Diversity of radial glial cells is formed along the dorso-ventral axis in the developing mouse spinal cord.

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Abbreviations

A/P	anterior-posterior
bHLH	basic helix-loop-helix
BCIP	X-phosphatate/5-bromo-4-chloro-3-indolyl-phospatate
BLBP	brain-lipid-binding protein
BSA	bovine serum albumin
CNS	central nervous systems
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DSHB	Developmental Studies Hybridoma Bank
D/V	dorso-ventral
E	embryonic days
GFAP	glial fibrillary acidic protein
GLAST	astrocyte-specific glutamate transporter
NBT	4-nitro blue tetrazolium chloride
PBS	phosphate-buffered saline

PCR polymerase chain reaction

PFA paraformaldehyde

RT room temrerature

VZ ventricular zone

ABSTRACT

The central nervous system (CNS) of the mammalian embryo is organized according to the expression of region-specific transcription factors along the anterior-posterior (A/P) and/or dorso-ventral (D/V) axis. For example, the dorsal ventricular zone (VZ) of the embryonic spinal cord expresses Pax3 and Pax7, the ventral VZ expresses Pax6, and the more ventral VZ expresses Nkx2.2. Properties of neuronal precursors located in the VZ are determined by the characteristic expression patterns of these transcription factors, leading to the generation of distinct classes of neurons. Recent studies demonstrated that radial glial cells produce neurons in addition to glia during CNS development. Thus, neuronal precursor diversity may reflect the diversity of radial glial cells. If the radial glial cells show diversity, astrocytes generated from them may also. To investigate this hypothesis, we analyzed the expression of radial glial cell markers and transcription factors in the mouse embryonic spinal cord. I show that radial glial cells vary in expression of the astrocyte-specific glutamate transporter (GLAST) and the brain-lipid-binding protein (BLBP) at embryonic day 12.5 (E12.5). The region where GLAST is strongly expressed in the ventral radial

glial cells is closely related to the Pax6-expressing domain, and the weakly expressed region corresponding to the Nkx2.2-expressing domain. Furthermore, dorsal radial fibers expressed ephrin-B1, where GLAST expression is negative. Thus, different types of radial glial cells exist according to the transcription factors at E12.5. Since GLAST is considered to be expressed when radial glial cells are generating astrocytes, there may also be diversity in glial precursor cells along the D/V axis in the mouse spinal cord.

INTRODUCTION

Most radial glial cells are present only transiently in the embryonic central nervous system (CNS). These cells are identified morphologically by their long radial fibers extending toward the pia mater and their cell bodies located in the ventricular zone (VZ)(Fig. 1) (Schnizer et al., 1981; Liuzzi and Miller, 1987). In fetal human and macaque cerebra, these cells exhibit glial characters, such as expression of glial fibrillary acidic protein (GFAP) and possession of glycogen granules (Choi et al., 1978 and 1986; Levitt et al., 1980; Rakic, 2003; deAzevedo et al., 2003). Radial glial cells have also been shown to differentiate into cells of the astrocyte lineage after an active neuronal migration period (Schmechel et al., 1979; Voigt, 1989; Misson et al., 1991; Yang et al., 1993). Therefore, it is conceivable that radial glial cells are a specialized cell type belonging to the astrocyte lineage.

The preexisting view was that neurons are generated from neuronal precursor cells that are distinct from radial glial cells, and newly generated neurons migrate along radial glial fibers (Fig. 2A) (for review, see Bentivoglio et al., 1999; Rakic, 2003). The VZ is a configured domain structure divided by the expression of

different homeodomain and basic helix-loop-helix transcription factors. Domain-specific transcription factors control the differentiation of neuronal precursor cells into specific types of neurons. For example, the developing spinal cord is broadly divided into dorsal and ventral domains. Pax7 is expressed in the dorsal spinal cord (Fig. 2A-a and 3), and Pax6 is expressed in the ventral spinal cord (Fig. 2A-b and 3) (Goulding et al., 1993; Tanabe et al., 1996; Mansouri et al., 1998). The VZ is divided into six domains (dp1-6) in the dorsal half and five domains (p0-2, pMN and p3) in the ventral half of the developing spinal cord (for review, see Tanabe et al., 1996; McMahon et al., 2000; and Helms et al., 2003). This heterogeneity in the VZ is thought to be formed by the neuronal precursor cells in the VZ—not by the radial glial cells (Fig. 2A). In fact, radial glial cells, as well as the astrocytes they generate, have been considered to be homogenous.

Recent studies established that radial glial cells generate not only glial cells but also neurons. By retroviral vector (Noctor et al., 2001), adenoviral vector (Tamamaki et al., 2001), or DiI (Miyata et al., 2001; Götz et al., 2002) labeling methods, radial glial cells were labeled, and their linage was traced in the developing cortex for a short term. These experiments confirmed the presence of

labeled neurons, indicating that some neurons are derived from radial glial cells. Thus, during cortical neurogenesis, radial glial cells play at least two roles—to serve as neuronal precursor cells and to guide neuronal migration. In a long-term, lineage-tracing experiment using retroviral vectors (Reid et al., 2002) or Cre-recombinase-mediated chromosomal labeling expressed from the human GFAP promoter in radial glial cells (Malatesta et al., 2003), both neurons and glial cells are labeled; thus, these two cell populations are derived from radial glial cells in the cortex. In the developing CNS, gliogenesis follows neurogenesis. Therefore, these results show that there are two stages during radial glial cell development: a neurogenic stage (committed to generate neurons and to guide neuronal migration; neurogenic radial glial cells) (Weissman et al., 2003) and a gliogenic stage (committed to generate glial cells; gliogenic radial glial cells). If this also happens in the spinal cord, the cells in the VZ responsible for the formation of domains encoded by the transcription factors would be neurogenic radial glial cells, and should have distinct characteristics according to the domain structure in the developing spinal cord.

It is unclear whether radial glial cells in the spinal cord exhibit diversity

depending on domain-specific transcription factors, as for neuronal precursor cells. Domain-specific transcription factors influence differentiation of neuronal as well as glial precursor cells within the domain. For example, the basic helix-loop-helix transcription factor Olig2 is located at the pMN domain in the spinal cord, and this factor determines the fate of motoneurons and oligodendorocytes (Lu et al., 2002; Zhou et al., 2002; Takebayashi et al., 2002). If radial glial cells express region-specific transcription factors, they should have distinct characteristics depending on where they are located (Fig. 2B). Thus, the aims of this study were (1) to investigate whether the region-specific transcription factors that define the domain structure of the developing spinal cord are expressed in radial glial cells, and (2) to investigate whether this diversity continues to be present in the following gliogenic stage of radial glial cells.

Radial glial cells can be identified by several markers. RC1 and RC2 are the most frequently used monoclonal antibodies for immunohistochemical analysis, identifying almost all radial glial cells (Misson et al., 1988ab). Brain-lipid-binding protein (BLBP) and the astrocyte-specific glutamate transporter GLAST is expressed in a subpopulation of radial glial cells (Hartfuss et al., 2001). In rodent

spinal cord, the GLAST gene starts to be expressed in radial glial cells at a later stage of development, when gliogenesis starts, and continues to be expressed in astrocytes (Shibata et al., 1997). Therefore, it is conceivable that GLAST is expressed in the radial glial cells committed to glial cell lineage in the spinal cord (gliogenic radial glial cells).

In this report, I show that radial glial cells exhibit region-specific characteristics. At embryonic day 12.5 (E12.5), region-specific transcription factors Pax7 (dorsal spinal cord) and Pax6 (ventral spinal cord) were located in the nucleus of dorsal and ventral radial glial cells, respectively. GLAST was expressed only in the Pax6-positive radial glial fibers at E12.5. The Pax7-positive radial glial cells located in the dorsal spinal cord did not express GLAST at E12.5, but they expressed ephrin-B1. Thus, the expression patterns of GLAST and ephrin-B1 were complementary. Ephrin-B1 and GLAST are cell surface proteins. The ephrin/Eph system shows bidirectional signaling (for review, see Kullarnder et al., 2002). Therefore, these results imply that radial glial cells not only have an effect on the extracellular environment, but also are influenced by their extracellular environment in a distinct manner through the cell surface proteins expressed in a

domain-specific manner.

EXPERIMENTAL PROCEDURES

Materials:

ICR mice were purchased from Japan SLC, Inc (Hamamatsu). Sey mice were provided from Dr. N. Osumi (Tohoku University, Sendai, Japan). The day of detection of a vaginal plug was considered embryonic day 0.5 (E0.5). Mouse ephrin-B1 was amplified by PCR and cDNA cloned into pBluescript (gene accession number, Z48781; nucleotide position 225-1141; Bouillet et al., 1995) and mouse GLAST cDNA (gene accession number, AF330257; nucleotide position 1746-2478; Tanaka, 1993) were used for in situ hybridization study. RC2 monoclonal antibody (Misson et al., 1988a) was a gift from Dr. M. Yamamoto (Tsukuba University, Tsukuba, Japan). Polyclonal anti-GLAST antibody (Shibata et al., 1997) and anti-BLBP antibody (Yamada et al., 2000) were gifts from Dr. M. Watanabe (Hokkaido University, Sapporo, Japan). Polyclonal anti-Pax6 antibody (Inoue et al., 2000) was a gift from Dr. N. Osumi (Tohoku University, Sendai, Japan). Monoclonal anti-Pax7 antibody and anti-Nkx2.2 antibody 74.5A5 were obtained from Developmental Studies Hybridoma Bank (DSHB) in University of

Iowa (IA, USA). Polyclonal anti-ephrin-B1 antibody was purchased from R&D systems (MN, USA). Alexa 488 conjugated anti-mouse IgG (cat #A-11029), Alexa 633 conjugated anti-mouse IgG (cat #A-21052), Alexa 488 conjugated anti-mouse IgM (cat #A-21042), Alexa 488 conjugated anti-rabbit IgG (cat #A-11008), Alexa 594 conjugated anti-rabbit IgG (cat #A-11012), Alexa 594 conjugated anti-guinea pig IgG (cat #A-11076) and Alexa 594 conjugated anti-goat IgG (cat #A-11080) were purchased from Molecular Plobes (OR, USA). Cy5 conjugated anti-mouse IgM (cat #AP128S) was purchased from CHEMICON (CA, USA). DIG labeling mix (cat #1277 073), proteinase K (cat #1373 196), alkaline phosphatase conjugated anti-DIG antibody (cat #1093 274), NBT (cat #1383 213), BCIP (cat #1383 221) were purchased from Roche (Mannheim, Germany). Tissue-Tek OCT compound (cat #4583) was purchased from SAKURA (Tokyo, Japan). General reagents were purchased from Wako Pure Chemicals Industries (Osaka, Japan).

