Published in final edited form as:

Dev Biol. 2017 March 01; 423(1): 77–91. doi:10.1016/j.ydbio.2017.01.008.

Knockdown of CXCL14 disrupts neurovascular patterning during ocular development

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Abstract

The C-X-C motif ligand 14 (CXCL14) is a recently discovered chemokine that is highly conserved in vertebrates and expressed in various embryonic and adult tissues. CXCL14 signaling has been implicated to function as an antiangiogenic and anticancer agent in adults. However, its function during development is unknown. We previously identified novel expression of CXCL14 mRNA in various ocular tissues during development. Here, we show that CXCL14 protein is expressed in the anterior eye at a critical time during neurovascular development and in the retina during neurogenesis. We report that RCAS-mediated knockdown of CXCL14 causes severe neural defects in the eye including precocious and excessive innervation of the cornea and iris. Absence of CXCL14 results in the malformation of the neural retina and misprojection of the retinal ganglion neurons. The ocular neural defects may be due to loss of CXCL12 modulation since recombinant CXCL14 diminishes CXCL12-induced axon growth in vitro. Furthermore, we show that knockdown of CXCL14 causes neovascularization of the cornea. Altogether, our results show for the first time that CXCL14 plays a critical role in modulating neurogenesis and inhibiting ectopic vascularization of the cornea during ocular development.

SUMMARY STATEMENT

Robust expression of CXCL14 in ocular tissues during development regulates innervation of the cornea and iris, neurogenesis and projection of retinal ganglion cells, and it prevents corneal vascularization.

Keywords

CXCL14; neurogenesis; vasculogenesis; ocular nerves; cornea; chemokine

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COMPETING INTERESTS

No competing interests to declare.

INTRODUCTION

The vertebrate eye forms as a result of interactions between the neural and surface ectoderm, and periocular mesenchyme of neural crest origin. Subsequently, nerves from the trigeminal and ciliary ganglia project into the anterior eye and innervate the cornea and iris (Bee et al., 1982; Lwigale and Bronner-Fraser, 2007; Pilar et al., 1980; Simpson et al 2013), and the inner layer of the optic cup differentiates into photo sensory cells and neurons of the neural retina (Adler and Hatlee, 1989; Cepko et al., 1996). Also, during ocular development, endothelial cells located in the periocular region form a dense limbal vascular network (Kwiatkowski et al., 2013; McKenna et al., 2014).

In the chick, trigeminal sensory nerves project from the ventrotemporal region of the eye and form a pericorneal nerve ring prior to radial innervation of the cornea (Bee, 1982; Lwigale and Bronner-Fraser, 2007). Sensory nerves are attracted to the anterior eye by neurotrophic factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor (GDNF) that are secreted by the cornea (Lambiase et al., 1997; You et al., 2000) and iris (Simpson et al 2013). However, nerve projection into the cornea is regulated by Semaphorins secreted mainly by the lens (Lwigale and Bronner-Fraser, 2007; McKenna et al., 2012) and Robo-slit signaling (Kubilus and Linsenmayer, 2010; Schwend et al., 2012).

Ocular vasculogenesis commences with angioblast migration into the periocular region followed by the formation of the dense vascular network that provides oxygen and nutrients. This process is influenced by VEGF and possibly, other angiogenic factors expressed by the ocular tissues (Kwiatkowski et al., 2013). Similar to neural guidance, vasculogenesis in the anterior eye is regulated by Semaphorin signaling through Neuropilin-1 and PlexinD receptors that are expressed by the angioblasts and nascent vasculature (Kwiatkowski et al., 2013; McKenna et al., 2014; and Kwiatkowski et al., 2016).

The above studies indicate that neurovascular development in the anterior eye is initially regulated by cross-talk between molecules that are expressed in the ocular tissues and receptors expressed by the forming vasculature and sensory nerve projections. Interestingly, this parallel patterning of the periocular vasculature and nerve ring is uncoupled at later stages of ocular development when only the sensory nerves are permitted to project into the cornea, which results in a highly innervated but avascular tissue (Lwigale and Bronner-Fraser, 2007; McKenna et al., 2014). In the adult, the sensory nerves play a critical role in maintaining corneal transparency (Müller et al., 2003; Shaheen et al., 2014), whereas neovascularization caused by injury or disease results in corneal opacity and vision impairment (Ellenberg et al., 2010; Abdelfattah et al., 2016). Although separation of the neurovascular congruence is crucial for proper corneal transparency and vision, the molecular mechanism involved in this process during development is not clear.

CXCL14 (also known as BRAK based on its constitutive expression in the breast and kidney) is a homeostatic chemokine that can function independent of the immune system (Allen et al., 2007; Mortier et al., 2012). CXCL14 is classified in a subgroup of chemokines without the three conserved amino acids Glu-Leu-Arg (non-ELR), which are considered to

have antiangiogenic properties (Mehrad et al., 2007; Kiefer and Siekmann, 2011). In addition to its expression in immune cells, CXCL14 is constitutively expressed in normal epithelial tissues such as skin, breast, kidney, brain, muscles, and lungs but it is absent or down regulated in malignant cells (Hromas et al., 1999; Izukuri et al., 2010; Long et al., 2000; Meuter and Moser, 2008; Chen et. al., 2010; Hara and Tanegashima, 2012). Although the receptor for CXCL14 and intracellular signaling cascade is not known, studies have shown its function in immune and immature dendritic cell migration (Hromas et al., 1999; Shellenberger et al., 2004; Izukuri et al., 2010). Reduced levels of CXCL14 in cancer tissues (Hromas et al., 1999), suppression of tumor growth due to reduced vasculature in CXCL14 transgenic mice (Izukuri et al., 2010), and its prevention of endothelial cell migration in vitro (Shellenberger et al., 2004), suggest a potential role as an antiangiogenic factor. CXCL14 knockout mice show reduced birth rate, lower body weight, altered feeding behavior and glucose metabolism, and no severe immune defects, suggesting that CXCL14 is an important metabolic regulator, but dispensable for the recruitment of immune cells (Meuter et al., 2007; Nara et al., 2007; Tanegashima et al., 2010).

Expression of *CXCL14* has been reported in Zebrafish, *Xenopus*, chick, and mouse embryos. In Zebrafish, *CXCL14* is expressed in the vestibular-acoustic system, mid-hindbrain, diencephalon and cerebellum (Long et al., 2000). In *Xenopus*, *CXCL14* is expressed in the dorsal retina, the embryonic ectoderm and its derivatives, and it is regulated by BMP and Wnt signaling (Park et. al., 2009). In chick and mouse, *CXCL14* is widely expressed in the ectoderm, cranial ganglia, mesonephros, neural tubes and limbs (Garcia-Andres and Torres, 2010; Gordon et al., 2011). Despite the localization of CXCL14 in various tissues during development, its role CXCL14 in these processes remains unknown.

