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Expression of axon guidance ligands and their receptors in the cornea and trigeminal ganglia and their recovery after corneal epithelium injury

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Abstract

Axon guidance proteins are essential for axonal pathfinding during development. In adulthood, they have been described as pleiotropic proteins with multiple roles in different organs and tissues. While most studies on the roles of these proteins in the cornea have been performed on the Semaphorin family members, with few reports on Netrins or Ephrins, their function in corneal epithelium wound healing and functional nerve regeneration is largely unknown. Here, we studied the expression of ligands belonging to three distinct axon guidance families (Semaphorins, Ephrins, and Netrins) and their most commonly associated receptors in the cornea and trigeminal ganglia (TG) using immunofluorescence staining and RT-qPCR. We also evaluated how their expression recovers after corneal epithelium injury. We found that all ligands studied (Sema3A, Sema3F, EphrinB1, EphrinB2, Netrin-1, and Netrin-4) are abundantly expressed in both the TG and corneal epithelium. Similarly, their receptors (Neuropilin-1, Neuropilin-2, PlexinA1, PlexinA3, EphB2, EphB4, Neogenin, UNC5H1 and DCC) are also expressed in both tissues. Upon corneal epithelium injury, quick recovery of both ligands and receptors was observed at the protein and gene expression levels. While the timing and expression levels vary among these proteins, in general, most of them remained upregulated for several weeks after injury. We propose that the initial protein expression recovery may be related to corneal epithelium recovery since Sema3A, EphrinB2 and Netrin-4 accelerated corneal epithelial cells wound healing. The sustained high expression levels may be functionally related to nerve regeneration and/or patterning. Whilst further studies are required to test this hypothesis, this work contributes to unraveling their function in normal and injured cornea.

Keywords

Semaphorin; Netrin; Ephrin; Axon guidance proteins; Cornea wound healing

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Declaration of competing interest
none.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2022.109054.

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1. Introduction

The cornea possesses the highest sensory innervation in the human body. Nerve fibers from the ophthalmic and maxillary branch of the trigeminal ganglia reach the corneal stroma as unmyelinated nerve bundles and travel anteriorly to the cornea surface where they divide to form a dense subbasal plexus (Muller et al., 2003). These nerve endings in the corneal epithelium provide sensory and trophic functions for corneal homeostasis and visual acuity. Corneal nerves can be damaged by a myriad of conditions, such as infection, surgery or trauma, diabetes, and chemical burns (Belmonte et al., 2004; Yu et al., 2008). Although the peripheral nerves have a significant capacity for regeneration, the reinnervation after these injuries may lead to altered functional sensation, changes in trophic maintenance, and more permanent sequelae, such as dry eye syndrome (Erie et al., 2005; Pajoohesh-Ganji et al., 2015; Shaheen et al., 2014).

The regeneration of corneal nerve density and patterning requires intrinsic and extrinsic factors, as well as an adequate microenvironment for axonal regrowth (Bosse, 2012). Several growth factors, lipids, and microRNAs have been described to contribute to the maintenance, renewal, and recovery from injury of both the corneal epithelium and nerve endings (Bazan, 2005; Guaiquil et al., 2014; Jhanji et al., 2021; Klenkler and Sheardown, 2004; Pan et al., 2013; Thiede-Stan and Schwab, 2015). However, the recovery of damaged nerve fibers not only requires axonal growth but also appropriate spatial distribution for an effective functional role. Several axon guidance molecules have been described to participate in the target localization of corneal nerves during development and early postnatal growth in mice (Bouheraoua et al., 2019). Proteins belonging to the Semaphorin family, such as Sema3A and Sema3F, are reported to have key roles during patterning and density of nerves in the embryonic mouse and chick cornea by restricting their axonal growth or directing them to the appropriate target (Kitsukawa et al., 1997; Lwigale and Bronner-Fraser, 2009; McKenna et al., 2012). Other axon guidance proteins from the Netrin and Ephrin families have also been identified in the developing chick and mouse cornea, but their role has not yet been determined (Conrad et al., 2008; Li et al., 2012).

In the adult cornea, the expression of several members of these axon guidance protein families has been established (Hogerheyde et al., 2013; Kojima et al., 2007; Li et al., 2012; Morishige et al., 2010). However, their trophic and neuroregenerative roles are not completely understood and their expression recovery during corneal wound healing has not been well established. Semaphorins are a glycoprotein family with pleiotropic activities in adulthood (Alto and Terman, 2017; Fard and Tamagnone, 2021). In the injured mouse cornea, supplementation of Sema3A, Sema3C, and Sema7A results in improved neuroepithelium regeneration (Lee et al., 2019; Namavari et al., 2012; Zhang et al., 2018a). Similarly, Netrins, which are laminin-related secreted proteins with multiple tissue functions (Chaturvedi and Murray, 2021; Lai Wing Sun et al., 2011), have also been described to decrease neovascularization and inflammation in the adult rat and mouse cornea (Han et al., 2012, 2015; Maier et al., 2017) and induce epithelium and nerve wound healing in diabetic mouse corneas (Zhang et al., 2018a, 2018b). Ephrins are the largest known receptor tyrosine kinase family; these membrane-associated proteins mediate cell–cell communication by interacting with their membrane-associated Ephrin ligands and are involved in many tissue

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functions, such morphogenesis, angiogenesis, synaptic plasticity, and neurite outgrowth (Cramer and Miko, 2016; Yang et al., 2018). In the cornea, Ephrins regulate epithelial and endothelial cell migration and limbal–corneal epithelial compartmentalization along with the response of these tissues to injury in mice (Kaplan et al., 2012, 2018; Walshe et al., 2018).

Overall, there is a limited understanding of the role of these axon guidance proteins in the cornea and how their expression levels change after injury. In this study, we analyzed the expression of axon guidance proteins and their most common receptors both in the trigeminal ganglia and cornea and evaluated their expression recovery after subbasal corneal nerve injury. We focused on determining the expression of ligands belonging to the Semaphorins (Sema3A and Sema3F), Ephrins (EphrinB1 and EphrinB2), and Netrins (Netrin-1 and Netrin-4). We also determined the expression of the most common receptors that interact with these ligands (Barton et al., 2004), such as Neuropilin-1 and 2, PlexinA1 and A3 that interact with Semas; EphB2 and EphB4 that interact with Ephrins; and Neogenin, UNC5H1, and DCC that interact with Netrins.

2. Materials and methods

2.1. Animals

Work with animals has been approved by the University of Illinois-Chicago IACUC and is covered under the approved protocol # 20–222. All experiments were performed according to the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision and in compliance with the Arrive guidelines. The C57BL/6 mouse strain was acquired from Jackson Laboratories (Bar Harbor, ME), and mice were fed a standard diet ad libitum and maintained on a 12-h light-dark cycle.