Procedures:

Immunohistochemistry

Spinal cords of E12.5 to E14.5 mouse embryos were fixed with 4%

paraformaldehyde (PFA) in PBS for 3 hours at 4˚C, cryoprotected with 20% sucrose, embedded in Tissue-Tek O.C.T. (SAKURA), and frozen on dry ice powder. Blocks were cut using a cryostat (Leica) to obtain 25 μm sections, and the sections were placed on APS-coated slides (Matsunami). Sections were washed with phosphate-buffered saline (PBS) and blocked with blocking buffer (10% normal goat serum, 0.3% Triton X-100 in PBS) for 1 hour at room temperature (RT). After blocking, sections were stained at 4˚C overnight with the following primary antibodies: mouse monoclonal anti-Pax7 antibody (1:20), rabbit polyclonal anti-Pax6 antibody (1:1000), mouse monoclonal anti-Nkx2.2 antibody (1:50), goat polyclonal anti-ephrin-B1 antibody (1:100), mouse monoclonal anti-RC2 antibody (1:10), and rabbit or guinea pig polyclonal anti-GLAST antibody (1:2000, Shibata et al., 1997). For detection of primary antibodies, the sections were then incubated for 2 hours at RT with the following secondary antibodies: donkey-anti-goat IgG Alexa-594 (1:2000), goat-anti-rabbit IgG Alexa-488 (1:2000), goat-anti-rabbit IgG Alexa-594 (1:2000), goat-anti-mouse IgG Alexa-488 (1:2000), goat-anti-mouse IgG Alexa-594 (1:2000), goat-anti-mouse IgG Alexa-633 (1:2000), goat-anti-mouse IgM Alexa-488 (1:2000), goat-anti-guinea pig IgG Alexa-594

(1:2000), anti-mouse IgM Cy5 (1:100), or goat-anti-mouse IgG Cy5 (1:100). Slides were mounted with 80% glycerol and covered with coverslips (Matsunami). Images were obtained using confocal laser microscope (Zeiss, LSM-510).

In situ hybridization

The distribution of mRNAs was visualized by *in situ* hybridization (ISH) using DIG-labeled cRNA antisense probes. Mouse ephrin-B1 cDNA and mouse GLAST cDNA were cloned into the pBluescript vector and used for riboprobe synthesis. For ISH, I used a modification of the methods described by Wilkinson (1992). Samples were fixed overnight in 4% PFA/PBS (pH 7.4) (4% PFA) at 4°C and washed 2 times for 10 minutes in PBS. The sections were air-dried prior to fixation in freshly prepared 4% PFA. The slides were rinsed with DEPC-PBS and incubated in proteinase K solution (1 μg/ml in 50 mM Tris-HCl (pH 7.6) and 0.5 mM EDTA) for 5 minutes at RT. After digestion, the sections were refixed in 4% PFA and 0.1% glutaraldehyde (Sigma) and acetylated.

The slides were then rinsed in DEPC-PBS, placed in prehybridization buffer for at least 1 hour at 65°C, and hybridized overnight. After hybridization, the slides

were washed with 2X SSC to remove excess probe, blocked in blocking buffer (20% sheep serum, 2% BSA, 0.1% Triton X-100) and incubated in anti–digoxygenin –AP-conjugated antibody at 4˚C overnight. The slides were rinsed to remove nonspecific antibody binding, and the color was developed using NBT/BCIP as a substrate.

RESULTS

RC2-positive radial glial cells expressed domain-specific transcription factors

In the developing spinal cord, Pax7 is expressed in the dorsal domains, and Pax6 is expressed in the ventral domains. To analyze whether radial glial cells express domain-specific transcription factors, I immunolabeled radial glial cells with RC2 and studied whether region-specific transcription factors are present in RC2-positive cell bodies.

The RC2 antigen was expressed in radial glial cells of all regions of the mouse spinal cord at E12.5 (Fig. 4A). Pax7 was expressed in the dorsal half of the VZ, and Pax6 was expressed in the ventral half of VZ (Fig. 4A). These transcription factors were localized in the nuclei of RC2-immunopositive cell bodies (Fig. 4B and C). Therefore, RC2-positive radial glial cells were expressing transcription factors localized along the D/V axis.

GLAST-positive radial fibers were located in the ventral domains

GLAST expression was detected in the radial glial fibers of the ventral spinal cord

at E13.5 and expanded into the entire spinal cord at later stages (Shibata et al., 1997). To examine whether GLAST expression patterns are associated with the domain structure, I compared the expression pattern of region-specific transcription factors with that of GLAST.

Immunohistochemical study of the E12.5 mouse spinal cord showed that GLAST was present in the ventral radial glial fibers (Fig. 5A), and *in situ* hybridization analysis showed that GLAST-expressing cell bodies were located in the VZ (Fig. 5B).

The location of GLAST-positive radial glial cells in the ventral region overlapped closely with Pax6-expressing domains, but did not colocalize with the Pax7-expressing domain at E12.5 (Fig. 6A). At a higher magnification, GLAST-positive radial glial cell bodies in the ventral regions were also positive for Pax6 (Fig. 6B). However, GLAST expression was very weak in a narrow region above the floor plate, a region that was identified to be the Nkx2.2-expressing domain known as the p3 domain (asterisks in Fig. 6C, D).

At E14.5, GLAST-positive radial glial fibers were detected both in the dorsal and ventral spinal cord (Fig. 7A), however, region specific transcription factors

were still expressed in both regions (Fig. 7A, B, D and F). Thus region specific expression of GLAST was lost at E14.5, as described previously (Shibata et al., 1997), while the domain structure still existed.

Ephrin-B1-positive radial fibers were located in the dorsal domains

The GLAST signal was found in the ventral domains of the mouse spinal cord at E12.5, but not in the dorsal domains. To search for a molecule whose expression is restricted to the radial glial cells in the dorsal domains of the spinal cord, I examined the localization of ephrin-B1, which was known to be restricted to the VZ by *in situ* hybridization (Imondi et al., 2000).

Immunohistochemical analysis showed that ephrin-B1 was present in the radial glial fibers in the dorsal domains of spinal cord, so that ephrin-B1 signals were complimentary to the GLAST signals in the spinal cord at E12.5 (Fig. 8A and B). Localization of ephrin-B1 in the dorsal radial fibers was confirmed by double-labeling with RC2 (Fig. 9A and B), and ephrin-B1-expressing cell bodies were located in the dorsal ventricular zone by *in situ* hybridization analysis (Fig. 9C).

Ephrin-B1 positive cells colocalized with Pax7 positive cells in the dorsal spinal

cord at E12.5 (Fig. 10A). A higher magnification demonstrated that ephrin-B1-positive radial glial cells in the dorsal region contained Pax7-positive nuclei (Fig. 10B). Radial fibers and the VZ of Pax7-negative domains were ephrin-B1 negative (Fig. 10A).

These results demonstrate that there is diversity in radial glial cells in the mouse spinal cord at E12.5. However, it was still unclear whether the ephrin-B1-expressing radial glial cells belonged to the same linage as the GLAST-expressing radial glial cells found in the dorsal spinal cord at E14.5.

At later stages, ephrin-B1-positive radial glial cells began to express GLAST

To determine whether ephrin-B1-positive radial glial cells in the dorsal region belong to the same lineage as the GLAST-positive radial glial cells at E14.5, ephrin-B1 and GLAST expression were studied at E13.5.

Weak GLAST signals were detected in the radial fibers of the dorsal spinal cord at E13.5 (Fig. 11A). Cell bodies and proximal radial fibers were double-positive for ephrin-B1 and GLAST (Fig. 11B-D). At E14.5, ephrin-B1 expression was no longer detectable in the spinal cord (data not shown).

These results clearly demonstrated that at least some of the ephrin-B1-positive radial glial cells at E12.5 began to express GLAST at E13.5 and down-regulate ephrin-B1-expression by E14.5. Therefore, the ephrin-B1-positive radial glial cells are the direct precursors of the glialcommitted (GLAST-positive) radial glial cells.

BLBP was strongly expressed in the p3 domain where GLAST-expression was weak.

I have shown that GLAST expression was weak in p3 domain at E12.5 (Fig. 6). I searched for a molecule that was expressed in this region, like the ephrin-B1 in the dorsal spinal cord. In the developing brain, BLBP is another marker that detects radial glial cells and shows heterogeneity in its expression (Hartfuss et al., 2001). Previous studies have shown that BLBP is required for the establishment and maintenance of the radial glial fiber system in the developing brain (Feng et al., 1994; Kurtz et al., 1994). Therefore, to analyze whether BLBP-expression pattern is different from GLAST expression pattern according to the domain structures defined by region specific transcription factors expression, I compared

BLBP-expression pattern with that of GLAST in the mouse spinal cord at E12.5.

BLBP was expressed intensely and homogeneously in the radial fibers of ventral spinal cord, similarly to the GLAST-expression. BLBP was expressed also in the dorsal spinal cord with a gradient formed from roof plate to the ventral border of dorsal domain (Fig. 12B and C). GLAST signal was weak in the p3 domain (Fig. 6D and Fig. 12D), but BLBP was expressed as strongly as in other ventral domains (Fig. 12E and F). Nkx2.2 is expressed in the p3 domain, and the cell bodies of the BLBP-positive radial glial fibers (RC2+) contained Nkx2.2 nuclei (Fig. 13E and F).

Thus, radial glial cells independently expressed Pax7, Pax6, or Nkx2.2 in E12.5 mouse spinal cord, and the characteristics of the radial glial cells (including their fibers) were distinct among the three. Pax7-positive radial glial cells were mostly ephrin-B1 (+)/GLAST (-). Gradient expression of BLBP suggests further subtyping of Pax7 (+) radial glial cells. Pax6-positive radial glial cells were mostly GLAST (+)/ BLBP (+)/ ephrin-B1 (-), except for the dp6 domain, as described above. Nkx2.2-positive cells were ephrin-B1- and GLAST-negative and BLBP-positive.

The GLAST expression pattern did not change in Pax6 mutant mice

GLAST was expressed in Pax6-positive cells; therefore, we analyzed GLAST expression in the spinal cord of the Pax6 mutant mice, Small eye (*Sey*). Contrary to our expectation, the GLAST expression pattern was not changed in these mice (Fig. 14A, B); the boundary between GLAST and Pax7 was similar to that in the wild-type mice (Fig. 14C).

DISCUSSION

In this study, I have shown that radial glial cells express region-specific transcription factors that define the domain structure of the developing spinal cord, and that this diversity continues to be present in the following gliogenic stage of radial glia.

Diversity of radial glial cells along the dorso-ventral axis

Neuronal precursors located in the VZ of the embryonic spinal cord express region-specific transcription factors that define the domain structure. These transcription factors determine cell fate and contribute to the generation of diverse neuronal subtypes by regulating downstream genes (for review, see Tanabe et al., 1996; McMahon, 2000; Helms et al., 2002). For example, VZ cells in the dp4 and dp5 domains in the dorsal embryonic spinal cord are defined by Mash1 and Pax7 expression, and migrating postmitotic neuronal precursors, called dI4 and dI5, express homeodomain factor, Lbx1 (Helms et al., 2002).

I showed that radial glial cells labeled by RC2 were present in the entire spinal cord and expressed region-specific transcription factors, such as Pax6 and Pax7

(Fig. 4). These data are consistent with the recent paradigm change that radial glial cells have neurogenic potential in the developing cortex, and suggest that radial glial cells in the spinal cord also have neurogenic potential. Furthermore, they raise the possibility that radial glial cell diversity may influence glial cell fate at later stages. Therefore, I searched for proteins that are regionally expressed in radial glial cells and found that ephrin-B1 is expressed in the radial glial cells of the dorsal spinal cord at E12.5. GLAST had already been reported to be expressed in the ventral spinal cord at E12.5 (Shibata et al., 1997). I found that ephrin-B1 and GLAST are complimentarily expressed in the spinal cord at this stage. Interestingly, ephrin-B1-positive radial glial cells were located in the Pax7-expressing domain (dp1-6) and roof plate (Fig. 10A and 15), while strongly GLAST-positive radial glial cells were located in Pax6-expressing domains (dp6 and p0-pMN) (Fig. 6A and 15) and the floor plate, and weakly positive cells were located in the Nkx2.2-expressing domain (p3) (Fig. 6C, D and 15). BLBP-positive radial glial fibers were present homogeneously in the Pax6/Nkx2.2-expressing domains (dp6 and p0-p3) and have gradient signals in the dorsal spinal cord of Pax7-expressing domains (dp1-6) (Fig. 12B and 15). These results

demonstrated that radial glial cells express transcription factors and proteins specific for each domain. From the roof plate, radial glial cells were divided in RC2/ephrin-B1 (BLBP gradient signal)-positive regions (dp1-6), RC2/GLAST/BLBP-positive regions (dp6 and p0-pMN), and a RC2/ GLAST (weak)/BLBP-positive region (p3) at E12.5 (Fig. 15). Therefore, radial glial cells have diversity according to the region-specific transcription factors they express. Previously, neuronal precursors distinct from the radial glial cells (Fig. 2A) had been considered to exhibit this diversity.

