ROLES FOR EphA4-MEDIATED INTERCELLULAR SIGNALING IN CORTICOGENESIS AND IN THE DEVELOPMENT OF THE PERIPHERAL SOMATOSENSORY SYSTEM

A Dissertation
submitted to the Faculty of the
Graduate School of Arts and Sciences
of Georgetown University
in partial fulfillment of the requirements for the
degree of
Doctor of Philosophy
in Neuroscience

By

Hilary North, M.A.

Washington, DC
September 17, 2010
ROLES FOR EPHA4-MEDIATED INTERCELLULAR SIGNALING IN CORTICOGENESIS AND IN THE DEVELOPMENT OF THE PERIPHERAL SOMATOSENSORY SYSTEM

Hilary North, M.A.
Thesis Advisor: Maria Donoghue, Ph.D.

ABSTRACT

The Eph family of receptor tyrosine kinases and its ligands, the ephrins, serve a variety of purposes during the development of many biological systems. Here, we have examined their role in nervous system development. Specifically, we used mice lacking one receptor, EphA4, to determine the function of that family member during normal development of the mouse. While many neurological phenotypes such as improper corticospinal and anterior commissure decussation, dendritic spine formation and auditory brainstem axon targeting have been attributed to insufficient EphA4 function during embryonic and neural development, we discovered two novel roles over the course of our investigation: EphA4 is essential to the proliferation of cortical progenitor cells during the development of the mammalian cerebral cortex and to the proper formation of the trigeminal somatosensory system’s primary sensory organ, the maxillary vibrissae. Our investigation of these two systems revealed a number of noteworthy
additional findings including a novel Eph receptor/ephrin ligand binding pair, EphA4 and ephrin-B1, and possible evidence that neuronal presence may induce formation of target organs during vibrissal development. The two distinct studies presented in this doctoral dissertation have generated excitement in their respective fields, underscoring the continued relevance of the Eph/ephrin family of molecules to mammalian nervous system development.
DEDICATION

To my Dad, who taught me to think like a scientist before I knew what science was, to take the time to do things right the first time, and to assemble my thoughts with logic, purpose, and humor.

To my Mom, whose unfailing assertion that I can do anything was sometimes more needed than she will ever know, and whose interest in every miniscule datum I reported repeatedly reinvigorated my motivation.

To my mentor, Maria, without whose patience, attention, affection, and dedication to teaching I would be, at this point, three years into a post-master’s degree career.

To my friend Melissa, whose intellect and unparalleled, deeply sincere love for the brain are breaths of fresh air in a thick fog of challenges, frustrations and politics. Conversations with you motivate me to be a better scientist.

MANY THANKS

To my IPN classmates, especially Alexis, Ana, Melissa and Danielle; our great group made this fun along the way.

To my lab mates, Meredith and Xiumei; I am thankful for your help, support, patience, and friendship. It goes without saying that these papers would not exist without you.

To the Donoghue lab undergraduate machine for brightening my day-to-day, and especially to Addie for helping me solve “the case of the missing whiskers.”

To Ed Monuki and his students, my hosts at UC Irvine; my time spent in your laboratory showed me a different perspective on research and invaluably helped prepare me for my postdoctoral years.

To my thesis committee, Drs. Casey, Pak and Rebeck, and the professors who taught our classes; your diversity of input helped shape my project and the work I will do in the future.

To our funding institutions (Georgetown University, the NIH and the NSF), and all of our collaborators.

To the creatures who made the ultimate sacrifice in the name of science.

And above all, I am forever thankful to my beautiful sister Victoria who will never cease to amaze or challenge me, and to my husband John who can always bring me to laughter, even through science-induced tears and madness.
# Table of Contents

Overview of mammalian cerebral cortical neurogenesis ........................................... 1  
Local influences on cortical progenitor cells during expansion of the mammalian cerebral wall ......................................................... 10  
Promotion of proliferation in the developing cerebral cortex by EphA4 forward Signaling .......................................................... 23  
Development of the Murine Somatosensory System .................................................. 52  
EphA4 is necessary for spatially selective peripheral somatosensory topography ................................................................. 58  
Conclusions and Future Directions ........................................................................... 74  
Materials and methods ............................................................................................. 76  
References ................................................................................................................. 87
OVERVIEW OF MAMMALIAN CEREBRAL CORtical NEUROGENESIS

The mammalian neocortex is aptly named, as it is the most recent evolutionary addition to the brain. Its elegant six layers of functionally distinct and morphologically diverse neuronal groupings are responsible for complex functions such as language, sensory perception and integration, awareness, working memory, and even personality. In order to accomplish such a myriad of high-level tasks, the cortex is divided into dozens of functional areas (Figure 1-1) (Brodmann, 1909). Despite the complexity of the neocortex and the importance of the functions it accomplishes, few, if any, new neurons have been shown to form in adulthood (Au and Fishell, 2006). Thus, the precise formation of this structure during embryonic development is crucial to the proper functioning of the mature structure and the animal it supports.

Figure 1-1: Brodmann’s map of the functional areas within the human cortex (Brodmann 1909).
Development of the cortex begins when the neural tube folds inward, laying a blueprint for the ventricular system on its interior and demarcating the boundary of the telencephalon, the anterior-most portion of the neuraxis. The thin epithelium of the dorsal telencephalon will ultimately become the cerebral neocortex, and rapid proliferation of neuroepithelial (NE) cells begins approximately halfway through the gestational period. During development of the mouse, the animal model from which the data in this thesis were gathered, these neural progenitor cells expand their population beginning around embryonic day (E) 10 (Figure 1-3) (Dehay and Kennedy, 2007). The proliferation of these NE cells will eventually produce the excitatory neurons that populate the cerebral cortex and its six functional layers. However, most inhibitory interneurons are generated in the ventral telencephalon’s ganglionic eminence and migrate tangentially to join the expanding cerebral wall (Figure 1-4) (Marin and Rubenstein, 2001, Dehay and Kennedy, 2007).
Around mouse E9.5, the epithelial cells that line the ventricle begin to express nestin, detectable by the antibody RC2, and are coupled by notch-dependent formation of tight junctions (Misson et al., 1988, Malatesta et al., 2000, Hartfuss et al., 2001, Malatesta et al., 2003, Hatakeyama et al., 2004, Mori et al., 2005). Due to these neural properties, the epithelial cells are now deemed NE cells, the first iteration of cortical neural progenitors (Gotz and Barde, 2005). The NE cells divide rapidly to produce additional NE cells. These divisions are termed symmetrical, as both offspring are also progenitors that will undergo mitosis themselves. This period of rapid proliferation is known as the NE expansion phase, as it serves to expand the pool of cortical progenitor cells (Gotz and Huttner, 2005, Miller and Gauthier, 2007, Kriegstein and Alvarez-Buylla, 2009). By E12, some of the NE cells also begin to display astroglial characteristics such as glycogen granules, the glutamate transporter GLAST, glutamine synthase, vimentin, and tenascin-C (Alvarez-Buylla et al., 2001, Gotz and Barde, 2005, Mori et al., 2005). With the expression of these glial properties, this subset of NE cells has become radial.

**Figure 1-3: Time course of cortical development in the mouse embryonic brain.** E11: NE cell population in the VZ expands rapidly. E12: preplate (PP) begins to form. E13: SVZ begins to form. E14: VZ radial glia and SVZ IPCs are populous; PP has split to from marginal zone (MZ) and subplate (SP); cortical plate (CP) begins to form in between, populated by the postmitotic cells migrating radially from the VZ. E15: CP continues to grow in an inside-out fashion; Fiber layer (FL) appears. E16: Postmitotic VZ cells continue to migrate radially; CP continues to expand. E17: VZ progenitor pool depletes and SVZ progenitors proliferate; CP expands maximally. (Dehay and Kennedy, 2007.)
By E14, nearly all cells lining the lateral ventricles are radial glia cells (RGCs), and the NE expansion stage has given way to the neurogenic phase of cell generation (Gotz and Huttner, 2005, Miller and Gauthier, 2007, Kriegstein and Alvarez-Buylla, 2009).

During neurogenesis, the progenitor cells have become polarized and display long basal process that reaches the perenchymal basal lamina superficial to the ventricular zone. A short apical process also extends towards the ventricular surface. The progenitor cells are now termed RGCs, although they are not truly glia, but specialized neural progenitor cells. The theories underlying the significance of this morphology are covered in the following section, but this and other concomitant changes, such as signaling through Notch, Dll1, ErbB, neuregulin1, FGF receptors and Wnts facilitate the switch from the symmetric divisions of NE cells, which yielded two progenitor cells, and the asymmetric divisions displayed by some RGCs, which yield one additional RGC and one postmitotic daughter neuron (Gaiano et al., 2000, Schmid et al., 2003, Hatakeyama et al., 2004, Hirabayashi et al., 2004, Yoon et al., 2004, Anthony et al., 2005, Guillemot, 2007, Kawaguchi et al., 2008, Sahara and O'Leary, 2009). The postmitotic cell will not divide again; instead, it begins to differentiate into a cortical neuron.

The appearance of RGCs and the generation of the first postmitotic neuron mark the beginning of neurogenesis. However, many symmetric divisions producing additional
radial glial progenitors also occur after E14. As time progresses, asymmetrical divisions increase in frequency and begin to take the place of symmetric divisions (Calegari et al., 2005). The decision of a daughter cell to remain a progenitor or become a neuron is accompanied with geographical placement: progenitor daughter cells destined to re-enter the cell cycle remain physically housed in the ventricular zone (VZ) while postmitotic, differentiating newborn neurons undergo radial migration superficially towards the forming cortical plate. However, it appears that the choice facing a daughter cell in mammals (but not in other vertebrates) is not as simple as stated: a number of cells migrating radially from the ventricular surface linger in the subventricular zone where they undergo another division (Privat, 1975, Sturrock and Smart, 1980, Bayer et al., 1991, Tarabykin et al., 2001, Miyata et al., 2004, Noctor et al., 2004, Wu et al., 2005). These cells, termed Intermediate Progenitors (IPCs), arise from NE cells (Haubensak et al., 2004) and divide symmetrically to yield two daughter neurons, though sometimes to generate additional IPCs (Noctor et al., 2004, Attardo et al., 2008). Unlike apical (VZ-housed, radial glial) progenitors, the IPCs, or basal progenitors do not exhibit interkinetic nuclear migration, the brief movement of a mitotic cell basally for S-phase followed by a return to the apical surface for mitosis (Sauer, 1935, Dehay and Kennedy, 2007). The proliferation of these additional progenitor cells provides another opportunity for the mammalian cortex to expand its size (Hevner, 2006, Martinez-Cerdeno et al., 2006) and is thought by some to have evolutionary significance in the origination of this specialized structure (Lukaszewicz et al., 2005, Kriegstein et al., 2006, Lukaszewicz et al., 2006).
With newborn neurons emerging from both the VZ and the SVZ, the cortical plate expands over time as these differentiating neurons insert themselves in an “inside-out” fashion: later-born neurons migrate past previously deposited neurons and rest superficially (Figure 1-5) (RIKEN news website; (Molyneaux et al., 2007). There is therefore a temporal correlation between when a neuron is “born” (i.e., the day on which the asymmetric division yielding the postmitotic cell occurs) and its ultimate cortical layer. (Polleux et al., 1997a, Polleux et al., 1997b, Polleux et al., 1998, Polleux et al., 2001). Indeed, carefully timed BrdU pulses in the developing primate cortex reveal an elegant time course of laminar-specific neuronal production (Figure 1-6) (Rakic, 1974).

The precise timing of the switch from neuroepithelial expansion to neurogenesis will dictate the number of neurons that populate the resulting cortical structure (Gotz and Huttner, 2005, Kriegstein et al., 2006, Pontious et al., 2008). Hypothetically, if NE expansion ends one day before it should, each progenitor cell will have had the opportunity to divide one fewer time,

**Figure 1-5:** Top: earliest born neurons (pink, 1) migrate radially towards the pial surface. Subsequently generated neurons (2, 3, 4 red) migrate past the first neurons (pink, 1) and rest superficial to the existing layer, and so on. Bottom: Schematic demonstrating birth dates, in mouse embryonic days, of neurons destined for the subplate (blue), layer VI (purple), layer V (teal), layer IV (olive), and the upper layers II/III (pink).
yielding a progenitor pool half the intended size. Likewise, if the switch to neurogenesis occurs one day too late, each progenitor will undergo one extra cell division and there will be twice the intended number of progenitor cells and ultimate neurons. Indeed, a lag in the onset of neurogenesis has been demonstrated in primates compared to mice and is thought to account for the larger cortex size in the former (Rakic, 1995).

Given the consequences of the exact onset of neurogenesis and the rate at which progenitors either proliferate or generate neurons, much attention has been given to the mechanisms controlling these processes. One hypothesis explaining how cell divisions switch from symmetric to asymmetric involves the length of the G1

Figure 1-6: Left: Cresyl violet-stained representative visual cortex section of juvenile Rhesus monkey that had been injected with [H3]dT at various days of gestation. Top: Injection time points. Right: Diagrammatic representation of the positions of labeled neurons. Horizontal markers on each vertical line indicate positions labeled neurons. Roman numerals indicate cortical layers according to Brodmann's classification. (Rakic, 1974)
portion of the cell cycle. A number of groups have demonstrated that G1, the phase of the cell cycle during which the cell prepares to replicate its DNA, is longer in duration during neurogenic divisions versus symmetric, progenitor cell yielding divisions (Caviness et al., 1995, Takahashi et al., 1995). Indeed, G1-slowing genes such as Tis21/Btg2 and BM88/Cend1 are selectively expressed in neurogenic divisions (Iacopetti et al., 1999, Tirone, 2001, Koutmani et al., 2004, Georgopoulou et al., 2006). When progenitor cells were experimentally treated with the mitogenic factor bFGF, G1 length was shortened in vitro; conversely, the differentiating factor NT3 increased G1 duration (Lukaszewicz et al., 2002), and experimentally slowing the cell cycle leads to premature neurogenesis (Calegari and Huttner, 2003). It has been suggested that differentiating factors may act on dividing cells during the G1 phase, which could explain why cells intending to yield two progenitor daughters may pass through G1 quickly- to diminish exposure to such factors (Mummery et al., 1987, Burdon et al., 2002, Kioussi et al., 2002, Baek et al., 2003, Oliver et al., 2003, Dehay and Kennedy, 2007). However, it remains to be shown whether these are correlative or causally related occurrences (Dehay and Kennedy, 2007, Lange et al., 2009).

Regardless of the importance of cell cycle length and its correlation to rates of neurogenesis, it is clear that the precise timing of the switch from progenitor proliferation to neurogenesis is crucial in determining the size of the resulting cortex, and that the proportion of cells born at given time points throughout neurogenesis will affect the balance of viable, functional cortical layers. Indeed, perturbations to cell generation and subsequent initiation of radial migration during corticogenesis are a basis for a number of clinical conditions including microcephaly, lissencephaly and periventricular nodular
heterotopia (Feng and Walsh, 2004, Mochida and Walsh, 2004). The experiments performed, reviewed and addressed in this thesis contribute to the scientific community’s understanding of the mechanisms that drive proper cortical neurogenesis.
Local Influences on Cortical Progenitor Cells During Expansion of the Mammalian Cerebral Wall

Proper development of the mammalian neocortex requires specific numbers of neurons to be formed at precisely the right time points during neurogenesis. Given the need for this impressive coordination, it is not surprising that a host of transcription factors, growth factors, morphogens and other molecules has been implicated in the control of this process (McConnell, 1995b, Schuurmans and Guillemot, 2002, Casanova and Trippe, 2006, Hevner, 2006). Communication between cells plays a role across nervous system development, from the secretion of growth factors by one structure to induce the shaping of another (Borello and Pierani, 2010) to the pathfinding of pioneering axons by guidance factors (Plachez and Richards, 2005). In some cases, a messenger is soluble and can target cells located remote distances away from the structure of origin. But in other communication paradigms, the cell involved must be neighboring the other cell or structure: physical interaction must occur. One type of molecule that is recognized in proliferation studies comprises cell surface-bound proteins that facilitate the physical interactions between progenitor cells or their immediate environment. The following is a review of the ways in which physical association with progenitors, the molecules facilitating contact and the resulting junctions are believed to affect proliferation rate and allow proper expansion of the progenitor population in the developing cerebral wall.

