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The Role of EphA4 in Glial Scar Formation Following Injury

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Abstract

Although many areas of the brain lose their regenerative capacity with age, stem cell niches have been identified in both the subventricular zone (SVZ) along the lateral walls of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (Gage, 2000; Alvarez-Buylla et al., 2001; Alvarez-Buylla and Lim, 2004). The SVZ niche utilizes many mechanisms to determine the migration patterns of neuroblasts along the RMS into the olfactory bulb, one being Eph/ephrin signaling (Conover et al., 2000; Holmberg et al., 2005). EphA4-mediated signaling is necessary for axon guidance during development, and its continued expression in the SVZ niche suggests a regulatory role throughout adulthood. Previous studies have suggested that EphA4 plays a role in the regulation of astrocytic gliosis and glial scar formation, which inhibits axonal regeneration in these areas following spinal cord injury (Goldshmit et al., 2004). Blood vessels may also play an important role in SVZ cell proliferation and neuroblast migration following injury (Tavazoie et al., 2008; Yamashita et al., 2006). The goal of this project is to examine glial scar formation as well as the relationship between SVZ vasculature, neuroblasts, and neural stem cells in EphA4 +/+, EphA4 +/-, and EphA4 -/- mice following a needle stick injury in the cortex or striatum. The outcome of these experiments will determine whether invasive procedures such as injections will affect neuroblast migration and/or the organization of the SVZ.

Introduction

i. Subventricular Zone

The adult brain contains two regenerative areas that persist throughout adulthood, the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone
SVZ located along the lateral walls of the lateral ventricles (Gage, 2000; Alvarez-Buylla et al., 2001; Alvarez-Buylla and Lim, 2004). The SVZ is the larger of these two regions, and is composed of four main cell types: astrocytes, neuroblasts, transit amplifying cells, and ependymal cells (Fig. 1, and (Doetsch et al., 1997; Alvarez-Buylla et al., 2002; Lim et al., 2007)). Astrocytes are supportive glial cells, and a subpopulation of these cells is thought to be the neural stem cells of the region (Doetsch et al., 1999). The neuroblasts are neuronal precursors that form astrocyte-ensheathed chains and migrate along the rostral migratory stream (RMS) from the SVZ to the olfactory bulb where they integrate as interneurons (Fig. 2, and (Lois and Alvarez-Buylla, 1994; Luskin and Boone, 1994; Doetsch and Alvarez-Buylla, 1996)). The transit amplifying cells, also known as type C cells, are highly proliferative cells that can give rise to neurons and glia. Ependymal cells are ciliated cells that line the lateral wall of the lateral ventricle and form a tight barrier between the SVZ and the cerebrospinal fluid located within the ventricle (Doetsch et al., 1997). The SVZ also contains a large planar vascular plexus that provides oxygen, essential nutrients, and signaling molecules to the cells of the region (Palmer et al., 2000; Shen et al., 2004; Wurmser et al., 2004; Baker et al., 2006).

**Figure 1: SVZ Composition.** Neuroblasts are neuronal precursors that migrate in chains from the SVZ to the olfactory bulbs within the rostral migratory stream. Astrocytes ensheathe the neuroblast chains and are the putative stem cells of this region. Transit amplifying cells are highly proliferative and give rise to neuroblasts. Ependymal cells are ciliated epithelial cells that separate the SVZ from the lateral ventricles. v, ventricle.
ii. SVZ Niche Regulation

The cells of the SVZ are tightly controlled by a complex integration of signaling from many different sources. Diffusible molecules such as neurotransmitters and growth factors can travel through the blood and provide regulatory signals to SVZ cells. For example, it has been shown that dopamine released from A9 neurons that terminate in the striatum adjacent to the SVZ affect proliferation in the SVZ (Baker et al., 2004). Also, growth factors such as fibroblast growth factor-2 (FGF2) and vascular endothelial growth factor (VEGF) increase neurogenesis in the SVZ (Kuhn et al., 1997; Schänzer et al., 2004).

Figure 2: SVZ in the Murine Brain. (a) Newly generated neuroblasts migrate along the rostral migratory stream, RMS, (in red) from the SVZ to the olfactory bulb. (b) Neuroblasts migrate from the lateral ventricles to the olfactory bulb, OB, along the RMS. They then integrate into the OB and form mature interneurons. (c) Schematic showing the cytoarchitecture of the SVZ along the lateral ventricle. Ependymal cells (gray) form a monolayer along the ventricle and astrocytes (green), neuroblasts (red), and transit amplifying cells (purple) comprise the SVZ. (d) Schematic of neuroblasts migrating along the RMS. Astrocytes (green) ensheath neuroblasts (red) and are thought to restrict these cells on the path to their final destination. (e) Upon arrival at the olfactory bulb, neuroblasts enter the OB and give rise to granule or periglomerular cells. LV, lateral ventricle.

