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Distribution of EphB Receptors and ephrin-B1 in the Developing Vertebrate Spinal Cord

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Abstract

Contact dependent interactions between EphB receptors and ephrin-B ligands mediate a variety of cell-cell communication events in the developing and mature central nervous system (CNS). These predominantly repulsive interactions occur at the interface between what are considered to be mutually exclusive EphB and ephrin-B expression domains. We previously used receptor and ligand affinity probes to show that ephrin-B ligands are expressed in the floor plate and within a dorsal region of the embryonic mouse spinal cord, while EphB receptors are present on decussated segments of commissural axons that navigate between these ephrin-B domains. Here we present the generation and characterization of two new monoclonal antibodies, mAb EfB1-3, which recognizes EphB1, EphB2 and EphB3 and mAb efrnB1, which is specific for ephrin-B1. We use these reagents and polyclonal antibodies specific for EphB1, EphB2, EphB3 or ephrin-B1 to describe the spatiotemporal expression patterns of EphB receptors and ephrin-B1 in the vertebrate spinal cord. Consistent with affinity probe binding, we show that EphB1, EphB2 and EphB3 are each preferentially expressed on decussated segments of commissural axons *in vivo* and *in vitro*, and that ephrin-B1 is expressed in a dorsal domain of the spinal cord that includes the roof plate. In contrast to affinity probe binding profiles, we show here that EphB1, EphB2 and EphB3 are present on the ventral commissure, and that EphB1 and EphB3 are expressed on axons that compose the dorsal funiculus. In addition, we unexpectedly find that mesenchymal cells, which surround the spinal cord and dorsal root ganglion, express ephrin-B1.

Introduction

Eph receptors are members of the largest known family of receptor tyrosine kinases and interact with membrane-associated ligands referred to as ephrins (Flanagan and Vanderhaeghen, 1998). Both Eph receptors and their ephrin ligands are divided into A and B subclasses based on structural characteristics and binding affinities. Ephrin-A ligands are tethered to the membrane through a glycosylphosphatidyl inositol (GPI) anchor whereas ephrin-B ligands possess transmembrane domains. Typically, EphA receptors bind ephrin-A ligands and EphB receptors bind ephrin-B ligands. However, EphA4 can activate B-type ephrins and ephrin-A5 is capable of binding and functionally interacting with EphB2 (Flanagan and Vanderhaeghen, 1998; Himanen et al., 2004; Pasquale, 2004). A unique feature of Eph-ephrin interactions is that they can facilitate bi-directional communication between cells. In addition to classical forward signaling, where a signal is transmitted into the Eph receptor-bearing cell, an Eph receptor-expressing cell is capable of transmitting a “reverse” signal into the ephrin-bearing cell (Cowan and Henkemeyer, 2002; Murai and Pasquale, 2003; Davy and Soriano, 2005). Surprisingly, both transmembrane ephrin-Bs and

the GPI-linked ephrin-As appear to participate in reverse signaling (Cowan and Henkemeyer, 2002; Holmberg and Frisen, 2002).

Eph-ephrin interactions mediate a variety of contact-dependent phenomena in both the developing (Wilkinson, 2001; Cooke and Moens, 2002; Kullander and Klein, 2002; Tepass et al., 2002; Poliakov et al., 2004) and mature (Klein, 2001; Murai and Pasquale, 2002; Yamaguchi and Pasquale, 2004) central nervous system (CNS). While these interactions predominantly generate a repulsive response, they can also facilitate attraction or adhesion (Holmberg and Frisen, 2002; Eberhart et al., 2004). Given their transmembrane structure, ephrin-Bs are especially well suited to function as highly localized repellents/attractants. Accordingly, in the developing vertebrate nervous system, repulsive or attractive short-range interactions between ephrin-Bs and their receptors give rise to forward or reverse signaling events that regulate: neural crest cell migration (Krull et al., 1997; Smith et al., 1997; Wang and Anderson, 1997; Santiago and Erickson, 2002; Davy et al., 2004), hindbrain segmentation (Mellitzer et al., 1999; Xu et al., 1999; Cooke et al., 2001), neuroblast migration/proliferation (Conover et al., 2000), morphogenetic movements during gastrulation (Chan et al., 2001), brain commissure formation (Henkemeyer et al., 1996; Orioli et al., 1996; Cowan et al., 2004), axon guidance in the inner ear (Cowan et al., 2000; Bianchi and Gray, 2002; Brors et al., 2003), retinal ganglion cell axon guidance (Birgbauer et al., 2000; Nakagawa et al., 2000; Birgbauer et al., 2001; Hindges et al., 2002; Mann et al., 2002; McLaughlin et al., 2003; Williams et al., 2003), and axon bundling in the hippocampus (Chen et al., 2004).

In contrast to other regions of the CNS, relatively little is known about the roles of ephrin-B-dependent signaling in the spinal cord proper. However, it has been shown that ephrin-B3 is expressed at the ventral midline of the developing mouse spinal cord (Gale et al., 1996a; Bergemann et al., 1998; Imondi et al., 2000) where it acts as a repulsive barrier, which prevents EphA4-expressing commissural axons from crossing over to the contralateral side of the spinal cord (Coonan et al., 2001; Kullander et al., 2001; Yokoyama et al., 2001) and that ephrin-B3-induced EphA4 signaling helps coordinate locomotion (Kullander et al., 2003). In addition, ephrin-B1 and ephrin-B2 are capable of repelling embryonic spinal motor axons and collapsing their growth cones *in vitro* (Wang and Anderson, 1997). In the adult rodent spinal cord, EphB-ephrin-B signaling modulates pain processing-associated synaptic efficacy (Battaglia et al., 2003) and may be activated in response to injury (Miranda et al., 1999; Bundesen et al., 2003).

In their best-understood roles, Eph receptors and ephrin ligands exhibit reciprocally compartmentalized or complementary expression patterns in developing neural systems, where functionally important interactions are thought to occur at the interface between distinct cell populations (Flenniken et al., 1996; Gale et al., 1996b; Holder and Klein, 1999; Wilkinson, 2000; Cooke and Moens, 2002). In many cases, ligand and receptor affinity probes; recombinant proteins representing the extracellular domains of Ephs and ephrins fused to an alkaline phosphatase (Flanagan et al., 2000) or immunoglobulin Fc tag (Gale et al., 1996b), have been used to visualize Eph and ephrin expression domains. These reagents are particularly well suited for detecting naturally occurring ligand-receptor interactions and they have provided key insights into the global distribution of Eph and ephrin proteins (Cheng et al., 1995; Gale et al., 1996b). However, the well-documented promiscuity of Ephs and ephrins has made it difficult to determine the identities of the specific receptors or ligands recognized by a given affinity probe. Another potential limitation of this approach is that endogenous receptor-ligand interactions can mask fusion protein binding sites. Therefore, the affinity probe approach may effectively exaggerate the complementarity of Eph and ephrin expression patterns by failing to reveal potential sites of overlapping expression (Sobieszczuk and Wilkinson, 1999; Flanagan, 2000). Consequently, other

techniques such as *in situ* hybridization and immunohistochemistry are required to accurately interpret affinity probe binding profiles. Since the mRNA expression pattern detected by *in situ* hybridization may not closely correlate with the distribution of the corresponding protein (especially in neural tissue where protein in axons may be quite distant from mRNA in cell bodies), immunolabeling in tissue sections is the method of choice. However, the unavailability of antibodies that can be used effectively in immunohistochemical applications has precluded a detailed analysis of the distribution of particular EphB and ephrin-B proteins, especially in the nervous system.

We previously used a battery of affinity probes to show that ephrin-B proteins are expressed in the floor plate and dorsal spinal cord and that the corresponding receptors are reciprocally expressed on axons, which assemble into longitudinal tracts between these presumably inhibitory domains (Imondi et al., 2000; Imondi and Kaprielian, 2001). The results of our *in situ* hybridization analyses further suggested that the EphB1 receptor, in particular, is likely to be preferentially expressed on decussated commissural axons and that ephrin-B1 and ephrin-B2, but not ephrin-B3, may be broadly expressed in the dorsal spinal cord (Imondi et al., 2000). Here we use newly generated monoclonal antibodies (mAbs) and commercially available polyclonal antibodies to document the distribution of individual EphB receptors and ephrin-B1 in the embryonic rodent spinal cord. The results of these analyses are generally consistent with our previous findings, however, they reveal additional sites of EphB and ephrin-B expression, which suggest new roles for EphB-ephrin-B1 interactions in the developing spinal cord.

