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## The Expression And Function Of Netrin-4 In Murine Ocular Tissues

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### Abstract

Netrin-4, a member of the netrin family, is a potent regulator of embryonic development. It promotes neurite extension and regulates pulmonary airway branching, vasculogenesis patterning, and endothelial proliferation in pathological angiogenesis. The initial characterization of netrin-4 expression was focused on epithelial-derived organs (kidney, lung and salivary gland) and the central nervous system. Ocular development is an ideal system to study netrin-4 expression and function, as it involves both ectodermal (cornea, lens and retina) and mesodermal (sclera and choroid) derivatives and has an extensive and well-characterized angiogenic process. Netrin-4 is expressed in all ocular tissues. It is a prominent component of the basement membranes of the lens and cornea, as well as all three basement membranes of the retina: the inner limiting membrane, vascular basement membranes, and Bruch's membrane. Netrin-4 is differentially deposited in vascular basement membranes, with more intense anti-netrin-4 reactivity on the arterial side. The retinal microcirculation also expresses netrin-4. In order to test the function of netrin-4 *in vivo*, we generated a conventional mouse lacking *Ntn4* expression. Basement membrane formation in the cornea, lens and retina is undisrupted by netrin-4 deletion, demonstrating that netrin-4 is not a major structural component of these basement membranes. In the *Ntn4* homozygous null (*Ntn4*<sup>-/-</sup>) cornea, the overall morphology of the cornea, as well as the epithelial, stromal and endothelial stratification are normal; however, epithelial cell proliferation is increased. In the *Ntn4*<sup>-/-</sup> retina, neurogenesis appears to proceed normally, as does retinal lamination. In the *Ntn4*<sup>-/-</sup> retina, retinal ganglion cell targeting is intact, although there are minor defects in axon fasciculation. In the retinal vasculature of the *Ntn4*<sup>-/-</sup> retina, the distribution patterns of astrocytes and the vasculature are largely normal, with the possible exception of increased branching in the deep capillary plexus, suggesting that netrin-4 may act as a negative regulator of angiogenesis. These data, taken together, suggest that netrin-4 is a negative regulator of corneal epithelial cell

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proliferation and retinal vascular branching in vivo, whereas netrin-4 may be redundant with other members of the netrin family in other ocular tissue development. *Ntn4*<sup>-/-</sup> mice may serve as a good model in which to study the role of netrins in vivo of the pathobiologic vascular remodeling in the retina and cornea.

## Keywords

Extracellular matrix; netrin; angiogenesis; cornea; retina; axonal pathfinding

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## 1. Introduction

Ocular development involves neural tube-derived components (retina) and ectoderm-derived components (lens and cornea) (Cvekl and Tamm, 2004). In mouse, retinal ganglion cells (RGCs) are generated prenatally (E11 to E19) in an approximately centrifugal order (Drager, 1985; Young, 1985). Their axons exit the eye starting at E11.5 also in a centrifugal order; thus, axons of the later-born RGCs arise from more peripheral regions and travel greater distances before they turn to exit into the optic nerve (Mann et al., 2004). Along their elongation path, RGC axons are in close contact with Müller cell endfeet and with the inner limiting membrane (ILM) (Mann et al., 2004; Stuermer and Bastmeyer, 2000).

The anterior segment follows parallel pathways of development. At E12.5-13.5, cells derived from the neural crest begin to migrate into the space between the anterior epithelium of the lens vesicle and the surface ectoderm. By E14.5-E15.5, the cells closest to the lens form an endothelial monolayer; the surface ectoderm becomes the corneal epithelium; cells between the endothelium and epithelium differentiate into keratocytes of the corneal stroma (Cvekl and Tamm, 2004).

During the development of these ocular structures, many extracellular matrix (ECM) molecules play important roles. For example, netrin-1, laminin  $\alpha1\beta1\gamma1$ , and ephrin-B are all implicated in RGC axon pathfinding (Hopker et al., 1999; Mann et al., 2004), and ECM molecules are thought to have a role in corneal development and maturation (Kabosova et al., 2007).

