Introduction

The cornea is a transparent, avascular, and highly innervated tissue located at the most anterior part of the eye. It is one of the most innervated tissues of the body, with the nerves providing both sensory information and trophic factors that maintain corneal health and normal vision [1]. Aberrant corneal innervation due to viral infection, trauma or elective surgery can cause neurotrophic keratitis, a condition that leads to degeneration of the cornea [2]. The anatomy and physiology of adult corneal nerves have been very well characterized in many animal models including mouse [3], rats [4], rabbits [5], and humans [6]. These studies show that nerve bundles enter the cornea periphery and radially project towards the center while bifurcating repeatedly into several branches that ultimately innervate the cornea epithelium. Although the development of corneal nerves has been characterized in chick [7,8] and mouse [9], the molecular mechanisms regulating this process are not well understood.

In the chick embryo, sensory nerves approach the cornea from the ventrotemporal region. These nerves initially avoid the cornea and project dorsally and ventrally forming a ring of nerve fascicles surrounding the developing cornea, also known as the pericorneal nerve ring [7,8]. In the mouse embryo, nerve bundles approach the cornea from four quadrants situated along the dorsal-ventral and nasal-temporal regions. Unlike corneal innervation in avian embryos, in the mouse, presumptive corneal nerves project directly into the developing cornea without forming a pericorneal nerve ring [9]. Major nerve bundles from all quadrants branch repeatedly as they grow into the corneal stroma and subsequently project anteriorly to innervate the epithelium.

Sensory nerves of the cornea originate from the ophthalmic and maxillary branches of the trigeminal ganglion [10,11,12]. Axon projections from the trigeminal ganglion are guided towards their targets by attractive and repulsive signals. Neurotrophins including nerve growth factor (NGF), brain-derived growth factor (BDNF), and neurotrophin-3 (NT-3) play a critical role in attracting sensory nerves to their targets and are expressed by the developing cornea [13]. Conversely, repulsive cues within the craniofacial environment determine their spatiotemporal behavior as they grow towards and innervate their targets. A group of axon guidance molecules known as Semaphorin (Sema), which signal through Neuropilin (Npn) receptors, play a major role in guiding trigeminal axons. Mutant mice lacking proper function of Sema3A or its receptor Npn1 [14,15,16], and Sema3F or its receptor Npn2 [17,18,19,20], show severe defasciculation of nerves projecting from the trigeminal ganglion. However, few studies have examined innervation of the target tissues in these mutants at later stages of development.

Several class 3 secreted semaphorins are expressed in the developing chick eye during early development. Of these, Sema3A and Sema3F are expressed in patterns consistent with a role in cornea development. Sema3A is expressed in the lens epithelium, while Sema3F is expressed in the adjacent presumptive cornea epithelium [21,22]. Inhibition of Sema3A signaling in the chick, either pharmacologically or by lens ablation abrogates the formation of the pericorneal nerve ring, which results in precocious and disorganized innervation of the cornea. [9]. Mutant mice lacking Sema3A exhibit misguided axon projections into the eye [15], but have not been studied in detail with respect to...
corneal innervation. Similarly, the role of Sema3F/Npn2 signaling has not been described during this process. Given that innervation of the mouse cornea is different than chick in that it lacks a pericorneal nerve ring [9], the role of Sema3A/Npn1 and Sema3F/Npn2 signaling in innervation of the mouse cornea remains unclear.

In this study, we investigated the role of Sema3A/Npn1 and Sema3F/Npn2 signaling during mouse corneal innervation. We analyzed the ocular expression of Sema3A and Sema3F, and their respective receptors Npn1 and Npn2 by the trigeminal ganglion over time. To determine the functional significance of Sema3A/Npn1 and Sema3F/Npn2 signaling during mouse corneal innervation, we compared Npn1<sup>−/−</sup> and Npn2<sup>−/−</sup> mutant corneas with wild type litter mates and observed that mutants 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 distinct and important role for Sema3A/Npn1 and Sema3F/Npn2 signaling during innervation of the mouse cornea.

