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The Role of Semaphorin/Neuropilin Signaling in the Development of

Innervation and Avascularization of the Cornea

by

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ABSTRACT

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Millions of corneal procedures performed every year and a multitude of corneal diseases can damage the corneal nerves or induce neovascularization into the cornea; a complete understanding of the molecular regulators of nerve and blood growth into the cornea is necessary to help keep this tissue properly innervated and avascular during a disease state. Identifying the developmental regulators that establish avascularity and innervation have great potential for therapeutic use in the adult.

The development of corneal innervation has only been studied in the avian system, but current molecular investigations into regulators of innervation and avascularity are done in the mammalian system. I performed a detailed analysis of the development of mammalian corneal innervation. This information can now be used for any future studies in this area. My work has also identified molecular regulators that help to control the development of corneal innervation and vascularization in both the avian and mammalian system. Semaphorin3A (Sema3A) is a known axon repellent and inhibitor of endothelial cell migration. This protein is known to...
affect avian cornea development, but it is unknown if this molecule affects mammalian cornea development or avascularity. Through the use of immunostaining, in situ hybridization, qRT-PCR, genetic and pharmacological inhibition of Sema3A, and implantation of recombinant Sema3A, I have discovered that Sema3A plays a key role in the timing of innervation into the mammalian cornea, and the development of an avascular cornea in both the avian and mammalian systems. This is the first time Sema3A has shown to be essential in both the mammalian cornea development and in establishing an avascular cornea. This information could serve as a springboard for further investigation into potential therapeutic drugs for diseases of cornea innervation or neovascularization.
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# TABLE OF CONTENTS

Abstract ii
Acknowledgments iv
Table of Contents v
Abbreviations x
List of Figures and Tables xii

**Chapter 1: Introduction** 1

1.1 Corneal Structure and Function 1
   1.1.1 Cornea Epithelium 2
   1.1.2 Bowman’s Layer 4
   1.1.3 Corneal Stroma 4
   1.1.4 Descemet’s Membrane 5
   1.1.5 Cornea Endothelium 7

1.2 Development of the Cornea 7
   1.2.1 Avian Corneal Development 8
   1.2.2 Murine Corneal Development 8

1.3 Corneal Innervation 9
   1.3.1 Development of Avian Cornea Innervation 13
   1.3.2 Molecular Guidance of Corneal Innervation 15
   1.3.3 Semaphorins in Axon Guidance 16

1.4 Corneal Avascularity and Neovascularization 18
   1.4.1 Molecular Regulators of Angiogenesis 20
1.4.1 The Role of Sema3A/Npn1 in Angiogenesis 22

1.5 Development of Corneal Avascularity 24

1.5.1 Development of Avian Pericorneal Blood Vessels 24

1.5.2 Development of Murine Pericorneal Blood Vessels 25

1.6 Summary 27

Chapter 2: Materials and Methods 29

2.1 Animals 29

2.2 Antibodies and Immunostaining 31

2.3 Classification of Innervation 32

2.4 Quantitative Analysis of Innervated Quadrants 32

2.5 In Situ Hybridization 32

2.6 Quantitative Analysis of Axon Lengths 34

2.7 Quantitative Real-Time PCR 36

2.8 Lens Ablation 36

2.9 Peptide Injection 37

2.10 Bead Implantation 37

2.10.1 Quantification of Anterior Eye Vasculature following Bead Implantation 37

Chapter 3: Development of Innervation in the Mouse Cornea 40

3.1 Early Innervation of the Eye 40

3.2 Innervation of the Corneal Stroma 43

3.3 Innervation of the Corneal Epithelium and Formation of the Sub-basal
Chapter 4: Distinct Roles for Semaphorin3A/Neuropilin1 and Semaphorin3F/Neuropilin2 Signaling During Mouse Corneal Innervation

4.1 Spatiotemporal Expression of Npn1 and Npn2 by the Trigeminal Ganglion and Sema3A and Sema3F by Ocular Tissues during Mouse Eye Development

4.1.1 Expression Pattern of Npn1 and Sema3A during Mouse Corneal Innervation

4.1.2 Expression Pattern of Npn2 and Sema3F during Mouse Corneal Innervation

4.2 Npn1, but not Npn2, Regulates Innervation of the Mouse Corneal Stroma

4.3 Innervation of the Corneal Quadrants in Npn1Sema-/- and Npn2-/-

4.4 Npn1 and Npn2 Play a Role during Innervation of the Corneal Epithelium

4.5 Corneal Innervation Defects are Enhanced in Npn1Sema-/-;Npn2/-
Double Mutants 68

4.6 Discussion 71

4.6.1 The Expression Patterns of Npn1, Npn2, Sema3A and Sema3F 71

4.6.2 Innervation of Npn1\(^{Sema-/-}\) Mutant Embryos 74

4.6.3 Innervation of Npn2\(^{-/-}\) Mutant Embryos 75

4.6.4 The Necessity for Both Sema3A/Npn1 and Sema3F/Npn2 Signaling in the Development of Corneal Innervation 76

4.6.5 Conclusions 78

Chapter 5: Semaphorin3A is Necessary to Establish an Avascular Cornea during Development 79

5.1 Npn-1 is Expressed by Angioblasts and Blood Vessels that Form Adjacent to the Developing Cornea 80

5.2 Localization of Sema3A and VEGF-A mRNA and Protein in the Developing Anterior Eye 83

5.3 The Lens Plays a Role in Establishing an Avascular Cornea 85

5.4 Sema3A Inhibition \textit{in vivo} Results in Ectopic Migration of Endothelial Cells into the Presumptive Cornea 87

5.5 Sema3A Inhibits VEGF Induced Endothelial Cell Migration in the Anterior Eye 92

5.6 Discussion 94

5.6.1 The Role of Npn1 in Corneal Avascularity Development 94
5.6.2 The Location and Expression of Sema3A and VEGF-A in Cornea Avascularity Development 95
5.6.3 The Role of the Lens in Corneal Avascularity Development 97
5.6.4 Sema3A is Required to Develop an Avascular Cornea 98
5.6.5 Sema3A Can Inhibit Pro-Angiogenic Molecules in the Anterior Eye 99

Chapter 6: Summary, Significance and Future Directions 101
6.1 Summary and Significance 101
6.2 Future Work 107
Bibliography 110
Abbreviations

DN; Dorsal-nasal quadrant of the anterior eye
DT; Dorsal-temporal quadrant of the anterior eye
DAPI; 4,6-diamidino-2-phenylindole, a nuclear stain
E; embryonic day
EPI; epithelium
FACIT; fibril-associated collagens with interrupted triple helices
IR; iris
LASIK; laser assisted in situ keratomileusis
LL; lower eyelid
NCA; nasal long posterior cilliary artery
Npn; neuropilin
OC; optic cup
P; post-natal day
PBS; phosphate buffered solution
PBT; phosphate buffered solution containing 0.1% (v/v) Tween
PC; presumptive cornea
PFA; paraformaldehyde
PR; periocular region
PRK; photorefractive keratectomy
Sema; semaphorin
sflt-1; soluble vascular endothelial growth factor receptor 1
ST; stroma

TCA; temporal long posterior ciliary artery

TVL; tunica vasculosa lentis

UP; upper eyelid

VEGF; vascular endothelial growth factor

VEGFR1; vascular endothelial growth factor receptor 1, also known as Flt-1

VEGFR2; vascular endothelial growth factor receptor 2, also known as KDR or FLK-1

VHP; vasa hyaloidea propria

VN; Ventral-nasal quadrant of the anterior eye

VT; Ventral-temporal quadrant of the anterior eye

WP; whisker pad
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Microphotograph of human cornea</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>Collagen fibrils in the corneal stroma</td>
<td>6</td>
</tr>
<tr>
<td>1.3</td>
<td>Innervation of the corneal stroma and epithelium</td>
<td>12</td>
</tr>
<tr>
<td>1.4</td>
<td>Structure of Npn1 and its binding sites</td>
<td>23</td>
</tr>
<tr>
<td>1.5</td>
<td>E15.5 mouse ocular vasculature</td>
<td>26</td>
</tr>
<tr>
<td>2.1</td>
<td>Orientation of the mouse embryo anterior eye quadrants</td>
<td>33</td>
</tr>
<tr>
<td>2.2</td>
<td>Quantification of axons using NeuronJ</td>
<td>35</td>
</tr>
<tr>
<td>2.3</td>
<td>Quantification of the iridial ring artery and any corneal endothelial cells</td>
<td>39</td>
</tr>
<tr>
<td>3.1</td>
<td>Innervation of the anterior ocular region at E12.5</td>
<td>42</td>
</tr>
<tr>
<td>3.2</td>
<td>Projection of nerve bundles towards the presumptive cornea at E13.5</td>
<td>44</td>
</tr>
<tr>
<td>3.3</td>
<td>Innervation of the mouse cornea stroma</td>
<td>46</td>
</tr>
<tr>
<td>3.4</td>
<td>Innervation of the mouse corneal epithelium</td>
<td>49</td>
</tr>
<tr>
<td>4.1</td>
<td>Expression of Npn1 and Npn2 and their ligands Sema3A and Sema3F</td>
<td>58</td>
</tr>
<tr>
<td>4.2</td>
<td>Innervation of the corneal stroma is defective in Npn1Sema-/- but normal in Npn2-/- mutant embryos</td>
<td>62</td>
</tr>
<tr>
<td>4.3</td>
<td>Loss of Npn1 signaling through Sema disrupts the pattern of innervation of the corneal quadrants</td>
<td>65</td>
</tr>
<tr>
<td>4.4</td>
<td>Timing of the innervation of the corneal epithelium is disrupted in Npn1Sema-/- and Npn2-/- mutants</td>
<td>67</td>
</tr>
</tbody>
</table>
Figure 4.5 Increased disruption of corneal innervation in $Npn1^{Sema-/-};Npn2^{-/-}$ double mutants 70

Figure 4.6 Proposed model for Sema/Npn signaling during mouse corneal innervation 77

Figure 5.1 Anterior eye vasculature and $Npn1$ expression during cornea development 81

Figure 5.2 Presence of Sema3A and VEGF-A protein and quantification of their respective mRNA in the developing anterior eye 84

Figure 5.3 Aberrant endothelial cells are present in the developing cornea after lens ablation 86

Figure 5.4 Lens injection of Sema3A blocking peptide induces endothelial cell presence in the developing cornea 89

Figure 5.5 $Npn1^{Sema-/-}$ mice display endothelial cells in the cornea during development 91

Figure 5.6 Effects of protein soaked beads on the endothelial cells proximal to the cornea during development 93

Fig 6.1 Comparison of key time points in avian and murine innervation and avascularity 105
INTRODUCTION

1.1 Corneal Structure and Function

The cornea is the anterior most segment of the eye and is essential for proper eyesight as it is responsible for two-thirds of the total refraction of light traveling to the retina. Clinically important refractive error, such as nearsightedness, farsightedness or astigmatism, affects half of the US population 20 years or older (Vitale et al., 2008). The cornea’s powerful refractive property has made it one of the most surgically altered tissues in the body with procedures such as laser-assisted in situ keratomileusis (LASIK), in which the refractive error is restored by reshaping the corneal stroma using advanced laser techniques. To maintain the proper refractive index, the cornea must remain completely transparent and avascular. The presence of blood vessels and/or opaque areas obstructs the light path through the eye, causing poor eyesight or blindness. If a cornea becomes too diseased or scarred, a corneal transplant may become necessary. The cornea is also one of the most highly transplanted tissues in the world, with approximately 40,000 cornea transplants annually in the United States (Stark et al., 1992).

The cornea also serves to protect the eye. This smooth but durable tissue acts as a physical barrier to keep dust, germs or other foreign material away. It also serves to help protect the posterior eye from ultraviolet radiation by absorbing 92% of UV-B and 60% of UV-A radiation (Zigman, 1993). Excessive amounts of UV radiation will start to alter the cornea structure, but the cornea is
designed to absorb the majority of UV-B radiation before it starts to damage the more susceptible interior parts of the eye (Zigman, 1993). The cornea performs these functions through five specialized tissue layers: the epithelium, the Bowman’s layer, the stroma, the Descemet’s membrane, and the endothelium (Fig 1.1).

1.1.1 Corneal Epithelium

The cornea epithelium is the most anterior layer of the eye. It plays the largest role in protecting the cornea from foreign objects, aids in absorption of oxygen from the atmosphere and its transport to posterior layers. This stratified epithelium comprises about ten percent of total corneal thickness (Dohlman et al., 1968). Cells closest to the basement membrane are more round, but as cells are pushed up toward the anterior surface the cells start to elongate, flatten and form many cell-cell desmosomal attachments. This forms a tight protective barrier from substances that are not lipid soluble, and makes this layer proficient as a mechanical barrier to bacteria and fungi. While an important barrier, the epithelium must also be well hydrated and extremely smooth to maintain proper optical transparency. This is most likely accomplished with the help of the tear film, produced by meibomian glands, the lacrimal gland and goblet cells in the conjunctiva. Tiny villi are located on the surface cells of the outer most layer of the epithelium and are thought to play a role in keeping the tear film in place (Dohlman, 1971). Surface irregularities are common in many corneal diseases and contribute to a significant reduction in vision. The epithelium is also the most
Figure 1.1 Microphotograph of human cornea (haematoxylin and eosin staining, magnification ×100).
The outermost layer of the cornea is the epithelium, Ep, followed by the Bowman's layer, BM. The corneal stroma (substantia propria), SP, consists mainly of collagen lamellae. The inner surface of the cornea is formed by endothelial cells, En, which are supported by Descemet's membrane, DM. Modified from (Hornof et al., 2005).
innervated surface tissue of the body, with 300-600 times the innervation density of the skin (Rozsa and Beuerman, 1982). Corneal nerves are essential for proper maintenance of a healthy epithelial surface (Muller et al., 2003), and create a highly sensitive tissue. The importance, function and anatomy of corneal innervation will be discussed later in this dissertation.

1.1.2 Bowman’s Layer

The Bowman’s layer is acellular and located between the epithelial basement membrane and the corneal stroma. The major components of this membrane are collagen fibrils consisting of type I and V collagens (Linsenmayer et al., 1998). The cornea stroma and Bowman’s layer are the only layers of the cornea to contain collagen fibrils. The collagen fibrils in the Bowman’s layer do not form bundles, like the stroma, but instead run in various directions to form a sheet about 8-12 μm thick (Komai and Ushiki, 1991).

The Bowman’s layer is non-regenerative and is destroyed when people undergo surgeries, however, no adverse consequences have been reported from the loss of this layer. It is therefore speculated that this membrane is not an important layer in the health or function of the cornea (Wilson and Hong, 2000).

1.1.3 Corneal Stroma

The corneal stroma comprises the majority of the corneal thickness. It consists of neural crest derived keratocytes embossed in a collagen-rich extracellular matrix. Collagens make up more than 70% of its dry weight (Leonard and Meek, 1997). Collagen type I and type V are the main collagen
types that form the collagen fibrils, with types VI, IX, XII, and XIV acting as FACIT collagens (fibril-associated collagens with interrupted triple helices), that associate with the collagen fibril surface and help link the fibrils together (Linsenmayer et al., 1998). These fibrils run parallel to each other with somewhat regular spacing to form flat orthogonally arranged corneal lamella (Fig 1.2) (Bard and Bansal, 1987; Trelstad and Coulombre, 1971). Corneal transparency, strength, elasticity and form depend on how these fibrils are ordered. Along with FACIT collagens, proteoglycans, macromolecules with a protein core and covalently linked glycosaminoglycan side chains, also play an important role in modifying the structure and function of the fibrils. The predominant glycosaminoglycans of the cornea are dermatan sulfate (~50%) and keratan sulfate (~50%), with smaller amounts of heparan sulfate (Michelacci, 2003). Within each stromal lamellum, the collagen fibrils are packed in an orderly, parallel array with keratocytes present in between the lamellae and proteoglycans decorating the fibrils (Almubrad and Akhtar, 2011).

### 1.1.4 Descemet's Membrane

The Descemet's membrane is a basement membrane for the corneal endothelium, which is the most posterior layer of the cornea. It is produced by the endothelium, continues to grow as humans age, is extremely resistant to infection and is easily regenerated after injury. This layer is weakly attached to the stroma and is a successful transplant tissue for diseases such as Fuchs'
**Figure 1.2 Collagen fibrils in the corneal stroma.**
Collagen fibrils in the corneal stroma are arranged in the same direction within the lamellae and each lamellae is organized orthogonally to an adjacent lamellae (indicated with parallel fibrils running horizontally in one layer and the adjacent layer showing the diameter of the fibrils running into in the plane 90° away). K = keratocyte (X32,000). Modified from (Komai
endothelial dystrophy and pseudophakic bullous keratoplasty (Guerra et al., 2011).

1.1.5 Corneal Endothelium

The corneal endothelium is a thin layer of cells lining the posterior side of the cornea and plays a vital role in fluid maintenance and nutrition uptake for the cornea. The cornea is avascular, therefore many of the nutrients necessary for cellular survival are acquired from the anterior chamber and must pass through the endothelium. The endothelium acts as a pump, allowing the appropriate amount of fluid and nutrients into the cornea to maintain the cornea at the appropriate homeostatic conditions. Endothelial cells have a limited capacity to regenerate in the adult, so when the endothelial layer is injured or damaged, the only effective remedy available is the corneal transplant (Murphy et al., 1984).

1.2 Development of the Cornea

Many aspects of corneal development are shared among species with some differences, which are discussed below. Corneal development is initiated by the formation of the adjacent lens tissue. The lens invaginates and detaches from the surface ectoderm, also known as the primitive corneal epithelium, and plays an important role in the start of corneal development. The corneal epithelium has altered thickness during development depending on the presence or absence of eyelid closure with more cellular layers in the absence of lid closure. In humans, at six weeks of development, mesenchymal cells proliferate
and migrate into the stroma. The periphery of the cornea develops mesenchymal cells first with the middle third of the stroma remaining acellular. As development progresses, the stroma continues to accumulate mesenchymal cells and forms several layers. The majority of stromal cells condense to a thick layer that will eventually become the endothelial layer with tight junctions forming at about four months of development. The remaining stromal cells continue to proliferate and migrate from the pericorneal area to fill the future cornea (Cvekl and Tamm, 2004; Sevel and Isaacs, 1988).