Previously, we showed that *CXCL14* mRNA is transiently expressed in several ocular tissues during development (Ojeda et al., 2013). Here we show that CXCL14 protein expression in the developing chick eye corresponds with innervation of the anterior ocular tissues and neurogenesis in the retina. Our results demonstrate that CXCL14 regulates the timing and modulates sensory nerve projections into the cornea and iris, and that it is involved in patterning of the neural retina. Furthermore, we show that CXCL14 inhibits neovascularization of the cornea during late stage ocular development. Therefore, CXCL14 signaling plays a critical role in regulating neurovascular patterning during ocular development.

MATERIALS AND METHODS

Animals

Fertilized White Leghorn chicken eggs (*Gallus gallus domesticus*) were obtained from Texas A&M (College Station, TX) and *Tg(tie1:H2B:eYFP)* transgenic quail eggs (*Coturnix japonica*) were obtained from Ozark Egg Company (Stover, MO). Eggs were incubated at 38°C under humidified conditions. Embryos were injected at Hamburger-Hamilton stage (HH) 6–9 (Hamburger and Hamilton, 1951), re-incubated, and collected for analysis between embryonic day (E)7 and E17. All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Rice University.

Histology and Immunohistochemistry

Heads or eyeballs were fixed overnight (O/N) in Modified Carnoy's or 4% paraformaldehyde. Tissues were processed as whole-mount, in 7.4% gelatin for cryosectioning, or in ethanol series for paraffin embedding. Modified Carnoy's sections were stained for hematoxylin and eosin (H&E) staining, or *in situ* hybridization as previously described (Etchevers et al. 2001). The following primary antibodies were used at indicated concentrations for PFA fixed whole-mount or cryosectioned tissues: Mouse neuron-specific-tubulin-Tuj1 (1:500; IgG, Covance), rabbit anti-GFP (1:500; IgG, Invitrogen), mouse anti-GFP (1:500 IgG1, Covance), mouse anti-avian myoblastosis virus-3C2 (1:500; IgG1, DSHB), rabbit anti chicken-CXCL14 (1:100, IgG, GenScript). Signals were detected using secondary antibodies (anti-rabbit Alexa Fluor 488 or 594, anti-mouse IgG1 594, or anti-mouse IgG2a 594, Invitrogen) at a concentration of 1:200. Sections were counterstained using 4,6-diamidino-2-phenylindole (DAPI) to label all nuclei.

RNA Isolation and Semi-quantitative Reverse Transcription PCR

Total RNA was extracted from DF-1 cells or E12 corneas with Trizol reagent (Life technologies) and cDNA was synthesized with reverse transcriptase (SuperScript III RT First-Strand Synthesis Systems, Invitrogen) following the manufacturer's protocol. Primer sequences for *GAPDH*, *CXCL14*, *GFP*, *Sema3A*, *NRP1*, *TrkA*, *TrkB*, *NT-3*, *NGF*, *BDNF*, *CXCL12*, *CXCR4*, *CXCR7*, *VEGF* and *VEGFR2* are listed in supplementary table 1. The expected size of PCR products was confirmed by agarose gel electrophoresis.

Primary Culture of Dissociated Trigeminal neurons for neurite outgrowth

Trigeminal ganglia were dissected from E10 and E11 chick embryos and incubated in 0.25% trypsin/EDTA (Corning) for 10 minutes. Cells were mechanically dissociated and resuspended in Dulbecco's modified eagle medium (DMEM) supplemented with 10% of fetal bovine serum (Invitrogen) and penicillin/streptomycin (Gibco) referred to here after as complete media. For neurite outgrowth, coverslips were coated with 100 $\mu\text{g ml}^{-1}$ of poly-llysine (Sigma) at room temperature for 1 hour and subsequently with 2 $\mu\text{g ml}^{-1}$ of laminin (Sigma) at 37°C for 2–3 hours. Cells were seeded at a final density of 4×10^3 cells per cm^2 in 24 well-plates and incubated in complete medium alone, or complete medium containing 10 ng/ml of NGF (Sigma), 100 ng ml^{-1} of human recombinant (hr) CXCL14 (R&D system), 100 ng ml^{-1} of hr CXCL12 (R&D system), or a combination of NGF and CXCL12, or NGF and CXCL14 for 24 hours.

Bead Implantation

Cibacron blue 3GA beads (Sigma-Aldrich) were rinsed in sterile PBS and soaked overnight at 4°C in 10 μl of 5 $\mu\text{g ml}^{-1}$ CXCL14, 5 $\mu\text{g ml}^{-1}$ of hrVEGF 164, or a combination of both. Protein-soaked beads were implanted between the presumptive cornea and the lens of E3 *Tg(tie1:H2B:eYFP)* transgenic quail embryos (Sato et al., 2010) and reincubated for two days. Quantification of the vascularized area was done in the region covered by fluorescently labeled endothelial cells from the iridial ring artery and the cornea using ImageJ software (Schneider et al., 2012).

Generation of RCAS-based vectors, virus production, and in ovo injection

The avian retroviral vector RCASBP(B) or RCASBP(A) (Hughes et al., 1987) were used to knockdown chick *CXCL14* mRNA. Using online shRNA design tools (Invitrogen, Dharmacon, Genscript), four *CXCL14*-specific sequences were selected and designated as 170, 210, 268 and 308 based on the starting sequence of *Gallus gallus* *CXCL14* mRNA (Fig. S2). A scrambled (SCR) shRNA sequence (Ferrario et al., 2012) or RCAS containing only the GFP reporter were used as controls. pSLAX-GFP-cU6-(*CXCL14*-shRNA) and pSLAX-(mCherry)-cU6-(SCR-shRNA) shuttle vectors were generated from a pSLAX-GFP-cU6-Sox9-shRNA (Deneen et al., 2006), a gift from David Anderson (Caltech). GFP-cU6-(*CXCL14*-shRNA), mCherry-cU6-(SCR-shRNA) or GFP fragments were cloned into the ClaI restriction site of RCASBP(A) or RCASBP(B) (referred as RCAS(A) and RCAS(B), respectively), using PCR-based cloning kits (*InFusion*, Clontech or *CloneEZ*, Genscript), to generate RCAS(A) and RCAS(B)-*CXCL14*shRNA, RCAS(A) and RCAS(B)-SCRshRNA, RCAS(A) and RCAS(B)-GFP. For the overexpression, RCAS(A)-*CXCL14* viral construct was generated by cloning a 296-bp cDNA fragment containing the coding region of *Gallus gallus* *CXCL14* (NCBI accession number: NM_204712.2) into RCAS-IRES-GFP vector using PCR-based cloning kits (*InFusion*, Clontech or *CloneEZ*, Genscript).