Materials and Methods

Mouse embryos

Generation of Npn1<sup>Sema<sup>−/−</sup></sup>, Npn2<sup>−/−</sup>, and double mutant mice lacking Semaphorin signaling through Neuropilins (Npn1<sup>Sema<sup>−/−</sup></sup>; Npn2<sup>−/−</sup>) was described previously [18,23,24]. Briefly, the Npn1<sup>−/−</sup> mice express normal levels of a modified Npn1 protein that binds to VEGF<sub>164</sub> but due to a 7 amino acid substitution in the binding domain, its interaction with Semaphorin is completely disrupted. Npn2<sup>−/−</sup> mice were generated with a complete deletion of the first exon and 1.7 kb of upstream sequence. Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Rice University (approval #A09081401). The mouse lines were maintained through heterozygous matings as separate breeding colonies. Mice with visible vaginal plugs the morning after mating were designated as embryonic day 0.5 (E0.5). For corneal innervation studies, embryos were collected at E12.5–E16.6 in sterile Ringer’s solution. Mutant embryos were identified by PCR genotyping using primers for Npn1<sup>Sema<sup>−/−</sup></sup> [23] and Npn2<sup>−/−</sup> [18] with the following modifications to the Npn2<sup>−/−</sup> primers: primers used to identify a 400 bp wild type band were (F, 5′-TCAGGACAG-GAAGTTGAGAAG-3′, RV, 5′-GGGA-TAGTGTTCTGCTTCA-3′) and primers used to identify 1 Kb mutant band were (F, 5′-CGCATGTGCTAGCCATGAT-3′, RV, 5′-GGAAGATGTGCTACTCTCTCTTGA-3′). 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.

In situ hybridization

Freshly isolated mouse heads (E12.5–E14.5) or eyes (E16.5) were fixed overnight at 4°C in 4% paraformaldehyde. Whole-mount immunostaining of heads and eyes was performed following standard procedures with minor modifications. Briefly, samples were washed in phosphate-buffered saline containing 0.1% Triton-X (PBT) and blocked in PBT containing 0.1% BSA and 5% heat-inactivated sheep serum. Mouse heads between E12.5–E13.5 were incubated overnight at 4°C and eyes from E14.5–E15.5 were incubated overnight at room temperature in antibody solution containing rabbit anti-neuron–specific β-tubulin (TuJ1) IgG antibody (Covance, Richmond, CA) diluted 1:500 in blocking solution. After extensive washes in PBT, samples were blocked for one hour then incubated overnight at 4°C in secondary antibody (Alexa 594 goat anti-rabbit IgG, Invitrogen, Carlsbad, CA) diluted 1:200 in blocking solution. Samples were washed and mounted in PBS, then imaged with a Zeiss AxioImager 2 fluorescence microscope with ApoTome and Axiocam (Carl Zeiss AG, Germany). After imaging, samples were embedded in gelatin, cryo-sectioned at 8–10 μm and counterstained with 4,6-diamidino-2-phenylindole (DAPI) to label all nuclei.

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 and therefore no effort was made to distinguish between stroma and epithelial nerves. Images were processed in ImageJ [28] and nerves were quantified using the NeuroJ plug [29]. 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.

Results

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 [12,30,31]. 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 [9]. Surprisingly, mice lack a pericorneal nerve ring and instead have direct projection of sensory afferents into the presumptive cornea [9]. Given these species differences in axon patterning, here we examine the role of Npn/Sema signaling in the mouse during corneal innervation.

As an initial step, we established the expression of Sema3A and Sema3F, and their respective receptors Npn1 and Npn2 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 [9]. The expression patterns of these mRNAs at E13.5 (data not shown) are similar to E12.5 (Fig. 1A, 1B, 1F and 1G).

Expression pattern of Npn1 and Sema3A during mouse corneal innervation

In situ hybridization on sections through the craniofacial region at E12.5 shows strong expression of Npn1 mRNA by the trigeminal...
Expression pattern of Npn2 and Sema3F during mouse corneal innervation

To determine whether Npn2/Sema3F, like Npn1/Sema3A, are candidates for mediating axonal guidance during mouse corneal innervation, we examined the expression of Npn2 and Sema3F mRNA 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 [17,18,20] and overlaps with Npn1 expression at these stages [14,32]. Interestingly, we found that Npn2 is expressed at relatively low levels in the trigeminal ganglion at E12.5 (Fig. 1F), compared to Npn1 expression at the same stage (Fig. 1A). However, similar to Npn1, Npn2 is expressed in the periorcular mesenchyme and hyaloid vasculature. Npn2 is also strongly expressed in the mesenchyme around the optic nerve (Fig. 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. 1H, 1I, and 1J; asterisk).

