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Astroglial Boundary Formation and EphA4 Signaling in Neuroblast Migration

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Approval Page

Honors Scholar & University Scholar Bachelor of Science Thesis

Astroglial Boundary Formation and EphA4 Signaling in Neuroblast Migration

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Nicholas Biron Gallo
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Abstract:

Adult neurogenesis, the process of generating new neurons from neural precursors, is a highly complex process that is limited to two specific areas of the brain, the dentate gyrus of the hippocampus and the subventricular zone (SVZ). Despite continued research investigating neurogenesis in these two regions, we still lack a fundamental understanding of the molecular mechanisms of neural cell division, migration, differentiation, and integration in the postnatal brain. In particular, the rostral migratory stream (RMS), which is a cellular migratory route for newly generated neuronal precursors that travel from the SVZ to the olfactory bulb, will provide a useful model to address these critical questions concerning postnatal neurogenesis and cell migration. Specifically, research involving the class of membrane-bound proteins known as Ephs and ephrins, which have many regulatory roles in development that persist in postnatal neurogenic regions, has helped to elucidate the complex regulatory network that governs neural stem cell migration in the developing RMS. Currently, it has been suggested that Ephs and ephrins are key regulators of RMS development, as they participate in contact mediated signaling involved in regulating the migration of neural cell precursors from the SVZ to the olfactory bulb. Out of this large class of receptor tyrosine kinases (Ephs) and associated ligands (ephrins), the receptor EphA4 appears to maintain this restriction and play a crucial role in regulating proper RMS development in mammals. Research performed has helped determine the expression profile of EphA4 in this developing brain region, its crucial role in organizing astrocytes around tangentially migrating stem cells in the RMS, and its role in cellular proliferation.

Introduction:

As the general understanding and appreciation for the field of neuroscience continues to grow, scientists are beginning to acknowledge the significant impact stem cell biology will have on current research. As a result, investigators across the globe have begun to investigate such topics as stem cell therapy, cloning, and the use of stem cells for drug screening and disease modeling in the hope that such research will provide the appropriate foundation for applications in humans. Similarly, groundbreaking research concerning the potential usage of stem cell therapy in the treatment of neurodegenerative diseases has also gained popularity as researchers have begun to explore the concept of neurogenesis in animal models. To be more specific, neurogenesis, or the birth of new neurons, is the process by which neurons are generated from neural stem/progenitor cells in the central nervous system. Interestingly though, the actual concept of neurogenesis and neural stem cells was first introduced and rejected by researchers at the beginning of the twentieth century. Dr. Santiago Ramón y Cajal, commonly referred to as the father of neuroscience, actually claimed, “In the adult centers the nerve paths are something fixed, ended and immutable. Everything may die, nothing may be regenerated” [1]. Interestingly, research by Joseph Altman in the 1960s utilizing tritiated thymidine as a mitotic label found that constitutive neurogenesis does occur in the hippocampus and olfactory bulb of the adult mammalian brain [1]. This breakthrough in neuroscience has led to the characterization and present understanding of adult neurogenesis and neural stem cells. As a result, current research has established the three fundamental features of adult CNS “stem cells”:

1. They are “self-renewing” with the theoretical ability to produce daughter cells that are indiscernible from themselves.
2. They are proliferative and continue to undergo mitosis
3. They are multipotent for the varying neuroectodermal lineages, including the differing neural and glial subpopulations [1].

Furthermore, subsequent research has revealed the probable role of growth factors for regulating neurogenesis, including epidermal growth factor (EGF) and fibroblast growth factor (FGF), which act as mitogens for neural progenitors and stem cells *in vitro* [2].

Ultimately, this fundamental research has provided the basis for the investigation of neurogenesis as it continually reinforces the underlying fact that neural stem cells reside in the adult central nervous system.

As additional research continues to elucidate the complexities of adult neurogenesis, the scientific community has concluded that this phenomenon occurs in two specific areas of the adult brain: the subgranular zone (SGZ) in the dentate gyrus of the hippocampus and the sub-ventricular zone (SVZ) of the lateral ventricles [3]. Research concerning the dentate gyrus of the hippocampus has revealed that immature neuronal cells generated in the subgranular zone (SGZ) migrate to the granular layer of the hippocampus where they differentiate into mature neuronal cells [2]. On the other hand, research has found that in SVZ neurogenesis, neuroblasts are generated in the anterior part of the SVZ and migrate through the rostral migratory stream (RMS) to the olfactory bulb (OB) where they differentiate into OB interneurons [2].

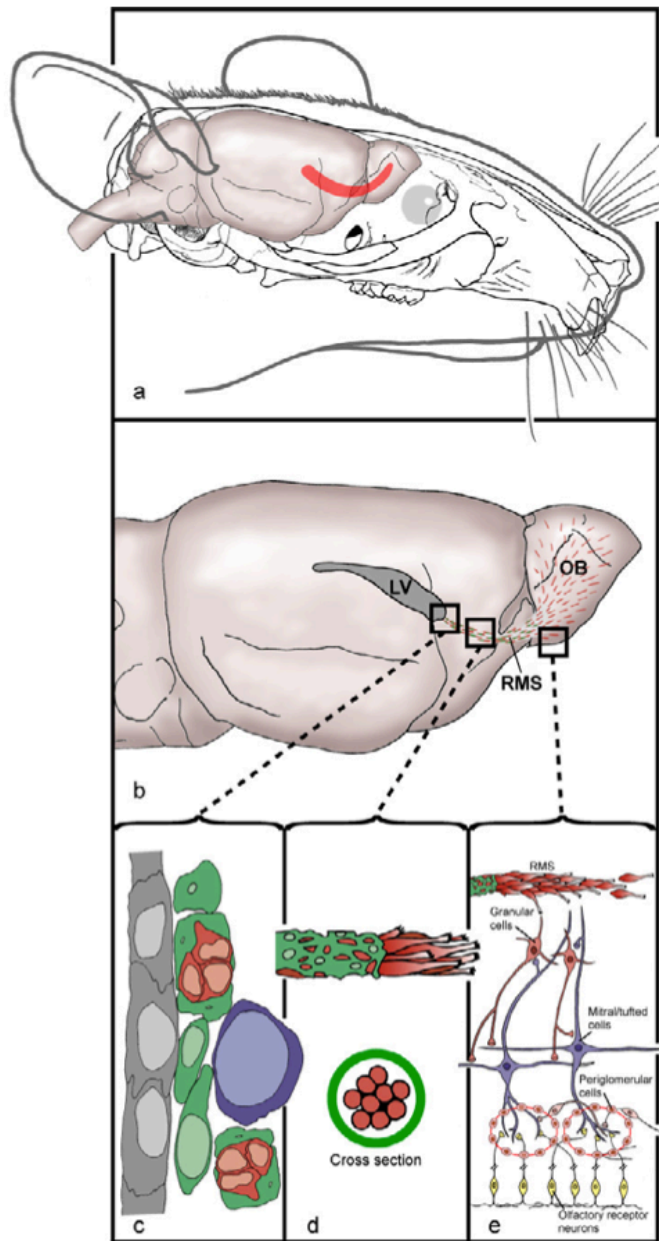


Figure 1. Overview of the SVZ/RMS/OB Neurogenic Niche. (a) The head of a mouse indicating the position of the brain and the rostral migratory stream (RMS; highlighted in red), which acts as the pathway along which neuroblasts migrate tangentially from the subventricular zone (SVZ) of the lateral ventricle to the olfactory bulb (OB). (b) Newly generated neuroblasts migrate from the lateral ventricle through the RMS to the OB, where the neuroblasts differentiate into mature interneurons. (c) A simplified diagram based on electron microscopy that depicts the cytoarchitecture of the SVZ along the ventricle. In this schematic, the ependymal cells (gray) form a monolayer along the ventricle wall with neuroblasts (red), astrocytes (green), and transit amplifying progenitors (TAPs; purple) forming the SVZ. (d) Sagittal and coronal diagrams of tangential neuroblast chain migration in the RMS. Astrocytes (green) form a complex network around the neuroblasts (red) and are believed to restrict the migrating neuroblasts to their pathway to the OB. (e) Upon reaching the OB, neuroblasts migrate radially and differentiate into granule or periglomerular cells. *Adapted from "Neural stem cells and the regulation of adult neurogenesis" Conover et al. 2003.*

Central to the growing field of stem cell biology is the phenomenon of stem cell migration and its possible application in future stem cell therapies. Fortunately, the RMS in the mammalian forebrain is a highly relevant model system for the investigation of endogenous stem cell migration as it is the site of PSA-NCAM-dependent neuroblast chain migration from the SVZ to the olfactory bulb [4]. Essential to the development and function of the RMS is the regulatory network of glial tubes that ensheath the tangentially migrating neuroblasts [4]. It is believed that various developmental guidance molecules, such as Ephs and ephrins, which are typically associated with boundary formation and axonal pathfinding [5], continue to provide cues for stem cell differentiation and neuroblast migration within the RMS. As a result, research concerning the RMS will likely reveal potential molecular targets for therapeutic intervention that enable the manipulation of endogenous neural stem cells or autologous/artificially cultured pluripotent cells *in vivo*.

Before outlining the numerous factors involved in the regulation of astrocyte-neuroblast interactions and the control of astroglial permissivity, it is important to stress the differences in the development of the rostral migratory stream between non-human mammals and humans. To begin, the SVZ of numerous adult non-human mammals, including rodents, has been found to generate a large number of neurons that travel and integrate in the olfactory bulb (OB). On the other hand, research investigating the adult human SVZ has found this region to contain a hypocellular gap layer that separates the ependymal lining from a periventricular ribbon of astrocytes [6]. In addition, it has been noted that a portion of these SVZ astrocytes can function as neural stem cells *in vitro*, but *in vivo* functioning remains unknown [6]. Based on research performed by Alvarez-Buylla et al., the infant human SVZ and RMS contain a large population of migrating immature

neurons before 18 months of age. Unlike rodents though, this corridor of migrating neural precursors appears to subside significantly in older children and is practically nonexistent by adulthood [6]. Despite the apparent decrease in neuronal migration in the rostral migratory stream of adult humans, research investigating this prolonged phenomenon in mouse models is highly relevant as it may lay the foundation for the molecular control of stem cell movement and integration following administration for therapeutic purposes.