2.2. Corneal epithelium debridement

Male and female C57BL/6 adult mice, 8–10 weeks old, were anesthetized with a mix of ketamine/xylazine, and one drop of 0.5% proparacaine hydrochloride ophthalmic solution (Bausch & Lomb, Tampa FL) was applied to the eye to deliver local corneal anesthesia before injury. A 2-mm circular area of corneal epithelium was demarcated using a trephine and gently removed using an Algerbrush II rotator burr without damaging the underlying stroma. Debridement was performed in one eye keeping the contralateral eye as control. The eyes were collected at Days 0 (immediately after debridement), 1, 3, 5, 7, and 14 using 3 mice per time point. Mice were sacrificed by CO_2 inhalation followed by cervical dislocation. The eyes were enucleated, washed once in PBS, immediately embedded in OCT compound (Tissue-Tek, Sakura, Torrance, CA), and stored at −80 °C until processing for cryosection.

2.3. Processing of trigeminal ganglia and cornea tissue for immunostaining

Trigeminal ganglia and eye globes were collected from adult male and female C57BL/6 mice, 8–10 weeks old. Mice were sacrificed as described above and the TG collected as described (Malin et al., 2007). The ganglia were washed twice in cold PBS and immediately embedded in OCT and stored as described above. Human corneas obtained from the

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Eversight Eye Bank were dissected and mounted over OCT compound. The collected mouse TG and eyes and the human corneas were sectioned using a Cryostar NX50 microtome (Thermo Fisher scientific, Waltham, MA, USA). The 8 μm sections were quickly dried at room temperature and stored at −20 °C. For immunofluorescence staining, sections were fixed in ice-cold methanol for 5 min, washed 3 times in PBS, blocked (2% BSA, 2.5% donkey or goat serum in PBS) for 1h at room temperature and incubated overnight with primary antibody (see Table 1) at 4 °C. Sections were washed 3 times in PBS and incubated at room temperature for 30 min with secondary antibody (see Table 1). Sections were washed 3 times with PBS, air dried and mounted in Vectashield mounting medium with DAPI (Vector labs, Burlingame, CA). Sections were imaged at 20x magnification using an AxioObserver fluorescent microscope (Carl Zeiss Microimaging GmbH, Jena, Germany) and merged using Photoshop at the same settings. Samples without the primary antibody were used as controls to determine any background effect by the secondary antibodies (see Supplementary Fig. S2).

2.4. Immunofluorescent quantification

The quantification of intensity staining in the cornea sections was performed using Fiji. For this, images were opened in Fiji then the image color was split into channels (red or green depending on the fluorophore used for staining). The image threshold was then adjusted to exclude pixels outside of the corneal epithelium. Three regions of interest (left, center, and right) using the polygon selection tool were analyzed for each image and the measurements were plotted as mean grey value that reflects the staining intensity. While informative, this semiquantitative analysis depends on the quality of the antibodies used for immunostaining and imaging technique.

2.5. Corneal epithelial cells wound healing assay

The effect of Sema3A, Netrin-4 and EphrinB2 on corneal epithelial cells was tested by in vitro scratch assays on human corneal limbus epithelial (HCLE) cells. Cells were incubated on K-SFM media supplemented with EGF and BPE (cat#10724–011) and antibiotic-antimycotic solution (Corning cat#30-004-CL) (Lawrence et al., 2012). Cells were seeded at a concentration of $2x10^5$ cells/well on a 24-well plate and grown until 100% confluent monolayers. A SPL 24 well Scratcher Tip Width 0.50 mm (ProLab Cat #201924) was used to create a straight cell-free line in the middle of the well. Media was aspirated and each well was washed with PBS twice before adding the treatments. Cells were left untreated (control) or treated with 50 ng/ml rhSema3A (R&D systems cat #1250-S3-025), 25 ng/ml rmNetrin-4 (Cat# 1132-N4-025/CF) or 25 ng/ml rhEphrinB2 (Cat#7397-EB-050). The plate was then placed in an AxioObserverZ1 microscope provided with a microchamber incubator with controlled heat, humidity and CO_2 (Carl Zeiss Microimaging GmbH, Jena, Germany). Phase-contrast images were taken every 20 min for a 15 h period using a 5x objective. Three different fields per scratch were recorded and treatments performed in triplicate.

The time-lapse images were exported as tiffs files and analyzed in FIJI using a wound healing plugin (Suarez-Arnedo et al., 2020) which traced the wound area over each timepoint. The data was normalized for each well by dividing every timepoint by the area at

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time 0 and multiplying by 100 to obtain the percentage of wound closure. Experiments were performed in triplicate.

2.6. Processing of cornea tissue for real-time quantitative polymerase chain reaction (RT-qPCR)

Mice were subjected to total corneal epithelium debridement as described above without damaging the limbus. For basal expression (time 0), the corneal epithelium was collected from both eyes using an Algerbrush II rotator burr, and the scraped epithelium was directly placed into an ice-cold microcentrifuge tube containing 40 μl PBS and quickly frozen on liquid nitrogen and stored at −80 °C until further processing. The epithelium was collected from one mouse at a time using 5 mice per time point. For post-injury expression, the epithelium was collected again after 3, 7, 14, 21 and 28 days after initial debridement and the mice were sacrificed by CO2 inhalation followed by cervical dislocation. The collected tissues were homogenized on lysis buffer using the RNeasy plus Mini Kit (Cat # 74134, Qiagen, Valencia, CA, USA) and further processed according to the manufacturer instructions to obtain pure RNA. RNA concentration was determined by using a nanodrop spectrophotometer (Implen, Inc. Westlake Village, CA, USA). Reverse transcription was then performed with a High Capacity cDNA Reverse Transcription Kit (Cat # 4368814, Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's instructions, in a 20 μl reaction volume with T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) settings as follows: 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min. Gene expression was evaluated by quantitative PCR using the predesigned mouse TaqMan gene expression assays outlined in Table 2.

Samples were analyzed using TaqMan Gene Expression Master Mix (Cat # 4369016, Applied Biosystems) in the Core QuantStudio Flex 7 qPCR System (Thermo Fisher scientific, Waltham, MA, USA). Samples were assayed in triplicate in a 20 μl reaction volume with the following thermal cycler settings: 1 cycle of 50 °C for 2 min and 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. GAPDH was evaluated as an internal control to normalize expression. A negative control without cDNA was included.