For virus production, avian DF-1 cells (ATCC) grown to 60–70% confluence in complete media were transfected with 1 µg of RCAS plasmid using Lipofectamine following manufacture's protocol (Sigma). Virus-containing supernatants were collected when at least 95% of the cells were infected by RCAS virus, as determined by the expression of GFP, 3C2 or mCherry. Viral particles were concentrated by ultracentrifugation at 4°C for 1.5 hours at 21,000rpm in a 45Ti rotor (Beckman-Coulter Optia L-80 XP). Pellets containing the virus particles (approximately $1-3 \times 10^8$ infectious units ml^{-1}) were resuspended in DMEM and stored in 50 µl aliquots at –80°C until use.

In ovo infections were accomplished by injecting high-titer virus between the vitelline membrane and the cranial region of HH7–9 embryos using a Picospritzer III pressure-based microinjection system (Parker Hannifin). Eggs were sealed and re-incubated at 38°C until the desired stage for analysis.

Quantification of corneal vascularization

The area of corneal vascularization was quantified in E12 *Tg(tie1:H2B:eYFP)* *CXCL14* knockdown eyes and controls embryos using ImageJ. Delineating lines were drawn around the outside edge of the limbal vasculature and blood vessels that were located in the inner edge of the limbus and cornea. The area covered by angioblasts was divided by the total area (outside edge of the cornea) to calculate the percent area of corneal vascularization.

Imaging

Images of heads and dissected eyeballs were acquired using a Zeiss Axiocam mounted on a Zeiss dissecting scope. Images of stained whole-mounts and sections were acquired using a Zeiss Axiocam mounted on an AxioImager2 fluorescent microscope with ApoTome (Carl Zeiss AG, Oberkochen, Germany).

Quantitative analysis of corneal innervation and axon length

To quantify the extent of corneal innervation at E10, three concentric circles (ring1, ring2, and ring3) were superimposed over images of whole-mount corneas stained for nerves. The same template with constant distances was used for all images. Cornea nerves that reached the concentric rings in RCAS-CXCL14 shRNA and control embryos were quantified and graphed. Similarly, innervation of E12 central corneas following CXCL14 over expression was quantified by superimposing a ring over the corneal center.

For corneal epithelial innervation, optical cross-sections of whole-mount corneas stained for nerves were generated using the Apotome mode on the Zeiss Axioskop 2 microscope. Images acquired in the X/Z and Y/Z showing focal points of Tuj1-positive projections of terminal nerves that invade the basement membrane and the corneal epithelium were quantified.

Stromal and iris nerve density on cross-sections were measured on the converted binary images using ImageJ. Stromal nerve occupancy was determined by calculating the total area of the cornea section and the area covered by nerves, then expressed as percentage of stromal area covered by nerves.

To determine the extent of neurite outgrowth, 3–5 images were randomly taken per coverslip at 10X magnification and the axon extensions per field were quantified using NeuronJ software (Meijering et al., 2004). All the conditions were performed at least in triplicate.

Statistical analyses

All data are presented as the mean $\pm$ SEM of at least three independent experiments. Comparisons among data sets were conducted by Student t-test. $P < 0.05$ was considered statistically significant.

RESULTS

Expression and Knockdown of CXCL14 during development

Previously we showed that *CXCL14* mRNA expression in the cornea begins at the onset of keratocyte differentiation at E6 and it is maintained in the anterior stroma until E15 when it is not detectable by in situ hybridization (Ojeda et al., 2013). As a first step in determining the role of CXCL14, we examined the protein expression at different time points during cornea development. In contrast to transcript expression, CXCL14 protein was not detected in the cornea at E7 (Fig. 1A). CXCL14 was first detected at E10 and it was strongly expressed in the anterior stroma and epithelium by E12 (Fig.1B; Fig. S1). Similar to transcript expression, expression of CXCL14 protein in the cornea was transient and it declined at E17 (Fig. 1C).

To generate knockdown embryos, we designed and tested RCAS-shRNA constructs using sequences from the coding region of CXCL14 in combination with GFP as a reporter gene (Fig. 1D; Fig. S2). The construct with the highest efficiency of *CXCL14* knockdown in the cornea was chosen for the functional experiments (Fig. S2), and it is hereafter referred to as CXCL14-shRNA. Additional testing on DF-1 cells expressing RCAS-CXCL14 validated

CXCL14 knockdown in the presence of *CXCL14*-shRNA but it was not affected by RCAS-GFP (Fig. 1E). Embryos injected with either control or *CXCL14*-shRNA were collected at desired time points and initially screened based on robust GFP expression in the anterior eye (Fig. 1F,G). In contrast to control corneas, diminished expression of *CXCL14* was detected in corneas of embryos injected with *CXCL14*-shRNA (Fig. 1H,I). Thus *CXCL14* is transiently expressed in the chick cornea and it is attenuated by overexpression of *CXCL14*-shRNA.

To examine the function of *CXCL14* during embryonic development, we overexpressed viral constructs containing *CXCL14*-shRNA by targeting the cranial region of HH6-9 chick embryos. Morphological analysis of E12 embryos showed a reduction in body size of *CXCL14* knockdown embryos (Fig. 1L) compared with controls (Fig. 1J). In addition, several *CXCL14* knockdown embryos exhibited eyelid (n=7/22) and mandibular defects (n=5/22) (Fig. 1L, arrowhead and asterisk), which correspond to *CXCL14* expression in the eyelid ectoderm (Ojeda et al., 2013) and first branchial arch (Garcia-Andres and Torres, 2010; Gordon et al., 2011; Ojeda et al., 2013).

Despite the vivid expression of *CXCL14* in the cornea (Fig. 1B), we did not observe any morphological defects or reduction in corneal thickness in *CXCL14* knockdown eyes (Fig. 1K,M). However, compared to control, large voids were apparent in the lens fiber cells (Fig. 1M, asterisk), and regions of the retina were disorganized (Fig. 1M, arrows) in *CXCL14* knockdown eyes. Thus, the expression of *CXCL14* corresponds with the ocular defects observed in its absence, suggesting potential role in their development.