(Lennington et al., 2003)
One possible mechanism determining the migration patterns of neuroblasts along the RMS is Eph/ephrin signaling (Conover et al., 2000; Holmberg et al., 2005). There are two classes of ligands within this system: ephrin-A ligands, which bind EphA receptors, and ephrin-B ligands, which bind EphB receptors. Upon cell-cell contact, Eph/ephrin binding can trigger bidirectional signals to the receptor-presenting and the ligand-presenting cells (Fig. 3, and (Flanagan and Vanderhaeghen, 1998)). The EphA4 receptor is an exception to traditional Eph/ephrin binding in that it can bind across subfamilies to ephrin-B2 and ephrin-B3 ligands in addition to ephrin-A ligands (Martinez and Soriano, 2005). EphA4-mediated signaling is necessary for axon guidance during development, and its expression during adulthood suggests a continued role in regulation. EphA4 has been detected within the adult SVZ and may be involved in neuroblast migration to the olfactory bulb (Conover et al., 2000).

**Figure 3: Eph/ephrin bidirectional signaling.** Schematic of domain structure of Eph receptors and ephrin ligands. Ephrin-A ligands are attached to the membrane via a glycosylphosphatidylinositol anchor while ephrin-B ligands cross the membrane and have a cytoplasmic tail containing a PDZ-binding motif. The conserved structure of EphA and EphB receptors includes the intracellular cytoplasmic kinase. PDZ, postsynaptic density protein/disks large/zona occludens; SAM, sterile-α-motif.
iii. EphA4 and Glial Scar Formation

CNS axons rarely, if ever, regenerate following injury or ablation. Neurite outgrowth in the spinal cord and brain is repressed by myelin inhibitors such as Nogo (Caroni and Schwab, 1988; Shnell and Schwab, 1990, 1993; Bandtlow and Schwab, 2000), as well as glial scar formation. The main component of glial scarring is astrocytic gliosis (Stichel and Muller, 1998) in which normally quiescent astrocytes become hypertrophic, upregulate glial fibrillary acidic protein (GFAP), and form a dense network of glial processes at and around lesion site in response to injury. Accompanying formation of a glial scar is the secretion of various cytokines and extracellular matrix molecules that inhibit axonal regeneration, such as chondroitin sulfate proteoglycan (CSPG) and collagen IV (Goldshmit et al., 2004). It is possible that following downregulation or elimination of astrocytic gliosis, axon regeneration in the CNS will occur more readily in response to injury.

Eph/ephrin signaling appears to regulate axon guidance via a repulsive mechanism that causes the collapse of neuronal growth cones (Wahl et al., 2000; Kullander et al., 2001). Members of this signaling family are upregulated in adult mice following injury (Moreno-Flores and Wandosell, 1999; Rodger et al., 2001; Willson et al., 2002). This indicates that the presence or absence of Eph receptors could greatly influence neuronal repair after damage to the CNS. It has been shown that after spinal cord hemisection, many axons are able to grow across the lesion site in EphA4 -/- mice but not in EphA4 +/+ mice. This is due to the fact that EphA4 plays a crucial role in the recruitment of astrocytes during glial scar formation, and in EphA4 +/+ mice astrocytic gliosis inhibits neurite outgrowth at the site of injury (Goldshmit et al., 2004). We will
induce a needle stick injury in the cortex or striatum of EphA4 +/+, EphA4 +/-, and EphA4 -/- mice to examine the role of Eph/ephrin signaling in glial scarring.

iv. Injury-induced Neuroblast Migration

Experimental evidence suggests that ischemic stroke leads to increased proliferation of neural stem/progenitor cells in the ipsilateral SVZ (Zhang et al., 2001; Jin et al., 2001) followed by migration of neuroblasts to the injured striatal region (Arvidsson et al., 2002; Parent et al., 2002; Jin et al., 2003). These neurons then differentiate into mature neurons with an identical phenotype to that of striatal projection neurons (Arvidsson et al., 2002). It is believed that this striatal neurogenesis is closely linked to angiogenesis associated with stroke, which is most likely stimulated by hypoxic conditions that trigger the vascular endothelial growth factor system (Marti et al., 2000). During the first few weeks following stroke, neuroblasts migrate in close association with blood vessels (Yamashita et al., 2006). They also exhibit endothelial cell proliferation for a few days immediately following stroke (Ohab et al., 2006). It is possible that the neighboring SVZ becomes hypoxic during stroke and triggers angiogenesis in the SVZ and areas of injury. A mechanical injury to the brain, such as a needle stick, may also recruit neuroblasts from the SVZ to the site of insult in a pattern related to restructuring of the vasculature of the region. This could interfere with the normal tracking of neuroblasts from the SVZ to the olfactory bulb. We will use our injury model to investigate this hypothesis.