Materials and Methods

mAb Production

mAb EfB1-3—One six-week old BALB/c female mouse (Charles River Laboratories International, Inc, Wilmington, MA) was immunized via intraperitoneal (IP) injection with 100 µg of rat EphB1-Fc (cat. #1596-B1-200; R & D Systems, Minneapolis, MN), resuspended in phosphate-buffered saline (PBS; 0.9% NaCl, 0.1M Na₂HPO₄, pH 7.4) and diluted 1:1 with MPL + TDM Adjuvant (cat. # M-6536; Sigma, St. Louis, MO) at approximately three-week intervals over three months. Three days after the last injection, the mouse was sacrificed by exposure to compressed carbon dioxide (CO₂) and its spleen was removed. The Animal Institute Committee of the Albert Einstein College of Medicine approved the animal (mouse) use protocol. Hybridomas were generated by combining 5.9×10^7 dissociated splenocytes with 1.97×10^7 NS1 myeloma cells (obtained from S. Morton, Jessell lab, Columbia University, New York, NY), in the presence of polyethylene glycol (PEG 1500; cat. # 783641; Roche Diagnostics, Indianapolis, IN), as previously described (Schubert and Kaprielian, 2001). The resulting cell suspension was plated into 96 well plates at 2.5×10^5 cells/well under HAT (hypoxanthine, aminopterin, thymidine) selection (Schubert and Kaprielian, 2001) in Iscove's Modified Dulbecco's Media (Gibco-BRL, Gaithersburg, MD) containing 20% Fetal Clone I serum (Hyclone, Logan, UT), 15% Hybridoma Cloning Factor (HCF, cat. # IG500615; Fisher Scientific International, Inc., Hampton, NH) and penicillin-streptomycin-glutamine (Gibco-BRL). Once the wells reached approximately 75% confluence, hybridoma supernatants were screened for binding to spinal cord-containing transverse cryosections derived from E13 rat embryos (see Immunohistochemistry, below). The hybridoma secreting mAb EfB1-3 was subcloned by limiting dilution in Iscove's Modified Dulbecco's Media containing 20% Fetal Clone I serum, 15% HCF and penicillin-streptomycin-glutamine, and its isotype was determined to be an IgG₁ using the Mouse Monoclonal Isotyping Kit (Sigma). mAb EfB1-3 reacts with rat, mouse, chick, and zebrafish tissue (see Results). A 1:5 dilution of hybridoma supernatant (subcloning medium without HCF) was used as the source of mAb EfB1-3 for

immunohistochemistry, except where noted, and undiluted hybridoma supernatant was used for immunoblotting.

mAb efrnB1—One six-week old female Armenian Hamster (Cytogen Research & Development; West Roxbury, MA) was immunized via IP injection with 100 μ g of mouse ephrin-B1-Fc (cat. # 473-EB-200; R & D Systems) resuspended and diluted as above, at approximately three week intervals over four months and the spleen was obtained as described above. The Animal Institute Committee of the Albert Einstein College of Medicine approved the animal (hamster) use protocol. Hybridomas were generated by combining 2.78×10^7 dissociated splenocytes with 0.93×10^7 NS1 myeloma cells in the presence of PEG1500 and plated in HAT selection medium (see above). Supernatants from wells that had reached 75% confluence were screened for binding to spinal cord-containing transverse cryosections derived from whole E12 mouse embryos (see Immunohistochemistry, below). The hybridoma secreting mAb efrnB1 was subcloned as described above. mAb efrnB1 is likely to be an IgG since it is detected by an anti-Armenian hamster IgG secondary antibody (an anti-Armenian hamster IgM secondary antibody is not commercially available). mAb efrnB1 reacts with mouse, but not rat or chick tissue (data not shown). Undiluted hybridoma supernatant (subcloning medium without HCF) was used as the source of mAb efrnB1 for all experiments.

Other primary antibodies—The following mouse mAbs were obtained from the Developmental Studies Hybridoma Bank (DSHB; Department of Biological Sciences, University of Iowa, Iowa City, IA): mAb 4D7 (anti-TAG-1; cat. # mAb 4D7; IgM; mouse immunized with cell suspension of E14-E17 rat brain; reacts with rat, mouse, but not chick tissue; recognizes 135 kD protein, immunoglobulin superfamily member, in western blot), mAb 2H3 (anti-neurofilament; cat. # mAb 2H3; IgG¹; mouse immunized with adult rat brain membranes; reacts with rat and mouse tissue; recognizes 165 kD protein in western blot), mAb GAD-6 (anti-GAD65; cat. # mAb GAD-6; IgG_{2A}; mouse immunized with affinity purified GAD-glutamic decarboxylase from adult rat brain; reacts with rat, mouse and human tissues; recognizes 64 kD protein, GAD enzyme, in Western blot), and mAb ASCS4 [anti-L1; cat. # mAb ASCS4; IgG₁; mouse immunized with sympathetic neuron/rat SCG (superior cervical ganglion) membranes; reacts with rat tissue; recognizes 200 kD, 130 kD, 85 kD proteins, rat L1 in western blot]. We also employed the following commercially available mAbs: mAb anti-L1 [clone 324; Chemicon (Temecula, CA), cat. # mAb 5272; IgG; rat immunized with glycoprotein fraction from P8-P9 mouse cerebellum; reacts with mouse and rat tissue; recognizes 220–240 kD protein, mouse L1 in western blot], mAb anti-DCC [mAb Ab-1; EMD Biosciences; Oncogene Research Products (La Jolla, CA), cat. # OP45; IgG¹; mouse immunized with extracellular domain of human DCC; reacts with human and mouse tissue; recognizes 190 kD protein, DCC in western blot], and mAb anti- β -tubulin [clone TUB2.1; Sigma (St. Louis, MO), cat. # T 4026; IgG₁; mouse immunized with purified rat brain tubulin; reacts with human, bovine, rat, mouse, and chick tissue; recognizes 55 kD protein in western blot]. Undiluted hybridoma supernatant was used as the source of mAb 4D7, mAb 2H3, mAb GAD-6 and mAb ASCS4 for all experiments. mAb anti-mouse L1, mAb anti-DCC, and mAb anti- β -tubulin were used at concentrations of 10 μ g/ml, 25 μ g/ml, and 1:500 in blocking buffer (see below), respectively, for all experiments. The following polyclonal goat IgG antibodies were obtained from R & D Systems (Minneapolis, MN) and used at the indicated concentrations in blocking buffer (see below) for immunohistochemistry: anti-rat EphB1 (15 μ g/ml; cat. # AF542; goat immunized with extracellular domain of recombinant rat EphB1; reacts with rat and mouse tissue; recognizes 102 kD protein, recombinant rat EphB1-Fc, in western blot), anti-mouse EphB2 (10 μ g/ml; cat. # AF467; goat immunized with extracellular domain of recombinant mouse EphB2; reacts with mouse and rat tissue; recognizes 100–110 kD protein, recombinant mouse

EphB2-Fc, in western blot), anti-mouse EphB3 (20 µg/ml; cat. # AF432; goat immunized with extracellular domain of recombinant mouse EphB3; reacts with mouse and rat tissue; recognizes 100kD protein, recombinant mouse EphB3-Fc, in western blot) and anti-mouse ephrin-B1 (5 µg/ml; cat. # AF473; goat immunized with extracellular domain of recombinant mouse ephrin-B1; reacts with mouse and rat tissue; recognizes 60 kD protein, recombinant mouse ephrin-B1-Fc, in western blot). Each of these polyclonal antibodies was used at a concentration of 1 µg/ml for immunoblotting.

Immunohistochemistry

Rodent and chick embryos—Timed pregnant CD1 mice and Sprague Dawley rats were obtained from Charles River Laboratories International, Inc. (Wilmington, MA) and Taconic Farms, Inc (Germantown, NY), respectively. Pregnant dams were exposed to compressed carbon dioxide and killed by cervical dislocation and embryos were removed by cesarean section. Fertilized chick eggs were obtained from Charles River Spafas (Wilmington, MA). Chick embryos were removed and decapitated prior to tissue preparation (see below). The Animal Institute Committee of the Albert Einstein College of Medicine approved the corresponding animal use protocols.

Cryosection preparation—Whole rodent and chick embryos were immersion fixed in 4% paraformaldehyde (PFA) in PBS at 4° C for 3 h and cryoprotected in 30% sucrose in PBS for 16 h at 4° C. Embryos were then embedded in Tissue-Tek OCT compound (Miles Scientific, Elkhart, IN) and frozen on dry ice. Cryosections (16 µm for fluorescence immunolabeling and 36 µm for avidin-biotin immunolabeling) were cut on a Leica cryostat, mounted on Superfrost Plus microscope slides (Fisher Scientific International, Inc.) and allowed to air dry for 16 h at room temperature.

Fluorescence immunolabeling—Sections were rinsed in PBS for 5 min and then incubated in one of the following blocking buffers: 1. 10% goat serum (Gemini Bio Products; Woodland, CA,) in PBS (for mAb EfB1-3 and mAb efrnB1) or 2. 3% Fetal Bovine Serum (FBS) (Gemini Bio Products), 0.1% Triton X-100 in PBS (for anti-EphB1, anti-EphB2, anti-EphB3 and anti-ephrin-B1), for 30 min at room temperature. After three 5 min washes in PBS, the sections were incubated in primary antibody (see above) for 1 h at room temperature and then washed 3X (5 min per wash) in PBS and incubated in a 1:200 dilution of one of the following secondary antibodies (all from Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA): 1. fluorescein-conjugated goat anti-mouse IgG (for mAb EfB1-3), 2. Cy2-conjugated goat anti-Armenian Hamster IgG (for mAb efrnB1), or 3. Cy3-conjugated donkey anti-goat IgG (for anti-EphB1, anti-EphB2, anti-EphB3 and anti-ephrin-B1), for 45 min at room temperature. The sections were then washed 3X (5 min per wash) in PBS and coverslipped in the presence of Prolong Antifade (Molecular Probes; Eugene, OR). For the double labeling experiment, the sections were incubated with a 1:1 mixture of the two primary antibodies (mAb EfB1-3 and anti-ephrin-B1), and then with a 1:1 mixture of the two appropriate secondary antibodies (see above). In the pre-absorption experiments, a 1:1 mixture of mAb EfB1-3 supernatant (1:100 dilution) and 100 µg of Fc alone (cat. # 110-HG-100), rat EphB1-Fc (see above), mouse EphB2-Fc (cat. # 467-B2-200), EphB3-Fc (cat. # 432-B3-200), EphB4-Fc (cat. # 446-B4-200), EphB6-Fc (cat. # 611-B6-200), or EphB1-Fc, EphB2-Fc and EphB3-Fc combined, was incubated for 1h at room temperature (each of the recombinant proteins were purchased from R & D Systems). Subsequently, these mixtures were applied to serial transverse cryosections of E12 rat spinal cord and the binding of mAb EfB1-3 was detected as described above.