Knockdown of *CXCL14* exacerbates sensory innervation of the cornea

Corneal innervation in chick begins at about E9.5 when trigeminal afferents branch from the pericorneal nerve ring and radially innervate the stroma (Bee, 1982; Lwigale and Bronner-Fraser, 2007). Given that the expression of *CXCL14* in the cornea commences at E10, which coincides with its innervation (Fig. S1), we asked whether knockdown of *CXCL14* perturbed sensory nerve projection into the corneal stroma. Whole-mount immunostaining of E10 corneas with anti-neurotubulin antibody (Tuj1) showed that as expected at this time-point (Lwigale and Bronner-Fraser, 2007), only the periphery of the corneal stroma was innervated in controls (n=8, Fig. 2A,C). In contrast, sensory nerves projected further towards the center of *CXCL14* knockdown corneas (n=7, Fig. 2B,D, arrows). The extent of sensory nerve projections into the cornea was determined by superimposing a concentric ring template on each corneal innervation image (Fig. 2E), and the numbers of nerve bundles traversing each ring were counted. Our analysis revealed that a significantly large number of nerve bundles reached each concentric ring in *CXCL14* knockdown corneas than in controls (Fig. 2F). The highest number of nerve bundles were located in the corneal periphery (ring 1, $P=0.0001$), and they reduced towards the middle (ring 2, $P=0.0003$) and center (ring 3, $P=0.0012$). In addition, we did not observe any differences in size and morphology of the trigeminal and ciliary ganglia between control and *CXCL14* knockdown embryos (Fig. S3). Indicating that the increase in corneal innervation in *CXCL14* knockdown corneas was not caused by increased number of neurons in the ganglia. These results suggest that *CXCL14* in

the cornea plays an inhibitory role in sensory nerve projection during early stages of innervation.

CXCL14 regulates the magnitude of stromal innervation and timing of corneal epithelial innervation

Corneal innervation is a multistep process that involves initial nerve projection into the stroma towards the center, followed by outcrops towards the anterior surface and innervation of the epithelial layer (Müller et al., 1997; Riley et al., 2001). Since CXCL14 is localized in the anterior corneal region occupied by the nerve bundles during stromal and epithelial innervation (Fig. 1B), we asked whether its presence affects these processes. As expected in control E12 corneas (Lwigale and Bronner-Fraser, 2007), nerve bundles innervate the entire corneal stroma from the periphery to the center (Fig. 3A). A similar pattern of stromal innervation was observed in CXCL14 knockdown corneas (Fig. 3D). Since corneal epithelial innervation in chick begins from the corneal periphery at E12 (Bee, 1982), we examined this process by taking z-stack images at the periphery and central regions of the cornea. First, we confirmed previous observation in E12 control corneas by showing that only the peripheral epithelium is innervated at this stage (Fig. 3B,C). Consistent with this observation, there was no significant difference between peripheral epithelial innervation between control (n=19) and CXCL14 knockdown corneas (n=25, $P=0.3542$, Fig. 3G). However, epithelial innervation was observed throughout the entire surface in CXCL14 knockdown corneas (Fig. 3E,F), which also showed significant epithelial innervation in the center (n=22, $P<0.0001$, Fig. 3H). Therefore, the absence of CXCL14 in the cornea causes precocious innervation of the epithelium.

Next, we examined the localization and density of stromal nerves in cross-sections through E12 corneas. In control corneas stromal and epithelial nerves occupy the anterior third of the cornea (n=8, Fig. 4A, bracket; Riley et al, 2001; Müller et al., 2003; McKenna and Lwigale, 2011). Analysis of CXCL14 knockdown corneas revealed a similar anterior localization, but significant expansion of nerves into the mid-stroma region (n=14, $P<0.0001$, Fig. 4B, bracket; Fig. 4C). Further analysis also revealed significant increase in nerve density in the stroma of CXCL14 knockdown corneas (n=14, $P=0.0002$, Fig. 4D). In sum, these data indicate that corneal expression of CXCL14 during innervation delays sensory nerve projections into the stroma and subsequent innervation of their final target, the corneal epithelium. In addition they suggest that CXCL14 confines and limits the extent of stromal innervation.

Overexpression of CXCL14 impairs nerve projections into the central corneal stroma

Based on the exacerbated corneal innervation phenotype following CXCL14 knockdown, we predicted that overexpression of CXCL14 may attenuate nerve projections during corneal development. We overexpressed the full-length protein by injecting an RCAS-CXCL14-GFP construct in the cranial regions of HH stage 5–8 embryos. We confirmed overexpression of CXCL14 in the anterior eye by presence of GFP and immunostaining for CXCL14 (Fig. S5). Our analysis of corneal innervation at E12 when nerve bundles normally project into the central stroma in control corneas (Fig. 5A), revealed absence of nerves in this region of corneas in which CXCL14 was overexpressed (Fig. 5B). Quantification of nerve bundles

crossing a ring superimposed onto the central region of corneal images revealed a significant decrease in central innervation of CXCL14 overexpressing corneas (n=7, p=0.001, Fig. 5C). These results confirm the inhibitory role of CXCL14 on axon projections in the cornea. Surprisingly, our analysis of pericorneal nerves at E7 (n=4) during their projection in an environment of ectopic CXCL14 expression, and at E10 (n=4) during innervation of the corneal periphery, revealed no obvious alteration in nerve patterning relative to stage-matched controls (Fig. S5). The failure of CXCL14 overexpression to inhibit axon projections at E7 could be due to differential expression of growth factors between the periocular region and cornea. The low quantity of nerve bundles in the cornea at E10 may obscure any differences between the response to endogenous and excessive CXCL14 expression.

