal outgrowth is potentially responsible for the regenerative failure. To address this possibility, we have examined the expression of EphA4 after SCI in adult rats following a contusion SCI. EphA4 expression studies demonstrated a time-dependent change for EphA4 protein without alterations in β-actin. EphA4 was downregulated initially and upregulated 7 days after injury. Blockade of EphA4 upregulation with antisense oligonucleotides did not produce an anatomical or physiological response monitored with anterograde tracing studies or transcranial magnetic motor evoked potentials (tcMMEP), respectively. These results demonstrated that upregulation of EphA4 receptors after trauma is not related to axonal regeneration or return of nerve conduction across the injury site.

Keywords: EphA; Trauma; Regeneration; Ephrin; Sprouting; Tracing

The adult central nervous system (CNS) has the plasticity to promote axonal elongation after trauma, if a supportive substrate and a permissive environment are provided. However, many inhibitory factors within the CNS restrict axonal regeneration. Molecular cues of myelin origin, as NOGO, MAG, and OMgp, are the best-known and intensively studied neurite outgrowth inhibitors [12]. Neutralization of NOGO-A with antibodies against this repulsive molecule enhanced nerve regeneration over long distances [1], was not complete, suggesting the presence of additional blockers to neurite outgrowth.

Other molecular signals with repellent properties that may influence axonal regeneration after CNS injury include semaphorins/collapsins, tenascin, slit proteins, sulfated proteoglycans [24], and the Eph receptor protein tyrosine kinase (RPTK) [3,11,22,23] and its ligands, the ephrins [5]. Although these molecules are predominantly recognized as developmental signals, many are constitutively expressed in the adult CNS and show altered expression after trauma [8]. Recent publications [5,6,10,13,30,31] showed that Eph and ephrin expression are markedly altered after SCI.

The Eph receptor tyrosine kinase family is the largest RPTK family known, and is divided in two groups: EphA and EphB receptors [9]. They are classified according to the type of ligands that activates them. The ephrin-A ligands are anchored to the plasma membrane by a glycosylphosphatidylinositol group and the ephrin-B ligands are anchored by a transmembrane domain. Therefore, those receptors activated by ephrin-A ligands are called EphA receptors and those receptors activated by ephrin-B ligands are called EphB receptors [9]. An interesting exception to this rule is EphA4 RTK, which can be activated by ephrin-B2 and ephrin-B3 [16].

The main characteristic of the Eph receptors is their ability to mediate cell–cell repulsion through the binding of their ligand on

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an adjacent cell surface [16]. Many members of the Eph RPTK family are expressed exclusively in the CNS and their temporal expression patterns as well as functional activity suggest that they are involved in development, maturation, and maintenance of the CNS [16]. While the expression of Eph RPTK has been shown to be critical during early stages of neural development, it is unknown if re-expression of these molecules after SCI affect axonal regeneration.

Recently, Cruz-Orengo et al. [6] reported changes in EphA4 mRNA expression after SCI. Moreover, they observed that blockade of EphA4 expression with antisense oligonucleotides after injury did not improve locomotor behavior in treated rats. However, more sensitive assays like anterograde tracing or transcranial magnetic motor evoked potentials (tcMMEPs) may uncover whether EphA4 plays a subtle but relevant role in axonal outgrowth or the return of nerve conduction after injury. We hypothesize that EphA4 plays a role in the injury response of the adult CNS generating a repulsive environment and blockade of its expression could promote axonal outgrowth and tcMMEP responses.

Adult female Sprague Dawley rats (225 g) were anesthetized with a cocktail of Ketamine/Xylazine/Acepromazine, 40/4/0.9 mg/kg, respectively (Fort Dodge Animal Health, Fort Dodge, IO) and the T10 spinal cord segment exposed, as described previously [6,10,22]. Briefly, the animals received a moderate contusion (10 g, 12.5 mm) SCI using the NYU impactor device and sham control rats had only a laminectomy. Rats ($n = 5$) were allowed to recover for 2, 4, 7, 14 and 28 days post-injury (DPI) to analyze the protein expression profile of EphA4.