The migration of SVZ-derived neuroblasts is a unique phenomenon involving characteristic morphological changes in cellular phenotype and diverse signaling mechanisms. To begin, neuroblasts have been found to slide along each other in organized chains, commonly visualized by polysialylated neural adhesion molecule (PSA-NCAM) immunostaining [7]. Within the astroglial “tunnel” of the RMS, neuroblasts have been found to undergo distinct stereotypical phases of migration [8]. The first step of tangential neuroblast migration involves the extension of the leading process in the direction of migration, which is stabilized by forming contacts with other cells and/or the extracellular matrix (ECM) [8]. Following extension, neuroblasts form a dilation in front of the nucleus that acts as a reservoir for organelles and the centrosome and is the site where endocytic trafficking weakens adhesion [8]. Finally, the nucleus advances in the dilation, which is aided by myosin II-mediated contraction at the cell rear [8]. Overall, the repetition of this cycle over time results in the forward migration of neuroblasts toward the olfactory bulb [8].

In addition to the morphological changes associated with neuroblast migration, an array of factors regulate RMS neuroblast migration across development, including adhesion and ECM molecules, axon guidance molecules, neurotransmitters, and many others. These

varying factors have diverse actions in the developing RMS, which range from chemorepellant/chemoattractant to motogenic properties. In addition, these key extracellular signals are vital to the development of this cellular migratory pathway as they help control the different phases of neuroblast migration, which include detachment from the SVZ, migration along the rostral migratory stream, and radial migration upon reaching the olfactory bulb.

One critical class of transmembrane adhesion molecules involved in RMS development is the family of integrins, which are composed of $\alpha 1$, αv , $\beta 3$, $\beta 6$, and $\beta 8$ integrin subunits among many others [9, 10]. For instance, research has found that the $\beta 8$ integrin subunit is expressed in the RMS of mice across their lifespan with genetic deletion of this integrin subunit causing tangential chain migration to be disrupted likely due to the development of disorganized neuroblast clusters and increased numbers of GFAP-positive astrocytes in the brain [10]. Unlike $\beta 8$ integrin subunit, the integrin subunit $\alpha 1$ is expressed in neonatal mice along the entire RMS but is significantly downregulated from early postnatal stages to adulthood [9]. As implicated by the increased number of integrins present along the RMS, their ligands, known as laminins, also have a unique distribution in the SVZ niche [11]. For instance, several α , β , and γ laminin subunits are highly expressed in the RMS, including $\alpha 1$, $\alpha 2$, and $\alpha 4$ [12]. Interestingly, it has been found that RMS organization becomes disrupted in $\alpha 2/\alpha 4$ double knockout animals. In these animals, the RMS becomes less compact indicating that integrin/laminin interactions play a crucial role in the proper formation of organized neuroblast chains [12]. It is also important to stress that integrins can control the activity of N-cadherin, an essential cell adhesion molecule, which is highly expressed along the rostral migratory stream and downregulated in the

olfactory bulb, where neuroblasts transition to radial migration [13]. As a whole, integrins and their associated ligands play a crucial role in the proper formation and maintenance of the RMS in the developing mammalian forebrain.

Two additional molecules critical to RMS development, in particular radial migration within the OB, are Reelin and Tenascin-R. To begin, Reelin is a secreted glycoprotein that is necessary for the development of laminated structures in the brain [14]. After birth, Reelin plays a crucial role in causing RMS neuroblast chains to detach, which ensures appropriate radial migration within the olfactory bulb [15]. As expected, Reelin mutant mice typically have an abnormal accumulation of neuroblasts at the end of the RMS due to hindered radial migration of this cell type. As a result, the olfactory bulbs of these mice lack the stereotypical layer organization and have a considerable reduction of newly generated neurons [15, 16]. Unlike Reelin, Tenascin-R is an ECM component that contains epidermal growth factor (EGF)-like domains, fibronectin type III homologous repeats, a cysteine-rich amino terminal region, and a fibrinogen homologous domain [17]. Despite these structural differences, it has been found that Tenascin-R promotes the detachment of neuroblasts from the end of the RMS and their subsequent radial migration in the olfactory bulb. In addition, RMS architecture and tangential migration in *TNR* knockout mice appear normal, but neuroblasts tend to accumulate at the rostral portion of the RMS prior to entering the OB, which suggests an issue involving neuroblast chain dispersion [18]. Currently, possible interactions between the Reelin and Tenascin-R signaling pathways remain unknown despite their shared role in regulating neuroblast radial migration in the developing RMS/OB system.

In addition to adhesion and ECM molecules, numerous axon guidance molecules have been shown to play a significant role in shaping the development of the RMS. One common example is the family of Slit proteins and their associated Robo receptors [19, 20]. It has been found that two members of the Slit family, Slit1 and Slit2, are expressed in the adult brain choroid plexus and septum. In addition, these diffusible proteins repel SVZ-derived neuroblasts *in vitro* [19, 20]. Studies in *Slit1* knockout mice have shown that neuroblasts prematurely leave the RMS and migrate throughout the corpus callosum (CC) [21]. Further research has revealed that the Slit receptors Robo2 and Robo3 are located on the migrating RMS neuroblasts and on the surrounding astrocytes [22]. Slit1 released by the migrating neuroblasts modulates astrocyte morphology in the RMS, ultimately “repelling” the surrounding astrocytes through Robo signaling. This repulsion plays a crucial role in the proper formation and maintenance of RMS astrocyte tunnels, which are vital to long-range neuroblast migration [22].

Another family of axon guidance molecules is the class of Neuregulins (NRGs) and their associated receptors ErbB2, ErbB3, and ErbB4, which are receptor tyrosine kinases [23]. NRGs are multiple EGF-like domain-containing ligands that are recognized as classic regulators of synaptic plasticity that also play an important role in glial-guided neuronal migration in the cortex and cerebellum during development [24, 25, 26]. In regards to the developing RMS, it has been found that NRG1-3 all activate ErbB4, which is selectively expressed at increased levels in type A migratory neuroblasts, a subpopulation of GFAP-positive astrocytes, and type C proliferating progenitors in the RMS and SVZ [27]. Conditional deletion studies in which ErbB4 was deleted in nestin-expressing stem cells or in GFAP-expressing stem cells and astrocytes resulted in fragmented neuroblast chains and

jagged boundaries of the RMS. These findings reveal the crucial role of the ErbB4 receptor present on neuroblasts and astrocytes as it helps regulate the formation and organizational structure of the RMS *in vivo*. Further investigation has revealed the NRG1 type III isoform as the major ErbB4 ligand acting in the RMS, especially in early postnatal stages [28]. Localization studies of NRG1 have found that there is almost complete overlap between NRG1 staining and PSA-NCAM positive neuroblasts suggesting that the NRG1 ligand is expressed on migrating neuroblasts in the RMS [28]. As a whole, the signaling cascade initiated by the binding of NRGs to ErbB4 appears to be a crucial aspect of RMS development, but future research concerning the role of ErbB4 signaling in cellular migration remains to be performed.

In addition to Neuregulins, a class of axon guidance molecules known as Semaphorins have been shown to play a significant role in regulating neuronal migration by binding to Plexin receptors, which are divided into four subgroups (A-D) [29]. Research by Saha et al. has discovered that Plexin-B2 is highly expressed in Mash1-positive transit amplifying progenitors (TAPs) and GFAP-positive astrocytes in the SVZ [30]. In the rostral migratory stream though, Plexin-B2 is found on migrating neuroblasts but absent on the surrounding astrocytes. Upon transitioning to radial migration in the OB, neuroblasts appear to downregulate Plexin-B2 expression, as it is not detectable in this population of cells [30]. Interestingly, expression of Plexin-B2 does appear at lower levels in all olfactory axons, periglomerular cells, and mitral cells [30]. In the RMS, granular cell layer, and mitral cell layer, class IV Semaphorins (Sema4A, 4C, 4D, and 4G) act as ligands for Plexin-B2, which reinforces the current belief that canonical Semaphorin-Plexin signaling may act as a fine-tuning mechanism for neuroblast migration at critical points during migration, such as

the exit from the RMS and forming the layers of the olfactory bulb. Studies with *Plxnb2* knockout mice found an increase in aberrant neuroblast migration in non-neurogenic regions such as the corpus callosum and the septum [30]. Overall, Sema-Plexin-B2 interactions are a crucial factor in cellular migration and neural development, especially in regulating neuroblast migration in the RMS.

In addition to the extensive interactions between migrating neuroblasts and astrocytes in the developing RMS, the important role of vasculature in this brain region continues to gain support. Unlike the majority of the brain, which has a random distribution of blood vessels, it has been found that blood vessels develop in parallel to the RMS following birth [31]. This ordered development of blood vessels is likely influenced by the secretion of the angiogenic factor VEGF at early postnatal stages by the astroglial network within the RMS. As expected, *in vivo* downregulation of VEGF expression in astrocytes caused angiogenesis at the outer border of the RMS to be altered, which led to aberrant neuroblast migration [32]. Further evidence for the crucial role of vasculature in the RMS is the finding that neuroblasts migrate more efficiently when in intimate contact with the vasculature scaffold of the RMS, which is further reinforced by the increased speed of neuroblast migration in adult mice in comparison to early postnatal stages when the vasculature network is less developed. Interestingly, a unique relationship has been revealed between astrocytes, blood vessels, and migrating neuroblasts in which the astroglial network promotes blood vessel development through VEGF signaling [32] while endothelial cells secrete BDNF, which promotes neuroblast migration through p75NTR activity in the neuroblast population [31]. Despite these advances in understanding the complex interactions between vasculature and the cellular subpopulations of the RMS,

much research remains to fully elucidate their role in the development and proper functioning of the RMS.