Methods and Materials

i. Needle Stick Injury

Two-month-old EphA4 +/+, EphA4 +/-, or EphA4 -/- mice were anesthetized with
isofluorane and placed in a stereotaxic apparatus, and a needle was unilaterally inserted into the cortex using the stereotaxic coordinates of -0.5 mm anterior/posterior, 1.2 mm lateral, and -2.2 mm ventral relative to bregma. For the striatal injury, the coordinates were -0.5 mm anterior/posterior, 1.2 mm lateral, and -2.2 mm ventral relative to bregma. Mice were then placed under a heat lamp for ten minutes and then returned to their cages to recover for one week.

ii. Immunohistochemistry

Mice were transcardially perfused with 0.9% saline followed by 2% paraformaldehyde in PBS. Brains were dissected out and post-fixed overnight at 4°C in 2% paraformaldehyde. Brains were washed in PBS three times for forty minutes, prior to cutting 40µm sections with a vibratome (VT-1000S; Leica, Wetzlar, Germany). Sections were placed in PBS, then washed in 0.1% Triton X-100 (Sigma, St. Louis, MO) for ten minutes. Sections were then blocked in 10% horse serum in PBS or goat serum in PBS for one hour at room temperature followed by overnight incubation in primary antibody at 4°C. Combinations of the following primary antibodies were used (listed as concentrations or as dilution factors): anti-DCX (1:200, Cat No. SC-8066; Santa Cruz Biotechnology, Santa Cruz, CA), anti-GFAP (1:400, Cat No. MAB360; Chemicon International, Temecula, CA), and anti-PECAM-1 (1:50, Cat No. 553370; BD Biosciences/Pharmingen, San Diego, CA). Sections were rinsed with PBS/0.1% TX for ten minutes followed by two ten minute washes in PBS before incubation in the appropriate Alexa Fluor dye-conjugated secondaries (1:1000, Molecular Probes, Eugene, OR) for two hours at room temperature. Following mounting of sections, slides were rinsed in PBS and DAPI (Sigma) for two minutes each, followed by a final two-minute
rinse in PBS. Slides were coverslipped with aquapolymount (Polysciences, Inc., Warrington, PA). Imaging was done on a Leica TCS SP2 confocal microscope (Leica Microsystems, Inc., Bannockburn, IL).

Results

i. EphA4 +/+ and EphA4 +/- Mice Show Similar Glial Scarring Patterns

Previous studies have indicated that EphA4 plays an important role in promoting the formation of a glial scar following injury in the spinal cord (Goldshmit et al., 2004). To see if this holds true in the brain, we performed needle stick injuries in the cortex or striatum of the murine brain and examined resulting gliosis, indicated by GFAP immunolabeling. In the EphA4 +/+ mouse, a needle stick injury in the cortex resulted in the formation of a glial scar lateral and above the ventricle and directly to the right of the site of the insult (Fig. 5, (a) and (b)). A glial scar formed in a similar location in the EphA4 +/- mouse (Fig. 5, (c) and (d)) but to the left of the needle tract. The density of GFAP+ cells in the glial scar were comparable in both the EphA4 +/+ and EphA4 +/- mice. DCX+ cells were found on the lateral wall of the ventricle in the EphA4 +/+ mouse, indicating the presence of neuroblasts in this area.

Following a needle stick injury to the striatum of both an EphA4 +/+ and EphA4 +/- mouse, astrocytic gliosis was seen adjacent to the lateral ventricle (Fig. 6, (a), (b), (c) and (e)). Although the needle tract was not visible, the clustering of GFAP+ cells near the striatum indicates that glial scarring took place next to the site of injury. GFAP+ cells were located closer and more lateral to the ventricle than with the cortical injury, reflecting the different stereotaxic coordinates used for both locations. The density of astrocytic gliosis was once again similar in the EphA4 +/+ and EphA4 +/- mice. DCX+
cells indicate neuroblasts along the wall of the ventricle.