2.7. Statistics

Data are presented as mean ± SD. All data were analyzed using GraphPad Prism version 8. The significance of differences was evaluated using analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. For any statistical test, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Axon guidance proteins and their associated receptors are expressed in the mouse trigeminal ganglia and cornea

The immunostaining of mouse trigeminal ganglia sections revealed that all the axon guidance ligands (Sema3A, Sema3F, EphrinB1, EphrinB2, Netrin-1 and Netrin-4) and the receptors (Neuropilin-1, Neuropilin-2, PlexinA1, PlexinA3, EphB2, EphB4, Neogenin, UNC5H1, and DCC) analyzed are present within the ganglia. All of these proteins were

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found in the neuronal cell bodies (Fig. 1). Interestingly, Neuropilin-1, EphB2 and EphB4 receptors were the only ones that also presented high intensity staining in the axonal fibers (data not shown) of the TG. In general, both ligands and receptors were observed covering the whole neuronal somas (Fig. 1A), while the receptors NRP1, NRP2 and PlexinA3 showed a more membrane-bound staining pattern (Fig. 1B). This was most likely due to our fluorescent technique since we did not observe this pattern in our co-immunostaining (Figs. S3-S5). We found that most of the ligands colocalized with the receptors in our co-immunostaining data, except for ephrinB2-EphB2 which showed minimal colocalization (Figs. S3-S5). We then evaluated the expression of the axon guidance ligands and receptors in the cornea. We found that all of these proteins are abundantly expressed in the corneal epithelium (Figs. 2 and 3). The immunostaining showed that the level of expression is in general evenly distributed through the layers of the epithelium for both ligands and receptors, and no differences were found between male and female mice.

3.2. Axon guidance proteins quickly recover their expression after cornea injury

The effect of wound healing on the expression of these proteins and their ability to recover over time was evaluated in a corneal epithelium injury model. For this, mice were subjected to a 2 mm central corneal epithelium debridement and then the eyes were collected at different time points for immunostaining analysis of corneal cryosections. We observed that after corneal injury, the expression recovery for the ligands and receptors increases as the epithelium heals. The staining of these proteins localized at the basal epithelium layer in the early post-procedure days and then in general was present in all the epithelium layers (Figs. 2 and 3 and S1). By day 14, the expression was comparable to that observed in the uninjured cornea, and high expression was still observed in the basal layer at this time point for Neuropilin-1 and PlexinA3 while PlexinA1 showed more apical expression (Fig. 3). Our immunostaining quantification showed that most ligands and receptors recovered their basal expression at day 14, while some such as Sema3A, EphrinB1, EphrinB2, NRP1, NRP2, PlexinA1 and Neogenin had overexpression and only DCC did not recover its initial basal expression (Figs. 2 and 3). The co-immunostaining data showed that most of the ligands colocalize with the receptors in the normal corneal epithelium. Only NRP1 showed little or no colocalization with Sema3A and Sema3F and EphrinB1 did not colocalize with EphB4. We observed colocalization of the Netrin ligands with all the receptors mostly in the basal layers of the epithelium (Figs. S6-S8).

We next corroborated the observed recovery of these proteins after corneal injury in our gene expression analysis. We found a variable response: while most of these ligands quickly recovered their initial basal expression, some were highly upregulated early on, such as EphrinB2, and others maintained upregulated levels well above their normal expression, such as Sema3A and EphrinB1. Netrins and Sema3F slowly recovered their expression over time (Fig. 4). Similar findings were observed for the receptors, with the Neuropilins, Plexins, EphB2, and Neogenin quickly reaching the basal levels and maintaining upregulation over time (Fig.5). The levels of expression of NRP2 were ten times higher than the aforementioned receptors, quickly upregulated after 3 days from injury, and remained higher until day 28. The expression of EphB4 fluctuated but was still significantly higher than the basal level at 28 days post injury. The only receptor

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that maintained downregulated levels was UNC5H1 but with a tendency to recovery, and it followed the exact pattern observed for Netrin-1 and Netrin-4. Unfortunately, we were unable to detect any gene expression for DCC using several Taqman gene expression assays and a Qiagen assay. Interestingly, PlexinA1 and A3 showed similar recovery patterns, as both were downregulated on day 3, upregulated on day 7 and had comparable expression levels thereafter. The recovery patterns of EphB2 and EphB4 were similar as well. A certain degree of correlation was observed between some ligands and receptors, as Sema3A and NRP1/NRP2 showed their highest expression on day 14, while Netrin-1 and Netrin-4 were similarly expressed to UNC5H1 through the time course of analysis (Figs. 4 and 5).

3.3. Axon guidance proteins are expressed in human cornea

To determine the relevance of our protein and gene expression analysis, we performed immunostaining of human cornea samples obtained from the Eversight Eye Bank. We found that these axon guidance ligands are also expressed in human cornea (Fig. 6). The receptors studied were also found in the human corneal epithelium. Most were highly visible through the epithelium layers, and only Neogenin was found restricted to the basal layer (Fig. 6B).

3.4. Axon guidance proteins accelerate corneal epithelial wound healing

The functional role that axon guidance proteins may have in the cornea was evaluated using scratch assays on HCLE cells. For this, the corneal epithelial cells were grown to confluency and a scratch injury induced. Cells were then treated with various doses of Sema3A, EphrinB2 or Netrin-4 recombinant proteins. The most effective doses inducing faster wound closure were selected and shown in Fig. 7. We found that Sema3A at 50 ng/ml, EphrinB2 and Netrin-4 at 25 ng/ml accelerated wound healing compared to untreated control. At 8h post treatment, cells treated with Sema3A or EphrinB2 showed complete wound closure while those treated with Netrin-4 reached complete closure at 10h, still faster than control (Fig. 7).

4. Discussion

Axon guidance proteins play a crucial role during development (Bagnard, 2007; Stoeckli, 2018). However, they are not restricted to embryonic stages and are expressed in many adult tissues and organs including the eye. They are involved in nerve regeneration, myelination, the vascular and the immune systems (Kalmarzi et al., 2020; Song et al., 2021; Van Battum et al., 2015), and play a role in many cellular functions such as neurogenesis, angiogenesis, immune modulation, proliferation, and cancer (Bruikman et al., 2019; Toledano et al., 2019; Yang et al., 2007, 2018). In the cornea, their role is not well understood, and although the expression of many of these proteins and receptors has been investigated previously in the cornea (Han et al., 2012; Kojima et al., 2007; Maier et al., 2017; McKenna et al., 2014; Zhang et al., 2018b), our study is the first report to examine their recovery after corneal epithelium injury and to investigate simultaneously the expression changes that occur in distinct axon guidance family members.