Many cell surface-bound proteins act to form specialized intercellular junctions. Among these are gap junctions, intercellular connections that directly link the cytoplasm of two cells through the binding of connexons, which provide an avenue for the passage of ions, second messengers, and even molecules such as transcription factors (Sohl et al.,
They are well known in the mature nervous system for their ability to facilitate the transmission of action potentials in the absence of chemical synapses, but they are important in nervous system development, as well (Sutor and Hagerty, 2005). In addition to affecting myelination (Sutor et al., 2000, Menichella et al., 2003, Theis et al., 2005), circuit formation (Sutor and Hagerty, 2005) and migration (Fushiki et al., 2003) during cortical development, several studies suggest a role for gap junctions in regulating proliferation of cortical progenitor cells (Lo Turco and Kriegstein, 1991, LoTurco et al., 1995, Bittman et al., 1997, Rozental et al., 2000).

Gap junctions were first observed in the cortical ventricular zone (VZ) when it was noticed that the input resistance of the progenitor cells was much lower than expected for their small size. Dye tracing experiments thereafter confirmed the presence of cytoplasmic coupling between VZ cells. (Lo Turco and Kriegstein, 1991, Mienville et al., 1994). In 1997, Bittman et al. reported that these linked progenitor cells, which had first been described as “clusters” by LoTurco in 1991, comprise progenitors undergoing cell division: from S-phase to G2-phase, proliferating cells join gap junction-mediated clusters until they progress to M-phase (Bittman et al., 1997). Cells that have become post-mitotic and will not re-enter the cell cycle also will not re-join these gap junction-mediated clusters. Nearly 100% of RGS display gap junctions early in corticogenesis, and this frequency decreases as neurogenesis proceeds and the production of post-mitotic neurons increases.

A potential explanation for this correlation between gap junctions and cortical progenitor proliferation was suggested by Weissman in 2004: proliferation is influenced by the ability of calcium waves to propagate between progenitors, or radial glia (RGCs),
in the VZ (Weissman et al., 2004). Elevated intercellular calcium may help proliferating cells move from G1 to S phase and bias them towards re-entering the cell cycle, a decision that is made when a cell is in G1 (Figure 2-1). The ability of high calcium levels to diffuse into neighboring progenitor cells would induce those neighbors to re-enter the cell cycle as well. Additionally, clusters of progenitor cells will divide in synchrony, leading to isochronic neuron production, or the production of neurons that will eventually reside in the same cortical layer, since laminar fate correlates with birth date.

As gap junctions have become increasingly relevant to the understanding of cortical neurogenesis, the molecules underlying their formation have been studied as well. Multiple reports have specifically implicated connexin43 as crucial to the regulation of progenitor cell behavior in the VZ. The clustering of RGCs that Rozental et al. demonstrated to be essential for cell cycle progression is dependent upon Cx43 (Rozental et al., 2000). Indeed, expression of that particular connexin is elevated in progenitors but decreases when a progenitor becomes postmitotic and begins to express other connexins (Cx26, Cx36, Cx45), along with
neuronal markers, instead (Nadarajah et al., 1997). Cx43 is also crucial to the radial migration of these postmitotic neurons: one investigation of Cx43-null mice revealed BrdU⁺ cells, cells that have recently divided, accruing in the intermediate zone (IZ) rather than joining the expanding cortical plate (CP) (Fushiki et al., 2003). Thus, connexins have demonstrated cell communication capabilities in addition to their role in establishing intercellular junctions.

While gap junctions are an important and direct mode of communication between neighboring progenitor cells in the VZ, adherens junctions and numerous cell surface-bound molecules may play a role in orchestrating the symmetry- or asymmetry- of dividing RGCs that is crucial to the fate of their offspring. With the emergence of electron microscopy in the 1970’s, the physical structure of progenitor cells proliferating in the VZ came under scrutiny. One early study observed that 70-80% of dividing cells in the VZ were oriented horizontally along the ventricular surface (Smart, 1973). When mitosis occurs at the ventricular surface, the spindles’ plane of division will be either parallel or perpendicular to the ventricle, or somewhere in between those two orientations. The angle at which the DNA segregates will dictate the angle at which the whole cell divides, and thus what morphological features (such as cytoplasmic processes) and distribution of molecules each daughter cell will inherit (Figure 2-2). There is agreement in the field that a symmetrical division in the VZ, which has a perpendicular plane of spindle cleavage with respect to the ventricular surface, will yield two new apical progenitor cells. But there are a number of different theories attempting to explain the link between non-perpendicular planes of spindle cleavage and the fate of the resulting cellular offspring. Chenn and McConnell proposed that divisions occurring
outside this horizontal constraint, with an oblique or even parallel plane of mitotic spindle cleavage, yield a neuron in addition to a progenitor, the latter of which remains at the apical ventricular surface (Chenn and McConnell, 1995). Some argue that daughter cell inheritance of an “apical complex” is what is necessary for the heir to remain in the progenitor pool (Chenn et al., 1998, Kosodo et al., 2004), while others suggest that only inheritance of both the apical and basal processes will leave a daughter cell in the apical progenitor pool (Konno et al., 2008). Some groups have observed that a loss of proper orientation, at the expense of cells that would cleave horizontally (perpendicularly), neuroepithelial and radial glial cells exit the cell cycle early and deplete the progenitor pool (Feng and Walsh, 2004). Still other groups demonstrate that the asymmetry of particular proteins within a dividing cell is what matters, and the cleavage plane is secondary in importance to this (Rasin et al., 2007).

Although researchers continue to tease apart the intricacies of asymmetrical

Figure 2-2: Cleavage orientation determines daughter cell fate. Left: symmetric divisions yield two new apical progenitors. Middle: Chenn and McConnell propose that asymmetric divisions yield one new apical progenitor, from the apical portion of the parent cell, and one neuron, from the basal portion. Right: Kosodo et al. propose that inheritance of both the apical and basal process is necessary for apical progenitor fate. (Adapted from Shioi et al., 2009.)
divisions and cleavage orientation during progenitor cell proliferation, it is clear that the process holds both evolutionary (Fish et al., 2006) and clinical (Feng and Walsh, 2004) significance. Thus, an increasing amount of attention has been paid to the mechanisms that may arrange the asymmetry of a subcellular entity or direct cell fate when inherited unequally. It appears that many of these factors have the unique property of facilitating direct interaction between a progenitor and its neighbors or surroundings via cell surface-bound molecules capable of both cross-talk and the exertion of physical tension.

Integrins are a family of cell surface-bound receptors that allow a cell to physically interact with its environment by binding to the molecules that comprise the extracellular matrix (ECM). Expressed by RGCs (Hirsch et al., 1994), some integrins (α5β1) are highly expressed in progenitor cells but are downregulated upon neuronal differentiation (Yoshida et al., 2003), suggesting a potential role in dividing, but not postmitotic cells. Integrins have been shown to be important for the proliferation many cell types (Grashoff et al., 2003, Terpstra et al., 2003), including cortical progenitor cells in the subventricular zone (SVZ) (known as basal, or intermediate progenitors). Interruption of integrin function by the addition of disintegrins or integrin antibodies resulted in a decrease in intermediate progenitor proliferation (Fietz et al., 2010). In this study, B3-integrin was shown to selectively affect the basal progenitors that would divide to yield a proliferative daughter cell, rather than the basal progenitors that would undergo their final division to yield two postmitotic neurons. Shedding additional light onto the importance of radial process inheritcance to daughter cell fate determination, Fietz et al suggest that the integrin-mediated proliferative effect they observed is due to the retention of the basal process: a SVZ progenitor cell will remain proliferative only if it
retains the basal process after cell division, and this retention requires B3-integrin cell-ECM attachment. Despite compelling evidence of a role in the basal progenitor population, several studies have shown that integrins do not significantly affect proliferation of apical progenitor cells in the VZ (Niewmierzycka et al., 2005, Haubst et al., 2006). Although integrins affect morphology of the radial process in VZ RGCs, there remains debate over whether radial process inheritance directs the ability of a daughter cell to self-renew (Fishell and Kriegstein, 2003, Miyata et al., 2004, Fietz et al., 2010).

While integrins can affect morphology, inheritance and proliferative capacity of basal progenitors, adherens junctions are largely credited with maintaining the polarity of apical progenitors in the VZ. These adhesion points are crucial to the maintenance of the epithelium early in gestation, and loss of adherens junctions facilitates the loss of epithelial cell polarity during epithelial-mesenchymal transition (Thiery, 2002). During neurogenesis, adherens junctions facilitate polarity by attaching neighboring RGCs to each other’s apical end feet (Noles and Chenn, 2007, Rasin et al., 2007). Adherens junctions are formed between members of the cadherin family of homophilic adhesion molecules, especially E- and N-cadherin, or Cdh1 and Cdh2, respectively, and create adhesion between cells by directing catenins to arrange actin microfilaments. These junctions are expressed by RGCs in the VZ during neurogenesis, but many of them are lost, along with the cells’ apical contacts, once neurogenesis gives way to the formation of astrocytes (Rasin et al., 2007). As adhesion to the apical, ventricular surface is crucial to the ability of a daughter cell to remain in the proliferative population, adherens junctions have been implicated in the promotion of proliferation (Rasin et al., 2007, Marthiens and ffrench-Constant, 2009). Beyond a physical role in maintaining apical
adhesion, cadherins may also affect the size of the progenitor pool by balancing symmetric and asymmetric divisions during neurogenesis through partitioning various fate-determining proteins between the two daughter cells either evenly or unevenly (Chenn and Walsh, 2002, Mizuhara et al., 2005, Lien et al., 2006, Kadowaki et al., 2007, Rasin et al., 2007, Marthiens and ffrench-Constant, 2009).

Given the importance of RGC polarity, the role adherens junctions play in establishing this polarity and the problems that arise when adherens junctions-mediated adhesion is lost, it is important to determine the processes that are responsible for the proper formation of these intercellular entities. The search for such processes has brought together yet another set of cell surface-bound players: the cell-fate determinant Numb and its close relative, Numb-like (Numbl). Numb is known to asymmetrically bias itself towards the basal pole of dividing neural progenitors in *Drosophila*, promoting a neuronal fate in the encompassing daughter cell by inhibiting the expression of non-neural genes (Doe et al., 1998, Jan and Jan, 2001, Berdnik et al., 2002). In 2007, Rasin et al. demonstrated that Numb and Numbl accomplish a similar determination of daughter cell fate in the murine VZ by directing the expression pattern of cadherins, thereby maintaining the polarity of RGCs through adherens junctions. Loss of Numb and Numbl in RGCs during neurogenesis disrupted adherens junction placement and compromised the proper polarity of the RGCs. Likewise, ectopic overexpression artificially maintained cadherin-based RGC polarity, extending the period of neurogenesis past its normal endpoint (Rasin et al., 2007).
Finally, the Eph/ephrins are another group of cell surface-bound proteins that have been shown to affect proliferation in the VZ. The Ephs are receptor tyrosine kinases whose ligands, the ephrins, are also cell surface-bound. Cells must come into contact with one another for signaling through either the receptor, the ligand or both to occur. While many members of the Eph/ephrin family have been implicated in the proliferation of cancer cells (Pasquale, 2008), EphA4 and ephrin-B1 in particular have surfaced as players in the cortical VZ. EphA4 promotes RGC proliferation (North et al., 2009, Sawada et al., 2010), a kinase domain-dependent event brought upon by the binding of the ephrin-B1 ligand and exerted through the receptor-containing cell (North et al., 2009). Consistent with a role as EphA4’s binding partner in proliferation promotion, ephrin-B1 itself has been shown to inhibit differentiation (Qiu et al., 2008, Wu et al., 2009, Qiu et al., 2010). Some downstream effectors have been suggested as mechanisms for this effect of EphA4 and ephrin-B1 (Qiu et al., 2010, Sawada et al., 2010), and preliminary data from our laboratory suggests a possible role in adherens junction distribution (Figure 2-3). Indeed, Ephs and ephrins are important for the arrangement of adherens junctions in a

Figure 2-3: Ephs may affect adherens junction distribution in the VZ. a, b: Cdh1 (green) distribution in WT (left) and EphA4−/− (right) VZ. c, d: Cdh1 (red) distribution in HEK293 cells transfected stably with EphA4 (c, top), ephrin-B1 (c, bottom), or untransfected (d), plated on opposing sides of combs and placed together for 24 hours. Dashed lines represent comb interface.
number of other systems (Winning et al., 2001, Cooper et al., 2008), and the possibility that this mechanism is responsible for the depleted proliferation seen in $EphA4^{-/-}$ mice must be further examined.

Though the bulk of this review has focused on regulation of cortex size by the exact onset of neurogenesis and the balance between symmetric and asymmetric divisions during the neurogenic phase, a final parameter— the moment at which cortical neurogenesis ends— will also influence the size of the resulting brain structure. One pair of cell surface-bound molecules, Notch and its ligand Delta-like-1 (Dll1), has a particularly dynamic role in the regulation of progenitor proliferation, uniquely executing mechanisms that subsequently promote proliferation, neural differentiation and, finally, astrocyte formation. When Notch binds Dll1 on a closely neighboring progenitor cell in the VZ, it is cleaved and releases its intercellular domain, which travels to the nucleus of the Notch-containing cell. There, the Notch intercellular domain binds to the transcription factor CBF1, changing it from a transcription repressor to an activator. This in turn allows the expression of a family of transcription factors termed Hes1, which are transcription repressors, attenuating the expression of pro-neural genes. In short, activation of Notch ultimately leads to the repression of neural differentiation (Kato et al., 1997, Bertrand et al., 2002). In this sense, Notch/Delta is another example of cell surface-bound receptor/ligand pairings that promote progenitor proliferation (Figure 2-4). But there is more to this pairing than a unique mechanism for promoting proliferation; Notch/Delta interaction provides a way for proliferating RGCs to time their progression into neurogenesis and neural differentiation, as well as glial differentiation, which ends neurogenesis.
While Notch stimulation leads to a suppression of proneural genes, the expression of Hes1 transcription factors also decreases the expression of Notch ligands, including Delta. A negative-feedback loop is therefore established: the more Delta activates Notch signaling, the more Delta expression itself is attenuated. With less Delta-mediated Notch expression, there is less inhibition of proneural genes, and the progenitor cells are more apt to quit the progenitor pool and become neurons. In other words, at the cellular level, the more progenitor cells are present, the less they will produce factors that encourage them to remain progenitors. The gradual diminution of Notch signaling encourages progenitor cell cycle exit by decreasing Notch’s inhibition of the cell cycle inhibitor p27^Kip1. It is an elegant system that incorporates the proliferation of progenitor cells and the progression to neurogenesis in one molecular cascade; furthermore, it is relevant to the end of neurogenesis, as well.