Netrins are a family of ECM molecules that have homology with the short arm of laminin molecules. Five members of the netrin family have been identified in mammals: netrin-1 (Kennedy et al., 1994), netrin-3 (Van Raay et al., 1997; Wang et al., 1999), netrin-4 (Koch et al., 2000; Yin et al., 2000), netrin-G1 and netrin-G2 (Nakashiba et al., 2000; Nakashiba et al., 2002). All netrin genes encode secreted proteins, but in contrast to netrin-1, -3 and -4, netrin-G1 and -G2 are bound to the membrane via a glycosylphosphatidylinositol (GPI) linkage.

Netrins were first recognized as long range guidance cues regulating the migration of neurons and the behavior of axon growth cones (Barallobre et al., 2005; Kennedy et al., 1994). However, they have wide-spread expression outside the nervous system, and netrins are involved in a wide variety of developmental events, including angiogenesis (Lu et al., 2004; Wilson et al., 2006), cell-cell adhesion (Srinivasan et al., 2003; Yebra et al., 2003), and branching morphogenesis of lung, mammary gland and salivary glands (Liu et al., 2004; Schneiders et al., 2007; Srinivasan et al., 2003).

In the embryo, netrin-1 is deposited at the exit of the optic nerve, and RGC axons express the netrin-1 receptor, DCC (de la Torre et al., 1997; Deiner et al., 1997; Keino-Masu et al., 1996; Livesey and Hunt, 1997) suggesting a role in guidance. In both netrin-1-hypomorphic

and DCC-null mouse retinas, RGC axons navigate to the optic disc. Some axons fail to exit into the optic nerve, indicating that netrin-1 is not required for guiding retinal axons to the optic disc, but may be required for proper exit of RGC axons (Deiner et al., 1997). Importantly, DCC-null mice have a more severe phenotype than netrin-1 hypomorphic mice, suggesting there may be additional ligands for the DCC receptor (Fazeli et al., 1997).

We first identified netrin-4 ( $\beta$ -netrin), and demonstrated its expression in multiple tissues (Koch et al., 2000). We showed that, in vitro, netrin-4 promoted the neurite growth from E14 rat olfactory bulb explants, suggesting netrin-4 might be important as a haptotactic factor in promoting axonal elongation or as a substrate for neuronal migration. Netrin-4 has also been shown to regulate epithelial branching and endothelial proliferation (Lejmi et al., 2008; Liu et al., 2004; Park et al., 2004; Schneiders et al., 2007). This study details netrin-4 expression in the mouse eye, provides the first characterization of a *Ntn4*<sup>-/-</sup> mouse, and uses that netrin-4-null mouse to define the role of netrin-4 in the development of ocular components in vivo.

## 2. Materials and Methods

All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Tufts University and State University of New York - Downstate Medical Center and were in accordance with the National Institute of Health Guide for the Care and Use of Animals.

### 2.1 Recombinant expression and purification of murine netrin-4 fragment

A truncated netrin molecule,  $\Delta$ netrin-4 (AF281278, nucleotides 311-1,672), was amplified by PCR and subcloned into a modified pCEP-Pu expression vector (an 8 histidine tag and a thrombin cleavage site were introduced either at the N-terminal or the C-terminal end of the protein sequence). The expression vector was transfected into 293-EBNA cells with FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN). Clones with the highest protein expression were expanded for large-scale production. The purification of the secreted proteins was performed as previously described (Koch et al., 2000).

### 2.2 Antibody production

KR1, a rabbit anti-netrin-4 antibody, was produced as described previously (Koch et al., 2000). Recombinant mouse  $\Delta$ netrin-4 (AF281278, nucleotides 311-1,672), from which the His tag was removed, was injected intradermally into a rabbit following standard procedures (Harlow and Lane, 1988). The KR1 antiserum was purified over a protein G column (Amersham Biosciences, Piscataway, NJ) and eluted with triethylamine (Sigma, St. Louis, MO). The neutralized elute was affinity-purified on a netrin-4 protein column. To prepare this column,  $\Delta$ netrin-4, from which the His tag was removed, was coupled to activated CNBr-Sepharose (Amersham Biosciences). Bound antibodies were eluted with triethylamine and immediately neutralized. Recombinant DCC (NM\_007831, amino acids 26-1,097) was expressed and purified similarly. Guinea pig polyclonal antibody against purified recombinant DCC protein was produced similarly.