The Semaphorins are a large family of proteins that mostly have a transmembrane domain that must be cleaved to release these ligands. An exception to this is the class 3 family

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members which are soluble and can interact with receptors that are not uniquely exclusive for these proteins (Alto and Terman, 2017; Potiron and Roche, 2005). Their role is still a matter of controversy and may be more dependent on their microenvironment than on a particular function. These proteins as well as Netrins and Ephrins are promiscuous, and many different roles have been associated with them. They are clearly needed for guidance of axons during development, but in adulthood, that role may change to more proliferative effects (Fard and Tamagnone, 2021; Zhang et al., 2018a). In the cornea, several members of the Semaphorin family are involved in nerve regeneration, and direct growth promoting effects have been described in isolated neurons (Lee et al., 2019; Namavari et al., 2012; Zhang et al., 2018a). However, their roles in patterning, distribution, density, and nerve function in the normal and injured cornea still need to be determined. In this study we found that two members of the class 3 proteins, Sema3A and Sema3F, are abundantly expressed in the TG and corneal epithelium. Similarly, the receptors that usually have been described to interact with these ligands, such as Neuropilins and Plexins, are also expressed in these tissues. Importantly, we found that after injury of the corneal epithelium, these proteins quickly recovered their expression levels, indicating that they may be required for wound healing. In the cornea, they may directly participate in neuroepithelial regeneration or regulate the growth promoting effects of other agents. We tested the effect of Sema3A on corneal epithelial wound healing and found that it accelerated the wound closure in scratch assays, indicating that at least one mechanism of action could be its direct effects on the epithelium recovery. The fact that Sema3A, NRP1, NRP2, and PlexinA3 have highly upregulated gene expression for several weeks after injury indicates that they may be required not only for the initial effects on wound healing, but for the long-term healing of the cornea as well. This could be related to nerve regeneration and perhaps the spatial distribution and density of the nerve endings in the cornea.

Ephrins (Erythropoietin-producing human hepatocellular proteins) are the largest protein tyrosine kinase family, and they have been described in many cellular systems with a variety of functions ranging from embryogenesis, angiogenesis, cell motility and proliferation, synaptic plasticity and neurite outgrowth (Cramer and Miko, 2016; Islam et al., 2010; Yang et al., 2018). Studies in the cornea have shown that members of the Ephrin family of ligands and receptors are expressed in the epithelium (Kaplan et al., 2012; Kojima et al., 2007), and EphA1 and EphA2 are involved in the migration of cultured corneal endothelial cells (Walshe et al., 2018). Additional studies have found abundant gene expression for several members of the Ephrin family of proteins in cells derived from human corneal tissues, but only EphrinA1 and EphB4 have been detected by immunostaining (Hogerheyde et al., 2013). However, the change in expression after cornea injury has not been evaluated. Here we corroborated this previous work and found that EphrinB1 and EphrinB2 are expressed in the corneal epithelium in both human and mouse cornea, and they are quickly upregulated after corneal epithelium injury. Furthermore, the receptors that interact with these ligands were highly upregulated after injury and remained elevated well after epithelium healing. Since these proteins interact in a paracrine way, their presence could be related to the initial cell-cell contact in the growing epithelium and our data support this notion since recombinant EphrinB2 accelerated the corneal epithelial cell wound closure in the scratch assays. The high expression levels after injury could also be related to the corneal wound

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healing mechanisms involved in nerve regeneration and patterning of nerves fibers into the corneal epithelium layers. However, further studies are required to test this hypothesis.

Netrins were initially described as the classical axon guidance proteins with a clearly defined role during development. However, similar to the other families of axon guidance proteins, they have shown different roles that are context-dependent, and the microenvironment dictates their function. These versatile proteins mediate cell migration, cell-cell interactions, and cell-extracellular matrix adhesion in multiple tissues (Lai Wing Sun et al., 2011). In this aspect, all of the axon guidance proteins studied may have pleiotropic effects, seem to be functionally flexible, and can perform different roles depending on the needs of where they are placed. In adulthood, Netrins have been described to participate in cell-cell organization beyond the CNS, and Netrin-1 is involved in angiogenesis, cancer progression, and inflammation (Lai Wing Sun et al., 2011; Wu et al., 2017; Yang et al., 2007). Using the cornea as a model for angiogenesis, Han et al., 2012, 2015 demonstrated Netrin-1 and Netrin-4 expression in rat epithelium and stroma. This group showed that topical treatment with both proteins independently, on alkali-injured corneas, reduced both inflammation and neovascularization, as well as infiltration of neutrophils and macrophages, resulting in faster corneal epithelium healing. Similar observations were found in corneas of diabetic mice subjected to complete epithelium limbus debridement, and additional enhanced nerve regeneration was shown after subconjunctival Netrin-1 treatment (Zhang et al., 2018b). Furthermore, Netrin-4 in the cornea has been implicated as an anti-angiogenic factor in mouse model of suture induced neovascularization (Maier et al., 2017). However, its role in corneal nerve regeneration has not been elucidated, and the role of Netrins in general in the recovery of corneal functional sensation is unknown. In our study, we found clear expression of both Netrin-1 and Netrin-4 in the corneal epithelium as well as in the TG. The recovery of protein and gene expression after injury indicates that they may have a functional role independent of angiogenesis or inflammation, since our model lacked these effects. We observed a slow gene expression recovery when compared to Semaphorins or Ephrins, and this could be more related to a function in nerve regeneration than epithelium healing. However, we found that Netrin-4 accelerated corneal epithelial wound healing in scratch assays but not as potently as Sema3A or EphrinB2. It may be possible that Netrin-4 could also induce nerve regeneration as shown for Netrin-1. However, this still need to be determined.

We found that all of the ligands and receptors studied are also expressed in the trigeminal ganglia and in general they colocalize in the neuronal soma. Since the sensory neurons that innervate the cornea reside there, additional roles and levels of communication must be present which may impact normal corneal homeostasis and wound healing. Retrograde studies to establish the changes that occur in the TG after corneal injury may shed some light on how these axon guidance ligands and their receptors sense and respond to signals coming from the distal nerve endings.

Taken together, our data demonstrate that members of different families of axon guidance proteins are most likely implicated in the normal and repair response in the cornea and may act in concert or independently in a time-dependent manner during the corneal wound healing process. Additionally, since our injury model uses a 2 mm central epithelium

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debridement wound, we do not believe that this wound size impacts the limbal region. The immunofluorescence and quantification studies suggest roles for these molecules in the wound healing recovery process, and co-localization staining showed that most ligands colocalize with the studied receptors, indicating that the elements for signaling are accessible. The immunofluorescence staining and qPCR data were not perfectly matched, which could be a result of the quality of antibodies used and the high sensitivity of the qPCR. Importantly, at least one mechanism of action of these proteins is the direct effect on the healing of injured cornea epithelial cells, as our wound healing assays showed that Sema3A, EphrinB2 and Netrin-4 accelerated corneal epithelial wound healing.