Though many mechanisms thought to end neurogenesis have been studied such as STAT activation, suppression of ErbB signaling and feedback from newly formed neurons (Takizawa et al., 2001, Yanagisawa et al., 2001, Fan et al., 2005, He et al., 2005), Notch/Delta demonstrates a role for cell surface-bound intercellular communication in this process as well. Newly born neurons, as they begin their migration from the VZ, express ligands for Notch including Dll1 and JAG1. These ligands bind the Notch receptor on neighboring RGCs, and the resulting Notch activation leads to expression of the transcription factor nuclear factor 1 (NF1). NF1 in turn binds to and demethylates the promoters of astrocyte specific genes, potentiating the genes for subsequent expression (Namihira et al., 2008). Thus the presence of young neurons in the VZ communicates to remaining progenitor cells, through Notch and its cell surface-
bound ligands, that astrocyte generation has become appropriate. As more neurons are generated, more RGCs will receive the Notch-mediated message that allows them to become astrocytes. The Notch system of intercellular communication therefore encourages progenitor proliferation, neuron generation, and a switch to astrocyte formation in succession throughout corticogenesis in an elegantly intertwined system of feedback loops.

Figure 2-4: Overview of Notch activity in the VZ. Dll1 binds Notch on a neighboring progenitor cell (blue + purple), leading to cleavage of the Notch intracellular domain (purple). The intracellular domain travels to the nucleus where it increases expression of Hes1 (yellow), inhibiting expression of neuronal genes and promoting proliferation of progenitors. However, Hes1 also acts to inhibit expression of Dll1 (blue), and with enough Hes1 presence, the Notch pathway fails to inhibit neuronal expression (black neuron). Newborn neurons express Dll1 (blue) and activate Notch while exiting the VZ. This activates NF1 (red), which in turn demethylates astrocytic genes, allowing for the generation of astrocytes on the heels of neurons.

Cortical development requires extensive coordination for proper formation, and the mechanisms assuring the correct number of progenitor cells available are complex and diverse. A progenitor cell’s position within the expanding cerebral wall is crucial to
its proper proliferation rate and differentiation time course, as interactions with its neighbors and surroundings affect its morphology, its plane of cell division, and the molecular profile of its offspring. However, the specific contributions of intercellular communication are still being elucidated. It is possible that a progenitor cell must acquire a sense of its surroundings to gauge whether it is no longer needed in the proliferative population. Such a model for cortical cell generation would argue in favor of a dynamic, adaptable path to cortex formation, rather than a pre-prescribed blueprint for number of cell cycles and date of radial migration initiation. It is feasible that short-range communication and a sense of surroundings is not crucial to the proper expansion of the progenitor pool, and that the mechanisms outlined in this review are each simply efficient means of directing an action within discreet cells. But as new examples of mechanisms affecting cells by changes in their immediate surroundings frequently come to light, a community-oriented group effort to build the brain emerges. Thus, as we consider the choreography of cortical development through appropriate cell division and differentiation, appreciating the influences of neighbors on cellular choices will be essential.
Promotion of Proliferation in the Developing Cerebral Cortex by EphA4 Forward Signaling

Hilary A. North, Xiumei Zhao*, Sharon M. Kolk*, Meredith A. Clifford, Daniela M. Ziskind and Maria J. Donoghue

Development 136, 2467-2476 (2009)

*These authors contributed equally to this work

Abstract

Eph receptors are widely expressed during cerebral cortical development, yet a role for Eph signaling in the generation of cells during corticogenesis has not been shown. Cortical progenitor cells selectively express one receptor, EphA4, and reducing EphA4 signaling in cultured progenitors suppressed proliferation, decreasing cell number. In vivo, EphA4-/- cortex had a reduced area, fewer cells and less cell division compared with control cortex. To understand the effects of EphA4 signaling in corticogenesis, EphA4-mediated signaling was selectively depressed or elevated in cortical progenitors in vivo. Compared with control cells, cells with reduced EphA4 signaling were rare and mitotically inactive. Conversely, overexpression of EphA4 maintained cells in their progenitor states at the expense of subsequent maturation, enlarging the progenitor pool. These results support a role for EphA4 in the autonomous promotion of cell proliferation during corticogenesis. Although most ephrins were undetectable in cortical progenitors, ephrin-B1 was highly expressed. Our analyses demonstrate that EphA4 and ephrin-B1 bind to each other, thereby initiating signaling. Furthermore, overexpression of ephrin-B1 stimulated cell division of neighboring cells, supporting the hypothesis that ephrin-B1-initiated forward signaling of EphA4 promotes cortical cell division.
INTRODUCTION

The intricate organization of the mature cerebral cortex is dependent on precisely tuned programs in the cells that seed this structure during development. To ensure proper formation, cortical cells are shaped by intrinsic genetic programs, signaling via soluble factors, interactions with substrates and differential cell adhesion as they undergo their stereotyped development during corticogenesis (McConnell, 1995a). Here, we examine a contribution of intercellular communication via Eph family members in regulating progenitor proliferation in cortical germinal zones.

Eph receptors and ephrin ligands, surface bound signaling molecules that mediate communication between cells, are broadly expressed within the forming cerebrum (Mackarehtschian et al., 1999, Yun et al., 2003). Receptors and ligands must be tethered to the cell surface and clustered (Davis et al., 1994) in order to engage, generating autophosphorylation-dependent forward signaling in receptor-containing cells and/or reverse signaling in ligand-expressing cells (Holland, 1996, Davy et al., 1999, Kullander et al., 2001b, Holmberg et al., 2005). Initial studies designated A and B subclasses of Eph/ephrins and proposed that interactions occurred largely within each subgroup (Gale et al., 1996); however, subsequent studies demonstrated extensive engagement, even between EphA and ephrin-B molecules (Kullander et al., 2001a, Kullander et al., 2003, Himanen et al., 2004).

EphA4 exemplifies the flexibility of this family of signaling molecules. Initially characterized as a binding partner of ephrin-A ligands (Cheng and Flanagan, 1994, Drescher et al., 1995, Dottori et al., 1998, Walkenhorst et al., 2000, Swartz et al., 2001),
additional studies demonstrated that EphA4 actually interacts with both A and B ligands (Gale et al., 1996, Kullander et al., 2003). Activation of EphA4 influences diverse cellular processes in the nervous system; neuronal migration, differentiation and connectivity can all be guided by EphA4 activation (Conover et al., 2000, Helmbacher et al., 2000, Walkenhorst et al., 2000, Swartz et al., 2001, Dufour et al., 2003, Goldshmit et al., 2006). Furthermore, engagement of EphA4 can produce both positive and negative consequences, depending upon levels of stimulation and characteristics of participating cells (Castellani et al., 1998, Connor et al., 1998, Eberhart et al., 2004, Hansen et al., 2004). Moreover, EphA4 is broadly expressed, often coexists with ephrin ligands and can be involved in both cis- and trans-interactions (Hornberger et al., 1999, Yin et al., 2004, Marquardt et al., 2005). Finally, a variety of downstream effectors of EphA4 have been identified that act to translate an extracellular signal into an intracellular response (Shamah et al., 2001, Murai et al., 2003, Knoll and Drescher, 2004, Egea et al., 2005, Sahin et al., 2005, Beg et al., 2007, Bourgin et al., 2007, Fawcett et al., 2007, Fu et al., 2007, Iwasato et al., 2007, Richter et al., 2007, Shi et al., 2007, Wegmeyer et al., 2007). Thus, EphA4 is a multifunctional receptor, and its effects are still being discerned.

In this study, we use in vitro and in vivo genetic and epigenetic approaches to characterize the role of EphA4 signaling in cortical proliferation during development. Our results support a role for EphA4-mediated forward signaling in the control of cell number in the forming cortex.
RESULTS

Expression of EphA4 in cortical proliferative zones

Cells of the developing cerebral cortex are generated in a precise temporal pattern that is translated into the spatial layout of the embryonic cerebral wall: immature, dividing cells populate the proliferative zone [PZ; comprises the ventricular zone (VZ) and subventricular zone (SVZ) in this study] close to the ventricle, migrating postmitotic cells exist in the intermediate zone (IZ) and more mature neurons reside in the cortical plate (CP) beneath the pia. The expression of Eph receptors in the embryonic cerebrum mirrors this developmental progression, with particular molecules marking specific locations, thus producing a complex signaling landscape (Fig. 3-1 A; Fig. 3-2) (Mackarehtschian et al., 1999, Yun et al., 2003) and supporting the concept that certain Eph signaling combinations uniquely impact discrete populations of cells within the forebrain. Despite widespread expression of several Eph receptors in the forebrain, cells of the cortical PZ selectively express only one receptor, EphA4 (Fig. 3-1 A; Fig. 3-2 (Kullander et al., 2001b, Yun et al., 2003). Analyses of RNA and protein distribution reveal that EphA4 is especially abundant within the proliferative zones of the cerebral cortex, with lower levels in the IZ and CP (Fig. 3-1 A,B,C,F; Fig. 3-2 E,O). Indeed, overlapping expression of EphA4 with Sox2 or phospho-histone H3 (pH3) reveals localization with markers of proliferating cells (Fig. 3-1 C-H). The strong expression of EphA4 in the cortex is in contrast to the weaker and more diffuse embryonic expression of EphA7 (Fig. 3-3), a receptor capable of affecting cell survival during corticogenesis (Depaepe et al., 2005). EphA4 is expressed by cells of the PZ throughout cortical
development and is coexpressed with markers of cortical progenitor cells, such as RC2 (Ifaprc2 – Mouse Genome Informatics) and nestin (Fig. 3-4 A-C,G-I), but not with mature neuronal markers (Fig. 3-4 D-F). Thus, based upon its level of expression and localization, EphA4 is a candidate for affecting neural proliferation during corticogenesis.

**Figure 3-1: Eph receptor expression in the embryonic cortex.** (A) Levels of Eph receptor expression, quantified from *in situ* hybridizations (see Fig. S1A-N in the supplementary material), of EphA receptors (red) and EphB receptors (blue) in the proliferative zones (PZ), intermediate zone (IZ) and cortical plate (CP) of the E14.5 cerebral wall. Color intensity corresponds to density of silver grains from *in situ* hybridization samples. (B) *In situ* hybridization signal of EphA4 in E14.5 cerebral cortex. (C-H) Immunohistochemical analyses of the E14.5 cortical proliferative zone reveal expression of EphA4 (C,F; red) and Sox2 (D) or phospho-histone H3 (pH3; G) (green), with merged images in E and H, respectively.
Figure 3-2: Eph receptor expression in the developing cortex. (A-N) E14.5 cerebral walls stained for Nissl (A,I) and hybridized with cRNA probes for EphA1 (B), EphA2 (C), EphA3 (D), EphA4 (E), EphA5 (F), EphA6 (G), EphA7 (H), EphB1 (J), EphB2 (K), EphB3 (L), EphB4 (M) and EphB6 (N). (O) Quantification of the expression of EphA receptors (green) and EphB receptors (blue) in the embryonic zones of the E14.5 cerebral wall. CP, cortical plate; IZ, intermediate zone; PZ, proliferative zone.

Figure 3-3: EphA4 and EphA7 expression in developing brain. Images of in situ hybridizations of EphA4 (A) and EphA7 (B) in sagittal sections of an E12.5 brain. Similar levels of expression but in opposing gradients are visible within the tectum (T), but levels of EphA4 are much higher than EphA7 in the cortex. A, anterior; D, dorsal; LV, lateral ventricle.
Figure 3-4: EphA4 is expressed by cortical progenitors. (A-I) E14.5 cerebral wall stained for EphA4 (A,D,G; green) and RC2 (B), Tuj1 (E) or nestin (H) (red) with merged images (C,F,I). EphA4 overlaps with RC2 and nestin (C,I) but not Tuj1 (F).

Figure 3-5: EphA receptor expression and activation. (A-C) RT-PCR of EphA receptors in E14.5 cortex (A), neurosphere (Nsph) cultures (B) and HEK293T cells (C). Patterns in Nsph (B) mirror the E14.5 cortex profile (A), whereas there is little detectable EphA expression in HEK293T cells (C). (D) Western analysis of EphA activation in HEK293T cells transfected with YFP (C, left panel), YFP and EphA4 (EphA4, middle panel), or YFP, EphA4 and DN (EphA4 & DN, right panel) before stimulation (0’) or upon incubation with clustered ephrin A5 for 2 or 10 minutes (2’ and 10’, respectively). There is no detectable EphA activation in control cells (left). Transfection with EphA4 results in some autoactivation (0’, middle), with elevated levels in the presence of ephrin A5 (2’ and 10’, middle). Receptor activation is eliminated when DN is coexpressed (right), demonstrating the ability of DN to silence ephrin A5-mediated EphA4 stimulation. Actin (bottom) reveals equal loading.
In vitro analyses of EphA4 signaling in neurosphere cultures

To study EphA4 function, neurosphere (Nsph) cultures (Tropepe et al., 1999), Fgf2-responsive cortical progenitors, were used as an in vitro model, as Eph receptor expression patterns within E14.5 cortex were largely mimicked in these cultures (Fig. 3-5 A,B). Nsph cultures derived from control and EphA4−/− cortex that had been exposed to the nucleotide analog BrdU during their last day in culture (Fig. 3-6 A,B) demonstrated that DNA synthesis was at lower levels in Nsphs derived from EphA4−/− (70±6%) than from control cortex (Fig. 3-6 C). Parallel experiments of Nsph cultures transfected with YFP (control) or a function-reducing DN (Fig. 3-5 B,D), demonstrated that transfected cell number was comparable shortly after transfection (Fig. 3-7), but DN+ cells were significantly less abundant (69±8%) than YFP+ cells two days later (Fig. 3-6 D-F, left). To determine whether the reduction in cell number was due to a change in proliferation, BrdU+ cells were quantified in these cultures. Numbers of BrdU+ cells in the DN-transfected Nsphs were significantly lower (74±5%) than in control Nsphs (Fig. 3-6 F, middle) and rare DN+ cells colabeled with BrdU were also less abundant than YFP+/BrdU+ cells (Fig. 3-6 F, right). No differences in cell survival were observed in any of the Nsph cultures (data not shown). Together, these results support a role for EphA4 in cortical proliferation.

Despite Nsph cultures being a popular in vitro model, analyses of a role for Eph function in proliferation may be complicated in this experimental paradigm by interactions between Eph receptors and Fgf2, the requisite growth factor, as Fgf2 signaling promotes cortical proliferation on its own (Vaccarino et al., 1999, Mizutani et al., 2007, Ever et al., 2008) and EphA4 can physically interact with Fgf2 receptors.
(Yokote et al., 2005, Arvanitis and Davy, 2008). We therefore turned to in vivo models to more reliably assess physiological EphA4 function in the cortex.

![Figure 3-6: EphA4 signaling in neurosphere (Nsph) cultures.](image)

Analysis of EphA4<sup>-/-</sup> cortex

To examine how EphA4 affects corticogenesis in vivo, the cortices of EphA4<sup>-/-</sup> mice were examined. Animals were similarly sized at birth, the gross appearance of EphA4<sup>-/-</sup>
brains, including the cerebral cortex, was normal (Fig. 3-8 A,B) (Helmbacher et al., 2000), and the anteroposterior (AP) length of the cortex was similar to that of control (WT) mice (Fig. 3-9 A). The thickness of the cerebral wall (CW width), however, was considerably less in EphA4−/− than control mice (526±38 µm for WT versus 419±20 µm for EphA4−/−; Fig. 3-8 C), resulting in smaller cortical area in EphA4−/− mice (Fig. 3-8 D).