One candidate transcription factor that regulates GLAST expression was Pax6, because the GLAST-positive region was closely associated with the Pax6-positive region. To investigate this possibility, I examined GLAST expression in the spinal cord of *Sey* mice (Pax6 mutant mice). Contrary to my expectations, the GLAST-expressing region remained in the ventral domains, as in the wild-type mice. Therefore, GLAST expression was not dependent on Pax6, but it is conceivable that GLAST coexpression was dependent on other transcription factor(s) coexpressed in the Pax6 domain.

At later stages, GLAST immunoreactivity expanded to entire spinal cord,

thereby, restriction of GLAST expression in the Pax6-expressing domain was lost (Fig. 7A). Shibata et al. (1997) showed that GLAST was continuously expressed in the radial glial cell-astrocyte lineage. Thus, GLAST-positive radial glial cells can be classified as "gliogenic radial glial cells." I showed that ephrin-B1-positive radial glial cells were restricted to the dorsal spinal cord and were complementary to GLAST-positive cells at E12.5 (Fig. 9A). They begin to express GLAST in the dorsal spinal cord at E13.5 (Fig. 11). Thus, GLAST-positive radial glial cells can be divided into two types: ephrin-B1 positive type or ephrin-B1 negative type.

Furthermore, my result showed that GLAST was expressed at pMN domain but weak at p3 domain (Fig. 15). Recent studies show that oligodendrocyte progenitor have two origins, Olig2-positive pMN domain and Nkx2.2-positive p3 domain, in the spinal cord at early stage, and each oligodendrocyte progenitor expresses different markers, Olig2+/PDGFRα+ and Nkx2.2+/PDGFRα- (Fu H. et al., 2002) respectively. Therefore, it is conceivable that the oligodendrocyte progenitor derived from each radial glial cell type have distinct domain-specific characters.

Possible roles of membrane proteins differentially expressed in radial glial cells

Ephrin-B ligands interact with Eph receptors, resulting in intracellular bidirectional signaling (Fig. 16). Therefore, the presence of ephrin-B1 on the dorsal radial glial fibers implies that intracellular signaling through the ephrin-B1 plays some roles in dorsal radial glial cell development when ephrin-B1 binds to Eph receptors. The intracellular domain of ephrin-B ligands contains five conserved tyrosine residues, which is important for reverse signaling from Ephs to ephrins. It is unclear which subtype of Eph receptors binds with ephrin-B1 in the dorsal spinal cord. Ephrin-B1 can bind with high affinity to Eph receptors, EphB1, EphB2, EphB3, and EphA4. EphB1 mRNAs are found in the ventral domains at E12 (Imondi et al., 2000). EphB2 (Imondi et al., 2000) and EphB3 (data not shown) mRNAs are found in both the dorsal and ventral VZ of the E12.5 mouse spinal cord. EphA4 is expressed in the ventral horn, including motor neurons and the dorsal medial area at E12 (Greferath et al., 2002). It is possible that ephrinB1 binds to EphB2, EphB3, and/or EphA4 at E12.5 in the spinal cord.

Ephrin ligands and Eph receptors are involved in vascular development, tissue-border formation, cell migration, and axonal guidance (for review, see Kullander et al., 2002; Huynh-Do et al., 2002). Since ephrin-B1-positive radial glial fibers were restricted to the dorsal half of the spinal cord at E12.5, ephrin-B1 should play a dorsal-specific role during development.

Dorsal radial glial fibers receive signaling via ephrin-B1 from Eph receptors, which should occur specifically in the dorsal spinal cord. Interestingly, a recent study showed that CD44 (HCAM) is expressed in astrocytes of the dorsal spinal cord (Liu et al., 2002) from the embryonic stage to the newborn stage in rats, which is consistent with previous data in chicks (Alfei et al., 1999). My present results, together with the above-mentioned results, suggest that dorsal astrocytes possess characteristics distinct from those of ventral astrocytes.

Diversity of radial glial cells and heterogeneity of astrocytes in the adult CNS

Classically, astrocytes are divided into two types by morphologic features at the adult stage. Fibrous astrocytes have a stellate, process-bearing morphology and contain large numbers of intermediate filaments in the cell body, whereas

protoplasmic astrocytes have sheetlike processes associated with neurons and relatively few intermediate filaments in their cell bodies (Vaughn et al., 1967; Mori et al., 1969). Recent studies have shown that astrocytes have functional heterogeneity in the adult mammalian spinal cord and other parts of the CNS (Wilkin et al., 1990; Miller et al., 1994; Song et al., 2002). For example, neurosphere cultures treated with ventral mesencephalon astrocyte-conditioned medium give rise to many more spheres with high densities of tyrosine hydroxylase (a maker of dopaminergic neuron)-positive cells and neurites (for review, see Hall et al., 2003). In this experiment, cultured astrocytes did not lose regional information, and regional diversities of astrocytes influenced region-specific neuronal phenotypes.

There are two possible mechanisms for generating heterogeneity in astrocytes in the adult brain: 1) glial cells may have diversity depending on their generated domains like neurons, and 2) astrocytes are produced without specific characteristics, and later ocome to exhibit specific characteristics depending on the region where they settle. My data show that the regional diversity of radial glial cells is tightly associated with region-specific transcription factors. Since radial

glial cells transform into astrocytes at later stages (Schmechel and Rakic, 1979; Voight, 1989; Misson et al., 1991; Yang et al., 1993), the diversity of radial glial cells may influence glial development at later stage. Oligodendrocytes are produced from a restricted region of the ventral VZ called the pMN domain under the control of Olig2 and are a good example of the generation of a specific type of glial cell tightly regulated by a specific transcriptional factor (Lu et al., 2002; Zhou et al., 2002; Takebayashi et al., 2002). A recent report showing that region-specific transcription factors, such as Nkx6.1 and Pax6, continue to be expressed in the VZ of the developing spinal cord at least until E18 (Fu et al., 2003) also supports this idea. Although I cannot rule out the latter possibility, it is conceivable that, at least in part, the diversity of radial glial cells will influence the diversity of astrocytes during development.

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FIGURE LEGENDS

Fig. 1. Radial glial cells in the mouse spinal cord are labeled with RC2 at E10.5. RC2 positive cell bodies are located in the VZ, and radial glial fibers extended to the pia matter.

Fig. 2. Schematic representation of diversity in the developing spinal cord. Model A: Pre-existing view of neural development in the spinal cord. Radial glial cells guide the migration of neuronal precursors at the stage of neurogenesis and later transform into astrocytes, but do not generate neurons. Radial glial cells are drawn in dark blue (A-a, A-b, A-c, and A-d). Neuronal precursors are drawn in light blue (A-a, dorsal) or blue (A-b, ventral). Glial cells are drawn in pink. From neurogenesis to gliogenesis, homogeneous radial glial cells are generated throughout the spinal cord. During neurogenesis, neuronal precursors are located in the VZ among the radial glial cells. These two populations are distinct. Neuronal precursors have diversity depending on region-specific transcription factors, for example dorsal neuronal precursors (A-a, light blue) express dorsal-specific transcription factors, and ventral neuronal precursors (A-b, blue) express ventral-specific transcription factors. These neuronal precursors migrate along radial glial fibers (locomotion). Later, during gliogenesis, radial glial cells transform into astrocytes (A-c and A-d).

Model B: A newly proposed model. Radial glial cells play two roles in the stage of neurogenesis—one in guidance of neuronal migration, and the other in

generation of neurons. Thus, in this model, radial glial cells are the neuronal precursors themselves. Radial glial cells express transcription factors depending on the domains in which they are located, and exhibit regional diversity; drawn in purple (B-e, dorsal) and in blue (B-F, ventral). Some of the daughter cells generated from neurogenic radial glial cells inherit the radial glial fibers and use "somal translocation" (Nadarajah et al., 2001) to reach their final destination. Other neuronal precursors locomote along the radial glial fibers. During gliogenesis, radial glial cells have diversity depending on the domains in which they are located, drawn in red (B-g, dorsal) and in the dark red (B-h, ventral).

Fig. 3. Diagram of an embryonic spinal cord showing the organization of domain structure and neuronal populations. Neuronal precursors are located in the VZ and defined by expression of bHLH and/or homeodomain factors. The VZ is divided into six domains (dp1-6) at the dorsal half and five domains (p0-2, pMN and p3) at the ventral half of developing spinal cord. Out of ventricular zone, distinct subtypes of interneurons and motoneurons (MN) can be identified by transcription factors. Pax7 is highly expressed in the dorsal domains of dp4 and 5, and at a low level in dp1-3 and 6. Pax6 is expressed in the dp6 and the ventral domains of and p0-2, and at low level in pMN domains. Nkx2.2 is expressed in p3 domain, and Olig2 is expressed in pMN domain.

Fig. 4. Region-specific transcription factors were expressed in the radial glial cells of the E12.5 spinal cord. RC2 (blue, A-C), Pax7 (green, A and B), and Pax6 (red, A and C) were detected immunohistochemically in transverse sections of the E12.5 spinal cord. RC2 (blue) stained radial fibers in all regions of the spinal cord (A). Pax7 (green) was expressed in the VZ of dorsal domains (A and B), and Pax6 was expressed in the VZ of ventral domains (A and C). B and C represent higher magnifications of A. Both Pax7- and Pax6-immunoreactive nuclei were closely apposed to RC2-positive radial glial fibers (B and C). Scale bars: 200 μm (A), 20 μm (B and C).

Fig. 5. GLAST expression in radial glial cells of the E12.5 mouse spinal cord. Expression of GLAST was detected immunohistochemically (A) or by *in situ* hybridization analysis (B) in transverse sections of the E12.5 spinal cord. GLAST-positive radial fibers were restricted to the ventral spinal cord (A). Strong *GLAST* signals were detected in the VZ of the ventral domains and weak signals were detected in the VZ of the dorsal domains by *in situ* hybridization (B). Scale bars: 200 μm (A and B).