1.2.1 Avian Corneal Development

The avian cornea is a highly studied tissue due to its ease of accessibility during development in the egg. The initiation of corneal development in the avian eye is similar to the human process of initiation with both processes starting with the invagination of the lens. An early acellular stroma forms consisting of about 30 strata of collagen fibrils located between the epithelium and the lens during early development. Neural crest derived mesenchymal cells migrate from the periphery of the cornea between the acellular stroma and the lens during embryonic day 4-5 (E4-5) forming the corneal endothelium and the anterior chamber. At about E5.5 a second wave of mesenchymal cells migrate to form the cells of the stroma, which eventually becomes the keratocytes. Thus, the avian system differs from the mammalian system in the development of an endothelial layer that forms first before the migration of stromal cells occurs (Hay, 1979).

1.2.2 Murine Corneal Development
Murine eye development is very similar to human eye development with the only major difference being the developmental timing of the ciliary body and iris. The murine cornea development follows the same pattern as human, with the start of cornea formation occurring when the lens invaginates and separates from the surface ectoderm. The mesenchyme cells migrate between the lens and ectoderm to form as many as 4-7 layers of presumptive stromal cells at E12.5. As more cells enter the cornea from E14.5-15.5, the stromal cells condense and flatten into layers, but are separated from other stromal cells by a loose fibrillar extracellular matrix. Between E14.4-15.5 the posterior layers of these stromal cells, closest to the lens, flatten and form continuous bands of junctional complexes with adjacent cells and this eventually forms the endothelial layer. By E15.5 all layers of the future cornea have been defined (Cvekl and Tamm, 2004; Pei and Rhodin, 1970; Qazi et al., 2011).

1.3 Corneal Innervation

A dense population of corneal nerves is crucial for a variety of purposes in the cornea: for a proper response to irritation and pain, to activate brainstem circuits that stimulate reflex tear production and blinking, and to secrete neuropeptides that maintain the functional integrity of the cornea (Belmonte and Gallar, 1996; Marfurt et al., 1998; Marfurt et al., 1989; Morgan et al., 1987; Tervo, 1978). Damage to cornea nerves can cause transient or chronic neurotrophic deficits and decreased integrity of the ocular surface.
The cornea is unique as the majority of its nerves are dedicated to sensory perception (Marfurt et al., 1989). The sensory nerves are supplied by the trigeminal ganglion, mainly from the ophthalmic division (Lwigale, 2001). The innervated cornea contains a higher density of free nerve endings than any other peripheral tissue (Rozsa and Beuerman, 1982), making it one of the most sensitive tissues of the body. The conduction velocities and responses to both mechanical and thermal stimulation of corneal nerves are similar to other sensory receptors associated with pain, known as nociceptors (Tinsley et al., 1988). These sensory nerves express a variety of active neurochemicals: substance P, calcitonin gene-related peptide, pituitary adenylate cyclase-activating peptide, and galanin (Muller et al., 2003). To date, there are a total of 17 different neuropeptides and neurotransmitters detected within corneal nerves, and it seems likely that some corneal nerves do not express any currently recognized neuropeptides (Muller et al., 2003).

Nerve-derived tropic factors serve an important role in epithelial cell maintenance. When nerves are absent, there is a significant decrease in the epithelium’s ability to heal after injury, and increased spontaneous epithelial erosions (Araki et al., 1994; Beuerman and Schimmelpfennig, 1980). The neurons and epithelial cells seem to support one another trophically (Muller et al., 2003). Trigeminal neurons release neurotransmitters and neuropeptides that stimulate corneal epithelial cell growth, proliferation, differentiation and collagen production (Baker et al., 1993; Garcia-Hirschfeld et al., 1994). In turn, corneal
epithelial cells release soluble factors that promote neurite extension and survival (Chan and Haschke, 1982).

Innervation of the adult cornea has been widely studied in mammals (Jones and Marfurt, 1991; Marfurt et al.; Muller et al., 2003; Zander and Weddell, 1951). These studies revealed that there are no major differences in adult corneal innervation among species including mouse, rabbit, dog, cats, and humans. In adult corneas, nerves originate from the limbus, the area bordering the cornea and the sclera (Muller et al., 2003). As the nerves project into the stroma and extend centrally, the nerve fibers are positioned in the anterior third of the stroma. Some research suggests that adult murine corneas can have nerves present as close as a few micrometers from the endothelium (Henriksson et al., 2009). The murine system may allow corneal nerves to be present in deeper layers of the corneal stroma than other models. The importance of this attribute is unknown. As the major stromal nerves travel centrally into the cornea they arch toward the anterior. As they reach the anterior layers, the nerves either branch 90° to travel anteriorly toward the epithelium, or bifurcate into a subepithelial nerve plexus (Auran et al., 1995). The nerves that enter the epithelium then run parallel to the surface of the cornea and form the basal epithelial nerve plexus. This plexus is composed of smaller nerves bundles or “leashes” that run parallel to each other (Fig 1.3). The nerve leashes travel towards the center of the cornea and form a swirling pattern near the apex of the cornea (Auran et al., 1995). This pattern has been identified in multiple species and can form in either a clockwise
Figure 1.3 Innervation of the corneal stroma and epithelium.
Innervation enters the cornea in the mid stroma from the limbus region and innervated the upper third of the stroma. Innervation in the epithelium forms basal epithelial leashes that travel parallel to the center of the cornea. Intraepithelial terminals are located throughout the epithelial surface of the cornea. Modified by (Chan-Ling, 1989).
or counterclockwise formation. A small percentage of corneas do not show a prominent swirl in either direction but rather a convergence and seam-like gathering near the apex (Dua et al., 1993; Leiper et al., 2009; Marfurt et al., 2009; Patel and McGhee, 2005).

While nerve damage from injury or disease can lead to neurotrophic keratitis, a condition that leads to the degeneration of the cornea (Bonini et al., 2003), there is a dramatic increase in cornea nerve related pain in recent years due to the increase of contact lens wear and refractive correction surgeries such as photorefractive keratectomy (PRK) and LASIK. In PRK the surface epithelium of the cornea is completely removed along with the sub-basal nerves and some superficial stromal nerves. In LASIK a corneal flap is created that cuts through the sub-basal nerves and the deeper stromal nerves and during the laser ablation the deep mid stromal nerves beneath the flap can be destroyed as well. The ablation of these nerves is thought to increase the occurrence of dry eye. In most cases, corneal sensation returns to normal in 3-12 months, however, 20-60% of patients that receive LASIK report worse or significantly worse eye dryness at least six months after their surgeries than before (Bailey and Zadnik, 2007; Hovanesian et al., 2001). It can take years for nerves to fully restore to pre-surgery densities (Erie et al., 2005).

1.3.1 Development of Avian Corneal Innervation

The development of corneal innervation is best documented in the avian system. No previous research has sought to determine if this development may
be different in other species, and the detailed work done by James Bee and others in the chick (Bee, 1982; Bee et al., 1986) is often cited for corneal nerve development irrespective of the model system being used. Bee used modified Bodian gold histological staining procedures and found that prior to the radial entry of nerves into the cornea, the nerves approach the eye from the ventrotemporal region and start to surround the cornea with a dorsal and a ventral branch until a ring of nerve fascicles is formed that completely surrounds the cornea in just a few days at E8. After the ring is formed, the fascicles start to innervate the cornea in a radial manner at multiple positions that are very specifically defined. As the nerves extend into the cornea the fascicles start to bifurcate, and it becomes apparent that these bifurcations happen at distinct distances from the nerve ring, forming concentric zones of bifurcation. Nerve bifurcation is not dependent on a fixed distance along the fascicle, but on a fixed distance from the periphery of the cornea. This indicates that the corneal microenvironment may play a role in controlling the pattern of innervation once the nerves enter the cornea. However, no current studies have investigated the molecular mechanisms of this control. Fascicles enter at approximately the mid-stroma and travel toward the center of the cornea where they start to innervate the epithelium. The nerves that pass through the Bowman’s membrane and enter into the epithelium appear only to do so in the periphery of the cornea. However in human adult studies, nerves pass through the membrane to the epithelium throughout the cornea, not just the periphery (Zander and Weddell, 1951). It is
unknown when the transition of penetration of the Bowman’s layer switches from the periphery to the entire cornea.

These studies established that avian innervation development occurs in stages. The first stage is the arrival of nerves to the periphery of the cornea and the formation of a pericorneal nerve ring. The nerves entering the corneal stroma establish the second stage and the final stage is the growth of nerves into the corneal epithelium. It is thought that through the orchestration of positive and negative molecular growth factors that the nerves are repelled from the cornea to form a pericorneal nerve ring and then are allowed to enter the cornea at a specific time during development.

1.3.2 Molecular Guidance of Corneal Innervation

During development, axons often travel amazingly far distances away from the cell body; sometimes the distance is more than a thousand fold the diameter of their cell body. This incredible task is accomplished by guidance cues in the nerve’s extracellular environment. Guidance cues can be either attractive or repulsive and either short (i.e. cell bound) or long range (secreted or diffused). As a nerve travels toward a destination, it can be attracted through a tissue corridor by attractive cues from the cells lining the corridor as well as respond to negative cues, thus preventing innervation of inappropriate areas in surrounding tissues. Some growth factors including neurotrophins such as Nerve Growth Factor Brain-Derived Neurotrophic Factor and morphogens such as Hedgehog or Wnt play a role in axon guidance. Four other major groups of axon guidance molecules that
have been the most investigated: the semaphorins, netrins, ephrins, Slits, and
their receptors also play important roles (Carmeliet and Tessier-Lavigne, 2005).
Specifically, the semaphorins play an important role in axon guidance in the
cornea (Kubilus and Linsenmayer, 2010; Lwigale and Bronner-Fraser, 2007;
Schwend et al., 2012).

1.3.3 Semaphorins in Axon Guidance

Semaphorins (Semas) are a large family of proteins divided into eight
classes. Classes 3-7 are found in vertebrates. All Semas have a conserved
cysteine-rich approximately 420 amino acid Sema domain. Semas signal through
multimeric signaling complexes, usually made up of Neuropilins (Npn) and
plexins. Npn has a short (40 amino acid long) cytoplasmic domain, and the
absence of this domain does not affect Sema signaling ability (Nakamura et al.,
1998). Npn forms complexes with plexins and the plexin provides the signal
transduction in this complex (Rohm et al., 2000). Plexins are unable to bind
directly to Semas and lack a tyrosine-kinase activity of their own, but their
intracellular domain undergoes phosphorylation on tyrosine residues and its
presence increases the affinity of Sema to Npn compared to Npn alone
(Takahashi et al., 1999; Tamagnone et al., 1999). Once Semaphorins bind the
Npn/plexin complex, a signal-transduction event occurs that ultimately leads to
modification in the cytoskeleton (Schwarz and Ruhrberg, 2010). Filopodia and
lamellipodia are retracted and a localized rearrangement of the actin cytoskeleton
occurs (Fournier et al., 2000).
The secreted class 3 Semas are capable of long range diffusion and are one of the most studied axon guidance molecules. Sema3A signals through Npn1-plexinA4 and Sema3F signals through Npn2-plexinA3 complexes (Suto et al., 2005; Yaron et al., 2005). Mutant mice lacking proper function of Sema3A or its receptor, Npn1, show drastic defasciculation of the trigeminal ganglion during development (Kitsukawa et al., 1997; Taniguchi et al., 1997; Ulupinar et al., 1999). The defasciculations are quite severe such that the ophthalmic and maxillary subdivisions of the trigeminal ganglion are indistinguishable from one another. Because the cornea is only innervated by the trigeminal ganglion, and this ganglion appears to rely on Sema3A/Npn1 for proper axon extension and navigation, there is a strong possibility that this signaling is required for proper corneal innervation. Sema3A-/- mice show premature corneal innervation, and presence of nerves within the normally nerve devoid lens vesicle at E12.5 (Taniguchi et al., 1997). This information defines the extent of previous observation for how Sema3A affects the development of mammalian corneas. In the avian system, Sema3A has been more thoroughly investigated in corneal development.

Sema3A plays a distinct role in avian corneal innervation (Kubilus and Linsenmayer, 2010; Lwigale and Bronner-Fraser, 2007). Expression patterns of Sema3A and its receptor Npn1 suggest a role in the formation of a pericorneal nerve ring as well as in timing of innervation into the cornea epithelium. There is strong Sema3A expression in the chick lens throughout cornea development.
When the lens is ablated at E4 in the chick, the nerve ring that surrounds the avian cornea becomes diminished and ectopic nerves enter the cornea prematurely. Similar results were observed when a Sema3A inhibitory peptide was injected into the lens at E4 (Lwigale and Bronner-Fraser, 2007). These findings indicate that Sema3A derived from the lens plays a critical role during pericorneal nerve ring formation and is required for proper patterning of corneal innervation.

The Npn family consists of two single span transmembrane proteins, Npn1 and Npn2. While Sema3A bind to Npn1, Sema3F binds to Npn2 (Giger et al., 1998). Npn2 has similar domain structures as Npn1, but the two share only a 44% amino acid homology (Chen et al., 1997). Both Npn1 and Npn2 play important roles in axon guidance during development (Chen et al., 2000; Huber et al., 2005; Walz et al., 2002). Similar to Sema3A mutant mice, mice homozygous for Npn1 or Npn2 deletions have defasciculations in the branches of the trigeminal ganglion in early development (Chen et al., 2000; Giger et al., 2000). The necessity of Npn1 and Npn2 for the appropriate development of the trigeminal ganglion suggests that this receptor plays a role during corneal innervation.

1.4 Corneal Avascularity and Neovascularization

The cornea has an “angiogenic privilege,” a distinct avascular state, which must be maintained through the delicate balance of pro- and anti-angiogenic molecules (Azar, 2006; Cursiefen, 2007). However, this equilibrium of pro- and
anti-angiogenic molecules is not unique to the cornea. The loss of this balance in numerous organs can lead to malignant, ischemic, inflammatory, infectious and immune disorders (Carmeliet, 2003). Cancer, autoimmune disorders, obesity, psoriasis, endometriosis, and arthritis are common examples when a tip in the angiogenic balance enhances disease progression (Carmeliet, 2003). When a cornea is wounded, hypoxia or inflammation affects the balance of these molecules, which can tip towards the pro-angiogenic side and blood vessels can enter the cornea causing disease and visual impairment when they persist (Azar, 2006). An estimated 1.4 million Americans suffer vision impairment due to cornea neovascularization (Lee et al., 1998). Also of concern is the high risk of rejection in corneal transplants. The cornea is the most common and successful tissue transplant in humans with a two-year survival rate of 90 percent on uninflamed corneas, but this rate drastically drops to less than 50 percent if the cornea is inflamed and/or vascularized (Cursiefen et al., 2003; Maguire et al., 1994). To date, there is no effective treatment for this situation, and current pharmacotherapy of corneal transplant rejections are fraught with side effects such as glaucoma, cataracts, and opportunistic infections (Chen, 2009).

Research into how the cornea uses anti-angiogenic molecules to establish an avascular state may lead to therapeutic opportunities in ophthalmology as well as in oncology and other angiogenic diseases.

The cornea has been used to study neo-vascularization and angiogenesis since the early 1970’s. Recent investigations of how the cornea maintains its
avascularity in a healthy state have concluded that avascularity is not a passive process, but rather an active process of balancing anti-angiogenic factors with pro-angiogenic factors. Several conditions, such as wounding, hypoxic state, inflammation or infectious disorders of the ocular surface, can lead to neovascularization of the cornea. When there is a decrease of anti-angiogenic factors and/or an increase of pro-angiogenic factors in the cornea, there is an induction of neovascularization into the corneal matrix. To initiate the neovascularization process, metalloproteinases degrade the extracellular matrix of the cornea and endothelial cell migration can arise from the capillaries and venules of the pericorneal plexus in the adjacent limbal region (Ellenberg et al., 2010).

1.4.1 Molecular Regulators of Angiogenesis

The growth of new blood vessels requires a tight coordination among cell proliferation, differentiation, migration, matrix adhesion and cell-cell signaling (Adams and Alitalo, 2007). The literature contains many identified regulators of angiogenesis: growth factors, chemokines, cytokines, lipid mediators, hormones and neuropeptides (Carmeliet, 2003). The most studied and important molecule to control blood vessel morphogenesis is Vascular Endothelial Growth Factor-A (VEGF-A).

VEGF-A is a 40-45K glycoprotein that increases blood vessel permeability, endothelial cell growth, and angiogenesis (Keck et al., 1989). Multiple VEGF-A isoforms are produced by alternative splicing from a single
gene containing eight exons (Poltorak et al., 1997; Tischer et al., 1991). These splice sites can have important functional consequences. The most abundant isoforms are VEGF\textsubscript{121} and VEGF\textsubscript{165}, with VEGF\textsubscript{165} having 100-fold higher mitotic activity on endothelial cells compared to VEGF\textsubscript{121} (Keyt et al., 1996). Splice sites determine which receptors these isoforms can bind. VEGF\textsubscript{165} and \textsubscript{121} can bind VEGFR-1 (Flt-1) and VEGFR-2 (KDR or FLK-1). Binding of VEGF to VEGFR-2 elicits endothelial cell proliferation, differentiation and sprouting, but this can be offset by VEGFR-1, which has a higher affinity for VEGF but weaker tyrosine-kinase activity (Adams and Alitalo, 2007). The precise control of VEGF signaling is critical for vascular development and organism survival. The deletion or overexpression of a VEGF isoform in mammalian development produces an embryonic lethal phenotype (Carmeliet et al., 1996; Ferrara et al., 1996; Miquerol et al., 2000). Deletion of either of the VEGF receptors also leads to disruption of vascular development and early embryo death (Fong et al., 1995; Shalaby et al., 1995).

A pivotal study conducted by Ambati and colleagues (Ambati et al., 2006) sought to determine which molecular mechanisms keep the adult cornea avascular. Researchers identified soluble VEGF receptor-1 (sflt-1) as a key player in this process. Through the use of neutralizing antibodies, RNA interference and Cre-lox-mediated gene disruption, the group disrupted corneal avascularity in mice by inhibiting an endogenous VEGF-A trap. In further detail, this group examined the expression levels and binding of VEGF to sflt-1 in
normal and neovascularized corneas (Ambati et al., 2007). In healthy corneas, \( \text{VEGF} \) was strongly expressed in the corneal epithelium where it was bound to sflt-1, however neovascularized corneas have decreased expression of sflt-1 and reduced VEFG bound to sflt-1.