5. Conclusions

Axon guidance proteins belonging to the Semaphorin, Ephrin, and Netrin families and their associated receptors are highly expressed in the cornea and trigeminal ganglia. After corneal epithelium injury, they quickly recover their expression, and some remain highly upregulated for several weeks post-injury. Their differential timing of gene expression recovery after injury may be related to their role in epithelial wound healing and/or nerve regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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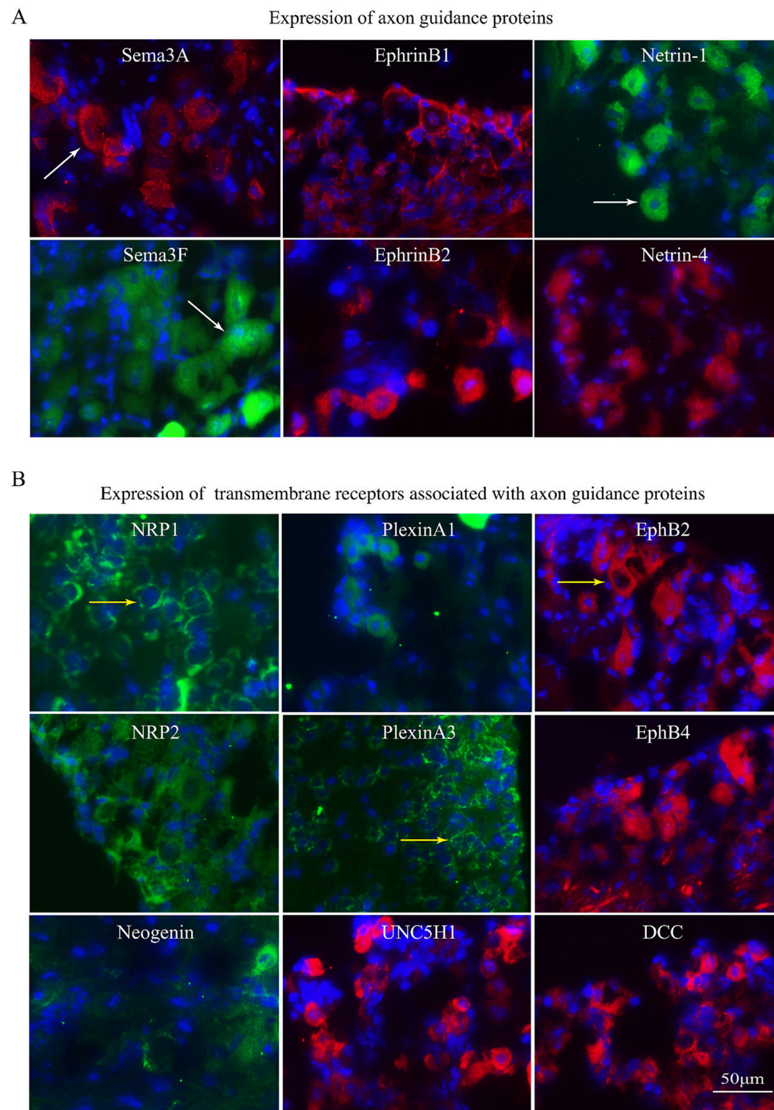

Fig. 1.

Expression of axon guidance proteins and their receptors in the mouse trigeminal ganglia. (A) Ligands belonging to the Semaphorin, Ephrin, and Netrin families of axon guidance proteins are expressed in the soma (white arrows) in the trigeminal ganglia. (B) Receptors that commonly associate with these ligands are also expressed in the TG neurons (yellow arrows). Representative images of longitudinal trigeminal ganglia section of n = 3 mice. Scale bar = 50 μm.

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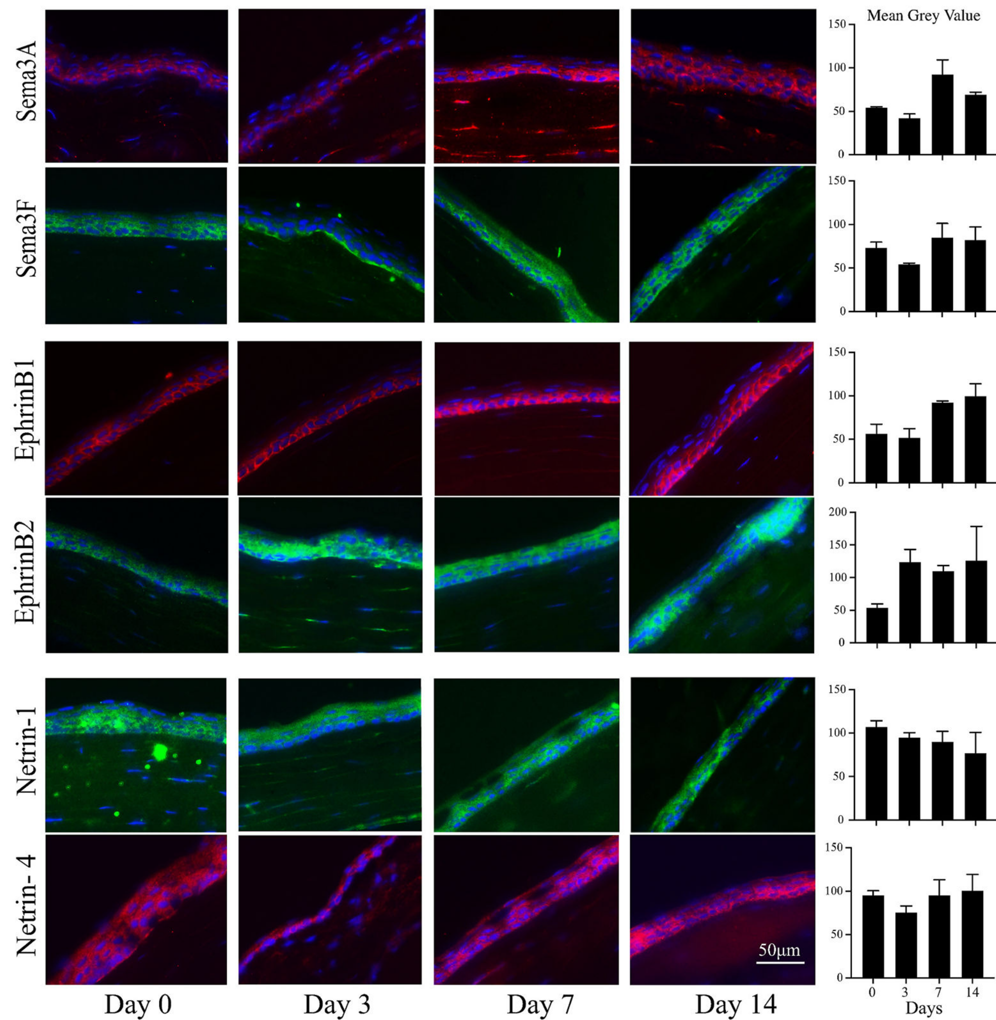

Fig. 2.
Basal and post-injury expression of axon guidance proteins in mouse cornea. Mice were subjected to corneal epithelium debridement, and the expression of these proteins was followed up in a time-dependent manner. All of these proteins are well-expressed in the uninjured cornea. After debridement, they quickly recovered, and their expression was visible from day 3 to day 14 post-injury. When compared to uninjured corneas, EphrinB1 was highly expressed at all post-injury days, while EphrinB2 and Sema3A where highly expressed from day 7 to day 14. The expression of Sema3F, Netrin-1 and Netrin-4 at day 14 was similar to the basal conditions. Quantification of immunostaining described in Methods is shown in the graphs. Representative images of the central debrided cornea, n = 3 mice per time point. Scale bar = 50 µm.