**ii. The Absence of EphA4 Reduces Glial Scarring**

It has been proposed that EphA4 is necessary for the proper formation of a glial scar following injury (Goldshmit et al., 2004). To test this theory, we performed a needle stick injury in the cortex of an EphA4 -/- mouse. Following cortical injury, a needle tract was visible but only a few GFAP+ cells could be seen (Fig. 5, (e) and (f)). This signifies a diminished astrocytic response to injury in the EphA4 -/- mouse brain.

**iii. Neuroblasts Do Not Migrate from the SVZ to the Site of a Cortical or Striatal Needle Stick Injury**

Studies have shown that following stroke in adult mice, neuroblasts migrate from the SVZ to the site of occlusion and differentiate into mature neurons (Yamashita et al., 2006). To see if this migration takes place following an invasive procedure to the brain, we examined the position of neuroblasts relative to the SVZ following a needle stick injury in the cortex or striatum. One week after a cortical injury in the EphA4 +/- mouse (Fig. 5, (a)) neuroblasts remained localized along the walls of the ventricle as shown by the location of DCX+ cells. Absence of migration was also seen in both the EphA4 +/- and EphA4 -/- mice following a cortical needle stick injury (Fig. 5, (c) and (e)), as there were no DCX+ cells around the site of injury. Following a striatal needle stick injury, neuroblasts remained along the ventricle wall in the EphA4 +/- and EphA4 +/- mice as shown by the restriction of DCX+ cells to the ventricle wall (Fig. 6, (c) and (f)).
Figure 5: Glial scar formation following cortical injury. (A, C, E) Glial scarring in EphA4 +/+, EphA4 +/-, and EphA4 -/- one week after cortical needle stick injury. Doublecortin (DCX) marks neuroblasts and is shown in red, GFAP marks astrocytes and is shown in blue. (B, D, F) Glial scarring in EphA4 +/+, EphA4 +/-, and EphA4 -/- one week after cortical needle stick injury. GFAP is contrasted in white. There is no migration of neuroblasts from the SVZ to the site of injury. Asterisk (*) indicates site of needle stick injury. v, ventricle. Scale bar is 300 µm.

Figure 6: Glial scar formation following striatal injury. (A, D) Glial scarring in EphA4 +/+ and EphA4 +/- mice one week after striatal needle stick injury. Doublecortin marks neuroblasts and is shown in red, GFAP marks astrocytes and is shown in blue. (B, E) Glial scarring in EphA4 +/+ and EphA4 +/- mice one week after striatal needle stick injury. GFAP is contrasted in white. There is no migration of neuroblasts from the SVZ to the site of injury. St, striatum. Scale bar is 300 µm.
Discussion

The present data show that following a cortical or striatal needle stick injury in the mouse brain, astrocytic gliosis occurs to a similar degree in EphA4 +/+ mice and EphA4 +/- mice, while it is reduced in EphA4 -/- mice. This suggests that the response to injury in the brain is similar to that in the spinal cord, in which EphA4 -/- mice lack the glial scarring seen in EphA4 +/+ mice (Goldshmit et al., 2004). The importance of EphA4 in recruitment of astrocytes to the site of injury is made apparent by this result. In theory, mice lacking functional EphA4 should be able to recruit neurons following an injury such as a stroke, while in wild-type mice neuron recruitment would be hindered by glial scarring. This proposed conclusion is relevant in that inhibition or reduction of EphA4 expression immediately following injury could potentially increase neurogenesis and recovery following insult to the brain.

Although previous studies suggest that neuroblasts migrate from the SVZ to an injury in the brain (Zhang et al., 2001; Jin et al., 2001), our experiments show that DCX+ cells remain localized along the lateral wall of the lateral ventricle following a needle stick injury in the cortex or striatum of EphA4 +/+ and EphA4 +/- mice. This implies that future procedures involving injections into the murine brain will not cause neuroblasts to move in a way that would affect the tracking of neuroblast migration from the SVZ to the olfactory bulbs. In order to study the role of EphA4 in SVZ neuroblast migration, it will most likely be possible to perform reciprocal transplants of wild-type SVZ cells into an EphA4 -/- SVZ niche and vice versa without neuroblast migration being altered by the presence of an injury.
The results presented above are preliminary; more data must be obtained from EphA4 +/+, EphA4 +/-, and EphA4 -/- mice to make concrete conclusions. In order to verify our results, we must quantify GFAP+ cells in a glial scar following each needle stick injury and relate this value to the presence or absence of EphA4 expression in numerous mice. It is important to consider that more invasive injuries, such as the use of a thicker needle, may cause formation of a denser glial scar or induce neuroblast migration to the site of injury in all mice. Although more studies need to be conducted, our data provides a foundation on which to base future investigations of the role of EphA4 in the SVZ.
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