Sema3F is ubiquitously expressed at low levels in the ocular and frontal-nasal regions of the mouse between E9.5 and E11 [18,20,33]. As previously shown, we found that at E12.5, Sema3F is ubiquitously expressed at low levels in the presumptive cornea, optic cup, and ocular mesenchyme (Fig. 1G). Between E14.5 and E15.5 expression of Sema3F remains at low levels in the eyelid mesenchyme, optic cup, periorcular mesenchyme and presumptive corneal stroma, but becomes prominent in the eyelid and corneal epithelium (Figs. 1H and 1I). By E16, expression of Sema3F persists in the corneal epithelium and becomes strong in presumptive iris and iridocorneal angle, optic cup, and in the eyelid mesenchyme adjacent to the ectoderm (Fig. 1J). The low levels of Npn2 expression by the trigeminal ganglion coupled with low levels of Sema3F expression by 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 corneal epithelium at E16.5 may play a role during epithelial innervation.

Npn1 but not Npn2 regulates innervation of the mouse corneal stroma

Mouse embryos lacking either Npn1 [14,23,34,35] or Npn2 [18,20], exhibit defects in trigeminal ganglion development including severe defasciculation of nerve projections. Similar trigeminal defects are observed in mice lacking Sema3A [16] or Sema3F [20] function. Given that Sema3A and Sema3F are expressed in ocular tissues in the pathway of trigeminal sensory projections, we asked whether Npn1 and Npn2 are required for nerve guidance during mouse corneal innervation. Since Npn1/Npn2−/− null mutant mice die at E8.5 from vascular defects due to lack of

ganglion, central region of the optic cup, hyaloid vasculature, and periorcular mesenchyme (Fig. 1A). Strong expression of Npn1 persists in the trigeminal ganglion throughout the period of corneal innervation (Figs. 1C−1E). 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.

Figure 1. Expression of Npn1 and Npn2 and their ligands Sema3A and Sema3F during eye development. Levels of mRNA 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), periorcular 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 periorcular 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 periorcular mesenchyme at E12.5. (H−I) 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. doi:10.1371/journal.pone.0037175.g001
signaling from vascular endothelial growth factor [36], we used a line of Npn1 mutant mice that is only defective in Npn1/Sema signaling [23,24] and is viable during the period of corneal innervation. Nerve projections were examined in wild type, Npn1Sema2−/−, and Npn2−/− mutant embryos that were stained with the TuJ1 antibody to identify axonal projections. Previously, we showed that in wild type mice, presumptive corneal nerves first appear in the anterior region of the eye at about E12.5 [9]. At this stage, anterior nerve projections are 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. 2A and J). Npn1Sema2−/− eyes (n = 6) are innervated in all quadrants including the DT quadrant (Fig. 2D; arrow). The DT quadrant is never innervated at this time in wild type eyes. Npn1Sema2−/− eyes display ectopic nerve bundles (Fig. 2D; asterisks) projecting between the entry points of the four major nerve bundles which are found in wild type eyes, and subsequently innervate the entire corneal stroma [9].

Although the major nerve bundles from the DN, VN, and VT quadrants (n = 11/35; Figs. 2A and J) were apparently not affected and resembled wild type at E12.5 (Figs. 2G and 2P). By E13.5, axons from the DN, VN, and VT major nerve bundles extend as far as the corneal periphery (Fig. 2D; asterisks) projecting between the entry points of the four major nerve bundles which are found in wild type eyes, and subsequently innervate the entire corneal stroma [9]. In addition, the nerve bundles project into the corneal periphery of E12.5 Npn1Sema2−/− eyes (Figs. 2D and M; arrowheads), which normally occurs later at about E13.5 in wild type (Fig. 2B). In marked contrast to Npn1Sema2−/−, axon projections into Npn2−/− eyes (n = 8) were apparently not affected and resembled wild type at E12.5 (Figs. 2G and 2P). By E13.5, axons from the DN, VN, and VT major nerve bundles extend as far as the corneal periphery and the DT quadrant is minimally innervated in some of the wild type eyes (n = 15, Fig. 2B). At this stage in Npn1Sema2−/− eyes (n = 8), nerve bundles continue to project from all quadrants and innervate the stroma (Figs. 2E and N). Nerve bundles in the DT quadrant projected further and innervated the stroma of Npn1Sema2−/− corneas (Fig. 2E; arrowhead). In some cases nerve bundles grew past the corneal center and projected across its diameter (Fig. 2E; asterisk). Npn2−/− eyes (n = 6) showed similar patterns of innervation as wild type at this stage (Figs. 2H and 2Q).

