Neuroscience 144 (2007) 1266–1277

CLASS A PLEXIN EXPRESSION IN AXOTOMIZED RUBROSPINAL AND FACIAL MOTONEURONS

E. D. SPINELLI, L. T. McPHAIL,[1] L. W. OSCHIPOK,[1] J. TEH AND W. TETZLAFF*

International Collaboration on Repair Discoveries, 6270 University Boulevard, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4

Abstract—The semaphorin family of guidance molecules plays a role in many aspects of neural development, and more recently semaphorins have been implicated to contribute to the failure of injured CNS neurons to regenerate. While semaphorin expression patterns after neural injury are partially understood, little is known about the expression of their signal transducing transmembrane receptors, the plexins. Therefore, in this study, we compared the expression patterns of all class A plexins (Plxn-A1, A2, A3, A4) in mouse CNS (rubrospinal) and peripheral nervous system (PNS)-projecting (facial) motoneurons for up to two weeks following axonal injury. Using *in situ* hybridization, immunohistochemistry, and Western blot analysis, in rubrospinal neurons, Plxn-A1 mRNA and protein and *Plxn-A4* expression did not change as a result of injury while *Plxn-A2* mRNA increased and *Plxn-A3* mRNA was undetectable. In facial motoneurons, *Plxn-A1*, *-A3* and *-A4* mRNA expression increased, *Plxn-A2* mRNA decreased while Plxn-A1 protein expression did not change following injury. We demonstrate that with the exception of the absence of *Plxn-A3* mRNA in rubrospinal neurons, both injured rubrospinal (CNS) and facial (PNS) neurons maintain expression of all plexin A family members tested. Hence, there are distinct expression patterns of the individual plexin-A family members suggesting that regenerating rubrospinal and facial motoneurons have a differential ability to transduce semaphorin signals.

Key words: plexins, semaphorins, regeneration, rubrospinal neurons, facial motoneurons, spinal cord injury.

Semaphorins play a crucial role during development by acting as cues guiding the correct patterning of the nervous system (reviewed in De Wit and Verhaagen, 2003). These findings led to the hypothesis that the re-expression/misexpression of semaphorins at the injury site may contribute to the inability of injured CNS neurons to regenerate into and past the injury site (Luo et al., 1993). Of the 20 semaphorin families identified to date (Semaphorin Nomenclature Committee, 1999), the class 3 secreted semaphorins are the most frequently studied following axonal injury. The Sema3 receptor complex consists of two subunits: the neuropilin-1/2 (Nrp-1/2), the ligand binding portion (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Nakamura et al., 1998) and the signal transducing component, the class A plexins (Plxn-A1, A2, A3, A4) (Takahashi et al., 1999; Tamagnone et al., 1999). Class A plexins, Nrps and class 3 semaphorins interact in a 2:2:2 ratio (Antipenko et al., 2003) hinting at a diversity of semaphorin-receptor combinations. Furthermore, the class 3 semaphorin receptor complex has been shown to include the adhesion factors L1 cell adhesion molecule (Castellani et al., 2002) and neuronal cell adhesion molecule (NrCAM) (Julien et al., 2005) as well as the receptors for vascular endothelial growth factors (Soker et al., 1998; Fuh et al., 2000). Plexins form the crucial link between ligand binding and activation of intracellular signaling pathways including the modulation of the actin and microtubule cytoskeleton (Fan et al., 1993; Fritsche et al., 1999), loss of integrin-mediated substrate adhesion (Mikule et al., 2002) and possibly changes of protein translation through the mitogen-activated protein kinase signaling pathway (Campbell and Holt, 2003; Guirland et al., 2004).