Essential to the crosstalk between migrating neuroblasts and the astrocytes of the RMS glial tubes is the Eph family of receptors and their transmembrane-associated ligands known as ephrins. Ephs are transmembrane tyrosine kinase receptors that bind to ephrin ligands, which are either transmembrane (ephrin-Bs) or tethered to the membrane by a GPI tail (ephrin-As) [32]. Eph/ephrin signaling is crucial during development, as it has been linked to the regulation of axon guidance and neural crest cell migration [34]. Research has elucidated two modes of signaling involving Ephs and ephrins: the 'classic' mode of signaling (forward signaling) is from ephrin ligands to their Eph receptors and typically results in cellular repulsion, while reverse signaling occurs from Eph receptors to ephrins and has been found to promote adhesion [33]. In regards to the development of the RMS, it has been noted that ephrin B2 and B3 ligands are located on astrocytes in the SVZ/RMS and the olfactory bulb. In addition, the disruption of normal EphB/ephrin-B signaling due to the infusion of truncated EphB2 and ephrin-B2 proteins into the lateral ventricle results in increased cellular proliferation in the SVZ and also disrupts neuroblast chain migration [35]. Overall, current research holds that ephrins are located on astrocytes in the SVZ and along the RMS and interact with the ephrin receptors EphA4, EphB1, and EphB2 along the RMS neuroblast migratory pathway. Currently, the exact location of these ephrin receptors in the developing RMS remains unknown, forcing a debate on whether they are present on astrocytes, neuroblasts, or both. In addition, it remains to be determined whether the disruption of proper neuroblast migration following changes in Eph/ephrin signaling is solely due to changes in astrocyte-neuroblast crosstalk or if

abnormal proliferation due to the disruption of ephrin signaling in the SVZ also plays a role. Despite recent advances in understanding cellular migration and Eph/ephrin signaling, much research remains to translate these findings to glial tube formation and neuroblast migration in the developing rostral migratory stream.

One Eph tyrosine kinase receptor that appears to be highly relevant in cellular migration and boundary formation is EphA4, which has the unique ability to bind ephrinB2 and ephrinB3 in addition to ephrinAs, consequently facilitating bidirectional signaling [36]. Research by Conover et al. has detected EphA4 within the SVZ, subsequently driving additional research to explore its potential role in regulating neuroblast migration and glial tube formation in the RMS [35]. Research by previous and current graduate students, in addition to my research as an undergraduate, has found EphA4 to be expressed in the SVZ and RMS where it acts to orient astrocytes into a complex astroglial meshwork around migrating neuroblasts. As a result, the EphA4-dependent formation of this astrocyte framework establishes a specific and well-defined pathway for neuroblast migration from the SVZ to the OB.

As a whole, my undergraduate research at the University of Connecticut was aimed at elucidating astrocyte infiltration and proliferation within the developing RMS and to compare RMS astrocyte morphology/phenotype to glial scar astrocyte morphology/phenotype. In addition to this research, I continued to explore the RMS developmental paradigm by building upon the research of current and past graduate students on the critical role of EphA4 in glial tube formation in the developing RMS. As a result, research by Dr. Conover's lab has found that disruption of EphA4 signaling leads to improper glial tube formation and irregular neuroblast migration. Our research has also

utilized MACS and MACS/FACS techniques to investigate the cell-specific location of EphA4, while also employing BrdU labeling to explore its possible role in regulating cellular proliferation in the SVZ and RMS.

Materials and Methods:

Animals:

Male and female *EphA4*, *EphA4^{ΔeGFP/ΔeGFP}*, and hGFAP:mRFP mice were bred and aged in Dr. Joanne Conover's vivarium at the University of Connecticut. Animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Connecticut and conform to the National Institute of Health and Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

Immunohistochemistry:

EphA4 mice were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in pH 7.4 phosphate buffered saline (PBS). Following perfusion, brains were removed from the mice and fixed overnight in a 4°C cold room in 4% paraformaldehyde in pH 7.4 PBS. Brains were then washed in PBS on a shaker table at room temperature three times for 40 minutes each. Brains were sectioned into 40-50 μm slices with a vibratome (VT-1000s, Leica, Wetzlar, Germany) and ordered into PBS-filled wells with extreme care to maintain the order of the serial sections. The tissue was then washed with PBS, blocked with 10% horse serum in PBS-0.1% Triton, and incubated with primary antibody (varied depending on specific experiment; included rat anti BrdU, rabbit anti GFAP, goat anti DCX, etc...) overnight on a shaker table in a 4°C cold room. Primary

antibody solution was removed and the brains were then washed with PBS three times for ten minutes each at room temperature. Alexa Fluor dye-conjugated secondary antibodies (varied depending on experiment; included donkey anti rat 488, donkey anti rabbit 546, donkey anti goat 546, donkey anti rabbit 647, etc...) were then applied to the tissue for one hour at room temperature on the shaker table. Brain sections were washed in PBS three times for 10 minutes each at room temperature on the shaker table and then mounted from PBS on slides. Coverslips were fixed on the slides using Aquapolymount and allowed to dry. Brain sections were then imaged and analyzed.

RMS Area Measurements:

Kasey Baker sliced coronal brain sections at 50 μ m and labeled the brain sections with DCX and GFAP. Following labeling, fluorescent images of the RMS were acquired (Axioskop 2+, Zeiss; Retiga EX, Q-Imaging System) at 3.67, 3.72, and 3.77 mm distal to the beginning of the OBs. Sample size for each group was at least three animals. The DCX⁺ areas were measured using Openlab 3.1.5 (Improvision, Lexington, MA). Mean values and \pm SEM were then calculated. Student's *t*-test was used for statistical analysis (* $P < 0.05$, ** $P < 0.001$).

BrdU Immunohistochemistry:

Mice were given an intraperitoneal injection with 300 mg BrdU/kg two hours prior to animal sacrifice and perfusion [37]. BrdU immunostaining of 40 μ m sections was performed as described by Cameron and McKay (2001) [37]. Sections were imaged (Axioskop 2+, Zeiss) and the BrdU⁺ cells were counted in coronal sections of the SVZ and

the RMS using ImageJ imaging software (National Institutes of Health, Bethesda, MD). SVZ sections (40 μ m) were analyzed from coordinates 1.70 mm bregma, 5.50 mm interaural to 0.00 mm bregma, 3.80 mm interaural and RMS sections (40 μ m) were analyzed from coordinates 2.71 mm bregma, 6.51 mm interaural to 1.98 mm bregma, 5.78 mm interaural. Number of mice varied for each group (see Results). Sections were labeled with anti-BrdU, anti-DCX, and anti-GFAP.

BrdU/EdU Pulse Labeling:

EphA4 WT mice (first experiment) were administered a 150 mg BrdU/kg IP injection at three days of age (P3). At P5, the same mice were given an IP injection of 150 mg EdU/kg two hours prior to animal sacrifice and perfusion. BrdU immunostaining of 40 μ m sections was performed as described by Cameron and McKay (2001). EdU immunostaining of the same sections was performed as described by the Click-iT[®] Plus EdU Imaging Kits protocol published by Life Technologies[™]. Sections were imaged with a Leica TCS SP2 confocal system together with a Leica DMIRE2 microscope and Leica confocal software (Leica Microsystems, Wetzlar/Marburg, Germany). Sections were labeled with anti-BrdU, anti-EdU, and anti-GFAP. An additional experiment was performed using hGFAP:mRFP mice following the same procedure to more clearly label astrocytes within the developing RMS. A third experiment using hGFAP:mRFP mice was conducted using a chase period of 10 days to provide additional time for dividing neuroblasts to escape the region, ultimately highlighting the proliferating astrocytes that remained. Preliminary results were obtained by analyzing three RMS sections from 2.34 mm bregma, 6.14 mm interaural to 2.22 mm bregma, 6.02 mm interaural from three WT brains. Total

BrdU⁺/GFAP⁺, EdU⁺/GFAP⁺, and BrdU⁺/EdU⁺/GFAP⁺ cells inside and outside the RMS were counted.

