The role of ephrin-A ligands and EphA receptors in the development and maintenance of somatosensory connectivity

Cynthia L. Kenmuir
Medical University of Ohio

Follow this and additional works at: http://utdr.utoledo.edu/theses-dissertations

Recommended Citation
Kenmuir, Cynthia L., "The role of ephrin-A ligands and EphA receptors in the development and maintenance of somatosensory connectivity" (2010). Theses and Dissertations. 876.
http://utdr.utoledo.edu/theses-dissertations/876

This Dissertation is brought to you for free and open access by The University of Toledo Digital Repository. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of The University of Toledo Digital Repository. For more information, please see the repository's About page.
Health Science Campus

FINAL APPROVAL OF DISSERTATION
Doctor of Philosophy in Biomedical Sciences
(Neuroscience and Neurological Diseases)

The Role of ephrin-A Ligands and EphA Receptors in the Development and Maintenance of Somatosensory Connectivity

Submitted by:
Cynthia Kenmuir

In partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biomedical Sciences

Examination Committee

Major Advisor: Richard Lane, Ph.D.

Academic Advisory Committee:
Nicolas Chiaia, Ph.D.
Richard Mooney, Ph.D.
John Wall, Ph.D.
Donald Godfrey, Ph.D.

Signature/Date

Senior Associate Dean
College of Graduate Studies
Michael S. Bisesi, Ph.D.

Date of Defense: June 2, 2009
The Role of ephrin-A Ligands and EphA Receptors in the
Development and Maintenance of Somatosensory Connectivity

University of Toledo, College of Medicine

Cynthia L. Kenmuir

2009
DEDICATION

I would like to dedicate this dissertation to my family, without whom none of this would be possible.

To my parents, Wayne and Nancy, your love, endless support, and constant encouragement throughout my journey have meant the world to me. You raised the four of us to be hard-working and well-rounded, which has brought us success in so many realms. I am truly grateful for all that you have taught us and for all of the sacrifices that you have made over the years.

To my siblings, Christina, Crystal, and David, although we have all chosen to pursue such separate career paths, I will always be there for you as you have all been there for me. Together, there’s nothing we can’t do.
ACKNOWLEDGEMENTS

I am very fortunate to have had an excellent advisor and advisory committee over the past three years of this work. The continual guidance and support of my advisor, Dr. Richard D. Lane, has been integral to the further development of my scientific independence. I am thankful for his constant encouragement to explore new laboratory techniques as well as for his endless patience that allowed me to continue to pursue my often competing interests in academics and medicine throughout my PhD years.

I would also like to express my gratitude to Dr. Nicolas L. Chiaia for his constant encouragement and mentorship and to Dr. Richard D. Mooney for sharing his wealth of knowledge, both of whom have helped me to become a better scientist.

I would also like to thank Drs. John Wall and Donald Godfey for their insight, and excellent comments as a part of my advisory committee.

Finally, I would like to thank Dr. G. Andrew Mickley, my undergraduate research advisor, for his continued support over the past ten years. As a freshman, it was Dr. Mickley’s passion for research that sparked my interests in neuroscience. His patience and dedication to each student’s success was essential to my scientific development. His continued support each year at the SFN meeting is a constant reminder of his encouragement and my spot in the “Mickley family tree”.

iii
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Preliminaries</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedication</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iv</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>II. Literature</td>
<td>4</td>
</tr>
<tr>
<td>III. Manuscript 1: Developmental expression of ephrin-A2 in Primary Somatosensory Cortex: A role in restricting the arborization of ventrobasal thalamic axons?</td>
<td>9</td>
</tr>
<tr>
<td>IV. Manuscript 2: Postnatal expression of ephrin-A3 and EphA4: A role in thalamocortical connections?</td>
<td>41</td>
</tr>
<tr>
<td>V. Manuscript 3: Expression of ephrin-A Ligands and EphA Receptors in the Development of the Peripheral Trigeminal Somatosensory System</td>
<td>79</td>
</tr>
<tr>
<td>VI. Manuscript 4: The Role of Ephrins in Thalamocortical Connectivity within the Rat Somatosensory System</td>
<td>110</td>
</tr>
<tr>
<td>VII. Discussion</td>
<td>145</td>
</tr>
<tr>
<td>VIII. Bibliography</td>
<td>151</td>
</tr>
<tr>
<td>IX. Abstract</td>
<td>172</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

The proper function of the nervous system depends on the development and maintenance of neural circuits. This requires that immature neurons are guided to their final destination, connected to their correct targets, and prevented from forming aberrant connections. The well described anatomical and physiological connectivity of the rodent somatosensory system from the periphery to the cortex makes it an attractive model for studying the molecular aspects of axonal guidance needed for the precise development of these connections. The orderly arrangement of the mystacial vibrissa on the rodent snout is maintained throughout each level of the neuraxis resulting in a topographic representation of the periphery within each of the brainstem nuclei, the ventrobasal thalamus (VB), and the parietal cortex.

Shortly after the neurons of the trigeminal ganglia (TG) are born, they extend their peripheral processes out toward the developing whisker pad (WP) and their central processes toward the brainstem. The peripheral processes of the maxillary division group together to form the infraorbital nerve (ION), which arrives at the back of the WP just after the vibrissal follicles have developed. At the posterior aspect of the WP, the ION splits into individual row nerves, each of which courses between the whisker rows. The row nerves further divide into superficial and deep vibrissal nerves that surround the outer epithelial root sheath (ORS) of each vibrissal follicle providing sensory innervations to each of the large sinus hairs. While this neural connectivity is occurring in the periphery, the central processes of the TG were also extending into the brainstem nuclei where they make topographic connections in the trigeminal brainstem complex.
Neurons from the brainstem nuclei then extend their axons towards the VB where they synapse on newly differentiated neurons, again in a topographic relationship to the periphery. Within days, the VB axons extend into the internal capsule (IC) and turn towards the developing cortex where they pause in the intermediate zone before extending upwards through the cortex. At this point, the thalamocortical axons (TCAs) extend through the newly formed cortical layers that have differentiated from the cortical plate (CP). TCAs make their primary synapses within layer IV in cell clusters representative of individual vibrissae. These axons fill the breadth of layer IV initially without extension into layers II/III or V, suggesting the presence of a repulsive guidance cue in those layers.

Although the development of these precisely organized connections from the periphery to the cortex are well described, many questions remain including the mechanisms involved in guiding axons at all levels of the somatosensory system to their next target region, establishing the topographic arrangement of those axons before the make their final connections, maintaining the laminar and areal boundaries of those regions, and providing for plasticity of the system following injury.

Several families of guidance molecules are involved in each of these processes, including netrins, semaphorins, neurotrophins, extracellular matrix molecules, and ephrins. In recent years, the Eph family of receptor tyrosine kinase molecules has become the focus of many laboratories investigating axonal guidance. The Eph receptors and their ligands, the ephrins, are a group of membrane bound molecules capable of bidirectional signaling. As such, they are well suited for precise guidance as they require transient
“synapse” formation between the Eph and the ephrin containing cells before downstream signaling mechanisms result in cytoskeletal rearrangement that leads to growth cone attraction or repulsion. Although the role of the ephrin family of guidance molecules has been fairly well described in the visual sensory system, we have only recently begun to understand their roles in the auditory, olfactory, and somatosensory systems. Results from our laboratory have added to our understanding of the role of ephrins and Ephs in thalamocortical connectivity. Furthermore, we provide the first evidence for a role of ephrins and Ephs in axon guidance associated with innervating the peripheral portions of the somatosensory system.

The following chapters will introduce the literature relevant to somatosensory connectivity in the central and peripheral portions of the nervous system, provide recent results from our laboratory that further our understanding of the expression of ephrins and Ephs in the developing somatosensory system, and discuss our findings relative to those from other laboratories in an effort to explain the potential role(s) of ephrins and Ephs in somatosensory connectivity.
CHAPTER 2: LITERATURE REVIEW

The large mystacial vibrissa on the rodent snout are arranged in orderly rows that are consistent between animals. Each vibrissae maintains individual connections to neuron clusters at each level of the neuraxis thus creating several neural representations of the vibrissal pad throughout the brain.

Review of Peripheral Connectivity

Peripheral processes of trigeminal ganglion cells display target-directed growth resulting in precise topographic innervation of the vibrissal pad. The mystacial vibrissae on the rodent snout serve as independent tactile sensors, sending information into the CNS via the infraorbital nerve (ION). The ION is formed from the peripheral processes of the TG neurons as they extend towards the mystacial vibrissal follicles. As early as embryonic day 12 (E12) TG neurons are arranged in rows according to their future position in the dorsal-ventral axis of the WP (Erzurumlu and Jhaveri, 1992; Rhoades et al., 1990). Their peripheral processes have extended out to the maxillary process (Erzurumlu and Killackey, 1983). The ION reaches the caudal edge of the whisker pad by E15 (Gunhan-Agar et al., 2000). Over the next two days, these axons form row nerves, which progress through the whisker pad to reach the rostral whisker follicles. The axon terminals of the ION fibers envelop individual follicles by E17 (Erzurumlu and Jhaveri, 1995; Erzurumlu et al., 1993). A vibrissae-related pattern of the central processes in the brainstem is not apparent until days after the arrival of the central TG processes at the
trigeminal brainstem nuclei (Belford and Killackey, 1979a; b; Erzurumlu and Jhaveri, 1992; Killackey et al., 1990), suggesting a directive role for the TG axons. Results from organotypic co-culture experiments suggest the presence and temporary expression of guidance molecules in the WP and TG that are capable of interacting perhaps through a repulsive mechanism to direct the organization of the TG peripheral processes (Gunhan-Agar et al., 2000).

The trigeminal afferents from the TG split into two main branches in the brainstem, one that ascends to the principal trigeminal nucleus, and one that descends to the spinal trigeminal nuclei. Brainstem neurons from each of these trigeminal nuclei then relay sensory information to the thalamus as reviewed in detail by (Killackey et al., 1990).

*Review of Thalamocortical Connectivity*

Neurons in the ventral posterior medial (VPM) thalamus are born in the rat as early as embryonic day 14 (E14) (McAllister and Das, 1977). These neurons quickly extend their axons (TCAs) out through the internal capsule (IC) and approach the developing cortical plate (CP) of the primary somatosensory area (SI) between E15 (Molnar et al., 1998) and E16 (Catalano et al., 1996; Molnar and Cordery, 1999). By E17, the first cortical layer (VIb) has differentiated and by E18 the TCAs have gathered beneath the subplate (Catalano et al., 1996; Molnar and Cordery, 1999). On E19, layer VIa has differentiated and by E20 the TCAs have invaded layer VIb as well as the ventral
portion of layer VIa. Layer V has differentiated by E21/P0 and TCAs are seen in this newly developed layer (Catalano et al., 1991; 1996; Schlaggar and O'Leary, 1994). Layer IV begins to differentiate from the cortical plate on postnatal day 1 (P1) and a few simple TCA branches are seen in the developing layer IV at this time (Senft and Woolsey, 1991; Shimogori and Grove, 2006). P2 reveals the first clusters of TCAs in layer IV (Erzurumlu and Jhaveri, 1990). By P3, most TCAs have arborized into cylindrical clusters extending the full thickness of layer IV. These cylindrical clusters, which also contain cortical neurons prominently along their walls are known as barrels (Jhaveri et al., 1991; Killackey and Belford, 1979; Rebsam et al., 2002; Rice and Van der Loos, 1977; Shimogori and Grove, 2006). The TCAs continue to expand in length, density, and complexity within individual layer IV barrels to form a dense meshwork by P7, without expansion above or below layer IV (Agmon et al., 1995; Catalano et al., 1996; Rebsam et al., 2002) with the exception that the VPM axon arbors form additional small clusters beneath each barrel, which are confined to layer VIa (Agmon et al., 1993; Catalano et al., 1996; Senft and Woolsey, 1991). Many of these TCAs also send collaterals into layer I (Lu and Lin, 1993; Oda et al., 2004; Rausell and Jones, 1991). From P12-20, the cortical neurons within the barrels increase in number so that by P20, the barrels no longer display a cell-sparse center (Rice, 1985). The TCAs in adult cortex also expand beyond the thickness of layer IV, a process that begins between P18 and P25 (Lorente de No, 1992). The highly coordinated and intricate nature of the developing somatosensory cortex suggests that transient expression of guidance molecules during the first two postnatal weeks may provide critical cues for proper termination of TCAs in layer IV and may temporarily limit axon arbors within layer IV. These data also suggest that the
downregulation of guidance molecules after P18 or P20 (Lorente de No, 1992; Rice, 1985) may be responsible for the subsequent supragranular extension and maturation of the adult rat barrel cortex.

Structure and Function of Ephs and ephrins

The ephrin ligands and their receptors, Ephs, comprise the largest family of receptor tyrosine kinases (RTKs) that are present in the developing nervous system (Tuzi and Gullick, 1994). There are two sub-families of ephrins based on their transmembrane/membrane linked domains. Ephrin-A ligands are GPI-anchored proteins, whereas ephrin-B ligands are transmembrane receptors (Bruckner and Klein, 1998). The binding of ephrin ligands with Eph receptors is largely promiscuous. However, ephrin-A ligands have the highest binding affinities for EphA receptors and ephrin-B ligands primarily bind to EphB receptors with a few exceptions (Himanen et al., 2004; Kullander and Klein, 2002; Murai and Pasquale, 2003). Currently, 10 EphA receptors and 6 ephrin-A ligands have been identified in total although only 9 EphA receptors and 5 ephrin-A ligands have been identified in humans and rodents (Pasquale, 2004).

The Eph receptors are characterized by highly conserved regions on their intracellular and extracellular domains. Their extracellular region includes the N-terminal ligand binding domain, a cysteine rich region, and two fibronectin type III repeats. Extending from the transmembrane region intracellularly are the juxtamembrane region with two tyrosine residues, a kinase domain, a sterile α-motif (necessary for
oligomerization), and PDZ-domain binding motif (necessary for interaction with other proteins) (Kullander and Klein, 2002; Stapleton et al., 1999). Eph receptors and ephrin ligands must be clustered before their interaction can lead to activation. Once an Eph receptor dimer binds to an ephrin oligomer, each receptor’s juxtamembrane tyrosine residues are autophosphorylated resulting in subsequent activation of the kinase domain of the partner receptor of the dimer, which leads to phosphorylation of downstream adaptor proteins. The majority of the adaptor proteins acted on by the activated Eph-ephrin complex function in the disassembly of the actin cytoskeleton triggering growth cone collapse and therefore axon repulsion, reviewed by (Kullander and Klein, 2002; Stapleton et al., 1999).

**Eph Family Involvement in Sensory Connectivity**

The literature concerning the involvement of ephrins and Ephs in axonal guidance within sensory systems, and especially within the somatosensory system is reviewed in Manuscript 4: The Role of ephrins in Thalamocortical Connectivity within the Rat Somatosensory System, Chapter VI.
Developmental expression of ephrin-A2 in Primary Somatosensory Cortex:

A role in restricting the arborization of ventrobasal thalamic axons?

Cynthia L. Kenmuir, Richard D. Mooney*, Nicolas L. Chiaia, and Richard D. Lane

Department of Neurosciences, University of Toledo College of Medicine,

Toledo, Ohio 43609

Number of Text Pages: 24

Number of Tables: 0

Number of Figures: 6

Running Title: Neonatal ephrin-A2 expression in Primary Somatosensory Cortex

Keywords: receptor tyrosine kinase, axon guidance, barrel, development, TCA

*Correspondence to: Dr. Richard D. Mooney, University of Toledo College of Medicine,
3000 Arlington Avenue - MS1007, Toledo, OH 43609.