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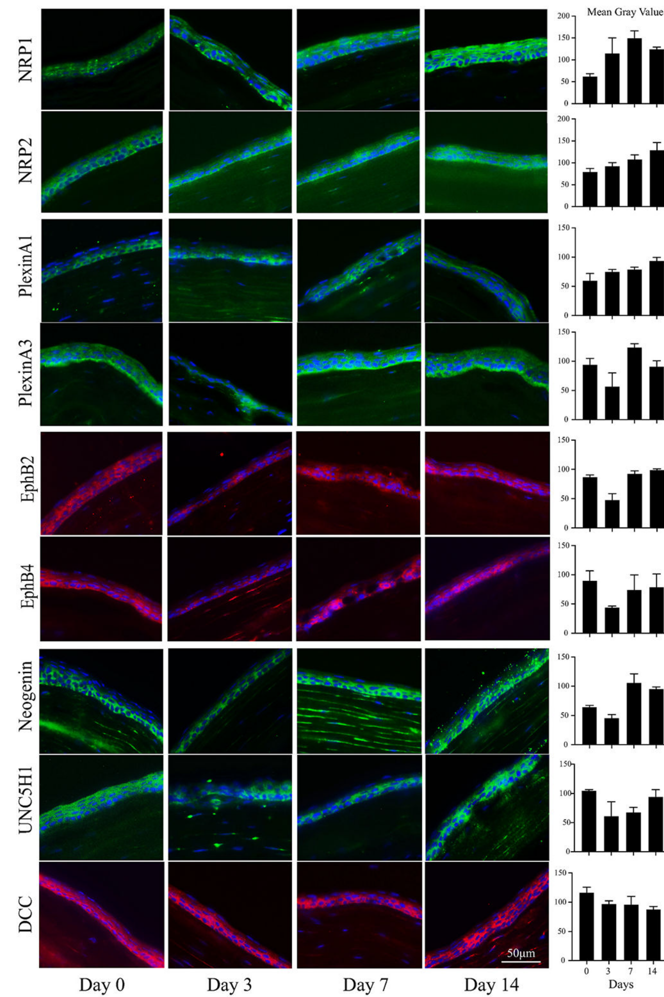

Fig. 3.

Normal and post-injury expression of receptors commonly associated with axon guidance proteins in mouse cornea. These receptors are expressed in the uninjured cornea, and similar to the ligand expression, they recovered as the epithelium healed, localizing mostly in the basal epithelial cells at day 3 to then be abundantly expressed in the epithelium at day 14. Compared to their initial basal expression, NRP1, NRP2, PlexinA1 and Neogenin showed higher expression at day 14, while only DCC had low expression pattern. NRP1 was highly expressed after cornea injury during all time points of analysis. Quantification of immunostaining described in Methods is shown in the graphs. Representative images of the central debrided cornea, n = 3 mice per time point. Scale bar = 50 μm.

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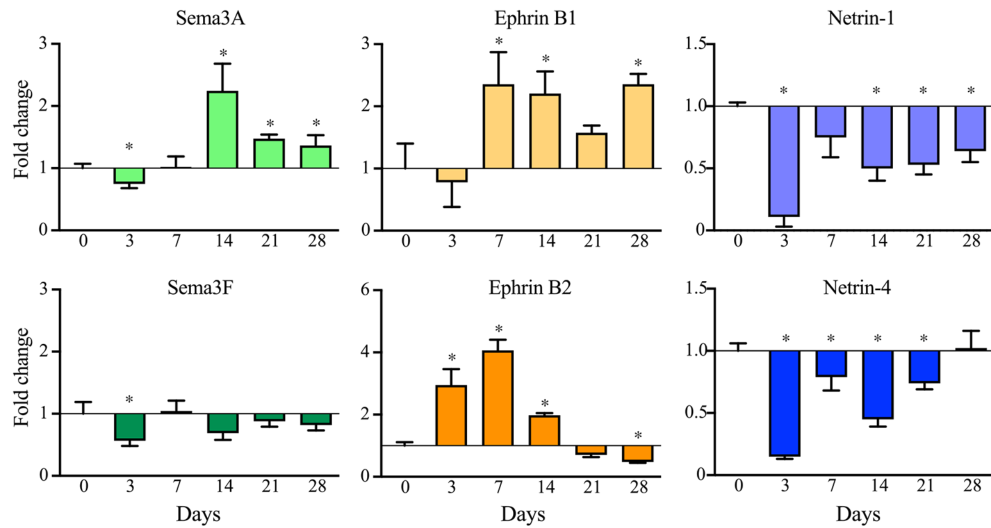

Fig. 4.

Variable gene expression recovery of axon guidance proteins in injured mouse cornea. Mouse corneal epithelium was harvested from uninjured mice corneas or animals that received cornea epithelium debridement. Gene expression was analyzed as described in Materials and Methods. We found that 3 days after injury, only the expression of EphrinB2 was significantly upregulated, while all other axon guidance ligands were downregulated. At later time points, Sema3A, EphrinB1, and EphrinB2 were upregulated as well, while Sema3F, Netrin-1 and Netrin-4 were downregulated. In general, all of these proteins trend to normalize their expression to the basal level, and only EphrinB1 was still showing high level of expression after 4 weeks post-injury. N = 5 mice per time point. * = $p < 0.05$.