### 2.3 Immunohistochemistry

For fresh-frozen samples, adult or embryonic tissues were dissected, embedded immediately in OCT compound (Sakura, Torrance, CA), and frozen by immersion in dry ice-cooled ethanol. For fixed tissues, mice were anesthetized by intramuscular injection of ketamine and xylamine, then transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Tissues were dissected and fixed in 4% PFA by immersion at 4°C overnight, then processed through 10%, 20% and 30% sucrose before being

embedded in OCT. For both fresh-frozen and 4% PFA fixed tissues, 12 $\mu$ m sections were cut with a Reichert-Jung 2800 Frigocut cryostat and placed onto Superfrost Plus slides (Brain Research Laboratory, Newton, MA). Slides were stored at  $-80^{\circ}\text{C}$  until use.

Upon use, slides were returned to room temperature and air dried. Fresh-frozen tissue slides were immersed in absolute methanol at  $-20^{\circ}\text{C}$  for 2min; 4% PFA fixed tissue slides directly went to the next step. Slides were then washed in PBS and blocked with 1% BSA and 5% goat or donkey serum in PBS for 1hr. The sections were incubated in primary antibody at room temperature for 2 hr or at  $4^{\circ}\text{C}$  overnight. The primary antibodies used were against: netrin-4 (KR1, rabbit polyclonal, 1:1,000; R33, rabbit polyclonal, 1:2,000) (Koch et al., 2000), DCC (guinea pig polyclonal, 1:1,000), perlecan (rat monoclonal, 1:50,000, Chemicon, Temecula, CA), vimentin (rabbit polyclonal, 1:5,000, Chemicon), calretinin (mouse monoclonal, 1: 1,000, Chemicon), glutamine synthetase (mouse monoclonal, 1:1,000, Chemicon), PKC $\alpha$  (MC5, mouse monoclonal, 1:1,000, Chemicon), and rhodopsin (RetP1, mouse monoclonal, 1:10,000, Sigma), PECAM-1 (CD31, rat monoclonal, BD Biosciences, San Jose, CA), Phospho-Histone H3 (Rabbit polyclonal, Upstate Biotechnology, Lake Placid, NY). The primary antibodies were diluted in PBS containing 1% BSA and 1% goat or donkey serum. After a PBS wash, slides were incubated in species-appropriate, affinity-purified, fluorescently labeled secondary antibodies (Invitrogen, Carlsbad, CA) at room temperature for 1hr. Some sections were counter-stained with DAPI (Sigma). After a PBS wash, slides were mounted in ProLong (Invitrogen).

Slides were examined and images were captured using OpenLab (4.0.2) or Volocity (5.0) software (Improvision Inc., Lexington, MA) with a Hamamatsu Orca camera on a Nikon E800 microscope. The figures shown here were created using Adobe Photoshop 7.0 and no adjustment other than contrast and brightness was made to the images. To make a montage image, series of images were taken using OpenLab 4.0.2 with a motorized X-Y stage and were put into a montage using GraphicConverter software (Lemke Software GmbH, Peine, Germany) after the background of these images was subtracted using OpenLab 4.0.2.

## 2.4 Mice

*Ntn4*<sup>-/-</sup> mice were generated using standard recombination methods. The targeting vector was designed to delete a 3.9kb fragment containing part of the first exon, the first intron and part of the second exon, inserting in its place a neomycin resistance (*Neo*) cassette flanked by *LoxP* sites and a *LacZ* reporter gene. The mutation was introduced into 129/SvJ embryonic stem (ES) cells (Li et al., 1992). Following neomycin selection and cloning, Southern analysis was used to identify ES cells for chimera production. Two probes were used one at the 5' end and one at the 3' end. Genomic DNA was digested with PvuIII for the 5' probe or HindIII for the 3' probe. The recombination event was confirmed in two lines (119 and 146) by the presence of mutant bands migrating above the wild-type bands. ES injection into blastocysts was performed in the transgenic mouse facility at the Cutaneous Biology Research Center, Massachusetts General Hospital.