E-mail: Richard.Mooney@utoledo.edu

Supporting Grants: Research supported by National Institutes of Health (P01-NS149048).
ABSTRACT

Ephrins and Eph receptor proteins play a variety of signaling roles in cortical development, extending from neurogenesis and process outgrowth to cellular migration, axon pathfinding, and topographic innervation of targets. Using a variety of techniques, we report the presence and developmental progression of ephrin-A2 mRNA and protein within the primary somatosensory cortex (SI) during the first two postnatal weeks. Immunohistochemistry revealed ephrin-A2 protein within SI from postnatal day 0 (P0) through P12. Ephrin-A2 protein expression was laminar, with maximal expression observed in the supragranular layers, infragranular layers, and in the subplate. Confocal microscopy was used to further localize the source of this ephrin-A2 protein expression to cortical neurons double-labeled with neuron-specific Hu RNA-binding protein. Additionally, we report that ephrin-A2 mRNA is expressed in the same three cortical layers as the protein and we show that ephrin-A2 mRNA levels, although present in SI throughout the first two postnatal weeks, are maximal at P4 dropping to background levels by P18. These findings are consistent with involvement of ephrin-A2, possibly in conjunction with EphA4, in axon terminal refinement and laminar maintenance during the first two postnatal weeks.
INTRODUCTION

The mammalian neocortex is separated into functionally discrete areas characterized by their topographic patterning as well as the laminar arrangement of their cellular components and connections. The primary somatosensory cortex (SI) is perhaps the best known example of the precision of cortical and subcortical connections that allows for proper interpretation of the peripheral location of an incoming stimulus. SI has a well-described topographic arrangement of cells and axons in the horizontal axis related to the pattern of innervation of the face (Woolsey and Van der Loos, 1970), while maintaining layer-specific connections in the vertical axis (Agmon et al., 1993; Lund and Mustari, 1977), which segregate input and output through highly stereotyped cortical modules. Thalamocortical axons (TCAs) from the ventrobasal (VB) thalamus run through the intermediate zone and then project outward through the cortex largely terminating in layer IV of SI (Agmon et al., 1995; Agmon et al., 1993; Bolz et al., 1992; Catalano et al., 1991; 1996; Molnar et al., 1998; Molnar and Blakemore, 1991; 1995; Yamamoto et al., 1989; Yamamoto et al., 1992). This precision targeting of thalamic input and somatotopic aggregation of postsynaptic cortical neurons most certainly requires instruction by molecular cues during development. Several families of guidance molecules have been implicated in the guidance of these axons from the ventrobasal (VB) thalamus into the cortex. Of those, several members of the Eph family of receptor tyrosine kinases (perhaps best known for their expression in complimentary gradients that drives the topography of the visual system) are expressed in gradients within VB and SI. Although the past few decades have seen a large number of studies investigating the molecules involved in
creating the patterning of the neocortex needed for proper function (Flanagan and Vanderhaeghen, 1998; Kullander and Klein, 2002; O'Leary and Wilkinson, 1999; Pasquale, 2005; van der Geer et al., 1994; Wilkinson, 2001; Zhou, 1998), several issues regarding the role of these molecules in guiding and constraining axon growth and targeting remain unresolved including their involvement in regulating the laminar distribution of TCAs during the first postnatal week of development.

Previous reports by Yamamoto and colleagues have shown that TCAs that normally stop upon reaching their target cells in layer IV no longer terminate in layer IV when treated with phosphatidylinositol phospholipase C (Yamamoto et al., 1997; Yamamoto et al., 2000). These results indicate that the ideal candidate molecule for the cortical stop signal for TCAs would be GPI anchored, located in the supragranular layers of SI, and would be repulsive to the incoming axon. The ephrin-A ligands are GPI-anchored and capable of repulsive interactions via reverse signaling upon interaction with an EphA receptor (Dufour et al., 2003; Hansen et al., 2004; Marquardt et al., 2005; Prakash et al., 2000; Sestan et al., 2001; Vanderhaeghen et al., 2000). One ephrin-A ligand, ephrin-A5, was suggested to fit this profile and has been reported to be expressed in defined lamina within SI (Donoghue and Rakic, 1999; Gao et al., 1998; Mackarehtschian et al., 1999; Mann et al., 2002); however, ephrin-A5 mutants and knockouts do not exhibit laminar disruption of the TCAs, although they do show disruptions in cortical area targeting (Dufour et al., 2003; Uziel et al., 2002) and decreased size of individual barrels as well as decreased size of the entire posterior medial barrel subfield (PMBSF) (Prakash et al., 2000; Vanderhaeghen et al., 2000).
Within the visual system, both ephrin-A5 and ephrin-A2 expression are required for proper retinotectal connectivity with both ligands being expressed in a gradient that peaks in the posterior tectum, while two of their receptors, EphA3 and EphA5, are expressed in a complimentary gradient within the retina (Cheng et al., 1995; Drescher et al., 1995; Feldheim et al., 1998; Wilkinson, 2001).

Our laboratory chose to further investigate the role of the ephrin-A guidance molecules in the postnatal somatosensory cortex when the TCA afferents are terminating and distributing in layer IV of SI. We report here that ephrin-A2 mRNA and protein are expressed by cortical neurons within the supragranular layers of SI throughout the first two postnatal weeks and ephrin-A2 is not expressed within layer IV. These results support a role for ephrin-A2 as one of the repulsive cues needed for the correct termination of the TCAs in layer IV of SI.

MATERIALS AND METHODS

Animals

Sprague Dawley breeder rats were obtained from Taconic Farms (Hudson, New York) and bred at the University of Toledo. Postnatal day 0 (P0) was defined as the day of birth. Rat pups were used for surgical manipulations, as described below, and euthanized at pre-defined ages from P0-P21 according to each experiment. All experiments were conducted in accordance with Institutional Animal Care and Use Committee Guidelines.
**Immunoreactivity**

For all brightfield immunohistochemistry (IHC), rats were euthanized and transcardially perfused with heparinized saline followed by 4% paraformaldehyde. Brains were removed and placed in 4% paraformaldehyde at 4°C overnight. A freezing microtome was used to create 50µm coronal or flattened tangential sections that were then treated with 3% H$_2$O$_2$, blocked with BSA (Sigma), permeabilized with Triton-X (Sigma) and incubated in 1:1000 ephrin-A2 primary antibody (Santa Cruz) overnight. Sections were washed and then incubated in 1:450 biotinylated secondary antibody (Chemicon) for two hours followed by washing and incubation with Vectastain avidin:biotin enzyme complex (Vector Labs) for two hours. After washing, sections were reacted with diaminobenzoate (Fluka), placed onto glass slides, coverslipped, and imaged using a Nikon SMZ-U microscope. For all experiments, olfactory bulb sections were processed in parallel and used as a positive control. Negative controls for each experiment included alternate cortical, thalamic, and olfactory bulb sections processed using either no primary antibody, a host-specific immunoglobulin in place of the primary antibody, or primary antibody adsorbed with the blocking peptide for that protein (Santa Cruz).

For fluorescent immunohistochemistry, tissue was collected and treated the same as described above except that 1:500 AlexaFlur -488 and -555 labeled-secondary antibodies (Invitrogen) were used. For double immunohistochemistry, sections were incubated with ephrin-A2 primary antibody in concert with either 1:40,000 HU primary antibody (gift of Dr. Marthe Howard, University of Toledo) or 1:1000 serotonin
transporter (SRT) primary antibody (Millipore). HU is a neuron-specific RNA binding protein ideal for localization of cortical neurons at all stages of maturation from early post-mitotic neurons exiting the ventricular zone to mature neurons in the cortex (Okano and Darnell, 1997). SRT selectively labels TCAs from VB thalamus and not those from the posterior nucleus or zona incerta (Hansson et al., 1998; Lebrand et al., 1996; Lebrand et al., 1998). Samples were imaged using a Leica SP5 confocal microscope (Leica, Mannheim, Germany) equipped with Argon-488 and diode pumped solid state-561 and 633 laser sources and a 63.0x 1.40 N.A. oil immersion objective. Resulting images were visualized using Leica LAS software and further analyzed using Image J (Rasband, 1997-2004). Colocalization values are reported as $R^2$ correlation coefficients.

**In Situ Hybridization (ISH)**

For ISH experiments, rats were euthanized, transcardially perfused using DEPC treated equipment and solutions. The brains were removed and placed into 4% paraformaldehyde at 4°C overnight and then cryoprotected in 30% sucrose for 48 hours. Brains were sectioned at 20 µm onto glass slides using a Leica CM3050S Cryostat. The sections were then dried, fixed, permeabilized with Proteinase K, and hybridized with digoxigenin-labeled ephrin-A2 antisense at 60°C overnight (plasmids obtained as gift from Dr. David Feldheim, University of California San Diego). Following washing, sections were blocked and incubated overnight in 1:2000 anti-digoxigenin antibody (Roche) and the next day reacted with NBT/BCIP. Slides were placed in fixative, then coverslipped, and imaged. Each antisense probe was preliminarily tested by hybridization
of dot blots including sense strands for ephrin-A2, ephrin-A3, ephrin-A5, EphA4, and EphA7. No cross hybridization to these additional ephrins and Ephs was observed.

Quantitative Real-Time Polymerase Chain Reaction (qRTPCR)

For qRTPCR experiments, the full cortical thickness of the PMBSF of SI cortex was dissected and placed into RNAqueous (Ambion). The remaining cortex was placed into 4% paraformaldehyde for fixation prior to histologic verification of the sampling sites. Histologically confirmed SI tissue blocks containing the full cortical thickness were homogenized, RNA isolated and reverse transcribed, and the resulting cDNAs were quantified on a NanoDrop and then frozen at -20°C. For qRTPCR, each ephrin or Eph and GAPDH (endogenous control) was analyzed simultaneously using the Applied Biosystems 7500fast system. For ephrin-A2 and GAPDH, a commercial primer-probe set was used (Applied Biosystems). Results from the qRTPCR experiments were first normalized to GAPDH levels, and then analyzed by calculating the ΔΔCt values for each ephrin at a particular age relative to P18.

RESULTS

Immunohistochemistry (IHC) was used to detect ephrin-A2 protein expression within SI in coronal and flattened tangential sections from rat brains at P0, 2, 4, 6, 8, 10, 12, and 14 (Figure 1). As early as P0, laminar expression of ephrin-A2 protein was observed in SI, with maximal expression seen in the subplate (SP) and within two discrete bands in the developing cortical plate (CP). As the CP develops into layers,
ephrin-A2 expression remained constant within the SP, and was expressed in two well-defined bands, one supragranular and the other infragranular. Throughout the first two postnatal weeks a relative lack of ephrin-A2 protein expression was observed in layer IV as identified by alternate CO sections (Figure 1E) or Nissl stained sections (not shown). Ephrin-A2 protein expression in the cortex began to decline at P12 and was minimal at P14.

In situ hybridization was used to confirm the presence of ephrin-A2 mRNA in SI (Figure 2). As with IHC results, ephrin-A2 mRNA was observed in the developing CP at P0. By P4, ephrin-A2 mRNA was observed in the SP and two discrete bands in the supragranular and infragranular layers of the developing CP. Taken together, these results show that cells located within discrete lamina within the SP and CP produce and express ephrin-A2 prior to the period of initial TCA connectivity into layers IV and VIa as well as throughout the period of terminal refinement of the VB TCAs in layer IV.

Quantitative RTPCR was used to assess the ephrin-A2 mRNA levels within the PMBSF of SI. For this analysis, full thickness blocks of cortical tissue containing PMBSF were collected from the cortices of P2, 4, 6, 8, and 18 pups. The remaining portions of the cortices were then reacted for CO to confirm the presence of PMBSF in the tissue blocks used for analysis. Interestingly, the ephrin-A2 mRNA reached maximal levels early in the first postnatal week, followed by a rapid decrease with levels dropping 10-20 fold lower by P18 (Figure 3).
The cellular localization of the ephrin-A2 protein expression was examined within SI over the first and second postnatal weeks. Confocal microscopy was used on sections from P8 cortices double-labeled for ephrin-A2 protein (green) and the serotonin transporter, a marker of TCAs (red) (Figure 4). The resulting images were analyzed for colocalization using Image J, which revealed that ephrin-A2 protein is not expressed on TCAs in SI ($R^2 = 0.2 – 0.3$). Employing the same strategy, we double-labeled ephrin-A2 (green) and HU RNA binding protein, a neuron-specific cell marker (red) (Figure 5). Ephrin-A2 and HU were significantly colocalized ($R^2 = 0.85 – 0.88$). These results indicate that ephrin-A2 protein seen in supragranular and infragranular SI cortex throughout the first two postnatal weeks is expressed by cortical neurons and not by TCAs.

**DISCUSSION**

The present study provides evidence that ephrin-A2, a GPI-anchored guidance molecule, is expressed by cortical neurons throughout the first two postnatal weeks during the time when VB axons arborize in layer IV of SI. Immunohistochemical localization further demonstrated that the ephrin-A2 protein is selectively expressed in the supragranular and infragranular layers as well as in the subplate.ISH at P4 showed that ephrin-A2 mRNA is also expressed in these three layers, indicating that the cortical cells in layers II/III and V as well as in the subplate are the source of the ephrin-A2 protein expression observed throughout the first two postnatal weeks. Using qRTPCR,
we have confirmed ISH results and quantitatively shown that the expression of ephrin-A2 mRNA in the PMBSF is maximal during the first four postnatal days and then sharply declines, decreasing by as much as 20-fold by P18. Thus, the limited temporal expression of ephrin-A2 suggests that it has a transient role in cortical development during this postnatal period. Confocal microscopy co-localization demonstrated that the source of ephrin-A2 expression observed in the cortex is the soma of cortical neurons and not VB axons or their terminals.

Molnar and Blakemore (1991) have previously suggested that a repulsive signal within the cortex could be necessary for confining TCA termination to layer IV during cortical development. Additional experiments using in vitro models of thalamocortical VB-SI connectivity provided further support. Co-culture experiments utilizing positionally variant thalamic and cortical explants have shown that TCAs approaching SI from either the pial or ventricular surface terminate in layer IV, but TCAs approaching SI from a trajectory parallel to the cortical layers do not terminate properly (Bolz et al., 1992; Yamamoto et al., 1997; Yamamoto et al., 1992). The ephrin-A family of guidance molecules were viewed as ideal candidates for this stop signal on the strength of a report from Yamamoto and colleagues who showed TCA overgrowth into supragranular cortical layers following phosphatidylinositol phospholipase C treatment, which disrupts GPI-anchored proteins (Yamamoto, 2002). Previous studies of ephrin-A involvement in VB-SI connectivity have focused on ephrin-A5. Although prenatal ephrin-A5 mRNA expression in SI cortex has been well described (Castellani et al., 1998; Dufour et al., 2006; Dufour et al., 2003; Gao et al., 1998; Mackarechtschian et al., 1999; Mann et al.,
2002; Vanderhaeghen et al., 2000; Yabuta et al., 2000), ephrin-A5 expression in SI during the postnatal period has received less attention. However, ephrin-A5 mRNA has been reported postnatally as a weak medial-lateral gradient in barrels within layer IV at P3 (Dufour et al., 2003; Vanderhaeghen et al., 2000) and within not graded within layer IV at P6 and P8 (Bolz et al., 2004; Castellani et al., 1998). The spatially graded expression of ephrin-A5 mRNA in layer IV with its abrupt edge at the boundary of somatosensory and limbic cortices (Gao et al., 1998) suggest that it may be a suitable candidate for interareal specificity and may play a role maintaining the topography of the VB axons within SI. However, unlike ephrin-A2, ephrin-A5 is not expressed in supragranular and infragranular cortical layers (Yabuta et al., 2000). Ephrin-A5’s temporal expression pattern and laminar distribution are also not well suited to constrain the termination of VB axons in layer IV on P0-P3. Consistent with this hypothesis, results from ephrin-A5 mutants show abnormalities in the areal size and shape of the PMBSF as well as in the barrel spacing (Dufour et al., 2003; Prakash et al., 2000; Uziel et al., 2002; Vanderhaeghen et al., 2000), but no deficits in laminar termination (Yabuta et al., 2000). A more recent investigation by Maruyama and colleagues suggests that ephrin-A5 may work cooperatively with semaphorin-7A and kit ligand for proper termination of TCAs in layer IV in addition to its role in interareal topography (Maruyama et al., 2008). Taken together, the results from these studies indicate that ephrin-A5 may be involved in, but is not sufficient for VB axon termination in SI. Our results suggest that supragranular ephrin-A2 expression may provide this additional, and potentially necessary, signal to constrain the laminar distribution of TCAs.
Although VB axons terminate in the newly formed layer IV between the first and third postnatal day (Catalano et al., 1996), we have detected ephrin-A2 protein in supragranular and infragranular layers into the second postnatal week. One possible explanation for this lengthy expression is that cortical ephrin-A2 may serve a role as a layer IV boundary molecule throughout the first two weeks of life. During the two weeks after the TCAs have made their synapses in layer IV, they continue to arborize and completely fill the laminar extent of layer IV. Then, around P20, the layer IV barrels continue to mature in the rat, eventually extending into the supragranular layers (Rice, 1985). This timing is consistent with the suggested role of cortical ephrin-A2 as a layer IV boundary marker. We’ve report detection of ephrin-A2 mRNA and protein within supragranular and infragranular SI layers as late as P18, which could be responsible for constraining the TCAs in layer IV. We were unable to detect ephrin-A2 mRNA (via qRTPCR) or protein (via IHC) in SI after P18, which could mean the elimination of the layer IV boundary molecule and may therefore explain the subsequent extension of TCA arbors through supragranular layers that begins around P20. Although it is also possible that cortical ephrin-A2 during the postnatal period serves additional roles, including the repulsion of invading axons from other cortical areas or layers, this possibility seems less likely due to its distinct pattern of expression at the boundaries of TCA arbors.

Surprisingly, we also detected consistent ephrin-A2 expression within the subplate. This may indicate that cortical ephrin-A2 has an additional role in the initial sensitization of VB axons rendering them more responsive to cortical guidance cues in the upper layers (Shimogori and Grove, 2006). It has previously been shown that TCAs
must interact with the SP in order to recognize their target (Ghosh et al., 1990; Ghosh and Shatz, 1993; O'Leary et al., 1994). It is possible that ephrin-A2 expressed in the SP may be one molecule that serves this role.