1.4.1 The Role of Sema3A/Npn1 in Angiogenesis

Interestingly, the VEGFRs may not act alone in vasculogenesis. The deletion of Npn1 is also embryonic lethal with neural and vascular defects (Kawasaki et al., 1999). Npn1 may induce endothelial cell migration and proliferation (Soker et al., 1998; Yamada et al., 2003). The precise mechanism of Npn1 action on endothelial cells is not fully understood, but it is hypothesized that Npn1 plays a receptor-clustering role with VEGFR2 and may promote the affinity of \( \text{VEGF}_{165} \) for VEGFR2 (Whitaker et al., 2001).

Sema3A is a ligand for Npn1, and Sema3A’s antiangiogenic properties have become of great interest. Sema3A null mice demonstrate blood vessel patterning defects (Serini et al., 2003) as well as hypertrophy and dilation of the heart (Behar et al., 1996). Because Sema3A shares the same receptor as VEGF, it was first assumed that Sema3A must interfere with VEGF/Npn1 signaling to inhibit angiogenesis. Npn1 has five extracellular domains. Four of the extracellular domains can bind either the Sema3s (a1a2 domains) or VEGF-A (b1b2 domains) (Fig 1.4). Structural studies suggest that there is no competitive binding between Sema3A and VEGF for Npn1 (Appleton et al., 2007). Also of note is the fact that Sema3A inhibits the proliferation of endothelial cells but not
Figure 1.4 Structure of Npn1 and its binding sites.
The Nrp ectodomain is comprised of tandem CUB (a1a2), tandem coagulation factor V/VIII (b1b2), and one MAM (c) domain. Sema3s bind to the a1a2 domains and VEGF binds to the b1b2 domains. Modified from (Appleton et al. 2007).
VEGF-induced phosphorylation of VEGFR-2 (Guttmann-Raviv et al., 2007).

Although Sema3A appears to not interfere with VEGF signaling, it can inhibit developmental angiogenesis (Bates et al., 2003), vascular branching (Serini et al., 2003), and induce apoptosis of endothelial cells (Guttmann-Raviv et al., 2007). More recently, exogenous Sema3A has shown to inhibit angiogenesis in vivo (Acevedo et al., 2008; Casazza et al., 2011).

1.5 Development of Corneal Avascularity

The anatomy of anterior eye vasculature has been investigated with interest in understanding the molecular regulators of the development and regression of the mammalian hyaloid artery, but little research has been devoted to understanding how the corneal avascularity is established. Development of cornea vascularization most likely arises from the pericorneal blood supply, making an understanding of the development and patterning of these vessels is necessary.

1.5.1 Development of Avian Pericorneal Blood Vessels

The development of avian eye vasculature was well documented by Hiruma and colleagues (Hiruma, 1996). The majority of ocular arteries that supply the anterior portion of the avian eye arise from the cerebral ophthalmic artery and the internal carotid ophthalmic artery. Long posterior ciliary arteries extend from these ophthalmic arteries to form the nasal long ciliary artery and the temporal long ciliary artery. These long ciliary arteries approach the edge of the cornea but do not enter. Instead, these arteries grow around the cornea and
merge with each other to form the iridial ring artery. Shortly after anastomosing, the nasal long ciliary artery begins to atrophy away from the iridial ring artery via a process known as vascular pruning or remodeling. The temporal ciliary artery becomes more prominent and supplies the presumptive iridial ring artery. The lens remains avascular throughout development.

1.5.2 Development of Murine Pericorneal Blood Vessels

The development of the murine eye vasculature is different from the avian eye vasculature and is described by Saint-Geniez and D’Amore (Saint-Geniez and D'Amore, 2004). During early eye development, the mouse develops a vascular system around the optic cup before the cornea begins to develop. As the cornea develops, the choroidal plexus anastomoses around the perimeter of the cornea and forms the annular vessel surrounding the cornea. Concurrently, the hyaloid artery enters into the posterior optic cup and begins to grow towards the lens. The hyaloid vessel system is a vital, but transient, intraocular circulatory system that regresses completely at later stages of ocular development. The hyaloid vasculature develops a dense capillary network on the posterior side of the lens called the tunica vasculosa lentis. This capillary network anastomoses with the pericorneal annular vessel to connect with the choroidal vasculature, which acts as venous vasculature for the arterial hyaloid vasculature. The tunica vasculosa lentis continues to develop over the anterior portion of the lens forming the pupillary membrane, which lies directly posterior to the developing cornea (Fig 1.5). The formation of a hyaloid vasculature in the mouse marks a stark
Figure 1.5 E15.5 mouse ocular vasculature.
The hyaloid artery enters the eye from the optic nerve head and extends branches through the vitreous. The hyaloid artery and all of its branches are collectively known as the hyaloid vascular system. The first branches lie suspended in the vitreous between the surface of the retina and the lens and are known as the vasa hyaloidea propria (VHP). Subsequent branches extend toward the lens and run along its surface. These vessels are part of the lens vasculature known as the tunica vasculosa lentis (TVL). The TVL does not penetrate the lens but instead lies adjacent to the lens capsule. The vasculature on the anterior lens capsule is known as the papillary membrane. Modified from (Ash and Overbeek, 2000).
difference from the development of the chick, which lacks this developmental characteristic. Similar to the avian cornea, the mammalian cornea remains avascular throughout development, however, the mammalian lens is vascularized during development, which does not happen in the avian system.

Many factors regulating angioblast migration during vasculogenesis and angiogenesis remain unknown. An investigation into how Sema/Npn signaling establishes an avascular cornea during development could provide a starting point for further investigation into the molecular pathways that regulate vasculogenesis and angiogenesis and lay a foundation for new therapeutic avenues for vision-threatening corneal neovascularization and potential applications as anti-cancer agents.

1.6 Summary

The developing cornea is a valuable tool in the search for axonal and vasculature regulatory molecules. This unique tissue is both avascular and highly innervated. The insights found in the study of corneal innervation and avascularity could be used in wound care, cornea neovascularization or angiogenic diseases such as cancer.

The mouse has become an important model for both cornea and eye diseases. Studies in corneal innervation in relation to development and wound healing are commonly performed in the adult and embryonic mouse. Previously, the early development of murine corneal innervation was assumed to be the same as avian corneal innervation. There has not been a detailed analysis of
how murine corneal innervation occurs. Through the use of antibodies and
immunostaining this dissertation characterizes the innervation of the murine
anterior eye, corneal stroma and corneal epithelium. This information is then
relevant to understand how Sema3A/Npn1 and Sema3F/Npn2 signaling
regulates the murine corneal innervation. The axon guidance molecule Sema3A
can also regulate endothelial cell migration. This dissertation also demonstrates
that Sema3A helps to establish an avascular cornea during development.
Chapter 2: Materials and Methods

2.1 Animals

Animal studies adhered to the Association for Research in Vision and Ophthalmology (ARVO) standards for the Use of Animals in Ophthalmic and vision research and were approved by the Institutional Animal Care and Use Committee (IACUC) of Rice University. Wild-type mice are the C57/B6 strain obtained from Charles River Laboratories International (Wilmington, MA).

Generation of \(Npn1^{Sema-/-}\), \(Npn2^{-/-}\), and double mutant mice lacking Semaphorin signaling through Neuropilins (\(Npn1^{Sema-/-};Npn2^{-/-}\)) was described previously (Giger et al., 2000; Gu et al., 2002; Gu et al., 2003). Briefly, the \(Npn1^{Sema-/-}\) mice express normal levels of a modified \(Npn1\) which is unaffected in its VEGF\(_{164}\)-Npn1 binding but due to a 7 amino acid substitution in the CUB domain, is completely disrupted in its Sema-Npn1 binding. \(Npn2^{-/-}\) mice were generated with a complete deletion of first exon and 1.7kb of upstream sequence. The mouse lines were maintained through heterozygous matings as separate breeding colonies. Mutant embryos were identified by PCR genotyping using primers for \(Npn1^{Sema-/-}\) (Gu et al., 2003) and \(Npn2^{-/-}\) (Giger et al., 2000) with the following modifications to the \(Npn2^{-/-}\) primers: primers used to identify a 400bp wild-type band were (F, 5'-TCAGGACACGAAGTGAGAAG-3', RV, 5'-GGGAGATGTGTTCTGCTTCA-3') and primers used to identify 1Kb mutant band were (F, 5'-CGCATTGCATCAGCCATGAT-3', RV, 5'-GGGAGATGTGTTCTGCTTCA-3'). All primers were produced by Sigma-Aldrich.
PCR reactions were carried out for 35 cycles at 95°C for 1 min, 60°C for 1 min 30 sec, and 72°C for 1 min.

Mice with visible vaginal plugs the morning after mating were designated as embryonic day 0.5 (E0.5). Embryos were collected between E12.5-E16.5. Postnatal animals were collected at P0, P10, and three weeks after birth. Whole heads (E12.5-E15.5) or eyes (E16.5-adults) were collected in ice-cold Ringer’s solution. Corneas were dissected from eyes and trimmed at the sclero-limbo region. To increase permeability of immunostaining reagents, trimmed corneas were incubated at room temperature in 1.5 mg/ml dispase (Atlanta Biologicals, Lawrenceville, GA) for 15-30 minutes. In some corneas, the epithelial layer was removed after enzyme digestion and immunostained separately. All tissues were fixed overnight at 4°C using 4% (w/v) paraformaldehyde (PFA) in phosphate buffered solution (PBS), with the exception of isolated corneal epithelial layers which were fixed at room temperature for 2 hours or overnight at 4°C. All tissues were briefly rinsed in PBS containing 0.1% (v/v) Tween (PBT) after fixation. Whole heads were placed in Ringer’s solution, held in place with forceps and a scalpel made a single cut sagittally down the center of the head creating two equal halves.

Fertile White Leghorn chick (*Gallus gallus domesticus*) eggs were obtained from (Texas A&M Poultry Center, College Station, TX) and Tg(tie1:H2B:eYFP) transgenic Japanese quail eggs, which naturally express enhanced yellow fluorescent protein (eYFP) in all endothelial cells (Sato et al.,
were obtained from Ozark Egg Company (Stover, Missouri). After oviposition, eggs are collected and shipped to the lab while in developmental pause. When arriving at the lab the eggs are considered E0 and incubated in a 38°C humidifying chamber until the desired embryonic stage. The embryos are staged matched to the Hamburger-Hamilton stages of chick development (Hamburger and Hamilton, 1951).

2.2 Antibodies and Immunostaining

Whole-mount immunostaining of tissues followed standard protocols. Briefly, samples were washed in PBT, and then blocked in antibody buffer (PBT containing 0.1% BSA (w/v) and 5% (v/v) heat-inactivated sheep serum). Rabbit anti-mammalian neuron-specific β-tubulin-TUJ1 (IgG, Covance, Dallas, TX) diluted at 1/500, rabbit anti-chick Sema3A antibody (Abcam, Cambridge, MA) diluted 1/50, mouse anti-chick VEGF antibody (Abcam) diluted 1/200, or rat anti-mouse endomucin (eBioscience, San Diego, CA) diluted 1/500 were used in blocking solution. Following overnight incubation at 4°C in primary antibody solution, tissues were extensively rinsed in PBT, blocked for 1 hr at room temperature then incubated overnight at 4°C in secondary antibody (Alexa 594 goat anti-rabbit IgG (Molecular Probes, Grand Island, NY); Alexa488 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA); Alexa488 goat anti-mouse IgG1 (Invitrogen); FITC goat anti-rat IgG2a (Bethyl, Montgomery, TX)) diluted at 1:200 in antibody buffer. Tissues were rinsed and mounted in PBS for imaging. Some immunostained tissues were cryosectioned at 8-10 μm and counterstained with
4,6-diamidino-2-phenylindole (DAPI) to label all nuclei. Images of whole-mount embryos and corneas or their sections were captured using a Zeiss Axiocam mounted on a Zeiss AxioImager 2 microscope with ApoTome (Carl Zeiss, Germany).

2.3 Classification of Innervation

Images of embryos between E12.5-E15.5 were oriented so that the whisker pad was facing towards the right side of the image and lowered 45° below the horizontal midpoint. Using the whisker pad nerves as a reference point, a horizontal line was drawn across the eye bisecting it into dorsal (D) and ventral (V) regions. A perpendicular line was drawn intersecting the first line at the center and dividing the eye into temporal (T) and nasal (N) regions. The resulting quadrants were designated dorsal-temporal (DT), dorsal-nasal (DN), ventral-temporal (VT), and ventral-nasal (VN) (Fig 2.1). Axons were analyzed and recorded based on their location in the designated quadrants.

2.4 Quantitative Analysis of Innervated Quadrants

Positions of major nerve bundles were recorded from digital images of similarly oriented corneas. Each innervated quadrant received a score of 1 and 0 for an uninnervated quadrant.

2.5 In Situ Hybridization

Freshly isolated mouse heads (E12.5-E14.5) or eyes (E16.5) were fixed overnight at 4°C in modified Carnoy’s fixative (60% ethanol, 30% formaldehyde, and 10% glacial acetic acid) and embedded in paraffin. In situ hybridization was
Figure 2.1 Orientation of the mouse embryo anterior eye quadrants.
The TuJ-1 (red) stained whisker pad (wp) was used to orient the embryo with the
dorsal side (D) up and nasal side (N) to the right. A line intersecting the eye from
the dorsal to the ventral (V) side was drawn, as well as a line intersecting the
temporal (T) to the nasal side. This made four quadrants; dorsal temporal, ventral
temporal, ventral nasal, and dorsal nasal. II, lower eyelid; ul, upper eyelid.
performed on 10-12 μm sections as described (Etchevers et al., 2001). Mouse
digoxigenin-labeled riboprobes for Npn1 (Fruttiger, 2002), Sema3A (Puschel et
al., 1995), and Sema3F (Giger et al., 2000) were transcribed as previously
described. Mouse Npn2 riboprobes were synthesized from cDNA isolated from
adult mouse trigeminal ganglia using the following primers; F, 5’-
GGACTGTACCTTCACCACCTGGC-3’ and RV 5’-
CTGGAAATGTTCATTGGGTTAG-3’. Chick Npn1 probes were used as
previously described (Lwigale and Bronner-Fraser, 2009).

2.6 Quantitative Analysis of Axon Lengths

To determine the extent of corneal innervation, a circle was digitally
superimposed over the whole mount captured image of the anterior eye to
delineate the cornea boundary (300 μm for E13.5, 500 μm for E14.5 and E15.5).
At these stages of development, there is no or minimal epithelial innervation,
therefore no effort was made to distinguish between stroma and epithelial nerves.
Images were processed in ImageJ (Abramoff et al., 2004) and nerves were
quantified using the NeuronJ plug-in (Meijering et al., 2004) (Fig 2.2). The total
length of corneal nerves was determined for each cornea. The values reported
are averages of each group. Statistical analysis was performed using a One-Way
Analysis of Variance (ANOVA) with a Tukey-Kramer Multiple Comparisons Post Test.
Figure 2.2 Quantification of Axons Using NeuronJ.
The TuJ1 (red) immunostained cornea was converted to a black and white image for processing by NeuronJ. A 300 or 500 μm ring was superimposed over the eye to ensure all eye at the same time stage had the same area of measurement. NeuronJ assisted in tracing the axons and then quantified the axon distance.
2.7 Quantitative Real-Time PCR

For qRT-PCR, E3-E7 chick lenses and E4-E7 chick corneas were dissected and pooled (n=24) for each time point and 2-3 biological pooled samples were used. RNA was isolated with TRIzol® Reagent (Invitrogen) and DNA was digested with TURBO DNA-free™ (Invitrogen). cDNA was reverse transcribed using qScript™ cDNA SuperMix (Quanta BioSciences, Gaithersburg, MD). Quantitative real-time PCR was carried out in triplicate using PerfeCTa® SYBR® Green SuperMix (Quanta BioSciences). Using the following primers:

- GAPDH Forward 5’-GATTCTACACACGGACACTT-3’;
- GAPDH Reverse 5’-TCTGCCCATTTGATGTTGCT-3’;
- Sema3A Forward 5’-AGGACAGCGGATCGTTGTGA-3’;
- Sema3A Reverse 5’-ATCGCTCCTCAGTCTTCTCC-3’;
- VEGF-A Forward 5’-GGTGGAGTACATATTCAGGCC-3’;
- VEGF-A Reverse 5’-CTATGTGCTGACTCTGATGGG-3’.

The fold change was calculated as the $\Delta\Delta Ct$ relative to E7 cornea Sema3A expression.

2.8 Lens Ablation

Lens ablations were performed as previously described (Lwigale and Bronner-Fraser, 2007). Briefly, a small window was cut in the shell of the quail eggs and embryos were made assessable at E2. At E3, a small incision was made in the cornea epithelium, the lens was gently detached from the optic cup
and overlaying epithelium and removed from the right eye, leaving the left eye as a control. Embryos were reincubated for 1-2 days and then collected for imaging.

2.9 Peptide Injection

Peptide injections were performed as described (Lwikale and Bronner-Fraser, 2007). Briefly, a Sema3A-Ig peptide that binds to and blocks Sema3A interaction with Npn1 (N-Ac-HAVEHGFMQTLLKVTLE-NH2), (Williams et al., 2005) or control scrambled peptide (N-Ac- AHLELLTQTGKVEVFHM-NH2) was pressure injected into the right lens of E3 quail embryos at a 10mM concentration. Embryos were reincubated for two days and then collected for analysis.

2.10 Bead Implantation

Beads were prepared as previously described (Bates et al., 2003) with minor modifications. Briefly, approximately twenty Cibacron blue 3GA beads (Sigma-Aldrich) were rinsed in Ringer’s solution and soaked overnight at 4°C in 5 μl of 90μg/ml recombinant mouse Sema3A Fc chimera protein (R&D Systems, Minneapolis, MN), 4.5μg/ml recombinant mouse VEGF 164 (this is the mouse equivalent isoform of the human VEGF 165 isoform) (R&D Systems), a combination of Sema3A-Fc and VEGF 164, or in Ringer’s solution. Beads were implanted between the presumptive cornea and the lens of E4 quail, reincubated for two days, then collected for analysis.