CXCL14 inhibits CXCL12-mediated trigeminal nerve growth in vitro

Previous studies have proposed that CXCL14 acts as an inhibitor for CXCL12 signaling in various cell types (Tanegashima et al., 2013). It has also been shown that *CXCL12* and *CXCL14* transcripts are expressed in complementary patterns in several tissues including the eye (Garcia-Andres and Torres, 2010; Ojeda et al., 2013). Given that CXCL12 has neurotrophic properties on various types of neurons (Chalasani et al., 2003a; 2003b; Ödemis et al., 2005; Opatz et al., 2009), we first asked whether a similar mechanism is involved in dissociated trigeminal neurons. We found that similar to control, neither treatment with CXCL12 nor CXCL14 stimulated neurite outgrowth in the absence of NGF (Fig. 6A–C). Treatment with CXCL12 and NGF led to a significant increase in neurite outgrowth compared to NGF alone (Fig. 6D,F,H, P=0.0018). However, treatment with CXCL14 and NGF did not promote or inhibit neurite outgrowth compared NGF alone (Fig. 6D,E,H). To determine whether CXCL14 has inhibitory effect on CXCL12 on dissociated trigeminal neurons, we treated the cultures with both chemokines in the presence of NGF. This treatment led to a significant decrease in neurite outgrowth compared to the combination of CXCL12 and NGF (Fig. 6F–H, P<0.0001). We also identified the presence of transcripts for *CXCL12* and its receptors *CXCR4* and *CXCR7* from trigeminal ganglia isolated at times corresponding with the period of nerve projection into the anterior eye and corneal innervation (E5, E7, E10 and E12, Fig. S4), indicating the presence of a functional CXCL12 signaling pathway. Combined, our results indicate that only CXCL12 has neurotrophic effects on trigeminal sensory nerves dependent on the presence of NGF and that CXCL14 attenuates CXCL12-induced nerve growth in vitro. A similar mechanism of CXCL14 inhibition may be involved in regulating sensory nerve growth during ocular development.

Knockdown of CXCL14 exacerbates iridial innervation

Similar to the cornea, *CXCL14* transcripts are expressed in the stroma of the iris during development (Ojeda et al., 2013). To further determine the function of CXCL14 during ocular neurogenesis, we examined iris innervation in control and CXCL14 knockdown eyes. First, we confirmed CXCL14 protein expression, which co-localized with the inner cells of the iridial stroma and also with the Tuj1-positive nerves (Fig. 7A,B, arrowheads). Then, we immunostained iris whole-mounts dissected from control and CXCL14 knockdown eyes with anti-Tuj1 antibody (Fig. 7C,E). Although the iridial nerve plexus and contributions from the ciliary nerve bundles were evident in both cases, additional major nerve bundles

were observed entering the iridial nerve plexus in the CXCL14 knockdown eyes (Fig. 7E, arrows). At high magnification, the control iris showed regular branching into smaller nerve bundles that covered the iris radius and few Tuj1-positive cells surrounding the iridial nerve plexus (Fig. 7D,D', arrowhead). By contrast, the major nerve bundles sporadically branched into finer nerve leashes in CXCL14 knockdown iridial nerve plexus (Fig. 7F), which was also surrounded by numerous Tuj1-positive cells with cell bodies and dendritic morphology typical of neurons (Fig. 7F,F', arrowheads). The large nerve bundles were also evident in cross sections through CXCL14 knockdown iris (Fig. 7H,I, arrowheads) compared with the punctate appearance of small nerve bundles in controls (Fig. 7G). To quantify the extent of iridial innervation, the CXCL14 knockdown iris sections were divided into moderate (Fig. 7H) and severe (Fig. 7I) phenotypes, and they both show significant innervation compared with control (Fig. 7J, $P=0.0001$ and $P<0.0001$, respectively). These results show that CXCL14 is expressed in the iris during ocular development and that it plays a role in regulating the extent of innervation and patterning of the iridial nerve plexus.

Knockdown of CXCL14 causes retinal nerve defects

Previously, we showed that *CXCL14* transcripts are initially expressed diffusely in the neural retina at E7, then later become restricted to the ganglion cell layer and inner nuclear layer at E12 (Ojeda et al., 2013). Similarly, we detected CXCL14 protein expression in the neural retina during early and late stages of retinogenesis and it co-localized with the Tuj1 expression in all the retinal layers (Fig. 8A,B; Fig. S6). Next, we examined the CXCL14 knockdown eyes for retinal defects. Initially, we observed massive ectopic projections from the retina into the vitreous in CXCL14 knockdown eyes ($n=6/13$, Fig. 8C, arrow). Immunoanalysis indicated that the ectopic projections were Tuj1-positive nerve bundles (Fig. 8D, arrow). Whole-mount staining of the neural retina revealed regions of voids and reduced nerve projections toward the optic nerve in CXCL14 knockdown eyes (Fig. 8F, asterisks), as well as ectopic projections (Fig. 8F, yellow arrow). In comparison, nerves were distributed in regular striations in control (Fig. 8E). In addition, cross sections through CXCL14 knockdown retinas revealed that the retinal layers were disrupted in several regions (Fig. 1M; Fig. S6C,E). Most especially, compared to control expressing only GFP (Fig. 8G), the ganglion cell layer in CXCL14 knockdown retinas appeared to contain more cells in the disrupted regions, and they were positive for Tuj1 staining (Fig. 8H, arrowheads). These results demonstrate that CXCL14 is required for proper neurogenesis of the retina and projection of the retinal ganglion axons.

CXCL14 is required to maintain corneal avascularity during development

Previously, it was demonstrated in corneal micropocket assays that CXCL14 inhibits corneal vascularization induced by multiple angiogenic factors including IL8 (CXCL8), bFGF, and VEGF (Shellenberger et al., 2004). Since expression of CXCL14 in the embryonic cornea (Fig. 1A–C) coincides with the period of sustained corneal avascularity during the formation of the dense vascular network in the adjacent limbus region (Kwiatkowski et al., 2013; 2016), we investigated whether it is involved in preventing corneal vascularization. First, we performed bead implantation experiments to test whether addition of recombinant CXCL14 would inhibit induced vascularization of the cornea during development. Consistent with VEGF's role as an angiogenic factor, implantation of VEGF-soaked beads induced extensive

aberrant vascularization of the cornea (n=10, Fig. 9B), compared to the avascular stage-matched control (Fig. 9A). However, implantation with beads soaked in VEGF+CXCL14 showed significant reduction in corneal vascularization in comparison to VEGF-soaked beads alone (n=7, Fig. 9C,D, $P<0.0001$). This result further confirms the antiangiogenic function of CXCL14.

Next, we tested whether CXCL14 expression in the cornea also functions as antiangiogenic factor during development. We knocked down CXCL14 by injecting *Tg(tie1-H2B:YFP)* quail embryos with RCAS(A)-CXCL14-shRNA or control viral constructs. Despite robust CXCL14-shRNA expression in the anterior eye during early development (E3-E9), we did not observe ectopic vascularization of the cornea (data not shown). This is not surprising given that CXCL14 protein is not expressed in the cornea at these times (Fig. 1A). However, analysis at E12 showed significant vascularization of CXCL14 knockdown corneas (n=10, Fig. 9F,H, arrows) compared with the avascular controls (n=9, Fig. 9E,G). Cross-sections through the vascularized corneas showed that the ectopic vasculature occupied the anterior region (Fig. 9H, arrow) where CXCL14 is localized (Fig. 1B). Combined, these data show that CXCL14 is an antiangiogenic factor that plays a role in preventing corneal vascularization during late stages of ocular development.