Avidin-Biotin immunolabeling—Sections were washed in PBS (buffer for mAb 4D7, mAb Ab-1; anti-DCC, and mAb EfB1-3), in Tris-buffered saline (TBS; 0.1M Tris

containing 1.4% sodium chloride, NaCl; buffer for mAb ASCS4), or in 0.02 M potassium PBS (pH7.4, KPBS; buffer for mAb GAD-6) for 5 min and then incubated in 0.3% hydrogen peroxide (H₂O₂) for 30 min. Subsequently, only those sections that ultimately would be labeled with mAb ASCS4 were incubated in 0.8% Triton X-100-containing buffer for 15 min. After washing each of the sections in their appropriate buffer for 15 min, the sections were then blocked in buffer containing: 1. 10% goat serum (for mAb 4D7, mAb Ab-1 and mAb EfB1-3), 2. 1.5% NHS, 0.1% Triton X-100 (for mAb ASCS4) or 3. 3% normal horse serum (NHS; Gemini Bio Products; for mAb GAD-6) for 1 h at room temperature. Following a 5 min wash in the appropriate buffer, the sections were incubated in primary antibody for 2 h at room temperature and then overnight at 4° C. The sections were then washed 3X (10 min per wash) in the appropriate buffer and incubated with the following biotinylated secondary antibodies (all from Vector Laboratories; Burlingame, CA): goat anti-mouse IgM (for mAb 4D7), goat anti-mouse IgG (for mAb Ab-1 and mAb EfB1-3), or rat adsorbed horse anti-mouse IgG (for mAb ASCS4 and mAb GAD-6) at a 1:200 dilution in the appropriate block for 1 h at room temperature. Following 3 washes (10 min each) in the appropriate buffer, the sections were incubated in avidin-biotin-peroxidase complex (Vectastain Elite standard kit; Vector Laboratories) for 1 h at room temperature. After this incubation, the sections were treated with 0.06% 3,3'-diaminobenzidine tetrachloride (DAB, DAB peroxidase substrate kit; Vector Laboratories) to reveal antibody binding. The reaction was stopped by incubating the sections in dH₂O. Subsequently, the sections were post-fixed in 4% PFA and mounted under a coverslip in a 1:1 glycerol:H₂O solution.

Zebrafish—Zebrafish were maintained using standard methods. Wild-type embryos were raised at 28.5° C in 0.2mM phenylthiourea to block pigment formation and then dechorionated and fixed in 4% PFA in PBS. Embryos were then embedded in 1.5% agar, 5% sucrose in PBS overnight at 4° C and 14–20 µm thick transverse cryosections were generated using a Leica cryostat. Immunostaining was then performed by incubating sections with mAb EfB1-3 in 10% heat-inactivated newborn calf serum (NCS; Gemini Bio Products), 0.1% Tween-20, 1% Dimethyl Sulfoxide (DMSO), in PBS at 4° C overnight. The sections were then washed 4 times (5 min per wash) in PBS and incubated in goat anti-mouse Alexa 488 (Molecular Probes; Eugene, OR) at 1:3000 in 10% heat-inactivated NCS, 0.1% Tween-20, 1% DMSO, in PBS for one hour at room temperature, washed again four times (5 min per wash) and then coverslipped with fluoromount-G (Southern Biotech; Birmingham, AL).

Open book preparation and immunolabeling—Open book spinal cord preparations were derived from E12 mouse and E13 rat embryos and labeled with mAb 4D7 (anti-TAG-1; labels both rat and mouse), mAb ASCS4 (anti-rat L1), mAb anti-mouse L1 or mAb EfB1-3 (labels both rat and mouse), all as previously described (Imondi et al., 2000). Primary antibody binding was detected with the following alkaline phosphatase (AP)-conjugated secondary antibodies (all from Jackson ImmunoResearch Laboratories, Inc.): mAb 4D7 (goat anti-mouse IgM; 1:500), mAb anti-mouse L1 (goat anti-rat IgG; 1:500), mAb ASCS4 and mAb EfB1-3 (goat anti-mouse IgG; 1:500).

Cultured spinal cord explant preparation and immunolabeling—Floor plate-attached half open book preparations were generated (see Zou et al., 2000) from E13 Sprague-Dawley rat embryos using tungsten microneedles as previously described (Imondi et al., 2000). These explants were dissected in ice-cold Dulbecco's Modified Eagle Medium (DMEM; Gibco-BRL) and positioned on Millipore (Billerica, MA) Isopore membrane filters (5 µm pore size, TMPT; cat. # TMTP02500) in 35 mm tissue culture dishes. After placing 20 µl of a rat-tail collagen solution (see Imondi and Kaprielian, 2001) on top of the explants,

they were allowed to dry for 5 min at room temperature. The embedded explants were then cultured for 48 h in DMEM supplemented with 10% FBS, Bottenstein's N2 supplement (Gibco-BRL) and penicillin-streptomycin-glutamine. For immunolabeling with mAb 4D7 (anti-TAG-1), mAb ASCS4 (anti-rat L1), EfB1-3 and mAb anti- β -tubulin the explants were fixed in 4% PFA in PBS for 1 hr at room temperature and then washed 4X (1hr per wash) in PBS, blocked for 3 h in PBS containing 1% heat-inactivated goat serum (Gemini Bio Products) and 0.1% (1.0% for anti-L1) Triton X-100, and then incubated in the appropriate primary antibody for 1 d at 4° C. The explants were then washed 5X (90 min per wash) in PBS at room temperature, incubated in PBS overnight at 4° C and then incubated in an isotype-appropriate fluorescein-conjugated secondary antibody (goat anti-mouse IgG for mAb ASCS4, mAb EfB1-3 and mAb anti- β -tubulin, and goat anti-mouse IgM for mAb 4D7; both from Jackson ImmunoResearch Laboratories, Inc) diluted 1:1000 in blocking buffer overnight at 4° C. Subsequently, the explants were washed 5X (90 min per wash) in PBS, the collagen pads were removed from the filters and the explants were mounted under a coverslip in a 1:1 glycerol:H₂O solution.

Photodocumentation

Immunolabeled cryosections and cultured spinal cord explants were visualized under epifluorescent or bright field optics (Nikon Eclipse TE300; Nikon, Melville, NY). Images were captured with a digital camera (Optronics; Bolton, MA) using compatible Magnafire software. Immunolabeled open-book preparations were visualized and photographed using a Zeiss Stemi-2000C stereo dissecting microscope. Color slides were digitally captured using an Agfa Duoscan flatbed scanner. Composites were assembled, annotated, and input levels were digitally adjusted using Adobe Photoshop 7.0 to highlight findings.

Electrophoresis and Immunoblotting

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8% running gel) and immunoblot analyses were performed and analyzed essentially as previously described (see Zhu et al., 1998). For the mAb EfB1-3, anti-EphB and anti-ephrin-B1 blots, protein samples were dissolved in 5X sample buffer containing 0.2 M β -mercaptoethanol and boiled for 5 min prior to loading. For the mAb efrnB1 blot, protein samples were dissolved in 5X sample buffer lacking 0.2 M β -mercaptoethanol (since we have determined that the mAb efrnB1 epitope is sensitive to reduction) and were not boiled prior to loading. The type and amount of protein loaded per lane for each of the immunoblotting experiments was as follows: mAb EfB1-3 blot (EphB-Fc reagents; 1 μ g), anti-EphB blots (EphB-Fc reagents; 250 ng), mAb efrnB1 blot (ephrin-B Fc reagents; 250 ng) and anti-ephrin-B1 blot (ephrin-B-Fc reagents; 100 ng). Primary antibody binding was detected with the following horseradish peroxidase (HRP)-conjugated secondary antibodies: mAb EfB1-3 (goat anti-mouse IgG; 1:500; Jackson ImmunoResearch Laboratories, Inc.), anti-EphB1, 2, 3 (rabbit anti-goat IgG; 1:1000; Sigma), mAb efrnB1 (goat anti-Armenian hamster IgG; Jackson ImmunoResearch Laboratories, Inc.; 1:500) and anti-ephrin-B1 (rabbit anti-goat IgG; Sigma; 1:500). Primary antibody binding was visualized through the use of ECL Western Blotting Detection Reagents (Amersham BioSciences; Piscataway, NJ) and autoradiography. Autoradiographs were digitally captured using an Agfa Duoscan flatbed scanner and composites were assembled and annotated using Adobe Photoshop 7.0.

Controls

In all immunohistochemical and immunoblotting analyses no specific signals were detected when each of the primary antibodies was omitted from the assay.