2.10.1 Quantification of Anterior Eye Vasculature following Bead Implantation
Although all beads were implanted in the same region, in some cases they were dislodged to the cornea periphery. To standardize their effect, only the beads localized within 300-600um of the edge of the iridial ring artery at E6 were quantified. To quantify the region covered by endothelial cells in the iridial ring artery and the cornea space, a delineating line was drawn around the outside and inside edges of the iridial ring artery and any of its extensions (Fig 2.3). The area of the outside boundary was calculated using ImageJ (Abramoff et al., 2004) and is considered the total area of the sample. The area between the outside boundary and inside boundary (the area covered by endothelial cells) was calculated using ImageJ. The area covered by endothelial cells was divided by the total area to determine the “percent area covered by endothelial cells.” The values reported are averages of each group. Statistical analysis was performed using an unpaired t test with a two-tailed p value.
Figure 2.3 Quantification of the iridial ring artery and any corneal endothelial cells.
A line was drawn around the outside of the iridial ring artery to measure the total area of the sample (outside red dashed line). A line was then drawn around the inside of the iridial ring artery (inside red dashed line). This included any endothelial cells that traveled into the cornea. The area of the inside circle was calculated. The inside area was subtracted from the outside area to find the area covered by endothelial cells. The area covered by endothelial cells was divided by the total area to determine the percent area covered by endothelial cells. white dashed circle, protein soaked bead; tca, temporal long posterior ciliary artery; nca, nasal long posterior ciliary artery. Scale bar, 100μm.
Chapter 3: Development of Innervation in the Mouse Cornea

Dense innervation of the cornea is important for maintaining its homeostasis and transparency. Although corneal nerves have been well studied in adults, little is known about mammalian corneal innervation during development. Through the use of an anti-neuron specific β-tubulin antibody, our results provide a detailed analysis of the development of mouse corneal innervation and reveal a novel pattern of cornea innervation that does not involve the formation of a pericorneal nerve ring, as was seen in the chick embryo. The results provide a platform for future studies of corneal nerves in the mouse, a species often used as a model for human ocular defects.

3.1 Early Innervation of the Eye

To determine the onset of sensory nerve projections into the anterior region of the eye, I immunostained mouse embryos starting at E11.5, corresponding to the time shortly after the formation of the lens vesicle and presumptive cornea epithelium (Pei and Rhodin, 1970). At this stage, trigeminal axons innervate the presumptive eyelids and the whisker pad, but no axons were observed in the anterior region of the eye.

By E12.5, nerve bundles began to appear at distinct locations in the periphery of the eye. To establish position identity of the pioneer axons, I partitioned each eye along the dorsal-ventral and temporal-nasal axes into four quadrants (VN, VT, DN, DT; Fig. 3.1). Similar to E11.5, the majority of the embryos (24/35) have innervation of the upper and lower eyelid region and
whisker pad, but not the eye (Fig. 3.1A). Nonetheless, some E12.5 embryos (11/35) exhibited an interesting variation in nerve staining along the edge of the eye. In some eyes, a single nerve bundle was present in the DN (Fig. 3.1B and C, arrowhead), VN (Fig. 3.1C, arrowhead) or VT quadrants. In other instances (Fig. 3.1D), a nerve bundle was present in each of the two quadrants of the same eye: DN and VN (1/35; Fig. 3.1C); VN and VT (1/35); and VT and DN (2/35). The VT quadrant was always innervated in combination with either the VN or DN quadrants. All three quadrants were never innervated in the same eye at E12.5.

Figure 3.1E shows the number of times each quadrant was innervated. The majority of the eyes did not show innervation of any of the four quadrants (24/35). For the eyes that were innervated, the majority of the pioneer nerve bundles were located in the VN quadrant (8/35), followed by the DN quadrant (4/35), then the VT quadrant (3/35). Interestingly, the DT quadrant was never innervated at E12.5 (0/35). A representative cross-section through an innervated quadrant at E12.5 revealed a single nerve bundle in the periocular region projecting towards the adjacent presumptive cornea (Fig. 3.1F, arrow). These results indicate that presumptive corneal nerves arrive in the periphery of the anterior surface of the eye as early as E12.5, the DN, VN, and VT quadrants are innervated prior to the DT quadrant, and a single nerve bundle is located in each innervated quadrant of the eye.
Figure 3.1 Innervation of the anterior ocular region at E12.5

(A) A mouse embryo showing innervation (red) of the upper and lower eyelids, and the whisker pad but no innervation of the eye. To determine the positioning of nerve bundles prior to cornea innervation, the eye was divided into quadrants along the dorsal-ventral and temporal-nasal axes. (B) Innervation of the DN quadrant (arrowhead). (C) Innervation of the DN and VN quadrants (arrowheads). (D) Venn diagram summarizing the number and overlap of innervated quadrants. (E) Quantification of eye innervation and position of pioneer nerve bundles. (F) Cross-section through an E12.5 eye counterstained with DAPI (blue) showing a nerve bundle (arrow) in the periocular region projecting toward the presumptive cornea. Image of Fig. 3.1F was taken from a similar location as the boxed region in Fig. 3.2D. D, dorsal; N, nasal; V, ventral; T, temporal; DN, dorsal-nasal; VN, ventral-nasal; II, lower eyelid; ul, upper eyelid; wp, whisker pad; pc, presumptive cornea; pr, periocular region; oc, optic cup. Scale bars, A-C 100μm; F, 50μm.
3.2 Innervation of the Corneal Stroma

Whole-mount immunostaining of eyes at E13.5 reveals that several nerve bundles project from each innervated quadrant and extend towards the presumptive cornea. The nerve bundles in each quadrant are possibly branches of a single major nerve projecting from the posterior region of the eye that is not visible in whole-mount corneas at this stage. The DN, VN, and VT quadrants are consistently innervated (15/15; Fig. 3.2 A, B, C, F). Innervation of the DT quadrant appeared to lag behind. The majority of E13.5 eyes (9/15) had no innervation of the DT quadrant (Fig. 3.2 A), although a few eyes exhibited slight innervation of this quadrant (5/15; Fig. 3.2 B, arrow; Fig. 3.2 F). Only one eye showed more extended nerve bundles in the DT quadrant that were nearly equivalent in size to the other three quadrants (Fig. 3.2 C, arrow; Fig. 3.2 F). The tips of the main nerve bundle in the DN, VN, and VT quadrants innervated the periphery of the presumptive cornea (Fig. 3.2 A-E, asterisk). Nerves from the DT quadrant never innervated the cornea at E13.5. Sections through E13.5 eyes show that nerves from the posterior region of the eye projected along the optic cup towards the anterior (Fig. 3.2 D, arrowheads). As they grew towards the presumptive cornea, nerve bundles projected almost parallel to the epithelium and optic cup before the bundles innervated the stroma (Fig. 3.2 D-E, asterisk). The results indicate that in the E13.5 mouse embryo, nerve bundles undergo several bifurcations as they extend from the periphery of the eye towards the
Figure 3.2 Projection of nerve bundles towards the presumptive cornea at E13.5

(A-C) Projection of nerve bundles from the DN, VN, and VT quadrants towards the presumptive cornea. Some nerve bundles (red) innervate the cornea periphery (asterisk). Innervation of the DT quadrant ranges from (A) none, (B) slight (arrow), compared to other quadrants, (C) and moderate (arrow), compared to other quadrants. (D) Cross-section of an immunostained E13.5 eye counterstained with DAPI (blue) showing that presumptive cornea nerves project along the optic cup (arrowheads) towards the anterior region of the eye. (E) Close up of the boxed area in D showing innervation of the mid-stromal region (asterisk) of the presumptive cornea. (F) Quantification of innervated quadrants at E13.5. DN, dorsal-nasal; VN, ventral-nasal; VT, ventral-temporal; DT, dorsal-temporal; oc, optic cup; pc, presumptive cornea. Scale bars, A-D, 100μm; E, 50μm.
cornea. Initial innervation of the cornea begins at this stage when nerve bundles project into the midstromal region.

To examine the development of the stromal plexus and determine when nerve bundles begin to project towards and innervate the cornea epithelium, corneas were immunostained between E14.5-E16.5. By E14.5 nerve bundles from the DN, VN, and VT quadrants extended further into the cornea than those from the DT quadrant. Only a few eyes (5/15) showed a single nerve bundle from the DT quadrant innervating the cornea periphery. Some of the smaller fascicles that branch from the nerve bundles innervated the cornea at different locations along the circumference. Between 2-3 nerve bundles each from the DN, VN, and VT quadrants innervated the cornea. Sections through the E14.5 cornea showed that nerve bundles from the stromal plexus begin to project anteriorly towards the cornea surface (Fig. 3.3 B).

At E15.5, nerve bundles from the DN, VN, and VT quadrants continued to branch as they projected closer to the cornea center (Fig. 3.3 C). Nerve bundles from the DT quadrant still lagged behind, only innervating the cornea periphery in half of the corneas (5/10). In the five corneas that were innervated in the DT quadrant, between one-two nerve bundles were visible. The other quadrants were each innervated by three-five nerve bundles that projected into the cornea periphery. Interestingly, the major nerve that bifurcated into the nerve bundles of the VN quadrant became visible when the eye was oriented at an angle (Fig. 3.3 D, arrow). In this case, the single major nerve bundle bifurcated into several
Figure 3.3 Innervation of the mouse cornea stroma

Whole-mount staining of (A) E14.5 and (C) E15.5 and (E) E16.5 corneas showing progressive extension and branching of nerves (red) as they innervate the entire cornea stroma. (D) Cornea in C imaged from an angle to show that a single major nerve bundle (arrow) from the posterior region of the eye bifurcates into several nerves (asterisks) in the VN quadrant. Cross-sections of (B) E14.5 counterstained with DAPI (blue) showing that nerve bundles innervate only the anterior two-thirds of the stroma and project towards the epithelium, which is innervated by (D) E16.5 (arrowheads). Dotted lines demarcate in (E) the boundary of the area covered by nerves from each quadrant, and in (F) the epithelium/stroma boundary. DN, dorsal-nasal; VN, ventral-nasal; VT, ventral-temporal; DT, dorsal-temporal; oc, optic cup; st, stroma; epi, epithelium; II, lower eyelid nerves. Scale bars; A, C, D and E, 100μm; B and F, 50μm.
branches (Fig. 3.3 D, asterisks) that innervated the anterior region of the eye including the cornea. Cross section through the cornea at this stage revealed that nerve bundles subdivided into smaller branches and extended closer to the epithelium.

By E16.5, major nerve bundles from all quadrants have undergone several bifurcations to form smaller branches that covered most of the cornea stroma (Fig. 3.3 E). The dotted lines demarcate the approximate area innervated by nerve bundles from each quadrant. The DT quadrant contributed relatively fewer nerves to the cornea, which was compensated by longer and more extensive nerve branching from the DN, VN, and VT nerve bundles. At this stage, several large nerve bundles and numerous smaller branches projected through the cornea periphery. Some of the nerve branches projected along the cornea circumference (Fig. 3.3 E, arrowheads). The earliest innervation of the cornea epithelium is observed at E16.5 (Fig. 3.3 F, arrowheads). Therefore, continuous branching of the nerve bundles from the four quadrants results in the formation of the stromal plexus and innervation of the anterior two-thirds of the entire cornea stroma, with visibly less contribution from the DT quadrant.

3.3 Innervation of the Corneal Epithelium and Formation of the Sub-basal Nerve Swirl

In the adult mouse cornea, sub-basal nerves orient towards the center where they form a distinct swirl pattern (Leiper et al., 2009; Yu and Rosenblatt, 2007). To determine when the sub-basal nerves form the swirl pattern, I
examined the innervation of the cornea epithelium at E16.5 and subsequent stages of eye development. To avoid interference of stromal nerve staining, I enzymatically isolated the epithelium from the rest of the cornea. Whole-mount immunostaining of E16.5 cornea epithelium demonstrated that each nerve bundle subdivided into several small branches that radially spread from the point of entry (Fig. 3.4 A). Cross-section of the cornea at this stage shows that the one-two cell thick epithelium was directly innervated (Fig. 3.4 B).

At post-natal day zero (P0), epithelial nerves extended further as compared to E16.5 from the point of entry and subdivided into smaller fascicles (Fig. 3.4 C). Cross-sections of corneas at this stage revealed that the epithelium was two-three cells thick, and small nerves innervated all of these layers (Fig. 3.4 D). At P10, nerves covered the entire surface of the cornea epithelium and ramified at several locations (Fig. 3.4 E). In the cross-section, nerves formed a sub-basal plexus from which individual strands perpendicularly grew through the three-four cell layer thick epithelium and towards the superficial apical layer (Fig. 3.4 F). By three weeks after birth, sub-basal nerves began to orient towards the center, where they started to form a swirl pattern (Fig. 3.4 G). The cornea epithelium was stratified and four-five cells thick at this stage. The sub-basal plexus was clearly visible below the basal epithelial layer (Fig. 3.4 H, arrowhead). Nerves branched from the sub-basal plexus and penetrated all layers of the epithelium and extended upwards into the superficial apical layer. In adults, sub-basal nerves oriented towards the center and the swirl pattern was distinct
Figure 3.4 Innervation of the mouse corneal epithelium
(A, C, E, G, I) Whole-mount immunostaining of corneal epithelia showing the distribution of epithelial nerves (red) at the corneal apex during different stages of development and in adult. (B, D, F, H, J) Cross-sections of corneas counterstained with DAPI (blue) at corresponding time points, showing innervation of the epithelium and the formation of the sub-basal plexus (arrowheads). epi, epithelium; st, stroma. Scale bars; A, C, E, G, I, 50μm; B, D, F, H, J, 20μm.
Cross-section of adult cornea revealed a nerve pattern that is similar to three weeks after birth (Fig. 3.4 J). These results suggest that the cornea epithelial cells are innervated prior to rearrangement of nerves to form the swirl pattern.

3.4 Discussion

The mouse is widely used to model genetic defects that reflect human ocular disorders. In this study I performed a detailed analysis of mouse cornea innervation during development and showed that nerve bundles grew directly into the cornea. This is different from the formation of a pericorneal nerve ring found in avian formation. Nerve bundles from four quadrants innervated the cornea, with the DT quadrant contributing relatively fewer nerves. This may be unique to the mammalian system and could be beneficial in determining where to place corneal incisions during surgeries to create the least amount of nerve damage. The cornea epithelium was first innervated at E16.5, subsequently forming a swirl pattern in the sub-basal nerve plexus at about three weeks after birth. The epithelial nerve swirl forms before the first documented epithelial cell swirl and may indicate that neuropeptides guide epithelial cell movement in the cornea.

3.4.1 Positioning of Major Corneal Nerves

During development, trigeminal sensory nerves innervate several tissues including the eyelids, eye, and maxillo-mandibular processes (Arvidson, 1977). Unlike chicks where presumptive corneal nerves always project from the ventrotemporal region, I found that the positioning varied from embryo to embryo.
in mice. This suggests that nerve axons arrive at the eye in very different manners in the avian and murine system. This could be caused because of different regulatory molecules or because of differences in developmental timing. Although the majority of embryos did not show innervation at E12.5, nerves mostly projected from the DN and VN quadrants first, because the VT quadrant was always innervated in combination with either of the two. This pattern was maintained during subsequent stages of development resulting in a large portion of the corneal stroma being innervated by nerve bundles from the DN, VN, and VT quadrants. Although fewer nerves grew into the stroma from the DT quadrant, it was apparent that nerves from the DN, VN, and VT quadrants compensated for this shortfall. The lack of axons approaching from the DT quadrant could indicate the presence of a regulatory molecule preventing the migration of the bundles, or conversely, there may be an attractive source located closer to the DN, VN, and VT regions encouraging axon growth. Further investigation into these potential regulatory molecules would be beneficial in understanding why differences in innervation occur between species and increase our understanding of axon guidance regulators.

### 3.4.2 Development of the Stromal Plexus

In mouse, the presumptive cornea stroma remained uninnervated until E13.5 when nerve bundles projected into the cornea periphery. Interestingly, unlike the chick (Bee, 1982; Lwigale and Bronner-Fraser, 2007) where nerve bundles form a pericorneal nerve ring prior to cornea innervation, nerves grew
directly into the mouse cornea. The lack of a pericorneal nerve ring in the murine eye may be because of the differences in cornea development between the avian and the mammalian systems. When nerves reach the cornea periphery in the avian system (E4), the cornea is composed of an endothelial layer and an acellular stroma. When nerves reach the cornea periphery in the murine system (E13.5), the cornea lacks an endothelial layer and the stroma is several layers thick. The increased mass of the murine cornea at the time of innervation may block any inhibitory signals from the lens, while the smaller avian cornea would have a greater chance of diffusion of any inhibitory signals from the lens and prevent any nerves from entering the cornea. The increased inhibitory signal in the avian cornea could produce the pericorneal nerve ring.

By E15.5, I found that nerve bundles from the VN quadrant originated from a single major nerve projecting from the posterior region of the eye. Since a single nerve bundle is present in each of the innervated quadrants at E12.5, it is possible that one major nerve from the posterior eye gives rise to the nerve bundles in one quadrant, suggesting that four major nerves, one in each quadrant, give rise to all the nerve bundles that innervate the anterior portion of the eye, including the cornea. This is distinctly different from the avian system and would need to be considered when studying innervation in the murine system.

All species studied to date have similar innervation patterns. Adult corneas have radial, approximately equally spaced branches of stromal axons. It could be
postulated that the formation of a pericorneal nerve ring is necessary for equally spaced radial innervation in the adult. My results would indicate otherwise. The embryonic mouse does not produce a pericorneal nerve ring, yet the adult mouse cornea contains equally spaced radial innervation. There is not a necessity to form a pericorneal nerve ring during development to create equally spaced stromal axons in the adult cornea.

3.4.3 Epithelial Innervation and Formation of Sub-basal Swirl

Innervation of the adult cornea epithelium has been well studied in mice (de Castro et al., 1998; Leiper et al., 2009; Yu and Rosenblatt, 2007) and human (Al-Aqaba et al., 2009; Marfurt et al.; Muller et al., 1997). Taken together, these studies demonstrate that stromal nerves project through the Bowman’s membrane and form small nerve bundles (leashes) that further bifurcate into individual terminals that innervate the epithelium. In the adult human (Patel and McGhee, 2005), mice (Leiper et al., 2009; Yu and Rosenblatt, 2007), and rat (Dvorscak and Marfurt, 2008), epithelial nerves form a swirl pattern near the apex of the cornea. My results indicate that the murine system is not an exception to the other examined model systems for epithelial innervation.

The factors regulating the projection of sub-basal nerves towards the central cornea remain unknown. Interestingly, epithelial cells similarly migrate towards the central cornea and form a swirl pattern at the apex (Collinson et al., 2002; Nagasaki and Zhao, 2003). Therefore it has been postulated that epithelial cell migration guides the nerves into a similar pattern. However, the epithelial
swirl does not form until after 4 weeks (Nagasaki and Zhao, 2003), indicating that
the swirl pattern of nerves is not dependent on epithelial cell migration. My results
confirm previous observations by Leiper and colleagues (Leiper et al., 2009)
showing that centripetal projection of nerves is independent of epithelial cell
migration. This is a break from standard tradition that cells guide nerve migration.
These results suggest that neuropeptides released from the nerves can guide
cellular migration. This is an important discovery and an area that could be
further investigated.