MACS/FACS Cell Enrichment and Sorting:

P3-P12 *EphA4*^{ΔeGFP/ΔeGFP} and *EphA4* WT (negative control) mice brains were dissected under cold DMEM, and the regions of interest were removed. The brain regions of interest were mechanically dissociated using forceps. Following mechanical dissociation, 0.025% trypsin was added to the tissue and then triturated using a 1000 μL filter-tipped pipet. The samples were then incubated for 20 minutes at 37°C with gentle shaking to facilitate dissociation. Following incubation, the samples were centrifuged at 350xg for three to five minutes, and supernatant was aspirated. The resulting pellet was resuspended in 2-7 mL 10% FBS in DMEM to neutralize the trypsin previously added to the solution. Solution was then filtered through 70μm cell strainer and collected. The resulting samples were centrifuged for five minutes at 350xg, and supernatant was removed. To perform MACS enrichment for neuroblasts, the pellet was resuspended in ice-cold 0.5% BSA in PBS ("buffer"). Solutions were aspirated into 2 mL eppendorf tubes (70-80 μL/sample) and incubated for 10 minutes at 4°C. To deplete cells with the A2B5 antigen (removal of glial progenitors), MACS antibodies (anti-A2B5 mouse IgM) and Anti-Mouse IgM Microbeads were added, and the solutions were incubated for 10-15 minutes at 4°C. Samples were rinsed in 1-2 mL buffer, centrifuged, and supernatant was aspirated. The resulting pellets were resuspended in buffer. Secondary antibody incubation was then performed for GLAST enrichment and the resulting samples were rinsed in 1-2 mL buffer, centrifuged, and supernatant was aspirated. The resulting pellets were resuspended in

buffer. MACS columns were prepared by setting up the magnet and then loading with 500 μ L ice-cold buffer, which was allowed to elute. 500 μ L of sample was loaded onto each column and 3x500 μ L ice-cold buffer was added to each column to rinse after each time it emptied. Flow-through was collected for analysis. The column was then removed and the sample was eluted with 500 μ L cold PBS or 5% serum in PBS (Concentration of samples should be below 10^6 cells/mL). For FACS, 1 μ L Alexa-fluor-conjugated antibody was added and then incubated with gentle agitation for at least 20 minutes at room temperature. Cells were rinsed in 1 mL PBS, centrifuged, and supernatant was aspirated. Cells were then resuspended in 500 μ L PBS and placed on ice. Labeled, unlabeled, or flow-through populations were sorted by FACS at the flow cytometry core. The FACS-sorted cell populations were then identified as neuroblasts, astrocytes, or endothelial cells based on comparative transcriptional analysis via qRT-PCR or by immunocytochemistry antibody labeling for cell-type specific proteins. Due to complications that arose in preliminary cell sorting methods, additional analysis required the cells to be fixed and permeabilized using 1% PFA and 0.1% Tx, 2% serum in PBS, which allowed the cells to be labeled using anti-GFP. Following this step, flow cytometry was performed using the same machine described previously.

qRT-PCR:

Following MACS-FACS, qRT-PCR was performed on cell populations according to Life Technologies™ SuperScript™ III First-Strand Synthesis System for RT-PCR protocol and BIO-RAD's iTaq™ Universal SYBR Green Supermix protocol.

Results:

Astrocyte Proliferation and Infiltration into the Neuroblast-Dense RMS

A hallmark of my undergraduate research at the University of Connecticut was focused on investigating the developmental time course of astrocyte invasion into the RMS of mice. In addition, a central question concerning this developmental phenomenon focuses on the proliferative capacity of invading astrocytes. In other words, I questioned whether astrocytes continue to divide during and/or after RMS glial tube formation or if these cells only divide outside of this region and subsequently invade and reorient in the RMS without any further division. Currently, it has been found that few astrocytes are present in the RMS core at P3 despite the large population of these cells densely surrounding this region at this time point. By P5, immunohistochemistry (IHC) studies have revealed that numerous astrocytes begin to invade and populate the developing RMS and form highly organized glial tubes. Additional IHC studies demonstrated that this phenomenon occurs through P16. The specific mechanism(s) for astrocyte infiltration and orientation within the RMS remain to be determined although and likely include signaling through Eph-ephrin interactions, which have been found to mediate cellular repulsion and adhesion in the developing nervous system. To address the temporal and spatial dynamics of astrocyte proliferation in the developing RMS, an experimental paradigm employing dual-pulse labeling with thymidine analogs was used. To be more specific, acute injections (150 mg/kg) of the proliferation markers 5-bromo-2'-deoxyuridine (BrdU) and 5-ethynyl-2'-deoxyuridine (EdU) were administered based on a specific experimental schedule highlighted in Figure 2a. Administration of BrdU at P3 was performed to label dividing astrocytes outside of the RMS, while the administration of EdU at P5/6 was employed to

label proliferating astrocytes within the RMS during development. BrdU/EdU tissue collected was also processed with GFAP antibodies to highlight astrocytes in this developing region. Administration of BrdU at P3 revealed a very small population of dividing astrocytes in the area surrounding the RMS. In addition, no BrdU⁺/GFAP⁺ double-labeled cells were found within the RMS core, indicating astrocyte division does not occur within the RMS at this time point. Following the administration of EdU at P5, IHC analysis revealed similar findings, with a reduced number of EdU⁺/GFAP⁺ double-labeled cells in the region surrounding the RMS and very few EdU⁺/GFAP⁺ cells within the core of the RMS. Triple-labeled BrdU⁺/EdU⁺/GFAP⁺ cells were also very rare; a low number were found in the region surrounding the RMS and none were observed within (Figure 2b). As a result, these experiments suggest that the increased number of astrocytes within the core of the early postnatal RMS results from the infiltration of proliferating astrocytes from the surrounding tissue. These experiments also address the question of astrocyte proliferation and infiltration by indicating that RMS astrocytes do not typically divide after forming glial tubes within this region. Instead, it appears that astrocytes divide in order to form, or while forming, glial tubes. Subsequent experimentation employing the same experimental schedule with transgenic GFAP:mRFP1 reporter mice used to aid in the visualization of nuclear proliferation markers has revealed similar findings (Figure 3). To further strengthen my findings, I performed an additional experiment in which the chase period for EdU administration was 10 days, which provided additional time for proliferating neuroblasts to exit the region. In doing so, I was able to visualize proliferating astrocytes in the developing RMS with reduced interference from BrdU and EdU labeling of dividing neuroblasts. Preliminary results, in which total BrdU⁺/GFAP⁺, EdU⁺/GFAP⁺, and

BrdU⁺/EdU⁺/GFAP⁺ cells inside and outside the RMS were counted, further reinforce the findings from the previous two experiments in which a greater number of triple-labeled cells were found outside of the RMS (Figure 4). Interestingly though, an increased number of EdU⁺/GFAP⁺ cells were found within the RMS using this experimental schedule, suggesting that division of astrocytes may occur within the RMS following invasion from the periphery. As a result, subsequent experimentation employing different pulse-chase time points will be performed in future studies.

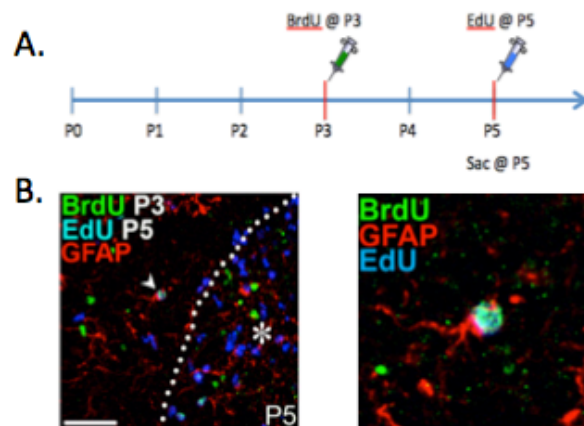


Figure 2. BrdU and EdU Pulse-Labeling to Investigate Astrocyte Proliferative Capacity in the Early RMS. (A) Diagram of initial dual-pulse labeling of proliferative S-phase cells within the anterior forebrain using the thymidine analogues BrdU and EdU. (B). Fluorescence microscopy of astrocyte proliferation in the region surrounding the RMS indicating a BrdU⁺/EdU⁺ proliferating GFAP⁺ cell within the tissue directly neighboring the developing RMS. Asterisk denotes the RMS, and its boundary is marked by the dotted line. Arrowhead marks a BrdU⁺/EdU⁺/GFAP⁺ triple-labeled cell. Scale bar 50 μ m.

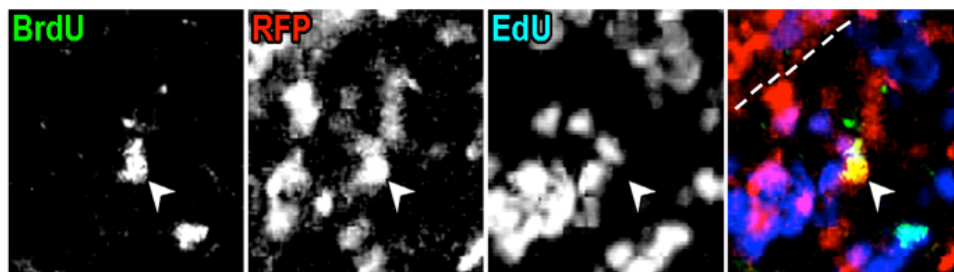


Figure 3. BrdU and EdU Pulse-Labeling in hGFAP:mRFP Mice. Fluorescence microscopy of astrocyte proliferation within the RMS indicating a RFP⁺ cell that is BrdU⁺ but not EdU⁺, suggesting a lack of multiple divisions. Arrowhead marks a BrdU⁺/RFP⁺ cell. The boundary of the RMS is marked by the dashed line.