Results

Generation and characterization of anti-EphB antibodies

In an attempt to generate mAbs against EphB1, we immunized a mouse with recombinant rat EphB1-Fc protein, representing the extracellular domain of EphB1 fused to human IgG Fc, and generated hybridomas according to previously described methods (see Materials and Methods; Schubert and Kaprielian, 2001). To select for antibodies that would be useful in immunohistochemical applications, hybridoma supernatants were screened for their ability to label E13 rat spinal cord-containing cryosections. The labeling pattern of one of the mAbs identified in this screen, mAb EfB1-3, closely resembled the distribution of EphB1 protein revealed in our previous ligand affinity probe binding and *in situ* hybridization analyses (Imondi et al., 2000). Specifically, mAb EfB1-3, brightly labeled the axon-rich ventral and lateral funiculi, as well as ventral and intermediate regions of the spinal cord that contain a variety of motor neurons and interneurons (Fig. 1A). In contrast to the binding of ephrin-B1-Fc (Imondi et al., 2000), mAb EfB1-3 also labeled the ventral commissure and the dorsal funiculus at this developmental stage (Fig. 1A). We next used an immunoblot analysis to determine whether mAb EfB1-3 recognizes EphB1 and, perhaps, other closely related EphB receptors. Specifically, we asked whether mAb EfB1-3 is capable of binding EphB-Fc recombinant proteins that represent the extracellular domains of EphB1, EphB2, EphB3, EphB4 and EphB6, linked to human IgG Fc. Consistent with the EphB-like labeling pattern we observed in tissue sections, mAb EfB1-3 recognized EphB1-Fc (Fig. 1B). However, mAb EfB1-3 also bound EphB2-Fc and EphB3-Fc, but not EphB4-Fc or EphB6-Fc (hence the designation, EfB1-3; Fig. 1B). While mAb EfB1-3 recognizes EphB1-Fc, EphB2-Fc and EphB3-Fc, we have consistently observed stronger binding to EphB1-Fc and EphB3-Fc (Fig. 1B and data not shown).

Based on the results of the immunoblot analysis it seemed reasonable to assume that the mAb EfB1-3 labeling pattern observed in tissue sections represented the combined distribution of EphB1, EphB2 and EphB3 proteins. Alternatively, mAb EfB1-3 might only be detecting a subset of these receptors. As a first step toward distinguishing between these possibilities, we confirmed the specificity of commercially available (R&D Systems) anti-EphB1, anti-EphB2 and anti-EphB3 polyclonal antibodies in immunoblots (Fig. 1C). We then labeled transverse cryosections of E13 rat spinal cord with each of these antibodies to selectively visualize the spatial distribution of EphB1, EphB2 and EphB3. This immunohistochemical analysis revealed that all three EphB receptors are expressed on longitudinally projecting axons contained within the ventral and lateral funiculi, as well as within ventral and intermediate regions of the spinal cord that are populated by a variety of motor neurons and interneurons (Fig. 1D–F). Each antibody also labeled the transversely projecting ventral commissure. Consistent with the mAb EfB1-3 labeling pattern, anti-EphB1 and anti-EphB3, but not anti-EphB2, labeled the dorsal funiculus. In contrast to anti-EphB1, anti-EphB3, and mAb EfB1-3, anti-EphB2 labeled the ventricular zone. These findings are generally consistent with the labeling of mAb EfB1-3 representing a composite of the EphB1, EphB2 and EphB3 expression patterns. However, the finding that mAb EfB1-3 strongly labeled the dorsal funiculus, but not the ventricular zone (see Fig. 1A), suggested that it may be primarily detecting EphB1 and EphB3 expression in tissue sections at this developmental stage. Consistent with this possibility, we have shown that pre-absorption of mAb EfB1-3 with EphB1-Fc or EphB3-Fc (or a mixture of EphB1-Fc, EphB2-Fc and EphB3-Fc), but not EphB2-Fc alone, significantly attenuates the binding of this antibody to tissues sections (see Supplementary Fig. 1).

EphB1, EphB2 and EphB3 are dynamically expressed in the developing rat spinal cord

We used each of the anti-EphB polyclonal antibodies, as well as mAb EfB1-3, to examine the spatiotemporal expression patterns of EphB1, EphB2 and EphB3 during early stages of embryonic rat spinal cord development. At E10, none of these antibodies labeled any region of the spinal cord (data not shown). While anti-EphB1 and anti-EphB3 immunoreactivity was also not observed at E11, both mAb EfB1-3 and anti-EphB2 labeled portions of the ventricular zone and early developing neurons and axons within ventral regions of the spinal cord (Fig. 2A,C). mAb EfB1-3 and anti-EphB2 also labeled the longitudinally projecting spinal accessory nerves that are positioned alongside the spinal cord, adjacent to the lateral exit points (Fig. 2A,C; **arrows**; see Schubert and Kaprielian, 2001; Dillon et al., 2005). The similarities between the anti-EphB2 and mAb EfB1-3 labeling patterns, together with the lack of anti-EphB1 and anti-EphB3 labeling, suggests that mAb EfB1-3 may selectively detect EphB2 expression at this developmental stage. By E12, each antibody labeled ventral regions of the spinal cord, the ventral commissure and the forming ventral and lateral funiculi (Fig. 2E–H). As noted above, anti-EphB1, anti-EphB3 and mAb EfB1-3, but not anti-EphB2, also labeled the dorsal funiculus, whereas only anti-EphB2 labeled the ventricular zone. At E13 (Fig. 2I–L), each of the antibodies displayed labeling patterns that were very similar to those observed at E12.

EphB1, EphB2 and EphB3 are preferentially expressed on decussated commissural axons in the embryonic rodent spinal cord

To verify the complement of EphB receptors expressed on commissural axons we used three approaches to compare the labeling patterns of anti-EphB antibodies with the distribution of several axonal markers. First, we used mAb EfB1-3 (Fig. 3A) to compare the expression of EphB receptors with the distribution of several well-established commissural axon markers; TAG-1, DCC, L1 and GAD-65 (Phelps et al., 1999; Tran and Phelps, 2000) in transverse cryosections of the E12.5 rat spinal cord. Consistent with previous findings (Phelps et al., 1999; Tran and Phelps, 2000), this analysis revealed that TAG-1 and DCC (Fig. 3B,C; **arrowheads**) are expressed on pre-crossing commissural axons emanating from dorsal regions of the spinal cord, whereas L1 and GAD65 (Fig. 3D,E; **arrowheads**) are expressed on a subset of ventrally located pre-crossing commissural axons. Antibodies specific for each of these proteins also labeled midline-crossing axons that compose the ventral commissure. On the contralateral side of the floor plate, DCC, L1 and GAD65 (Fig. 3C–E) were each expressed in both the ventral and lateral funiculi, but TAG-1 (Fig. 3B) expression was restricted to the ventral funiculi. In general agreement with the results of our previous ligand affinity probe binding studies, mAb EfB1-3 labeling demonstrated that EphB receptors are expressed at a low level on the ventral commissure, and at significantly higher levels on longitudinally projecting axons contained within the ventral and lateral funiculi. Many of these axons are likely to represent decussated commissural axons that express DCC, L1 and GAD65. Importantly, EphB receptors do not appear to be expressed at high levels on pre-crossing commissural axons. We next compared the distribution of EphB receptors with the distribution of TAG-1 and L1 in open book preparations derived from E13 rat and E12 mouse (as described below, mAb EfB1-3 cross-reacts with multiple species) spinal cord. As expected, TAG-1, but not L1 and EphB receptors, was expressed on pre-crossing commissural axons in the transverse plane, while L1 and EphB receptors, but not TAG-1, were expressed on longitudinally-oriented axons, many of which are decussated commissural axons, that compose the ventral and lateral funiculi (Fig. 4). Taken together, the results of these *in vivo* labeling studies confirm that EphB1, EphB2 and EphB3 are preferentially expressed on decussated commissural axons.

In a complementary approach aimed at localizing EphB receptors on commissural axons, we compared the labeling of mAb EfB1-3 to TAG-1 and L1 expression in cultured (for 48 h)

floor plate-attached half-open book explants derived from E13 rat spinal cord (Fig. 5; see Materials and Methods). Consistent with its expression being down-regulated on commissural axons during midline crossing *in vivo*, TAG-1 was selectively detected on pre-crossing commissural axons that extend in the transverse plane within the cultured explants (Fig. 5E; see also Zou et al., 2000). In contrast, EphB receptors (as detected by mAb EfB1-3) and L1 were expressed on axons that emerged from the cut edge of the explant through the floor plate (Fig. 5B,C). Without more specific markers of commissural axons, we cannot exclude the possibility that some of the emerging axons may not normally cross the floor plate *in vivo*. However, as has been previously noted, axons extending through the floor plate in these types of cultured explants are likely to be post-crossing commissural axons (see Zou et al., 2000). Thus, these observations are consistent with the preferential expression of L1 and EphB receptors on decussated commissural axons *in vivo*.