This study is the first to reveal that development of mouse corneal
innervation differs from chick. Unlike the pericorneal nerve ring formed in chick,
the development of mouse stromal innervation is by nerve bundles from four
quadrants, which branch irregularly to cover the entire cornea. These results may
be used to guide future studies of mouse corneal nerves and provide insight into
cornea development, wound healing, and disease.

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Chapter 4: Distinct Roles for Semaphorin3A/Neuropilin1 and Semaphorin3F/Neuropilin2 Signaling During Mouse Corneal Innervation

Trigeminal sensory innervation of the cornea is critical for protection and synthesis of neuropeptides required for normal vision. Little is known about axon guidance during mammalian corneal innervation. In contrast to the chick where a pericorneal nerve ring forms via Sema/Npn signaling, mouse corneal axons project directly into the presumptive cornea without initial formation of an analogous nerve ring.

In this study, I investigated the role of Sema3A/Npn1 and Sema3F/Npn2 signaling during mouse corneal innervation. I first analyzed the ocular accumulation patterns of Sema3A and Sema3F mRNA transcripts, and that of the genes encoding their respective receptors Npn1 and Npn2 in the trigeminal ganglion over time. To determine the functional significance of Sema3A/Npn1 and Sema3F/Npn2 signaling during mouse corneal innervation, I compared Npn1<sup>Sema-/-</sup> and Npn2<sup>-/-</sup> mutant corneas with corneas of wild-type litter mates and found that mutant corneas were prematurely innervated. Double mutant corneas exhibit increased severity of stromal and epithelial innervation defects as well as ectopic nerve projections between the optic cup and lens vesicle. The results reveal a separate and important role for Sema3A/Npn1 and Sema3F/Npn2 signaling during innervation of the mouse cornea.
4.1 Spatiotemporal Expression of *Npn1* and *Npn2* by the Trigeminal Ganglion and *Sema3A* and *Sema3F* by Ocular Tissues during Mouse Eye Development

Sensory innervation of the cornea is derived from the trigeminal ganglion (Arvidson, 1977; Lwigale, 2001; Marfurt and Echtenkamp, 1988). During chick development, inhibition of Sema3A signaling by the lens disrupts the formation of the pericorneal nerve ring resulting in the mispatterning of corneal nerves (Lwigale and Bronner-Fraser, 2007). Surprisingly, mice lack a pericorneal nerve ring and instead have direct projection of sensory afferents into the presumptive cornea (McKenna and Lwigale, 2011). Given these species differences in axon patterning, here I examined the role of Npn/Sema signaling in the mouse during corneal innervation.

As an initial step, I established the accumulation patterns of *Sema3A*, *Sema3F*, *Npn1* and *Npn2* mRNA transcripts during eye development. Embryos were analyzed between E12.5 and E16.5, a period corresponding to the time when presumptive corneal nerves first appear at the periphery of the anterior eye region until when the corneal stroma and epithelium are innervated (McKenna and Lwigale, 2011). The accumulation patterns of these mRNAs at E13.5 (data not shown) are similar to E12.5 (Fig. 4.1A, B, F and G).

4.1.1 Expression Pattern of *Npn1* and *Sema3A* during Mouse Corneal Innervation
In situ hybridization on sections through the craniofacial region at E12.5 showed strong accumulation of *Npn1* mRNA in the trigeminal ganglion, central region of the optic cup, hyaloid vasculature, and perioocular mesenchyme (Fig. 4.1A). Strong expression of *Npn1* persisted in the trigeminal ganglion throughout the period of corneal innervation (Figs. 4.1C’, D’, and E’), consistent with previous observations (Kawakami et al., 1996; Kitsukawa et al., 1997).

At E12.5, expression pattern of *Sema3A* was prominent in several ocular tissues including the lens vesicle, central region of the optic cup, in the presumptive eyelid ectoderm (Fig. 4.1B), and pericorneal epithelium (Fig. 4.1B; arrowhead). Although *Sema3A* was expressed in the ocular mesenchyme and eyelid, there was no expression from the region adjacent to the optic cup (Fig. 4.1B; arrows) along which trigeminal nerves project towards the presumptive cornea (McKenna and Lwigale, 2011). Apart from the pericorneal epithelium (arrowheads) and eyelid ectoderm where *Sema3A* mRNA accumulation was less, *Sema3A* expression pattern persisted in the ocular tissues up to E16.5 (Figs. 4.1C, D, and E). *Sema3A* expression became prominent in the presumptive iris and iridocorneal angle (region between the cornea and iris) at E16.5 (Fig. 4.1E). These results show that, similar to chick *Npn1* and *Sema3A* expression (Lwigale and Bronner-Fraser, 2007), mouse *Npn1* is expressed in the trigeminal ganglion and *Sema3A* is expressed in ocular tissues in patterns consistent with a possible role of Npn1/Sema3A signaling in guiding trigeminal afferents during corneal innervation.
Figure 4.1 Expression of Npn1 and Npn2 and their ligands Sema3A and Sema3F during eye development

Levels of mRNA expression were revealed by section in situ hybridization on transverse sections through the cranial region of wild-type mice. (A) Npn1 is expressed in the trigeminal ganglion (tg), periocular mesenchyme (pm), optic cup (oc) and hyaloid vasculature (black arrowhead) at E12.5. (C-E') The trigeminal ganglion remains positive for Npn1 during corneal innervation between E14.5-E16.5. (B) Sema3A is expressed in the lens, pericorneal epithelium, and in the ocular and eyelid mesenchyme (m), but void from the periocular region (arrows) at E12.5. (B-E) This pattern of Sema3A expression is maintained at E14.5-E16.5, except in the pericorneal epithelium (white arrowheads) where it is down regulated at E16.5. (F) In contrast with Npn1, Npn2 is expressed at low levels in the trigeminal ganglion but strongly expressed around the optic nerve (arrow), hyaloid vasculature, and periocular mesenchyme at E12.5. (H-J') Low levels of Npn2 expression are maintained in the trigeminal ganglion throughout E14.5-E16.5 with strong expression restricted to the trigeminal root (asterisks). (G) Expression of Sema3F is ubiquitous in the eye region at E12.5. (H-J) Between E14.5-16.5, expression of Sema3F is prominent in the corneal epithelium, optic cup, and eyelids. Scale bars: 200 μm.
4.1.2 Expression Pattern of Npn2 and Sema3F during Mouse Corneal Innervation

To determine if Npn2/Sema3F, like Npn1/Sema3A, are candidates for mediating axonal guidance during mouse corneal innervation, I examined the expression of Npn2 and Sema3F between E12.5 and E16.5 by in situ hybridization in tissue sections. Previous studies have shown that Npn2 is expressed by the trigeminal ganglion during gangliogenesis between E9 and E11.5 (Chen et al., 2000; Gammill et al., 2006; Giger et al., 2000) and overlaps with Npn1 expression at these stages (Kawakami et al., 1996; Kitsukawa et al., 1997). Interestingly, I found that Npn2 was expressed at relatively low levels in the trigeminal ganglion at E12.5 (Fig. 4.1F), compared to Npn1 expression at the same stage (Fig. 4.1A). However, similar to Npn1, Npn2 was expressed in the periocular mesenchyme and hyaloid vasculature. Npn2 was also strongly expressed in the mesenchyme around the optic nerve (Fig. 4.1F, arrow). Between E12.5 and E16.5, expression of Npn2 remained low in the trigeminal ganglion, but high expression persisted in the trigeminal nerve root where it attaches to the central nervous system (Figs. 4.1H, I, and J; asterisk).

Sema3F was ubiquitously expressed at low levels in the ocular and frontal-nasal regions of the mouse between E9.5 and E11 (Gammill et al., 2007; Gammill et al., 2006; Giger et al., 2000). As previously shown, I found that at E12.5, Sema3F was ubiquitously expressed at low levels in the presumptive cornea, optic cup, and ocular mesenchyme (Fig. 4.1G). Between E14.5 and
E15.5 expression of *Sema3F* remained at low levels in the eyelid mesenchyme, optic cup, periocular mesenchyme and presumptive corneal stroma, but became prominent in the eyelid and corneal epithelium (Figs. 4.1H and I). By E16, expression of *Sema3F* persisted in the corneal epithelium and became strong in the presumptive iris and iridocorneal angle, optic cup, and in the eyelid mesenchyme adjacent to the ectoderm (Fig. 4.1J). The low levels of *Npn2* expression in the trigeminal ganglion coupled with low levels of *Sema3F* expression in the ocular mesenchyme and cornea indicate that Npn2/Sema3F signaling may play a minor role in guiding axons during their projection into the corneal stroma. However, high levels of *Sema3F* expression in the cornea epithelium at E16.5 may play a role during epithelial innervation.

### 4.2 Npn1 but not Npn2 Regulates Innervation of the Mouse Corneal Stroma

Mouse embryos lacking either Npn1 (Fujisawa, 2004; Fujisawa and Kitsukawa, 1998; Gu et al., 2003; Kitsukawa et al., 1997) or Npn2 (Gammill et al., 2007; Giger et al., 2000), exhibit defects in trigeminal ganglion development including severe defasciculation of nerve projections. Similar trigeminal defects are observed in mice lacking Sema3A (Ulupinar et al., 1999) or Sema3F (Gammill et al., 2007) function. Given that *Sema3A* and *Sema3F* are expressed in ocular tissues in the pathway of trigeminal sensory projections, I asked if Npn1 and Npn2 are required for nerve guidance during mouse corneal innervation. Since Npn1^+/−^-null mutant mice die at E8.5 from vascular defects due to lack of signaling from vascular endothelial growth factor (Takashima et al., 2002), I used
a line of Npn1 mutant mice that is only defective in Npn1/Sema signaling (Gu et al., 2002; Gu et al., 2003) and is viable during the period of corneal innervation. The $Npn1^{Sema-/-}$ mice are unaffected in their Npn1/VEGF binding but their $Npn1$ contains a seven amino acid substitution in the CUB domain that prevents Npn1 from binding to Sema. Nerve projections were examined in wild-type, $Npn1^{Sema-/-}$, and $Npn2^{/-}$ mutant embryos that were stained with the TuJ1 antibody to identify axonal projections. Previously, I showed that in wild-type mice, presumptive corneal nerves first appeared in the anterior region of the eye at about E12.5. At E12.5, anterior nerve projections were absent in most wild-type eyes (n=24/35), but a few embryos show initial nerve projections in the dorsal-nasal (DN), ventral-nasal (VN), or the ventral-temporal (VT) quadrants (n=11/35; Figs. 4.2A and J). $Npn1^{Sema-/-}$ eyes (n=6) were innervated in all quadrants including the DT quadrant (Fig. 4.2D; arrow). The DT quadrant was never innervated at this time in wild-type eyes. $Npn1^{Sema-/-}$ eyes displayed ectopic nerve bundles (Fig. 4.2D; asterisks) that projected between the entry points of the four major nerve bundles that were found in wild-type eyes, and subsequently innervated the entire corneal stroma.

In addition, the nerve bundles projected into the corneal periphery of E12.5 $Npn1^{Sema-/-}$ eyes (Fig. 4.2D and M; arrowheads), which normally occurred later at about E13.5 in wild-type (Fig. 4.2B). In marked contrast to $Npn1^{Sema-/-}$ eyes, axon projections into $Npn2^{/-}$ eyes (n=8) were apparently not affected and resembled wild-type at E12.5 (Fig. 4.2G and P). By E13.5, axons from the DN, VN, and VT major nerve bundles extended as far as the corneal periphery and the DT
Figure 4.2 Innervation of the corneal stroma is defective in \textit{Npn1\textsuperscript{Sema\textendash}} but normal in \textit{Npn2\textendash} mutant embryos

Immunostaining for TuJ1 (red) was performed on whole-mount corneas (A-I) and sections (J-R) at E12.5, E13.5 and E14.5. (A-C) Innervation of wild type cornea showing the spatiotemporal projection of nerve bundles into the four quadrants of the stroma and minimal contribution from the DT quadrant (arrow in C). (D) At E12.5, \textit{Npn1\textsuperscript{Sema\textendash}} eyes show several aberrant projections (asterisks) that also innervate the DT quadrant (arrow) and extend close to the corneal periphery (black arrowheads). (E) By E13.5, nerve bundles from all quadrants including the DT quadrant (arrowhead) innervate the cornea and some project across the diameter of the cornea (asterisk). (F) At E14.5, extensively bifurcated nerve bundles cover most of the cornea. (G-I) Innervation of \textit{Npn2\textendash} eyes resembled wild type at these stages of corneal development. (J-R) Representative sections through wild type, \textit{Npn1\textsuperscript{Sema\textendash}} and \textit{Npn2\textendash} mutant corneas showing the extent of nerve projections at each developmental stage. All sections were counterstained with DAPI (blue). Arrowheads in J, M, and P show relative projection of nerve bundles in the periocular region at E12.5. (S) Quantification of nerves in wild type, \textit{Npn1\textsuperscript{Sema\textendash}} and \textit{Npn2\textendash} mutant corneas was carried out by measuring the lengths of nerve bundles in the cornea at E13.5-E15.5; for details see Methods section. For all samples n=8, except \textit{Npn2\textendash} mutants where n=6 for E14.5 and E15.5. Error bars=SEM. Scale bars: 100 \textmu m. ***, p<0.001.
quadrant was minimally innervated in some of the wild-type eyes (n=15, Fig. 4.2B). At this stage in Npn1Sema^-/- eyes (n=8), nerve bundles continued to project from all quadrants and innervated the stroma (Fig. 4.2E and N). Nerve bundles in the DT quadrant projected further and innervated the stroma of Npn1Sema^-/- corneas (Fig. 4.2E; arrowhead). In some cases nerve bundles grew past the corneal center and projected across its diameter (Fig. 4.2E; asterisk). Npn2^-/- eyes (n=6) showed similar patterns of innervation as wild-type at this stage (Fig. 4.2H and Q). By E14.5, major nerve bundles from the DN, VN, and VT quadrants innervated the stoma and projected towards the center (Fig. 4.2C and L). The major nerve bundle in the DT quadrant extended a relatively short distance compared to nerve bundles in the other quadrants, and at this time it innervated the corneal periphery (Fig. 4.2C; arrow). At E14.5, Npn1Sema^-/- corneas were innervated by numerous axons that bifurcated from the major nerve bundles and projected throughout the stroma (Fig. 4.2F and O). Innervation of Npn2^-/- corneas (n=6) appeared similar to wild-type (Fig. 4.2I and R).

Because of variations in the extent of axon projections into the cornea within wild-type and mutant embryos, I quantified axon lengths within the cornea during stromal innervation with the use of NeuronJ (Fig. 4.2S). I found that axons grew significantly further in Npn1Sema^-/- corneas at E13.5 (n=8; p<0.0001), E14.5 (n=8; p=0.0007), and E15.5 (n=8; p=0.0133) compared to wild-type corneas. Npn2^-/- corneas did not show significant difference in axon length at E13.5 (n=6; p=0.8833), E14.5 (n=6; p=0.0455) or E15.5 (n=8; p=0.7168) relative to wild-type.
My results indicate that Npn1 is required for axon guidance during the innervation of the mouse corneal stroma but Npn2 plays an insignificant role during this process.

4.3 Innervation of the Corneal Quadrants in $Npn1^{Sema^{-/-}}$ and $Npn2^{Sema^{-/-}}$ Mutant Mice

As shown above and described in the previous chapter, a unique characteristic of normal mouse corneal innervation is that all nerves originated from four major nerve bundles that repeatedly branched as they innervated the corneal stroma and epithelium. Although the major nerve bundles from the DN, VN, and VT quadrants projected into the cornea at the same rate and innervated approximately similar surface areas, the DT quadrant grew relatively slowly and therefore covered a smaller area. To further determine if there were aberrant nerve projections in $Npn1^{Sema^{-/-}}$ and $Npn2^{Sema^{-/-}}$ mouse corneas, we quantified the extent of stromal innervation in each quadrant by measuring the total length of axons using the NeuronJ plugin for the ImageJ software. Statistical analysis was performed on measurements taken from wild-type, $Npn1^{Sema^{-/-}}$ and $Npn2^{Sema^{-/-}}$ corneas at E13.5-E15.5.

During the onset of stromal innervation in wild-type E13.5 (n=8) and at E14.5 (n=8) corneas, I observed a significant bias of axons away from the DT quadrant compared to other quadrants (Fig. 4.3A and B). By E15.5 (n=8), nerves in the DT quadrant projected further towards the cornea center (Fig. 4.2M). Although there were relatively fewer nerve bundles in the DT quadrant at this
Figure 4.3 Loss of Npn1 signaling through Sema disrupts the pattern of innervation of the corneal quadrants

Whole mount corneas were immunostained with TuJ1 and imaged. The lengths of nerve bundles projecting into each quadrant were quantified as described in the Methods section. For all graphs, Y-axis is the average length of corneal nerves (μm) and X-axis is corneal quadrants. (A-C) Wild-type, (D-F) Npn1^{Sema/-} mutant, and (G-I) Npn2^{-/-} mutant eyes were analyzed at E13.5-15.5. ANOVA with a Tukey post test was performed on all data sets. For all samples n=8, except Npn2^{-/-} mutants where n=6 for E14.5 and E15.5. Error bars=SEM. No bracket indicates p>0.05; *, p<0.05; **, p<0.01; ***, p<0.001.
time, we observed no difference in the innervation of the DT and VT quadrants, but it remained less innervated than the VN and DN quadrants (p<0.05; Fig. 4.3C). Conversely, stromal innervation of Npn1\textsuperscript{Sema-/-} corneas at E13.5 (n=8) showed no bias away from the DT quadrant and the VT quadrant was more innervated (p=0.001; Fig. 4.3D). By E14.5 (n=8) all quadrants in Npn1\textsuperscript{Sema-/-} corneas were equally innervated (p<0.05; Fig. 4.3E). At E15.5 (n=8), the VN and DN quadrants were innervated at about the same level (p<0.05) but more than the DT and VT quadrants (p=0.001; Fig. 4.3F). At these stages of development, innervation of Npn2\textsuperscript{-/-} stromal quadrants was similar to wild-type (compare Fig. 4.3G-I and 4.3A-C). These results show random projection of nerve bundles during the innervation of Npn1\textsuperscript{Sema-/-} corneal stroma compared to Npn2\textsuperscript{-/-} and wild-type, further indicating the requirement of Npn1 but not Npn2 during stromal innervation.