DISCUSSION

In this study we sought to identify the function of CXCL14 by focusing on its role during chick ocular development. First, we identified that CXCL14 protein is expressed at critical times in the cornea and iris during innervation, and in the retina during neurogenesis. Then we found that knockdown of *CXCL14* caused neuropatterning defects during corneal and iridial innervation. Consistent with the innervation defects in the anterior eye, we also observed neural defects in the retina and aberrant projection of retinal ganglion nerves. We show in vitro that the neural defects are likely due to increased chemoattraction of CXCL12 in the absence of CXCL14. Our results also show that CXCL14 plays a crucial role in preventing corneal vascularization during late development. In line with these data, we observed significant upregulation of *TrkA*, *VEGFR2*, *CXCL12*, and *CXCR4* in CXCL14 knockdown corneas compared with control (Fig. 10A,B), possibly due to increased sensory innervation and ectopic vasculature in the knockdown corneas. Thus CXCL14 signaling ensures proper innervation and neurogenesis, and also maintains corneal avascularity during ocular development.

Previous studies in multiple organisms have shown that *CXCL14* is robustly expressed in various tissues during embryonic development (Long et al., 2000; Park et al., 2009; García-Andrés and Torres, 2010; Gordon et al., 2011). While *CXCL14* expression is evident at the onset of the formation of the corneal stroma at E6 in chick (Ojeda et al., 2013), our results show that protein expression occurs much later at E10 and it coincides with the projection of sensory nerves into the corneal stroma (Bee et al., 1982; Lwigale and Bronner-Fraser, 2007; Kubilus and Linsenmayer, 2010). Interestingly, CXCL14 protein is localized in the anterior stroma and corneal epithelium where the sensory nerves subsequently project. CXCL14 expression is also localized in the iris and neural retina during iridial innervation and

neurogenesis, respectively. Thus the high levels of expression of CXCL14 in these ocular tissues suggest a crucial role during their formation and innervation.

We observed that knockdown of CXCL14 in chick embryos caused a reduction in body size as well as abnormal mandibular and eyelid development. The reduced body size is consistent with the lower body weight observed in CXCL14 knockout mice (Meuter et al., 2007; Tanegashima et al., 2010). Despite the lower birth rate of homozygous breeding pairs and the lower number of CXCL14 mutants than the expected Mendelian ratio, the function of CXCL14 during murine embryonic development remains unexplored. Histological analysis of ocular tissues from CXCL14 knockdown chick embryos revealed malformation of the lens fiber cells and neural retina. Surprisingly, the cornea did not show apparent defects, indicating that CXCL14 is dispensable for the formation of the corneal stroma and epithelium. The above defects suggest that CXCL14 has pleiotropic functions during chick development and its role in each of the embryonic tissues will require further investigation. In the present study, we focused on the role of CXCL14 during neurovascular development in the eye.

Given that CXCL14 is localized in several neural tissues including the trigeminal ganglion (García-Andrés and Torres, 2010; Gordon et al., 2011; Park et al., 2012) that provides sensory innervation to the cornea, we first investigated its role during corneal innervation. Presumptive corneal nerves are initially repulsed by the cornea due to the presence of chemorepellents such as Sema3A and Slit2, secreted by the cornea and adjacent lens (Lwigale and Bronner-Fraser, 2007; Kubilus and Linsenmayer, 2010; Schwend et al., 2012). In contrast, CXCL14 is expressed in the anterior region of the cornea that is invaded by sensory nerves. Therefore it is tempting to speculate that CXCL14 plays a chemoattractive role for the sensory nerve projections. Although this would be consistent with a previous observation that CXCL14 acts as a chemoattractant for cerebellar granule cell migration in vitro (Park et al., 2012), in contrast, our results show that its absence in the cornea exacerbates stromal and epithelial innervation. The overexpression experiments confirmed the inhibitory role of CXCL14. However, absence of innervation phenotype following ectopic expression in the pericorneal nerve ring indicates that CXCL14 alone does not affect nerve projections, and that it may require growth factors that are differentially expressed between the cornea and periocular region.

Similar to CXCL14 knockdown in the cornea, we observed extensive iridial innervation, which originate from the trigeminal and ciliary ganglia (Kuwayama and Stone, 1987; Neuhuber and Schödl, 2011; Simpson et al., 2013). We also noticed numerous Tuj1-positive cells that remained unincorporated into the iridial nerve plexus in CXCL14 knockdown eyes. It is possible that these isolated cells are immature neurons that migrate, proliferate, or differentiate extensively in the absence of CXCL14. Combined, these results suggest that CXCL14 plays a role in controlling the extent of corneal and iridial innervation. At this point the receptor for CXCL14 remains unknown, so it is difficult to present a clear mechanism of how it regulates axon growth in the ocular tissues. Our attempt to identify the mode of action of CXCL14 on dissociated trigeminal axons in vitro suggest that it does not affect nerve growth stimulated by NGF. However, as shown in previous studies that CXCL12 stimulates neural migration in the central nervous system (Yang et al., 2013) and

axon growth and survival from dissociated dorsal root ganglia (Belmadani et al., 2005; Ödemis et al., 2005; Opatz et al., 2009), we observed significant increase in trigeminal axon growth in the presence of CXCL12. Interestingly, the CXCL12-induced increase in axon growth was abolished by CXCL14. Our results are in agreement with previous observations that CXCL14 functions as an inhibitor for CXCL12 (Tanegashima et al., 2013a,b; Hara and Tanegashima, 2014). This is likely through the CXCL12 receptors CXCR4 and CXCR7 given that they are expressed by the chick trigeminal neurons during late development and during gangliogenesis in *Xenopus* and Zebrafish (Theveneau et al., 2013; Knaut et al. 2005). It is possible that CXCL12 interacts with neurotrophins and other guidance cues in the anterior eye to promote axon growth, which is toned-down in the cornea and iris by CXCL14 to permit optimal iridial and corneal innervation.