Fig. 6. Immunohistochemical analysis of GLAST and region specific transcription factor expression in the E12.5 mouse spinal cord. Expression of GLAST (red, A-D), Pax7 (blue, A), Pax6 (green, A and B) and Nkx2.2 (green, D) was detected immunohistochemically in transverse sections of the E12.5 spinal cord. GLAST-positive domains (red, A and B) overlapped with Pax6-positive domain (green, A and B), but not with Pax7-positive domains (blue, A). B represents a higher magnification of A. GLAST-positive radial glial cell bodies (red, B) contained Pax6-positive nuclei (green, B). Weakly GLAST-positive domain (asterisks, red, C and D) corresponded to the Nkx2.2-expressing p3 domain (green, D). Scale bars: 100 μm (A, C and D), 20 μm (B)

Fig. 7. Immunohistochemical analysis of GLAST and region specific transcription factor expression in the E14.5 mouse spinal cord. Expression of GLAST (red, A, B, D and F, white, C, E and G), Pax6 (green, A and B), Pax7 (green, D) and Nkx2.2 (green, F) was detected immunohistochemically in transverse sections of the E14.5 spinal cord. At E14.5, GLAST signal extended dorsally into entire spinal cord (red, A). Pax6 (green, A and B), Pax7 (green, D) and Nkx2.2 (green, F) were expressed similarly as in E12.5 spinal cord. In the VZ, restricted expression of GLAST observed at E12.5 was lost at E14.5 (C, E and G). Scale bars: 200 μm (A), 100 μm (B-G)

Fig. 8. Ephrin-B1 and GLAST expression in the E12.5 mouse spinal cord. Expression of GLAST (green) and ephrin-B1 (red) was detected immunohistochemically in transverse sections of the E12.5 spinal cord (A and B). Ephrin-B1-positive fibers (red) were located complementary to the GLAST positive fibers (green) at E12.5 (A and B). B represents a higher magnification of A. Scale bars: 200 μm (A), 100 μm (B)

Fig. 9. Expression of ephrin-B1 detected immunohistochemically and by *in situ* hybridization in analysis the E12.5 mouse spinal cord. Expression of ephrin-B1 (A and B) and RC2 antigen (green, B) was detected immunohistochemically on transverse sections of the E12.5 mouse spinal cord, or by *in situ* hybridization (C). Ephrin-B1 was present the radial fibers of the dorsal spinal cord at the E12.5 (A). Ephrin-B1 (red) colocalized with the RC2 antigen (green) on the radial fibers (yellow, B). *Ephrin-B1* signals obtained by *in situ* hybridization were observed in the VZ of the dorsal domains (C). Scale bars: 200 μm (A, C), 20 μm (B)

Fig. 10. Immunohistochemical analysis of ephrin-B1 and region specific transcription factor expression in the E12.5 mouse spinal cord. Expression of ephrin-B1 (red) and Pax7 (green) was detected immunohistochemically in transverse sections of the E12.5 spinal cord (A and B). Ephrin-B1 (red) was detected in the dorsal domains, whose ventral border corresponded to the Pax7-positive domain (green) (A). B represents a higher magnification of A. Ephrin-B1-positive radial glial cell bodies (red) contained Pax7-positive nuclei (green) (B). Scale bars: 100 μm (A), 20 μm (B)

Fig. 11. Immunohistochemical analysis of ephrin-B1 and GLAST expression in the E13.5 mouse spinal cord. Expression of ephrin-B1 (red, A, B and D), GLAST (green, A-C) was detected immunohistochemically in transverse sections of the E13.5 spinal cord. GLAST signal extended to the dorsal spinal cord (red, A-C). B-D represent higher magnification of the boxed area in A. Ephrin-B1-positive radial fibers began to express GLAST in the dorsal domains (A, B and D). Scale bars: 200 μm (A), 50 μm (B-D)

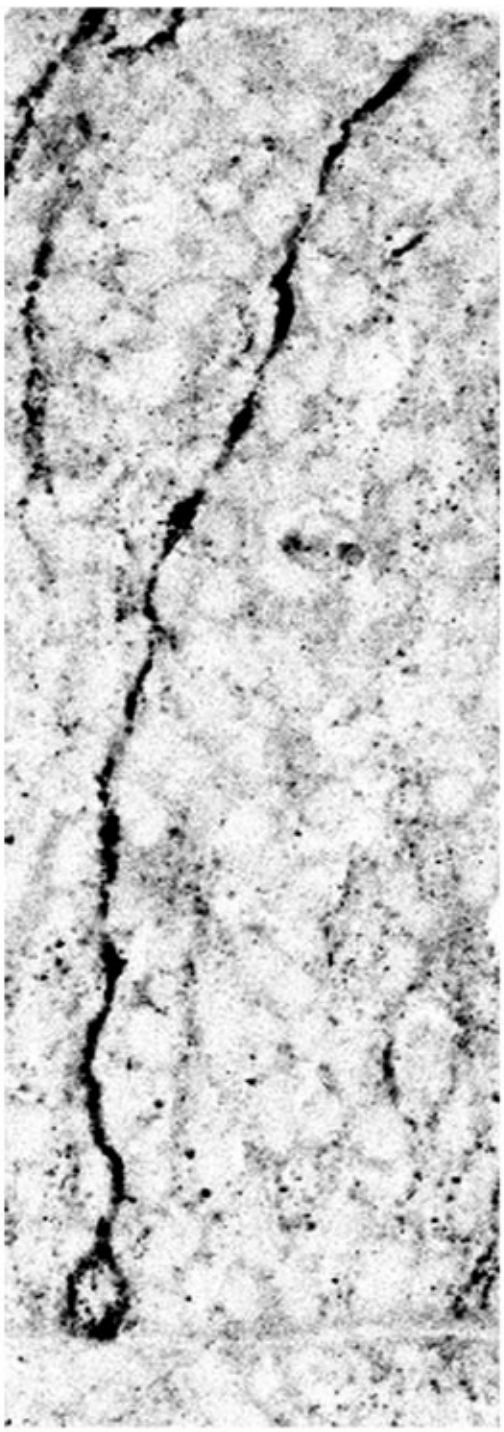
Fig. 12. Immunohistochemical analysis of BLBP and GLAST expression in the E12.5 mouse spinal cord. Expression of GLAST (green, A, C, D and F) and BLBP (red, B, C, E and F) was detected immunohistochemically in transverse sections of E12.5 spinal cord (A-F). Compared with GLAST (green, A and C), BLBP (red, B and C) was not only present in the radial fibers of ventral domain, but also showed gradient expression in the dorsal domains from roof plate to the edge of ventral domains. D-F represent higher magnification of ventral regions of A-C. BLBP-positive radial glial fibers (red, E and F) were present in the region where GLAST signal is weak (asterisks, green, D and F). Scale bars: 200 μm (A-C), 100 μm (D-E)

Fig. 13. Immunohistochemical analysis of BLBP-positive domains and region specific transcription factor expression in the E12.5 mouse spinal cord. Expression of BLBP (red, A-F), Nkx2.2 (green, B, C, E and F), RC2 antigen (blue, C and F) was detected immunohistochemically in transverse sections of the E12.5 spinal cord (A-F). BLBP (red, A-F) was expressed in all the ventral domains, and Nkx2.2 (green, B, C, E and F) was expressed strictly in the p3 domain. D-F represent higher magnification of A-C. Nkx2.2-immunoreactive nuclei (green) were closely apposed to BLBP-positive radial glial cell bodies (red) (E). BLBP-positive cells (red) were RC2 positive (blue, C and F). Scale bars: 50 μm (A-C), 20 μm (D-F)

Fig. 14. GLAST expression did not change in the Sey/Sey mouse. Expression of GLAST (red, A-C), and Pax7 (green, B and C) was detected immunohistochemically in transverse section of E12.5 spinal cord of Sey/Sey mice. GLAST expression was restricted to the ventral domains (red, A-C), and was not expressed in the VZ of dorsal domains expressing Pax7 (green, B). C represents a higher magnification of B. Scale bars: 200 μm (A and B), 100 μm (C)

Fig. 15. Diagram of diversity of radial glial cells in the embryonic spinal cord. Pax7 is highly expressed in the dorsal domains of dp4 and 5, and at a low level in dp1-3 and 6. Pax6 is expressed in the dp6 and the ventral domains of and p0-2, and at low level in the pMN domains. Nkx2.2 is expressed in the p3 domain, and Olig2 is expressed in the pMN domain. RC2 antigen is expressed in entire spinal cord (dp1-dp6 and p0-p3). GLAST is expressed in dp6, p0-2 and pMN domains. Ephrin-B1 is expressed in dp1-6 domains. BLBP have gradient signals in radial fibers of dorsal domains from roof plate to the ventral border of dorsal domain (dp1-dp5), and intensely and homogeneously in the ventral half of spinal cord (dp6 and p0-p3).

Fig. 16. Bidirectional signalling of ephrin-Bs and EphB receptors. Diagram shows an ephrin-B-expressing cell (bottom) interacting with EphB-expressing cell (top). After ligand-receptor interaction, ephrin-B/EphB family proteins transduce bidirectional signalings. The intracellular domain of ephrin-B ligands contains five conserved tyrosine residues. These tyrosine residues have been identified as the main tyrosine phosphorylation sites of activated ephrin-B. Ephrin-B reverse signaling is thought to transduce signaling through phosphorylation of the ephrin-B intracellular domain.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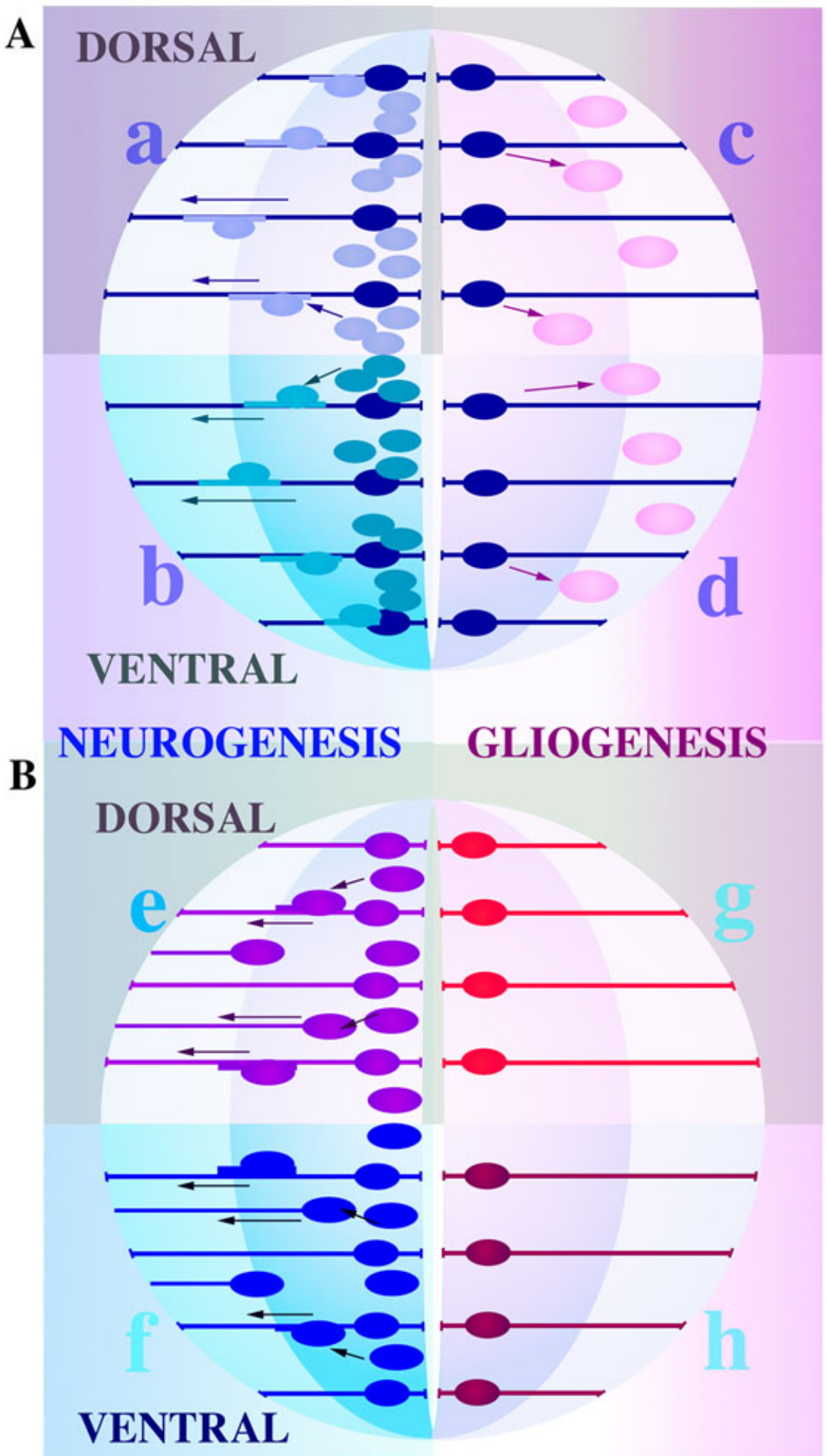
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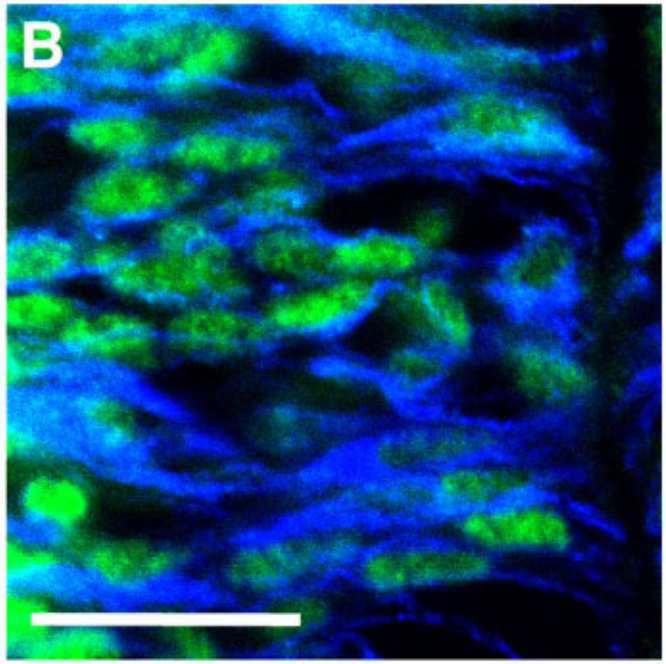
RC2 / Pax7 / Pax6