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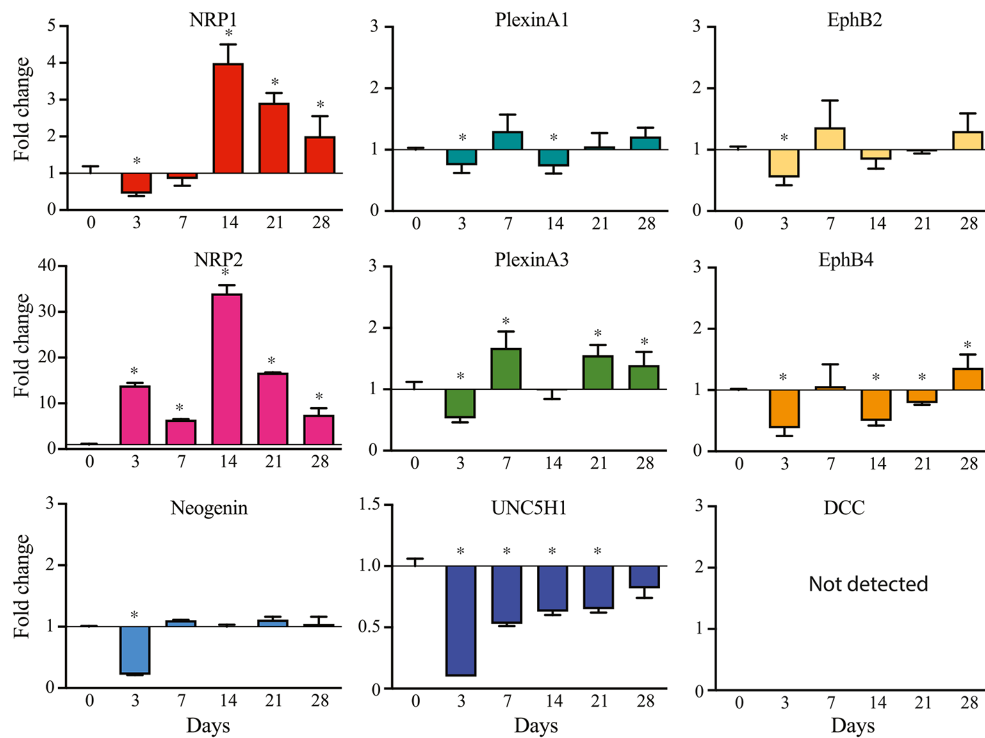

Fig. 5.

Variable gene expression recovery of receptors associated with axon guidance proteins in injured mouse cornea. We found that after cornea injury, NRP2 was always upregulated over the basal uninjured levels with values significantly higher than any other of the proteins analyzed. All other proteins were downregulated at day 3 and either recovered its expression quickly, such as Neogenin, or show variable expression along the time course of the analysis. UNC5H1 was the only receptor that was consistently downregulated but slowly trended to values close to basal levels. N = 5 mice per time point * = $p < 0.05$.

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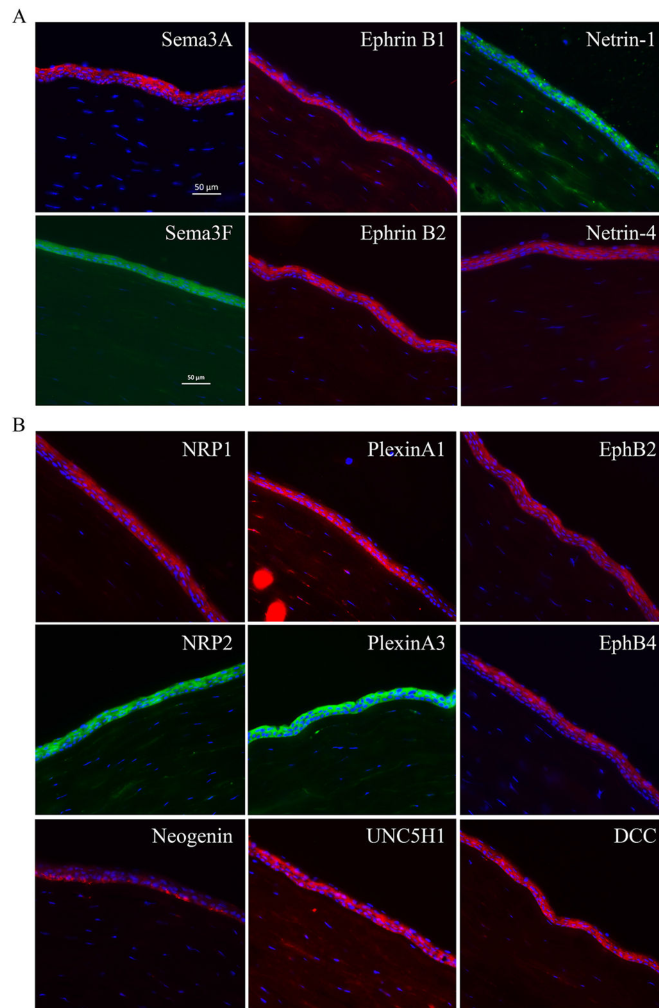

Fig. 6.

Axon guidance proteins and their associated receptors are expressed in human cornea. We found that all of the axon guidance ligands studied are expressed in cross sections of human corneas (A). Similarly, immunostaining for the receptors also shows abundant expression in the corneal epithelium for all the proteins studied (B). Representative images of n=2 corneas. Scale bar = 50 µm.

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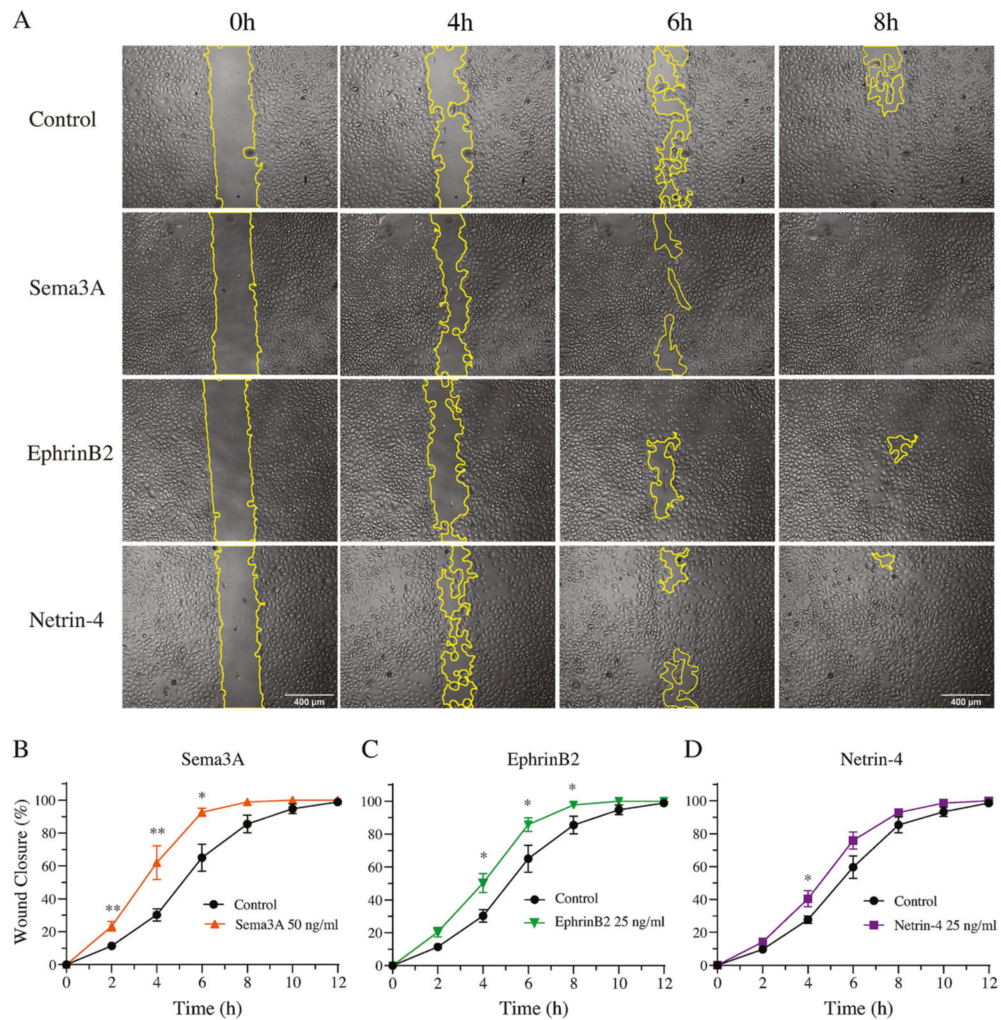