Because of variations in the extent of axon projections into the cornea within wild type and mutant embryos, we quantified axon lengths within the cornea during stromal innervation (Fig. 2S). We 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 = 0.7160) relative to wild type. Altogether, our 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.

**Innervation of the corneal quadrants in Npn1Sema−/− and Npn2−/− mutant mice**

As shown above and previously described [9], a unique characteristic of normal mouse corneal innervation is that all nerves originate from four major nerve bundles that repeatedly branch as they innervate the corneal stroma and epithelium. Although the major nerve bundles from the DN, VN, and VT quadrants project into the cornea at the same rate and innervate approximately similar surface areas, the DT quadrant grows relatively slowly and therefore covers a smaller area. To further determine if there were aberrant nerve projections in Npn1Sema−/− and Npn2−/− mouse corneas, we quantified the extent of stromal innervation in each quadrant by measuring the total length of axons using the NeuroJ plug-in for the ImageJ software.
Statistical analysis was performed on measurements taken from wild type, Npn1Sema−/− and Npn2−/− 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, we observed a significant bias of axons away from the DT quadrant compared to other quadrants (Figs. 3A and 3B). By E15.5 (n = 8), nerves in the DT quadrant project further towards the cornea center (Fig. 2M). Although there are relatively fewer nerve bundles in the DT quadrant at this time, we observed no difference in the innervation of the DT and VT quadrants. Nonetheless the DT quadrant remained less innervated than the VN and DN quadrants (P<0.05; Fig. 3C). Conversely, stromal innervation of Npn1Sema−/− 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. 3D). By E14.5 (n = 8) all quadrants in Npn1Sema−/− corneas were equally innervated (P<0.05; Fig. 3E). At E15.5 (n = 8), the VN and DN quadrants were innervated at the same level (P<0.05) but more than the DT and VT quadrants (P = 0.001; Fig. 3F). At these stages of development, innervation of Npn2−/− stromal quadrants was similar to wild type (compare Figs. 3G–3I and 3A–3C). Our results show random projection of nerve bundles during the innervation of Npn1Sema−/− corneal stroma compared to Npn2−/− and wild type, further suggesting the requirement of Npn1 but not Npn2 during stromal innervation.

Figure 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 average length of corneal nerves (µm) and X-axis is cornea quadrants. (A–C) Wild type, (D–F) Npn1Sema−/− 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. No bracket indicates P > 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001. doi:10.1371/journal.pone.0037175.g003

Npn1 and Npn2 play a role during innervation of the corneal epithelium

As nerve bundles grow toward the central region of the cornea, both large and small branches project anteriorly toward the epithelial layer at E15.5 (Fig. 4A, arrow), subsequently innervating it at E16.5 (Fig. 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 is prominent in the epithelial layer at these stages (Fig. 1H, 1I and 1J) and probably plays a role during its innervation. To determine whether Npn1 and Npn2 play a role during innervation of the corneal epithelium, we analyzed corneal sections from E15.5 and E16.5 Npn1Sema−/− and Npn2−/− mutant embryos. The first evidence of epithelial innervation in Npn1Sema−/− (Fig. 4G, asterisk; n = 5/7) and Npn2−/− (Fig. 4E, asterisk; n = 4/7) corneas was at E15.5, a day earlier than wild type (n = 0/13). By E16.5, there was continued innervation of the epithelium in both Npn1Sema−/− and Npn2−/− corneas (Fig. 4D and 4F; n = 5/5 each) without apparent difference in the number of fascicles compared to wild type (n = 11/11). Thus both Npn1 and Npn2 are required for the proper timing of epithelial innervation.
Corneal innervation defects are enhanced in Npn1Sema−/−Npn2−/− double mutants