Our results also indicate that CXCL14 is essential for proper development of the neural retina. CXCL14 protein is expressed throughout the neural retina during neurogenesis and it is later elevated in retinal ganglion cells. We demonstrate that knockdown of CXCL14 in chick causes severe defects in the neural retina including increase in the number of retinal ganglion cells and aberrant projection of neurons. This suggests that CXCL14 regulates either the proliferation or migration of retinal ganglion cells and their projection into the optic nerve. Since *CXCL14* transcript expression pattern in the retina is conserved in chick and mouse (Ojeda et al., 2013), it is possible that its function is as well. Adult CXCL14 mutant mice have inconsistencies in feeding behavior that may indicate poor vision (Tanegashima et al., 2010). As discussed above, the retinal defects could also be due to unmodulated effects of CXCL12 signaling in the absence of CXCL14. Previous studies have shown that *CXCL12* is expressed in the inner nuclear layer, whereas the retinal ganglion cells express *CXCR4* (Ojeda et al., 2013; Chalasani et al., 2003b), and that CXCL12 promotes their survival (Chalasani et al., 2003b). The misprojection of retinal ganglion cells could be a result of high numbers of cells in this layer in the absence of CXCL14, which may fail to follow the existing cues from CXCL12 required for proper neural guidance into the optic nerve (Li et al., 2005).

Another interesting observation from our findings is the possibility that CXCL14 expression in the cornea uncouples the neurovascular congruence that exists in the periocular region by completely inhibiting corneal vascularization while permitting optimal innervation. In chick, afferent trigeminal sensory nerves first appear at the periphery of the presumptive cornea at about E5 (Bee, 1982; Lwigale and Bronner-Fraser, 2007). Formation of the dense limbal vascular network is underway by this time (Kwiatkowski et al., 2013). The pericorneal nerves and vasculature both express *Nrp1* and they are repelled from the presumptive cornea by Semaphorins secreted by the cornea and adjacent lens (Lwigale and Bronner-Fraser, 2007; McKenna et al., 2012; 2014; Kwiatkowski et al., 2016). The cornea remains un-innervated and avascular until about E10 when only sensory nerves are permitted to project into its periphery. Our results show that this process correlates precisely with the onset of corneal expression of CXCL14 protein. Our bead implantation experiment confirmed a previous observation that CXCL14 inhibits VEGF-induced vasculogenesis in the cornea (Shellenberger et al., 2004). We also observed massive ectopic ingression of blood vessels into the corneas of CXCL14 knockdown embryos. Our findings are also consistent with a previous study showing that the efficacy of the VEGF inhibitor (Sorafenib) used in cancer

antiangiogenic therapy depends on the induction of CXCL14 (Rivera et al., 2015). Interestingly, neovascularization of the CXCL14 knockdown corneas occurred later in development, suggesting that CXCL14 is dispensable as an antiangiogenic factor during early corneal development, which is regulated by Semaphorins (Kwiatkowski et al., 2013; 2016; McKenna et al., 2015). We propose that CXCL14 sustains antiangiogenic signaling to maintain corneal avascularity as the repulsive Semaphorin signals weaken to permit innervation.

Collectively, our results show for the first time that CXCL14 plays a crucial role in maintaining proper innervation of the cornea and iris, neurogenesis and projection of retinal ganglion cells, and it inhibits corneal vascularization. Based on our data, we propose that its mode of action on ocular neurons and blood vessels during late stages of development is via inhibition of the trophic effects of CXCL12 and VEGF (Fig. 10C). Since transcripts of other angiogenic factors including *FGF*, *PDGFB*, and *Sema3G* are expressed in the anterior eye during development (Kwiatkowski et al., 2013), it is likely that CXCL14 may inhibit their function in the cornea as well, given its potent inhibition of angiogenesis induced by multiple growth factors in corneal micropocket assays (Shellenberger et al., 2004). Future studies will address the role of CXCL14 in other tissues where it is strongly expressed during development, and how it affects signaling from other growth factors associated with neurovascular patterning.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Benjamin Deneen and Stephen Hughes for the expression plasmids and RCAS vectors. We would like to thank James Spurlin, Sam Kwiatkowski, and members of Lwigale lab for the helpful discussions and suggestions on this project.

FUNDING

This work was funded by a CONICYT-Fulbright Fellowship to AO and by NEI Grant EY022158 to PYL.

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HIGHLIGHTS

- CXCL14 is expressed in ocular tissues during development.
- Knockdown of CXCL14 caused neuropatterning defects in the cornea, iris, and retina.
- Knockdown of CXCL14 caused neovascularization of the cornea.
- CXCL14 modulates response to signals involved in neurovascular development.

Figure 1. Corneal expression of CXCL14 protein and ocular defects caused by overexpression of CXCL14-shRNA

(A-C) Vivid expression of CXCL14 is detected in the cornea by E12 and it is diminished by E17. (D) Plasmid map for RCAS-CXCL14-shRNA vector. (E-I) In vitro and in vivo validation of mRNA and protein knockdown by overexpression of CXCL14-shRNA. *CXCL14* was knocked down in DF-1 cells transfected with RCAS-CXCL14-shRNA but not RCAS-GFP control (E). CXCL14-shRNA injected embryos showing robust expression of GFP in the anterior eye (F,G) and diminished expression of CXCL14 protein in the corneal

stroma and epithelium (**H,I**). (**J,L**) Gross anatomical images of chick heads showing reduction in the head size, eyelid (arrowhead) and mandibular (asterisk) defects in a CXCL14 knockdown embryo. (**K,M**) Histological analysis of the eye showing large gaps in the lens fibers cells (asterisk) and defects in the neural retina (arrows) in CXCL14 knockdown embryos. co, cornea; L, lens; ep, epithelium; st, stroma; en, endothelium; ret, neural retina. Scale bars: 100 μm (A–C); 50 μm (H,I); 200 μm (K,M).

Figure 2. Knockdown of CXCL14 increases axon projection into the stroma during corneal innervation

Whole-mount and cross section of E10 corneas: (A,C) control showing sensory innervation of only the corneal periphery. (B,D) CXCL14 knockdown showing precocious innervation of the corneal center (red arrows). (E) Diagram showing how the concentric ring template was superimposed on images of immunostained corneas to determine the extent of nerve projection. (F) The number of nerves traversing rings 1–3 were counted in control and CXCL14 knockdown corneas. Values are expressed as mean ± SEM. Statistical significance

was determined by Student's t-test. ** $P < 0.01$, *** $P < 0.001$. Dotted lines mark the corneal endothelium. Scale bars: 200 μm .