To determine a cause for the small EphA4−/− cortex, cell division was examined during corticogenesis. The number of mitotic cells was assessed in control and EphA4−/− embryos that had been exposed to BrdU two hours prior to sacrifice (Fig. 3-8 E,F). These analyses revealed that fewer cells incorporated BrdU in EphA4−/− cortex than in control cortex (14,452 versus 10,030 cells/mm2 in WT and EphA4−/−, respectively; Fig. 3-8 G), with decreases observed in both the PZ and IZ (Fig. 3-9 B). No changes in cell death were observed in EphA4−/− cortex (Fig. 3-9 C,D). To investigate the cellular and molecular basis of this difference, cortical cells in WT and EphA4−/− were labeled with YFP via in utero electroporation (IUE) (Tabata and Nakajima, 2001). Embryonic cortices were transfected in vivo at E14.5 and transfected cell numbers were evaluated at E17.5. Consistent with changes in BrdU incorporation, there were fewer transfected cells in the
PZ of EphA4−/− cortex than WT cortex 3 days after IUE (Fig. 3-8 H, compare dark gray and black bars). To confirm that this phenotype was dependent upon EphA4, full-length EphA4 (FL) was reintroduced into E14.5 EphA4−/− cortex by IUE and the number of transfected cells was similarly assessed. Expression of EphA4 significantly increased the number of transfected cells in the PZ of EphA4−/− cortex, demonstrating that EphA4 expression helps to overcome the EphA4−/− phenotype (Fig. 3-8 H, white bar). Importantly, this increase did not occur when the reintroduced receptor was kinase-inactive (KI; Fig. 3-8 H, light gray bar). Although the fully functional receptor increased cell number significantly, wild-type numbers were not achieved. This might be due to developmental changes in EphA4−/− cortex that cannot be overcome with transient gene expression, or to unregulated FL promoting cell division while also affecting death and differentiation (see below), thus limiting overall numbers.
Differences in cerebral wall thickness, cortical area, BrdU incorporation and transfected cell number between WT and EphA4−/− cortex were significant yet not overwhelming. It is possible that molecular compensation by other Eph receptors in the absence of EphA4 is responsible for these modest effects and complicates the rescue by FL. Indeed, we find evidence of upregulation of related Eph genes in EphA4−/− cortex. For example, EphA5 is normally expressed within the IZ during corticogenesis and is downregulated by P0 (Fig. 3-9 E,F) (Mackarehtschian et al., 1999, Yun et al., 2003).
Although EphA5 expression was properly initiated within the EphA4−/− cortex (Fig. 3-9 G), embryonic levels were low and the age-related decrease in expression in the cortex was not observed. Instead, elevated levels of EphA5 were present postnatally in EphA4−/− cortex (Fig. 3-9 H). These data suggest that Eph gene expression in the forming cerebral cortex is coordinated and that expression patterns of other Eph receptors are perturbed in EphA4−/− cortex.

**Figure 3-9: Quantification of EphA4−/− cortex.** (A) Anteroposterior length is similar between WT and EphA4−/− mice. (B). BrdU incorporation is decreased in the PZ and IZ of EphA4−/− cortex compared with WT. (C,D) TUNEL+ cells are rare in WT (C) and EphA4−/− (D) cortex. (E-H) In situ hybridization of EphA5 in coronal sections at E14.5 (E,G) and sagittal sections at P0 (F,H) in WT (E,F) and EphA4−/− (G,H) cortex revealed persistent expression of EphA5 after birth in EphA4−/− (H) but not in WT (F).

To avoid genetic compensation, IUE of wild-type animals was used to alter Eph signaling transiently. Exogenous DNA was introduced into cells of the ventricular zone of mouse somatosensory cortex, a large and easily identifiable cortical region. When IUE
of a control YFP vector was performed at the peak of corticogenesis (E14.5), transfected cells were visible within 24 hours, predominantly within the PZ (Fig. 3-11 A,C). Three days after transfection, YFP-expressing cells spanned the cerebral wall, including a proportion of cells residing in the CP 3 days after IUE (Fig. 3-11 B,C), confirming that

Figure 3-10: Analyses of electroporated brains after 3 days in utero. (A-D) Cerebral walls transfected with YFP control (A), shRNA for EphA4 (sA4, B), DN for EphA4 (C) or EphA4 full-length (FL, D), with transfected cells in green and Bisbenzamide in blue. (E) Quantification of transfected cells demonstrates that there are fewer sA4+ and DN+ cells than C-transfected cells and more FL+- than C-transfected cells. (F) There are fewer phospho-histone H3+ cells in sA4-and DN-transfected brains and more in FL- than C-transfected brains. (G) The distributions of transfected cells within the cerebral walls of YFP- (C), sA4-, DN- and FL-transfected cortex. C- and sA4-transfected cells were similarly distributed within the cerebral wall, whereas DN+ and FL+ cells were mislocalized, with proportionally more cells in the IZ and PZ, respectively. (H) Proportion of transfected cells that incorporated BrdU is significantly less with sA4 or DN versus control YFP, suggesting a cell-autonomous effect.
YFP-transfected cells undergo stereotyped maturation. Consistent with transfected cells undergoing division, total numbers of YFP+ cells increased over time (compare levels in Fig. 3-11 D and in Fig. 3-10 E).

To investigate the role of EphA4 in corticogenesis, forward signaling was reduced via transfection of an shRNA specific for EphA4 (sA4) (Fu et al., 2007) or a DN EphA4 (DN) (Fig. 3-5 D) (Xu et al., 1995). Initial transfection rates were similar (Fig. 3-11 D), but DN+ and sA4+ cells were less abundant than YFP+ cells (Fig. 3-10 A-C,E), and there were fewer dividing cells in those cerebral walls (Fig. 3-10 F) 3 days after transfection. TUNEL assay excluded cell death as an explanation for the diminished transfected cell count; cell death increased in the DN-transfected brains, but only in neighboring untransfected cells (Fig. 3-12). These data demonstrate that a reduction in EphA4
signaling suppresses normal cell proliferation. Levels of EphA4 signaling were also artificially elevated by transfection of a full-length (FL) construct (Fig. 3-10 D). There were modest increases in transfected cell number (Fig. 3-10 E) and cell division (Fig. 3-10 F) when FL was expressed.

Interestingly, at the later timepoint, whereas YFP+ and sA4+ cells were similarly distributed, DN+ and FL+ cells were abnormally dispersed within the cerebral wall. In mice transfected with YFP or sA4, the majority of transfected cells (~60%) resided within the PZ, with smaller proportions of transfected cells occupying the IZ and CP (~30% and ~10%, respectively; Fig. 3-10 G). When DN was transfected, proportionally fewer DN+ cells resided within the PZ and relatively more populated the IZ: 49% of DN+ cells were housed within the PZ, with 42% located in the IZ (Fig. 3-10 G). Conversely, overexpression of FL led to an increase in the proportion of transfected cells in the PZ and a decrease in the proportion within the CP (Fig. 3-10 G). To confirm that FL could overcome the effects of sA4 or DN, rescue experiments were performed (Fig. 3-13).
Together, these data suggest that EphA4 promotes cortical progenitor cell proliferation and reduces the proportion of differentiated cells.

To determine whether this effect of EphA4 was cell autonomous, through the receptor itself, or non-cell autonomous, through a potential ligand, we asked whether transfected cells incorporated less BrdU when their EphA4 signaling was reduced. Indeed, sA4- and DN-containing cells incorporated significantly less BrdU than YFP-containing cells (Fig. 3-10 H), supporting a model in which EphA4 forward signaling promotes cell division in receptor-containing cells.

**Figure 3-13: Rescue of knockdown phenotype.**
Proportion of transfected cells present in the PZ 3 days after IUE with YFP (C, gray bar), DN or sA4 (black bars), or DN or sA4 plus FL (white bars). The addition of FL increased the proportion of cells remaining in the proliferative zone.
Ligands for EphA4 in cortical proliferative zones

Expression levels of ephrins are low in the forming cortex, particularly within regions with dividing cells: ephrin-A2 is diffusely expressed throughout the cerebral wall and ephrins-A5 and -B2 are the most concentrated within the IZ and CP (Fig. 3-14 A; Fig. 3-15) (Mackarehtschian et al., 1999, Kullander et al., 2001b). By contrast, ephrin-B1 is abundantly expressed in the PZ (Fig. 3-14 A,B,C,F; Fig. 3-15 H), colocalized with

Figure 3-14: Ligand expression in cortical proliferative zones. (A) Levels of ephrin ligand expression, quantified from in situ hybridizations (see Fig. S8A-J in the supplementary material), of ephrin A ligands (green) and ephrin B ligands (yellow) in the proliferative zones (PZ), intermediate zone (IZ) and cortical plate (CP) of the E14.5 cerebral wall. Color intensity corresponds to density of silver grains from in situ hybridization samples. (B) In situ hybridization of ephrin B1 in the E14.5 cerebral cortex. (C-H) Immunohistochemical analyses of the cortical proliferative zone reveal expression of ephrin B1 (C,F; green) with EphA4 (D) or Sox2 (G) (red), with merged images in E and H, respectively.
EphA4 in the PZ throughout cortical development (Fig. 3-14 C-E) and coexpressed with markers of cortical progenitor cells, such as Sox2, nestin and RC2, but not neuronal markers (Fig. 3-14 F-H; Fig. 3-16). Thus, based upon both abundance and localization, ephrin-B1 is a candidate for engaging EphA4 in promoting corticogenesis.

Previous studies demonstrated that EphA4 binds ligands permissively and is capable of engaging both ephrin-A and -B ligands (Gale et al., 1996, Kullander et al., 2003). Indeed, recognition of binding partners for EphA4 has progressed: ephrin-A5s and ephrin-B2 were shown to engage EphA4 in vitro (Gale et al., 1996), but binding of ephrin-B3 to EphA4 was only detected later, in analyses of mutant mice in vivo (Kullander et al., 2003). To investigate whether an interaction between ephrin-B1 and EphA4 exists, COS7 cells were transfected with YFP in combination with either an inert plasmid (control, C) or expression vectors encoding EphA4 or ephrin-B1, and postfixation staining with ephrin and Eph reagents was performed. Compared with control-transfected cells, in which the low density of endogenous receptors and ligands was not detectable in this assay of fixed cells (Fig. 3-17 A,C), EphA4-transfected cells bound ephrin-B1, and ephrin-B1-transfected cells bound EphA4 (Fig. 3-17 B,D).

To assess the relative strength of EphA4/ephrin-B1 binding, COS7 cells were again transfected, but this time serial dilutions of binding reagent were used for detection. EphA4-transfected cells bound ephrin-A5, the cognate ligand, and ephrin-B1, the novel ligand, and in all cases the number of positive cells increased according to the amount of binding reagent present (Fig. 3-17 E). Still, there were more positive cells at each concentration of ephrin-A5 than of ephrin-B1, suggesting a weaker interaction with the latter ligand (Fig. 3-17 E). Cells transfected with ephrin-B1 bound EphB2, the previously
characterized receptor, and EphA4, the novel receptor, in a dose-dependent manner (Fig. 3-17 F), but in this case no obvious interaction preference was apparent.

Figure 3-15: Ephrin ligand expression in the developing cortex. (A-I) E14.5 cerebral walls stained for Nissl (A,G) and hybridized with cRNA probes for ephrin A1 (B), ephrin A2 (C), ephrin A3 (D), ephrin A4 (E), ephrin A5 (F), ephrin B1 (H), ephrin B2 (I) and ephrin B3 (J). (K) Quantification of the expression of ephrin A (purple) and ephrin B (red) ligands in the embryonic zones of the E14.5 cerebral wall. CP, cortical plate; IZ, intermediate zone; PZ, proliferative zone.
To confirm a molecular interaction between EphA4 and ephrin-B1, protein extracts were produced from either control-transfected cells or cells transfected with EphA4 or ephrin-B1 that were subsequently mixed and grown together. EphA4 was concentrated following pulldown with the EphA4-specific antibody (Fig. 3-17 G, top) and ephrin-B1 copurified with EphA4 (Fig. 3-17 G, bottom). EphA4/ephrin-B1 engagement in this system appeared to be exclusively due to trans binding; cells cotransfected with both EphA4 and ephrin-B1 and grown at low density to allow for cis- and prohibit trans-interactions did not result in coimmunoprecipitation of EphA4 and ephrin-B1 (data not shown). To determine whether EphA4/ephrin-B1 engagement occurs in vivo, similar analyses were performed using embryonic cortex. EphA4 was detectable in the cortical lysate and was enriched following precipitation with EphA4 antiserum (Fig. 3-17 H, top). Importantly, ephrin-B1 copurified with EphA4 (Fig. 3-17 H, bottom), confirming an interaction in embryonic cortex. Thus, EphA4 and ephrin-B1 interact both in vitro and in vivo.

**Figure 3-16:** Ephrin-B1 is expressed by cortical progenitors. (A-I) E14.5 cerebral wall stained for ephrin B1 (A,D,G; green) and RC2 (B), Tuj1 (E) or nestin (H) (red) with merged images in C, F and I. Ephrin B1 overlaps with RC2 and nestin (C,I), but not Tuj1 (F).
Figure 3-17: EphA4 interacts with ephrin B1. (A-D) COS7 cells transfected with nuclear-localized YFP alone (C, panel A) or in addition to EphA4 (B) or ephrin B1 (C,D) and incubated with ephrin B1 (A,B), control (C) or EphA4 (D) binding reagents. COS7 cells have little background binding (A,C), whereas cells transfected with EphA4 bind ephrin B1 (B) and cells transfected with ephrin B1 bind EphA4 (D). (E,F) The number of EphA4+ (E) and ephrin B1+ (F) cells per 10 field that bind ephrin A5 or ephrin B1 (E) or EphB2 or EphA4 (F) at 0.1, 1 or 10 µg/ml binding protein. (G) Coimmunoprecipitation of EphA4 and ephrin B1 from HEK293T cells transfected with YFP (C) or EphA4 and ephrin B1 separately, precipitated with anti-EphA4 and then blotted with either anti-EphA4 (top) or anti-ephrin B1 (bottom). Ephrin B1 purifies with EphA4 in EphA4/ephrin B1-transfected cultures. (H) Coimmunoprecipitation of EphA4 and ephrin B1 from E14.5 cortex lysates. Ephrin B1 copurifies with EphA4. (I) Western analysis of EphA phosphorylation following transfection of HEK293T cells with YFP (C) (left), EphA4 (middle) or a mix of EphA4 and DN (right) before stimulation (0) or upon incubation with clustered ephrin B1 for 2 or 10 minutes (2 or 10). Actin (bottom) confirms equal loading. (J) Quantification of stimulation of EphA4 with ephrin B1 (left) and ephrin A5 (right).
To assess the functional consequences of an EphA4/ephrin-B1 interaction, HEK293T cultures, cells that express few Eph receptors (Fig. 3-5 C), were treated with clustered ephrin-B1 and analyzed biochemically for EphA receptor activation. Parallel to previous ephrin-A5 stimulation results, owing to low levels of endogenous receptors (Fig. 3-5 D) cells transfected with YFP and treated with ephrin-B1 displayed little receptor activation (Fig. 3-17 I, left). Also similar to previous experiments (Fig. 3-5 D), owing to high receptor density following EphA4 transfection, receptor activation was apparent in the absence of ligand (Fig. 3-17 I, middle panel, left lane), but was elevated in the presence of ephrin-B1 (Fig. 3-17 I, middle panel, right lanes) (Davis et al., 1994, Gale et al., 1996). Importantly, coexpression of EphA4 and DN blocked ephrin-B1-stimulated EphA4 activation (Fig. 3-17 I, right panel).