RC2 / Pax7



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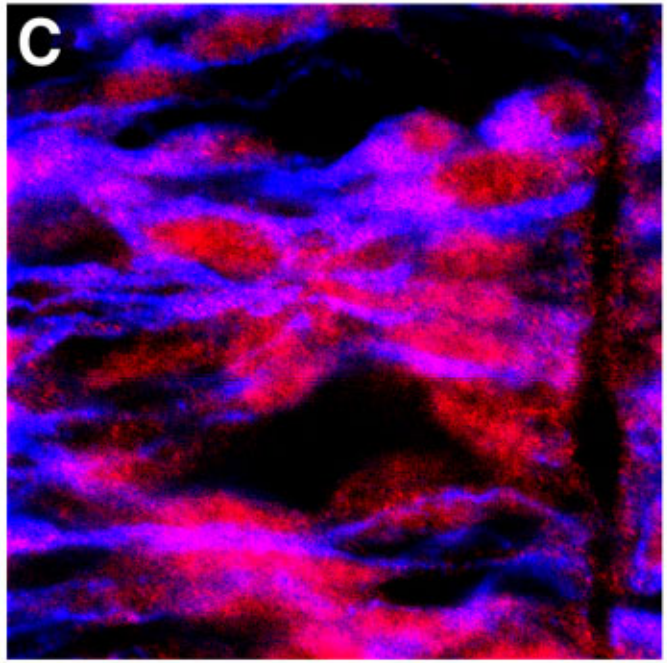