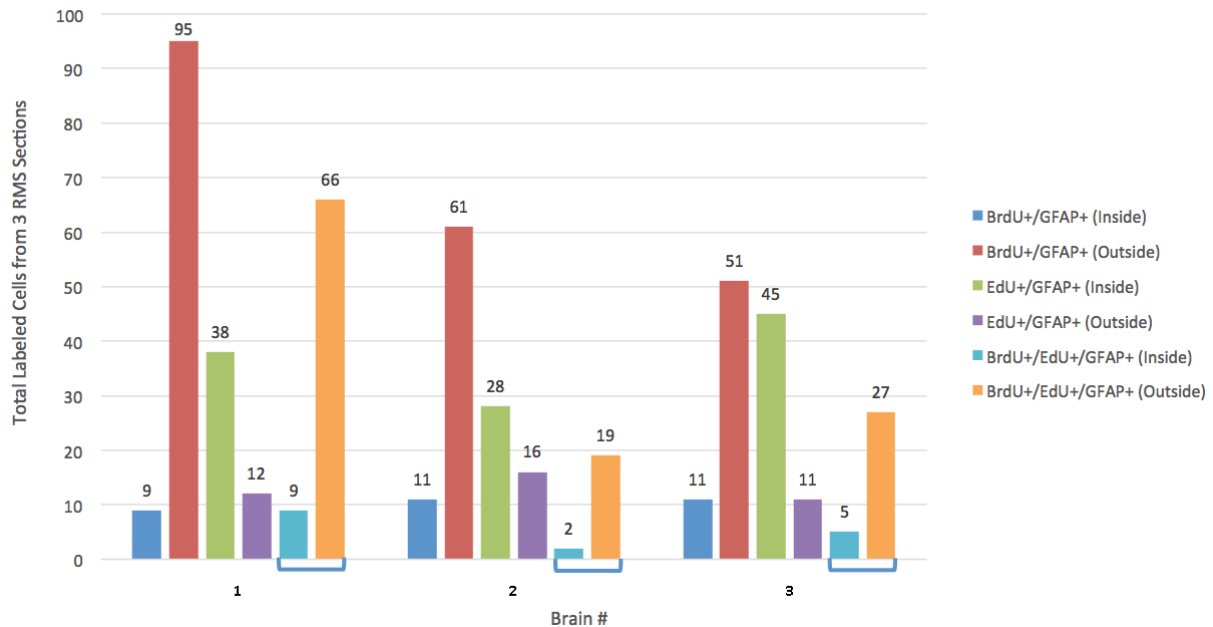


Figure 4. BrdU⁺, EdU⁺, and GFAP⁺ Total Cell Counts from the RMS of hGFAP:mRFP Mice. Total cell counts of BrdU⁺/GFAP⁺, EdU⁺/GFAP⁺, and BrdU⁺/EdU⁺/GFAP⁺ cells inside and outside the RMS were counted from 3 RMS sections from 3 WT hGFAP:mRFP mice (9 sections in total). As expected, the total number of BrdU⁺/EdU⁺/GFAP⁺ cells inside the RMS was much less than the total number of these triple-labeled cells outside the RMS. Blue brackets highlight triple-labeled cells inside and outside the RMS.

Astrocyte Morphology in the Rostral Migratory Stream Resembles Reactive Astrogliosis

While investigating astrocyte proliferation and infiltration in the developing RMS, it was noted that invading astrocytes in the RMS have distinct morphological features that strongly resemble reactive astrocytes, which play a crucial role in the formation of glial scars following CNS injury [38, 39]. To further investigate this initial observation, tissue samples were collected and analyzed using GFAP antibodies to visualize astrocyte morphology. In doing so, representative images of protoplasmic, fibrous, reactive, and RMS astrocytes were processed for initial comparison (Figure 5). Analysis of the representative images collected further reinforced the similarities between RMS and reactive astrocytes as

both cell populations exhibit hypertrophic fibrous processes and increased cellular packing density. Subsequent research will investigate astrocyte morphology at P3, P5, P7, P10, P12 in a minimum of n=3 mice per timepoint using IHC. Quantitative real-time polymerase chain reaction (qRT-PCR) against TGF β , AQP4, nestin, and GFAP δ , which have been shown to be upregulated in glial scarring, will be used for transcriptional analysis of RMS and reactive astrocytes [39, 40, 41]. GFAP:mRFP1 mice will also be used to aid in the visualization of reactive and RMS astrocytes and in fluorescence-activated cell sorting (FACS) for transcriptional analysis of these cell populations. Findings from preliminary data and future studies concerning glial tube astrocyte morphology and reactive astrogliosis will hopefully increase our current understanding of glial scar formation following CNS injury and subsequently lead to the development of novel therapies to control glial scarring.

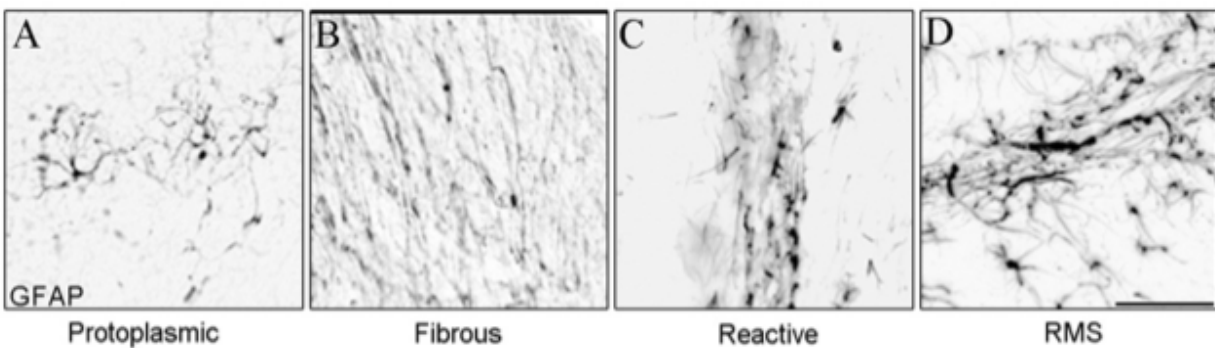


Figure 5. Astrocyte Morphology in the Brain. GFAP immunoreactivity of (A) protoplasmic astrocytes found in the cortex, (B) fibrous astrocytes present in the corpus callosum, (C) reactive astrocytes resulting from a cortical stab injury, and (D) glial tube astrocytes from the rostral migratory stream. Similar features are shared between reactive and RMS astrocytes, including hypertrophic fibrous processes and increased cellular packing density. Scale bar 50 μ m.

EphA4 Plays a Crucial Role in Glial Tube Formation and Neuroblast Restriction in the RMS

Currently, little research has investigated the molecular and cellular mechanisms involved in the arrangement of astrocytic processes into RMS glial tubes in early postnatal development. We hypothesize that the early development of the RMS is dependent on molecular signaling mechanisms similar to reactive astrogliosis. For instance, one critical member of the Eph family of tyrosine kinase receptors, EphA4, has been found on reactive astrocytes, but its role in the formation of glial scars remains to be determined [43-46]. In addition to glial scarring, a more comprehensive investigation of EphA4 and its role in glial tube formation and regulation in the postnatal and adult forebrain is crucial for a greater understanding of cellular migration and cell-cell contact-mediated interactions.

To first investigate the role of EphA4 in RMS development, cytoarchitectural morphology of transgenic EphA4^{-/-} and wild-type (WT) mice was analyzed at P6 and P12 (Figure 6). At the P6 time point, few differences were found between the two mouse strains. GFAP⁺ astrocytes from the surrounding tissue had begun to invade the RMS core and blood vessel development appeared to occur in a parallel fashion to the RMS. DCX⁺ neuroblasts also participated in tangential chain migration in EphA4^{-/-} and WT mice at P6. By P12 though, changes in RMS structure between EphA4^{-/-} and WT mice became much more apparent. In WT mice, it was observed that astrocytes within the RMS core were oriented in a parallel fashion to neuroblast migration, essentially forming a defined astroglial meshwork that enclosed the migrating neuroblasts. In the KO mouse though, astrocyte morphology and orientation appeared greatly changed, as hypertrophic and disorganized astrocytes were found within the RMS core. The restriction of neuroblasts to the RMS was also disturbed in EphA4^{-/-} mice as neuroblasts were noted to extend

processes radially and escape from the RMS. This was not found in WT mice, as neuroblasts appeared to be highly restricted within the developing RMS, which is shown through DCX IHC (red dotted line in Figure 6).