EphA4 protein levels after SCI was determined using standardized Western blot analysis. Tissue from the lesion epicenter (5 mm) was dissected out and homogenized in ice-cold lysis buffer consisting of 20 mM Tris (pH 7.5), 150 mM NaCl, 5 mM NaF, 1 mM EDTA, and 1 mM EGTA, and a cocktail of proteinase inhibitors: 2 μg/ml antipain, 10 μg/ml aprotinin, 5 mM benzamidine, 1 mM DTT, 10 μg/ml leupeptin, 1 mM Na3VO4, 1 mM PMSF, and 10 μg/ml trypsin inhibitors [18]. Lysates were centrifuged at 20,000 × g for 90 min at 4 °C. Pellets were resuspended in the same lysis buffer with 1% NP-40 and incubated at 4 °C for 45 min while shaking. Following centrifugation at 20,000 × g for 10 min at 4 °C, the protein concentration of the resulting supernatant was determined using DCprot with bovine serum albumin as a standard following the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

Protein lysates (25 μg) were analyzed on 6 and 10% SDS-PAGE gels, for EphA4 and actin immunoblots, respectively. Proteins were electroblotted to a nitrocellulose membrane and the membranes were stained with 0.1% Ponceus S to confirm equal loading and appropriate transfer. The membranes were blocked for 3 h in Blotto and incubated overnight with primary antibody (1:4000) sc-921 rabbit anti-EphA4 (Santa Cruz Biotechnologies, CA) or 1:1000 A4700 monoclonal anti-actin (Sigma–Aldrich, MO). Blots were incubated 1 h with HRP-conjugated secondary goat anti-rabbit IgG (Sigma–Aldrich, MO) or rabbit anti-mouse IgG. Chemiluminescence was activated using SuperSignal West Dura (Pierce Chemical Company, IL) following the manufacturer's directions. Membranes were exposed and developed using Kodak (Rochester, NY) films.

Antibody specificity against the EphA4 receptor was determined by preabsorbing the primary antibody with 0.5 μg sc-921P blocking peptide, which was used to generate the polyclonal antibody. A single band of 120 kDa was detected but no immunoreactivity was obtained after preabsorption with the blocking peptide. Western Blot autoradiograms were quantified by densitometry using the Bio-Rad Gel Doc 2000 (Bio-Rad Laboratories, CA) and QuantiScan software (Biosoft, Ferguson, MO). Immunoblots with a series of membrane protein concentrations were used to determine the linear range for analysis. Statistical analysis to compare sham versus injured groups was performed with ANOVA, followed by Bonferroni post hoc test using InStat Version 3.0 software (GraphPad Software Inc., San Diego, CA). Statistical significance was set at $p < 0.05$.

Intrathecal infusion of EphA4 antisense oligonucleotide (AS) to the contused spinal cord with a miniosmotic-pump blocked the expression of EphA4 by approximately 50%, but did not produce any locomotor recovery [6]. However, studies to analyze the effect of EphA4 gene expression blockade at more sensitive anatomical and physiological levels were not previously investigated. Therefore, rats were treated with AS oligonucleotides immediately after contusion for a period of 28 days to block EphA4 expression after SCI as described by Cruz-Orengo et al. [6] and axonal regeneration monitored with anterograde tracing studies or tcMMEP responses to determine any return of nerve conduction.

After 28 DPI, antisense and control treated rats were assessed by tcMMEP as previously described [10]. Briefly, two sterile platinum subdermal needle electrodes were introduced in both gastrocnemius muscles and the reference electrode in the distal tendon of the gastrocnemius. Once the magnetic coil was placed over the skull, magnetic pulses were generated and electromyograms recorded from the gastrocnemius muscles. tcMMEPs were recorded at 70, 85, and 100% intensity of the magnetic field to validate the obtained responses. Latency onset, defined as the time between stimulation and muscle evoked EMG, was monitored and analyzed for both hindlimbs. Analyses of the converted recordings were done using Axoscope 8.2 software (Molecular Devices Corporation).

The extent of axonal outgrowth across the lesion epicenter was monitored with anterograde tracing studies after 42 DPI, giving enough time to observe some neuronal regrowth in a permissive environment. Antisense- and control-treated rats were anesthetized, as described above, for sterotaxic surgery using the Stoelting Stereotaxic Apparatus model 51650 (Stoelting Co., IL). The skull was exposed through a linear skin incision allowing visualization of both bregma and lambda sutures. The anteroposterior (AP), mediolateral (ML), and dorsoventral (DV) stereotaxic coordinates for bregma were calculated. For tracing the right motor cortex, layer V (MCV) at three points, the coordinates selected were: (1) AP, −0.8; ML, −1.0; DV, −2.0 (2) AP, −1.8; ML, −1.2; DV, −2.0 and (3) AP, −2.8; ML, −1.7; DV, −2.0. The bilateral red nuclei (RN)