Several EphA receptor mRNAs (EphA3, EphA4, and EphA7) capable of binding ephrin-A2 (Gale et al., 1996) are expressed in VB during the late embryonic and early postnatal period (Bolz et al., 2004; Catalano et al., 1996; Dufour et al., 2003; Garel and Rubenstein, 2004; Mackarehtschian et al., 1999; Takemoto et al., 2002; Vanderhaeghen et al., 2000) when VB axons are migrating to the cortex, making connections in layer IV, and then further refining those connections (Rice, 1985). EphA3 and EphA4 mRNA have each been reported in VB from E17 to P1 (Mackarehtschian et al., 1999). At these early ages, EphA4 mRNA (Vanderhaeghen et al., 2000) and EphA7 mRNA (Sestan et al., 2001) are expressed in VB in a high medial to low lateral gradient that is complimentary to that of ephrin-A5 seen in the cortex. The present study provides data that support the proposal that later in the postnatal period from P0-P6 EphA4 mRNA and protein are no longer expressed in a gradient in VB, but instead are expressed in barreloid patches (Kenmuir et al., In review.). We suggest that cortical ephrin-A2 interacting with EphA receptors on TCA terminals may serve a distinct role in constraining the laminar termination of TCAs within SI. Experiments to test this hypothesis are currently underway.

In 1996, Catalano and colleagues described the connectivity of TCAs into layer IV of SI and reported that the TCAs “remain spatially restricted rather than by overbranching and retracting arbors.” We propose a model of ephrin-A2 involvement in
laminar termination within SI whereby the transient interaction of VB axons with cortical ephrin-A5 in layer IV encourages branch formation and elongation as previously suggested (Mann et al., 2002), but as those fibers elongate, they are restricted at the upper and lower borders of layer IV by ephrin-A2 (see Figure 6 A and B). Furthermore, we suggest that the substantial reduction in cortical ephrin-A2 and VB EphA expression that occurs by P18 (Figure 6 C), relieves the resistance and permits the TCAs to expand into layers II/III (Rice, 1985). Future studies will focus on testing these hypothetical functions of ephrin-A2 in the postnatal SI.
LITERATURE CITED


Rasband W. 1997-2004. ImageJ, National Institutes of Health, Bethesda, Maryland, USA.


FIGURE LEGENDS

Fig. 1. Neonatal ephrin-A2 protein expression in primary somatosensory cortex (SI).
Ephrin-A2 immunoreactivity was observed in SI cortex as early as postnatal day 0 (P0) when it can be seen throughout several layers (white arrows) of the developing CP (A). Over the first postnatal week as the cortex continues to separate into discrete layers, ephrin-A2 immunoreactivity shows maximal cellular expression in supragranular layers II/III and infragranular layer V and the subplate [white brackets in F] (B-D and F-H). An alternate section (E) reacted for cytochrome oxidase (CO), demonstrates the layer IV barrels [white brackets in E] of the PMBSF as well as the layer VIa arbors (white arrowheads). Scale bar represents 1mm.

Fig. 2. Neonatal ephrin-A2 mRNA expression within SI. ISH reveals expression of ephrin-A2 mRNA in discrete layers (white arrows) within the developing SI CP at P0 (A). At P4, ephrin-A2 mRNA is not expressed by cells of layer IV but is expressed in both supragranular layers II/II and in infragranular layer V and the subplate (B). Scale bar represents 1mm.

Fig. 3. Quantitative Real Time PCR (qRTPCR) of ephrin-A2 mRNA expression in PMBSF. The expression of ephrin-A2 mRNAs in PMBSF containing samples was maximum at P4 and declined sharply by P6 (A). Results are shown as fold changes relative to P18 (the oldest age sampled when neither protein is expressed at observable
levels). Error bars denote standard deviation of the ΔΔCt values. Panel B is a section through one of the P8 cortices reacted for cytochrome oxidase to confirm the presence of PMBSF in the samples. The contralateral hemisphere is also shown to demonstrate an undisturbed PMBSF (outlined by white box) within the corresponding region (C). Scale bar represents 1mm.

Fig. 4. Confocal microscopy images of sections through the layer IV/V border double-labelled with IHC for ephrin-A2 and the serotonin transporter (SRT). High power merged images from P8 show ephrin-A2 protein (green) expression in SI is not colocalized ($R^2 = 0.2 - 0.3$, panels A-F) with SRT (red), a specific marker of VB TCAs. Shown is a series of z-stack images collected at 1 µm increments from the top (A) through the bottom (F) of an ephrin-A2 immunoreactive cell that reveals branches of a TCA surrounding the ephrin-A2 positive cell. Scale bar represents 5 µm.

Fig. 5. Confocal microscopy was used to colocalize ephrin-A2 protein expression to neurons in the PMBSF. Cells from layer V in PMBSF of P8 cortices were imaged following double immunolabelling for ephrin-A2 protein (green) and HU RNA binding protein (red), a neuron-specific cell marker. Low and high power images from P8 cortices show ephrin-A2 protein expression (A, D) and HU protein expression (B, E) was colocalized ($R^2 = 0.85$ C, $R^2 = 0.88$ F) on layer V pyramidal cells (C, F merged images). Scale bar represents 10 µm (A-C) and 5 µm (D-F).
Fig. 6. Model depicting developmental expression of cortical ephrin-A2 and its potential role in restricting TCA arbors to layer IV and VIa. Several EphA receptors (EphA3, EphA4, EphA7) capable of binding ephrin-A2 are expressed in VB thalamus during the late embryonic period (A) (see Discussion for details). Their expression is graded from medial to lateral from E15 until birth. During the same developmental period, several Eph receptors and ephrin-A5 ligand are expressed within the developing SI cortex, which has not yet differentiated into layers. Ephrin-A5 expression during this period is graded complimentary to that of the Eph receptors in VB (A). Throughout the first two postnatal weeks, ephrin-A2 is expressed in three discrete laminae: the subplate (SP), layer V, and layers II/III. Maximal expression was identified in layer II at the layer I/II boundary and in SP. There have been no reports of EphA3 expression in VB after E19, and EphA7 is only expressed weakly in the most superior, lateral tip of VPM at P4. However, EphA4 is expressed from P2-P8 in VPM in barreloid patches and is expressed within the TC axons and terminals within layer IV barrels from P6-P10 (Kenmuir, et al, in review) (B). TCAs invade the developing cortex, migrate into layer IV, and elaborate dense arbors that fill the breadth of layer IV during the first postnatal week, but do not extend into layer V or II/III until after P18 when ephrin-A2 is no longer present in SI and EphA4 is no longer present in TCAs from VB. Taken together, these results suggest that cortical ephrin-A2, possibly interacting with EphA4 on TCAs, is involved in restricting layer IV TCA arbors until P18, which may delay their branching into layers II/III (C). Dashed lines represent the borders of SI. Primary motor cortex is labeled as M1.
Figure 1

A  ephrin-A2  P0

B  ephrin-A2  P2

C  ephrin-A2  P4

D  ephrin-A2  P6

E  CO  P8

F  ephrin-A2  P8

G  ephrin-A2  P10

H  ephrin-A2  P12
Postnatal expression of ephrin-A3 and EphA4:
A role in thalamocortical connections?

Cynthia L. Kenmuir, Richard D. Mooney*, Nicolas L. Chiaia, and Richard D. Lane
Department of Neurosciences, University of Toledo College of Medicine,
Toledo, Ohio 43609

Number of Text Pages: 28
Number of Tables: 0
Number of Figures: 8
Running Title: Neonatal EphA4 and ephrin-A3 expression in Primary Somatosensory Cortex
Keywords: receptor tyrosine kinase, axon guidance, barrel, development, TCA

*Correspondence to: Dr. Richard D. Mooney, University of Toledo College of Medicine,
3000 Arlington Avenue - MS1007, Toledo, OH 43609.
E-mail: Richard.Mooney@utoledo.edu
Supporting Grants: Research supported by National Institutes of Health (P01-NS149048).
ABSTRACT

During early stages of somatosensory system development, ephrins have a recognized role in guidance and topographic ordering of thalamocortical axons (TCAs) from ventrobasal thalamus (VB) to primary somatosensory cortex (SI). However, well after target innervation and terminal patterning have occurred, constitutive regulation of Eph and ephrin expression continues and may function in pattern maintenance or laminar segregation during postnatal development. In the current study, immunohistochemistry of cortical sections revealed expression of both ephrin-A3 and EphA4 in a barrel-like manner within layer IV of the postnatal SI cortex of the rat. Similarly, both ephrin-A3 and EphA4 are concurrently expressed in VB in a barreloid spatial pattern. However, in-situ hybridization of ephrinA-3 and EphA4 indicated that the mRNA for these proteins was present in supragranular, infragranular and subplate regions but not in layer IV. Robust ISH staining for both messages was seen in the barreloids of the VB. The expression of ephrin-A3 and EphA4 in SI barrels was dependent on the integrity of subcortical somatosensory pathways as VB lesions disrupted or abolished patterned ephrin-A3 and EphA4 immunoreactivity in SI, indicating that the TCAs of VB neurons are the source of these guidance molecules. This view is further supported by confocal microscopic results showing that ephrin-A3 and EphA4 proteins in SI co-localize with a known marker for VB TCAs, the selective serotonin transporter and do not co-localize with a neuronal soma marker, Hu RNA binding protein. Additionally, we describe distinct temporal and spatial expression of ephrin-A3 and EphA4 within SI and VB. Taken together, these data better characterize the precise location and timing of postnatal
EphA4 expression and reveal that a high affinity ligand, ephrin-A3 is co-expressed with this receptor on TCAs during postnatal development. The possible role(s) and relationships of EphA4 and ephrin-A3 to other cortical Ephs and ephrins in the postnatal maturation of the cortex are discussed.
INTRODUCTION

The guidance molecules involved in the development of the thalamocortical (TC) circuit from the ventrobasal thalamus (VB) to the primary somatosensory cortex (SI) have been the subject of many investigations and reviews over the past twenty years. Although several classes of molecules are involved in the guidance of thalamocortical axons (TCAs) to the cortex, a family of membrane bound receptor tyrosine kinases and their ligands, the Ephs and ephrins, have been a major focus of investigations into the topographic and laminar connectivity of these TCAs in SI.

The EphA4 receptor, which is capable of interacting with all of the ephrin-A ligands as well as with ephrin-B2 and ephrin-B3, reviewed by (Drescher et al., 1995; Pasquale, 2004), has been identified in the developing cortical plate (CP) in the region of the future SI as early as embryonic day 12 (E12) when its expression is diffuse within the CP (Yun et al., 2003). By E13, EphA4 mRNA is detectable in a gradient across the CP, highest in the rostromedial portions and lowest in the caudolateral portions (Dufour et al., 2003; Liebl et al., 2003; Vanderhaeghen et al., 2000). This gradient is maintained through E21 (Vanderhaeghen et al., 2000). During the early postnatal period, EphA4 mRNA shows laminar specificity, being expressed in two distinct bands in supragranular and infragranular SI on P0.5 (Liebl et al., 2003) and P1 (Vanderhaeghen et al., 2000). The latest that EphA4 mRNA has previously been detected in SI is on P3 where it is weakly expressed in layer IV in segments that appear to correspond to the SI barrels (Vanderhaeghen et al., 2000).
During embryonic development, EphA4 mRNA is also expressed in VB in a medial to lateral gradient. The gradient appears as early as E16.5 and lasts until P1 (Dufour et al., 2003; Garel and Rubenstein, 2004; Liebl et al., 2003; Mackarehtschian et al., 1999; Vanderhaeghen et al., 2000). EphA4 mRNA expression in VB, although no longer arrayed in an observable gradient, has been reported as late as P3 (Vanderhaeghen et al., 2000). Other EphA receptors have also been identified in VB during embryogenesis, notably EphA3 and EphA7, although their duration of mRNA expression seems to be briefer, lasting from E16.5 to E19 (Bolz et al., 2004; Dufour et al., 2003; Garel and Rubenstein, 2004; Mackarehtschian et al., 1999; Takemoto et al., 2002; Vanderhaeghen et al., 2000) though one study reported EphA7 to be present in the most superior-lateral portion of VB at P4 (Torii and Levitt, 2005). The transient and early expression of EphA3 and EphA7 suggests their potential role in guidance of the TCAs from their egress from VB, through the internal capsule, through the intermediate zone, and then towards the developing CP; whereas, the more protracted expression of EphA4 additionally makes it a suitable candidate molecule for involvement in TCA growth within SI in regards to laminar specificity, axon arborization, and/or topography.

Ephrin-A5, a ligand with high binding affinity for the EphA4 receptor, is expressed in a medial to lateral gradient across the developing CP of SI from E16-18, when the TCAs are approaching the CP (Bolz et al., 2004; Castellani et al., 1998; Gao et al., 1998). Although ephrin-A5 mRNA is still expressed from P0-P4 when the TCAs terminate and begin to arborize in layer IV, its graded expression is extremely weak (Dufour et al., 2003; Torii and Levitt, 2005; Yun et al., 2003) lasting as late as P8 when it
becomes segmented within layer IV (Bolz et al., 2004). Results from single and double knockout experiments have suggested that this cortical ephrin-A5 expression promotes branch formation of TCAs in layer IV (Mann et al., 2002) and contributes to both inter- and intra-areal topography (Miller et al., 2006; Prakash et al., 2000; Uziel et al., 2002; Vanderhaeghen et al., 2000). We have recently reported that an additional ligand, ephrin-A2 is expressed by supragranular and infragranular cortical neurons during the first two postnatal weeks and may serve a role in preventing TCAs from spreading beyond the breadth of layer IV during this period (Kenmuir et al., 2008; Kenmuir et al., In review.).

Collectively, these studies suggest that EphA4 expressed by cortical projecting VB neurons during the late embryonic to early postnatal period may serve multiple, distinct physiological roles by interacting with several ephrins each with distinct cortical distributions. Hence, we chose to further investigate the expression of EphA4 throughout the first two weeks of postnatal development, a time when TCA connectivity continues development and refinement. During this postnatal period the TCAs terminate in layer IV, arborize in layer IV and VIa, and send collaterals into layer I. Furthermore, since ephrin-A2 and A5 were the only previously known ephrin-A ligands expressed in SI during the postnatal period, we investigated the possible postnatal expression of ephrin-A3, another high affinity ligand for the EphA4 receptor. This study provides the first report of the postnatal developmental timecourse of the protein and mRNA expression of ephrin-A3 in the VB and SI. The significance of the locations, temporal expression patterns, and possible interactions of ephrin-A3 and EphA4 during postnatal development of the TC circuit are discussed.
MATERIALS AND METHODS

Animals

Sprague Dawley breeder rats were obtained from Taconic Farms (Hudson, New York) and bred at the University of Toledo. Postnatal day 0 (P0) was defined as the day of birth. Rat pups were used for surgical manipulations, as described below, and sacrificed at pre-defined ages from P0-P21 according to each experiment. All experiments were conducted in accordance with Institutional Animal Care and Use Committee Guidelines.

Immunoreactivity

For all brightfield immunohistochemistry, rats were euthanized and transcardially perfused with heparinized saline followed by 4% paraformaldehyde. Brains were removed and placed in 4% paraformaldehyde at 4°C overnight. A freezing microtome was used to create 50µm coronal or flattened tangential sections. All flattened tangential sections were processed from the cortical surface through underlying white matter, and representative sections through layer IV are shown. Sections were then treated with 3% H$_2$O$_2$, blocked with BSA (Sigma), permeabilized with Triton-X (Sigma) and incubated in 1:1000 ephrin-A3 or EphA4 primary antibody (Santa Cruz) overnight. Sections were washed and then incubated in 1:450 biotinylated secondary antibody (Chemicon) for two
hours followed by washing and incubation with Vectastain avidin:biotin enzyme complex (Vector Labs) for two hours. After washing, sections were reacted with diaminobenzoate (Fluka), placed onto glass slides, coverslipped, and imaged using a Nikon SMZ-U microscope. For all experiments, olfactory bulb sections were processed in parallel and used as a positive control. Negative controls for each experiment included alternate cortical, thalamic, and olfactory bulb sections processed using either no primary antibody, a host-specific immunoglobulin in place of the primary antibody, or primary antibody adsorbed with the blocking peptide for that protein (Santa Cruz).

For fluorescent immunohistochemistry (IHC), tissue was collected and treated the same as described above except that 1:500 AlexaFlur-488 and -555 labeled-secondary antibodies (Invitrogen) were used. For double immunohistochemistry, sections were incubated with ephrin-A3 or EphA4 primary antibody in concert with either 1:40,000 HU primary antibody (gift of Dr. Marthe Howard, University of Toledo) or 1:1000 serotonin transporter (SRT) primary antibody (Millipore). HU is a neuron-specific RNA binding protein ideal for localization of cortical neurons at all stages of maturation from early post-mitotic neurons exiting the ventricular zone to mature neurons in the cortex (Okano and Darnell, 1997). SRT selectively labels axons from VB and not those from the posterior nucleus or zona incerta (Hansson et al., 1998; Lebrand et al., 1996; Lebrand et al., 1998). Samples were imaged using a Leica SP5 Confocal microscope (Leica, Mannheim, Germany) equipped with Argon-488 and diode pumped solid state-561 and 633 laser sources and a 63.0x 1.40 N.A. oil immersion objective. Resulting images were
visualized using Leica LAS software and further analyzed using Image J (Rasband, 1997-2004).