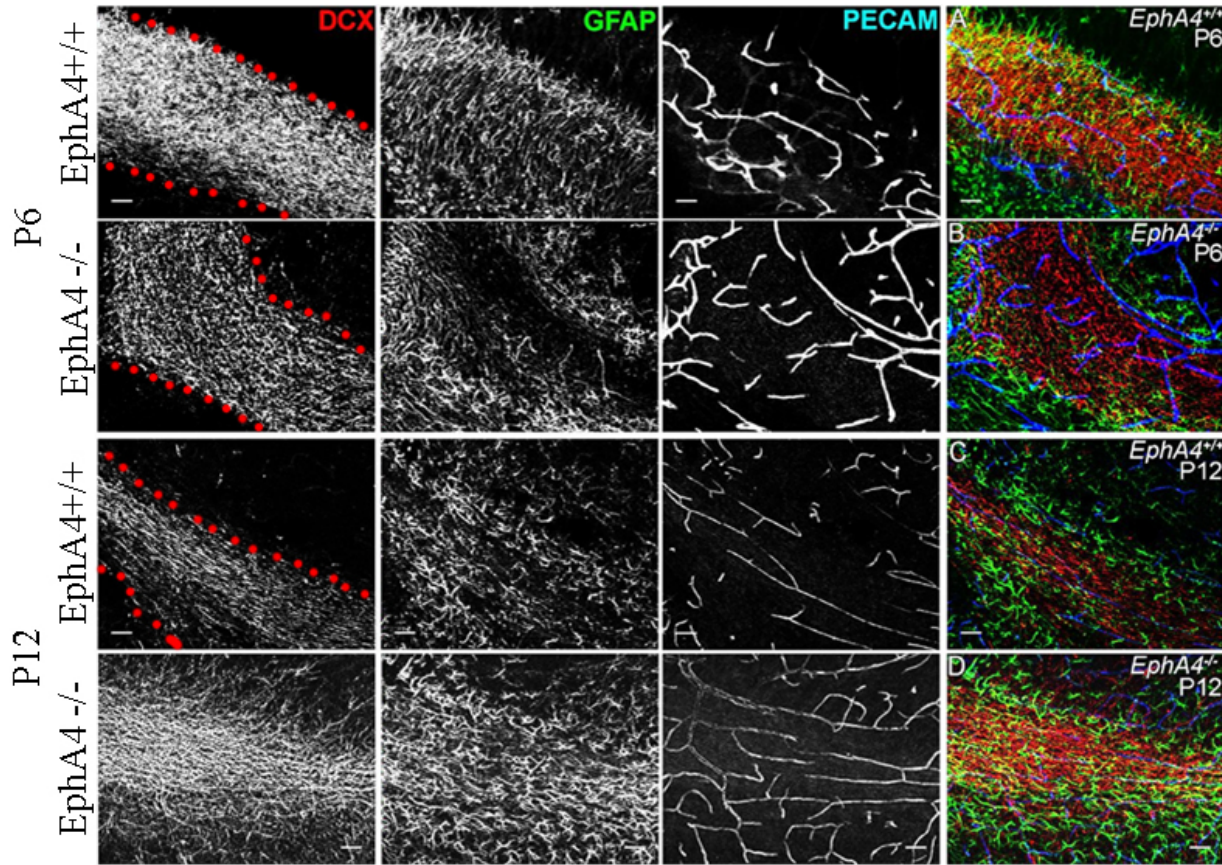


Figure 6. Postnatal Development of the RMS and Astrocyte Invasion. Fluorescence microscopy of the rostral migratory stream in early postnatal development. At P6, sagittal sections of the RMS reveal few astrocytes in the core of the RMS. In addition, the width of the RMS is comparable in EphA4^{+/+} and EphA4^{-/-} mice at P6. By P12, many astrocytes have invaded the developing RMS and oriented parallel to the direction of the migrating neuroblasts in EphA4^{+/+} mice. Astrocytes appear more disorganized and hypertrophic in EphA4^{-/-} mice at P12. RMS width (marked by red dotted lines) at P12 is greater in EphA4^{-/-} mice than in EphA4^{+/+} mice, with an increased number of neuroblasts extending radial processes into the parenchyma and escaping from the RMS. PECAM immunohistochemistry reveals a similar parallel orientation of blood vessels within the RMS of EphA4^{+/+} and EphA4^{-/-} mice. Scale bar 50 μ m.

To investigate further the developmental role of EphA4 in RMS boundary formation, coronal sections of WT, EphA4^{+/-}, and EphA4^{-/-} tissue were processed and cross-sectional areas of the RMS from each sample type were compared. In EphA4^{+/-} coronal sections, tangentially migrating neuroblasts were confined to a small and relatively compact area (Figure 7A). As expected, RMS cross-sectional analysis in EphA4^{-/-} mice revealed numerous neuroblast processes that extended radially into the surrounding parenchyma (Figure 7B). Area measurements at three different stereotaxic coordinates in each mouse revealed a significant increase in cross sectional RMS area in both EphA4^{+/-} and EphA4^{-/-} in comparison to EphA4^{+/+} mice (n=3 for each group, *P<0.05 and **P<0.001; Student's *t*-test; Figure 7E).

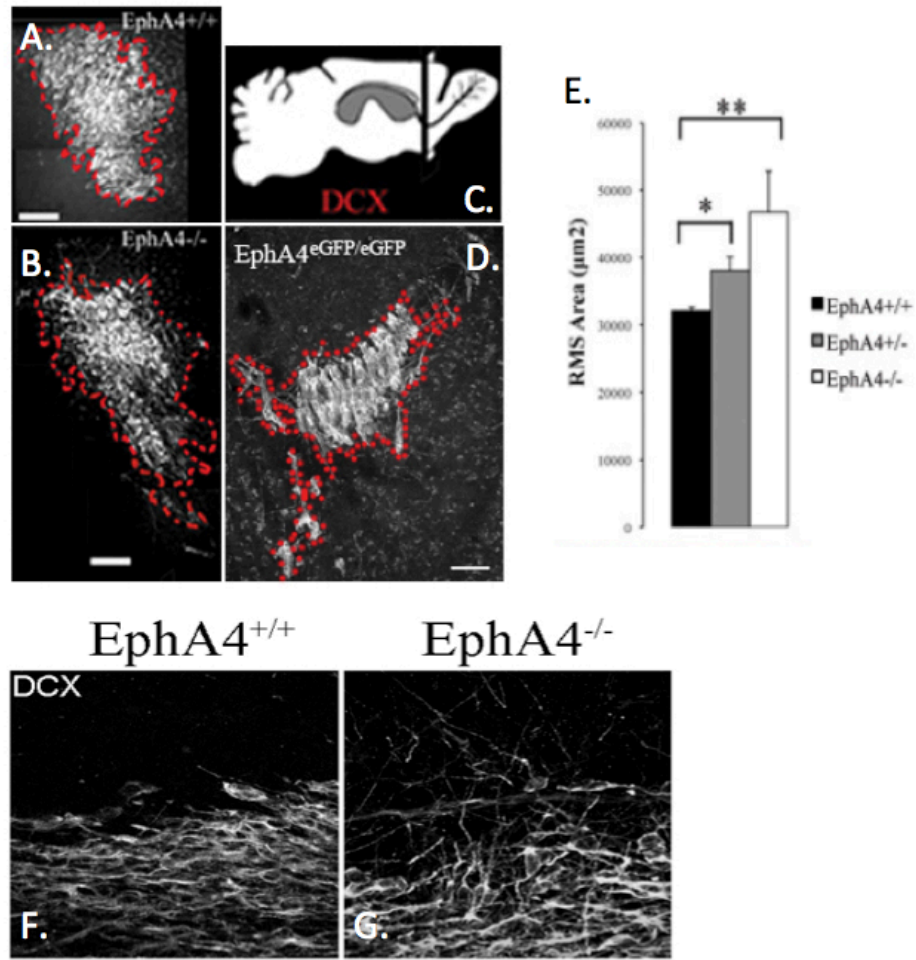


Figure 7. Persistent Role of EphA4 in Maintaining RMS Boundaries for Neuroblast Migration. Fluorescence immunohistochemistry of coronal sections of the RMS obtained from the descending limb and elbow region (indicated in C) reveal differences between the restriction of migrating neuroblasts in the adult RMS of *EphA4* WT and mutant mice. In A, DCX⁺ neuroblasts appear to be tightly confined within the forebrain of *EphA4* WT mice. In B and D, fluorescence immunohistochemistry of *EphA4*^{-/-} and *EphA4*^{ΔeGFP/ΔeGFP} sections revealed an increase in RMS cross-sectional area. DCX⁺ neuroblast chains are also more dispersed. Scale bars 100 µm (A & B) and 50 µm (D). Sagittal RMS sections further demonstrated changes in neuroblast restriction as an increased number of neuroblasts were observed to escape the RMS in *EphA4*^{-/-} mice (G) in comparison to *EphA4* WT mice (F). RMS area measurements, defined by DCX staining, found *EphA4*^{+/-} and *EphA4*^{-/-} RMS areas to be significantly larger than the RMS area of WT mice (*P<0.05 and **P<0.001; Student's *t*-test; Figure 7E).

Identification of EphA4-Expressing Cells Using a Novel EphA4-ΔeGFP MACS-FACS Approach

Despite EphA4's apparent role in defining RMS boundaries and regulating neuroblast migration, previous research remains in disagreement on its cellular location in the postnatal brain [45 – 48]. To address these discrepancies concerning EphA4 localization, we developed an experimental paradigm employing EphA4-ΔeGFP fusion protein to aid in the isolation and subsequent identification of cell(s) expressing the tyrosine kinase receptor EphA4.

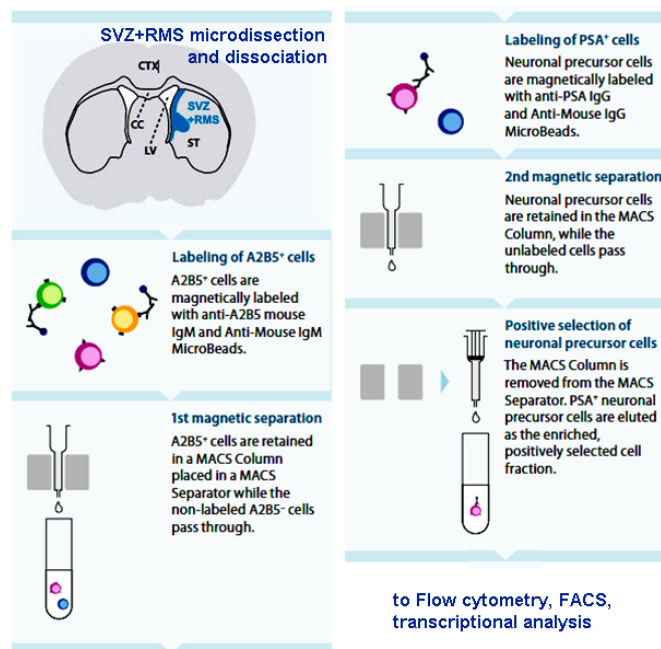


Figure 8. Schematic of MACS Enrichment for Populations of SVZ-RMS Cells. Multiple rounds of MACS depletion (A2B5) and enrichment (PSA-NCAM) lead to neuroblast enrichment. Astrocyte enrichment (GLAST) follows a similar method to PSA-NCAM enrichment. *Adapted from Miltenyi Biotec.*

In this experiment, microdissected mouse SVZ and RMS tissue from P3, P7, and P12 *EphA4^{ΔeGFP/ΔeGFP}* mice was dissociated with trypsin and filtered through a 70 μm and 35 μm cell strainer. The resultant suspensions underwent magnetic bead-conjugated antigen-based cell sorting (MACS) as described in the Materials and Methods section to enrich for neuroblasts. Antigen-enriched populations (neuroblasts) and flow-through samples were then analyzed with fluorescence activated cell sorting (FACS) or flow cytometry, which allowed EphA4⁺ cell populations to be separated due to the presence of GFP. Processing of

the neural progenitor enriched (PSA⁺-fraction) or flow-through samples for EphA4-ΔeGFP⁺ cells through FACS was followed by transcriptional analysis via qRT-PCR and immunocytochemistry (ICC) antibody labeling to identify the cells as neuroblasts, astrocytes, or endothelial cells. ICC antibody labeling of DCX⁺ neuroblasts and GFP expression (EphA4, via EphA4-ΔeGFP fusion protein) from P12 EphA4-ΔeGFP mice revealed co-localization of these signals on DCX⁺ neuroblasts, suggesting that EphA4 is normally expressed on migrating neuroblasts in the developing RMS (Figure 9A). Initial studies also demonstrate the efficacy of MACS-FACS techniques in separating GFP⁺ cells (Green Box in Figure 9C) from the collected MACS fractions. Mixed results were obtained for cell enrichment using EphA4-ΔeGFP MACS-FACS techniques though, which necessitated other experimental strategies.