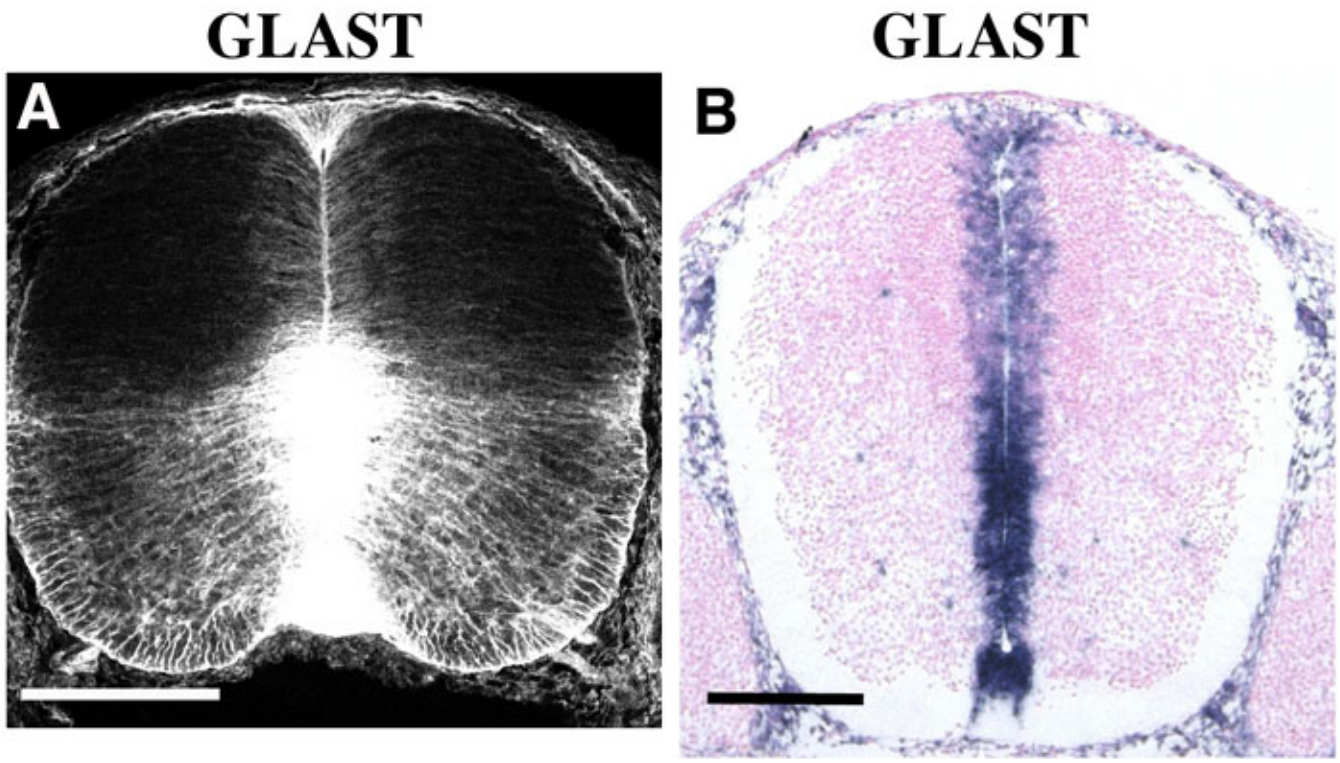
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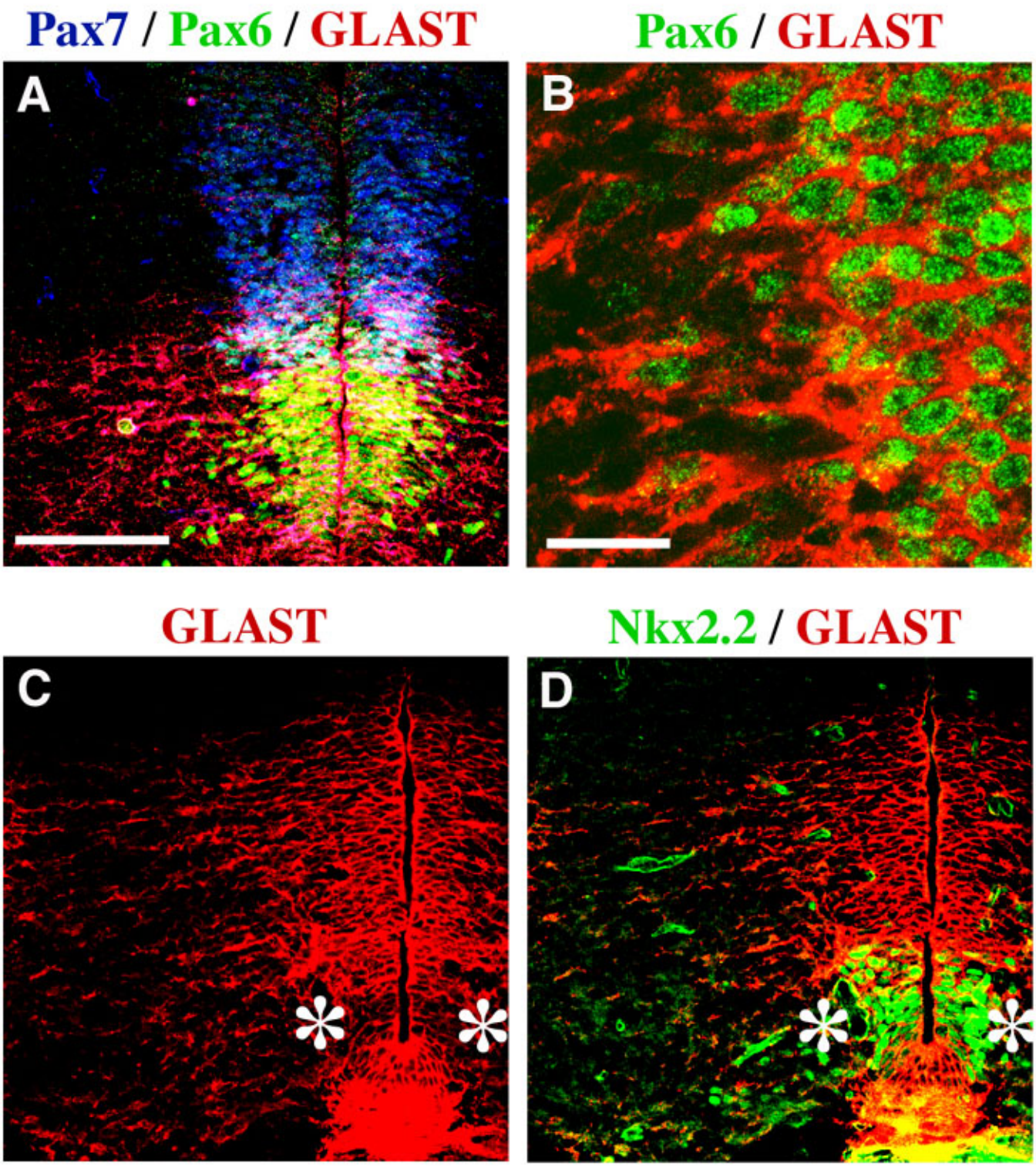
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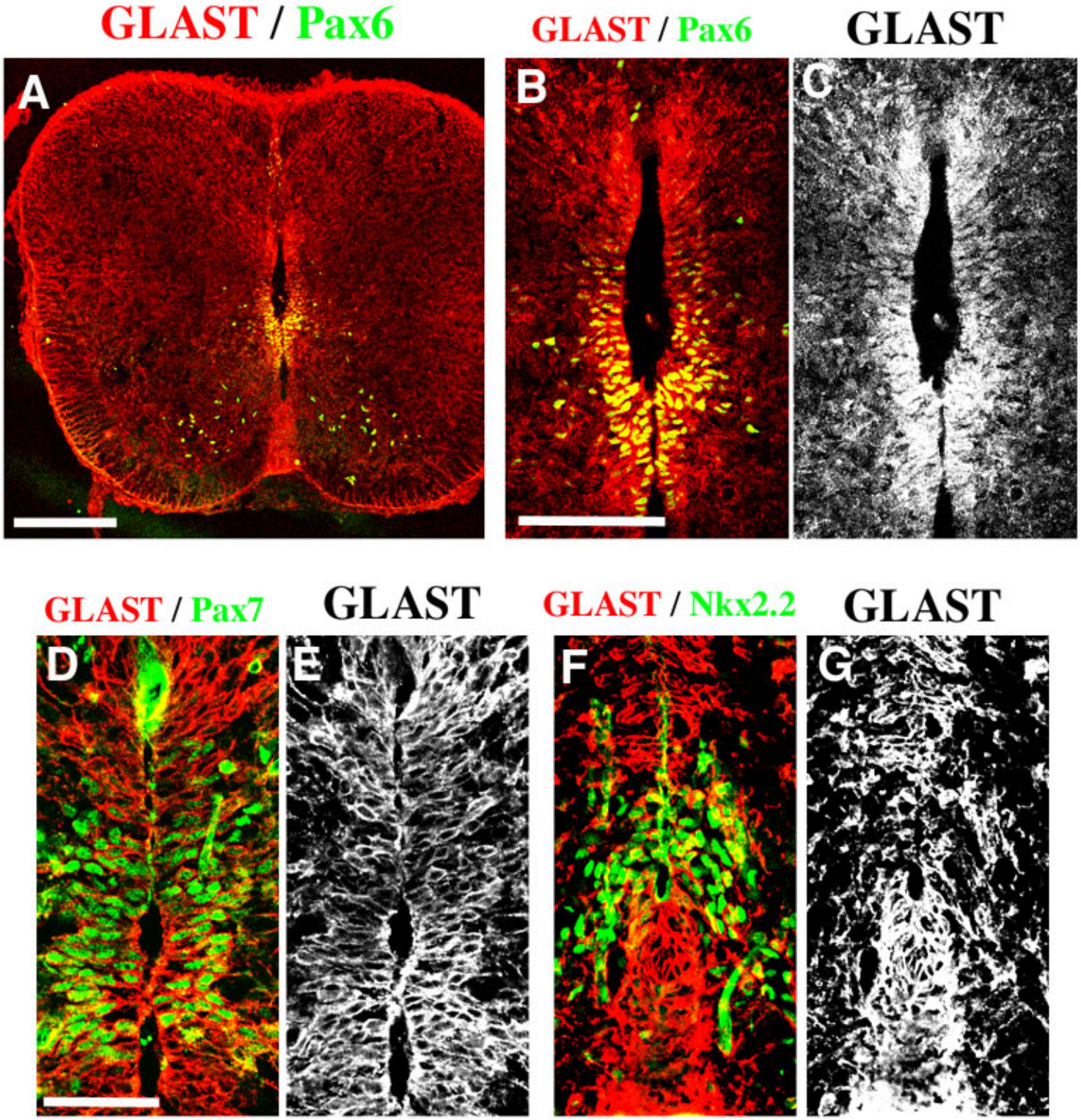
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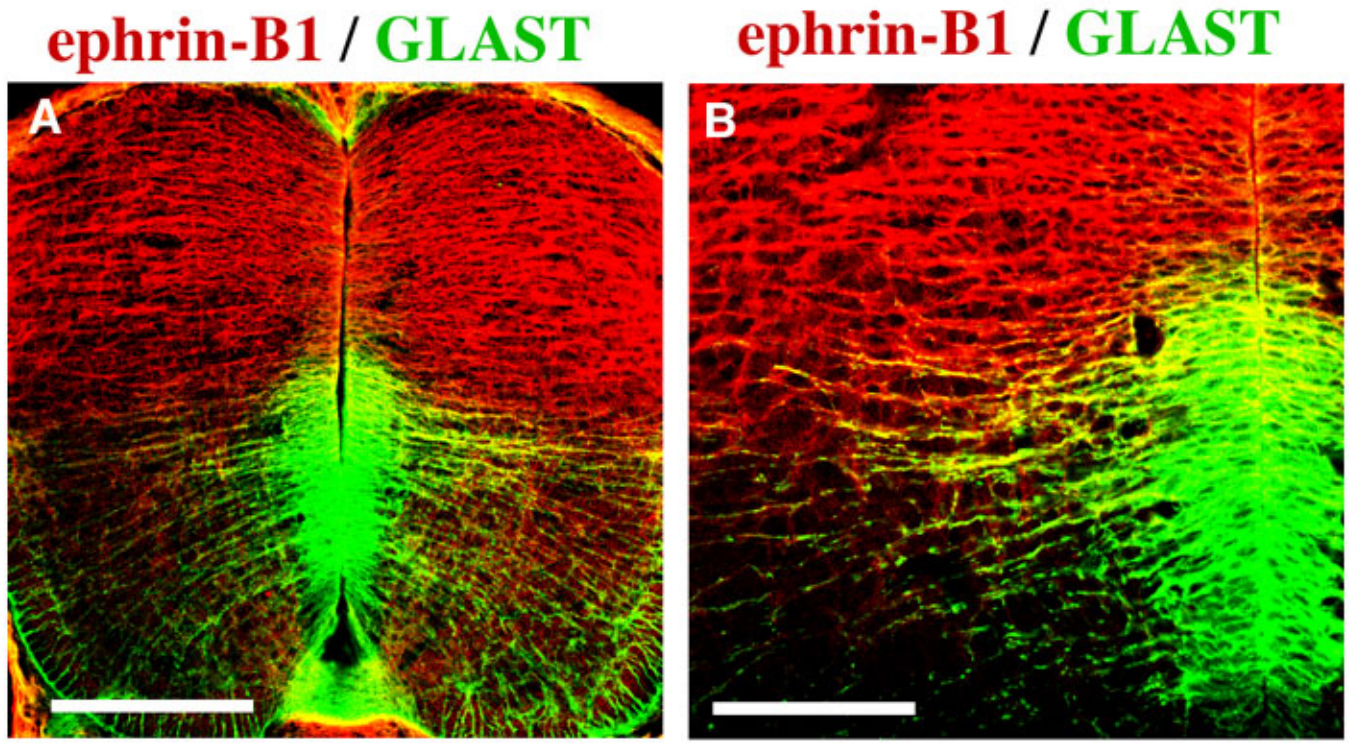
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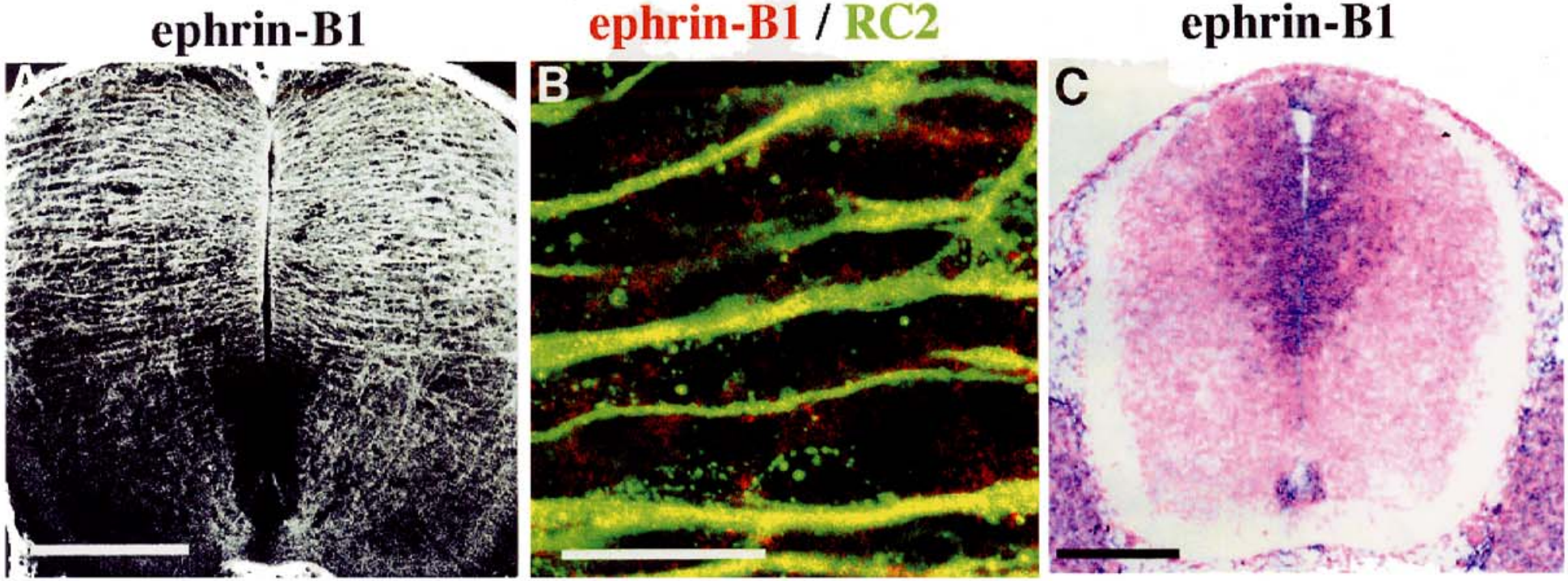