Double mutant mouse embryos lacking semaphorin signaling through Npn1 and Npn2 (Npn1Sema−/−;Npn2−/−) show increased disorganization of cranial ganglia including the trigeminal ganglion compared to Npn1Sema−/− and Npn2−/− single mutants [37]. Since corneal sensory nerves are derived from the trigeminal ganglion [12], we asked whether the synergistic defects observed in the trigeminal ganglion of double mutants were recapitulated during corneal innervation. We found that similar to Npn1Sema−/− (Fig. 2B), several axons projected into the anterior eye region of E12.5  Npn1Sema−/−;Npn2−/− double mutant embryos (Fig. 5A). Some axons projected into ectopic regions (Fig. 5A; asterisks) and the DT quadrant was innervated (Fig. 5A; arrow). Cross-sections through E12.5 double mutant eyes revealed that axons projected into the presumptive corneal stroma (Fig. 5B; arrowhead). Comparisons of nerve lengths revealed that Npn1Sema−/−;Npn2−/− double mutant eyes (n = 8) were more innervated than Npn1Sema−/− (p<0.01) and Npn2−/− (p<0.001) single mutants (Fig. 5C). At E13.5, we 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. 5D). Due to difficulties in obtaining Npn1Sema−/−;Npn2−/− embryos (1/16 from an average litter of 8 embryos), we were 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 project through the presumptive stroma, but also prematurely innervate the corneal epithelium (Fig. 5E, arrowhead). In addition, we observed ectopic nerve projections near the lens vesicle (Fig. 5E, arrow) and between the optic cup and lens vesicle (Fig. 5E, asterisks). Although aberrant innervation of the lens vesicle was previously reported in Sema3A−/− mutants [13], it was not evident in the Npn1Sema−/− (Fig. 2N), Npn2−/− (Fig. 2Q), or wild type (Fig. 2K) eyes at E13.5. Our results further suggest that both Npn1 and Npn2 are required for and play distinct roles during mouse corneal innervation.

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 [14,15,20,23,37], very little is known about its role 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 [8,38]. However, the mouse cornea develops differently, such that nerve bundles project directly into the presumptive cornea without initial formation of a pericorneal nerve ring [9].

This led us to investigate whether Npn1/Sema3A and Npn2/ Sema3F signaling play a role in guiding trigeminal afferents during

Figure 4. Timing of the innervation of the corneal epithelium is disrupted in Npn1Sema−/− and Npn2−/− double mutants. Cross sections of E15.5–E16.5 (A–B) Wild type, (C–D) Npn1Sema−/−, and (E–F) Npn2−/− corneas immunostained with TuJ1 and counterstained with DAPI. In wild type corneas, axons projection toward the epithelium at E15.5 (A) and innervate it at E16.5 (B). The corneal epithelium is innervated by E15.5 in Npn1Sema−/− (C, asterisk) and Npn2−/− (E, asterisk) mutant corneas. Scale bar: 50 μm.

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Figure 5. Increased disruption of corneal innervation in Npn1Sema−/−;Npn2−/− double mutants. Whole mount and sections of E12.5–E13.5 eyes were immunostained with TuJ1. Sections were counter stained with DAPI. (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 Npn1Sema−/−;Npn2−/− double mutant cornea than Npn1Sema−/− or 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. (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 μm. oc, optic cup; c, cornea.

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Figure 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+/− mutant embryos, stromal innervation is similar to wild type but the epithelium is prematurely innervated by E15.5. (C) In Npn1Sema−/− 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 Npn1Sema−/− and Npn2−/− mutants are exacerbated in the absence of Sema/Npn signaling (Npn1Sema−/−, Npn2−/− double mutants) by E13.5. In addition, nerve bundles project into ectopic regions of the eye between the optic cup and lens vesicle.

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mouse corneal innervation. Our 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.

Several studies have shown that both Npn1 and Npn2 are expressed in the trigeminal ganglion during early development of the mouse embryo [20,39]. 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. Since trigeminal afferent axons are guided by signals from their targets and surrounding tissues [8,40,41], we 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. We found that Npn1 is expressed prominently and continuously in the trigeminal ganglion. This pattern of expression is conserved between chick [8] and mouse. Suggesting that in addition to guiding neuron progenitor cells during gangliogenesis, Npn1 plays an important role in guiding axon projections towards their targets. Our results also show that Sema3A is abundantly expressed in the lens but absent from the mesenchyme adjacent to the optic cup through which axons traverse as they project towards the cornea [9]. During chick eye development, Sema3A is expressed in the lens vesicle, presumptive corneal epithelium, and mesenchyme of the presumptive iris and ciliary body [21,22,38]. In vitro, Sema3A from the lens repels trigeminal axonal growths whereas its inhibition in vivo results in precocious innervation of the cornea [8]. Therefore the conserved expression of Sema3A in the mouse lens suggests that it plays a similar role of guiding trigeminal axons during mouse corneal innervation. Unlike chick, Sema3A signaling by the mouse lens does not lead to the formation of a pericorneal nerve ring. 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 [7,9]. 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 [42], which 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 since inhibition of Sema3A during chick corneal innervation does not completely abolish the pericorneal nerve ring [8].