Following identification of founder mice, *Ntn4*<sup>+/-</sup> mice were back-crossed to C57BL/6 wild-type mice for 9 generations. The genotypes of the offspring were determined by PCR analysis of genomic DNA prepared from tail or toe samples. Primers used: wild type and null allele forward AGCAGCCTTTAAACATCCTGAG, wild type allele reverse GAAAGCTCCGGGACAGACTATGTG, and null allele reverse CAAATGTGTCAGTTTCATAGCC. B6.Cg-Tg(Thy1-YFPH)2Jrs/J mice (Feng et al., 2000) were crossed with *Ntn4*<sup>+/-</sup> mice to generate a line of *EYFP*<sup>+/+</sup>/*Ntn4*<sup>+/-</sup> mice. The primers used to detect the *EYFP* transgene were as described (Feng et al., 2000). All animals used in this study were littermates; both *Ntn4* lines (*Ntn4*<sup>+/-</sup> and *EYFP*<sup>+/+</sup>/*Ntn4*<sup>+/-</sup>) were maintained as heterozygotes.

## 2.5 Cornea whole-mounts

Mice were euthanized by carbon dioxide and the eyes were enucleated. The cornea was dissected and fixed in 4% PFA for 2hrs. Four radial cuts were made on the cornea to ease the flattening. The cornea was then flattened onto a glass slides. Processing followed the procedures for retinal whole-mounts below. Whole-mounts were imaged using Volocity 5.5.

## 2.6 Retinal whole-mounts

Mice were anesthetized by intramuscular injection of ketamine and xylamine and then then transcardially perfused by PBS followed by 4% PFA. Eyes were enucleated from the mice and the anterior segment was discarded.

The eyecups were further fixed in 4% PFA by immersion at room temperature for 30 min. The sclera was peeled away and the pigment epithelium was removed. The residual vitreous was carefully removed under a dissecting microscope to help flatten the retina. Four radial cuts were made to allow for further flattening. *EYFP+/Ntn4+/+* and *EYFP+/Ntn4-/-* retina whole-mounts were mounted in glycerol and the coverslips were sealed to the slides with nail polish.

The whole-mount immunohistology protocol was modified from Cellerino et al. (Cellerino et al., 1998). Retinas were dehydrated in ethanol with ascending concentrations (50%, 70%, 80%, 90%, 95%, and 100%) for 10min each, and then incubated in xylene at room temperature for 10min. They were rehydrated in ethanol with descending concentrations (100%, 95%, 90%, 80%, 70%, and 50%) for 10min each before being washed in PBS for 30min. The retinas were blocked in 1% BSA with 20% goat or donkey serum and 0.3% Triton X-100 overnight, and then incubated in primary antibodies in 1% BSA with 5% goat or donkey serum at 4°C for 3 days with continuous agitation. The primary antibodies used were against: netrin-4 (KR1, 1:5,000), neurotubulin (TuJ-1, mouse monoclonal, 1:500, Covance Innovative, Berkeley, CA) and integrin  $\beta$ 4 (346-11A, rat monoclonal, 1:100, Chemicon). After a PBS wash, the retinas were incubated in species-appropriate, affinity-purified, fluorescently labeled secondary antibodies (Invitrogen) at 4°C for 1 day with continuous agitation. After a PBS wash, the retinas were flattened onto slides with the ganglion cell layer side facing up and mounted in ProLong (Invitrogen). Whole-mount retina slides were stored at 4°C between examinations.

## 2.7 DiI labeling

Retinal organotypic cultures of P7 wild type and mutant mice were prepared as previously described (Pinzon-Duarte et al., 2000). DiI crystals were applied onto the retinas and cultured for 2 weeks. The retinas were then fixed in 4% PFA and mounted for photographing.

## 3. Results

### 3.1 Netrin-4 expression in the mouse eye

Netrin-4 is first detected in the BM of the presumptive corneal surface epithelium at E13 (Fig. 1; arrowheads). As development proceeds, netrin-4 immunoreactivity (IR) becomes more intense in the corneal epithelial BM, i.e., Bowman's membrane. Concomitant with the migration of mesenchymal cells, netrin-4 IR is found within the developing corneal stroma beginning at approximately E16. An abrupt boundary in netrin-4 expression at the corneal limbus is evident at this early age. This striking boundary is maintained throughout development and into adulthood (Fig. 1). Starting at E17, netrin-4 IR becomes detectable in the corneal endothelial BM, i.e., Descemet's membrane. At first the expression in

Descemet's membrane is discontinuous; however, it becomes a continuous sheet near birth (Fig. 1).