To examine the relative levels of activation, EphA phosphorylation was quantified following stimulation with ephrin-B1 (Fig. 3-17 J, left) or ephrin-A5 (Fig. 3-17 J, right) and compared with baseline levels of phosphorylation. EphA4 activation in response to ephrin-B1 was observed (150% at 5 minutes) and prolonged (sustained phosphorylation at 10 minutes; Fig. 3-17 J, top left), whereas stimulation in response to ephrin-A5 was stronger (200% at 5 minutes) but quicker (receptor became less phosphorylated at 10 minutes; Fig. 3-17 J, top right). In both cases, EphA4 activation was eliminated in the presence of DN (Fig. 3-17 J, bottom). Together, these data support an EphA4/ephrin-B1 interaction based upon results from (1) cell-based binding, (2) biochemical interactions in transfected cells in culture, (3) biochemical interactions between native proteins in the developing cortex and (4) specific EphA4 activation
following exposure to ephrin-B1. Although structural analyses or additional protein chemistry could be performed to more fully understand this interaction, we conclude that EphA4 and ephrin-B1 bind to one another and that the DN blocks ephrin-B1-induced receptor activation.

**Analysis of ephrin-B1 function in vitro and in vivo**

Ephrin-B1 has previously been shown to play a role in promoting progenitor cell fate and inhibiting cortical neuronal differentiation (Qiu et al., 2008). To understand the role of ephrin-B1 in regulating cortical proliferation, Nsph cultures transfected with YFP (Fig. 3-18 A) or ephrin-B1 (Fig. 3-18 B) and then exposed to BrdU prior to harvest were analyzed. Initial transfection rates were similar (Fig. 3-7, right) and there was no significant difference between the number of YFP+ and ephrin-B1+ cells per Nsph at any timepoint (Fig. 3-18 C, left). There was, however, a significant increase in the number of BrdU+ cells in ephrin-B1-transfected Nsphs (Fig. 3-18 C, right). Similarly, introduction of YFP (Fig. 3-18 F) or ephrin-B1 (Fig. 3-18 G) into an E14.5 cortex via IUE resulted in the same number of transfected cells (Fig. 3-18 D) and similar cell distributions (Fig. 3-18 E) in vivo. However, the number of cells expressing pH3 increased in ephrin-B1-transfected cortex compared with control (Fig. 3-18 F,G insets, Fig. 3-18 H). These results indicate a non-cell-autonomous effect in which ephrin-B1 overexpression promotes division in neighboring cells.
DISCUSSION

As the nervous system forms, Eph signaling affects several cellular processes including segregation (Mellitzer et al., 1999, Xu et al., 1999), migration (Conover et al., 2000, Karam et al., 2000) and parcellation (Vanderhaeghen et al., 2000, Miller et al., 2006) in early neurons, and axonal pathfinding (Castellani and Bolz, 1997, Frisen et al., 1998, Helmbacher et al., 2000, Dufour et al., 2003, Torii and Levitt, 2005) and synaptic...
transmission (Torres et al., 1998, Dalva et al., 2000, Kayser et al., 2006, Kayser et al., 2008) in more mature neurons. Eph signaling impacts neurogenesis in the adult olfactory system and hippocampus (Conover et al., 2000, Depaepe et al., 2005, Holmberg et al., 2005, Chumley et al., 2007), but the consequences of this mode of intercellular communication on the initial generation of cells in the cerebral cortex have not been clearly elucidated. Our genetic, epigenetic, histological and biochemical studies, both in vitro and in vivo, support a novel role for EphA4-mediated forward signaling, initiated by ephrin-B1 binding, in the promotion of progenitor cell proliferation during corticogenesis.

Within the PZ, EphA4 and ephrin-B1 are highly expressed and colocalize with radial glial markers. Although questions regarding heterogeneity of progenitor cells in the VZ (Malatesta et al., 2003, Gal et al., 2006) and the roles of intermediate progenitor cells within the SVZ (Mizutani et al., 2007, Noctor et al., 2007) exist, our results simply demonstrate that EphA4 signaling influences cortical proliferation. For example, when EphA4-mediated signaling is reduced, affected cells divide less, and when signaling is elevated, cells are more mitotically active. These results parallel findings that Eph signaling promotes cell division in the adult olfactory system (Conover et al., 2000) and the hippocampus (Chumley et al., 2007), as well as in non-neural stem cells (Holmberg et al., 2006, Fukai et al., 2008).

We demonstrate that ephrin-B1 is coordinately expressed with, binds to and initiates forward signaling through EphA4. Our in vitro and in vivo results are consistent with an enhancement of cortical cell division by ephrin-B1 via forward, but not reverse, signaling. Promotion of a cellular process by an EphA4/ephrin-B1 interaction clarifies
previous results that demonstrated that ephrin-B1 interacts repulsively with EphB receptors but not with EphA4 (Mellitzer et al., 1999). Furthermore, the broad coexpression of EphA4 with ephrins in development and maturity (Mackarehtschian et al., 1999, Yun et al., 2003) argues that EphA4 is not always a repulsive receptor. Our results support a system in which ephrin-B1 initiates forward signaling via EphA4 to stimulate cortical cell division. This role is consistent with the findings that overexpressed EphA4 (Fig. 3-10 E) or ectopic ephrin-B1 (Qiu et al., 2008) antagonize neuronal differentiation.

**Figure 3-19: Schematic of Eph signaling within the cerebral wall.** In the proliferative zones (PZ), ephrin B1 and EphA4 are expressed (light blue) and ephrin B1-EphA4 signaling (pink solid arrow) promotes cell proliferation (purple circles) in balance with other mitogens (pink dashed arrows). Embryonic zones containing postmitotic neurons, the intermediate zone (IZ) and cortical plate (CP), express unique subsets of Eph family members (yellow and green, respectively). Ectopic expression of ephrin B1 in postmitotic populations or EphA4 in progenitors antagonizes (black dashed inhibitory bar) neuronal differentiation (red arrow).

Engagement of Eph receptors and ephrin ligands can result in bidirectional events, with signaling cascades initiated in both receptor- and ligand-expressing cells (Holland,
1996, Kullander et al., 2001b, Davy et al., 2004), and the consequences of forward and reverse signaling can be functionally dissected (Kullander et al., 2001b, Davy et al., 2004, Holmberg et al., 2005). This was the case in our in vitro and in vivo studies: EphA4 forward signaling promoted cell division during corticogenesis in gain- and loss-of-function paradigms, whereas reverse signaling via ephrin-B1 had a minor effect on cell proliferation (Fig. 3-19). Consistent with this model, ephrin-B1 conditional mutants had fewer mitotic cells within their cerebral cortex, although it was not determined whether the effect was autonomous or not (Qiu et al., 2008). Differences in the results of overexpression studies are likely to be related to the cell types manipulated; our elevation of ephrin-B1 was in proliferating cells, whereas Qiu et al. overexpressed ephrin-B1 exclusively in differentiating neurons. Our results also support the idea that excessive and unbalanced reverse signaling can impact cell survival, consistent with other overexpression studies (Depaepe et al., 2005). From these results, we conclude that the major biological role of EphA4 signaling in corticogenesis is to regulate proliferation (Fig. 3-19).

Stereotyped placement of cortical cells of particular maturational states in specific locations is a hallmark in the construction of a functional cerebral cortex: dividing cells reside near the ventricle, more mature cells occupy the most superficial locations, and migrating cells are sandwiched in between. Unique combinations of Eph receptors are expressed by cells within each developmental niche (Yun et al., 2003), such that a spatial, stage-specific sequence of Eph signaling is apparent (Fig. 3-19). This developmental patterning supports a continuous role for Eph signaling, using different signaling partners at different times, in the maturation of cortical cells. Indeed, ectopic expression of EphA4
resulted in the maintenance of the proliferative state (Fig. 3-10), a result phenocopied when ephrin-B1 was ectopically expressed in postmitotic neurons (Qiu et al., 2008). Our finding that DN\(^+\) but not sA4\(^+\) cells are mislocalized within the cerebral wall suggests that a related receptor, silenced by the DN but not the sA4, or the excessive reverse signaling initiated by the DN but not the sA4, might mediate migration. In parallel, the build-up of FL\(^+\) cells in the PZ, at the expense of differentiated neurons in the CP, demonstrates that these states are linked. In these ways, our results reveal the coordination that exists between division and differentiation, and highlight possible roles for discrete Eph signals in maintaining this balance.

In summary, our study demonstrates that EphA4/ephrin-B1 signaling plays a role in the modulation of cortical progenitor cell proliferation. Characterizing the balance that exists between the generation of cells and their subsequent differentiation is essential for understanding the organization and function of the cerebral cortex, as well as for providing insights into instances of malfunction and their potential therapies.
DEVELOPMENT OF THE MURINE SOMATOSENSORY SYSTEM

The Eph/ephrins play pivotal roles in many systems beyond the development of the cortex. Our cortical studies led us to interesting observations regarding the effects of these molecules unrelated to our primary area of investigation. These chapters focus on one of these peripheral phenomena: the influence of EphA4 in the development of the murine somatosensory system.

Nocturnal animals negotiate their worlds primarily through touch, or somatosensation. In mice, somatosensation is mainly transmitted via the mystacial vibrissae, or whiskers, on the snout, capable of relaying both surface characteristics and vibration. The layout of vibrissae on the mouse nose is stereotyped, and little spatial variation exists between individuals (Davidson and Hardy, 1952, Dun and Fraser, 1958, Yamakado and Yohro, 1979, Van Exan and Hardy, 1980). Five rows of vibrissae comprise the mature whisker pad, with the most dorsal row designated as A, the most ventral as E, and rows B-D sandwiched in between (Figure 4-1). In addition to the main rows of vibrissae, there are also four whiskers, termed straddler vibrissae, positioned between the main rows caudally and a set of smaller auxiliary whiskers ventrally (Davidson and Hardy, 1952). Vibrissae are large, stiff hairs, which- unlike standard body hairs- have innervated follicles (Davidson and Hardy, 1952), and each whisker follicle is innervated by specific sensory axons derived from the maxillary branch of the trigeminal ganglion (Dorfl, 1985a) (Figure 4-2). Interestingly, trigeminal neurons that innervate particular vibrissae are spatially ordered within the ganglion (Erzurumlu and Jhaveri, 1992, Hodge et al., 2007) and these neurons project topographically into the central nervous system, such that positional accuracy is maintained in nuclei of the brainstem.
and thalamus, and in layer IV of the somatosensory cortex (Killackey et al., 1995) (Figure 4-3). The reliable organization of the whisker pads, and the stereotyped connections that exist within the trigeminal ganglion and throughout the neuraxis, support a model in which there is tight regulation of axon-target matching. Many of the mechanisms accomplishing this matching, however, remain obscure.

Mystacial vibrissae emerge during the second half of mouse gestation. Around embryonic day (E) 10, connections between the trigeminal ganglion and the prospective whisker pad are initiated as trigeminal neurons extend axons peripherally. The guidance of these axons is influenced by factors secreted by the target maxillary field, such as Brain-derived neurotrophic factor (BDNF), Neurotrophin-3 (NT-3) (O'Connor and Tessier-Lavigne, 1999), Transforming Growth Factor-β (TGF-β) (Pisano et al., 2003), Bone Morphogenetic Proteins (BMPs) (Guha et al., 2004), and Fibroblast Growth Factor-10 (FGF10) (Ohuchi et al., 2003). At the same time, five horizontal ridges emerge in the target whisker pad whose location corresponds to the position of future whisker rows, A-E (Van Exan and Hardy, 1980). Trigeminal axons begin to invade the target maxillary field between E11.5 and E12.5 (Davies and Lumsden, 1984, Davies and Lumsden, 1986, O'Connor and Tessier-Lavigne, 1999). As innervation proceeds, individual follicles become defined within the ridges, paired with fine axonal endings in the mesenchyme (E13.5). By E14.5, condensations of both mesenchyme and epidermis at the site of innervation exist, forming the papillae that
correspond to the location of mature vibrissae (Hardy, 1949, 1951). Consistent with the caudal-to-rostral invasion of trigeminal axons in each row, vibrissal development begins at the caudal-most regions of the whisker pad and progresses rostrally (Davidson and Hardy, 1952). By E15.5, the placement of the follicles is set and whisker growth begins, with whiskers emerging from the follicles a few days later. Notably, vibrissae emerge much earlier than the non-sensory hair that will eventually cover the entire body, as body hair is not obvious until after birth (Davidson and Hardy, 1952) (Figure 4-4).

![Figure 4-2](image)

**Figure 4-2:** “Diagram of active vibrissal follicle in adult mouse,” taken from Davidson and Hardy, Journal of Anatomy, 1952.

![Figure 4-3](image)

**Figure 4-3:** Hodge et al., (Neuron 2007) injected red DiI and green DiA into alternating rows on the E13 whisker pad (above). The dyes were allowed to migrate retrogradely to the maxillary lobe of the trigeminal ganglion, where they demonstrated topography congruent with their rows of origin.

Neurons of the trigeminal ganglion are derived from neural crest cells that migrate to and then condense to form the well-circumscribed grouping of neurons ventral to the forebrain and caudal to the snout (Hodge et al., 2007). Trigeminal neurons are bipolar,
such that a single neuron sends a sensory ending into the periphery and an afferent projection into the brainstem. The peripheral axons segregate into one of three major pathways, with the maxillary branch containing axons that innervate the whisker pad sandwiched between the dorsal ophthalmic branch and ventral mandibular branch. Neurons contributing axons to the maxillary nerve extend peripheral and central projections concurrently. During development, there is topographic correspondence between the location of neuron cell bodies within the trigeminal ganglia and the location of the vibrissal row a neuron innervates (Figure 4-3) (Klein et al., 1988, Erzurumlu and Jhaveri, 1992, Hodge et al., 2007), a positional correspondence that degrades in maturity (Jacquin et al., 1986, Jacquin and Rhoades, 1987, Klein et al., 1988).

**Figure 4-4: Developmental Innervation of Vibrissae.**
E10.5 (top). Axons begin to project from the maxillary lobe of the trigeminal ganglion (Mx) which is sandwiched between the ophthalmic (Op) and mandibular (Md) lobes. Rows of ridges begin to form on the snout. E12.5 (middle). Axon tract reaches snout and branches grow underneath each row. A plexus forms underneath each vibrissae site which displays a mesenchymal thickening. E14.5 (bottom). Individual follicles innervate the mesenchyme at the site of each vibrissa. (f, follicle; p, plexus) (Adapted from Hodge, 2007 and Davidson and Hardy, 1952).
Several cues that promote specific connection between trigeminal axons and the whisker pad have been identified. Neurotrophins can act as survival factors for trigeminal neurons (Pinon et al., 1996, Davies, 1997a, b): NT-3 and BDNF before and NGF after innervation (Buchman and Davies, 1993, Enokido et al., 1999), with NT-3 and BDNF specifically promoting the extension of maxillary axons over axons of the ophthalmic and mandibular tracts (O'Connor and Tessier-Lavigne, 1999). Epithelium-derived BMPs also modify trigeminal axons innervating the maxillary pad (Guha et al., 2004). Moreover, the guidance molecules slit and robo influence trigeminal innervation both peripherally and centrally (Ozdinler and Erzurumlu, 2002, Ma and Tessier-Lavigne, 2007). These factors apply generally to whisker pad innervation but do not explain region-selective topography. An elegant, recent study characterized molecular differences between trigeminal neurons that innervate particular rows of vibrissae but focused mainly on mechanisms responsible for maintaining central topography (Hodge et al., 2007). The subject of vibrissal topography has been addressed by reports of disorganized patterning or failed whisker formation spanning the entire maxillary pad (Juriloff and Harris, 1983, Juriloff et al., 1987, Harris and Juriloff, 1989, Ohuchi et al., 2003), and some strains have been characterized with altered whisker formation (Van der Loos et al., 1986). However, no clear picture of how trigeminal neurons innervate position-selective targets in the periphery or how the conserved topography of the vibrissae has emerged.