4.4 Npn1 and Npn2 Play a Role during Innervation of the Corneal Epithelium

As nerve bundles grew toward the central region of the cornea, both large and small branches project anteriorly toward the epithelial layer at E15.5 (Fig 4.4A, arrow), subsequently innervating it at E16.5 (Fig 4.4B, arrowheads). This change in the direction of axon projections during stromal innervation is probably in response to the repulsive Sema3A cues emanating from the lens. In addition, expression of Sema3F was prominent in the epithelial layer at these stages (Fig. 4.1H, I and J) and probably plays a role during epithelial innervation. To
Figure 4.4 Timing of the innervation of the corneal epithelium is disrupted in $Npn1^{Sema^-/-}$ and $Npn2^{-/-}$ mutants

Cross sections of E15.5-E16.5 (A-B) Wild-type, (C-D) $Npn1^{Sema^-/-}$, and (E-F) $Npn2^{-/-}$ corneas immunostained with TuJ1 (red) and counterstained with DAPI (blue). In wild-type corneas, axons project toward the epithelium at E15.5 (A) and innervate it at E16.5 (B). The corneal epithelium is innervated by E15.5 in $Npn1^{Sema^-/-}$ (C, asterisk) and $Npn2^{-/-}$ (E, asterisk) mutant corneas. Scale bar: 50 μm.
determine if Npn1 and Npn2 play a role during innervation of the corneal epithelium, I analyzed corneal sections from E15.5 and E16.5 \(Npn1^{Sema-/-}\) and \(Npn2^{-/-}\) mutant embryos. The first evidence of epithelial innervation in \(Npn1^{Sema-/-}\) (Fig. 4.4C, asterisk) and \(Npn2^{-/-}\) (Fig. 4.4E, asterisk) corneas was at E15.5, a day earlier than wild-type. Because these are cross sections, not all sections could be retrieved successfully, but of the 13 wild-type corneas sectioned there was never evidence of any epithelial innervation. In the seven corneas examined for the \(Npn1^{Sema-/-}\) and \(Npn2^{-/-}\) mice five and four corneas exhibited epithelial innervation respectively. By E16.5, there was continued innervation of the epithelium in both \(Npn1^{Sema-/-}\) and \(Npn2^{-/-}\) corneas (Fig. 4.4D and F) without apparent difference in the number of fascicles compared to wild-type. Thus both Npn1 and Npn2 are required for the proper timing of epithelial innervation.

4.5 Corneal Innervation Defects are Enhanced in \(Npn1^{Sema-/-};Npn2^{-/-}\) Double Mutants

Double mutant mouse embryos lacking semaphorin signaling through Npn1 and Npn2 (\(Npn1^{Sema-/-};Npn2^{-/-}\)) showed increased disorganization of cranial ganglia including the trigeminal ganglion compared to \(Npn1^{Sema-/-}\) and \(Npn2^{-/-}\) single mutants (Schwarz et al., 2008). Because corneal sensory nerves are derived from the trigeminal ganglion (Lwigale, 2001), we asked whether the synergistic defects observed in the trigeminal ganglion of double mutants were recapitulated during corneal innervation. It was found that similar to \(Npn1^{Sema-/-}\) (Fig. 4.2B), several axons projected into the anterior eye region of E12.5 \(Npn^{Sema-}\)
Some axons projected into ectopic regions (Fig. 4.5A; asterisks) and the DT quadrant was innervated (Fig. 4.5A; arrow). Cross-sections through E12.5 double mutant eyes revealed that axons projected into the presumptive corneal stroma (Fig. 4.5B; arrowhead). Comparisons of nerve lengths revealed that Npn1Sema-/-;Npn2-/- double mutant eyes (n=4) were more innervated than Npn1Sema-/-(p<0.01) and Npn2-/- (p<0.001) single mutants (Fig. 4.5C). At E13.5, I observed that defects in corneal innervation were further enhanced in double mutants. The double mutant cornea was innervated by several axons that projected towards the center (Fig. 4.5D). Due to difficulties in obtaining Npn1Sema-/-;Npn2-/- embryos (1/16 from an average litter of 8 embryos), I was unable to quantify the defects in axon lengths. However, transverse sections through an E13.5 Npn1Sema-/-;Npn2-/- cornea indicate that the nerve bundles not only projected through the presumptive stroma, but also prematurely innervated the corneal epithelium (Fig. 4.5E, arrowhead). In addition, I observed ectopic nerve projections near the lens vesicle (Fig. 4.5E, arrow) and between the optic cup and lens vesicle (Fig. 4.5E, asterisks). Although aberrant innervation of the lens vesicle was previously reported in Sema3A-/- mutants (Taniguchi et al., 1997), it was not evident in the Npn1Sema-/- (Fig. 4.2N), Npn2-/- (Fig. 4.2Q), or wild-type (Fig. 4.2K) eyes at E13.5. These results further indicate that both Npn1 and Npn2 are required for and play distinct roles during mouse corneal innervation.
Figure 4.5 Increased disruption of corneal innervation in \( \textit{Npn}^{\text{Sema-/-}; \textit{Npn2-/-}} \) double mutants

Whole mount and sections of E12.5-E13.5 eyes were immunostained with TuJ1 (red). Sections were counter stained with DAPI (blue). (A-B) Several nerve bundles projected into ectopic regions of the anterior eye (asterisks), the dorsal temporal region (arrow) and presumptive stroma (arrowhead) are prematurely innervated. (C) Quantification of axon lengths at E12.5 showed that more nerve bundles innervated the \( \textit{Npn}^{\text{Sema-/-}; \textit{Npn2-/-}} \) double mutant cornea than \( \textit{Npn}^{\text{Sema-/-}} \) or \( \textit{Npn2-/-} \) alone. The quantification method is described in the Methods section. No bracket indicates a relationship of \( p>0.05; **, p<0.01; ***, p<0.001 \). Error bars=SEM. (D-E) By E13.5, several nerve bundles project towards the central cornea. The presumptive corneal epithelium is innervated (arrowhead), and some nerve bundles project into ectopic regions near the lens vesicle (arrow) and between the lens and optic cup (asterisks). Scale bar: 100 \( \mu \text{m.} \) oc, optic cup; c, cornea.
4.6 Discussion

Sensory nerves originating from the trigeminal ganglion innervate the cornea and play a major role in maintaining its homeostasis and transparency. Although Sema/Npn signaling is critical to the formation of the trigeminal ganglion (Gammill et al., 2007; Gu et al., 2003; Kitsukawa et al., 1997; Schwarz et al., 2008; Taniguchi et al., 1997), very little is known about the role of Sema/Npn signaling during mouse corneal innervation. Previous studies in chick have shown that Sema3A/Npn1 signaling regulates the formation of the pericorneal nerve ring that is crucial for the proper patterning of corneal nerves (Kubilus and Linsenmayer, 2010; Lwigale and Bronner-Fraser, 2007). However, the mouse cornea develops differently, such that nerve bundles project directly into the presumptive cornea without initial formation of a pericorneal nerve ring.

This led me to investigate if Npn1/Sema3A and Npn2/Sema3F signaling play a role in guiding trigeminal afferents during mouse corneal innervation. The results show that Sema3A/Npn1 is required for proper innervation of the corneal stroma and epithelium, whereas Sema3F/Npn2 is only required during epithelial innervation. Thus Sema3A/Npn1 and Sema3F/Npn2 are both required and play distinct roles during mouse corneal innervation.

4.6.1 The Expression Patterns of Npn1, Npn2, Sema3A and Sema3F

Several studies have shown that both Npn1 and Npn2 are expressed in the trigeminal ganglion during early development of the mouse embryo (Gammill et al., 2007; Melendez-Herrera and Varela-Echavarria, 2006). However these
studies only addressed trigeminal expression of *Npn1* and *Npn2* during gangliogenesis up to E12.5. Furthermore, the expression of *Sema3A* and *Sema3F* during mouse ocular development was previously unknown. Because trigeminal afferent axons are guided by signals from their targets and surrounding tissues (Huber et al., 2005; Lwigale and Bronner-Fraser, 2007; Rochlin and Farbman, 1998), I first established the expression of *Npn1* and *Npn2* in the trigeminal ganglion, and the ocular expression of *Sema3A* and *Sema3F* during corneal innervation between E12.5-E16.5. I found that *Npn1* was expressed prominently and continuously in the trigeminal ganglion. This pattern of expression was conserved between chick (Lwigale and Bronner-Fraser, 2007) and mouse, indicating that in addition to guiding neuron progenitor cells during gangliogenesis, *Npn1* may play an important role in guiding axon projections towards their targets. Our results also show that *Sema3A* is abundantly expressed in the lens but expression is absent from the mesenchyme adjacent to the optic cup through which axons traverse as they project towards the cornea. In chick, *Sema3A* from the lens repels trigeminal axons in vitro and its inhibition in vivo results in precocious innervation of the cornea (Lwigale and Bronner-Fraser, 2007). Therefore the conserved expression of *Sema3A* in the mouse lens suggests that *Sema3A* plays a similar role of guiding trigeminal axons during mouse corneal innervation. *Sema3A* signaling by the mouse lens does not lead to the formation of a pericorneal nerve ring that is found in the chick. One possibility is that differences between chick and mouse cornea development may
play a part in the magnitude of lens-derived Sema3A signal the nerve bundles receive as they approach the eye. In chick, trigeminal afferents reach the pericorneal region at about E4.5 as the first wave of neural crest cells form the corneal endothelium and are repulsed dorsally and ventrally (Bee, 1982; Lwigale and Bronner-Fraser, 2007). This contrasts with the mouse where axons reach the corneal periphery at about E13.5, subsequent to the migration of numerous neural crest cells between the lens and corneal epithelium (Pei and Rhodin, 1970). The increased number of neural crest cells may buffer the Sema3A secreted by the lens. Alternatively, a combination of Sema3A and other guidance signals in the periocular region may be required for pericorneal nerve ring formation because inhibition of Sema3A during chick corneal innervation does not completely abolish the pericorneal nerve ring (Lwigale and Bronner-Fraser, 2007).

The trigeminal ganglion expresses Npn2 at about E9.5 where Npn2 plays a role during gangliogenesis (Gammill et al., 2007). These results showed low levels of Npn2 expression in the trigeminal ganglion by E12.5 and this level of expression was maintained during corneal innervation, except for the strong expression that was restricted to the trigeminal root where it attached to the central nervous system. Expression of Sema3F was diffuse in the ocular tissues at E12.5, but becomes restricted to the corneal epithelium at E14-E16.5. The low levels of Npn2 expression in the trigeminal ganglion coupled with the low expression of Sema3F in the corneal stroma indicate that Sema3F/Npn2
signaling is dispensable during innervation of the corneal stroma. However, the relatively high expression of *Sema3F* in the corneal epithelium may suffice in guiding axons during epithelial innervation.

### 4.6.2 Innervation of *Npn1*<sup>−/−</sup> Mutant Embryos

Given that *Npn1* and *Npn2* are expressed at different levels in the trigeminal ganglion and that their respective ligand encoding genes *Sema3A* and *Sema3F* are expressed in distinct patterns in ocular tissues, I analyzed *Npn1*<sup>−/−</sup> and *Npn2*<sup>−/−</sup> mutant embryos for development of corneal innervation. Previous studies of *Npn1*<sup>−/−</sup> mutant embryos have shown premature innervation of the limbs (Huber et al., 2005) and aberrant projection of axons in the ear (Fritzsch et al., 2005). Similarly, the work showed that axons are misguided in *Npn1*<sup>−/−</sup> mutants resulting in precocious, disorganized, and increased innervation of the corneal stroma. *Npn1*<sup>−/−</sup> mutant corneas were innervated by E12.5, and the patterning and the length of axon projections into the stromal quadrants was disorganized compared to wild-type littermates. Because *Sema3A* is expressed in the lens epithelium in close proximity to the corneal stroma, it is likely that Npn1/Sema3A signaling guides sensory axons during stromal innervation. In addition, I found that in *Npn1*<sup>−/−</sup> mutants, the corneal epithelium was prematurely innervated at E15.5, a day earlier than wild-type littermates. The corneal innervation defects are probably due to early projection of nerve bundles into the stroma because unlike chick, there is no pause in this process during normal development of the mouse cornea. Early innervation of the epithelium
may cause aberrations in the development of the epithelial cells since
neuropeptides have shown to effect epithelial cell proliferation and migration
(Belmonte and Gallar, 1996). When lens-derived Sema3A signaling is blocked,
neural crest cell progenitors fail to migrate appropriately resulting in the abnormal
development of the cornea (Lwigale and Bronner-Fraser, 2009). While there is a
possibility that malformation of the cornea may contribute to the corneal
innervation defects in chick, this does not appear to be the case in mouse. No
apparent defects in neural crest cell migration were observed during mouse
corneal development and all layers were formed normally in the Npn1Sema-/-
mutants (data not shown). Interestingly, periocular neural crest cells did not
express Npn1 during active migration into the presumptive cornea region
between E10.5-E11.5, which may explain their insensitivity to lens-derived
Sema3A signaling and the differences between chick and mouse corneal
development.

4.6.3 Innervation of Npn2-/ Mutant Embryos

In contrast to Npn1Sema-/ -, innervation of the corneal stroma was not
affected in Npn2-/ mutants. This is not surprising because Npn2 and Sema3F
were expressed at low levels in the trigeminal ganglion and corneal stroma
during this time (Fig. 4.1F-J and H'-j'). However, similar to Npn1Sema-/ -, the
epithelium was prematurely innervated at E15.5 in Npn2-/ mutant corneas. At this
time Sema3F was strongly expressed in the corneal epithelium; therefore
premature innervation of the epithelium could be due to lack of response to
Sema3F signaling in this layer despite basal levels of Npn2 expression in the trigeminal ganglion. Although \(Npn1^{Sema-/}\) and \(Npn2^{-/-}\) mutant embryos have defective trigeminal ganglia that are severely defasciculated at the sites of their targets (Giger et al., 2000; Schwarz et al., 2008), innervation of the corneal stroma was only affected in \(Npn1^{Sema-/}\). This suggests that further signaling at the target site is required for proper innervation since in the absence of Sema3F/Npn2 signaling, Sema3A/Npn1 signaling is sufficient to guide axons into the corneal stroma. However, these results cannot rule out that the defasciculation of the trigeminal ganglion in \(Npn1^{Sema-/}\) is a contributing factor to the ectopic nerve bundles in the cornea periphery and increased stromal innervation.

### 4.6.4 The Necessity for Both Sema3A/Npn1 and Sema3F/Npn2 Signaling in the Development of Corneal Innervation

Finally, I analyzed \(Npn1^{Sema-/};Npn2^{-/-}\) double mutants for corneal innervation. Defects in gangliogenesis of the trigeminal ganglion were increased in \(Npn1^{Sema-/};Npn2^{-/-}\) double mutants compared with single mutants (Schwarz et al., 2008). These results showed that the defects in mouse corneal innervation were exacerbated in the absence of Sema/Npn signaling. Both the stroma and epithelium were prematurely innervated by E12.5 in \(Npn1^{Sema-/};Npn2^{-/-}\) double mutants. In addition, double mutant eyes showed ectopic nerve projections between the lens and optic cup. Although Sema3A^{-/-} mutants show ectopic innervation in the lens vesicle (Taniguchi et al., 1997), innervation in the lens...
Figure 4.6 Proposed model for Sema/Npn signaling during mouse corneal innervation

(A) Schematic diagram showing the spatiotemporal expression of Sema3A and Sema3F in wild-type embryos, which guide axons during stromal and epithelial innervation. Innervation of the wild-type mouse corneal stroma and epithelium respectively commence at E13.5 and E16.5. (B) In Npn2<sup>−/−</sup> mutant embryos, stromal innervation is similar to wild-type but the epithelium is prematurely innervated by E15.5. (C) In Npn1<sup>Sema<sup>−/−</sup></sup> mutants, the stroma and epithelium are both prematurely innervated by several nerve bundles that are misguided into the cornea. (D) The corneal innervation defects shown in Npn1<sup>Sema<sup>−/−</sup></sup> and Npn2<sup>−/−</sup> mutants are exacerbated in the absence of Sema/Npn signaling (Npn1<sup>Sema<sup>−/−</sup></sup> ;Npn2<sup>−/−</sup> double mutants) by E13.5. In addition, nerve bundles project into ectopic regions of the eye between the optic cup and lens vesicle.
vesicle was not evident in \( \text{Npn1}^{\text{Sema-/-}} \) or \( \text{Npn2}^{\text{-/-}} \) single mutants. The severe phenotype seen by the double mutant embryos indicate that the Semas may have a redundant role during cornea formation and the loss of one Sema may be compensated by the other. These data further indicate that \( \text{Sema3A/Npn1} \) and \( \text{Sema3F/Npn2} \) signaling both are required for proper corneal innervation.

4.6.5 Conclusions

In summary, I examined the roles of \( \text{Sema3A/Npn1} \) and \( \text{Sema3F/Npn2} \) signaling during mouse corneal innervation (Fig. 4.6). I found that \( \text{Sema3A/Npn1} \) is required for the innervation of the corneal stroma and epithelium, whereas \( \text{Sema3F/Npn2} \) is only required for epithelial innervation. The distinctive functions in corneal innervation of \( \text{Sema3A/Npn1} \) and \( \text{Sema3F/Npn2} \) are further confirmed by the increase in defective axon projections in the absence of \( \text{Sema/Npn} \) signaling. Because adult corneal nerves are repulsed when \( \text{Sema3A} \) is ectopically added to the cornea (Tanelian et al., 1997), these results may provide insight into the mechanisms that control regeneration of corneal nerves in wounded and diseased corneas.

Chapter 5: Semaphorin3A is Necessary to Establish an Avascular Cornea during Development

The cornea has an “angiogenic privilege” that must be maintained by a distinct avascular state through the delicate balance of pro- and anti-angiogenic molecules (Azar, 2006; Cursiefen, 2007). When a cornea is wounded or becomes inflamed the balance of these molecules can incline to the pro-angiogenic side and blood vessels can enter the cornea causing disease and blindness (Azar, 2006). There are many pro- and anti-angiogenic factors identified in the adult cornea (Ellenberg et al., 2010), but little is known about the molecules responsible for establishing corneal avascularity during development.