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Figure 3. Knockdown of CXCL14 causes precocious innervation of the corneal epithelium (A,D) Whole-mount E12 corneas showing nerve projections in (A) control and (D) CXCL14 knockdown embryos. Higher magnification and Z-stack images of periphery and central regions in: (B,C) Control corneas show epithelial innervation of only the peripheral region. (E,F) CXCL14 Knockdown corneas show epithelial innervation of both the peripheral and central regions. (G,H) Quantification of epithelial nerve leashes (red arrows) showing significant increase in central innervation of CXCL14 knockdown corneas. Values are expressed as mean $\pm$ SEM. Statistical significance was determined by Student's test. n.s non-significant; ****P<0.0001. ep, epithelium; st, stroma. Scale bars: 500 μ m (A,D), 100 μ m (B,C,E,F).

Figure 4. Aberrant innervation of the corneal stroma following CXCL14 knockdown

Cross sections of immunostained E12 corneas showing: (A) Nerve projection in the anterior third of the corneal stroma in controls (red bracket). (B) Increased nerve density and occupation of the mid stroma in CXCL14 knockdown (red bracket). (C,D) Quantitative analysis shows significant increase in stromal nerve occupancy (C) and stromal nerve density (D) in CXCL14 Knockdown corneas. Statistical significance was determined by Student's t-test, *** $P < 0.001$, **** $P < 0.0001$. Dotted lines denote the limits of the corneal epithelium and endothelium. ep, epithelium; st, stroma; en, endothelium. Scale bar: 200 μm .

Figure 5. Overexpression of CXCL14 attenuates nerve projection into the corneal stroma

Whole-mount E12 corneas immunostained for Tuj1 to show extent of stromal innervation.

(A) Control cornea showing nerve projections into the corneal center. (B) Cornea with

CXCL14 overexpression showing diminished innervation of the corneal center. (C) The

number of nerves traversing a ring superimposed to the corneal center where counted in

control and CXCL14 overexpression corneas. Values are expressed as mean ± SEM.

Statistical significance was determined by Student's t-test. *** $P < 0.001$. Scale bar: 200 μm .

Figure 6. Effect of CXCL14 on dissociated trigeminal neurons
Negligible growth observed when neurons are cultured in (A) complete medium alone, (B) supplemented with 100 ng ml⁻¹ CXCL14, or (C) supplemented with 100 ng ml⁻¹ CXCL12. (D,E) Neurite outgrowth in presence of NGF is not affected by supplementation with CXCL14. (F) CXCL12 increases neurite outgrowth in presence of NGF. (G) CXCL14 diminishes neurite outgrowth associated with CXCL12. (H) Quantification of total neurite growth under different conditions. Statistical significance was determined by Student's t test, *P<0.05; ***P<0.001; ****P<0.0001. Scale bar: 200 μm.

Figure 7. Iris innervation defects caused by knockdown of CXCL14

(A,B) CXCL14 is expressed in the stroma of the iris and co-localizes with nerve staining. (C-F) Whole-mount immunostaining of iris at E12 showing nerve architecture: (C,D) in control. (E,F) Ectopic nerve branches in CXCL14 knockdown eyes (red arrows) and irregular axon branching, and substantial increase in thickness of the nerve bundles. (D', F') Higher magnification showing few Tuj1-positive cells in control compared to the relatively higher number in CXCL14 knockdown. (G-I) Cross sections of the iris showing, (G) normal innervation in control, and (H,I) different levels of increased innervation of CXCL14

knockdown. **(J)** Quantification of the area covered by nerves in control and CXCL14 knockdown irises. Statistical significance was determined by Student's t test, *** $P < 0.001$; **** $P < 0.0001$. ir, iris; cn, ciliary nerve; inp, iris nerve plexus. Scale bars: 100 μm (A,B); 500 μm (C,E); 200 μm (D,F-I); 50 μm (D',F').

Figure 8. Expression of CXCL14 in the neural retina and defects caused by CXCL14 knockdown (A,B) Cross section of E12 retina showing expression of CXCL14 and Tuj1. (C,D) Aberrant projections of Tuj1-positive neurons from the retina caused by CXCL14 knockdown (arrows). (E,F) Whole-mount staining of neural retinas with Tuj1 antibody showing: (E) Normal layout of nerve projections towards the optic nerve in control, and (F) Gaps in nerve projections (asterisks) caused by CXCL14 knockdown. (G,H) Cross sections showing GFP and Tuj1 staining of control (G) and CXCL14 knockdown (H) retinas. Arrowheads in (H) indicate defective neurogenesis in regions that express CXCL14-shRNA. GCL, ganglion cell

layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; on, optic nerve. Scale bars: 50 μm (A,B,C,D,G,H), 200 μm (E,F).

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Figure 9. Knockdown of CXCL14 causes neovascularization of the cornea
(A-D) CXCL14 inhibits VEGF-induced neovascularization. GFP staining of *Tg(tie1:H2B:eYFP)* quail eyes showing: **(A)** Avascular cornea. **(B)** Ectopic vasculature in the cornea induced by insertion of VEGF-coated bead. **(C,D)** Bead coated with a combination of VEGF and CXCL14 induce significantly less ectopic vasculature compared to VEGF alone. **(E-I)** CXCL14 is required to maintain corneal avascularity during late development. **(E,G)** *Tg(tie1:H2B:eYFP)* quail control eyes showing normal limbal vasculature (lv) and corneal avascularity. **(F,H)** CXCL14 knockdown eyes showing ectopic vascularization of the cornea (arrows). **(I)** Quantification of the extent of corneal neovascularization. Dotted lines delineate the boundary between the corneal periphery and adjacent limbus. Statistical significance was determined by Student's test. ** $P < 0.01$, *** $P < 0.0001$. co, cornea; lv, limbal vasculature. Scale bars: 200 μm (A,B,G,H); 500 μm (E,F).

Figure 10. Effect of CXCL14 knockdown on expression of candidate genes associated with neurovascular development

(A) RT-PCR analysis of the expression of *NGF*, *BDNF*, *TrkA*, *TrkB*, *NT3*, *VEGFA*, *VEGFR2*, *CXCL12*, *CXCR4*, *Sema3A*, and *Nrp1* RNA extracted from E12 control and CXCL14 knockdown corneas. (B) ImageJ software was used to determine band intensity normalized to GAPDH, which indicate significant increase in the expression of *TrkA*, *VEGFR2*, *CXCL12*, and *CXCR4* in CXCL14 knockdown corneas. Statistical significance was determined by Student's test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (C) Schematic diagram of the proposed inhibitory effect of CXCL14 on neural and vascular development via suppression of CXCL12 and VEGF signaling. Solid lines indicate data from this and previous studies, broken lines indicate interactions of CXCL14 inferred from our results.