In other neural systems, proper matching between axons and their targets relies on a combination of soluble and surface-bound cues (Tessier-Lavigne and Goodman, 1996). Among the latter, Eph/ephrins are a particularly large and versatile set of intercellular signaling molecules. They were initially defined as axon guidance cues
(Cheng et al., 1995, Drescher et al., 1995) but have subsequently been credited with accomplishing a wide range of developmental tasks (Klein, 2004). EphA4 is an example of an Eph receptor with particularly broad functions, including serving as either an inhibitory or permissive axon guidance cue in certain contexts (Kullander et al., 2001a, Zimmer et al., 2008) (Eberhart et al., 2004), a modulator of dendritic spine growth (Richter et al., 2007), and a promoter of proliferation (North et al., 2009). In this study, we investigated whether EphA4 signaling also directs positional connectivity in the murine trigeminal somatosensory system.

In this first description of spatial selectivity during vibrissal pad development, our data demonstrate that the ventral whisker pad expresses high levels of EphA4 while it is being innervated, and that trigeminal neurons express several ligands for EphA4, some in selective spatial patterns. This coincident expression raised the question of whether EphA4-mediated signaling influences trigeminal axon guidance to the peripheral field. Indeed, examination of EphA4<sup>−/−</sup> mice demonstrated that the large caudal vibrissae of the most ventral (E) row of whiskers were absent. Since a hallmark of the somatosensory system is its activity-dependence, this peripheral deficit was faithfully represented through the neuraxis, as anticipated. Developmental analyses demonstrate that trigeminal axons that normally innervate the ventral whisker pad are abnormal, failing to invade the presumptive E row terrain in a patterned fashion and defasciculating into a reticular pattern. Together, our results support a repulsive interaction between the EphA4-rich ventral whisker pad and innervating trigeminal axons, which, when lost, results in the incomplete innervation and perturbed vibrissal formation.
EphA4 is Necessary for Spatially Selective Peripheral Somatosensory Topography
Hilary A. North, Adnan Karim, Mark F. Jacquin, and Maria J. Donoghue
Developmental Dynamics 239, 630–638 (2010)

Abstract

Somatosensation is the primary sensory modality employed by rodents in navigating their environments, and mystacial vibrissae on the snout are the primary conveyors of this information to the murine brain. The layout of vibrissae is spatially stereotyped and topographic connections faithfully maintain this layout throughout the neuraxis. Several factors have been shown to influence general vibrissal innervation by trigeminal neurons. Here, the role of a cell surface receptor, EphA4, in directing position-dependent vibrissal innervation is examined. EphA4 is expressed in the ventral region of the presumptive whisker pad and EphA4−/− mice lack the ventroposterior-most vibrissae. Analyses reveal that ventral trigeminal axons are abnormal, failing to innervate emerging vibrissae, and resulting in the absence of a select group of vibrissae in EphA4−/− mice. EphA4’s selective effect on a subset of whiskers implicates cell-based signaling in the establishment of position-dependent connectivity and topography in the peripheral somatosensory system.

Introduction

Nocturnal animals negotiate their worlds primarily through somatosensation, which, in mice, is mainly transmitted via the mystacial vibrissae (whiskers) on the snout. Vibrissae, innervated by specific sensory axons of the maxillary branch of the trigeminal
ganglion (Davidson and Hardy, 1952, Dorfl, 1985b), are topographically organized into five rows with little variation between individuals (Davidson and Hardy, 1952, Dun and Fraser, 1958, Yamakado and Yohro, 1979, Van Exan and Hardy, 1980). The cell bodies whose axons innervate the vibrissae are also spatially organized within the developing trigeminal ganglion (Erzurumlu and Jhaveri, 1992, Hodge et al., 2007), and these neurons project topographically into the central nervous system such that positional accuracy is maintained in the brainstem, thalamus, and somatosensory cortex (Killackey et al., 1995). Although the reliable organization of vibrissae and neural topography throughout the neuraxis argue for tight spatial regulation during the formation of the somatosensory system, mechanisms responsible for local positioning remain largely unknown.

Mystacial vibrissae emerge during the second half of mouse gestation. Connections between the trigeminal ganglion and the prospective whisker pad are initiated as trigeminal axons extend peripherally around embryonic day (E) 10. At the same time, five horizontal ridges emerge in the target whisker pad whose locations correspond to the position of future whisker rows, A–E (Van Exan and Hardy, 1980). Trigeminal axons begin to invade the target maxillary field between E11.5 and E12.5 and individual follicles become defined within the ridges as they pair with fine axonal endings in the mesenchyme slightly later (E13.5) (Davies and Lumsden, 1984, Davies and Lumsden, 1986, O'Connor and Tessier-Lavigne, 1999). By E14.5, condensations of both mesenchyme and epidermis form the papillae in the locations of mature vibrissae (Hardy, 1949, 1951). Coincident with the arrival of trigeminal axons in each row, vibrissal development begins at the caudal-most regions of the whisker pad and progresses rostrally (Davidson and Hardy, 1952). By E15.5, the placement of the follicles is set and
whisker growth begins, with whiskers emerging from the follicles a few days later.

Several cues that promote connectivity between trigeminal axons and the whisker pad have been identified. Neurotrophins can act as survival factors for maxillary trigeminal neurons (Pinon et al., 1996, Davies, 1997b, a): NT-3 and BDNF before and NGF after innervation (Buchman and Davies, 1993, Enokido et al., 1999), with NT-3 and BDNF specifically promoting the extension of maxillary axons over axons of the ophthalmic and mandibular trigeminal tracts (O'Connor and Tessier-Lavigne, 1999). Epithelium-derived BMPs also modify trigeminal axons innervating the maxillary pad (Guha et al., 2004). Moreover, the guidance molecules slit and robo influence trigeminal innervation both peripherally and centrally (Ozdinler and Erzurumlu, 2002, Ma and Tessier-Lavigne, 2007). Vibrissal topography has been addressed in studies of disorganized patterning or failed whisker formation across the entire maxillary pad (Juriloff and Harris, 1983, Juriloff et al., 1987, Harris and Juriloff, 1989, Ohuchi et al., 2003), and strains have been characterized with altered whisker patterning (Van der Loos et al., 1986, Welker and Van der Loos, 1986). In general, these studies apply to whisker pad patterning and formation but do not address region-selective topography. In contrast, a recent, elegant study characterized molecular differences between trigeminal neurons that innervate particular rows of vibrissae, although the focus was mainly on mechanisms responsible for establishing central topography (Hodge et al., 2007). Despite our understanding of general vibrissal growth and central patterning, there is no clear picture of how trigeminal neurons innervate discrete peripheral targets or how the conserved topography of the vibrissae emerges.

In other neural systems, proper matching between axons and their targets relies on a
combination of soluble and surface-bound cues (Tessier-Lavigne and Goodman, 1996). Among the latter, Eph/ephrins are a particularly large and versatile set of intercellular signaling molecules. Indeed, Eph/ephrins have been shown to direct spatial topography in sensory systems, most notably the visual system (Frisen et al., 1998, Yates et al., 2001, Cang et al., 2008). The Eph/ephrins were initially defined as axon guidance cues (Cheng et al., 1995, Drescher et al., 1995) but have subsequently been credited with accomplishing a wide range of developmental tasks (Klein, 2004). EphA4 is an example of an Eph receptor with particularly broad functions, serving to inhibit or promote axon guidance in particular contexts (Kullander et al., 2001a, Eberhart et al., 2004, Zimmer et al., 2008), to modulate dendritic spine growth (Richter et al., 2007), and to promote proliferation (North et al., 2009). In this first investigation of spatial selectivity during vibrissal pad development, we asked whether EphA4 signaling directs positional connectivity in the murine trigeminal somatosensory system.

RESULTS

Eph Expression in the Developing Maxillary Trigeminal System

Within the trigeminal whisker system, EphA4 is the only receptor expressed at appreciable levels by the peripheral whisker pad (Fig. 5-1). EphA4 expression was detected with several methods: reporter gene expression in mice with either β-galactosidase (LacZ) or Alkaline Phosphatase (AP) inserted into the EphA4 locus via homologous recombination or gene trapping, such that reporter gene expression is present where EphA4 is expressed (Helmbacher et al., 2000, Leighton et al., 2001), immunohistochemistry (IHC) using antibodies specific for EphA4 (North et al., 2009), in
Figure 5-1: EphA4 is ventrally expressed in the developing whisker pad. A: Developmental schematics with trigeminal ganglia in black and whisker pad in grey, at E10.5 (top), when trigeminal axon extension initiates; at E12.5 (second), when the first axons have reached the maxillary pad and ridges corresponding to the five rows have formed; at E14.5 (third), when innervation by trigeminal neurons is extensive and follicles form along the ridges; and at E15.5 (bottom), when each follicle is innervated and hairs begin to develop. B, C: EphA4 expression at E10.5. Whole-mount LacZ staining of E10.5 EphA4−/− embryo (inset is magnification of maxillary protrusion) (B) and IHC of endogenous E10.5 EphA4 (C). D, E: E12.5 EphA4 expression. ISH (D) and IHC (E) of EphA4 in wild-type maxillary pad. F, G: E14.5 EphA4 expression and trigeminal innervation. Neurofilament (NF) IHC (brown) and PLAP staining (purple) of EphA4−/− (F) and NF IHC (green) and ephrin-A5 ligand body staining (red) of WT maxillary pad (G). H, I: EphA4 expression at E15.5. LacZ expression on sagittal (H) and coronal (I) whisker pad sections. R, rostral; D, dorsal; L, lateral; tel, telencephalon; np, nasal process; mx, maxillary protrusion; md, mandibular protrusion; op, ophthalmic protrusion.
situ hybridization (ISH) using a mouse EphA4 probe (Yun et al., 2003), and the use of a chimeric ligand reagent, capable of detecting Eph binding (Gale et al., 1996). Analysis began at E10.5, prior to the arrival of most trigeminal axons to the maxillary pad. At this stage, the whisker pad has thickened and trigeminal neurons have begun to extend sensory axons into the periphery (Fig. 5-1 A, top) (Davidson and Hardy, 1952). Both LacZ staining of whole-mount EphA4−/− embryos and IHC on sections revealed high levels of EphA4 expression on the ventral maxillary protrusion at E10.5 (Fig. 5-1 B, C). Similarly, both ISH and IHC revealed ventral-high EphA4 expression two days later, at E12.5 (Fig. 5-1 D, E). Ventral patterning was apparent at E14.5, with both AP staining and ligand binding (Fig. 5-1 F, G). By the time vibrissal development is nearly complete, at E15.5, ventral-high expression remained, visualized with LacZ staining of both a whole EphA4−/− whisker pad and a coronal section (Fig. 5-1 H, I). At this latest age, EphA4 was distributed in a ring around each vibrissa, consistent with the location of the outer sheath. This expression analysis, spanning embryonic vibrissal development and using multiple detection methods, revealed that the levels of EphA4 are highest in the ventral whisker pad during the specification and innervation of vibrissae.

**Ligands for EphA4 on Innervating Trigeminal Axons**

Since Eph receptors are activated by ligands on interacting cells, ephrin expression in the whisker pad’s synaptic partners, trigeminal ganglion cells, was examined. To localize expression of particular ligands, ISH was performed using probes corresponding to all of the ephrins and the topography of expression within the trigeminal ganglion was examined. Ephrin-A1 was present only on blood vessels, consistent with a
previous report (Luukko et al., 2005), and expression of ephrin-A4 and -B2 was not detectable in the trigeminal ganglion (data not shown). In contrast, Ephrin-A2, -A3, -A5, -B1, and -B3 were expressed by trigeminal neurons (Fig. 5-2). Of these, ephrin-B1 and -B3 were fairly uniformly expressed within the trigeminal ganglion (Fig. 5-2 A, B, F, G), ephrin-A2 was most concentrated dorsally (Fig. 5-2C), and ephrin-A3 and -A5 were well expressed dorsally and ventrally with low levels in the medial domain (Fig. 5-2 D, E).

Previous studies have demonstrated topography of cell bodies within the ganglion, with cells whose axons innervate the ventral maxillary pad located in the ventral portion of the middle, maxillary lobe (Hodge et al., 2007). This diverse and patterned ligand expression in the trigeminal supports the idea that trigeminal axons, including the maxillary subset, bear a variety of ligands, each of which has been shown to be capable of binding EphA4 (Gale et al., 1996, Kullander et al., 2001a, North et al., 2009).

![Figure 5-2: Ephrin expression in the trigeminal ganglion.](image)

**Figure 5-2: Ephrin expression in the trigeminal ganglion.** E12.5 trigeminal ganglion stained with Nissl (A), IHC for neurofilament (B), or ISH using probes for ephrin-A2 (C), -A3 (D), -A5 (E), -B1 (F), and -B3 (G). R, rostral; D, dorsal.

**Mice Lacking EphA4 Exhibit Perturbed Vibrissal Patterning**

To investigate the role of EphA4 in the organization of the trigeminal whisker system, mutant mice were examined. Comparison of control and EphA4 knockout mice revealed
that peripheral organization was altered: whisker rows A–D were normal, but low-numbered whiskers in the E row were missing (Fig. 5-3 A, B). Since central somatosensory representations reflect peripheral organization (Van der Loos and Woolsey, 1973) patterning throughout the somatosensory pathway was likely to be affected. Indeed, at two levels of the neuraxis- the brainstem (trigeminal nucleus principalis, spinal trigeminal subnucleus interpolaris, and spinal trigeminal subnucleus caudalis) (Fig. 5-4 A, B) and cortex (Fig. 5-4 C, D)- rows A through D were normal, but positions corresponding to caudal E-row whiskers were missing both shortly after birth (Postnatal day 3–7, P3–7) and in maturity (P60) (Fig. 5-4). Since caudal vibrissae develop first, tend to be the largest whiskers, receive unique innervation, and rely more heavily on innervation for their presence (Van Exan and Hardy, 1980, Dorfl, 1985b, Welker and Van der Loos, 1986), the possibility that the deficit in EphA4−/− mice was concomitant with a defect in the innervation of peripheral whiskers by trigeminal axons was examined.

**Figure 5-3: Vibrissal patterning is altered in EphA4−/− mice.** Under-side of whisker pads removed from the face and pinned surface-side down, from P14 control (A) and EphA4−/− (B) mice, with photomicrographs on top and tracings of the main rows of whiskers shown as lines on the bottom, and with whisker rows A through E labeled and straddler whiskers designated by circles. Position of missing E-row vibrissae is indicated by the dashed oval in B. R, rostral; D, dorsal.
Proper innervation by trigeminal axons corresponds with proper vibrissae formation, particularly for the larger, more caudal whiskers (Van Exan and Hardy, 1980), though the necessity of innervation for formation remains unclear. Because EphA4 guides axonal pathfinding in other developing systems (Helmbacher et al., 2000, Eberhart et al., 2004, Marquardt et al., 2005, Canty et al., 2006) and because the missing vibrissae in EphA4−/− are located in an area that is usually high in EphA4 expression, and are innervated by a different trigeminal fascicle than their caudal counterparts (Dorfl, 1985b),

Figure 5-4: Central whisker representations are perturbed in EphA4−/− mice. Cytochrome oxidase staining of three brainstem nuclei- trigeminal nucleus principalis (PrV), left; spinal trigeminal subnucleus interpolaris (SpVi), middle; spinal trigeminal subnucleus caudalis (SpVC), right (panels A, B)- and the primary barrel field in adult somatosensory cortex (panels C, D), in control (A, C) and EphA4−/− (B, D) mice, with rows A through E labeled. Positions of missing E-row vibrissae are indicated by the dashed oval and arrows in B and D.