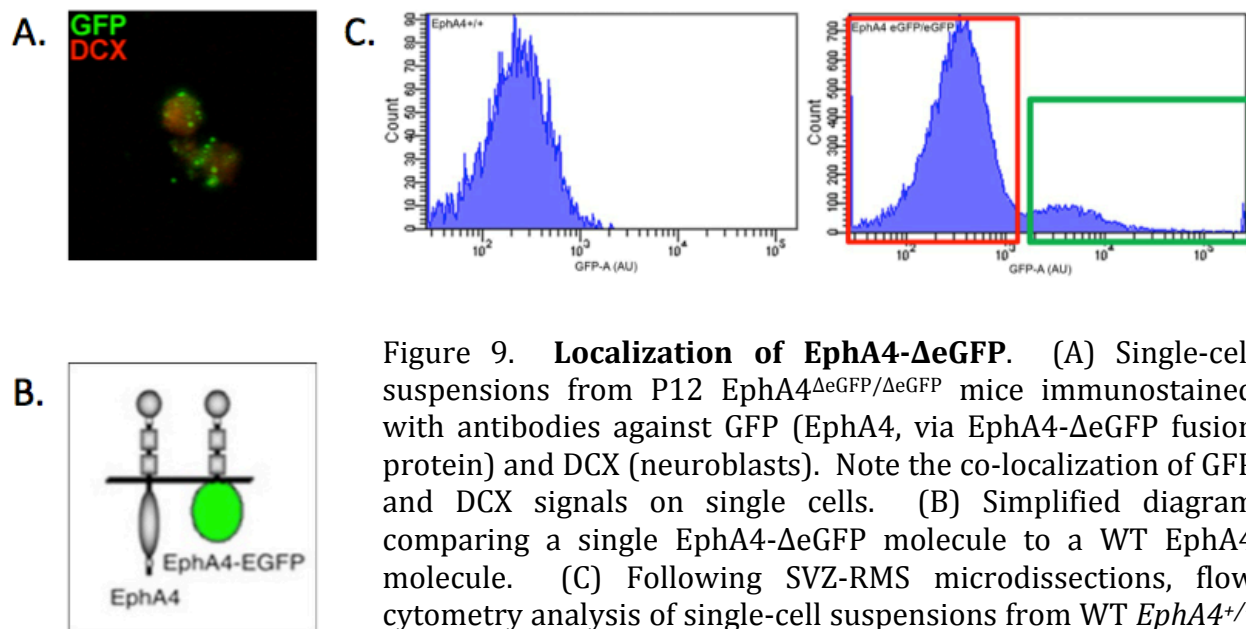


Figure 9. Localization of EphA4-ΔeGFP. (A) Single-cell suspensions from P12 EphA4^{ΔeGFP/ΔeGFP} mice immunostained with antibodies against GFP (EphA4, via EphA4-ΔeGFP fusion protein) and DCX (neuroblasts). Note the co-localization of GFP and DCX signals on single cells. (B) Simplified diagram comparing a single EphA4-ΔeGFP molecule to a WT EphA4 molecule. (C) Following SVZ-RMS microdissections, flow cytometry analysis of single-cell suspensions from WT *EphA4*^{+/+} mice and *EphA4-ΔeGFP* mice detected eGFP⁺ cells in EphA4-ΔeGFP mice. The detection of eGFP⁺ cells was revealed through increased GFP relative intensity (green box) in comparison to cells from WT mice (control GFP relative fluorescence levels, bound by red box).

To increase the specificity of MACS-FACS cell enrichment, additional experiments using GFAP:mRFP1 mice were used. By performing MACS enrichments for PSA and FACS sorting for RFP⁺ cells at P7, separate samples of neuroblasts and astrocytes were collected and confirmed through measurements of the relative normalized expression of DCX and GFAP (Figure 10A). As expected, the δ isoform of GFAP (GFAP δ), which is typically expressed in astrocytes of the SVZ and RMS, was detected in the RFP⁺ cell population (Figure 10A) [40]. Following separate GFAP:mRFP1 MACS enrichments for neural progenitors (PSA⁺ cells) and astrocytes (GLAST⁺ cells), FACS was used to either enrich for RFP⁺ cells or to remove RFP⁺ cells from PSA-enriched samples. The purified cellular populations underwent qRT-PCR for transcriptional analysis (Figure 10B), which revealed increased relative expression levels of EphA4 in the PSA⁺ cell fraction and ephrinA5 and ephrinB1 in the RFP⁺ cell population. Overall, these initial studies suggest that EphA4 is expressed at a higher level on migrating neuroblasts in the RMS with ephrins A5 and B1 more highly expressed on RMS astrocytes.

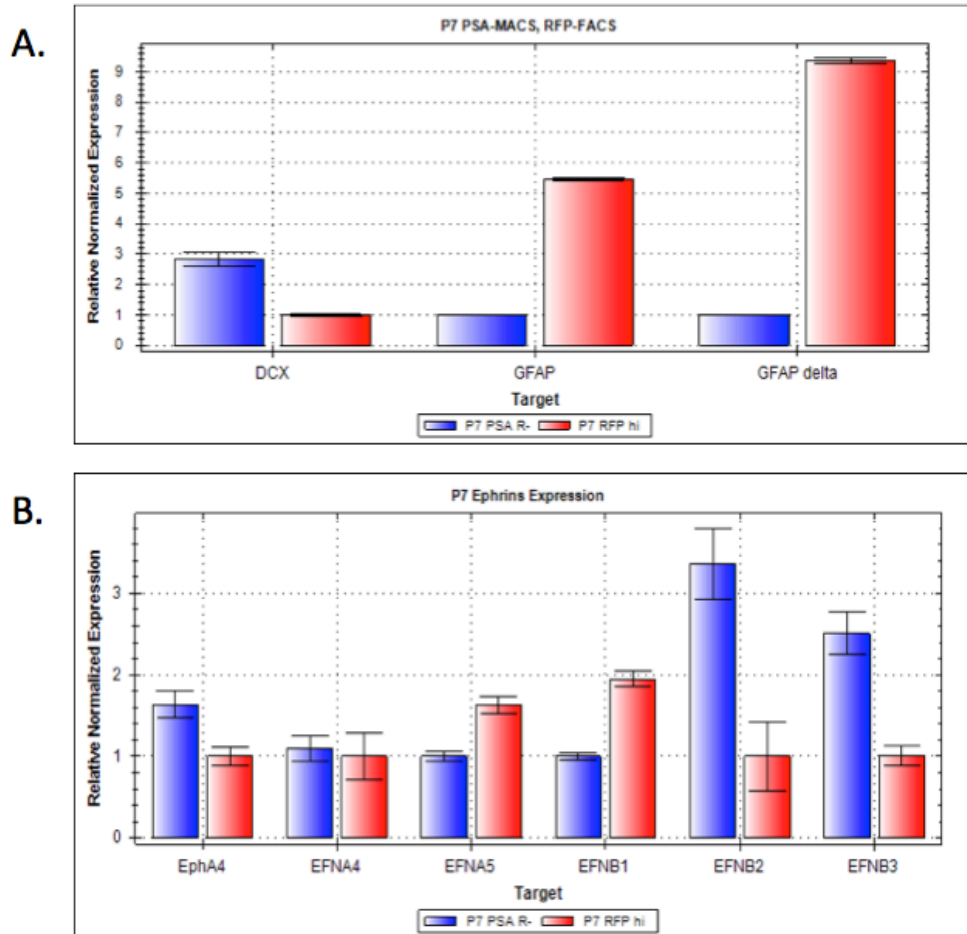


Figure 10. Transcriptional Analysis of SVZ-RMS Cell Populations Enriched by MACS-FACS Using qRT-PCR. (A) Relative normalized expression levels of transcript targets (DCX, GFAP, and GFAP delta) from enriched P7 GFAP:mRFP1 SVZ-RMS cell populations through MACS selection for PSA⁺ cells, followed by FACS. FACS was used to exclude RFP⁺ cells from the PSA-enriched fraction. RFP⁺ cells were selected via FACS in other trials. (B) Relative normalized expression levels of EphA4 and ephrin target transcripts from the same cell populations in (A). In the PSA⁺ cell fraction, the level of EphA4 expression is higher in comparison to the RFP⁺ cell fraction. Higher relative expression levels of ephrin A5 and ephrin B1 were found in RFP⁺ astrocytes.

The Role of EphA4 Signaling in Regulating Proliferation in the SVZ/RMS System

A final line of research addressed during my undergraduate career was the investigation of changes in cellular proliferation mediated by EphA4 in the SVZ/RMS system. Previous research by Holmberg et al. (2005) found that ephrin-A2 reverse

signaling negatively regulates neural progenitor proliferation and also neurogenesis [49]. To be more specific, it was found that EphA7 causes ephrin-A2 reverse signaling, which is involved in negatively regulating the proliferation of neural progenitor cells. In mice lacking ephrin-A2, cells in the adult neural stem cell niche were found to proliferate more and have a shorter cell cycle. In addition, the increased proliferation of progenitor cells led to a greater number of cells in the OB. By interfering with ephrin-A2 and EphA7 interactions in the adult brain of WT mice, proliferation was disinhibited, and an increase in neurogenesis was observed. Interestingly, mixed results have been obtained concerning the role of EphA4 signaling in regulating proliferation in the SVZ/RMS system, which subsequently encouraged us to perform preliminary experiments investigating this possible phenomenon.