The lens capsule is a specialized BM surrounding the lens (Seland, 1992). It serves as a basal attachment site for lens epithelial cells and provides stable anchoring sites for zonular fibers, filamentous structures that couple the ciliary muscle to the lens during accommodation reflexes (Kelley et al., 2002). Many extracellular matrix proteins have been found in the lens capsule, including entactin/nidogen (Cammarata and Spiro, 1985), laminin and fibronectin (Parmigiani and McAvoy, 1984), heparin sulfate proteoglycan (Mohan and Spiro, 1986), collagen XVIII (Halfter et al., 1998), and collagen IV (Schmut, 1978); indeed, laminin  $\beta 2$  was isolated from the lens capsule (Hunter et al., 1989). Netrin-4 is detectable in the BM of the developing lens placode as early as E11, the earliest time point that we examined (Fig. 1). Netrin-4 continues to be expressed in the lens BM as the lens placode detaches from the surface ectoderm, and is expressed through adulthood (Fig. 1). Thus, netrin-4 is deposited in the BM compartment separating the invaginating lens and the neural retina. Throughout development (E14 to P5) there appears to be a differential expression of netrin-4 in the lens capsule - netrin-4 deposition in the posterior capsule appears higher than in the anterior capsule (Fig. 1; e.g., compare anterior with posterior at E19). Although it may be that netrin-4 is important in this developmental gradient, it may also be that the apparent difference in the intensity of netrin-4 IR in the anterior versus the posterior lens capsule reflects the relative thickness of the anterior versus the posterior lens capsule during development (Kelley et al., 2002) rather than a change in concentration of the molecule.

### 3.2 Netrin-4 in retina and optic nerve head

Netrin-1 and its receptor, DCC, are expressed in the optic nerve and are critical components of the molecular mechanism guiding RGC axons out of the eye. Thus, we were interested in discovering if netrin-4 overlapped with these molecules in expression and deposition. From the earliest point in development that we assayed (E11), netrin-4 is deposited in the BM of the invaginating eye cup (i.e., the ILM), the BM of the RPE (Bruch's membrane) and the BM of the ciliary and iris epithelium (Figs. 1, 2). Its expression persists into adulthood (Fig. 1, P26; Fig. 2C). There is also continuous deposition of netrin-4 in the pars plana of the retina (Fig. 1, P26).

At E11-12, netrin-4 is present along the ILM and along the optic fissure, the exit point for RGC axons and the entry point for the hyaloid and retinal arteries (Fig. 1). Netrin-4 expression is not found surrounding RGC axons, i.e. in the endo- or perineurium, rather netrin-4 is associated with the hyaloid artery from E13 through E19 (Fig. 2A, B, arrows).

Retinal vascularization proceeds first by growth along the ILM/vitreous border (from P0 to P10), then arterial branches penetrate deep into the retina (from P4 to P14), forming a capillary bed at the synaptic bases of photoreceptors in the outer retina (Connolly et al., 1988; Stone et al., 1995). Netrin-4 IR remains as a component of the vascular BM throughout retinal vasculogenesis and persists in mature retina (Fig 2C).

We ascertained whether netrin-4 is evenly distributed over the retinal vascular tree using adult retina whole-mounts. Netrin-4 deposition appears more intense on the arterial side of the vascular tree and extends deeply into the capillary bed, whereas netrin-4 in the veins appears considerably less intense (Fig. 3). Netrin-4 IR is deposited in a circumferential pattern on the artery (Fig. 3B, arrow) with punctate regions of increased staining in the micro-vessels particularly at branch points, whereas netrin-4 is deposited longitudinally on the vein (Fig. 3B, arrowhead). This pattern matches the smooth muscle distribution or pericyte distribution on the respective vessels.

In addition to the vascular BM deposition, netrin-4 is a prominent component of the ILM. This distribution is apparent in whole-mounts (Fig. 3A) as well as radial sections (Fig. 4); moreover, netrin-4 is expressed in the epineurial sheath of the nerve (Fig. 4).