In the injured rodent spinal cord, semaphorins may act as a barrier to regeneration, as mRNA transcripts for almost all class 3 semaphorins (*Sema3A*, *3B*, *3C*, *3E* and *3F*, as well as Sema3A protein) are expressed in fibroblasts that invade the spinal cord injury site (Pasterkamp et al., 1999, 2001; De Winter et al., 2002; Lindholm et al., 2004). In addition, *Sema3B* mRNA and Sema4D protein are expressed in Schwann cells and oligodendrocytes (respectively) at the site of injury (Moreau-Fauvarque et al., 2003; De Winter et al., 2002). Nrp-1, Nrp-2 mRNA and protein expression as well as *Plxn-A1* mRNA expression are all either maintained or increased in many CNS neurons following injury (De Winter et al., 2002) suggesting that injured CNS neurons remain responsive to secreted class 3 semaphorins present at the injury site. In the regenerating peripheral nervous system (PNS) of adult rodents *Sema3A*, *3B*, *3C*, *3E* and *3F* mRNA expression is increased in endoneural fibroblasts distal to the site of a sciatic nerve crush (Scarlato et al., 2003; Ara et al., 2004) a compartment typically avoided by the regenerating axons that grow along the basal lamina tubes of the Schwann cells. However, in both facial and sciatic motoneurons, only *Nrp-1* mRNA has been studied and its expression does not change after axotomy (Pasterkamp et al., 1998).

Despite comprehensive data on semaphorin and Nrp expression following axonal injury, little is known about the expression of plexins in injured CNS or PNS motoneurons.

[1] These authors contributed equally.

*Corresponding author. Tel: +1-604-822-4956; fax: +1-604-822-2924.
E-mail address: tetzlaff@icord.org (W. Tetzlaff).

Abbreviations: ANOVA, analysis of variance; EDTA, ethylenediaminetetraacetic acid; ISH, *in situ* hybridization; Nrp, neuropilin; PBS, phosphate-buffered saline; PF, paraformaldehyde; Plxn, plexin; PNS, peripheral nervous system; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBST, tris-buffered saline Tween-20.

0306-4522/07$30.00+0.00
doi:10.1016/j.neuroscience.2006.10.057

Understanding plexin expression patterns in injured motoneurons may provide invaluable insight into the mechanisms possible in the responses of regenerating axons to semaphorins present at the injury site. To address this question, we compared the expression patterns of all class A plexins (Plxn-A1, A2, A3, A4) in mouse CNS (rubrospinal) and PNS (facial) motoneurons following axonal injury.

EXPERIMENTAL PROCEDURES

Animals

Male CD-1 mice (6–8 weeks old) were used in this study. All experiments were performed in accordance with the guidelines on the ethical use of animals set forth by the Canadian Council for Animal Care and were approved by the University of British Columbia Animal Care Committee. Animals were kept in a 12-h light/dark cycle and provided food and water *ad libitum*. For all surgical procedures, animals were anesthetized with a mixture of ketamine (135 mg/kg, Bimeda-MTC, Animal Health Inc., Cambridge, ON, Canada), and xylazine (6.5 mg/kg, Bayer Inc., Toronto, ON, Canada). Mice were killed with a lethal injection of chloral hydrate (900 mg/kg) at 3, 7 or 14 days post-surgery. Care was taken to minimize the number of animals used and their suffering.

Facial nerve axotomy

The facial nerve resection procedure was performed as previously described (Tetzlaff et al., 1991; McPhail et al., 2004). The left facial nerve was exposed and a 2–3 mm section of nerve was removed from all branches, 3 mm distal from the stylomastoid foramen, to prevent reconnection of growing axons to the distal nerve stump. The contralateral facial nerve was left intact as a control. The wound was closed with wound clips (Michel, Fine Science Tools, North Vancouver, BC, Canada) and the animals were returned to the housing unit.

Rubrospinal tract lesion

The spinal cord injury procedure used was performed as previously described (Tetzlaff et al., 1991; Fernandes et al., 1999). The spinal vertebrae were exposed at the third cervical level and partly removed. After opening of the dura, the dorsolateral funiculus of the spinal cord was cut with a pair of fine iris scissors. The wound was subsequently closed with wound clips and the animals were returned to their housing unit.

Immunohistochemistry

Animals were perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by an equal volume of 0.1 M phosphate buffered (pH 7.4) paraformaldehyde (4% PF in 1× phosphate buffer) and the brains removed. All samples were post-fixed overnight in 4% PF followed by cryoprotection in increasing sucrose gradients (14%, 18% and 22% sucrose in pH 7.4 0.1 M PBS). Each tissue sample was rapidly frozen in dry-ice cooled isopentane. Fourteen micrometer coronal sections were collected at −20 °C, mounted onto Superfrost Plus Slides (Fisher Scientific, Pittsburgh, PA, USA) and stored at −80 °C.