This study sought to determine if Sema3A plays a key role in endothelial cell migration during cornea development in vivo. Using Tg(tie1:H2B:eYFP) transgenic quail, I show that the cornea is avascular during development. Npn-1 expression is present in the pericorneal region where migrating endothelial cells form the iridial ring artery surrounding the cornea but do not enter the cornea. I examined mRNA levels of Sema3A and VEGF-A in the eye region during development, determined the location of these proteins during the same time points, and removed or inhibited Sema3A activity in the murine and avian systems. I found that Sema3A is highly expressed in the lens and when the lens is removed or Sema3A inhibited, endothelial cells migrate into the normally avascular cornea. To confirm the ability of Sema3A to inhibit VEGF-A’s pro-angiogenic effect in the developing eye I performed bead implantations in ovo
during chick cornea development. These results suggest that Sema3A plays an important anti-angiogenic role in establishing corneal avascularity during development.

5.1 Npn-1 is Expressed by Angioblasts and Blood Vessels that Form Adjacent to the Developing Cornea

I sought to determine the location of endothelial cells in the anterior eye during cornea development. Using Tg(tie1:H2B:eYFP) transgenic quail, which express H2B:eYFP in nuclei of endothelial cells, I documented the progression of vascularization from the beginning of cornea development (E3) up to when the cornea has formed all three of its cellular layers (E6).

At E3, the lens pore was closed and detached from the surface ectoderm. The surface ectoderm will become the cornea epithelium that rests on a basement membrane. Blood vessels formed in the proximal optic cup started to form two branches, the cerebral ophthalmic artery and the internal carotid ophthalmic artery, from the cranial ramus of the circle of Willis (Hiruma, 1996). These ophthalmic arteries started to become the primordia of the nasal and temporal long posterior ciliary arteries at E3 (Fig 5.1A), and endothelial cells were present on the anterior of the optic cup but not at the margin (Fig 5.1A'). At E5 the “brille” was created which is the combination of the cornea epithelium, acellular stroma and endothelium layer (Hagedoorn, 1928). The nasal (NCA) and temporal long posterior ciliary arteries (TCA) surrounded the cornea at this time and anastomosed to start the formation of the iridial ring artery (Fig 5.1B).
Figure 5.1 Anterior eye vasculature and \textit{Npn1} expression during cornea development

(A-C) Examination of Tg(tie1:H2B:eYFP) transgenic quail at E3, 5 & 6. Sections were counter stained with DAPI (blue). (A) Endothelial cells migrate into the periocular region adjacent to the optic cup at E3, but have not reached the tip of the optic cup (A'; white arrow) or entered the cornea (dashed line). (B) Endothelial cells aggregate to form the nasal and temporal ciliary arteries (nca & tca) and localize at the margin of the optic cup at E4 (B'; white arrow) but have not entered the brille. (C) The iridial ring artery (ira) forms around the cornea at E6 and endothelial cells remain at the margin of the optic cup but do not enter the cornea (C'; white arrow). (D) \textit{Npn1} is expressed in the periocular mesenchyme comprised of both neural crest cells and endothelial cells at E4. (E) By E5 \textit{Npn1} is expressed in the mesenchyme adjacent to the optic cup and forming ocular blood vessels (*) proximal to the cornea but some periocular cells not expressing \textit{Npn1} migrate into the region between the lens and epithelium to form the corneal endothelium (en). (F) At E6 \textit{Npn1} is expressed in the blood vessels and mesenchyme of the presumptive iris stroma not in the three layers of the developing cornea. oc, optic cup; epi, cornea epithelium. Scale bars, 100\,\mu m.
Endothelial cells were present at the margin of the optic cup, proximal to the cornea (Fig 5.1B'). The cornea was composed of three full cellular layers, including the endothelium, stroma and epithelium by E6 (Meyer and Orahilly, 1959). At this time the NCA started to atrophy while the TCA became more prominent and created a full ring around the cornea with the formation of the iridial ring artery (Fig 5.1C). In cross section, all three cellular layers of the cornea with endothelial cells just at the margin of the optic cup were present, but the cornea remained avascular (Fig 5.1C').

I investigated the expression pattern of Npn1 during these key times of development. Npn1 had a very similar pattern of expression in E3-4 in the pericorneal region of the anterior eye between the surface ectoderm and the optic cup (Lwigale and Bronner-Fraser, 2009) (Fig 5.1D). Interestingly this region overlaped with the location where endothelial cells were at this time. At E5 some cells that had turned off Npn1 expression had migrated between the cornea epithelium and the lens, while cells in the pericorneal area retained a strong level of expression evident also in the developing vasculature of the iridial ring artery (Fig 5.1E, asterisk). By E6 the cells that formed the cornea stroma and endothelium did not express Npn1, but expression remained in the anterior uvea that contained the pericorneal blood vessels (Fig 5.1F). The expression pattern of Npn1 indicated that the pericorneal endothelial cells had the Sema receptor, Npn1, during the time when vasculature was developing around the cornea but cells that were not expressing Npn1 migrated between the lens and epithelium to
form the cornea endothelium and stroma suggesting that Sema/Npn1 signaling may play a role in keeping the cornea avascular during development.

5.2 Localization of Sema3A and VEGF-A mRNA and Protein in the Developing Anterior Eye

Given that the migratory of endothelial cells avoid the presumptive cornea, I examined the spatiotemporal expression of Sema3A and VEGF-A mRNA and protein to determine if these molecules were present in the appropriate locations and time to play a role in inhibiting the pericorneal endothelia cells from entering the cornea.

Sema3A was present in the lens vesicle, optic cup, and epithelium at E3 and E4 (Fig 5.2A-B, A'-B'), but then was restricted in the cornea epithelium and lens with little or no protein presence in the cornea endothelium or stroma between E5 and E7 (Fig 5.2C-D, C'-D'). By E7, Sema3A appeared to only be in the cornea epithelium. It is interesting to note that the presence of Sema3A appeared decreased in the optic cup at the time that the endothelial cells reached the margin of the optic cup at E4 and that once the endothelial layer was established the presence of Sema3A switched from the lens to the cornea epithelium where the molecule could play a more important role in signaling to cells migrating into the cornea.

Similar to Sema3A, VEGF-A was present in the lens, cornea epithelium and the optic cup at E3 (Fig 5.2E, E'). Between E4-E5, VEGF-A was still present in the cornea epithelium, but it was diminished in the lens epithelium and optic
Figure 5.2 Presence of Sema3A and VEGF-A protein and quantification of their respective mRNA in the developing anterior eye

(A-H) Immunohistofluorescence of transverse sections from E3-5 and E7 chick eyes. Sections were counter stained with DAPI (blue). (A-D) Sema3A (green) is present in the lens and the cornea epithelium (epi) during early cornea development but decreases in the lens by E7. (E-H) VEGF-A (green) is also present in the lens and cornea epithelium during early cornea development. (I) qRT-PCR of chick E3-5 and E7 lens and cornea tissue. Between E3-E5, Sema3A mRNA levels are significantly higher than VEGF-A levels in the lens. By E7, the levels of Sema3A and VEGF-A are drastically decreased. Expression of VEGF-A is higher than Sema3A in the cornea at E4 and E5 but as in the lens, levels of both transcripts decrease at E7. (*) Indicates no data. The data is represented as the fold change compared to E7 cornea tissue. Error bars=SD. oc, optic cup; st, cornea stroma; en, cornea endothelium; ir, iris. Scale bars, 100μm.
cup (Fig 5.2F, G). By E7, VEGF presence was only maintained in the cornea epithelium (Fig 5.2H).

To quantify the steady state expression levels of Sema3A and VEGF-A transcripts at the same developmental time points, I performed qRT-PCR. As the lens developed, there was a significant decrease of Sema3A expression from E3 to E4 (p<0.001) and from E5 to E7 (p<0.001). Sema3A was most highly expressed in the E3 lens with approximately 15 fold more expression at this time than E7 (p<0.001). The amount of VEGF-A expression remained relatively unchanged from E3-5 (p>0.05) and decreased just slightly from E3-E7 (p<0.05). Corneal expression of Sema3A at E4-5 did not differ significantly (p>0.05) but decreased significantly at E7 (p<0.001). The expression of VEGF-A followed a similar pattern in the cornea with no significant difference in expression between E4-5 (p>0.05) followed by a significant decrease at E7 (p<0.001). The high level of expression of Sema3A from E3-5, a key time point when endothelial cells approached the cornea and established the iridial ring artery, are consistent with the concept that Sema3A helps to establish an avascular cornea during early eye development.

5.3 The Lens Plays a Role in Establishing an Avascular Cornea

Endothelial cells approached the cornea as early as E3, but did not travel past the optic cup into the presumptive cornea over the Sema3A-producing lens. When a lens ablation was performed in early cornea development, neural crest cells, which expressed Npn1 and would normally have been restricted to the
Figure 5.3 Aberrant endothelial cells are present in the developing cornea after lens ablation

(A) Control corneas at E4 have no endothelial cells present. (B) At 1 day post lens ablation, endothelial cells can become present in the normally avascular cornea space (arrows). (C) E5 control eyes have a clear delineation of endothelial cell presence outside of the cornea. (D) At 2 days post lens ablation, endothelial cells are present in the cornea space; apparent in the whole mount images and confirmed in cross sections (arrows). Sections were counter stained with DAPI (blue). tca, temporal long posterior ciliary artery; nca, nasal long posterior ciliary artery; d, dorsal; v, ventral; t, temporal; n, nasal; oc, optic cup. Scale bars, 100μm.
margin of the optic cup, migrated into the cornea (Lwigale and Bronner-Fraser, 2009). I sought to find out if endothelial cells were able to migrate into the cornea when the lens was ablated.

I tested the anti-angiogenic capacity of the lens by performing lens ablation at E3 on the right eye, leaving the left eye as a control, and examining corneas from E4-5 (1-2 days post ablation). At E4 and E5 the pericorneal region had a strong demarcation for where angioblasts were present on the proximity of the cornea but were not present in the presumptive cornea (Fig 5.2A, C). Angioblasts entered the cornea space as early as one day post lens ablation (Fig 5.2B). By two days post lens-ablation, 6/6 eyes had a large flux of cells from the pericocular mesenchyme, as previously described (Lwigale and Bronner-Fraser 2009). Angioblasts were present among this population in the corneal space (Fig 5.2D, F).

Strong angioblasts migration occurred in the dorsal/ventral regions of 5/6 corneas while only 3/6 displayed weak angioblast migration in the temporal/nasal regions. This suggests that the lens is not the sole barrier against angioblast migration. Although the lens provides a physical barrier as well as endogenous Sema3A expression, a separate force must be acting on angioblasts in order to prevent them from migrating freely into the cornea.

5.4 Sema3A Inhibition in vivo Results in Ectopic Migration of Endothelial Cells into the Presumptive Cornea
Sema3A, an axon repellent and anti-angiogenic factor, is present in the developing lens, regulates cornea innervation in both the avian and murine systems (Lwigale and Bronner-Fraser, 2007), and inhibits cell migration of Npn1 expressing pericorneal cells into the cornea space during development (Lwigale and Bronner-Fraser, 2009). I tested if this molecule is also playing a role in establishing an avascular cornea during development.

To inhibit Sema3A activity in the avian system I injected the lens with a Sema3A inhibitory peptide that blocks the Sema3A immunoglobulin domain from interacting with Npn1 (Williams et al., 2005). I injected this peptide at E3 into the right eye, leaving the left eye as a control, and examined the eyes at E5 (two days post injection). I performed the same injection method with an IgG control peptide and found that IgG injections had no affect on cornea development (Fig 5.3A), with no endothelial cells present in the presumptive cornea at E5 (Fig 5.3A'). However, two of the seven eyes injected with the Sema3A inhibitor peptide had endothelial cell migration into the presumptive cornea (Fig 5.3B). Because this is not a complete genetic knock-down, I did not expect every eye injected to show a drastic result. This was confirmed in cross section (Fig 5.3B'). It was interesting to observe that inhibiting Sema3A in the anterior eye also allowed increased migration of pericorneal cells into the cornea as had been observed previously (Lwigale and Bronner-Fraser, 2009).

The development of anterior eye vascularization in the mouse is different from the avian system since it develops a transient hyaloid vascular bed around
Figure 5.4 Lens injection of Sema3A blocking peptide induces endothelial cell presence in the developing cornea

(A) IgG control peptide lens injection do not allow endothelial cells in the developing cornea and appear like untreated control eyes at E5, two days after injection. (B) Lens injections of Sema3A blocking peptide can induce endothelial cell presence in the developing cornea; apparent in the whole mount images and confirmed in cross section (arrows). Sections were counter stained with DAPI (blue). tca, temporal long posterior ciliary artery; nca, nasal long posterior ciliary artery; oc, optic cup; epi, cornea epithelium; en, cornea endothelium; *, precocious neural crest migration. Scale bars, 100μm
the lens during the time that the cornea is developing. The hyaloid artery extends through the vitreous from the optic nerve and forms the tunica vasculosa lentis on the posterior and lateral sides of the lens where it connects with the choroidal vasculature at the margin of the optic cup and will continue to develop onto the posterior side of the lens forming the pupillary membrane (Saint-Geniez and D’Amore, 2004) (Fig 5.5B). In mouse eye development the lens pinches off from the surface ectoderm at E12.5 and the cornea has formed all three layers with a definitive anterior chamber by E15.5 (Pei and Rhodin, 1970).

To determine if the role of Sema3A signaling in pericorneal endothelial cell migration is conserved in mammals, I examined cornea development of Npn1Sema-/- mutant mouse embryos (Gu et al., 2002; Gu et al., 2003) between E12.5-E16.5. In the early stages of cornea development, E12.5-14.5, there were occasional endothelial cells present in the posterior cornea near the developing pupillary membrane but no evidence of stroma vasculature. There was no discernable differences bewtween Npn1Sema-/- or wild-type mice at this time (Wild-type E12, 13, 14 n=9, 9, 5 respectively and Npn1Sema-/- E12, 13, 14 n=4, 7, 5; data not shown). In E15.5-16.5 wild-type embryos, endothelial cells were not present in the cornea stroma (Fig 5.4A-B). However, in Npn1Sema-/- mice endothelial cells were present in the cornea during development. At E15.5, four of the six corneas examined displayed endothelial cells within the cornea stroma (Fig 5.4C, arrows) and at E16.5, six of the eight samples had endothelial cells
Figure 5.5 Npn1<sup>Sema-/-</sup> mice display endothelial cells in the cornea during development

(A-B) Immunostaining of E15-16 Npn1<sup>Sema-/-</sup> cross sections for endothelial cells (green) shows that in wild-type, the cornea is void of endothelial cells but rests on top of the papillary membrane (pm) which is present on the anterior of the lens during development. (C-D) Npn1<sup>Sema-/-</sup> mice have endothelial cells present within the cornea stroma (arrows) during its development at E15-16. Sections were counter stained with DAPI (blue). epi, cornea epithelium; st, cornea stroma; oc, optic up; cv, choroidal vasculature; tvl, tunica vasculosa lentis. Scale bars, 50µm.
present (Fig 5.4D, arrows). Interestingly, the vascular defects were transient, because the adult Npn1<sup>Sema-/-</sup> mice do not have vascularized corneas.

### 5.5 Sema3A Inhibits VEGF Induced Endothelial Cell Migration in the Anterior Eye

To determine if Sema3A elicits an anti-angiogenic response in the anterior eye, beads soaked in Sema3A were placed between the presumptive cornea epithelium and the lens at E4 and the embryo was allowed to develop for two days. At E6 the eyes were imaged and the boundaries of the iridial ring artery were measured, with the outer boundary representing the total area measured. Beads soaked in Ringers, blank beads, had an average of 32.0% of their pericorneal and corneal area present with endothelial cells (Fig 5.5 A, C). However, beads soaked in Sema3A had an average of 26.2% (Fig 5.5 B-C). When blank bead samples (n=4) were compared to Sema3A soaked beads (n=4) by an unpaired t test the p value was less than 0.05, which suggests a significant difference. When the beads were placed at E4 the eye continued to grow over the next two days, moving the bead, so the average bead placements fell within 300-600um from the iridial ring artery at time of collection. The distance of the Sema3A bead from the pericorneal ring may be why there was not a more significant decrease when compared to the blank beads.

The cornea must maintain a delicate balance of pro- and anti-angiogenic factors to establish avascularity. I wanted to ensure that Sema3A could counteract a tip in balance by a pro-angiogenic factor in the anterior eye. I
Figure 5.6 Effects of protein soaked beads on the endothelial cells proximal to the cornea during development

Protein soaked beads were implanted between the cornea and the lens at E4 and embryos were allowed to develop for 2 days. (A) Beads (white dashed circle) soaked in Ringers (Blank) solution showed no effect on the endothelial cells proximal to the cornea (red dashed outline). (B-C) Beads soaked in Sema3A protein exhibited a smaller area of endothelial cells proximal to the cornea when compared to Blank beads. (D) Beads soaked in VEGF-A protein induced a large number of endothelial cells into the cornea space (arrows). (E-F) Beads soaked in both Sema3A and VEGF-A protein showed an increase of endothelial cells present in the corneal space (arrow), but there was significantly less area of endothelial cells present in the cornea and pericorneal area when compared to VEGF-A samples. Dashed red circles represent the outer and inner boundaries of endothelial cell presence. tca, temporal long posterior ciliary artery; nca, nasal long posterior ciliary artery; oc, optic cup; ir, iris; epi, cornea epithlium; en, cornea endothelium; white dashed circle, protein soaked beads; *, p value 0.0429; ***, p value 0.0096. Error bars=SEM. Scale bars, 100µm.
induced vascularization in the eye by implanting VEGF-A soaked beads. These beads induced a generous response of aberrant migration of endothelial cells into the normally avascular cornea space with an average of 49.8% of the pericorneal/corneal space present with endothelial cells (Fig 5.5 D,F). Beads soaked in VEGF-A combined with Sema3A (90μg/ml Sema3A to 4.5μg/ml VEGF-A) did not show such a robust migration of endothelial cells with an average area of 30.4% (Fig 5.5 E-F). When the VEGF-A samples (n=6) were compared to the VEGF-A+Sema3A sample (n=5) in an unpaired t test, it suggested the samples varied significantly with a p value less than 0.01 (Fig 5.5F).

5.6 Discussion

Understanding how corneal avascularity is established during development is of critical importance to understanding pathological corneal neovascularization. There has been little investigation into which molecules establish this avascularity during development. Previous research has shown that Sema3A signaling from the lens plays a dramatic role in cornea development and that this molecule also may play significant roles in vasculogenesis. This led me to investigate Sema3A signaling as a mechanism in regulating ocular vasculogenesis. These results show that Sema3A is required to establish an avascular cornea during development and that it is capable of inhibiting endothelial cell migration into the developing cornea.