For Western blots of PMBSF tissue homogenates, rats brains were removed, the cortices embedded in 4% agarose and flattened between glass slides on dry ice. Once the agarose had solidified, each cortex was sectioned at 75 µm using a vibratome and the sections were placed into ice cold PBS. Free floating sections were illuminated with back lighting and imaged on a Nikon SMZ-U microscope in order to select the section with the PMBSF visible within layer IV. A glass tube with a 2 mm internal diameter was used to selectively remove the PMBSF which was flash frozen using liquid nitrogen. PMBSF samples from 4 litters of P8 rat pups were pooled for each Western blot. Frozen tissue was then homogenized using a glass pestle and following centrifugation the proteins were extracted with SDS-sample buffer at 95-100°C for 5 minutes. Samples were loaded onto precast 10% Tris-HCl gels (BioRad). Following gel electrophoresis, the gel was transferred to nitrocellulose membrane, which was blocked using 2% Carnation milk, incubated overnight in 1:1000 primary antibody, washed, incubated for 1 hour in alkaline phosphatase conjugated secondary antibody (Zymed), washed, and then reacted with an alkaline phosphatase substrate solution containing NBT, BCIP and MgCl₂. Once again, negative control blots were processed in parallel with the exception that the primary antibody was excluded, replaced with normal serum immunoglobins, or adsorbed with the appropriate blocking peptide.
In Situ Hybridization (ISH)

For ISH experiments, rats were sacrificed, transcardially perfused using DEPC treated equipment and solutions. The brains were removed and placed into 4% paraformaldehyde at 4°C overnight and then cryoprotected in 30% sucrose for 48 hours. Brains were sectioned at 20 µm onto glass slides using a Leica CM3050S Cryostat. The sections were then dried, fixed, permeabilized with Proteinase K, and hybridized with digoxigenin-labeled ephrin-A3 or EphA4 antisense at 60°C overnight (plasmids obtained as gift from Dr. David Feldheim, University of California San Diego). Following washing, sections were blocked and incubated overnight in 1:2000 anti-digoxigenin antibody (Roche) and the next day reacted with NBT/BCIP. Slides were placed in fixative, then coverslipped, and imaged. Each antisense probe was preliminarily tested by hybridization of dot blots including sense strands for ephrin-A2, ephrin-A3, ephrin-A5, EphA4, and EphA7. No cross hybridization to these additional ephrins and Ephs was observed.

Quantitative Real-Time Polymerase Chain Reaction (qRTPCR)

For qRTPCR experiments, the full cortical thickness of the PMBSF of SI cortex was dissected and placed into RNAqueous (Ambion). The remaining cortex was placed into 4% paraformaldehyde for fixation prior to histologic verification of the sampling sites. Histologically confirmed SI tissue blocks were homogenized, RNA isolated and reverse transcribed, and the resulting cDNAs were quantified on a NanoDrop and then frozen at -20°C. For qRTPCR, each ephrin or Eph and GAPDH (endogenous control)
was analyzed simultaneously using the Applied Biosystems 7500 fast system. For ephrin-A3 and GAPDH, a commercial primer-probe set was used (Applied Biosystems). For EphA4, primers were designed using Accelrys DS Gene software and candidates were tested on rat olfactory bulb samples. An EphA4 primer probe set was then designed using Primer Express software (Applied Biosystems). Results from the qRTPCR experiments were first normalized to GAPDH levels, and then analyzed by calculating the ΔΔCt values for each ephrin at a particular age relative to P18.

**Thalamic lesions**

On P4, pups were individually anesthetized by Isoflurane inhalation. The skull was then exposed using a midline cranial incision. A small (.3mm x .3mm) flap of bone overlying the ventral posterior medial (VPM) thalamus was then reflected and removed. Stereotaxic measurements relative to Bregma were used to locate VPM (3mm posterior, 3mm lateral, 3.5mm deep). Two techniques were used to lesion the VPM, electrolytic or chemical. For electrolytic lesions, a tungsten probe was inserted into the VPM and cauterized for 10-30 seconds. For chemical lesions, a glass microelectrode was loaded with 6.5μg/μl Ibotenic Acid and a micromanipulator was used to deliver 1.6 μg over 30 seconds. Finally, the incision site was cleaned and sealed using Vetbond surgical adhesive. Pups were allowed to recovery and were returned with their litters until sacrifice on P8. On P8, pups were sacrificed, transcardially perfused, midbrain sectioned and stained for Nissl substance to determine the extent of the lesion and cortices processed for ephrin-A3 or EphA4 immunohistochemistry.
RESULTS

Immunohistochemistry (IHC) was used to detect ephrin-A3 protein expression within SI in coronal and flattened tangential sections and VB coronal sections from rat brains at P0, 2, 4, 6, 8, 10, 12, 14, and 21 (Figure 1). From P0 - P4 ephrin-A3 protein expression was not observable above background levels in SI (Figure 1A). At P6, ephrin-A3 was observed in some layer IV barrel clusters related to the large mystacial vibrissae as well as in the clusters representing the rostral sinus hairs (Figure 1B). Ephrin-A3 protein expression was maximal from P8 to P12 where it was observed in all barrels of the PMBSF as well as in other body representations including forelimb and hindlimb (Figure 1C-F). By P14, ephrin-A3 protein expression was reduced in PMBSF to levels similar to surrounding cortical regions, but was still elevated in the rostral sinus hair clusters (Figure 1G). At P21 ephrin-A3 protein was no longer detectable as levels in SI had dropped below that of the surrounding cortical regions (Figure 1H). Throughout the first postnatal week ephrin-A3 protein was observed in VB in barreloids within both the medial and lateral subcompartments from P4-P8 and there was no apparent expression within the IC (Figure 1I).

IHC was also used to detect the EphA4 receptor in SI and VB (Figure 2). Similar to ephrin-A3 expression, no EphA4 was observed in SI from P0 - P4 and at P6 expression was observed in the rostral sinus hair clusters with only some of the PMBSF barrels being immunoreactive (Figure 2A-B). Unlike ephrin-A3 expression, EphA4 immunoreactivity appeared maximal in PMBSF barrels and labeled layer VIa aggregates on P8 (Figure 2D). On P10, EphA4 was present in a few barrels and in the rostral sinus
hairs (Figure 1E). By P14, no EphA4 was present in SI barrels and its expression in the barrels had fallen below that of the surrounding tissue. IHC of VB revealed maximal EphA4 reactivity in barreloids of the medial subcompartment on P2 that was reduced by P6 and not detectable by P10. We also observed robust EphA4 immunoreactivity in the IC from P2-10 (Figure 2G-I).

Western blot of layer IV PMBSF samples from P8 pups revealed a discrete band of approximately 32 kDa that was immunoreactive for ephrin-A3. This size is consistent with that previously reported for ephrin-A3 (Xu et al., 2003). The ephrin-A3 immunoreactive band was blocked by adsorbing the antibody with ephrin-A3 peptide (Figure 3A; optical density values normalized to background showed an 88% reduction in mean OD with adsorption). Western blot of the same tissue samples revealed several large bands ranging from 120 - 70 kDa in size that were immunoreactive for EphA4. The ≈120 kDa band is consistent with the intact EphA4 protein observed by others on western blots (Martone et al., 1997; Takemoto et al., 2002) and the two smaller bands likely correspond to partially degraded EphA4 and are consistent with those observed in inner ear extracts (van Heumen et al., 2000). These three EphA4 immunoreactive bands were blocked by antibody adsorption with the EphA4 peptide (Figure 3B; optical density values normalized to background in order of decreasing size showed a 98%, 92%, 99% reduction in mean OD with adsorption, respectively).

Results from ISH on tissue sections from P0 - P8 brains show ephrin-A3 and EphA4 mRNA expression within the developing cortex. At P4, an age where the cortical plate continues to segregate into discrete layers and layer IV has fully differentiated, both
mRNAs were observed in supra- and infra-granular bands as well as the subplate, but not within layer IV (Figure 4 A, C). ISH revealed ephrin-A3 and EphA4 mRNA in VB from P0 - P8. Both ephrin-A3 and EphA4 mRNA were detected in a barreloid distribution within VB at P4 (Figure 4 B, D).

Due to relatively weak signal strength in SI in our ISH experiments, we used qRTPCR to confirm the endogenous expression of ephrin-A3 and EphA4 mRNA in PMBSF from full thickness cortical samples taken at P0, 2, 4, 6, 8, and 18. Evidence from our ISH and IHC experiments suggest that levels of ephrin-A3 and EphA4 in PMBSF fall to or below background between P14-P18; therefore, data was normalized to P18 levels. During the first two postnatal weeks, both ephrin-A3 and EphA4 mRNA were maximal in PMBSF at P4 and each was reduced by more than 30 fold by P18 (Figure 5 A, B).

Using IHC, we have demonstrated the presence of ephrin-A3 and EphA4 protein within layer IV barrels during the postnatal period. However, ISH indicated the presence of mRNA for both ephrin-A3 and EphA4 in SI was above and below, but not within layer IV. In order to investigate the source of the cortical ephrin-A3 and EphA4 protein, we utilized double immunofluorescence and confocal microscopy. We observed that ephrin-A3 and EphA4 protein both colocalize with the serotonin transporter (SRT), which specifically labels TCAs from VB ($R^2 = .90$, $R^2 = .86$, respectively) and not with HU, which specifically labels the somas of post-mitotic neurons regardless of developmental stage (Figure 6). These results suggest that the specific pattern of layer IV barrel immunoreactivity of ephrin-A3 and EphA4 is due to labeling of the protein on TCAs.
distributed in the barrels and not on the cortical layer IV neurons, which also cluster in a barrel pattern.

To confirm that VB is the source of ephrin-A3 and EphA4 protein observed in layer IV, we conducted a series of lesion experiments where VB was partially or completely ablated using electrolytic or chemical methods on P4 or P6 and then ephrin-A3 and EphA4 immunoreactivity in the cortex was assessed two or four days later. Complete ablation of VB at P4 resulted in elimination of ephrin-A3 and EphA4 immunoreactivity in layer IV of SI on P8 (Figure 7A and A’, Figure 8A and A’, respectively) as compared to the contralateral hemisphere (inset of Figure 7A’ and 8A’). Precise lesions limited to VB alone resulted in selective regional elimination of ephrin-A3 and EphA4 immunoreactivity in layer IV of SI (Figure 7B and B’, Figure 8B and B’, respectively) as compared to the contralateral hemisphere (inset of Figure 7B’ and 8B’). Lesions sparing VB were used as procedural controls and showed normal ephrin-A3 and EphA4 immunoreactivity in layer IV barrels (Figure 7C and C’, Figure 8C and C’, respectively). Additionally, VB lesions created on P4 using ibotenic acid injections showed similar results at P8: ephrin-A3 and EphA4 immunoreactivity in layer IV of SI was eliminated (not shown).

**DISCUSSION**

The present study expands our understanding of ephrin/Eph involvement in the development of TCA connectivity by defining the expression of ephrin-A3 and EphA4 in
thalamus and cortex during the first two postnatal weeks. Both proteins are expressed by TCAs terminating in layer IV and not by cortical neurons. Furthermore, using thalamic lesions, we were able to verify that VB is the source of the ephrin-A3 and EphA4 protein expression in SI. Finally, we have defined the timecourse of expression of both ephrin-A3 and EphA4 in the thalamus and cortex during the first two postnatal weeks. Ephrin-A3 protein was detectable in barreloid clusters across the medial and lateral subcompartments of VB during the first postnatal week. EphA4 expression in VB progressively decreased across the first postnatal week and was localized to the barreloids of VPM. Unlike ephrin-A3, EphA4 protein was prominently detected in the IC even after levels in VB were reduced to background. Ephrin-A3 protein was detectable in layer IV of PMBSF from P6 to P14 although uniform expression throughout all barrels was only seen from P8-P12. EphA4 protein expression was also detectable in layer IV, but with a shorter timecourse peaking at P8 and dropping below background cortical levels by P14. Unlike ephrin-A3, EphA4 protein was also detected in small aggregates in layer VIa underlying each layer IV barrel. These aggregates correspond to additional TCA arbors observed in layer VIa (Catalano et al., 1996).

Results from our ISH experiments demonstrated a lack of ephrin-A3 and EphA4 mRNA in layer IV of SI, which supports the hypothesis that ephrin-A3 and EphA4 protein expression in layer IV is derived from TCA axon terminals. Although many cellular systems show overlap of mRNA and protein expression, it is known that this is not necessarily the case in neuronal systems where axon terminal protein expression may be distant from the site of the soma (Sutton and Schuman, 2005). Recent work suggests
that although axon terminals are capable of local translation independent of the soma (Lin and Holt, 2008), local translation and protein synthesis at the axon terminal are not necessary for responsiveness to guidance cues (Roche et al., 2009). Using ISH, we detected additional ephrin-A3 and EphA4 mRNA in supra- and infragranular SI cortex. These endogenous sources of cortical ephrin-A3 and EphA4 may have physiological roles distinct from those expressed by TCAs.

The coexpression of ephrin-A3 and EphA4 proteins on VB axons raises questions of their possible physiological role in TCA development. A cell culture model using ephrin-A2 and EphA4 co-transfected cells showed reduced responsiveness to simulation by ephrin-A5 or EphA3 suggesting that co-expression of an ephrin ligand and Eph receptor can attenuate or even silence their ability for intracellular signaling following binding (Carvalho et al., 2006; Yin et al., 2004). These results were somewhat challenged by a report that co-expressed ephrins and Ephs on axons in the motor system do not show attenuation of one another’s signaling, but instead mediate opposite effects depending on whether stimulated by an ephrin or Eph (Marquardt et al., 2005). Recently, these differences have been explained based on the relative location of the ephrin and Eph within the membrane where they are co-expressed. If the co-expressed ephrin and Eph are physically close enough within the membrane to interact in cis, mutual attenuation of intracellular signaling will result. However, if the ephrin and Eph are localized in separate non-interacting microdomains, no attenuation will occur, and both molecules are able to signal independently (Egea and Klein, 2007). Hence, the partially overlapping and transient nature of ephrin-A3/EphA4 co-expression and their relative membrane
distribution may provide mechanisms that allow the TCAs to temporarily penetrate existing ephrin barriers within SI at selected times of development.

Although the embryonic expression of EphA4 in VB, where its expression is graded from medial to lateral, has been implicated in establishing the topography of the TCAs in SI through its interactions with the cortical ephrin-A5 gradient (Dufour et al., 2003; Garel and Rubenstein, 2004; Liebl et al., 2003; Mackarehtschian et al., 1999; Vanderhaeghen et al., 2000), the postnatal EphA4 expression that we report here may have additional roles. EphA4 protein expressed postnatally on TCAs could have a role in laminar termination of the TCAs, as a boundary molecule preventing axons from other cortical areas or layers from invading the TCA territory in layer IV, or may even serve to maintain topographic order of the axons into the second postnatal week. It is possible that coexpression of EphA4 and/or ephrin-A3 on TCAs contribute to their laminar segregation. In their 2008 paper, Gallarda and colleagues report that EphA3/EphA4 double knockouts display misguided motor axons with no effect on sensory axons in mixed sensory-motor peripheral nerves. These results allowed the authors to speculate that EphA3 and EphA4 expressed on motor axons may interact with an ephrin ligand expressed on sensory axons. Hence a similar role for postnatal ephrin-A3 expression may be to help maintain axonal segregation of sensory and motor fibers in the IC.

Although we cannot dismiss the possibility that ephrin-A3 or EphA4 protein in layer IV serves a boundary role preventing axons from other cortical areas (ipsilateral or contralateral) from invading SI, this seems unlikely considering their specific laminar localization and that their area of expression is patched in barrels. An effective inter-areal
boundary molecule would likely be expressed more uniformly across laminae or be
confined to the exterior bounds of one cortical area. Rather, we suggest an intra-areal role
of ephrin-A3 and EphA4 in SI. Given the specificity of ephrin-A3 and EphA4 protein
within the TCA terminals, they may have roles in preventing other cortical cells (like
layer II/III pyramidal cells) from projecting into layer IV, or in establishing and
maintaining the laminar bounds of TCAs in layer IV.