To determine whether the netrin receptor, DCC, is expressed near netrin-4 in the retina, we labeled sections with antibodies against DCC and netrin-4; co-localization of DCC and netrin-4 signals is present only at the very margins of the optic nerve and at the junction of the ILM and nerve fiber layer of the mouse retina (Fig. 4). This arrangement is also present in rat retina (data not shown). Thus, embryonic RGC axons (expressing DCC) run along the netrin-4-rich ILM until the RGC axons turn and exit into the optic nerve. This co-localization of netrin-4 with DCC is consistent with immunoprecipitation data demonstrating that netrin-4 directly binds to DCC in retina (data not shown).

### 3.3 Generation and verification of *Ntn4*<sup>-/-</sup> mice

To understand the function of netrin-4 in the mouse CNS, we generated *Ntn4*<sup>-/-</sup> mice. We confirmed that *Ntn4*<sup>-/-</sup> mice did not express netrin-4 protein using immunohistological methods (Fig. S1). Although netrin-4 is widely expressed in wild-type retina, no netrin-4 IR was detectable in *Ntn4*<sup>-/-</sup> retina at any stage in development. Therefore we conclude that there is no netrin-4 protein in *Ntn4*<sup>-/-</sup> mice. These antibodies against netrin-4 have been additionally characterized elsewhere (Koch et al., 2000; Schneiders et al., 2007).

### 3.4 Proliferation in cornea of *Ntn4*<sup>-/-</sup> mice

Netrin-4 is present in the cornea (Figs. 1, 2). Because netrins may play roles in cellular proliferation, we assayed whether proliferation was altered in the cornea of the netrin-4-null mouse. Cellular proliferation was assayed by Phospho-Histone H3 expression. In both whole-mount and radially sectioned cornea, low levels of proliferation are detectable (Fig. 5 A,B). In the cornea of *Ntn4*<sup>-/-</sup> mice, Phospho-Histone H3-expressing cells are considerably more numerous than in wild-type mice (compare Fig. 5C to A). The proliferation appears to occur in the apical levels of the epithelium (compare Fig. 5D to B). These data are quantified in Fig 5E.; the increase is statistically significant. This increase in proliferation in cornea in the absence of netrin-4 likely reflects a negative regulation of proliferation in cornea by netrin-4 during normal corneal development and maintenance. However, although there is increased proliferation in the cornea in the absence of netrin-4, there is no change in the thickness of the cornea, suggesting that an increase in cell death leads to an overall homeostasis in the thickness of the cornea.

### 3.5 RGC axon fasciculation and pathfinding in *Ntn4*<sup>-/-</sup> mice

Because RGC axons are in proximity to the netrin-4-rich ILM while running in fascicles toward the optic nerve head, we asked whether RGC axon pathfinding was affected by netrin-4 ablation. We used three parallel sets of experiments; we first labeled all RGCs non-selectively and in subsequent experiments we selectively labeled RGCs with either a transgenic marker or DiI.

We used an anti-neurotubulin antibody (TuJ1) to label all RGC axons (Fig. 6A). In the *Ntn4*<sup>+/+</sup> retina, RGC axon fascicles were formed in the periphery and ran centripetally to reach the optic nerve head following the nearest path. A similar pattern was seen in the *Ntn4*<sup>-/-</sup> retina; i.e., there was no gross misrouting of RGC axon fascicles in the *Ntn4*<sup>-/-</sup> retina. In addition, the diameter of the *Ntn4*<sup>-/-</sup> optic nerve is similar to that of the *Ntn4*<sup>+/+</sup> optic nerve (Fig. S2).