5.6.1 The Role of Npn1 in Cornea Avascularity Development
Npn1 is a well-established receptor for Sema3A axon guidance throughout development, and has more recently shown to play an important role on corneal nerves and corneal neural crest cell migration (Lwigale and Bronner-Fraser, 2007, 2009). Npn1 is expressed in endothelial cells (Soker et al., 1998), migratory neural crest cells adjacent to the hindbrain (Eickholt et al., 1999), and neural crest cells in the pericorneal region during early cornea development (Lwigale and Bronner-Fraser, 2009). This research found an interesting pattern of Npn1 expression at key time points during cornea development that indicates this receptor may also play a role in the development of cornea avascularity. During ocular vasculogenesis, endothelial cells migrated anteriorly around the optic cup toward the presumptive cornea, but do not reach the margin of the optic cup at E3, where there was a strong presence of Npn1 expression. The expression of Npn1 remained in the margin of the optic cup through E6, but only in the posterior portion adjacent to the optic cup where the anterior uvea was forming with pericorneal blood vessels. Only cells that downregulated Npn1 can pass over the margin of the optic cup and enter the cornea space (Lwigale and Bronner-Fraser, 2009). These results suggest that expression of Npn1 in blood vessels at the posterior margin of the optic cup make these blood vessels sensitive to the repellent force of Sema3A in the lens and are therefore unable to enter the cornea space during cornea development.

5.6.2 The Location and Expression of Sema3A and VEGF-A in Cornea Avascularity Development
Sema3A is expressed in the lens during development and has a well-documented affect on corneal innervation (Chilton and Guthrie, 2003; Kubilus and Linsenmayer, 2010; Lwigale and Bronner-Fraser, 2007). VEGF-A is also expressed in the developing lens (Ash and Overbeek, 2000; Potts et al., 1993). The presence of VEGF-A may result from the fact that the lens is in a constant state of relative hypoxia, increasing the activation and transcription of hypoxia-inducible factor, which stimulates the transcription of VEGF-A (Shui et al., 2003), or VEGF-A may play an important role in the development of the lens and the mammalian hyloid vasculature (Ash and Overbeek, 2000). I sought to discover the mRNA levels and spatiotemporal locations of Sema3A and VEGF-A in relation to the development of ocular vascularization in the chick. I found that Sema3A was present in the lens, cornea and optic cup when endothelial cells were approaching the pericorneal region (E3) but decreased in location as the cornea had developed and pericorneal blood vessels were established. This result matches previous work, which showed that Sema3A expression is absent in E13 corneas (Kubilus and Linsenmayer, 2010). This strong repulsive cue at the beginning of corneal development may be necessary to inhibit angioblast migration, but become less necessary once the cornea is established. Similar to Sema3A, the presence of VEGF-A in the lens and cornea did not decrease until the cornea had developed the three cellular layers. However, VEGF may play a more important role in the cornea since the cornea does express significantly more transcript compared to the lens during E4-5. This may be unique to the
avian, because it does not develop a hyaloid vasculature and may not require a significant increase of expression in the lens compared to the cornea.

5.6.3 The Role of the Lens in Cornea Avascularity Development

The lens plays a crucial role in the development of the eye and cornea (Beebe and Coats, 2000; Coulombre and Coulombre, 1964), but it is unknown if the lens plays a role in keeping the cornea avascular. I ablated the lens at E3, and as early as one day post ablation, endothelial cells had migrated into the periphery of the cornea space. By E5, two days post ablation, the cornea space had major streams of endothelial cells from specific corneal regions. This migration did not occur randomly, but was more prevalent in the dorsal and ventral regions. These results suggest that the lens is not acting as a simple barrier of endothelial cell migration, because if this was true there would most likely be migration from all regions equally. Because the cornea space did not completely fill with endothelial cells this suggests that the lens does not act alone in repelling endothelial cells during development. The E4-5 cornea expressed Sema3A and could be helping in this inhibitory action, or other known corneal antiangiogenic molecules could help orchestrate the development of an avascular cornea such as sflt-1, angioatin or endostatin (Ellenberg et al., 2010). The lens may play a small role as a physical barrier during development, as evident by the large number of precocious neural crest cells migrating past the optic cup into the cornea space, or this may be due to the lose of an inhibitory signal provided by the lens.
5.6.4 Sema3A is Required to Develop an Avascular Cornea

I next sought to investigate the role of lens-derived Sema3A by inhibiting binding of Sema3A to Npn1. A Sema3A inhibitory peptide that binds to and inhibits Sema3A action was injected into the lens (Williams et al., 2005). When the peptide is injected into the lens at E3, I found that blocking Sema3A interaction with Npn1 prevented the inhibition of endothelial cells into the cornea, similar to what I observed in lens ablation. I also observed an increase of precocious neural crest cell migration between the cornea epithelium and the lens, but to a much smaller degree compared to lens ablation. This result confirms the role Sema3A plays in preventing precocious neural crest cell migration (Lwigale and Bronner-Fraser, 2009) and indicates that Sema3A plays a critical role in keeping the cornea avascular during development.

The mammalian system has shown to have the same sensitivities in the corneal nervous system to Sema3A as the avian system, and as shown previously in this dissertation, Sema3A was expressed in the developing lens during key times of cornea development in the mouse. The murine eye started to develop all of its corneal layers and innervation from E12.5-16.5. I examined the anterior eye vasculature during this time to determine if Npn1^{Sema-/-} mice had any vascular defects due to lost Sema signaling through Npn1. I found no apparent defects in embryos from E12.5-14.5 with the choroidal or hyloid vasculature. However I did begin to see aberrant corneal vascularization in embryos from E15.5-16.5. Interestingly VEGF-A is expressed in the mouse lens at the start of
the development of the hyloid vasculature at E12.5 (Shui et al., 2003), but when a transgenic mouse was created that expresses human VEGF-A, its highest expression of transcript in the lens occurred at E15.5 (Ash and Overbeek, 2000). If there is a peak of VEGF-A expression in the lens at E15.5, this might explain the tip in the angiogenic scale to the proangiogenic side when Sema3A is not present. When adult corneas are wounded, there is an increase of VEGF-A, and if Sema3A is able to inhibit such an increase, it could be a potential therapeutic drug target for neovascularized corneas, wound healing or other vascularization diseases.

5.6.5 Sema3A Can Inhibit Pro-Angiogenic Molecules in the Anterior Eye

To investigate further if Sema3A can inhibit VEGF-A endothelial cell migration into the anterior eye, I performed bead implantation experiments in the developing cornea. Previous research in developing chick limbs found that implanted beads bounds to Sema3A repelled both axons and vessels (Bates et al., 2003). Using the same technique, I implanted beads soaked in VEGF-A, Sema3A, combination of the two or in Ringer’s to serve as a control. I first discovered that Sema3A, even when placed as far as 300-600um away from the iridial ring artery, influenced the development of the iridial ring artery compared to control beads. The pericorneal iridial ring artery is the vasculature that would provide endothelial cells if any migrated into the cornea. Sema3A can affect this artery in an inhibitory way, suggesting that Sema3A can inhibit cornea
vascularization. When VEGF-A was introduced to the cornea, there was a predictable pro-angiogenic affect on the iridial ring artery, and endothelial cells were able to enter the cornea space. However, when Sema3A was combined with VEGF-A there was a significant decrease in endothelial cell migration into the anterior eye. Sema3A was able to inhibit the VEGF-A’s proangiogenic affect on the iridial ring artery. A delicate balance between multiple pro and antiangiogenic molecules maintains the avascularity of the cornea. It can be assumed that a similar balance is required to establish avascularity. Sema3A has shown to be a key player in this balance. With the absence of Sema3A, the inhibitory affect of this molecule on the endothelial cells into the cornea is weakened, and when the tip of the scale falls to the proangiogenic side with an increase of VEGF-A, Sema3A is able to balance the scale back toward avascularity.

It is my belief that Sema3A is acting in the developing cornea as an antiangiogenic factor to help establish avascularity. This role may become diminished as the cornea matures. This interesting attribute may make Sema3A a potential therapeutic target for adult neovascularization. VEGF-A is increased in vascularized corneas. Sema3A has now been shown to inhibit the pro-angiogenic migration of endothelial cells caused by VEGF-A. Sema3A’s limited role or transcript level in the adult cornea could easily be therapeutically increased during adult neovascularization, potentially decreasing the amount of blood vessel attraction in this diseased state.
Chapter 6: Summary, Significance and Future Directions

6.1 Summary and Significance

This work represents an important advance in our understanding of cornea development and the contribution of Sema/Npn signaling in this developing tissue. Proper innervation and maintenance of avascularity in the cornea is crucial for adult vision, however, the analysis of the molecular regulators that help to establish these characteristics in development are lacking in the literature.

Although the development of corneal innervation has been characterized in the avian system, the innervation process in the developing murine cornea was undocumented. Previous to my work, murine innervation was presumed to follow a similar developmental pattern as compared to avian development. In this dissertation I have used immunofluorescence to label neurons in the murine system to define the timing and pattern of corneal innervation in the stroma and epithelium of the developing mouse cornea. This contributes to a better understanding of cornea development, as this data support a model in which nerves progress directly into the mouse cornea, rather than forming a pericorneal nerve ring found in the avian systems.

Murine cornea development consists of distinct positioning of stromal nerves around the cornea with the majority of nerve bundles originating from the DN, VN, and VT quadrants. A significant decrease of bundles in the DT quadrant may be significant as the animal matures. There may be fewer terminal nerve endings in this quadrant in the adult and this information may be beneficial when
determining placement of surgical wounds such as where to make a LASIK flap or incision for cataract or elective lens replacement surgeries.

These results also demonstrate that cornea epithelial innervation occurs in two distinct phases with the ramification of stromal nerves into radial patterns that directly innervate the developing epithelial layer and the arrangement of nerves into leashes that project toward and form an epithetical swirl in the cornea apex at about three weeks after birth. Previous research has established that corneal epithelial cells form a swirl in the adult cornea, and that there is a suspected correlation in movement between cornea epithelial cells and epithelial nerves (Collinson et al., 2002). Previous research is unclear on whether epithelial cells guide nerve migration or if nerves guide the cellular migration. The formation of a swirl in the epithelial nerves at three weeks of development occurs before the documented epithelial cellular swirl at 4 weeks (Nagasaki and Zhao, 2003), indicating that the nerves may be guiding the epithelial cells toward the apex of the cornea and also that nerves project independently of epithelial cell migration.

The development of the murine cornea is distinct from the avian cornea, but many of the same signaling molecules that help to control development are common to both models. The next group of research experiments demonstrates that Sema/Npn signaling, which is an important signaling pathway for avian development, is also important for the development of the murine system, and that this well known axon signaling pathway also plays an important role in corneal avascularity during development.
I have demonstrated that similar to the avian system, Sema3A is strongly expressed in the lens and its receptor, Npn1, in the trigeminal ganglion throughout murine cornea development. I also have shown that Npn2, expressed in the trigeminal ganglion during early development, and a strong expression pattern of Sema3F in the cornea epithelium place these molecules in key positions to regulate epithelial innervation. The similarities in expression of these regulatory molecules and the differences seen in the development of innervation between the chick and the mouse may be due to the differences in cornea development. The mouse corneal nerves arrive in the proximity of the Sema3A producing lens after a large influx of neural crest cells into the cornea has already occurred. This may buffer the repulsive force of the Sema3A signal and prevent the formation of a pericorneal nerve ring. However, this research demonstrates that Sema3A/Npn1 signaling has a significant repulsive affect on the developing corneal nerves of the mouse and Sema3F/Npn2 signaling in epithelial innervation. Interestingly, in Npn1\textsuperscript{Sema-/-};Npn2\textsuperscript{2/-} double mutants there is increased ectopic innervation of the cornea stroma, epithelium and lens space suggesting that these signaling pathways may play a redundant role during cornea formation. This is significant for therapeutic purposes. If only one pathway is targeted for therapeutic treatment, the other pathway may compensate for this lose making the treatment less effective.

The avian and murine systems develop differently in the timing of neural crest cell migration, innervation and avascularity. The most important stage of
development for the timing of innervation and avascularity in the avian system occurs at E5. At this time, \textit{Npn1} expression is prominent in the pericorneal region, but is absent in any neural crest cells that have migrated over the lens and established the corneal endothelium. At this same time point (E5), \textit{Npn1}-expressing endothelial cells have stopped their migration at the tip of the optic cup and have started to form the vasculature of the iridial ring artery around the cornea, and axons originating from the \textit{Npn1}-expressing trigeminal ganglion have arrived at the perimeter of the cornea but are starting to be repelled to form the pericorneal nerve ring (Fig 6.1A). In the avian system, endothelial cells and axons arrive and are subsequently prevented from entering the cornea at approximately the same time. The murine system has different developmental timing. In the mouse, endothelial cells are present in the eye region at E10.5 (Fig 6.1B), well before the arrival of axons (E13.5) and the initiation of corneal development (E11.5). The murine cornea develops in the presence of proximal endothelial cells from the start. By the time axons have reached the perimeter of the cornea at E13.5, the anterior eye has already formed much of the hyaloid and annular vasculature (Fig 6.1C). Previous research has suggested that blood vessels can guide peripheral nerves during development (Bates et al., 2003). The early arrival of endothelial cells in the anterior eye, and presence throughout corneal development in the mouse may be a reason why nerves are not initially repealed from the cornea and do not form a pericorneal nerve ring. Guidance molecules released from the anterior eye vasculature could be playing a role in
Fig 6.1 Comparison of key time points in avian and murine innervation and avascularity.

(A) At E5 in the avian system, both the axons and endothelial cells are repelled from the cornea (dashed yellow circle) at approximately the same time. (B) In the mouse, endothelial cells arrived in the anterior eye and presumptive cornea (dashed yellow circle) at E10. This is before the cornea has started to form. (C) By the time murine innervation reaches the cornea (E13.5), there are numerous corneal stroma layers and the presence of established vasculature. Sections are stained with DAPI (blue).
the timing and regulation of innervation in the mouse. The results of this
dissertation and previous research indicate that Sema3A from the lens plays a
large role in preventing premature innervation and vascularization of the cornea
during development. In the avian system, the repulsive force of Sema3A from the
lens is working on a single endothelial layer at E5, allowing for a possible
maximum impact on axons and endothelial cells at this time. This may be why
axons and endothelial cells are both repelled from the cornea at approximately
the same time. However, in the murine system, the neural crest cells from the
mesenchyme have already migrated into the stroma and formed several layers
before axons have arrived. The extra corneal layers and presence of endothelial
cells may buffer the amount of repulsive Sema3A that reaches the approaching
axons at E13.5 and allow the axons to travel directly into the cornea.

The contribution that Sema3A plays in keeping the cornea avascular
during development is a novel finding and represents an important molecular
regulator of avascularity. This research emphasizes the important role that the
lens plays in cornea development and to specifically determine the role that lens-
derived Sema3A plays in both the avian and mammalian systems. Although the
avian lens is avascular, and the murine lens is highly vascular, lens derived
Sema3A appeared to function as an important antivascular role in the cornea of
both systems. This suggests that Sema3A may play a universal antivascular role
across divergent species. This could be a significant advance in our
understanding of the role that Sema3A plays in cornea development and also for its potential role as an antivascular therapeutic agent.

6.2 Future Work

The work put forward in this thesis clearly shows that Sema/Npn signaling is important for the development of a properly innervated and avascular cornea, but several important questions remain. For instance it is still unclear if the human cornea develops corneal innervation through branching similar to the mouse or by a radial manner after the formation of a pericorneal nerve ring similar to the chick. The equal distribution of adult corneal stromal branches suggests the formation of a pericorneal nerve ring, but further work should be performed to determine if this is the case. Additionally, since in the development of nerves in the murine eye there is a decrease in the contribution of nerves in the DT quadrant, it would be of interest to determine if there is a difference in density of stromal or epithelial nerves in the quadrants of the adult mouse.

Corneal limbal stem cells are an important source of epithelial cells in the adult cornea, and limbal stem cell deficiencies can cause decreased vision, chronic inflammation and hyperemia, and recurrent episodes of pain (Dua and Azuara-Blanco, 2000). The hypothesis suggested by these results is that the epithelial nerve swirl forms before the epithelial cells form their swirl, and implicates the neuropeptides play a role in epithelial cell migration. This investigation would be an interesting area of further research with potential broad ranges of therapeutic benefits for limbal stem cell deficiencies.
Recently, it has been discovered that other signaling molecules, such as the Roundabout (Robo)/Slit family of nerve guidance molecules, play a role in axon guidance in the chick, suggesting that Sema3A/Npn1 signaling does not act alone in controlling axon growth into the cornea (Kubilus and Linsenmayer, 2010; Schwend et al., 2012). Studies to investigate if these molecules play a similar regulatory role in mouse corneal innervation would be beneficial in understanding differences in nerve patterning. Again, little is known about the mechanisms of corneal innervation or how to regenerate a damaged stromal nerve bundle. Any additional insight into how Sema/Npn or Robo/Slit function during corneal innervation across species should be considered when determining therapeutic nerve growth stimulants for nerve regeneration.

In the work performed on the Npn1Sema-/− mouse, it was found that the formation of the cornea during development was not affected compared to wild-type littermates. This finding is interesting because the disruption of Sema signaling in the chick led to presumptive migration of pericorneal neural crest cells into the cornea stroma in early development. Differences in the expression patterns of Npn1 in these pericorneal neural crest cells in the mouse exist compared to the chick. The chick pericorneal neural crest cells express Npn1 and the cells are only able to enter the cornea ectopically when Sema3A is not present (Lwigale and Bronner-Fraser, 2009). In contrast, expression of Npn1 in the pericorneal neural crest cells of the mouse was not seen. This may suggest why corneal development defects were not seen in Npn1Sema-/− mutants, but this
may also suggest a mechanism through which the corneal development is different in these systems. It would be interesting to investigate the role Sema/Npn signaling plays in preventing stromal cell migration into the avian cornea during early development and test the conservation of this same mechanism in regulating mouse pericorneal cell migration into the stroma of the developing cornea and creating the documented phenotypic differences in developmental patterning in these two systems.

Identification of other molecular signals in corneal development is also necessary. The research in this dissertation demonstrates that Sema3A is required to establish an avascular cornea during development, but it does not appear to be the only inhibitory molecule to establish avascularity. Further research can elucidate all of the molecular players in avascularity. These types of studies will not only expand our understanding of the development of the cornea, but also can be used to investigate the mode of action of a wide range of angiogenic therapeutics.
BIBLIOGRAPHY