In other studies, we have recently identified ephrin-A2 protein and mRNA
expression confined to the supra- and infragranular layers of SI continuously throughout
the first two postnatal weeks (Kenmuir et al., In review.). Taken together, we suggest a
model where postnatal expression of EphA4 on TCAs may interact with ephrin-A5
within layer IV (Bolz et al., 2004) to promote branch formation (Mann et al., 2002),
whereas their interaction with supra- and infra-granular ephrin-A2 may serve as a
boundary preventing these terminals from expanding beyond layer IV. Furthermore, co-
expression of ephrin-A3 and EphA4 on the TCAs may help to temporally modulate the
TCA response to cortical ephrin barriers. Moreover, it is also possible that ephrin-A3 on
TCAs may have a role in preventing supragranular pyramidal neurons from arborizing
within layer IV via interaction with EphA5 expressed on their surface (Castellani et al.,
1998). Future studies in our laboratory will focus on testing these hypothetical functions
of ephrin-A3 and EphA4 in the developing postnatal SI.
LITERATURE CITED


Kenmuir C, Chiaia N, Mooney R, Lane R. In review. Developmental expression of ephrin-A2 in Primary Somatosensory Cortex:

A role in restricting the arborization of ventrobasal thalamic axons?.


Rasband W. 1997-2004. ImageJ, National Institutes of Health, Bethesda, Maryland, USA.


FIGURE LEGENDS

Fig. 1. Neonatal ephrin-A3 protein expression in primary somatosensory cortex (SI) and ventrobasal thalamus (VB). No ephrin-A3 immunoreactivity was observed in somatosensory cortex on postnatal day 0 (P0), P2, (not shown) or P4 (A). Ephrin-A3 protein expression was first observed in SI on P6 where it is expressed in layer IV and arrayed in a vibrissae-related pattern within posterior medial barrel subfield (PMBSF) and the region corresponding to the representation of the rostral sinus hairs [white bracket] (B). From P8-P12, ephrin-A3 protein expression is maximal in layer IV where it is present in all body representations within SI including the PMBSF as seen in panels C-F. Coronal section shows ephrin-A3 immunoreactivity is confined to layer IV and concentrated in the barrels. Note, no ephrin-A3 staining was observed in layer VIa (D). At P14, limited expression was observed in layer IV, and it was confined to the rostral sinus hair clusters (G). By P21, no ephrin-A3 protein expression was apparent in SI (H). From P4-P8, ephrin-A3 protein expression was also observed in the ventrobasal (VB) thalamus (I) and the expression was clustered within the somatosensory barreloids.

Panels A-C and E-H: PMBSFs with immunoreactive barrels are outlined with white boxes in flattened tangential sections and section orientation is shown in panel A. Panel D: Layer IV with immunoreactive barrels is labeled with white arrows. Panel I: ventroposteriomedial section of VB with immunoreactive barreloids is outlined in white and broken black line outlines Internal Capsule (IC). Scale bar represents 1mm.
Fig. 2. Neonatal EphA4 protein expression in primary somatosensory cortex (SI) and ventrobasal thalamus (VB). Similar to ephrin-A3 immunoreactivity, no EphA4 protein was observed in somatosensory cortex on P0, P2 (not shown), or P4 (A). Unlike ephrin-A3 protein expression, at P6 EphA4 protein was more apparent in SI layer IV PMBSF barrels as well as rostral sinus hairs [white bracket] (B). Unlike ephrin-A3 immunoreactivity, EphA4 protein was observed in layer IV barrels as well as layer VIa aggregates underlying the barrels (C). From P8-P10, EphA4 protein is expressed in all body regions of SI (D-E). By P14, no EphA4 protein expression was observed in the barrel clusters of layer IV, in fact, a negative image of the barrels is present (F). EphA4 protein was also observed in VB. EphA4 expression in VB was highest from P0-P2 and was reduced by P6, but remained clustered within the somatosensory barreloids (G-H). By P10, EphA4 protein expression was no longer detected in VB (I). Panels A-B and D-F: PMBSFs with immunoreactive barrels are outlined with white boxes in flattened tangential sections and section orientation is shown in panel A. Panel C: Layer IV with immunoreactive barrels is labeled with large white arrows. Small white arrows label layer VIa immunoreactivity. Panel G-I: ventroposteriomedial section of VB with immunoreactive barreloids is outlined in white and broken black lines outline IC. Scale bar represents 1 mm.

Fig. 3. Western blot of ephrin-A3 and EphA4 immunoreactivity from PMBSF layer IV tissue homogenates. Immunoblot for ephrin-A3 shows one predominate band at approximately 32 kDa, which was blocked by antibody adsorption using ephrin-A3
peptide (A). EphA4 immunoreactivity revealed several larger bands between 70 and 120 kDa, which were also blocked by antibody adsorption using EphA4 peptide (B).

Fig. 4. In situ hybridization (ISH) indicates that ephrin-A3 and EphA4 mRNA is present in SI and VB. ISH revealed a laminar distribution of ephrin-A3 mRNA at P4 in SI in the supragranular and infragranular laminae [white brackets] as well as in the subplate (white arrow) (A) and barreloid clusters in VB medial and lateral subcompartments (A’). EphA4 mRNA is also expressed in the supragranular layers, infragranular layers [white brackets], and subplate (white arrow) (B). Unlike ephrin-A3, EphA4 signal was prominent in barreloid clusters in VB only within the medial compartment (outlined in white) (B’). Scale bar represents 1mm.

Fig. 5. Quantitative Real Time PCR (qRTPCR) of PMBSF homogenates shows that ephrin-A3 and EphA4 protein expression peaks at P4. ephrin-A3 (A) and EphA4 (B) mRNA expression was quantified using qRTPCR. The expression of both mRNAs was maximal at P4 and quickly returned to baseline levels. Results are shown as fold changes relative to P18 (the oldest age sampled and an age where neither protein is expressed at observable levels) with error bars reflecting standard deviation of the ΔΔCt values. Surface to depth cortical samples from the PMBSF were analyzed following confirmation of their location by cytochrome oxidase reactivity of the residual cortices. Shown in C is a section through one of the residual P8 cortices. The contralateral hemisphere reacted for
cytochrome oxidase is shown in D to demonstrate the location of PMBSF (outlined in white) within the sample collected for qRTPCR analysis. Scale bar represents 1mm.

Fig. 6. Confocal microscopy was used to colocalize the protein expression of ephrin-A3 and EphA4 in layer IV of PMBSF. Low power images from P8 SI show ephrin-A3 and EphA4 protein expression are both most dense in the layer IV barrels and are colocalized ($R^2 = .90$, $R^2 = .86$, respectively) with the serotonin transporter (SRT), a marker of thalamocortical afferents (TCA) (Row A). High power images of single cells reveal colocalization of ephrin-A3 and EphA4 with SRT (Rows B-C), but not with HU RNA binding protein, a neuron specific cell marker (Rows D-E). Scale bars represent 250 µm in Row A, and 5 µm in Rows B-E.

Fig. 7. Electrolytic lesion of VB at P4 resulted in a disruption of ephrin-A3 immunoreactivity in PMBSF at P8. Nissl stained coronal sections show damage [lesions identified by *] to VB in extreme hemispheric lesion (A), selective VB lesion (B), and control lesion medial to VB (C). Both the hemispheric lesion including VB, as well as the selective VB lesion resulted in a selective loss of ephrin-A3 immunoreactivity in PMBSF in coronal section (A’) and flattened horizontal section (B’). Insets in A’ and B’ show contralateral hemisphere with positive ephrin-A3 immunoreactivity. PMBSF is outlined in white (A’-C’). The control lesion showed no disruption of ephrin-A3
immunoreactivity in the ipsilateral cortex (C’). Panels B’, C’: section orientation is shown in panel B’ for flattened tangential sections. Scale bar represents 1mm.

Fig. 8. Electrolytic lesion of VB at P4 resulted in a disruption of EphA4 immunoreactivity in PMBSF at P8. Nissl stained coronal sections show damage [lesions identified by *] to VB in extreme hemispheric lesion (A), precise VB lesion (B), and control lesion medial to VB (C). Both hemispheric lesion including VB as well as precise VB lesion show a selective loss of ephrin-A3 immunoreactivity in PMBSF in coronal section (A’) and flattened horizontal section (B’). Insets in A’ and B’ show contralateral hemisphere with positive EphA4 immunoreactivity. PMBSF is outlined in white (A’-C’). The control lesion showed no disruption of EphA4 immunoreactivity in the ipsilateral cortex (C’). Panels B’, C’: section orientation is shown in panel B’ for flattened tangential sections. Scale bar represents 1mm.
Figure 3

A

B

- bp + bp
Anti- ephrin-A3

- bp + bp
Anti- Eph A4

-40 kDa
-30 kDa
-120 kDa
-70 kDa
Figure 4

A  ephrin-A3  P4
B  Eph A4  P4
A'  B'

[Images of A, A', B, B']
Figure 5
Figure 7
CHAPTER 5: MANUSCRIPT 3

Expression of ephrin-A Ligands and EphA Receptors in the
Development of the Peripheral Trigeminal Somatosensory System

Cynthia L. Kenmuir, Richard D. Mooney*, Nicolas L. Chiaia, and Richard D. Lane
Department of Neurosciences, University of Toledo College of Medicine,
Toledo, Ohio 43609

Number of Text Pages: 17

Number of Tables: 0

Number of Figures: 7

Running Title: ephrin-A expression in the peripheral trigeminal system

Keywords: receptor tyrosine kinase, axon guidance, barrel, development, TCA

*Correspondence to: Dr. Richard D. Mooney, University of Toledo College of Medicine,
3000 Arlington Avenue - MS1007, Toledo, OH 43609.

E-mail: Richard.Mooney@utoledo.edu

Supporting Grants: Research supported by National Institutes of Health (P01-NS149048).
ABSTRACT

Peripheral processes of trigeminal ganglion cells display target-directed growth resulting in precise topographic innervation of the vibrissal pad. We and others have shown that ephrins and Ephs are selectively expressed in patterns that indicate their involvement in the guidance of thalamocortical fibers into SI cortex and formation of the barrel pattern corresponding to the vibrissae. At the periphery, afferent fibers of the infraorbital branch of the trigeminal nerve selectively innervate the vibrissae hair follicles of the rodent whisker pad. It is reasonable to speculate that ephrins and Ephs might be involved in this peripheral somatosensory organization as well. Using immunohistochemistry, we have identified distinct expression patterns of several ephrin ligands and Eph receptors within the peripheral innervation territory of the trigeminal nerve. At embryonic day 15 (E15), when the axons of the infraorbital nerve are known to have migrated into the caudal portion of the vibrissal pad, we detected ephrin-A2 and ephrin-A3 expressed on follicular trigeminal fibers, and EphA7 in cutaneous trigeminal fibers whereas EphA4 was detected in facial nerve axons. Consistent with their location on trigeminal fibers, ephrin-A2, ephrin-A3, and EphA7 was also observed in the trigeminal ganglia. Furthermore, ephrin-A2 was expressed within the inner epithelial root sheath, while EphA4 was detected within the outer epithelial root sheath. A similar expression patterns were observed in the vibrissal pads on E17 and postnatal day 0 (P0) with the exception that EphA4 was transiently expressed in trigeminal fibers only on E17. By P4, No EphA expression was observed in trigeminal or facial nerve fibers, but ephrin-A expression remained in trigeminal fibers until P8. These results describe the presence
of several ephrins and Ephs differentially localized around both the nerve fibers and the 
vibrissal follicles during the critical vibrissae follicle innervation period. This expression 
suggests a role for ephrin-Eph interactions in establishing correct topographic 
connections within the peripheral somatosensory system.
INTRODUCTION

The rodent somatosensory system maintains exquisite topographic representations of its peripheral connections throughout all levels of the neuraxis making it an ideal model for investigating guidance molecules involved in establishing and maintaining patterning. Although numerous investigations of guidance molecules involved in somatosensory topography have focused on thalamocortical connectivity, reviewed in (Lopez-Bendito and Molnar, 2003), relatively little is known about their role in peripheral somatosensory axon guidance. Several classes of guidance molecules have been detected in the developing trigeminal ganglia (TG) and whisker pad (WP) including semaphorin 3A, neuropilin-1 and -2, laminin, β1-integrin, NGF, BDNF, and neurotrophin-3, neurotrophin 4/5, and Slit2, reviewed in (Erzurumlu et al., 2006; Kury et al., 2000; O'Connor and Tessier-Lavigne, 1999; Tessier-Lavigne and Goodman, 1996). However, none of these are expressed differentially within the ganglion or at the vibrissae follicle suggesting that additional guidance cues may be needed for trigeminal axons to make proper connections to their peripheral targets.

The mystacial vibrissae on the rodent snout serve as independent tactile sensors, sending information into the CNS via the infraorbital nerve (ION). The ION is formed from the peripheral processes of the TG neurons as they extend towards the mystacial vibrissal follicles. As early as embryonic day 12 (E12) TG neurons are arranged in rows according to their future position in the dorsal-ventral axis of the WP (Erzurumlu and Jhaveri, 1992; Rhoades et al., 1990) and their peripheal processes have reached the maxilla (Erzurumlu and Killackey, 1983). The ION reaches the caudal edge of the
whisker pad by E15 (Gunhan-Agar et al., 2000). Over the next two days, these axons form row nerves, which continue through the whisker pad to reach the rostral whisker follicles. The axon terminals of the ION fibers envelop individual follicles by E17 (Erzurumlu and Jhaveri, 1995; Erzurumlu et al., 1993). A vibrissae-related pattern of the central processes of the trigeminal ganglion cells is not apparent until days after the arrival of the central TG processes (Belford and Killackey, 1979a; b; Erzurumlu and Jhaveri, 1992; Killackey et al., 1990), suggesting a directive role for the peripheral TG axons. Results from organotypic co-culture experiments indicate the temporary expression of putative guidance molecules in the WP and TG that are capable of interacting through a repulsive mechanism (Gunhan-Agar et al., 2000).

The Eph family of receptor tyrosine kinases, largely capable of repulsive interactions, are involved in topographic patterning within the central portions of the somatosensory system (Dufour et al., 2006; Dufour et al., 2003; Kenmuir et al., In review.-a; b; Uziel et al., 2006; Uziel et al., 2008; Vanderhaeghen et al., 2000; Vanderhaeghen and Polleux, 2004) as well as peripheral portions of the visual (Cheng et al., 1995; Feldheim et al., 2000; Feldheim et al., 2004; Feldheim et al., 1998; Fraser and Hunt, 1980; Holash and Pasquale, 1995; Lukehurst et al., 2006), olfactory (Chen and Flanagan, 2006; Cutforth et al., 2003; St John et al., 2002; Tisay and Key, 1999), auditory (Cramer et al., 2002; Cramer et al., 2000; Ellsworth et al., 2005), and motor systems (Eberhart et al., 2004; Eberhart et al., 2002; Gallarda et al., 2008; Helmbacher et al., 2000; Iwamasa et al., 1999; Kania and Jessell, 2003; Kilpatrick et al., 1996; Ohta et al., 1997; Wang and Anderson, 1997). Hence, the variety of systems where ephrin-Eph interactions are involved in
axonal guidance during development suggests that ephrin and Eph proteins may also have a role in axon guidance in the peripheral portion of the somatosensory system. In this paper, we report the spatial and temporal expression of ephrin-A2, ephrin-A3, EphA4, and EphA7 protein in the developing TG and WP from E15 through the first postnatal week.

MATERIALS AND METHODS

Animals

Sprague Dawley breeder rats were obtained from Taconic Farms (Hudson, New York) and bred at the University of Toledo. Postnatal day 0 (P0) was defined as the day of birth. Rat pups were used for surgical manipulations, as described below, and euthanized at pre-defined ages from embryonic day 15 (E15) to P8. All experiments were conducted in accordance with Institutional Animal Care and Use Committee Guidelines.

Embryonic Tissue

For time-pregnant rats, breeder rats were paired for 12 hours overnight and the female’s weight monitored throughout pregnancy. The morning of separation was defined as E0. At E15 or E17, pregnant rats were anesthetized with 75mg/kg sodium pentobarbital. Following a midline laparotomy, the uterus was exteriorized and individual embryos were removed, euthanized, and then perfused as described below. Following removal of the embryos, the female was euthanized.
Immunoactivity

For all brightfield immunohistochemistry (IHC), rats were euthanized and transcardially perfused with heparinized saline followed by 4% paraformaldehyde. Brains were removed and placed in 4% paraformaldehyde at 4°C overnight. A freezing microtome was used to create 50μm coronal or flattened tangential sections that were then treated with 3% H₂O₂, blocked with BSA (Sigma), permeabilized with Triton-X (Sigma) and incubated in primary antibody overnight (1:10,000-1:1000 protein gene product 9.5 (PGP9.5, UltraClone Limited); 1:1000 ephrin-A2, ephrin-A3, and EphA4 (Santa Cruz); 1:1000 EphA7 (Abgent)). Localization of PGP 9.5 was used to identify the precise location of trigeminal and facial nerve fibers in the developing whisker pad from E15 to P8. Sections were washed and then incubated in 1:450 biotinylated secondary antibody (Chemicon) for two hours followed by washing and incubation with Vectastain avidin:biotin enzyme complex (Vector Labs) for two hours. After washing, sections were reacted with diaminobenzoate (Fluka), placed onto glass slides, coverslipped, and imaged using a Nikon SMZ-U microscope. For all experiments, olfactory bulb sections were processed in parallel and used as a positive control. Negative controls for each experiment included alternate cortical, thalamic, and olfactory bulb sections processed using either no primary antibody, a host-specific immunoglobulin in place of the primary antibody, or primary antibody adsorbed with the blocking peptide for that protein (Santa Cruz).
RESULTS

Immunohistochemical detection of several ephrin and Eph proteins revealed differential spatial and temporal expression patterns within the developing WP, although we did not detect spatial expression differences across the dorsal-ventral or anterior-posterior axis of the WP for any single ephrin or Eph. Localization of PGP 9.5 was used to identify the precise location of trigeminal and facial nerve fibers in the developing whisker pad from E15 to P8. PGP9.5 at higher antibody concentrations, was used to identify facial nerve fibers (Figure 1 A, C, C’). At lower concentrations PGP9.5 labeled trigeminal fibers including the vibrissal branches as the enveloped individual follicles (Figure 1 B, B’).