In the wild-type retina, some RGC axons switch from one fascicle to another, a process called selective fasciculation (Raper et al., 1983). Thus, in order to look more closely at the

events of fasciculation, we turned to a transgenic approach to allow us to selectively visualize individual axons. We crossed the *Ntn4*<sup>+/-</sup> mouse with a Thy1.1-*EYFP* transgenic mouse, in which ~10-30% RGCs express the *EYFP* transgene and, thereby, have endogenous fluorescence (Feng et al., 2000). The resultant *EYFP*<sup>+/Ntn4</sup><sup>+/+</sup> and *EYFP*<sup>+/Ntn4</sup><sup>-/-</sup> mouse retinas are compared in Figure 5B. In both genotypes, some of the EYFP (+) RGC axons fasciculate with one another. In *EYFP*<sup>+/Ntn4</sup><sup>+/+</sup> retinas, the EYFP (+) fascicles are largely stable: no or only minor defasciculation was present before the axons reach the optic nerve head. However, in *EYFP*<sup>+/Ntn4</sup><sup>-/-</sup> retina, EYFP (+) fascicles sometimes defasciculated to form 'fork-like' structures before reaching the optic nerve (Fig. 6B, arrows), suggesting the RGC axon fasciculation in the *EYFP*<sup>+/Ntn4</sup><sup>-/-</sup> retina may be less stable.

We also labeled adjacent quadrants of the retina using DiI. Consistent with results using other methods (Figs. 6A,B), RGC axon targeting to the head of the optic nerve assayed with DiI was not affected by *Ntn4* ablation. However, dense fascicles of axons were present in the wild-type retina, most containing multiple RGC axons; rarely did we observe a single axon in isolation (Fig. 6C); in contrast, the fascicles were smaller in the *Ntn4*<sup>-/-</sup> retina and appeared to be composed of fewer axons, with frequent single axons present (Fig. 6C).

### 3.6 Retinal structure in the *Ntn4*<sup>-/-</sup> mouse

We also investigated whether retinal cell types other than RGCs were affected in the *Ntn4*<sup>-/-</sup> retina (Fig. 7). We observed no gross changes in retinal structure or lamination in the *Ntn4*<sup>-/-</sup> retina. The *Ntn4*<sup>-/-</sup> retina was well laminated and photoreceptors were normal in morphology and expressed and localized rhodopsin normally. Bipolar cells, visualized with antibodies to PKC $\alpha$ , were unchanged either in density or stratification of the axonal terminals in the deepest layers of the inner plexiform layer (IPL). Subsets of amacrine and ganglion cells were revealed with calretinin immunoreactivity. In the wild type retina, calretinin-expressing cell bodies were present in both the inner nuclear layer (INL) and the ganglion cell layer (GCL); there was a tri-laminate expression of calretinin in the IPL. Calretinin immunoreactivity was unchanged in the *Ntn4*<sup>-/-</sup> retina.

We also examined the glial cells of the retina - Müller cells and astrocytes. Müller cells are radial glial elements that span the entire thickness of the neural retina; their basal endfeet lie in close contact with the ILM, where netrin-4 is richly deposited. Müller cells were visualized by examining the presence of two different Müller cell proteins, glutamine synthetase (GS, Fig. 7A) and vimentin (Fig. 7B).

The orientation, density and organization of Müller cells were not altered in the netrin-4 null retina. The apical ends of the Müller cell (photoreceptor side) form tight junctions with each other and with photoreceptors, forming the external limiting membrane (ELM). The ELM was not disrupted in the netrin-4 null retina. The basal ends of the Müller cells sit on the perlecan-expressing ILM (Fig. 7B). In the netrin-4 null retina, perlecan expression was not affected and the Müller cell endfeet were well organized and properly oriented (Fig. 7B).

Astrocytes invade the neural retina via the optic nerve and their entry precedes and parallels vasculogenesis in the retina (Gariano, 2003). Astrocytes are the only cells that express GFAP in the normal murine retina; therefore, we used this marker for them to determine whether the number of astrocytes was altered in the netrin-4 null retina. In radial sections, GFAP-expressing cells formed a sparse layer of cells over the nerve fiber layer of the retina, and there was no difference between wild type and netrin-4 null retinæ (data not shown). In retina whole-mount preparations, astrocytes invested the vascular supply of the retina and we observed no apparent difference in the number and the branching structure of astrocytes in all regions of the netrin-4 null retina (Fig. 8).