Fig. 7.

Axon guidance proteins accelerated corneal epithelial wound closure. Human corneal limbal epithelial (HCLE) cells were grown to confluency on a 24-well plate and then a scratch was performed using a scratcher tip as described in Methods. Cells were then treated with recombinant Sema3A, EphrinB2, Netrin-4 or left untreated as control. Cells were immediately placed in a chamber incubator on a microscope and images were collected every 20 min for 15 h and analyzed using Fiji software. All the axon guidance proteins accelerated the wound closure when compared to control. (A) Representative images of scratch area. Sema3A (B) and EphrinB2 (C) induced faster healing with complete closure at 8 h, while Netrin-4 (D) did at 10 h, still faster than control. Each treatment was performed in triplicate and 3 images were obtained per treatment. Experiments were performed three times. Scale bar = 400 μm. * = $p < 0.05$; ** = $p < 0.01$. Values are mean ± S.E.M.

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Table 1

Antibodies used for immunofluorescence staining of trigeminal ganglia and cornea sections.

Primary antibody	Source	Dilution	Tissue	Vendor	Secondary antibody	dilution	Vendor
Semaphorin3A	Rabbit	1:300	all	Proteintech 21925	Donkey anti rabbit Cy3	1:400	Jackson Immunoresearch 711-025-152
Semaphorin3F	Rabbit	1:400	all	EMD Millipore AB5471	Goat anti rabbit Alexa green 448	1:400	ThermoFisher A11008
EphrinB1	Goat	1:200	all	R&D Systems AF473	Donkey anti goat TRITC	1:400	Jackson Immunoresearch 705-025-147
EphrinB2	Goat	1:200	mc hc, tg	R&D Systems AF496	Donkey anti goat Alexa green 488 Donkey anti goat TRITC	1:400 1:400	ThermoFisher A11055 Jackson Immunoresearch 705-025-147
Netrin-1	Chicken	1:200	mc, tg	Neuromics CH23002	Goat anti chicken Alexa 488	1:400	Invitrogen A11039
	Rabbit	1:100	hc		Donkey anti rabbit Alexa green 488	1:800	
				Bioss - BS-1858R-TR			ThermoFisher A11008
Netrin-4	Rabbit	1:200	all	Bioss BS110062R-TR	Donkey anti rabbit Cy3	1:400	Jackson Immunoresearch 711-025-152
Neuropilin-1	Rabbit	1:200	mc, tg	Santa Cruz SC-5541	Goat anti rabbit Alexa green 448	1:400	ThermoFisher A10037
	Mouse	1:200	hc		Donkey anti mouse Alexa red 568	1:400	
				Proteintech 60067			ThermoFisher A10031
Neuropilin-2	Rabbit	1:200	all	Santa Cruz SC-5542	Donkey anti rabbit Alexa green 448	1:400	ThermoFisher A11008
PlexinA1	Goat	1:200	all	R&D Systems AF4309	Donkey anti goat Alexa green 448	1:400	ThermoFisher A11055
PlexinA3	Rabbit	1:400	all	Santa Cruz SC-25641	Goat anti rabbit Alexa green 448	1:400	ThermoFisher A11008
EphB2	Goat	1:200	all	R&D Systems AF467	Donkey anti goat TRITC	1:400	Jackson Immunoresearch 705-025-147
EphB4	Goat	1:200	all	R&D Systems AF446	Donkey anti goat TRITC	1:400	Jackson Immunoresearch 705-025-147
Neogenin	Mouse	1:50	hc	Santa Cruz SC-514872	Donkey anti mouse Alexa red 568	1:400	ThermoFisher A10031
	Goat	1:200	mc, tg		Donkey anti goat Alexa green 448	1:400	ThermoFisher A11055
				R&D Systems AF1079			
UNC5H1	Goat	1:200	mc	R&D Systems AF1405	Donkey anti goat Alexa green 488	1:400	ThermoFisher A11055
			hc, tg		Donkey anti goat TRITC	1:400	
							Jackson Immunoresearch 705-025-147
DCC	Goat	1:400	all	R&D Systems AF844	Donkey anti goat TRITC	1:400	Jackson Immunoresearch 705-025-147
Whole IgG	Rabbit			Jackson Immunoresearch 011-000-003			

hc = human cornea; mc = mouse cornea; tg = trigeminal ganglia; all = hc, mc, tg.

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Table 2

Gene expression assays used for qPCR analysis of axonal guidance proteins in the corneal epithelium.

Assay ID	Gene name	Species
Mm00436469_m1	Semaphorin 3A	*Mus musculus*
Mm00441325_m1	Semaphorin 3F	*Mus musculus*
Mm00438666_m1	Ephrin B1	*Mus musculus*
Mm00438670_m1	Ephrin B2	*Mus musculus*
Mm00500896_m1	Netrin 1	*Mus musculus*
Mm00480462_m1	Netrin 4	*Mus musculus*
MmO0435372_m1	Neuropilin 1	*Mus musculus*
Mm00803099_m1	Neuropilin 2	*Mus musculus*
Mm00501110_m1	Plexin A1	*Mus musculus*
Mm00501170_m1	Plexin A3	*Mus musculus*
Mm01181021_m1	Eph receptor B2	*Mus musculus*
MmO1201157_m1	Eph receptor B4	*Mus musculus*
Mm00476326_m1	Neogenin	*Mus musculus*
Mm00462368_m1	Unc5H1	*Mus musculus*
Mm01262259_m1	DCC	*Mus musculus*
Mm00514509_m1		
Mm99999915_g1	GAPDH	*Mus musculus*