At E15, both ephrin-A2 and ephrin-A3 were prominently expressed in the trigeminal axons as they invade the caudal portion of the WP (Figure 2 A, B). Both ephrins were present in the superficial and deep vibrissal nerves as they approached the follicles (Figure 3 A, B). Additionally, we observed strong immunoreactivity for ephrin-A2 in the inner epithelial root sheath (IRS) surrounding the vibrissa (Figure 2 A). No Eph receptors were detected within the trigeminal nerves in the caudal WP (Figure 2 C, D), although EphA7 was observed in cutaneous trigeminal fibers (Figure 2 D, 3 D) and within some deep trigeminal fibers at the base of individual follicle (Figure 3 D). In contrast, EphA4 was observed in the outer epithelial root sheath (ORS) surrounding the vibrissa (Figure 2 C) and in the facial nerve fibers that innervate the whisking muscles (Figure 3 C). Consistent with their presence on trigeminal afferents, we detected ephrin-A2, ephrin-A3, and EphA7 in the trigeminal ganglion cells at E15 (data not shown).
At E17, both ephrin-A2 and ephrin-A3 immunoreactivity remained prominent within the trigeminal nerve fibers throughout the WP (pictured at the caudal WP in Figure 4 A, B). Although EphA4 expression remained strong within the ORS (Figure 4 C, inset) and facial nerves (Figure 4 C), it was also observed in the trigeminal nerves (Figure 4 C). EphA7 expression changed markedly from that observed at E15. While still observed within the cutaneous trigeminal fibers (Figure 4 D, inset), EphA7 was also now prominent within the facial nerve and its branches (Figure 4 C).

On the day of birth (P0), ephrin-A3 expression remained prominent in the trigeminal fibers within the row nerves as well as within their branches as they enveloped individual follicles (Figure 5 A, A’). EphA4 expression was no longer observed in trigeminal fibers, although its expression remained prominent in the ORS and facial nerve branches (Figure 5 B, B’) similar to that observed on E15 and E17.

On P4, ephrin-A3 protein expression was still prominent in the trigeminal fibers within the row nerves as well as within their branches as they wrap and ascend the follicles (Figure 6 A). Although EphA4 protein was still expressed within the ORS (Figure 6 B), it was no longer observed in facial or trigeminal nerve fibers of the WP.

On P8, both ephrin-A2 and ephrin-A3 protein expression remained in the trigeminal fibers within the row nerves and those enveloping individual follicles (Figure 7 A, B). EphA4 expression remained as on P4. EphA4 protein was still prominent within the ORS (Figure 7 C), and no immunoreactivity was observed in nerve fibers of the WP.
DISCUSSION

The peripheral processes of trigeminal ganglion cells display target-directed growth resulting in precise topographic innervation of the WP. These processes are known to reach the caudal extent of the WP by E15 and to have enveloped the follicles by E17 (Erzurumlu and Jhaveri, 1995; Erzurumlu et al., 1993; Gunhan-Agar et al., 2000). Our laboratory has previously shown that ephrin-A2, ephrin-A3 and EphA4 may play a role in the guidance of thalamocortical fibers into SI cortex (Kenmuir et al., 2008; Kenmuir et al., In review.-a; b). Additionally, EphA7 was previously observed in the peripheral processes of trigeminal axons exiting the ganglion between E12.5 to E14.5 (Rogers et al., 1999). In this paper, we report the first evidence of ephrin and Eph at the WP during the period of development when the vibrissae follicles become innervated by trigeminal afferents.

Using immunohistochemistry, we have identified differential spatial and temporal expression patterns of ephrin-A2, ephrin-A3, EphA4, and EphA7. We did not observe gradients in their expression across either the dorsal-ventral or anterior-posterior axis of the WP. This suggests that these ephins and Ephs are not be involved in establishing or maintaining the topographic connections between the ganglion cells and the individual follicles. Both ephrin-A2 and ephrin-A3 protein expression were prominent within the trigeminal nerves innervating the vibrissa and remained constant from E15 to P8. EphA4 protein expression was variable from E15 to P8, but was localized to the ORS throughout that time. Taken together, these results suggest the possibility that ephrin-A in the trigeminal axon tips interacts with EphA4 in the ORS to prevent the trigeminal axons
from invading the ORS. This suggestion is consistent with reports that note the consistent
termination of the sensory nerve endings on the exterior of the ORS (Dorfl, 1985; Rice
and Munger, 1986).

Additionally, ephrin-A2 protein was also prominently expressed within the IRS as
early as E15. This expression may serve additional roles within the maturing follicle,
which may include the promotion of epithelial cell proliferation as observed with
exogenous application of ephrin-As to human hair follicles (Midorikawa et al., 2004). In
addition to EphA4 expression in the ORS, it was also prominently expressed on the facial
nerve from E15 to P0. This suggests that EphA4 has a role in the formation of
neuromuscular synapses between the facial nerve fibers and the whisking muscles. This
result is consistent with reports indicating that ephrin-As are expressed in sensory axons
and EphAs are expressed in motor axons (Helmbacher et al., 2000; Kilpatrick et al.,
1996; Ohta et al., 1997; Wang and Anderson, 1997). Furthermore, EphA4 has been
previously reported in trigeminal and facial motor neurons in the chicken (Kury et al.,
2000), which is consistent with the transient expression of EphA4 in the trigeminal nerve
that we observed. We observed strong EphA4 immunoreactivity within the trigeminal
nerve only at E17; however, we did not detect EphA4 immunoreactivity in the sensory
fibers enveloping the follicles from E15 to P8. It is possible that the transient EphA4
expression that we observed in the trigeminal nerve was expressed by motor fibers that
innervate the muscles of mastication.

Finally, we observed the presence of EphA7 in cutaneous trigeminal fibers as well
as in those innervating the vibrissal follicles on E15. On E17, we were no longer able to
detect EphA7 in trigeminal fibers at the follicles, although some immunoreactivity remained in the cutaneous trigeminal fibers. These results were expected based on previous reports observing EphA7 protein expression in the peripheral processes of the trigeminal ganglia at E12.5 and E14.5, but not at P1 (Rogers et al., 1999).

These results identify the presence of several ephrins and Ephs in the developing peripheral somatosensory system, the expression of which is differentially localized around the vibrissal follicles and is temporally regulated. Unexpectedly, we have additionally identified the presence of EphA receptors on the peripheral motor axons of the face. The differential expression of these proteins suggest a role for the ephrin-Eph system in establishing correct connectivity of the trigeminal axons to their follicular targets as well as roles in promoting epithelial cell growth and motor axon connectivity. Ongoing experiments in our laboratory are directed toward investigating these functional possibilities.


Dorfl J. 1985. The innervation of the mystacial region of the white mouse: A

EphA-dependent signaling mechanisms controlling topographic mapping in vivo.
Development 133(22):4415-4420.

JG, Polleux F, Vanderhaeghen P. 2003. Area specificity and topography of
thalamocortical projections are controlled by ephrin/Eph genes. Neuron

Eberhart J, Barr J, O'Connell S, Flagg A, Swartz ME, Cramer KS, Tosney KW, Pasquale
EB, Krull CE. 2004. Ephrin-A5 exerts positive or inhibitory effects on distinct


conserved mapping mechanisms in visual and auditory thalamic targets. J Comp
Neurol 488(2):140-151.
Erzurumlu RS, Chen ZF, Jacquin MF. 2006. Molecular determinants of the face map
development in the trigeminal brainstem. Anat Rec A Discov Mol Cell Evol Biol
288(2):121-134.

Erzurumlu RS, Jhaveri S. 1992. Trigeminal ganglion cell processes are spatially ordered

Erzurumlu RS, Jhaveri S. 1995. Target influences on the morphology of trigeminal

Erzurumlu RS, Jhaveri S, Takahashi H, McKay RD. 1993. Target-derived influences on
axon growth modes in cultures of trigeminal neurons. Proc Natl Acad Sci U S A
90(15):7235-7239.


Genetic analysis of ephrin-A2 and ephrin-A5 shows their requirement in multiple

Feldheim DA, Nakamoto M, Osterfield M, Gale NW, DeChiara TM, Rohatgi R,
Yancopoulos GD, Flanagan JG. 2004. Loss-of-function analysis of EphA


Kenmuir C, Chiaia N, Mooney R, Lane R. In review.-a. Developmental expression of ephrin-A2 in Primary Somatosensory Cortex: A role in restricting the arborization of ventrobasal thalamic axons?.


Fig. 1. Flattened tangential section through the whisker pad of a P0 rat labeled with PGP9.5 immunohistochemistry show the orderly array of the large mystacial vibrissae in the rat. In this section, most of the vibrissae are present and are labeled with the conventional row-arc classification (A). Scale bar represents 1 mm. High power images from flattened tangential sections show individual vibrissae follicles with corresponding nerve fibers (B-C). The trigeminal (CN V) nerve fibers from the deep branch of each vibrissal row nerve ascend and wrap (large black arrow) around the outer epithelial root sheath (ORS, dotted outline) of individual follicles as shown in B. A muscle sling used for whisking wraps around each of the large follicles (small white arrows) and is innervated by motor fibers of the facial nerve (small black arrow), which span the caudal gap between the muscle attachments in C. High power images from longitudinal sections through the whisker pad (B’-C’) show several deep vibrissal nerves (large black arrow) as they approach individual follicles, ascend the follicles, and distribute around the ORS (large white arrow) of each follicle (B’). In adjacent sections, branches of the facial nerve (small black arrows) are shown innervating the whisking muscles (m) (C’). Scale bar for B-C’ represents 1 mm. Similar orientation and arrow schema was maintained in all subsequent figures for comparison.

Fig. 2. Flattened tangential sections through the superficial portion of the whisker pad on embryonic day 15 (E15) show differential ephrin and Eph protein expression. Prominent
expression of ephrin-A2 was observed in the growth cones of trigeminal axons (white arrow) at the caudal extent of the whisker pad and in the inner epithelial root sheath (black arrow) of individual follicles (A). Expression of ephrin-A3 was also observed in the growth cones of trigeminal axons (white arrow), but was less prominent within the follicular root sheaths (B). At this age, expression of EphA4 and EphA7 were not observed in growth cones of trigeminal fibers in the caudal portion of the whisker pad although both were observed in fibers of the facial nerve in deeper sections (not shown). However, EphA4 was observed in the ORS (white arrow) of individual follicles (C) and EphA7 was present in cutaneous sensory fibers (white arrow) of the trigeminal nerve (D) as well as in some deep trigeminal fibers (see Fig.3). Scale bar represents 1 mm.

Fig. 3. Longitudinal sections through the whisker pad on embryonic day 15 (E15) show differential ephrin and Eph expression in the superficial (SVN) and deep (DVN) vibrissal nerves. Prominent expression of ephrin-A2 and ephrin-A3 was observed in the growth cones of the SVN (white arrow) and in the DVN (black arrow) as they approached individual follicles (A-B). EphA4 expression was not observed in trigeminal fibers, but was observed in the ORS (white arrow) and in fibers of the facial nerve (black arrow) between the whisking muscles (C). Similar to that of the ephrins, EphA7 was present in the SVN (white arrow) and DVN (black arrow), although to a lesser extent (D). Scale bar represents 1 mm.
Fig. 4. Flattened tangential sections through the superficial (A-B and insets C-D) and deep (C-D) portions of the whisker pad on E17 showing ephrin and Eph expression. Similar to their expression at E15, ephrin-A2 (A) and ephrin-A3 (B) were observed in trigeminal axons (white arrow). At this age, EphA4 was still present in the ORS (white arrow in inset) and facial nerve (wide black arrows), but was also now apparent in the trigeminal nerve (wide white arrow) at the posterior of the whisker pad (C). EphA7 expression was still observed in the cutaneous trigeminal fibers between follicles (large white arrow in inset) and was prominent on the branches of the facial nerve (black arrows) (D). Scale bar represents 1 mm.

Fig. 5. Flattened tangential sections through the superficial (A, B) and deep (A’, B’) portions of the whisker pad on postnatal day 0 (P0) show continued and distinct ephrin-A3 and EphA4 expression. Prominent expression of ephrin-A3 was observed in the trigeminal axons as they travel along the row nerves and envelope the follicles (white arrows) in superficial (A) and deep (A’) sections. EphA4 remained prominent in the ORS (white arrow) in superficial sections (B) and in facial nerve fibers (black arrows) in deeper sections (B’). Scale bar represents 1 mm.

Fig. 6. Flattened tangential sections through the superficial portion of the whisker pad on P4. Prominent expression of ephrin-A3 was observed in the trigeminal axons (white arrows) as they travel in the row nerves and envelope the follicles (A). EphA4 protein
remained prominent in the ORS (white arrow) but was no longer observed in the facial nerves (B). Scale bar represents 1 mm.

Fig. 7. Alternate sections from the superficial portion of a P8 whisker pad show continued distinct ephrin and Eph localization. Prominent expression of ephrin-A2 (A) and ephrin-A3 (B) was observed in the trigeminal axons as they form the row nerves and supply individual follicles (wide white arrows). EphA4 protein remained prominent in the ORS (white arrows) and not in the nerve fibers (B). Scale bar represents 1 mm.
Figure 4
Figure 6

A

P4

ephrin-A3

B

EphA4
CHAPTER 6: MANUSCRIPT 4

The Role of Ephrins in Thalamocortical Connectivity

within the Rat Somatosensory System

Cynthia L. Kenmuir, Richard D. Mooney*, Nicolas L. Chiaia, and Richard D. Lane

Department of Neurosciences, University of Toledo College of Medicine,

Toledo, Ohio 43609

Number of Text Pages: 24

Number of Tables: 0

Number of Figures: 3

Running Title: ephrins in somatosensory thalamus and cortex

Keywords: receptor tyrosine kinase, axon guidance, barrel, development, TCA

*Correspondence to: Dr. Richard D. Mooney, University of Toledo College of Medicine,
3000 Arlington Avenue - MS1007, Toledo, OH 43609.

E-mail: Richard.Mooney@utoledo.edu
ABSTRACT

Paramount to the proper function of the nervous system is the development and maintenance of neural circuits. This requires that immature neurons are guided to their final destination, connected to their correct targets, and often bounded in that target to prevent the formation of aberrant connections. The developmental timecourse and topographic arrangement of the rodent whisker-barrel somatosensory system has been well described making it an attractive model for studying the molecular aspects of axonal guidance needed for the precise development of these connections. In this paper, we provide a current review that relates the timecourse of thalamocortical connectivity with the temporal and spatial expression of the Eph/ephrinA family of guidance molecules. The possible roles of these guidance molecules in the development and organization of the somatosensory cortex are discussed.
Paramount to the proper function of the nervous system is the development and maintenance of neural circuits. This requires that immature neurons are guided to their final destination, connected to their correct targets, and often bounded in that target to prevent the formation of aberrant connections. The developmental timecourse and topographic arrangement of the rodent whisker-barrel somatosensory system has been well described making it an attractive model for studying the molecular aspects of axonal guidance needed for the precise development of these connections. In this paper, we first review the development of the thalamocortical pathway and then focus on summarizing the current literature regarding the temporal expression of members of the Eph/ephrinA family of guidance molecules along this sensory pathway.