Aberrant Trigeminal Innervation of Maxillary Pads in EphA4 Mutant Mice

Proper innervation by trigeminal axons corresponds with proper vibrissae formation, particularly for the larger, more caudal whiskers (Van Exan and Hardy, 1980), though the necessity of innervation for formation remains unclear. Because EphA4 guides axonal pathfinding in other developing systems (Helmbacher et al., 2000, Eberhart et al., 2004, Marquardt et al., 2005, Canty et al., 2006) and because the missing vibrissae in EphA4−/− are located in an area that is usually high in EphA4 expression, and are innervated by a different trigeminal fascicle than their caudal counterparts (Dorfl, 1985b),
we investigated properties of trigeminal axon in control and EphA4<sup>−/−</sup> whisker pads during vibrissal development. Since the snout is curved, thick sections were used and adjacent sections were examined in order to gain a full sense of trigeminal axon patterning; the images selected are representative of all sections. At E11.5, the trigeminal nerve has invaded the entire dorsoventral extent of the whisker pad in control mice (Fig. 5-5A). In EphA4<sup>−/−</sup>, dorsal invasion of the nerve was similar to control, while ventral invasion was less evident (Fig. 5-5D). Indeed, dorsal axons were well fasciculated in both control and EphA4<sup>−/−</sup> mice (Fig. 5-5 B, E, E'), but ventral axons appeared less tightly packed and less ordered in the mutant (Fig. 5-5 C, F, F'). One day later, at E12.5, dorsal axons were again present in tight accumulations in both genotypes (Fig. 5-5 G, H, J, K, K'), while ventral trigeminal axons, which had by now invaded the mutant whisker pad more extensively (Fig. 5-5 G, J), still displayed an abnormal and poorly ordered appearance (Fig. 5-5 I, L, L').

**Figure 5-5:** Initial trigeminal innervation of the vibrissal pad is perturbed in EphA4<sup>−/−</sup> mice. Neurofilament staining of trigeminal axons in the whisker pad at E11.5 (A–F') and E12.5 (G–L') mice. Low-magnification images of the entire whisker pad (A, D, G, J) and high-magnification images of dorsal (B, E, E', H, K, K') or ventral (C, F, F', I, L, L') axons. R, rostral; D, dorsal; A and E separated by a dashed line indicates the position of prospective whiskers at E11.5 and E12.5.
By E14.5, when the control whisker pad is innervated throughout its dorsoventral extent, with five rows apparent (Fig. 5-6 A), trigeminal axons in the ventral whisker pad of EphA4<sup>−/−</sup> remained sparse (Fig. 5-6 B). In addition, in contrast to the tightly fasciculated E-row axons in control whisker pad (Fig. 5-6 C), axons in the E-row region in EphA4<sup>−/−</sup> were defasciculated, with a reticular appearance (Fig. 5-6 D). Moreover, a caudal deficit was apparent when emerging follicles were examined. Follicles ringed by axons were present throughout the rostrocaudal extent of both D and E rows in control animals (Fig. 5-6 E), but only D and the rostral E follicles were present in EphA4<sup>−/−</sup> (Fig. 5-6 F). While D-row follicles appeared less pronounced at this age, the deficit seems transient since D-row whiskers and their representations exist later (Figs. 5-3, 5-4).

Figure 5-6: Ventral axons defasciculate abnormally and do not associate with follicles in EphA4<sup>−/−</sup>. Neurofilament staining of trigeminal axons in the whisker pad at E14.5, with low-magnification images of the entire whisker pad (A, B), high-magnification images of the caudal E-row tract (C, D), or newly formed D- and E-row follicles (E, F). R, rostral; D, dorsal; letters and numbers indicate the position of each vibrissal row at E14.5.
Finally, in contrast to the tight axon bundles innervating control caudal E-row follicles (arrowheads in Fig. 5-6 E), the axons present in the EphA4<sup>−/−</sup> E row were disorganized and not coupled with a follicle (Fig. 5-6 F). Thus, in the absence of EphA4, axonal characteristics were perturbed and follicles did not form. Interestingly, these results parallel data supporting a role for EphA4-mediated signaling between axons and the environment they traverse in the maintenance of tight axon fascicles in the corticospinal system (Canty et al., 2006). Analogous with these results, EphA4 appears to act normally by limiting ventral trigeminal defasciculation, thus promoting the matching of ventral axons with caudal E-row targets.

**DISCUSSION**

The generation of topography within neural systems by positionally-based molecular cues was postulated by Roger Sperry (Sperry, 1963) and his hypothesis was elegantly illustrated by studies characterizing Eph receptor interactions with ephrin ligands in establishing topographic maps in the retinotectal system (Cheng et al., 1995, Drescher et al., 1995). We speculated that Eph/ephrins may play a similar role in positional specification within the developing trigeminal maxillary system since there were high levels of one receptor, EphA4, in the maxillary target field and several ephrin ligands in trigeminal neurons. Our findings implicate EphA4 as influencing the spatial wiring of peripheral representation. Strikingly, the absence of EphA4 also resulted in a loss of particular sensory structures: a subset of the ventral vibrissae. These results firmly implicate Eph signaling in the establishment of peripheral somatosensory topography.

Because EphA4, a known axon guidance cue, was enriched in the ventral maxillary
pad, we investigated whether ventral trigeminal axons were abnormal in EphA4−/− mice. Indeed, E-row axons were less substantial, more disorganized, and aberrantly defasciculated in mutant whisker pads. While innervation by trigeminal axons and emergence of peripheral vibrissae are coincident processes, it remains unclear whether the former relies on the latter, or if these processes are merely coincident. Results from several studies suggest that axon innervation is necessary for vibrissal formation, supporting the concept that either a threshold number of endings or a relative difference of nerve endings between locations is required for a follicle to form (Hardy, 1949, 1951, Davidson and Hardy, 1952, Welker and Van der Loos, 1986). Our results, that the ventral EphA4−/− maxillary pad is improperly innervated and lacks follicles, is consistent with a model in which the presence of a threshold number of endings induces whisker follicles, a process that for caudal E-row vibrissae requires EphA4.

Eph signaling is generally considered a repulsive cue for axon guidance; however, EphA4 can both promote and inhibit axon growth (Eberhart et al., 2004). At first glance, our results- loss of innervation in the absence of EphA4- appear most consistent with an attractive mechanism (Fig. 5-7, E14.5B). Indeed, the observed delay in ingrowth of ventral axons in EphA4−/− whisker pads may indicate weak attraction. A substantial number of ventral axons, however, do eventually invade the EphA4−/− whisker pad, suggesting that lack of an attractive cue is not the explanation for the observed deficit. Rather, ventral axon targeting appears askew, with improper organization of E-row tracts. A recent study of the corticospinal tract demonstrated that EphA4 in the tissue surrounding the descending tract was necessary to repulse the axons, thus preventing early defasciculation and allowing proper target innervation (Canty et al., 2006). Absence
of EphA4 in this system led to premature axon defasciculation and degradation of the tract before the target organ was reached. Our data support a similar system in the developing maxillary pad, a classical repulsive mechanism with an interesting twist: ventral axons are repulsed from the surrounding ventral whisker pad in order to prevent E-row fascicles from prematurely separating (Fig. 5-7, E14.5A). In this scenario and consistent with our data, the presence of EphA4 in the whisker pad serves to prevent inappropriate branching of E-row trigeminal axons so that the proper terminals innervate each vibrissa.

**Figure 5-7: Stages of trigeminal innervation of the forming whisker pad.** Early in development (top), the newly formed whisker pad (left) is largely unspecified, but weak patterning exists in the trigeminal ganglion (right). In control mice (far left), combinations of molecules, including EphA4 and ephrins, positionally pattern the whisker pad and the trigeminal ganglion, respectively, and trigeminal neurons extend axons, including the middle maxillary tract (red) (E10.5). Row tracts emerge as the maxillary nerve reaches the whisker pad (E11.5) and invade the whisker pad topographically (E12.5), eventually ringing whisker follicles (E14.5). In the absence of EphA4 peripheral patterning (middle column), ventral deficits are observed: the ventral-most row tract is slow to arrive (E11.5) and axons invasion is light (E12.5). Premature axon defasciculation is proposed to explain the deficits, with ventral vibrissae not forming (E14.5A). Other possible mechanisms are less consistent with the data. A lack of attraction is predicted to result in axons halting (E14.5B), while lack of peripheral prepatterning is predicted to result in disorganized and nonspecific innervation (E14.5C).
Another possibility is that EphA4 acts to pattern the whisker pad itself (Fig. 5-7, E14.5C). In this case, an improperly patterned maxillary pad may result in nonspecific innervation that might not support vibrissal formation. We examined E10.5 maxillary protrusions for signs of gross morphological differences and found no clear evidence of a prepatterning deficit (data not shown). These observations, together with the arrival of pioneer axons to wild type but not EphA4−/− maxillary protrusion prior to vibrissal formation, suggest a system in which the axons are required for vibrissal growth. The question of whether aberrant axonal guidance causes or is a consequence of missing vibrissae is an important one, however, and future studies must investigate pre-patterning in EphA4−/− on a molecular level to rule peripheral changes as a cause for the E-row abnormalities (Fig. 5-7). Until then, our data demonstrate that EphA4 is necessary for the conserved spatial topography of whiskers and support a relationship between axonal innervation and peripheral sensory organ formation.

The establishment and maintenance of spatial maps in the nervous system is governed by a myriad of molecular cues, in both target fields and innervating neurons, that serve to match appropriate synaptic partners, strengthen subsets of synapses, and guide activity-dependent plasticity. Our data contribute new insight into the developmental processes by which such maps are generated by addressing how positionally selective connections are guided, in our case by a surface-based Eph receptor. In particular, the selective dependence of the most caudal E-row vibrissae on EphA4 for their presence is a striking example of the specification of a subset of sensory structures within a whole. Since at least some vibrissae may require an innervating axon, the EphA4−/− mouse may represent a unique instance in which incomplete innervation
leads to a position-dependent deletion within a topographic system. In conclusion, the peripheral somatosensory system seems especially well poised for future investigations of the interplay between attractive and repulsive forces in the definition of spatial maps, intersections between patterning and guidance systems, and the behavioral consequences of altered somatosensory representations.
CONCLUSIONS AND FUTURE DIRECTIONS

Our investigation of the role of EphA4 in cortical and peripheral somatosensory system development has unveiled additional ways in which the diverse and multifunctional Eph/ephrin family contributes to the formation of the mature nervous system. As the embryonic cortex expands, EphA4 activation promotes the proliferation of the progenitor cells that will seed the adult somatosensory cortex. This activation is triggered by the binding of the ligand ephrin-B1, a binding partner for EphA4 that was previously unrecognized. But many questions remain unanswered regarding the mechanisms surrounding this receptor-ligand binding pair and its promotion of proliferation.

Our results indicate that proliferation is promoted through the receptor-containing cell, but it is unclear to us whether the receptors and ligands are expressed on distinct cells in the ventricular zone (VZ), or if some combination of EphA4 and ephrin-B1 is present on all VZ cells. Furthermore, it is unclear whether either or both of these molecules are preferably expressed on the cell bodies of progenitors undergoing mitosis at the ventricular surface, or on the apical end feet of progenitor cells resting in the G1 phase of the cell cycle. High-resolution electron microscopy must be performed to answer these questions.

Most of our molecular analyses focused on the binding of EphA4 and ephrin-B1, as this finding was heavily contested by a number of peer reviewers. But molecular analyses still need to be performed in order to determine the mechanisms downstream of
the cell-surface molecules that are responsible for exacting the proliferative end state of the binding event.

Finally, our manipulations and observations were all made at a singular, albeit crucial, time point for cortical progenitor proliferation. The question of whether EphA4 affects proliferation evenly throughout both neuroepithelial expansion and radial glial neurogenesis remains. It is possible that EphA4 is dedicated to one process or the other, or, even more specifically, to the generation of cells fated for particular cortical layers. Precisely timed experiments during development and investigation of laminar proportions in adulthood must be performed to determine temporal relevance of EphA4 and ephrin-B1.

Our examination of the EphA4 mutant cortex led to the discovery that the peripheral somatosensory system is affected by the presence of this receptor tyrosine kinase, as well. While our analyses of the mutant whisker pad revealed a potential mechanism for the resulting missing whiskers, in contrast to the proliferation studies, the complimentary ligand involved was not elucidated. Future studies must determine not only which ephrin within the trigeminal neurons repels EphA4 and maintains fasciculation during innervation, but also the other molecules that direct the same process in rows A through D. More importantly, scientists intimately familiar with vibrissal development must consider whether our findings provide evidence for induction of vibrissal growth by neuron presence. The role that the trigeminal axons play in forming the vibrissae remains a major question in the study of peripheral sensory organ development, and our work may be relevant to the argument over this mechanism.
MATERIALS AND METHODS

Animals

All animal use and care was in accordance with institutional guidelines (the Yale IACUC protocol 2002-10098 and the Georgetown GUACUC protocols 06-022 and 09-020) in compliance with federal policy. Wild-type CD-1 (Charles River Laboratories) or EphA4 mutant mice (P. Charney, INSERM, Paris, France), maintained on a C57Bl/6 strain (for 5-15 generations) and bred as heterozygotes (Helmbacher et al., 2000), were used. PLAP EphA4 mutant mice were kindly provided by M. Tessier-Lavigne (Genentech, South San Francisco, CA) and were maintained on a CDI background (5–15 generations), bred in homozygote/wild type crosses (Leighton et al., 2001). Wild-type, heterozygous and mutant alleles of EphA4 were generated in mendelian ratios. Body sizes of all genotypes were similar on the days of analyses. The day of the vaginal plug was embryonic day 0.5 (E0.5) and the day of birth, postnatal day 0 (P0). Comparisons between mutant and control mice were performed using littermates.

Expression vectors and expression analyses

Control vectors were either pCMV-eYFP-N1 (Clontech) or CAG-DsRed (Akiko Nishiyama, University of Connecticut, Storrs, CT, USA). The full-length (FL) EphA4 construct and shRNA for EphA4 (sA4) were provided by Mustafa Sahin (Harvard Medical School, Boston, MA, USA) and Nancy Ip (Hong Kong University of Science and Technology, Hong Kong, China), respectively, and were previously characterized (Fu et al., 2007). The dominant negative (DN) construct was produced using a DNA fragment generated by RT-PCR corresponding to the ectodomain and transmembrane
region cloned in frame with YFP in pCMV-eYFP-N1, based upon previous studies (Hu et al., 2003). An additional shRNA for EphA4 targeting the 3’ UTR was generated using the pSUPER system and used in Fig. 3-13. Ephrin B1 coding sequence was produced via RT-PCR and cloned into an expression vector with the RSV LTR (Davy et al., 2004). COS7 cells were transfected with each construct and expression was evaluated by autofluorescence, immunohistochemistry, western blotting, live and fixed ligand binding, and receptor activation assays (Cheng and Flanagan, 1994, Gale et al., 1996). RT-PCR using primers corresponding to the ectodomain of each Eph receptor in either HEK293T or mouse [E14.5 cortex and neurospheres (Nsph)] was used to assess expression of endogenous genes. All amplified products were approximately 500 bp in length.