mAb EfB1-3 labels embryonic mouse, chick and zebrafish spinal cord

mAb EfB1-3 was produced by immunizing a mouse with recombinant rat EphB1-Fc protein. Nevertheless, mAb EfB1-3 labels both the rat and mouse spinal cord. mAb EfB1-3 labeling of the E12 mouse spinal cord (Fig. 6A) closely resembles the distribution of EphB receptors observed in the rat spinal cord. However, more pronounced labeling of the ventral commissure and pre-crossing segments of commissural axons was apparent in the mouse spinal cord (Fig. 6A; **vc**, **arrowheads**). To determine whether mAb EfB1-3 cross-reacts with other vertebrates, we labeled transverse cryosections derived from E5 chick and 36 hours post fertilization (hpf) zebrafish spinal cord. mAb EfB1-3 labeled the chick spinal cord (Fig. 6B) in a manner consistent with the labeling patterns observed in rat and mouse. As in the rat, only weak mAb EfB1-3 labeling of the ventral commissure and pre-crossing segments of commissural axons was observed in the E5 chick spinal cord (Fig. 6B). Consistent with the distribution of rat EphB2, in particular, mAb EfB1-3 also labeled the ventricular zone (Fig. 6; **vz**). Notably, labeling of the dorsal root ganglia (DRG) was more pronounced than in the rodent (rat or mouse) spinal cord. In the zebrafish spinal cord, the most conspicuous mAb EfB1-3 labeling was associated with the neuropil located at the lateral edges of the spinal cord (Fig. 6C; **np**). This marginal zone contains longitudinally projecting axons that compose the medial longitudinal fasciculus (MLF), ventral longitudinal fasciculus (VLF) and the dorsal longitudinal fasciculus (DLF; Lauderdale et al., 1997). mAb EfB1-3 also weakly labeled a more medial region of the ventral half of the spinal cord that extends from the neuropil to the ventricular zone (Fig. 6C). The results of these inter-species labeling studies suggest that mAb EfB1-3 recognizes an evolutionarily conserved epitope, which resides within the extracellular domain of EphB proteins.

Generation and characterization of anti-ephrin-B1 antibodies

We previously proposed that a dorsal domain of ephrin-B expression demarcates the position at which EphB receptor-bearing decussated commissural axons execute a final stereotypic turn into the longitudinal axis (Imondi and Kaprielian, 2001). Our previous receptor affinity probe binding analyses, which demonstrated that ephrin-B ligands are expressed in the floor plate and dorsal regions of the embryonic mouse spinal cord including the roof plate, provided support for this hypothesis (Imondi et al., 2000). *In situ* hybridization studies revealed that while *ephrin-B3* mRNA is selectively expressed in the floor plate and roof plate, *ephrin-B1* and *ephrin-B2* mRNA is also present in a broad dorsal region of the spinal cord (Imondi et al., 2000). Thus, ephrin-B1 and ephrin-B2 are good candidates for specifying the dorsoventral position of a longitudinally oriented commissural axon tract on the contralateral side of the floor plate. In an attempt to generate reagents that could be used to specifically localize ephrin-B1 protein, we immunized an Armenian hamster with recombinant mouse ephrin-B-Fc (represents extracellular domain of mouse ephrin-B1 fused to human IgG Fc) and generated hybridomas. The resulting hybridoma

supernatants were screened for their ability to bind transverse cryosections of the E13 mouse spinal cord. The labeling of one of the mAbs identified in this screen, mAb efrnB1, resembled the distribution of ephrin-B1 revealed by our previous receptor affinity probe binding and *in situ* hybridization analyses (see Imondi et al., 2000). Most notably, mAb efrnB1 labeled the roof plate and a broad dorsal region of the spinal cord (Fig. 7A). While we detected no labeling of the floor plate, mAb efrnB1 immunoreactivity was also present all around the spinal cord and surrounded the DRG (Fig. 7A). In contrast to mAb EfB1-3, mAb efrnB1 did not label the embryonic rat or chick spinal cord (data not shown). We next used an immunoblot analysis to demonstrate that mAb efrnB1 is capable of binding ephrin-B1-Fc, but not ephrin-B2-Fc or ephrin-B3-Fc (Fig. 7B). It should be noted that we have not been able to generate mAbs against ephrin-B2 and ephrin-B3 using a similar immunization scheme.

To verify that mAb efrnB1 is likely to detect ephrin-B1 in tissue sections, we first used an immunoblot analysis to confirm the specificity of a commercially available (R & D Systems) polyclonal antibody, anti-ephrin-B1 raised against mouse ephrin-B1-Fc (Fig. 7C). We then demonstrated that anti-ephrin-B1 labels the roof plate and dorsal spinal cord in E13 mouse cryosections just as does mAb efrnB1 (Fig. 7D). Further consistent with mAb efrnB1, anti-ephrin-B1 labeling surrounded the spinal cord and DRG but was not detected in the floor plate. Thus, mAb efrnB1 and anti-ephrin-B1 revealed an essentially identical distribution of ephrin-B1 protein in the E13 mouse spinal cord. However, stronger labeling was consistently observed with anti-ephrin-B1 (see also below).

Spatiotemporal distribution of ephrin-B1 in the embryonic mouse spinal cord

We used both mAb efrnB1 and anti-ephrin-B1 to investigate the spatiotemporal expression pattern of ephrin-B1 in the developing mouse spinal cord. At E10, anti-ephrin-B1 labeled the dorsal half of the spinal cord including the roof plate, while mAb efrnB1 labeling was restricted to the roof plate (data not shown). Similar patterns of mAb efrnB1 and anti-ephrin-B1 immunoreactivity were observed at E11 (Fig. 8A,B). The lack of mAb efrnB1 labeling in the dorsal spinal cord (excluding the roof plate) may indicate that relatively low levels of ephrin-B1 are expressed at these early developmental stages, since mAb efrnB1 appears to bind more weakly than anti-ephrin-B1 in immunohistochemical applications. Notably, neither anti-ephrin-B1 nor mAb efrnB1 labeling was observed around the spinal cord and DRG at E10 or E11 (Fig. 8A–D). By E12, both mAb efrnB1 and anti-ephrin-B1 detected ephrin-B1 expression within the dorsal spinal cord from the ventricular zone to its lateral edges and in the roof plate, as well as around the entire spinal cord and DRG (Fig. 8E,F). Using either antibody, a very similar expression pattern was observed at E13. However, at this age, labeling in the dorsal spinal cord was restricted to the ventricular zone and the roof plate (Fig. 8G,H). By E14, mAb efrnB1 and anti-ephrin-B1 immunoreactivity was clearly observed around the spinal cord and DRG, and the only remaining labeling within the spinal cord was localized to a circumscribed region of the dorsal ventricular zone (Fig. 8I,J).

Complementary expression of ephrin-B1 and its EphB receptors in the developing mouse spinal cord

In a variety of developing systems, Eph receptors and their ligands are expressed in reciprocal and mutually exclusive domains (see Introduction). We previously used receptor and ligand affinity probes to demonstrate complementary expression of transmembrane ephrin-B ligands and their EphB receptors in the embryonic mouse spinal cord (Imondi et al., 2000). These studies revealed that while ephrin-B ligands are present in the dorsal spinal cord, as well as in the roof plate and floor plate, EphB receptors are preferentially expressed on longitudinal axon tracts and within ventral regions of the spinal cord. We used anti-ephrin-B1 and mAb EfB1-3 to directly determine whether ephrin-B1 and EphB receptors,

respectively, are expressed within non-overlapping regions of the embryonic mouse spinal cord. Specifically, we double-labeled an E12 mouse spinal cord-containing cryosection with anti-ephrin-B1 and mAb EfB1-3. Consistent with the results of the single labeling experiments described above, this revealed that ephrin-B1 and EphB receptors are expressed in essentially reciprocal domains within the spinal cord; EphB receptors are concentrated in the ventral half and ephrin-B1 in the dorsal half (Fig. 9A,B). Furthermore, the anti-ephrin-B1 immunoreactivity surrounding the spinal cord is immediately adjacent to and outside of the marginal zone, which is composed of EphB-expressing axons contained within the ventral and lateral funiculi (Fig. 9A; **arrowheads**). Despite the relative complementarity, however, some overlap does appear to exist between EphB and ephrin-B1 expression in dorsolateral regions of the spinal cord outside of the ventricular zone. This suggests that EphB receptors and ephrin-B1 may be co-expressed on newly differentiated interneurons (Fig. 9C).

Discussion

EphB receptors and their ephrin-B ligands mediate a wide variety of cell-cell communication events in both the developing and mature nervous system. In most cases, the underlying contact-dependent interactions are thought to occur along the interface of complementary EphB and ephrin-B expression domains. Due to the unavailability of appropriate antibodies, these domains have been identified mainly through the binding of receptor and ligand affinity probes. However, this approach is incapable of revealing the distribution of individual EphB and ephrin-B proteins and may accentuate the complementarity of ligand and receptor expression patterns. Here, we use newly generated mAbs and commercially available polyclonal antibodies to describe the spatiotemporal distribution of several EphB receptors and ephrin-B1 in the developing vertebrate spinal cord. In general agreement with the results of our previous affinity probe binding and *in situ* hybridization analyses (Imondi et al., 2000; Imondi and Kaprielian, 2001), we find that EphB proteins and ephrin-B1 are reciprocally expressed on decussated segments of commissural axons and within dorsal regions of the spinal cord. However, we also identify additional sites of EphB and ephrin-B expression that were not revealed by affinity probe binding analyses. Most notably, significant levels of EphB proteins are associated with axons that compose the dorsal funiculi and ephrin-B1 is expressed in mesenchymal cells that envelop the spinal cord and adjacent DRG. These new findings suggest additional roles for EphB-ephrin-B1 interactions in spinal cord development.