The trigeminal ganglion expresses Npn2 at about E9.5 where it plays a role during gangliogenesis [20]. Our results show low levels of Npn2 expression in the trigeminal ganglion by E12.5 and this level of expression is maintained during corneal innervation, except for the strong expression that is restricted to the trigeminal root where it attaches to the central nervous system. Expression of Sema3F is diffuse in the ocular tissues at E12.5 but becomes restricted to the corneal epithelium at E14–E16.5. Similarly, expression of Sema3F is prominent in the presumptive corneal epithelium of the chick [21], but was not examined at later stages of corneal development. The low levels of Npn2 expression in the trigeminal ganglion coupled with the low expression of Sema3F in the corneal stroma suggest 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.

Given that Npn1 and Npn2 are expressed at different levels in the trigeminal ganglion and that their respective Sema3A and Sema3F ligands are expressed in distinct patterns in ocular tissues, we analyzed Npn1Sema−/− and Npn2−/− mutant embryos for development of corneal innervation. Previous studies of Npn1Sema−/− and Npn2−/− mutant embryos have shown premature innervation of the limbs [40] and aberrant projection of axons in the ear [43]. Similarly, we show that axons are misguided in Npn1Sema−/− mutants resulting in precocious, disorganized, and increased innervation of the corneal stroma. Npn1Sema−/− 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 [9]. Since 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, we found that in Npn1Sema−/− 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. When lens-derived Sema3A signaling is blocked, neural crest cell progenitors fail to migrate appropriately resulting in abnormal development of the cornea [22]. While there is a possibility that malformation of cornea may contribute to the corneal innervation defects in chick, this does not appear to be the case in mouse. There were no apparent defects in neural crest cell migration during mouse cornea development and all layers were formed normally in the Npn1Sema−/− mutants (data not shown). Interestingly, unlike chick where Npn1 is expressed early in the periocular region [21,22], it is not expressed in the mouse during active migration into the presumptive cornea region between E10.5–E11.5 (data not shown). This may explain the insensitivity of mouse periocular neural crest cells to lens-derived Sema3A signaling and the differences between chick and mouse cornea development.
In contrast to \(Npn1^{Sema-/-}\), innervation of the corneal stroma was not affected in \(Npn2^{-/-}\) mutants. This is not surprising since \(Npn2\) and \(Sema3F\) are expressed at low levels in the trigeminal ganglion and corneal stroma during this time (Fig. 1F–I and H–J). However, similar to \(Npn1^{Sema-/-}\), the epithelium is prematurely innervated at E13.5 in \(Npn2^{-/-}\) mutant corneas. At this time, \(Sema3F\) is strongly expressed in the corneal epithelium, therefore its premature innervation could be due to lack of response to \(Sema3F\) signaling in this layer despite basal levels of \(Sema3F\) expression in the corneal epithelium (Fig. 6). We find that \(Sema3A/Npn1\) is required for the innervation of the corneal stroma and epithelium, whereas \(Sema3F/Npn2\) is only required for epithelial innervation. The distinctive functions in corneal innervation of \(Sema3A/Npn1\) and \(Sema3F/Npn2\) are further confirmed by the increase of defective axon projections in the absence of \(Sema/Npn\) signaling. Since adult corneal nerves are regenerable when \(Sema3A\) is ectopically added to the cornea [44], our results may provide insight into the mechanisms that control regeneration of corneal nerves in wounded and diseased corneas.

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**Author Contributions**

Conceived and designed the experiments: CCM RPM PYL. Performed the experiments: CCM RPM PYL. Analyzed the data: CCM RPM PYL. Wrote the paper: CCM RPM PYL.

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