Sections containing the red nucleus or facial nucleus were analyzed for protein expression with antibodies against Plexin-A1 (1:200, kindly provided by Dr. Yanagi, Laboratory of Molecular Biochemistry, School of Life Science, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo, Japan) (Mitsui et al., 2002) or an anti-Nrp-2 antibody (1:200, Zymed Laboratories Inc., South San Francisco, CA, USA). Both antibodies have been raised against an amino terminal, extracellular epitope of the protein of interest. All antibodies used in histochemical analysis were diluted in 0.01 M PBS and all washes were performed three times for 5 min in 0.01 M PBS. After rehydration in 0.01 M PBS, sections were incubated overnight with primary antibody at 4 °C. After washing, sections were blocked for 20 min at room temp. with 10% normal donkey serum (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) and then incubated with a cyanin-3 conjugated donkey anti-goat secondary (1:200, Jackson Immunoresearch Laboratories Inc.) for 2 h at room temperature (RT) and subsequently washed. To identify motor neurons, sections were counterstained with the Nissl stain Neurotrace (1:200, 500/525 nm; Molecular Probes, Eugene, OR, USA) for 5 min at RT. Fluorescent images of Plxn-A1 or Nrp-2 along with Neurotrace immunoreactivity were captured using Northern Eclipse image analysis software (Empix Imaging Inc., Mississauga, ON, Canada) using a digital camera (Q-Imaging Systems, Burnaby, BC, Canada) mounted on a Zeiss Axioplan2 microscope (Carl Zeiss Canada Ltd., Toronto, ON, Canada).

Western blotting

For protein isolation, a total of 10 facial nuclei were micro-dissected and pooled from fresh-frozen mouse brains. Total protein was extracted using radio immunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 5 mM EDTA, 1% tert-octylphenoxy poly(oxyethylene)ethanol (IgePalCA630), 1% sodium deoxycholate, 0.1% sodium-dodecyl-sulfate, 1 μg/ul each of aprotinin, phenylmethylsulfonylfluoride, leupeptin, pepstatinA; pH 7.4). Pooled nuclei were homogenized and stored on ice for 30 min, centrifuged at 11,500 r.p.m. for 5 min at 4 °C and the supernatant collected. Protein concentration of supernatant was quantified by bicinchoninic assay (BCA, Pierce, Rockford, IL, USA, as per manufacturer's instructions) and samples immediately aliquoted and stored at −80 °C.

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), total protein was mixed 1:2 with Laemmli buffer (Bio-Rad Laboratories, Hercules, CA, USA), boiled for 5 min and each lane loaded with 40 μg of protein sample. Proteins were resolved SDS-PAGE (4% stacking gel, 7.5% resolving gel) for 2 h and electrophoretically transferred onto polyvinylidene fluoride (Immobilon-P, Millipore, Billerica, MA, USA) membranes overnight. After transfer, membranes were dipped in 100% methanol and allowed to dry at room temp for a minimum of 3 h. For immunodetection, membranes were initially blocked in 5% milk powder with tris-buffered saline containing 0.1% Tween-20 (TBST, pH 8.0) for 2 h at RT, followed by incubation with primary antibody diluted in 0.5% milk powder with TBST (1:3000 for Plexin-A1) overnight at 4 °C. After washing, membranes were incubated with horseradish peroxidase–conjugated donkey anti-goat antibody diluted in 5% milk powder with TBST (1:30,000; Jackson Immunoresearch Laboratories Inc.) incubated for 1 h at RT and subsequently exposed to electrogenerated chemiluminescence reagent for 1 min (electrochemoluminescence, ECL, Amersham Biosciences, Pharmacia Biotech, UK; as per manufacturer's instructions). The membranes were then exposed to autoradiographic film (Kodak Biomax, Eastman Kodak Company, Rochester, NY, USA) to visualize the protein. All membranes were washed six times for 5 min (TBST, pH 8.0) between each step. To determine equal loading of samples, membranes were stripped, blocked and re-probed with an anti-actin antibody (1:1000, ICN Biomedicals, Costa Mesa, CA, USA), followed by a horseradish peroxidase–conjugated donkey anti-rabbit secondary antibody (1:10,000, Jackson Immunoresearch Laboratories Inc.) and protein bands visualized by chemiluminescence as described above.

ISH

Two 50-mer oligonucleotide probes were used for each gene of interest to decrease developing time. All probes were created using the web-based primer design program Primer3 (Rozen and Ska-