EphB expression

We previously used ephrin-B affinity probes to show that EphB receptors are localized to decussated segments of commissural axons in the embryonic mouse and chick spinal cord (Imondi et al., 2000; Imondi and Kaprielian, 2001). However, the binding promiscuity of these reagents precluded us from determining whether particular EphB receptors or multiple receptors are expressed on these axonal segments. Several findings we present in this study support our conclusion that each of the three EphB receptors, EphB1, EphB2 and EphB3, are preferentially localized to post-crossing commissural axons. First, mAb EfB1-3, which recognizes EphB1, EphB2 and EphB3, as well as anti-EphB1, anti-EphB2 and anti-EphB3 specific polyclonal antibodies rather selectively, labels the decussated commissural axon-rich ventral and lateral funiculi in transverse sections of E13 rat spinal cord. Second, mAb EfB1-3 labeling of transverse sections of E12.5 rats spinal cords overlaps with the marginal zone-associated labeling of the established commissural markers, anti-DCC, anti-L1, and anti-GAD65 (Phelps et al., 1999; Tran and Phelps, 2000), but not with anti-TAG1 and anti-DCC labeling of pre-crossing commissural axons. While we do occasionally detect EphB protein expression on what appear to be pre-crossing segments of commissural axons in cryosections derived from rat and mouse spinal cord, significantly higher levels are

consistently observed on the decussated portions of these axons. Third, mAb EfB1-3 labels longitudinally oriented axonal tracts that compose the lateral and ventral funiculi, but not transversely oriented anti-TAG1-positive pre-crossing commissural axons, in open book preparations derived from E13 rat and E12 mouse spinal cord. These *in vivo* findings suggest that EphB proteins are rather selectively expressed on decussated segments of commissural axons. However, both the ventral and lateral funiculi also contain ipsilaterally projecting axons, which never cross the ventral midline. Thus, consistent with our interpretation of previous affinity probe binding analyses (Imondi et al., 2000), it is perhaps more accurate to conclude that EphB receptors are present on decussated segments of commissural axons and longitudinally oriented ipsilateral axons located within the spinal cord marginal zone. Fourth, mAb EfB1-3 appears to label anti-L1-positive commissural axons that have extended out of E13 rat, floor plate–attached half spinal cord explants *in vitro*. Further consistent with previous affinity probe (Wang and Anderson, 1997; Imondi et al., 2000) and antibody (Koblar et al., 2000) binding studies carried out in a variety of vertebrates, mAb EfB1-3, anti-EphB1, anti-EphB2 and anti-EphB3 each also label ventrally exiting spinal motor axons.

The distribution of EphB proteins revealed using immunohistochemistry in this study is generally consistent with the results of our previous affinity probe binding analyses (Imondi et al., 2000). However, antibody labeling identifies additional sites of spinal cord-associated EphB expression that were not previously observed using ephrin-B affinity probes to localize EphB proteins. For example, while both methods detect EphB receptors on decussated segments of commissural axons, we show here that mAb EfB1-3, anti-EphB1, anti-EphB2 and anti-EphB3, also label the ventral commissure, which is comprised of commissural axons in the act of crossing through the floor plate. It is important to note in this regard that affinity probes, but not antibodies, selectively detect functionally available forms of molecules that are capable of participating in natural ligand-receptor interactions (Sobieszczuk and Wilkinson, 1999; Flanagan, 2000). Thus, if EphB receptors that reside on midline crossing commissural axons are selectively unavailable for ephrin-B binding, this could account for the discrepancy between the affinity probe- and antibody-binding data. In one possible scenario, just as has been reported for EphA2 (Brittis et al., 2002) and as we have previously suggested (Imondi et al., 2000), EphB receptor expression may be up-regulated on commissural axons as they course through the floor plate. These receptors would then be well positioned to engage floor plate-associated ephrin-B proteins in trans (see Imondi et al., 2000) and effectively mask ephrin-B-Fc binding sites. In contrast to its best-understood role as a contact-dependent repellent, ephrin-B1 is also capable of mediating positive/attractive cell migration (Santiago and Erickson, 2002) and guidance (Mann et al., 2002; McLaughlin et al., 2003) events. Thus, interactions between commissural axon-associated EphB receptors and ephrin-B1 in the floor plate could potentially facilitate midline crossing.

We also show here that mAb EfB1-3, and the polyclonal antibodies, anti-EphB1 and anti-EphB3 (but not anti-EphB2) intensely label longitudinally projecting axons, which compose the dorsal funiculus. Therefore, EphB1 and EphB3, but not EphB2 are likely to represent the complement of EphB receptors expressed on these axons. It is interesting to note in this regard that mAb EfB1-3, anti-EphB1 and anti-EphB3 only weakly label DRG neurons and their axons as they extend toward the spinal cord. Taken together, these observations suggest that EphB1 and EphB3 expression must be selectively upregulated on DRG neuron axons after they make the transition from growth in the transverse to the longitudinal plane and assemble into the dorsal funiculus. This is in accord with our observation that EphB receptors are preferentially expressed on the longitudinally projecting decussated segments of commissural axons. In contrast to the results of the antibody labeling analyses presented here, affinity probes designed to detect EphB proteins fail to decorate dorsal funiculus-

associated axons (Imondi et al., 2000). Given that ephrin-B proteins do not appear to be expressed on these axons (Imondi et al., 2000), masking of ephrin-B binding sites is unlikely to account for the lack of affinity probe labeling.

Ephrin-B1 expression

Our immunohistochemical studies show that ephrin-B1 is expressed in the roof plate and within a dorsal domain of the embryonic mouse spinal cord. Importantly, both mAb efrnB1 and an anti-ephrin-B1 polyclonal antibody label these regions of the spinal cord between E10 and E13. These findings are generally consistent with the distribution of *ephrin-B1* mRNA revealed by *in situ* hybridization (Bouillet et al., 1995; Imondi et al., 2000; Dionne et al., 2002) and the expression pattern of ephrin-B protein visualized through affinity probe binding (Imondi et al., 2000; Imondi and Kaprielian, 2001). However, very low levels of *ephrin-B1* mRNA have also been detected in the floor plate (Bouillet et al., 1995; Imondi et al., 2000), whereas the anti-ephrin-B1 antibodies employed in this study fail to label this ventral midline structure. The presumably limited amount of ephrin-B1 protein expressed by floor plate cells might account for this apparent discrepancy. In contrast to the results of our *in situ* hybridization (Imondi et al., 2000) and immunohistochemical analyses (this study) designed to localize *ephrin-B1* mRNA and ephrin-B1 protein, respectively, EphB affinity probes detect significant levels of ephrin-B protein in the floor plate (Imondi et al., 2000; Imondi and Kaprielian, 2001). Notably, very low levels of *ephrin-B2* mRNA (Imondi et al., 2000; Dionne et al., 2002), but high levels of *ephrin-B3* mRNA (Bergemann et al., 1998; Imondi et al., 2000) are expressed at the ventral midline. Therefore, it seems likely that the affinity probes mainly detect ephrin-B3 protein in the floor plate. Unfortunately, we have not been able to directly confirm this hypothesis, due to the unavailability of anti-ephrin-B2 and anti-ephrin-B3 antibodies that work well in immunohistochemical applications. In more rostral regions of the embryonic mouse CNS, both mAb efrnB1 and anti-ephrin-B1 strongly and preferentially label anterior and lateral regions of the telencephalon (data not shown). This observation is consistent with the previously described labeling pattern of mAb 25H11, an independently generated anti-ephrin-B1 mAb (Stuckmann et al., 2001).

Mesenchymal cells surrounding the developing vertebrate brain and spinal cord are rich sources of inhibitory cues that constrain the growth of several classes of axons (Tosney and Oakley, 1990; Keynes et al., 1991; Oakley and Tosney, 1991; Lieberam et al., 2005). For example, the cranial mesenchyme associated with rhombomere 3 in the embryonic mouse hindbrain possesses a chondroitin sulphate proteoglycan-binding inhibitory activity for trigeminal ganglion axons (Golding et al., 1999). Also, mRNA encoding the Bone Morphogenetic protein, BMP5 (William et al., 2003) or the chemokine, Cxcl12/SDF-1 (Chalasani et al., 2003) is expressed in mesenchymal cells that surround the spinal cord and DRG, as well as the dorsal funiculus/dorsal root entry zone. Consistent with an inhibitory role for mesenchymal cell-associated BMP5, several BMPs function as chemorepellents for pre-crossing commissural axons *in vitro* (Augsburger et al., 1999) and presumably *in vivo* (Butler and Dodd, 2003). More directly, the chemokine, Cxcl12/SDF-1, is thought to prevent dorsally projecting motor axons from exiting the spinal cord through the ventral roots (Lieberam et al., 2005) and inhibits the premature entry of collaterals emanating from dorsal funiculi-associated axons into the spinal cord (Chalasani et al., 2003).