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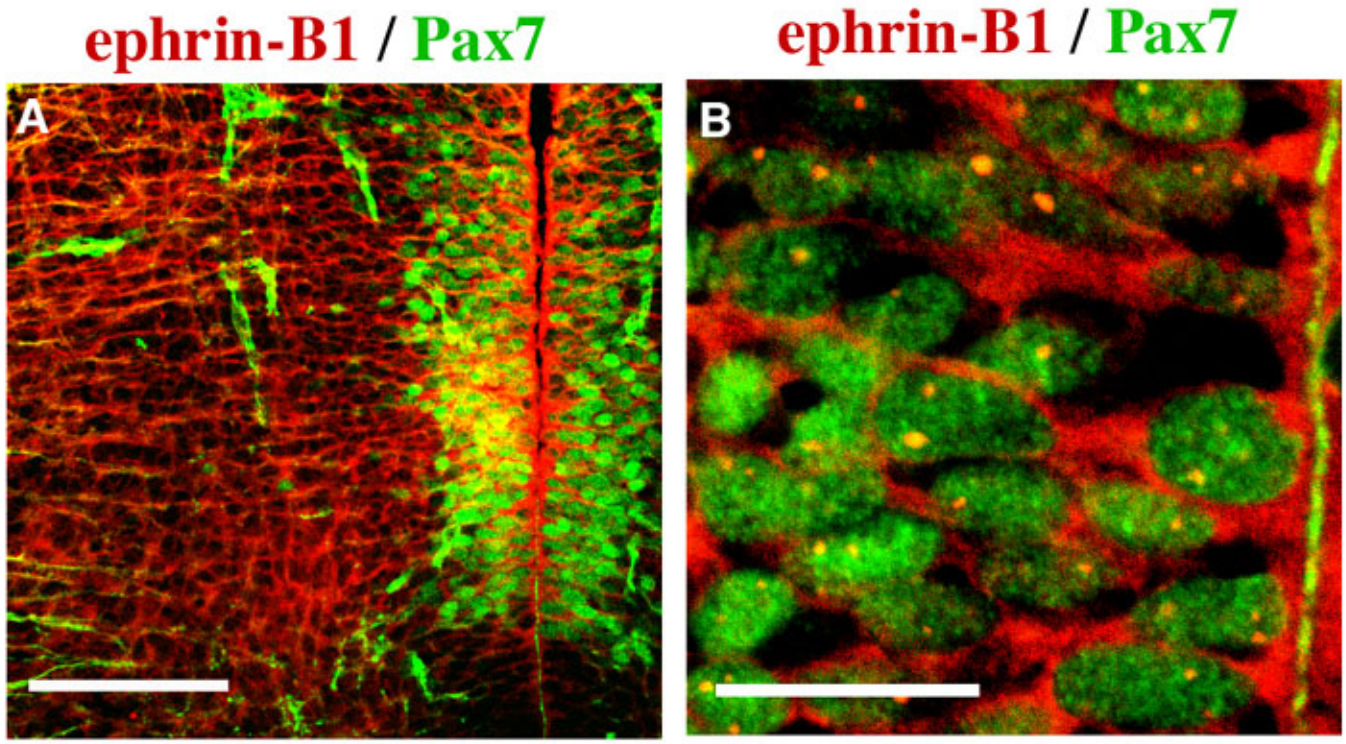
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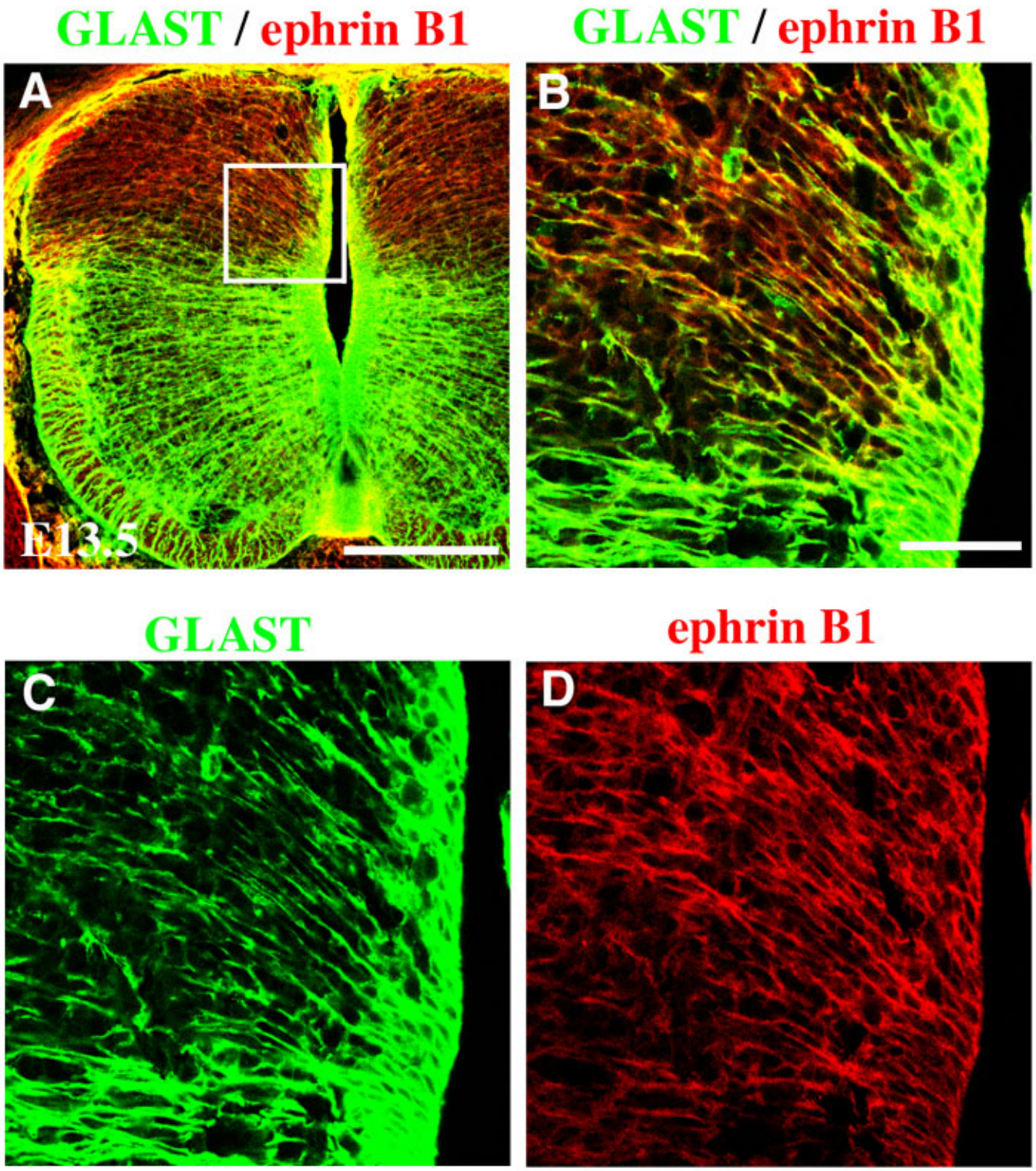
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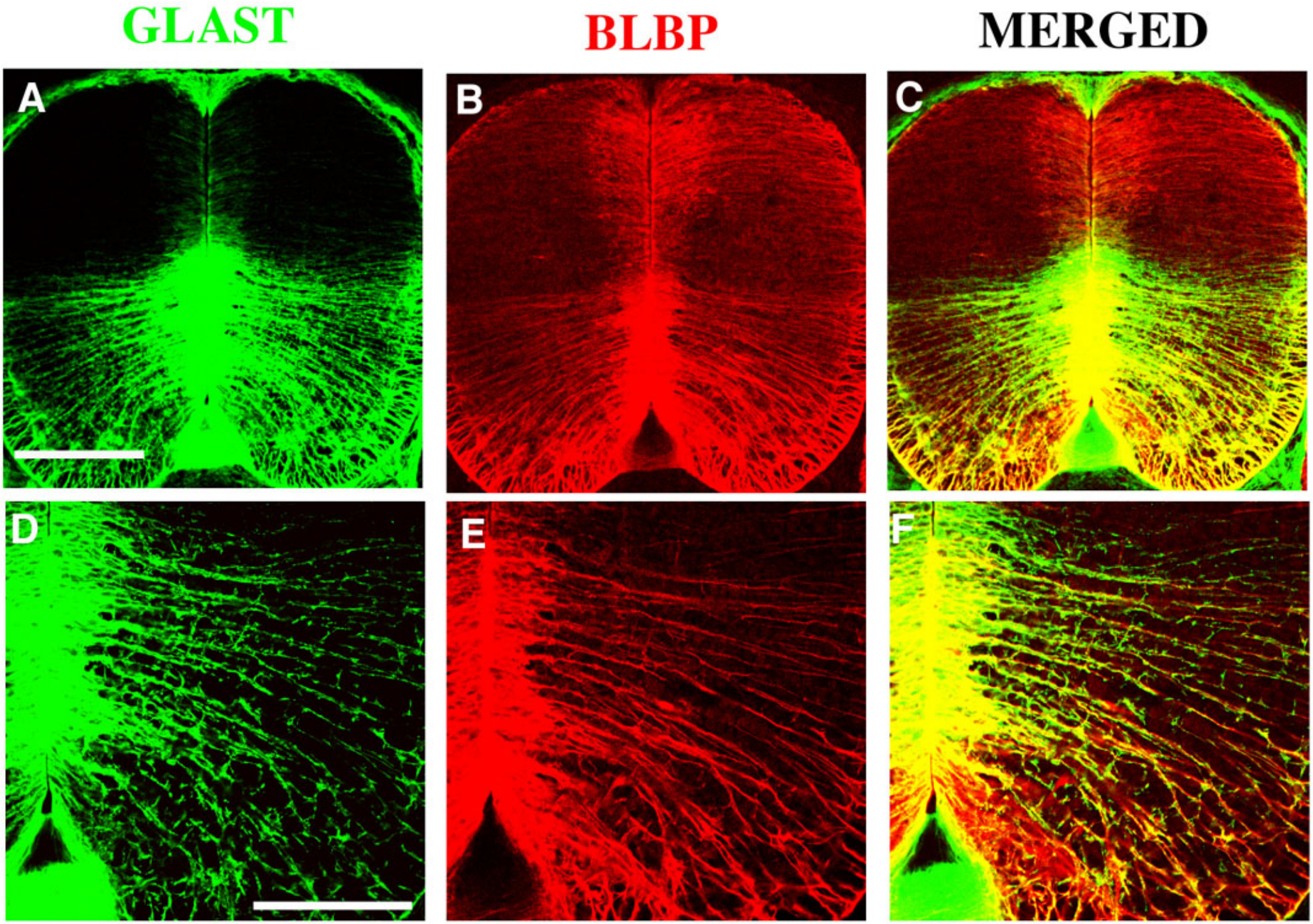
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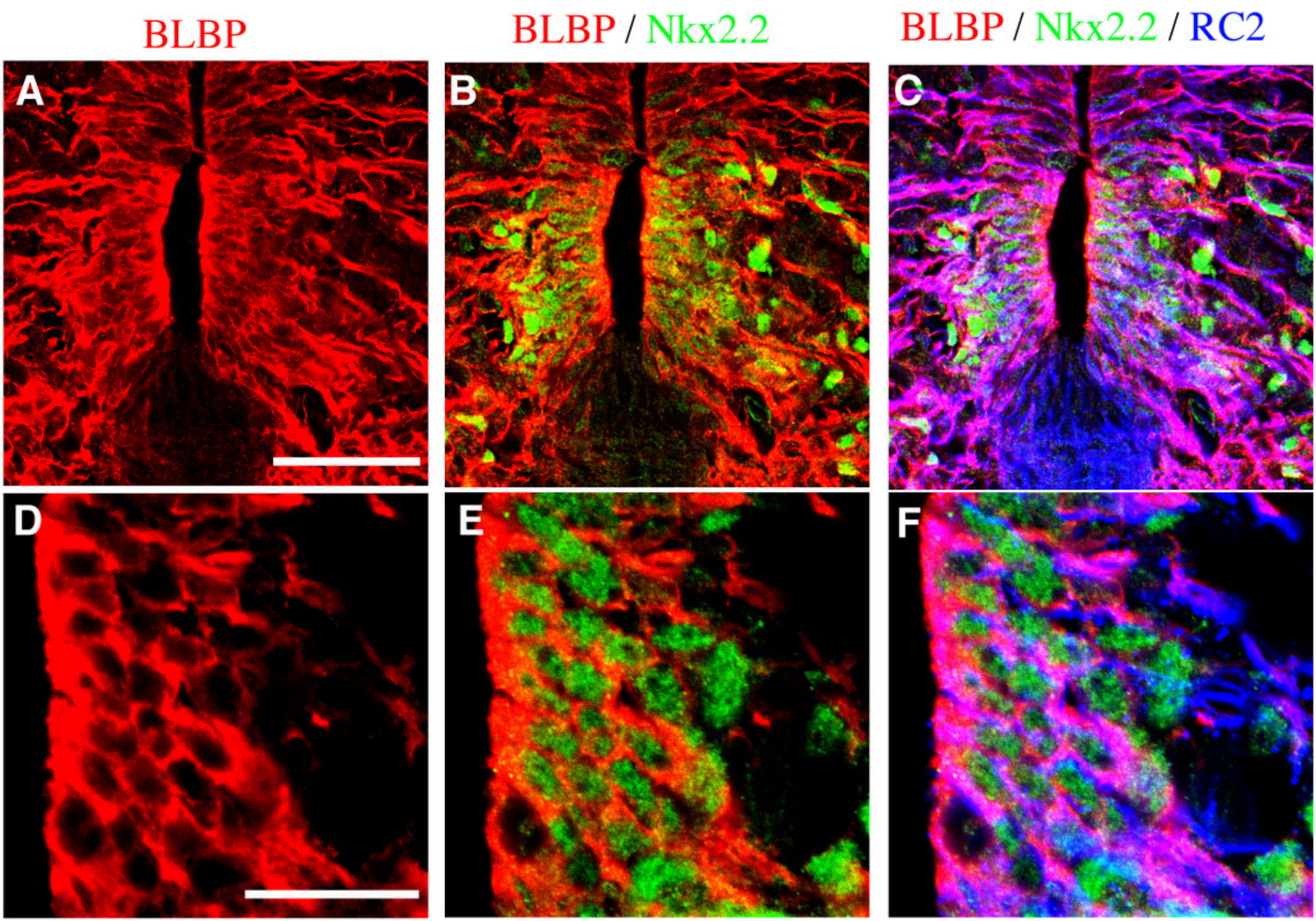
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BLBP / Nkx2.2 / RC2