To address this important question and provide a conclusive answer in response to the field's mixed results, we developed a comprehensive plan to analyze cellular proliferation in the developing SVZ/RMS system. We first administered 2-hr BrdU pulses to six-month old EphA4 WT and KO mice. Unfortunately, pup births limited our first experiment to a sample population of $n=1$ for EphA4 WT and $n=1$ for EphA4 KO mice. Following perfusion, serial sectioning, and antibody labeling, ten 40 μ m RMS sections from coordinates 2.71 mm bregma, 6.51 mm interaural to 1.98 mm bregma, 5.78 mm interaural and eighteen 40 μ m SVZ sections from coordinates 1.70 mm bregma, 5.50 mm interaural to 0.00 mm bregma, 3.80 mm interaural were imaged, and BrdU⁺ cells were counted. In the RMS, 520 BrdU⁺ cells were found in the EphA4 WT mouse in comparison to 1167 BrdU⁺ cells present in the EphA4 KO mouse, denoted by a cellular proliferation ratio of 2.24:1 (BrdU⁺ EphA4 KO cells: BrdU⁺ EphA4 WT cells). SVZ counts from these two mice revealed a

similar phenomenon as 1870 BrdU⁺ cells were found in the SVZ of EphA4 WT mice, while 4324 BrdU⁺ cells were counted in the SVZ of EphA4 KO mice, resulting in a cellular proliferation ratio of 2.31:1. As a whole, this initial experiment indicated that EphA4 may play an important role in regulating cellular proliferation in the SVZ/RMS system. To complement this data, we performed a similar pilot experiment in which cellular proliferation was investigated in two-month old EphA4 WT and EphA4^{+/-} mice. Due to similar restrictions in mouse colony size, a sample population size of n=1 was used for EphA4 WT and EphA4^{+/-} mice. Following a similar methodology to the first proliferation study, cell counting in the RMS found 777 BrdU⁺ cells in the EphA4 WT sample, while 813 BrdU⁺ cells were counted in the EphA4^{+/-} sections, resulting in a cellular proliferation ratio of 1.21:1 (BrdU⁺ EphA4^{+/-} cells: BrdU⁺ EphA4 WT cells). In addition, analysis of SVZ tissue revealed 9796 BrdU⁺ cells in the EphA4 WT mouse and 13529 BrdU⁺ cells in the EphA4^{+/-} tissue, resulting in a cellular proliferation ration of 1.38:1. By combining the data collected from the initial proliferation studies with this data, it becomes clear that EphA4 may play an important dosage-dependent role in regulating cellular proliferation in the SVZ/RMS system (Figure 11). Overall, our results suggest that disruption of EphA4 signaling may disinhibit cellular proliferation in a manner similar to the disruption of ephrin-A2/EphA7 interaction. On the other hand, the reported increase in cellular proliferation may have resulted from a 'bottleneck effect,' in which decreased cell migration speed allowed an increased number of cells to be labeled with the proliferative marker. Future studies investigating cellular proliferation are aimed at increasing the sample population size to establish statistical significance and gain a deeper understanding of the role of EphA4 in regulating proliferation in the SVZ/RMS neurogenic niche.

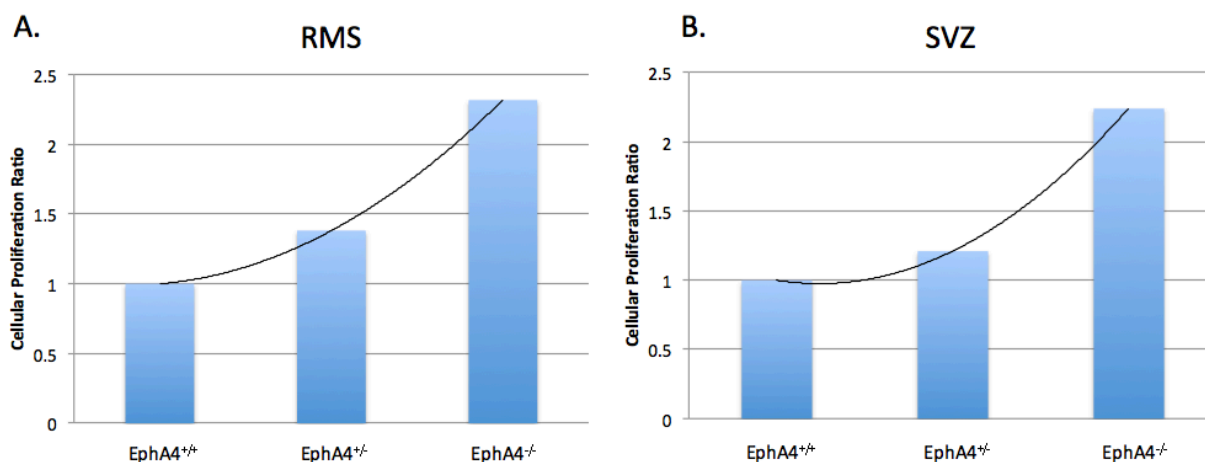


Figure 11. EphA4 May Regulate Cellular Proliferation in the SVZ/RMS System in a Dosage Dependent Manner. After BrdU⁺ cells were counted in EphA4^{+/+}, EphA4^{+/-}, and EphA4^{-/-} tissue, cellular proliferation ratios (BrdU⁺ EphA4 KO or BrdU⁺ EphA4^{+/-} cells: BrdU⁺ EphA4 WT cells) were calculated. Figure 11A (RMS) and Figure 11B (SVZ) both demonstrate an increase in cellular proliferation in EphA4^{+/-} and EphA4^{-/-} mice in a dosage dependent manner. Polynomial trend line added using Microsoft Excel.

Conclusions:

Studies over the past several decades have demonstrated the unique complexity of the SVZ/RMS/OB neurogenic niche and its relevant role as an important model system for the study of neurogenesis and cellular migration. By investigating astrocyte proliferation and infiltration in this system, we have gained a greater understanding of the dynamics of RMS development in the mammalian forebrain. In addition, our study of RMS astrocyte morphology and our future research investigating expression levels of GFAP δ will help draw comparisons between glial tube and reactive astrocyte morphology, hopefully laying a strong foundation for the development of glial scar treatments.

Our research concerning the tyrosine kinase receptor EphA4 has revealed its crucial role in the development and maintenance of the SVZ/RMS system. In the RMS of mutant EphA4 mice, we found disorganized and hypertrophic astrocytes, which led to improper astroglial boundary formation and abnormal neuroblast migration into the surrounding parenchyma. Additional studies found increases in cross-sectional RMS area in EphA4^{-/-} mice, further reinforcing the crucial role of EphA4 in directing the tangential migration of neuroblasts to the olfactory bulb. To determine the exact location of EphA4 in the SVZ/RMS neurogenic niche, a novel combination of immunocytochemistry and MACS/FACS techniques were utilized on EphA4-ΔeGFP and hGFAP:mRFP1 mice. From these experiments, we revealed an increased level of expression of EphA4 in the PSA⁺ cell fraction (neuroblasts). Due to the complexity of this system though, it is likely that EphA4 is still expressed on astrocytes but at a much lower level. Future studies aimed at refining our cell sorting techniques will likely provide definitive answers to the question of exact expression levels throughout RMS development.

Finally, our study addressed the possible role of EphA4 signaling in regulating proliferation in the SVZ/RMS system. By using an experimental technique employing BrdU to label cells undergoing S phase of the cell cycle, we were able to collect a representative picture of cellular proliferation in EphA4^{+/+}, EphA4^{+/-}, and EphA4^{-/-} mice. From our initial findings, it appears that EphA4 negatively regulates cell proliferation in the SVZ/RMS system as EphA4 knockout transgenic mice strains show an increased number of BrdU⁺ cells in this system. In addition, findings from EphA4^{+/-} mice suggest that EphA4-mediated repression of proliferation is dosage dependent as EphA4^{+/-} proliferation levels fall between WT and KO mice. Another possible explanation for the increase in cellular

proliferation is the 'bottleneck effect,' in which decreased cell migration speed allowed an increased number of cells to be labeled with the proliferative marker. Overall, our experimental paradigm investigating EphA4-dependent changes in proliferation provides substantial evidence that cellular proliferation and/or migration speed in this neurogenic niche is dependent upon the EphA4 tyrosine kinase receptor.

Despite the molecular and cellular nature of the experiments that I performed throughout my undergraduate career, investigation of astroglial boundary formation and EphA4 signaling in the RMS is a crucial stepping-stone for future stem cell therapeutics. By gaining insight into the molecular mechanisms involved in glial tube formation in the RMS, substantial progress is made toward understanding and eventually treating glial scars formed after traumatic brain injury. In addition, investigations into the molecular mechanisms involved in the restriction of neuroblast migration in the developing RMS may unveil potential molecules for therapeutic intervention that act either alone on endogenous neural stem cells or together with autologous or artificially cultured pluripotent cells. By discovering regulators of neuroblast migration, these molecules can hopefully be used to direct stem cells to sites of injury in future stem cell therapeutic treatments. Our investigation into neuroblast migration within the central nervous system will also provide valuable information that can be related to the metastasis of cancerous cells in the CNS, as in the case of glioblastoma. Finally, the novel research techniques developed and refined over the course of my undergraduate studies are of significant importance as they lay a solid foundation for further investigation into the highly complex SVZ/RMS/OB system. As a whole, our research into astroglial boundary formation and the critical role of EphA4 in

the developing RMS has laid a solid foundation for future work concerning developmental neurobiology, adult neurogenesis, and stem cell therapeutics.

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