We show here that both mAb efrnB1 and anti-ephrin-B1 label mesenchymal cells, which envelop the spinal cord and DRG and ensheath sensory axons as they extend toward the dorsal root entry zone. These observations are consistent with the previously reported distribution of *ephrin-B1* mRNA (Bouillet et al., 1995) and ephrin-B1/ephrin-B2 protein (Stuckmann et al., 2001; Dionne et al., 2002) and suggest new inhibitory roles for ephrin-B1. Specifically, mesenchymal cell-associated ephrin-B1 surrounding the spinal cord may hem in EphB receptor-bearing ipsilateral, commissural and motor axons through a contact-

dependent repulsive mechanism. Ephrin-B1 may also maintain the integrity of DRG and the dorsal funiculi in the same way that Semaphorin 5A is thought to operate in neuroepithelial cells, which surround retinal axons at the optic disk and along the optic nerve (see Oster et al., 2003). By analogy with a recently described role for SDF-1 in sensory axon guidance (Chalasanian et al., 2003), ephrin-B1 may also preclude the precocious entry of EphB receptor-bearing, dorsal funiculi-associated axon collaterals into the dorsal spinal cord. These proposed functional interactions might help explain, at least in part, why EphB-Fc affinity probes fail to detect ephrin-B protein in the mesenchyme outside and adjacent to the spinal cord. If, in fact, EphB receptors residing on these various axons are normally bound to ephrin-B1 expressed by adjacent mesenchymal cells, these particular ephrin-B1 molecules would be inaccessible to exogenously applied affinity probes.

Complementary and co-expression of EphB receptors and ephrin-B1

The immunohistochemical findings we report here are in general agreement with the results of our previous affinity probe binding analyses (Imondi et al., 2000). Both approaches reveal complementary patterns of receptor and ligand expression, with an interface between EphB and ephrin-B1 expression residing in an intermediate/dorsal domain of the spinal cord. The additional sites of EphB and ephrin-B1 expression that we describe in this study extend the complementarity to regions, which are immediately adjacent to the spinal cord. Specifically, a mesenchymal cell-associated ephrin-B1 domain abuts an EphB receptor-rich marginal zone along the lateral edges of the ventral spinal cord. In addition, EphB receptors located within the axons that compose the dorsal funiculus are located adjacent to a domain of ephrin-B1 expression in the dorsal spinal cord. Despite these complementary expression patterns, we also report here that EphB receptor and ephrin-B1 expression domains appear to overlap in a dorsolateral region of the spinal cord, located outside and adjacent to the ventricular zone (see Fig. 9). The intermingling of EphB proteins and ephrin-B1 that gives rise to a less than sharp interface in this region of the spinal cord likely reflects receptor and ligand co-expression within a variety of interneuron populations. Whether the apparently co-expressed EphB receptors and ephrin-B1 are segregated to distinct compartments within these cells (Marquardt et al., 2005) or whether functionally relevant *cis* interactions (Hornberger et al., 1999) occur between co-expressed EphB receptors and ephrin-B1 remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AP	Alkaline Phosphatase
CNS	Central Nervous System

DAB	3, 3' Diaminobenzedine
df	dorsal funiculi
dlf	dorsal longitudinal fasciculus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
drg	dorsal root ganglion
EB1	EphB1-Fc
eB1	ephrin-B1-Fc
EB2	EphB2-Fc
eB2	ephrin-B2-Fc
EB3	EphB3-Fc
eB3	ephrin-B3-Fc
EB4	EphB4-Fc
EB6	EphB6-Fc
FBS	Fetal Bovine Serum
fp	floor plate
GPI	Glycosylphosphatidyl inositol
HAT	Hypoxanthine, Aminopterin, Thymidine
HCF	Hybridoma Cloning Factor
hpf	hours post fertilization
HRP	Horseradish Peroxidase
IP	Intraperitoneal
KPBS	Potassium-Phosphate Buffered Saline
lf	lateral funiculi
mAb	Monoclonal Antibody
mlf	medial longitudinal fasciculus
mn	motor neurons
NCS	Newborn Calf Serum
NHS	Normal Horse Serum
np	neuropil
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
PFA	Paraformaldehyde
rp	roof plate
SAN	Spinal Accessory Nerve
SDS-PAGE	Sodium-Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis

TBS	Tris Buffered Saline
vc	ventral commissure
vf	ventral funiculi
vlf	ventral longitudinal fasciculus
vr	ventral root
vz	ventricular zone

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Figure 1. Characterization of mAb Efb1-3 and anti-EphB polyclonal antibodies

A. Photomicrograph of a transverse cryosection obtained from the forelimb level of an E13 rat embryo labeled with mAb Efb1-3. In the ventral spinal cord, mAb Efb1-3 labels the ventral funiculi (vf) and lateral funiculi (lf), which contain longitudinally projecting commissural axons, as well as motor neurons (mn) and their ventral roots (vr). Weaker mAb Efb1-3 labeling is detected on the ventral commissure (vc), which is comprised of commissural axons that project in the transverse plane through the floor plate. mAb Efb1-3 also labels the dorsal funiculi (df), which are comprised of longitudinally projecting sensory axons, and to a lesser extent, the dorsal root ganglia (drg). mAb Efb1-3 binding was detected with a fluorescein-conjugated goat antimouse IgG secondary antibody. Scale bar = 100µm in **A**. **B.** In an immunoblot analysis performed under reducing conditions, mAb Efb1-3 labeled EphB1-Fc (EB1), EphB2-Fc (EB2) and EphB3-Fc (EB3), but not EphB4-Fc (EB4) and EphB6-Fc (EB6). Each of the recombinant EphB-Fc fusion proteins (1 µg loaded per lane) are approximately 100–117kD and represents the extracellular domain of the particular receptor linked to human Ig Fc. The weakly labeled lower molecular weight bands in the EB1 and EB3 lanes are likely to be degradation products since they are more prominent in samples that have been subjected to repeated freeze-thaw cycles. mAb Efb1-3 does not recognize Fc alone (data not shown). mAb Efb1-3 binding was detected with a HRP-conjugated goat anti-mouse IgG secondary antibody. Specific signals were not detected when mAb Efb1-3 was omitted from the analyses. **C.** In an immunoblot analysis performed under reducing conditions, anti-EphB1, anti-EphB2 and anti-EphB3 polyclonal antibodies selectively bind EphB1-Fc (EB1), EphB2-Fc (EB2) and EphB3-Fc (EB3), respectively. The anti-EphB-positive proteins migrate at the same molecular weights as the corresponding mAb Efb1-3-positive bands in **B**. None of the anti-EphB antibodies recognized EphB4-Fc, EphB6-Fc or Fc alone (data not shown). For these immunoblots, 250 ng of a given recombinant EphB-Fc fusion protein was loaded per lane. Anti-EphB binding was detected with a HRP-conjugated rabbit anti-goat IgG secondary antibody. (**D–F**). Photomicrographs of serial transverse cryosections obtained from the forelimb level of an E13 rat labeled with anti-EphB1 (**D**), anti-EphB2 (**E**) or anti-EphB3 (**F**). All three antibodies label the ventral and lateral funiculi contained within the marginal zone (arrows), the ventral commissure (vc), motor neurons (mn) and the ventral roots (arrowhead). Both anti-EphB1 and anti-EphB3, but not anti-EphB2, brightly label the dorsal funiculi (df), whereas only anti-EphB2 labels the ventricular zone (vz). Anti-EphB binding was detected with a Cy3-conjugated donkey anti-goat IgG secondary antibody. Specific signals were not detected in the absence of primary antibody. Scale bar in **F** = 100 µm in **D–F**.

Figure 2. EphB receptors are dynamically expressed in the developing rat spinal cord (A–L). Photomicrographs of transverse cryosections obtained from the forelimb level of E11, E12 or E13 rat embryos labeled with mAb EfB1-3, anti-EphB1, anti-EphB2 or anti-EphB3. At E11 (A–D), both mAb EfB1-3 (A) and anti-EphB2 (C) label the ventricular zone (vz) and longitudinally projecting spinal accessory nerves (SAN; long arrows), which lie adjacent to and alongside the lateral exit points positioned midway along the dorsoventral axis of the spinal cord. No specific labeling was obtained with either anti-EphB1 (B) or anti-EphB3 (D). In the E12 (E–H) and E13 (I–L) spinal cord, each of the antibodies labeled the marginal zone (short arrows), ventral commissure (vc), motor neurons (mn) and, to some extent, ventrally-located pre-crossing commissural axons (arrowheads). mAb EfB1-3 (E, I), anti-EphB1 (F, J) and anti-EphB3 (H, L), but not anti-EphB2 (G, K), also label the dorsal funiculi (df) at these embryonic ages. mAb EfB1-3 binding was detected with a fluorescein-conjugated goat anti-mouse IgG secondary antibody. Anti-EphB binding was detected with a Cy3-conjugated donkey anti-goat IgG secondary antibody. Scale bar in L = 100 μ m (A–D); 120 μ m (E–H); 140 μ m (I–L).

Figure 3. EphB receptors are preferentially expressed on decussated commissural axons in E12.5 rat spinal cord

(A–E). Photomicrographs of semi-serial transverse cryosections obtained from the forelimb/trunk level of an E12.5 rat embryo labeled with either mAb EfB1-3 (A) or several well-established commissural axon markers (B–E). mAb EfB1-3 (A), anti-DCC (C), anti-L1 (D), and anti-GAD65 (E) label the marginal zone (arrows), which contains longitudinally projecting, decussated commissural axons. While each of these antibodies also labels motor neurons (mn) and the ventral commissure (vc), mAb EfB1-3 (A) does not significantly label pre-crossing commissural axons. In contrast, anti-L1 (D) and anti-GAD65 (E) label ventrally located pre-crossing commissural axons (arrowheads), whereas anti-TAG1 (B) and anti-DCC (C) label pre-crossing commissural axons that emanate from more dorsal regions of the spinal cord (arrowheads). Anti-GAD65 (E) and, to some extent, anti-DCC (C), conspicuously label small clusters of cell bodies located in ventral and intermediate regions of the spinal cord (asterisks). Primary antibody binding was detected with biotinylated secondary antibodies. Scale bar in A = 100 μ m (A–E).