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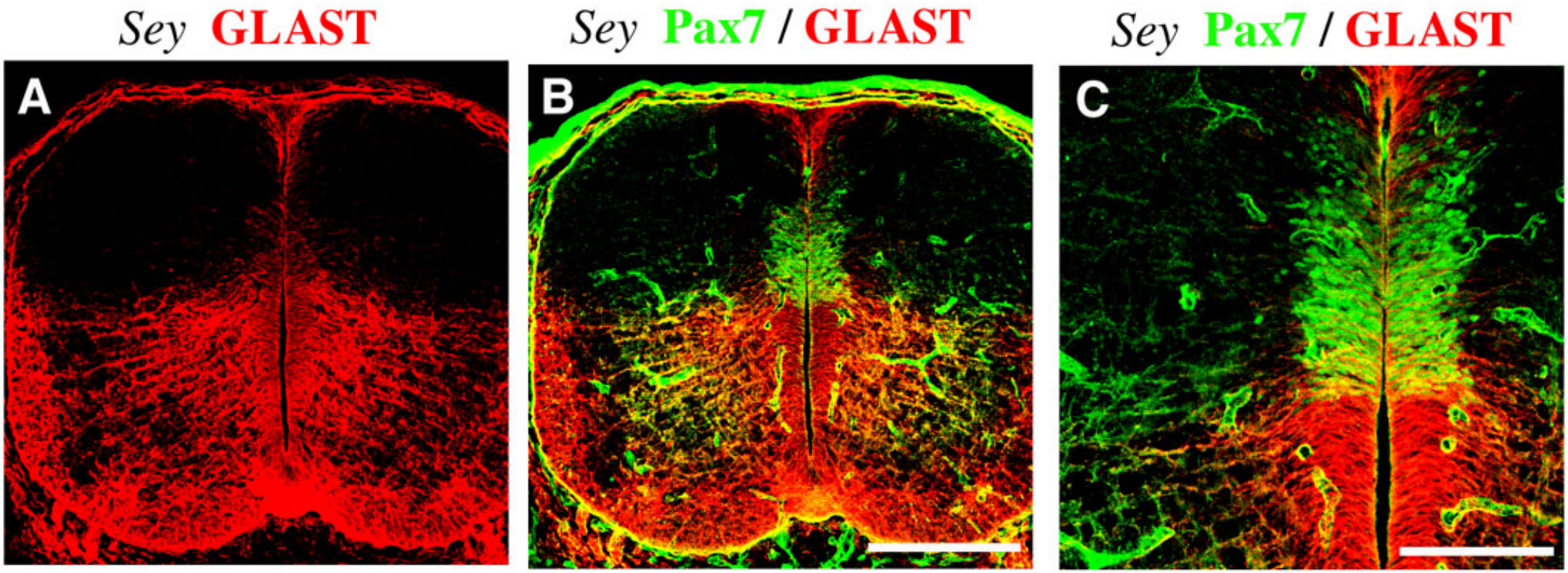
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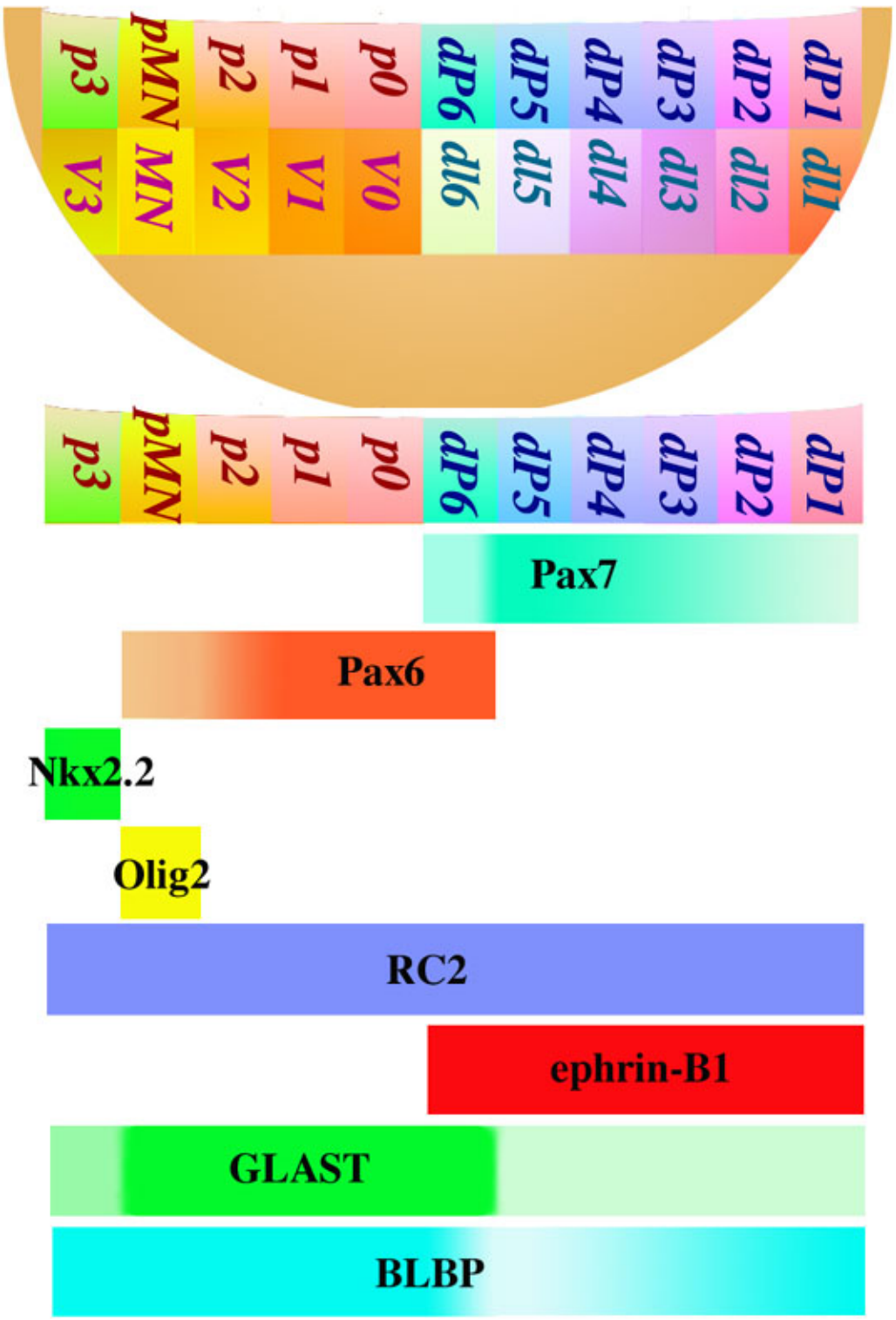
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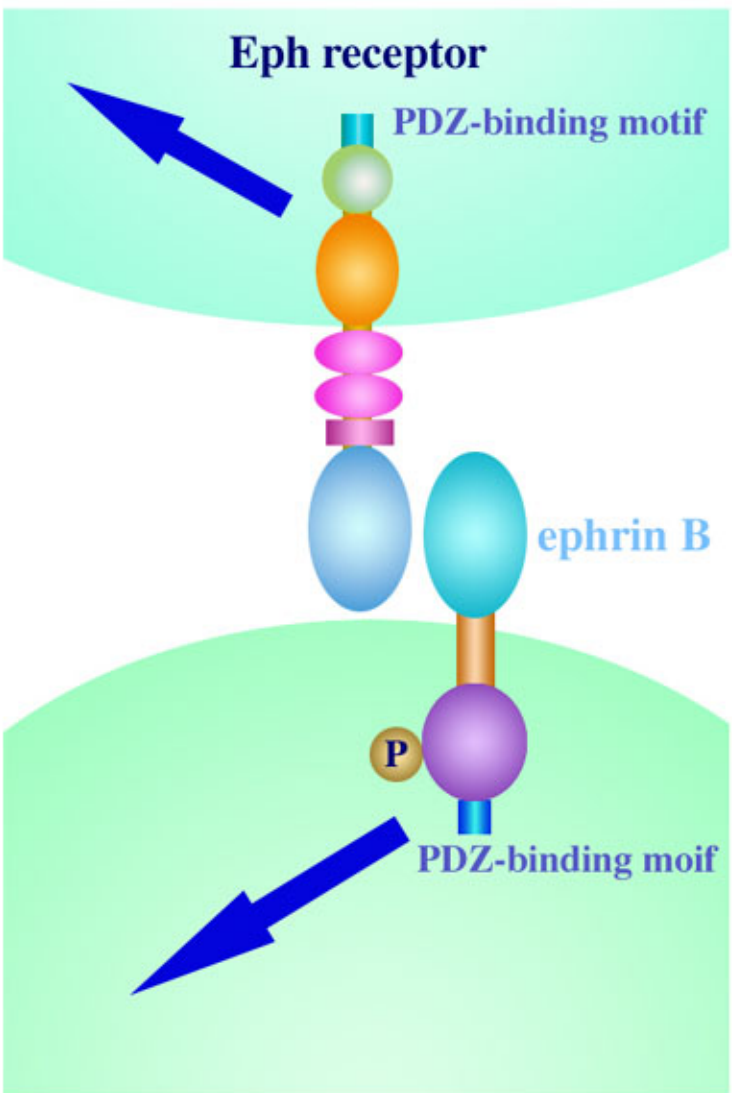
Pax7
Pax6
Nkx2.2
Olig2
RC2
ephrin-B1
GLAST
BLBP


Eph receptor
PDZ-binding motif
ephrin B
P
PDZ-binding moif