**Thalamocortical Development**

Neurons in the ventral posterior medial (VPM) thalamus are born in the rat as early as embryonic day 14 (E14) (McAllister and Das, 1977). These neurons quickly extend their axons (TCAs) out through the internal capsule (IC) and approach the developing cortical plate (CP) of the primary somatosensory area (SI) between E15 (Molnar et al., 1998) and E16 (Catalano et al., 1996; Molnar and Cordery, 1999) [Fig.1A]. By E17, the first cortical layer (VIb) has differentiated and by E18 the TCAs have gathered beneath the subplate (Catalano et al., 1996; Molnar and Cordery, 1999) [Fig.1B]. On E19, layer V1a has differentiated and by E20 the TCAs have invaded layer VIb as well as the ventral portion of layer V1a [Fig.1C]. Layer V has differentiated by
E21/P0 and TCAs are seen in this newly developed layer (Catalano et al., 1991; 1996; Schlaggar and O'Leary, 1994) [Fig.1D]. Layer IV begins to differentiate from the cortical plate on postnatal day 1 (P1) and a few simple TCA branches are seen in the developing layer IV at this time (Senft and Woolsey, 1991; Shimogori and Grove, 2006) [Fig.1E]. P2 reveals the first clusters of TCAs in layer IV (Erzurumlu and Jhaveri, 1990). By P3, most TCAs have arborized into cylindrical clusters extending the full thickness of layer IV [Fig.1F]. These cylindrical clusters, which also contain cortical neurons prominently along their walls are known as barrels (Jhaveri et al., 1991; Killackey and Belford, 1979; Rebsam et al., 2002; Rice and Van der Loos, 1977; Shimogori and Grove, 2006). The TCAs continue to expand in length, density, and complexity within individual layer IV barrels to form a dense meshwork by P7, without expansion above or below layer IV (Agmon et al., 1995; Catalano et al., 1996; Rebsam et al., 2002) with the exception that the VPM axon arbors form additional small clusters beneath each barrel, which are confined to layer VIa (Agmon et al., 1993; Catalano et al., 1996; Senft and Woolsey, 1991). Many of these TCAs also send collaterals into layer I (Lu and Lin, 1993; Oda et al., 2004; Rausell and Jones, 1991) [Fig.1G]. From P12-20, the barrels still span the full thickness of layer IV; but on P20, the barrels begin to further mature such that there is no longer a cell-sparse center. Rather, the cortical neurons and TCAs within each mature barrel become evenly distributed within the layer IV cylinders and the TCAs eventually extend upward into the supragranular layers (Rice, 1985) [Fig.1H]. TCAs in adult cortex also expand beyond the thickness of layer IV, a process that begins between P18 and P25 (Lorente de No, 1992). Taken together, these data suggest that the transient expression of guidance molecules during the first two postnatal weeks may provide critical cues for
proper termination of TCAs in layer IV and may temporarily limit axon arbors within layer IV. These data also suggest that downregulation of these molecules after P18 or P20 (Lorente de No, 1992; Rice, 1985) respectively) may be responsible for the subsequent supragranular extension and maturation of the adult rat barrel cortex.

*Repertoire of ephrin-Eph interactions in thalamocortical connectivity*

Although the result of the interaction between an ephrin-A expressing cell and an EphA expressing cell was once thought to be that of simple repulsion of the EphA positive cell, it is now known the ephrin-A ligands are also capable of reverse signaling through intracellular pathways involving their GPI linked domain. Therefore, interactions between ephrin-A ligands and EphA receptors may have bidirectional effects including, but not limited to, repulsion of the axon with the EphA receptor and branch promoting effects within the ephrin-A positive cell. To make matters more complicated, ephrin ligands and Eph receptors can interact in cis when expressed within the same microdomain on a cell membrane. For instance, bidirectional reduction of intracellular signaling has been reported when ephrin-A2 and EphA4 interact in cis on co-transfected cells (Yin et al., 2004). Furthermore, complete silencing of intracellular signaling is also possible as was reported with ephrin-A5/EphA3 co-transfected cells. The cis interaction between ephrin-A5 and EphA3 on one cell resulted in silencing of the subsequent forward signaling through the EphA3 receptor upon its binding to another ephrin ligand in trans (Carvalho et al., 2006). The proposed mechanism for this silencing interaction is that once an ephrin-A and Eph interact in cis, ADAM10’s (A Disintegrin And Metallopeptidase 10) cysteine rich region is able to recognize the complex, which re-
positions the proteinase domain to cleave the ephrin-A5 in cis and then will cleave the
next ephrin-A in trans (Egea and Klein, 2007; Janes et al., 2005). But, there are other
possibilities for ADAM10’s cleavage. It is also possible that reverse signaling will
remain after an ephrin-Eph interaction in cis if the ephrin-Eph complex next encounters
an Eph rather than an ephrin, reviewed by: (Egea and Klein, 2007; Himanen et al., 2007).
Moreover, ephrins and Ephs expressed by the same cell may be expressed in separate
microdomains, which allow them to signal independently depending on whether the cell
encounters an ephrin or Eph in its environment. Evidence for this has been reported in the
motor system where ephrins and Ephs on the same axon can mediate opposite effects
(Marquardt et al., 2005). The complexities of the ephrin-Eph interaction are intriguing in
light of the recent report from our laboratory showing EphA4 and ephrin-A3 protein co-
expression on TCAs from VB in layer IV of SI from P6-P12. The identification of the
ephrin-A3 ligand on TCAs in the postnatal period may have additional signaling roles
through interaction with any of the EphA receptors expressed in SI cortex, or may serve
to silence the effects of EphA4 also expressed on the TCAs (Kenmuir et al., In review.-
b).

**Molecular guidance during development**

An array of signaling molecules has been implicated in the development and
subsequent maintenance of topographic arrangement at all levels of the somatosensory
pathway, for reviews see: (Hatten, 1999; Kaprielian et al., 2001; Song and Poo, 2001;
Tessier-Lavigne and Goodman, 1996). Extracellular matrix molecules, semaphorins, netrins, neurotrophins, and ephrins have all been reported to be needed for proper connectivity within the developing somatosensory system (Dickson, 2002; Flanagan and Vanderhaeghen, 1998). Most of these are soluble molecules capable of freely diffusing to form extracellular gradients. However, the ephrin family of receptor tyrosine kinases (RTKs) are membrane bound and once oligomerized are capable of bidirectional signaling (Davis et al., 1994; Massague and Pandiella, 1993; Pasquale, 1997; Taipale and Keski-Oja, 1997). In a sensory system where guidance must be so tightly regulated as to allow for one-to-one cellular connections, the ephrins, with their precise, short-range signaling capability, have ideal properties to guide the establishment and maintenance of proper topographic and laminar connectivity.

The ephrin ligands and their receptors, Ephs, comprise the largest family of RTKs that are present in the developing nervous system (Tuzi and Gullick, 1994). There are two sub-families of ephrins based on their transmembrane/membrane linked domains. Ephrin-A ligands are GPI-anchored proteins, whereas ephrin-B ligands are transmembrane receptors (Bruckner and Klein, 1998). The binding of ephrin ligands with Eph receptors is largely promiscuous. However, ephrin-A ligands have the highest binding affinities for EphA receptors and ephrin-B ligands primarily bind to EphB receptors with a few exceptions (Himanen et al., 2004; Kullander and Klein, 2002; Murai and Pasquale, 2003). Currently, 10 EphA receptors and 6 ephrin-A ligands have been identified in total although only 9 EphA receptors and 5 ephrin-A ligands have been identified in humans and rodents (Pasquale, 2004).
The role of these signaling molecules in the development of mammalian sensory systems has been described most extensively in the rodent visual system where tangentially oriented spatial gradients of ephrin-A family molecules and ephrin-B family molecules dictate retinal ganglion cell topography in the tectum (Braisted et al., 1997; Cheng et al., 1995; Feldheim et al., 2000; Feldheim et al., 1998; Fraser and Hunt, 1980; Holash and Pasquale, 1995; Lukehurst et al., 2006). Although members of both the ephrin-A and ephrin-B classes may also be involved in somatosensory connectivity, most investigations within the somatosensory system have focused on ephrin-A family members. Ephrin-A ligands and EphA receptors have also been recognized as guidance molecules in the auditory (Cramer et al., 2002; Cramer et al., 2000; Ellsworth et al., 2005), olfactory (Chen and Flanagan, 2006; Cutforth et al., 2003; St John et al., 2002; Tisay and Key, 1999), and motor systems as well (Eberhart et al., 2004; Eberhart et al., 2002; Gallarda et al., 2008; Helmbacher et al., 2000; Iwamasa et al., 1999; Kania and Jessell, 2003; Ohta et al., 1997). In addition to their roles in axonal guidance, Eph-ephrin interactions have numerous other roles throughout the body associated with normal physiology, pathologic processes, and repair following injury, all of which have been reviewed by others: (Calo et al., 2006; Pasquale, 2008; Wilkinson, 2001).

*EphA receptors in VB thalamus*

The ephrin/Eph family of guidance molecules is well known for their role in axonal guidance in the CNS. Within the somatosensory system, several ephrin-A ligands
and EphA receptors have been investigated for their potential role in thalamocortical connectivity. In 1992, it was first reported that Eph receptors were present on developing axons in the somatosensory system (Pasquale et al., 1992). In situ hybridization studies have been used to report the graded expression of EphA3, EphA4, and EphA7 receptor mRNAs in ventrobasal (VB) thalamus as early as E16.5 [Fig.2 A], the age when TCAs are found in the intermediate zone beneath the developing CP (Bolz et al., 2004; Dufour et al., 2003; Garel and Rubenstein, 2004; Mackarehtschian et al., 1999; Takemoto et al., 2002; Vanderhaeghen et al., 2000). Although each of these receptors is initially expressed in a medial to lateral gradient across VB [Fig.2 A], their temporal expression profiles are quite different from one another. EphA3 mRNA has been reported to decrease to background levels by P1 (Mackarehtschian et al., 1999). However, EphA7 mRNA has been reported as late as P7 when it is expressed in only the most superior portion of VPM (Torii and Levitt, 2005). EphA4 mRNA has been reported to decrease to background levels as early as E19 (Mackarehtschian et al., 1999), yet others have observed it expressed diffusely across VB at P3 (Vanderhaeghen et al., 2000), and we have observed it expressed in barreloid patches during the postnatal period from P2-P10 (Kenmuir et al., In review.-b). The multi-phasic expression of EphA receptors in VB suggests that they play several distinct roles in TCA connectivity, topography, and boundary formation. In order for these receptors to be effective in any of these roles, ephrin-A ligands capable of interaction with the receptors would need to be expressed by other cells or axons along the path from thalamus to cortex.
**Cortical ephrin-A5**

In 1998, Castellani and colleagues reported that ephrin-A5 ligand was expressed in the cortical plate (CP) from E16-18 [Fig.3 B] and in layer IV at P7 [Fig.3 E,F] while the EphA5 receptor was expressed earlier in the CP from E13-16 [Fig.3 A] and its expression shifted later to the intermediate zone (IZ) and subplate (SP) at E18 [Fig.3 B] and to layers II/III and V at P7 [Fig.3 E,F]. Gao and colleagues (1998) further reported that ephrin-A5 was expressed in infragranular SI at P3 [Fig.3 D] and that in culture models it inhibited the limbic axons from the medial thalamus (that expressed EphA5 receptor) from invading SI territory. Although the laminar cortical location of the ephrin-A5 expression has been debated, expression in layer IV (Bolz et al., 2004; Castellani et al., 1998; Mackarehtschian et al., 1999; Shimogori and Grove, 2006; Vanderhaeghen et al., 2000) versus infra- and supra-granular expression (Gao et al., 1998; Yabuta et al., 2000), ephrin-A5 expression has consistently been reported as graded from medial to lateral across SI [Fig.3 A-F]. It is thought that this graded expression of cortical ephrin-A5, which is higher in medial SI [Fig.3 A-C], repulses the EphA positive TCAs, helping to maintain topography within SI (Prakash et al., 2000; Vanderhaeghen et al., 2000). It has also been suggested that cortical ephrin-A5 expression may serve roles in inter-areal topography or laminar termination (Maruyama et al., 2008; Yabuta et al., 2000).
Ephrin-A5 knockouts

Many laboratories have since investigated these possible roles of ephrin-A5 in thalamocortical connectivity using knock-out and cell culture models. Collectively, these studies have reported a range of mild TCA abnormalities in ephrin-A5 knockouts that has lead to the concept that cortical ephrin-A5 may play a role in maintaining the topographic arrangement of the TCAs once in the PMBSF, but is not necessary for laminar termination of the TCAs in layer IV. One of these ephrin-A5 knockout models was reported to show normal location and spacing of the TCAs in SI barrels, but decreased branching and complexity of their arbors in layer IV (Uziel et al., 2008) suggesting that ephrin-A5 expressed in layer IV is not a stop signal, but is rather a branch promoting factor. This role for ephrin-A5 is further supported by reports that ephrin-A5 stimulation induces branching of TCAs in culture (Mann et al., 2002). Additional reports show that ephrin-A5 knockouts have closer, more overlapping TCA arbors in only the medial barrels of layer IV (where ephrin-A5 expression is highest) while all barrels remained normal in size and shape (Prakash et al., 2000). However, it should be noted that these data are challenged by a report that the PMBSF and individual barrels had decreased in size in ephrin-A5 knockouts (Miller et al., 2006). Furthermore, it has been reported that TCAs from VB thalamus maintained normal connections to SI in ephrin-A5 knockouts, but that the TCAs from the limbic system were now able to invade SI (Uziel et al., 2002). Collectively, these ephrin-A5 knockout experiments suggest a dual role for ephrin-A5 expression in the developing cortex as a repulsive signal to keep limbic thalamic axons from invading SI and as a branch promoting signal for VB TCAs in layer IV.
It should also be noted that ephrin-A5 may have additional roles that single knockout experiments are unable to reveal due to redundancy in the ephrin family or compensatory upregulation of additional ephrins in response to the lack of ephrin-A5. A recent paper used microarray techniques to identify mRNAs upregulated after ephrin-A5 knockout. They reported significant upregulation of ephrin-A2 in SI during the postnatal period in ephrin-A5 knockout mice, which was confirmed using qRTPCR (Peuckert et al., 2008). These increased levels of ephrin-A2 may serve to replace the missing ephrin-A5 and thus may mask additional roles of ephrin-A5 in SI.

Several double knockouts (DKOs) have been designed to further investigate the role of the ephrin-Eph interaction in thalamocortical connectivity. Dufour and colleagues reported that single ephrin-A5 knockouts showed no disruption of SI topography while their single EphA4 knockouts showed some deficits that were inconsistent across animals. However, their ephrin-A5/EphA4 DKO resulted in a significant disruption of intra-areal guidance within SI. TCAs from medial VB that normally terminate in lateral SI were found projecting to both medial and lateral portions of SI. This report suggested that the interaction between the graded expression of ephrin-A5 in SI and the EphA4 expressed in VB may be necessary for establishing the topographic arrangement of TCAs in SI. Furthermore, they reported a consistent inter-areal defect where limbic TCAs projected to SI as well as their normal target (MI) (Dufour et al., 2003). Additionally, Miller and colleagues reported that EphA7 and ephrin-A5 single knockouts showed mild decreases in the size of SI and of individual barrels without topographic deficits. However, their ephrin-A5/EphA7 DKO showed a further reduction in the size effects.
(Miller et al., 2006). These results suggest several possibilities. It is possible that cortical ephrin-A5 stimulates EphA7 positive thalamic axons to branch within layer IV. Without ephrin-A5, compensatory upregulation of ephrin-A2 may provide this branching stimulus. Likewise, compensatory upregulation of an additional EphA receptor may fill the role of missing EphA7 in single knockouts. Taken together with the results from single ephrin-A5 knockouts reported by others (Prakash et al., 2000; Vanderhaeghen et al., 2000), these descriptive and knockout experiments suggest that cortical ephrin-A5 through its interaction with EphA7 serves to stimulate branching of TCAs in layer IV of SI and through its interactions with EphA4 helps to establish or maintain topographic terminations of the TCAs. A role of ephrin-A5 in arborization is further supported by in vitro experiments that showed that ephrin-A5 induces branch formation in TCAs in culture (Mann et al., 2002).

Cortical ephrin-A2

Previous reports by Yamamoto and colleagues have shown that TCAs that normally stop upon reaching their target cells in layer IV no longer terminate in layer IV when treated with phosphatidylinositol phospholipase C, which disrupts GPI anchored molecules including the ephrin-As (Yamamoto et al., 1997; Yamamoto et al., 2000). These results suggest that a GPI-anchored molecule, perhaps an ephrin-A, restrains the incoming axons within layer IV. Although ephrin-A5 has been reported to be expressed in defined lamina within SI (Donoghue and Rakic, 1999; Gao et al., 1998;
Mackarehtschian et al., 1999; Mann et al., 2002), ephrin-A5 mutants and knockouts do not exhibit laminar disruption of the TCAs. Alternatively, a recent report from our laboratory shows that the ephrin-A2 is also expressed within discrete laminae in SI throughout the early postnatal period. Ephrin-A2 is not expressed in layer IV, but was observed at the boundaries of layer IV in layer II/III and V [Fig.3 C-F]. We propose that while ephrin-A5 may promote branching of TCAs in layer IV, that ephrin-A2 restricts branching to the breadth of layer IV (Kenmuir et al., In Review.-a).