Figure 4. EphB receptors are localized to longitudinally oriented axonal tracts in open book preparations of the developing rodent spinal cord (A–F). Open book preparations (see Materials and Methods) derived from E13 rat (A–C) and E12 mouse (D–F) spinal cord were labeled with mAb EfB1-3 (A, D), anti-L1 (B, E) and anti-TAG1 (C, F). In both the rat and mouse preparations, EphB receptors (A, D) are expressed on longitudinally projecting, decussated commissural axons contained within ventral funiculi (arrowheads) located immediately adjacent to the floor plate (fp). EphB receptors (A, D) are also expressed on decussated commissural axons that extend significant distances away from the floor plate and compose the lateral funiculi (arrows). In contrast, TAG1 (C, F) is expressed on pre-crossing commissural axons, which project in the transverse plane toward the floor plate. mAb EfB1-3 (A, D), anti-L1 (B, E) and anti-TAG1 (C, F) each label the dorsal funiculi (df). In the anti-L1 labeled E13 rat (B) and mAb EfB1-3 labeled E12 mouse (D) open-book preparations, intense labeling of the ventral funiculi obscures the unlabeled floor plate. Primary antibody binding was detected with AP-conjugated secondary antibodies. Omission of primary antibodies resulted in no specific labeling of the open-book preparations. Scale bar in F = 250 μ m (A–F).

Figure 5. EphB receptors are expressed on commissural axons that extend through the floor plate *in vitro*

(A–E). Half-open book explants derived from E13 rat spinal cord were cultured for 48 h and then labeled as whole mount preparations with mAb EfB1-3 (B), anti-L1 (C), anti- β tubulin (D) or anti-TAG1 (E). mAb EfB1-3 (B), anti-L1 (C) and anti- β tubulin (D) label axons that have grown through the floor plate (fp) and extended out of the explants. Anti-L1 (C) also labels longitudinally oriented axons (perpendicular to those that extend out of the explants) within the explant that presumably correspond to ipsilaterally projecting axons *in vivo*. Anti-TAG1 (E) selectively labels pre-crossing segments (confined to the explant) of transversely projecting axons that extend to the floor plate. Primary antibody binding was detected with fluorescein-conjugated secondary antibodies. Omission of primary antibodies resulted in no specific labeling of the cultured explants. Scale bar in E = 100 μ m (A–E).

Figure 6. mAb EfB1-3 labels the mouse, chick and zebrafish spinal cord (A–C). Photomicrographs of spinal cord-containing transverse cryosections obtained from E12 mouse (**A**), E5 chick (**B**) and 36 hours post fertilization (hpf) zebrafish (**C**) labeled with mAb EfB1-3. In both the mouse and chick spinal cord, mAb EfB1-3 (**A, B**) labels the marginal zone (arrows), ventral commissure (vc), dorsal funiculus (df) and a subset of pre-crossing commissural axons (arrowheads). mAb EfB1-3 also labels the ventricular zone (vz) in the chick (**B**), but not the mouse (**A**) spinal cord. In the zebrafish spinal cord (**C**), mAb EfB1-3 prominently labels the neuropil (np), which contains longitudinally projecting axons that compose the ventral longitudinal fasciculus (VLF, arrowhead), medial longitudinal fasciculus (MLF, arrowhead), and the dorsal longitudinal fasciculus (DLF, arrowhead). mAb EfB1-3 binding was detected with a fluorescein-conjugated goat anti-mouse IgG secondary antibody. Scale bar in **A** = 100 μ m (**A, B**) and 25 μ m (**C**).

Figure 7. Characterization of mAb efrnB1 and an anti-ephrin-B1 polyclonal antibody

A. Photomicrograph of a transverse cryosection obtained from the forelimb region of an E13 mouse embryo labeled with mAb efrnB1. mAb efrnB1 labels a dorsal region of the spinal cord that includes the roof plate (rp) and ventricular zone (vz). mAb efrnB1 immunoreactivity also surrounds the spinal cord and dorsal root ganglia (drg), where it is presumably associated with mesenchymal cells (arrowheads). mAb efrnB1 binding was detected with a Cy2-conjugated goat anti-Armenian Hamster IgG secondary antibody. Scale bar = 100 μ m in **A**. **B.** In an immunoblot analysis performed under non-reducing conditions, mAb efrnB1 labeled ephrin-B1-Fc (eB1), but not ephrin-B2-Fc (eB2) or ephrin-B3-Fc (eB3). Each of the recombinant ephrin-B-Fc fusion proteins (250 μ g loaded per lane) migrates at approximately 207 kD under non-reducing conditions and represents the extracellular domain of the particular ligand linked to human Ig Fc. The weakly labeled higher molecular weight bands in the eB1 lane are likely to be aggregates of ephrin-B1-Fc because the sample was not reduced or boiled. mAb efrnB1 does not recognize Fc alone (data not shown). The epitope recognized by mAb efrnB1 is destroyed in the presence of 0.2 M β -mercaptoethanol (reducing condition; data not shown). mAb efrnB1 binding was detected with a HRP-conjugated goat anti-Armenian Hamster IgG secondary antibody. No

specific signals were detected when mAb efrnB1 was omitted from the immunohistochemical and immunoblot analyses. **C.** In an immunoblot analysis performed under reducing conditions, anti-ephrin-B1 polyclonal antibody binds ephrin-B1-Fc (eB1; migrates at approximately 60kD under reducing conditions), but not ephrin-B2-Fc (eB2), ephrin-B3-Fc (eB3) or Fc alone (data not shown). For this immunoblot analysis, 100 ng of a given recombinant ephrin-B-Fc fusion protein was loaded per lane. Anti-ephrin-B1 binding was detected with a HRP-conjugated rabbit anti-goat IgG secondary antibody. **D.** Photomicrograph of a serial transverse cryosection obtained from the forelimb level of an E13 mouse embryo labeled with anti-ephrin-B1. Just as mAb efrnB1, anti-ephrin-B1 labels a dorsal region of the spinal cord that includes the roof plate (rp) and ventricular zone (vz), as well as mesenchymal cells (arrowheads) that surround the spinal cord and dorsal root ganglia (drg). Anti-ephrin-B1 binding was detected with a Cy3-conjugated donkey anti-goat IgG secondary antibody. Scale bar = 100 μ m in **D**. No specific signals were detected when the primary antibodies were omitted from the immunohistochemical and immunoblot analyses.

Figure 8. Ephrin-B1 is dynamically expressed in the developing mouse spinal cord (A–J). Photomicrographs of transverse cryosections obtained from the forelimb level of E10 (A, B), E11 (C, D), E12 (E, F), E13 (G, H) or E14 (I, J) mouse embryos labeled with mAb efrnB1 (A, C, E, G, I) or anti-ephrin-B1 (B, D, F, H, J). As detected by both mAb efrnB1 (A, C, E, G) and anti-ephrin-B1 (B, D, F, H), ephrin-B1 is expressed in the roof plate (rp) from E10–E13. While mAb efrnB1 also labels a dorsal region of the spinal cord (*) at E12 (E), anti-ephrin-B1 dorsal spinal cord labeling (*) is evident as early as E10 (D) and persists until E13 (H). Between E12 and E14, bright mAb efrnB1 (E, G, I) and anti-ephrin-B1 (F, H, J) immunoreactivity (arrowheads) surrounds the spinal cord and dorsal root ganglia (drg). At E14, mAb efrnB1 (I) and anti-ephrin-B1 (J) labeling of the spinal cord proper is

dramatically reduced. mAb efrnB1 and anti-ephrin-B1 binding was detected with a Cy2-conjugated goat anti-Armenian Hamster IgG and a Cy3-conjugated donkey anti-goat IgG secondary antibody, respectively. Scale bar in **J** = 100 μm (**A–D**); 130 μm (**E–H**); 160 μm (**I, J**).

Figure 9. Ephrin-B1 and EphB receptors are expressed in complementary domains within the E12 mouse spinal cord

(A–C). Photomicrographs of a transverse cryosection derived from the forelimb level of an E12 mouse embryo double-labeled with anti-ephrin-B1 to visualize ephrin-B1 expression and mAbEfB1-3 to detect the collective expression of EphB1, EphB2 and EphB3. Ephrin-B1 is expressed in the dorsal spinal cord (*), and by mesenchymal cells (arrowheads) that surround the entire spinal cord and dorsal root ganglia (drg). EphB receptors are mainly expressed in the ventral spinal cord by axon tracts that compose the marginal zone (arrows) and those that form the ventral commissure (vc). Despite these complementary expression patterns, some overlap between mAb EfB1-3 and anti-ephrin-B1 labeling is apparent in dorsolateral regions of the spinal cord (C). Anti-ephrin-B1 and mAb EfB1-3 labeling was detected with a Cy3-conjugated donkey anti-goat IgG and fluorescein-conjugated goat anti-mouse IgG secondary antibody, respectively. df = dorsal funiculi. Scale bar in C = 100 μ m (A–C).