**Binding studies**

To examine ligand and receptor binding, COS7 cells were transfected with vectors encoding either EphA4 or ephrin-B1. Two days later, cells were washed in PBS, fixed for 5 minutes with 4% PFA and incubated with 3 μg/ml of ephrin-B1, ephrin-A5, EphA4 or EphB2 reagents (ectodomains fused in frame with a human Fc fragment), or a control chimeric protein (Gale et al., 1996) for 1 hour at RT. Coverslips were washed with PBS, fixed in 4% PFA for 1 hour, washed with PBS again, incubated with an Alexa-conjugated anti-human Fc antibody for 1 hour, washed in PBS and mounted immediately. The number of positive cells per 10x field in each condition was recorded in five randomly designated positions on each of four coverslips.

**Activation studies**
To examine receptor activation, parallel plates of HEK293T cells were transfected with (1) YFP, (2) EphA4 (10%) and pSK+ (90%), or (3) EphA4 (10%) and DN (90%) expression vectors. Two days after transfection, plates were either harvested immediately (0 minutes) or incubated with 5 µg/ml of clustered ephrin-B1 or ephrin-A5 reagents for 2 or 10 minutes. Cells were scraped in PBS and homogenized in RIPA buffer with protease inhibitors, and cell extracts were produced. Protein samples (15 µg/lane) were resolved on 10% SDS-PAGE gels, transferred to a nitrocellulose membrane and incubated with primary antibody for phosphorylated EphA (provided by Mustafa Sahin and Michael Greenberg, Harvard Medical School, Boston, MA, USA) overnight at 4°C in Blocking Solution [BS; 2.5% skimmed milk, 2.5% normal goat serum (NGS) in Tris-buffered saline (TBS)]. Following TBS washes, detection was carried out using a species-specific HRP-conjugated secondary antibody and chemiluminescence. Blots were stripped and reprobed for actin to confirm equal loading. Experiments were performed in triplicate. Band intensity was quantified and relative stimulation was calculated.

**Neurosphere cultures**

Neurospheres (Nsphs) were derived from dorsal telencephalons free of membranes and dissected from either CD-1 or EphA4 mutant E15.5 mouse embryos into Hank’s buffered salt solution (HBSS). A single cell suspension was produced by trituration and plated into serum-free Neurobasal medium containing 25 ng/ml human recombinant FGF2, 2% B27 supplement, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were plated at a density of 50,000-75,000 cells/ml, maintained at 37°C and supplemented with fresh media every other day.
Following tail PCR, cells of similar EphA4 genotypes were pooled. Every 5-7 days cells were passed, dissociated by trituration and replated (Reynolds and Rietze, 2005). Following the third passage, wild-type cultures were transected with YFP, YFP and DN or YFP and ephrin-B1, using Lipofectamine 2000 (Invitrogen). One day before harvest, BrdU was added to the media (10 µM final concentration). Cells were harvested, fixed in 4% PFA, processed for BrdU and YFP immunocytochemistry, and counterstained with Bisbenzamide.

**In utero electroporation (IUE)**

Timed pregnant CD-1 or EphA4+/− (E14.5) mice were anaesthetized with 100 mg/kg Ketamine and 10 mg/kg Xylazine. Following laparotomy, a solution containing plasmid DNA (2-4 µg/µl of YFP, DsRed, sA4, DN or FL EphA4, or ephrin-B1 in Tris-buffered 0.02% Fast Green) was injected through the uterine wall into the lateral ventricle of each embryo. Current pulses were delivered across the embryonic head using Tweezertrodes (BTX). Embryos were kept hydrated, placed back into the abdomen of the mother and gestation was allowed to proceed for 1-3 days. The survival rate was ~84% and the rate of transfection in surviving embryos was ~71%. Only embryos showing effective transfection in the somatosensory cortex were analyzed.

**Cell proliferation analysis**

Pregnant mice received an intraperitoneal injection of BrdU (50 µg/g) 2 hours before euthanasia. Embryonic brains were dissected, fixed, frozen and sectioned as described in the Immunohistochemistry methods. Sections were postfixed in 4% PFA for
10 minutes, washed in PBS, treated with 0.1% trypsin for 10-30 seconds and incubated in 100% fetal calf serum. Sections were rinsed in PBS, incubated in 2 N HCl for 30 minutes at 37°C, rinsed in 0.1 M sodium borate pH 8.14, and incubated overnight with mouse α-BrdU and rabbit α-GFP in BB⁺. Standard immunohistochemistry was then performed. Sections (3-5) in 3-5 individuals were analyzed.

**Coimmunoprecipitation**

HEK293T cells were transfected with DsRed (control), EphA4 or ephrin-B1. One day later, cells were trypsinized. The DsRed control cells were replated at a high density (100,000 cells/cm²). The EphA4 and ephrin B1 cells were mixed together and plated at the same high density. Three hours later, cells were harvested in immunoprecipitation (IP) buffer [1% NP-40, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, Complete Protease Inhibitors (Roche)], incubated for 10 minutes at 4°C, and spun. The supernatant was then incubated with 20 µl protein A sepharose (GE Healthcare) and 5 µg of either α-EphA4 or α-IgG overnight at 4°C. Following washes with IP buffer, proteins bound to protein A were eluted by boiling and subjected to western blot analyses using antiserum for EphA4 or ephrin-B1.

Coimmunoprecipitation from brain tissue was performed as in Buchert et al. (Buchert et al., 1999). Briefly, E14.5 cortex was homogenized in 1 ml neurolysis (NL) buffer (20 mM Hepes pH 8.0, 1% NP-40, 0.2 M NaCl, 2 mM EGTA, and Complete Protease Inhibitors) using a dounce homogenizer, shaken vigorously at 4°C for 15 minutes, spun at 100,000 g for 1 hour and supernatant was collected. Brain lysate (1 mg) was precleared with protein A sepharose beads for 30 minutes at RT. In parallel, 20 µl
sepharose was incubated for 30 minutes at RT with 10 µg of either α-EphA4 or α-IgG to allow antibody binding to beads. Precleared lysate was then mixed with prebound antibody/sepharose complexes, incubated overnight at 4°C and beads were washed in NL buffer. Proteins bound to protein A were eluted by boiling and subjected to western blot analysis using either EphA4 or ephrin-B1 antiserum.

In situ hybridization

(Radioactive) Mouse embryos were collected, fixed, dehydrated, embedded in paraffin and sectioned sagittally (10-15 µm) for analysis of gene expression. In situ hybridizations were performed with 33P-labeled antisense probes to Eph family members, as previously described (Yun et al., 2003). Slides were dipped in autoradiographic emulsion, exposed for 2-6 weeks and developed. Our laboratory previously published an image of EphA4 expression at E12.5 (Yun et al., 2003). To obtain a profile of Eph family gene expression in cortical development, the density of silver grains was quantified in the proliferative zone (PZ), the intermediate zone (IZ) and the cortical plate (CP) of 3-5 sections of somatosensory cortex at E14.5 using ImageJ software. Probes with the same specific activity were used (Figs 3-2 and 3-15). Silver grain density was converted to color intensity (Fig. 3-1 A and Fig. 3-14 A) or represented graphically (Fig. 3-2 O and Fig. 3-15 K).

(Non-radioactive) Tissue sections, prepared as described in the immunohistochemistry methods, were subjected to non-radioactive in situ hybridization. Digoxygenin-labeled antisense probes corresponding to Ephrin ligands were generated from mouse templates (Yun et al., 2003). Sections were post-fixed in 4% PFA, washed in
PBS, acetylated, washed in PBS, incubated in pre-hybridization solution for several hours at room temperature (50% formamide, 5 x SSC, 1 x Denhardt, 250 mg/ml tRNA, 500 mg/ml fish sperm DNA), hybridized overnight at 65°C in hybridization buffer (50% formamide, 300 mM NaCl, 20 mM Tris-HCl, pH 8, 5 mM EDTA, 10 mM Na₂HPO₄ pH 7.2, 10% Dextran Sulfate, 1 x Denhardt, 500 mg/ml tRNA, 200 mg/ml fish sperm DNA) containing 300–500 ng probe/ml, and washed. IHC for the labeled nucleotide was performed using AP detection.

**Immunohistochemistry**

Brains (E14.5-17.5) were fixed by immersion for 1-2 hours in 4% paraformaldehyde (PFA) at 4°C, washed in phosphate-buffered saline (PBS), cryoprotected in 30% sucrose overnight and frozen. Brains were stored at −80°C until 14 µm coronal cryosections were produced. Sections were mounted, air dried and stored desiccated at −20°C. Sections and other tissue preparations were incubated in block buffer plus (BB⁺; 2.5% goat serum, 2.5% donkey serum, 1% BSA, 1% glycine, 0.1% lysine, 0.4% Triton X-100 in PBS) for 30 minutes at room temperature (RT) and then incubated in primary antibody or ephrin-fc diluted with BB⁺ overnight at 4°C. After PBS washes, sections were incubated with Alexa-conjugated secondary antibodies (1:800), counterstained with fluorescent Nissl (1:500) or Bisbenzamide (1:1000), and mounted. The primary antibodies used, their source and dilutions are as follows: rabbit α-GFP, Molecular Probes, 1:3000; mouse α-betaIII-tubulin (Tuj1, also known as Tubb3), Babco, 1:500-1000; mouse α-RC2, Developmental Studies Hybridoma Bank (DSHB; under the auspices of the NICHD and maintained by The University of Iowa, Department of...
Biological Sciences, Iowa City, IA 52242, USA), 1:5; mouse α-nestin (rat-401), DSHB, 1:10; rabbit α-EphA4, Zymed, 1:300; mouse α-EphA4 Zymed, 1:500; rabbit α-ephrin B1, Zymed, 1:250; rabbit α-phospho-histone H3, Calbiochem, 1:200; goat α-Sox2, R&D Systems, 1:20; mouse α-BrdU, BD Biosciences, 1:100; rabbit α-NF (Sigma, St. Louis, MO; 1:1,000), chicken α-NF (Millipore, Billerica, MA; 1:1,000). The ligand body was used at 30 mg/ml.

**Cytochrome Oxidase Staining**

Postnatal mice (P3–60) were anesthetized and perfused with saline followed by 4% paraformaldehyde (PFA) in phosphate buffer (0.1M, pH 7.4) (PBS). Brains were sometimes post-fixed overnight. For brainstem preparations, the tissue at the base of the skull was embedded in gelatin and sectioned horizontally at 40–100 mm (Henderson et al., 1994). For cortical samples, brains were fixed by immersion in PFA and embedded in 4% suprasieve agarose/PBS. The cortex was freed from subcortical structures, flattened between glass slides, and sectioned at 200 mm (Miller et al., 2006). All sections were pre-treated with 10% sucrose/0.12M phosphate buffer solution at 37°C and then incubated in the CO reaction (30 mg cytochrome C, 50 mg dianminobenzidine, 2 mg catalase, and 10 g sucrose per 100 ml of 0.12M PB) for 6–24 hours at 37°C. Stained sections were post-treated by immersion in increasingly dilute sucrose solutions (10, 5, 0% sucrose in 0.12M PB), mounted, dried, dehydrated through graded ethanols (50, 70, 95 and 100% for 5 minutes each), cleared in xylenes and coverslipped.

**Whole-Mount Whisker Pad Examination**

83
P10-adult wild-type and EphA4<sup>+</sup> littermates were euthanized, and the skin covering the snout was carefully removed, pinned surface-side down, fixed with 4% PFA for 1 hour, cleared of excess tissue enzymatically using dilute trypsin or manually to reveal vibrissae, and photographed.

**Reporter Gene Detection**

β-galactosidase expression was detected using standard staining conditions: sections were post-fixed in 4% PFA, 0.2% glutaraldehyde and then incubated in 4 mM potassium ferrous cyanide, 4 mM potassium ferric cyanide, 0.8 mg/ml Xgal, in PBS containing 2 mM MgCl<sub>2</sub> at 30°C for 1 hour to overnight. Following a PBS wash, sections were post-fixed in 4% PFA for 15 minutes, washed in PBS, and either mounted or subjected to immunohistochemistry as described above. For alkaline phosphatase staining, sections were incubated in PBS at 65°C for 1 hour to inactivate endogenous phosphatases, pre-treated in Buffer 3 (150 mM NaCl, 100 mM Tris pH 9.5) for 30 minutes, and incubated in reaction buffer (Buffer 3 with 0.35 mg/ml NBT and 0.175 mg/ml BCIP) for 10 minutes to 3 hours, rinsed with TE, fixed in 4% PFA for 10 minutes, washed with water, and mounted.

**In vitro comb analyses of Cdh1**

In conjunction with Elliot Hui, University of California, Irvine, Department of Bioengineering, HEK293 cells that had been stably transfected with EphA4 or ephrin-B1 were plated onto a polystyrene comb apparatus treated with lamanin and housed inside a standard tissue culture 12-well plate. Once confluency was reached, combs were
rearranged such that EphA4 cell-seeded combs were snapped together with ephrin-B1 cell-seeded combs. Untransfected HEK cells were also used as controls. 24 hours later, comb pairs were washed, fixed, and subjected to immunocytochemistry for Cdh1.

**Data analysis**

In the *in vitro* studies, 20-50 Nsphs per condition were imaged in each of at least three experiments. Both the number of transfected cells and BrdU\(^+\) cells were quantified. To compare between experiments, values were converted to a percentile of the maximal level in each experiment and then averaged between experiments. To analyze EphA4 mutant cortex, images of several similar mediolateral positions were obtained of Nissl-stained wild-type and EphA4\(^{-/-}\) cortex and processed in parallel (n=4 and 5, respectively). ImageJ was used to measure anteroposterior length, thickness of the cerebral wall and cortical area. Following IUE, 2-8 embryos per group (control, sA4, DN, FL or ephrin-B1) were analyzed at particular times after transfection or BrdU introduction. Only animals with transfected somatosensory cortex were included in the analyses. Images of three to five well-spaced sections per animal were assessed using Adobe Illustrator. A 0.1 mm-wide rectangle that spanned the cerebral wall was placed over transfected cortex. Embryonic zones (PZ, IZ and CP) were identified using Tuj1 immunoreactivity and fluorescent Nissl staining, and were demarcated within this rectangle. The area of each zone was measured and the numbers of transfected or BrdU\(^+\) cells within each zone were recorded. Data were normalized to total cell number per mm\(^2\), averaged for each embryo and data from several individuals were pooled. Data were tested for significance by one-way analysis of variance (ANOVA; \(P=0.05\)) and expressed as mean ± s.e.m.


Buchman VL, Davies AM (Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic sensory neurons. Development 118:989-1001.1993).


Kawaguchi D, Yoshimatsu T, Hozumi K, Gototo Y (Selection of differentiating cells by different levels of delta-like 1 among neural precursor cells in the developing mouse telencephalon. Development 135:3849-3858.2008).


Marthiens V, ffrench-Constant C (Adherens junction domains are split by asymmetric division of embryonic neural stem cells. EMBO Rep 10:515-520.2009).


Mummery CL, van den Brink CE, de Laat SW (Commitment to differentiation induced by retinoic acid in P19 embryonal carcinoma cells is cell cycle dependent. Dev Biol 121:10-19.1987).


Yamakado M, Yohro T (Subdivision of mouse vibrissae on an embryological basis, with descriptions of variations in the number and arrangement of sinus hairs and cortical barrels in BALB/c ( nu/+; nude, nu/nu) and hairless (hr/hr) strains. Am J Anat 155:153-173.1979).

Yates PA, Roskies AL, McLaughlin T, O'Leary DD (Topographic-specific axon branching controlled by ephrin-As is the critical event in retinotectal map development. J Neurosci 21:8548-8563.2001).