EphA receptors in SI

Several EphA receptors have been reported in SI using ISH, although some controversy exists concerning their specific site(s) of expression during development. From E12-E16, EphA3 and EphA4 mRNA has been reported to be expressed diffusely across the SI CP (Yun et al., 2003), in a medial to lateral gradient within the CP (Dufour et al., 2003), or in a lateral to medial gradient within both the CP and SP (Bolz et al., 2004) [Fig.3 A]. During this same time, EphA5 mRNA is expressed evenly across the SI CP (Yun et al., 2003) and EphA7 is expressed in a medial to lateral gradient within the SI CP (Dufour et al., 2003) [Fig.3 A]. From E16-E18, EphA5 is expressed in the SP of SI (Castellani et al., 1998) [Fig.3 B]. Just before birth from E19-E21, EphA4 mRNA expression in the CP becomes graded medial to lateral (Vanderhaeghen et al., 2000) [Fig.3 B]. From P0-P1, EphA4 mRNA expression has been reported to be diffuse within the SI CP (Yun et al., 2003), or within the suprgranular and infragranular layers but not in
the CP (Liebl et al., 2003; Vanderhaeghen et al., 2000) [Fig.3 C]. During this time, EphA3 mRNA is expressed in supragranular layers whereas EphA5 expression is observed in supragranular and infragranular layers (Yun et al., 2003) [Fig.3 C]. On P3-P4, when layer IV has fully differentiated and barrels are visible, EphA4 mRNA has been observed in a weak medial to lateral gradient distributed in patches within layer IV corresponding to the barrels (Vanderhaeghen et al., 2000) [Fig.3 D]. Further, EphA7 has been reported to be expressed in supragranular and infragranular layers of SI again in a lateral to medial gradient (Torii and Levitt, 2005) [Fig.3 D]. From P6-P8 EphA5 mRNA is expressed in layers II/II and V (Castellani et al., 1998); whereas, EphA4 protein is expressed in layer IV and VIa in TCAs (Kenmuir et al., In review.-b) [Fig.3 E].

Although the role of ephrin-Eph interactions in thalamocortical connectivity have been investigated for several decades, proper interpretation of results is often difficult due to the complex interactions of these molecules, leaving us with many unanswered questions. However, building on all of these reports, we propose a partial model for ephrin-A2 and ephrin-A5 involvement in somatosensory connectivity where EphA4 positive axons from VB migrate through the developing cortex until encountering graded ephrin-A5 expression in layer IV. The stimulation by ephrin-A5 induces branch and collateral formation within layer IV, which is restricted by interaction with ephrin-A2 in layers II/III, V, and SP. We propose that ephrin-A2 expression in the postnatal SI cortex may serve an integral role in laminar termination of the TCAs in layers IV, I, and VIa until P18, when ephrin-A2 levels are no longer detectable. This decline in ephrin-A2 expression during the third postnatal week would presumably relieve the restrictive
boundary of TCAs permitting their extension into supragranular layers, which has been reported to occur around P20.

**Conclusions**

Ephrin and Eph proteins are known to play a variety of signaling roles in cortical development from neurogenesis and process outgrowth to cellular migration, axon pathfinding, and topographic innervation of targets. Similar to other sensory systems, several ephrin-A ligands and EphA receptors have been identified in the pre and postnatal somatosensory system, which are necessary for proper TCA connectivity in SI. This review compiles what is known about ephrin and Eph expression (often from ISH studies without protein data) within VB and SI from E12 to P18 and highlights current theories of their functional roles in establishing and maintaining proper connectivity within the rodent somatosensory system.
**FIGURE LEGENDS**

**Fig.1.** Between E14 and E16, VPM axons exit the thalamus, enter the IC, and extend through the intermediate zone (IZ) where they maintain their topographic arrangement in the medial-lateral dimension (A). By E18, the TCAs can be seen gathered beneath the subplate (SP) (B). Over the next two days the TCAs extend through the SP into layers VIa and b (C). By P0, the infragranular layers have fully differentiated from the CP and the TCAs are observed in layers V and VIa (D). On P1, a few simple TCA branches are seen in the developing layer IV (E). By P3, the TCAs have begun to arborize in pre-barrel cylinders within layer IV (F). The TCAs continue to expand and branch for several days within layer IV forming the dense meshwork characteristic of the cortical barrels (G). Several TCAs also send collaterals into layer I around P6-7 (G). Between P18 and P25 the TCAs extend superiorly while forming short collateral branches through layers II/III and a few fibers terminate in layer I (H) where they remain through adulthood.

**Fig.2.** Several EphA receptor mRNAs are transiently expressed across the ventrobasal thalamus (VPM and VPL) beginning on E16.5. EphA3, EphA4, and EphA7 mRNAs are most prominent in the medial-ventral portion of VB and lowest in the lateral-dorsal VB (A). By P3, EphA4 expression has changed substantially, as it is present in barreloid patches (B) where it remains as late as P10. Postnatal EphA7 expression is substantially reduced and has only been reported in the most dorsal aspect of VB. During the early postnatal period, ephrin-A2 and ephrin-A3 are present within the barreloids of the VPM.
Ephrin-A3 is distributed consistently throughout the barreloids, whereas ephrin-A2 is expressed in only the most dorsal barreloid patches. Additionally, ephrin-A5 mRNA is expressed uniformly across VPL during this time. Note in Figure 2 and 3: for the sake of clarity, stripes are used in regions expressing multiple Ephs/ephrins and this does not denote striped expression, but rather homogenous expression within the defined region.

**Fig.3.** The expression of ephrin-A ligands and EphA receptors in SI cortex varies markedly depending on developmental stage. Early in embryogenesis (E12-16.5), EphA3, EphA4, and EphA5 are distributed evenly across SI. EphA7 mRNA is expressed in two discrete bands, both in a lateral to medial gradient. In an opposing gradient, ephrin-A5 is expressed in the across the cortical plate (CP), but in a medial to lateral gradient with an abrupt border at the medial edge of SI (A). Few reports have focused on ephrin expression between E16-E21. Of those, ephrin-A5 is no longer graded across the CP, EphA5 is now expressed only in the subplate (SP), and EphA4 expression has become graded in a medial to lateral distribution across the CP (B). By P0, EphA3, EphA4, and EphA5 are expressed evenly across the supra and infragranular layers of SI, with EphA4 also being expressed homogenously within the CP. EphA7 is still expressed in a lateral to medial gradient across the CP and ephrin-A5 expression, although reduced, is expressed in its early embryonic gradient. Expression of ephrin-A2 is now apparent in the supra and infragranular layers as well as the SP (C). On P3, ephrin-A5 is expressed in layer VI and the SP and ephrin-A2 expression remains unchanged. EphA7 is still expressed in a lateral to medial gradient, but in layers V and II/III rather than the CP as
on P0. Interestingly, EphA4 expression is now expressed weakly within layer IV patches, perhaps in a medial to lateral gradient (D). By P6, ephrin-A3 and EphA4 are expressed uniformly within the layer IV barrels. Additionally, ephrin-A5 is expressed homogenously within layer IV. EphA5 and ephrin-A2 expression remains distributed as they were on P0 (E). By P8, ephrin-A5 is present within layer IV barrels, and EphA4 is now expressed in clustered TCA arbors within layer VIa. EphA5 and ephrin-A2 continue to maintain their distribution patterns throughout the first postnatal week (F).
Figure 1

A

B

C

D

E 15/16

E17/18

E19/20

E21/P0

E

F

G

H

P1

P3

P6/7

P20
Figure 2

A

E16.5-19

VPL
VPM

B

P3-10

Eph A3
Eph A4
Eph A7
ephrin-A2
ephrin-A3
ephrin-A5
LITERATURE CITED


Fraser SE, Hunt RK. 1980. Retinotectal specificity: models and experiments in search of

Pfaff SL, Marquardt T. 2008. Segregation of axial motor and sensory pathways

neurite outgrowth by the Eph ligand ephrin-A5: implications in the development
of thalamocortical projections. Proc Natl Acad Sci U S A 95(9):5329-5334.

Garel S, Rubenstein JL. 2004. Intermediate targets in formation of topographic
projections: inputs from the thalamocortical system. Trends Neurosci 27(9):533-
539.

Hatten ME. 1999. Central nervous system neuronal migration. Annu Rev Neurosci
22:511-539.

Helmbacher F, Schneider-Maunoury S, Topilko P, Tiret L, Charnay P. 2000. Targeting of
the EphA4 tyrosine kinase receptor affects dorsal/ventral pathfinding of limb

Himanen JP, Chumley MJ, Lackmann M, Li C, Barton WA, Jeffrey PD, Vearing C,


Kenmuir C, Chiaia N, Mooney R, Lane R. In Review.-a. Developmental expression of ephrin-A2 in Primary Somatosensory Cortex: A role in restricting the arborization of ventrobasal thalamic axons?.


CHAPTER 7: DISCUSSION

The exquisite organization of the rodent whisker-barrel somatosensory system makes it an ideal model for studying axonal guidance. From the time that somatosensory neurons are born, they begin to encounter guidance molecules. Some of these cues are soluble and exist in diffuse chemoattractant or chemorepulsive gradients that are well suited for gross guidance throughout the brain. Others, including the ephrins, are membrane bound allowing for more precise interactions between two cells, which results in either facilitation of an axon to continue by the cell or disassembly of cytoskeletal elements resulting in repulsion of the axon. The preceding chapters reported recent results from our laboratory investigating the involvement of ephrin-A2, ephrin-A3, EphA4, and EphA7 in thalamocortical and peripheral somatosensory connectivity and discusses their possible mechanistic roles in shaping the development of the sensory circuits.

Molnar and Blakemore (1991) previously suggested that a repulsive signal within the cortex could be necessary for confining TCA termination to layer IV during cortical development. Additional experiments using in vitro models of thalamocortical VB-SI connectivity provided further support for this concept (Bolz et al., 1992; Yamamoto et al., 1997; Yamamoto et al., 1992). The ephrin-A family of guidance molecules are viewed as ideal candidates for this stop signal based on a report from Yamamoto and colleagues who showed TCA overgrowth into supragranular cortical layers following phosphatidylinositol phospholipase C treatment, which disrupts GPI-anchored proteins (Yamamoto, 2002).
Previous studies of ephrin-A involvement in VB-SI connectivity have focused on ephrin-A5. The results from these studies revealed that ephrin-A5 is a potent branching signal for TCAs in layer IV, but that it is not solely responsible for VB axon termination in SI. Our results suggest that supragranular ephrin-A2 expression may provide this additional, and potentially necessary, signal to constrain the laminar distribution of TCAs. We have shown that ephrin-A2, a GPI-anchored guidance molecule, is expressed by cortical neurons throughout the first two postnatal weeks during the time when VB axons arborize in layer IV of SI. Protein and RNA localization methods demonstrated that ephrin-A2 is selectively expressed in the cortical neurons of the layers II/III, V, and the subplate. Quantitative real-time polymerase chain reaction (qRT-PCR) further showed that the expression of ephrin-A2 mRNA in the PMBSF is maximal during the first four postnatal days and then sharply declines by as much as 20-fold by P18. The limited temporal expression of ephrin-A2 suggests that its role in cortical development is transient.

Our laboratory has also identified the expression of ephrin-A3 and EphA4 proteins in barrel patches within layer IV. Using protein and RNA localization methods, the source of both proteins was identified as TCAs and not cortical neurons. The coexpression of ephrin-A3 and EphA4 proteins on VB axons raises questions of their possible physiological role in TCA development. A cell culture model using ephrin-A2 and EphA4 co-transfected cells showed reduced responsiveness to simulation by ephrin-A5 or EphA3 suggesting that co-expression of an ephrin ligand and Eph receptor can attenuate or even silence their ability for intracellular signaling following binding.
These results were somewhat challenged by a report that co-expressed ephrins and Ephs on axons in the motor system do not show attenuation of one another’s signaling, but instead mediate opposite effects depending on whether stimulated by an ephrin or Eph (Marquardt et al., 2005). Recently, this controversy has been abrogated by the discovery that the relative location of the ephrin and Eph within the membrane is critical with respect to their signalling. If the co-expressed ephrin and Eph are physically close enough within the membrane to interact in cis, mutual attenuation of intracellular signaling will result. However, if the ephrin and Eph are localized in separate non-interacting microdomains, no attenuation will occur, and both molecules are able to signal independently (Egea and Klein, 2007). Hence, the partially overlapping and transient nature of ephrin-A3/EphA4 co-expression and their relative membrane distribution may provide mechanisms that allow the TCAs to temporarily penetrate established ephrin barriers within SI at selected times of development.

In 1996, Catalano and colleagues described the connectivity of TCAs into layer IV of SI and reported that the TCAs “remain spatially restricted rather than by overbranching and retracting arbors.” We propose a model of ephrin-A2 involvement in laminar termination within SI whereby the transient interaction of VB axons with cortical ephrin-A5 in layer IV (Bolz et al., 2004) encourages branch formation and elongation as previously suggested (Mann et al., 2002), but as those fibers elongate, they are restricted at the upper and lower borders of layer IV by the presence of ephrin-A2 in the adjoining lamina. Furthermore, we suggest that the substantial reduction in cortical ephrin-A2 and
VB EphA expression that occurs by P18, relieves the resistance and permits the TCAs to expand into layers II/III (Rice, 1985). Furthermore, as mentioned above, co-expression of ephrin-A3 and EphA4 on the TCAs may help to temporally modulate the TCA response to cortical ephrin barriers. Moreover, it is also possible that ephrin-A3 on TCAs may have a role in preventing supragranular pyramidal neurons from arborizing within layer IV via interaction with EphA5 expressed on their surface (Castellani et al., 1998). Future studies in our laboratory will focus on testing these hypothetical functions of ephrin-A3 and EphA4 in the developing postnatal SI.

Peripheral processes of trigeminal ganglion cells also display target-directed growth resulting in precise topographic innervation of the vibrissal pad. Our laboratory can now report the first evidence of ephrin and Eph involvement in the guidance of trigeminal axons to their peripheral targets from E15 to P8. Using immunohistochemistry, we have identified differential spatial and temporal expression patterns of ephrin-A2, ephrin-A3, EphA4, and EphA7. We did not detect any differences in protein expression across the dorsal-ventral or anterior-posterior axis of the WP indicating that their expression may not be involved in establishing or maintaining the topographic connections that match specific ganglion cells with individual follicles. Both ephrin-A2 and ephrin-A3 protein expression were prominent within the trigeminal nerves innervating the vibrissa and remained constant from E15 to P8. EphA4 protein expression was variable from E15 to P8, but did remain constant in the ORS throughout that period. These results suggest the possibility that ephrin-A expression in the trigeminal axon tips may interact with EphA4 expression in the ORS preventing the trigeminal axons from
invading the ORS. This suggestion is consistent with the development of the sensory nerve endings as they form a lattice network on the exterior of the ORS (Dorfl, 1985; Rice and Munger, 1986).

Conclusions

Ephrin and Eph proteins are known to play a variety of signaling roles in cortical development from neurogenesis and process outgrowth to cellular migration, axon pathfinding, and topographic innervation of targets. Results from our laboratory have revealed the presence and distinct distribution of ephrin-A2, ephrin-A3, EphA4, and EphA7 expression within the vibrissal pad, trigeminal ganglia, ventrobasal thalamus, and primary somatosensory cortex. Similar to other sensory systems, several ephrin-A ligands and EphA receptors have been identified in the pre and postnatal somatosensory system, which may be necessary for proper TCA connectivity in SI and trigeminal axon connectivity in the WP. Taken together with the results from other laboratories, we have developed a hypothetical model of thalamocortical connectivity that incorporates details of cortical development and TCA growth with temporal fiber and lamina specific expression of individual ephrins and Ephs. This model suggests that EphA4 positive TCAs interact with cortical ephrin-A5 in layer IV resulting in arborization that is confined to the breadth of layer IV by supra and infragranular ephrin-A2 expression. Furthermore, we have identified the presence of ephrin-A2 and ephrin-A3 in trigeminal axons as they invade the whisker pad and approach the vibrissal follicles. The outer
epithelial root sheath (ORS) cells that line the follicle express EphA4 protein, which likely prevents ephrin-A positive axons from penetrating the ORS resulting in the final distribution of their sensory axons encompassing the ORS. Although these results serve to address several of the outstanding questions concerning axon guidance in the developing somatosensory system, many more remain unanswered and our future work will continue to address these issues.
CHAPTER 8: LITERATURE CITED


Kenmuir C, Chiaia N, Mooney R, Lane R. In review.-a. Developmental expression of ephrin-A2 in Primary Somatosensory Cortex: A role in restricting the arborization of ventrobasal thalamic axons?.


Rasband W. 1997-2004. ImageJ, National Institutes of Health, Bethesda, Maryland, USA.


the developing mouse neocortex: regional patterns reveal intrinsic programs and

The proper function of the nervous system depends on the development and maintenance of exquisitely precise and often long-reaching neural circuits. This requires that immature neurons are accurately guided to their final destination, connected to their correct targets, and often restricted at that target to prevent the formation of aberrant connections. The developmental timecourse and precise topographic arrangement of the rodent whisker-barrel somatosensory system has been well documented making it an attractive model for studying the molecular aspects of axonal guidance needed for the accurate development of these connections. The membrane bound Eph family of receptor tyrosine kinase guidance molecules is well suited for the precise control of connectivity necessary in the somatosensory system. Using RNA and protein detection methods, the temporal and spatial expression patterns of ephrin-A2, ephrin-A3, EphA4, and EphA7 have been described within the vibrissal pad, trigeminal ganglia, ventrobasal thalamus, and primary somatosensory cortex. The possible roles of these guidance molecules in the development and organization of the somatosensory system are discussed.