Because astrocytes guide retinal vascularization (Stone et al., 1995) and netrins including netrin-4 regulate endothelial migration and proliferation in vitro (Larrivee et al., 2007; Lejmi et al., 2008), we analyzed potential changes in retinal vascularization in the netrin-4 null retina. We labeled the vascular tree with anti-PECAM-1 (CD31), and examined detailed branching structure of microvessels at the superficial (vitreal) surface, in the middle of the IPL, and deep in the retina at the level of the OPL in the adult retina (approximately 1 month old) (Fig. 9). While branching patterns appear largely normal at the surface of the retina, there appears to be some increased branching, particularly in the middle levels of the microvessels (compare Fig. 9C, G) and somewhat less in the deep levels of the microvessels (compare Fig. 9D, H). A more extensive examination of the potentially increased branching awaits further experiments.

## 4. Discussion

### 4.1 Conclusions and Summary of Principle Findings

We, and others, have suggested roles for netrin-4 by localization in vivo (Liu et al., 2004), by analyzing effects of netrin-4 on cells in vitro (Lejmi et al., 2008; Park et al., 2004), and by exogenous netrin-4 in vivo (Larrieu-Lahargue et al., 2010). However, this study is the first direct demonstration of a role for netrin-4 in vivo. Although data reported by others have suggested roles for netrin-4 by localization and exogenous application of netrin-4, our results are the first to demonstrate a role for netrin-4 in cellular proliferation and morphogenesis in vivo. Additional data on the role for netrin-4 in regulation of proliferation in the cerebellum are being prepared for publication (Li and Brunken, unpublished data).

Netrin-4 is expressed in all BMs of ocular tissue. This is particularly clear in early development, where netrin-4 is present as a continuous band around the eyecup and surrounding the lens placode. As development proceeds, netrin-4 remains as a prominent component of the BM underlying both ectoderm-derived neural tissues (the iris epithelium, the ciliary epithelium and the retinal pigmented epithelium; i.e., Bruch's membrane) and the neural retina (i.e., the ILM). Netrin-4 is also expressed in the vascular BM where it appears to be tightly associated with smooth muscle or pericytes.

### 4.2 Netrin-4 Is Not Required for BM Integrity

Structural integrity of the ILM is important for retinal histogenesis (Halfter et al., 2001). Components of the ILM include laminins, nidogens, agrin and collagens (Halfter et al., 2000). With this report, we add netrin-4 to these components.

The ILM may be a stable attachment site for both ganglion cells and Müller cell endfeet (Halfter et al., 2001). Simultaneous deletion of the laminin  $\beta 2$  and  $\gamma 3$  genes disrupts Müller cell adhesion to the ILM and causes ganglion cell ectopia and other more profound defects in the retina (Pinzón-Duarte et al., 2010). Other laminin mutations or deletions also produce profound disruptions in the organization of the BM, similar to those produced by collagen IV mutations (Poschl et al., 2004). However, deletion of netrin-4 in the *Ntn4*<sup>-/-</sup> mouse did not disrupt the BMs of the retina, cornea or lens, suggesting that netrin-4 is not a structural element of ocular BMs, but netrin-4 might function in other ways, regulating proliferation or migration.

### 4.3 Netrin-4 and retinal vasculogenesis

Netrin-4 is richly deposited in vascular BMs. Vascular endothelial cells are likely the cell source of netrin-4, as vascular endothelial cells have been reported as a source of netrin-4 (by in situ hybridization, Koch et al., 2000; and microarray, Ho et al., 2003). However, the spatial distribution of netrin-4 protein is similar to the distribution of smooth muscle cells in

arteries and veins, and pericytes in microvessels. Netrin-4 has been reported to be anti-adhesive to endothelial cells (Wilson et al., 2006). Given the uneven distribution of netrin-4 in vascular BMs, netrin-4 may contribute to the arterial-venous axis; however, we detected no disruption in this axis in the null mouse.

Two other observations concerning netrin-4 and retinal angiogenesis are worthy of further comment. First, netrin-4 expression is temporally linked to the maturation of the vascular system at the vitreal border. Angiogenesis is directed by astrocyte migration over the retinal surface. Netrin-4 is expressed over the ILM, and therefore is placed to contribute to astrocyte guidance. However, astrocyte numbers and distribution are not altered in the *Ntn4*<sup>-/-</sup> mouse, suggesting that netrin-4 is not involved in astrocyte migration or proliferation, and any modulation of vascular development by netrin-4 is not via a direct action on astrocyte migration or proliferation. In addition, superficial angiogenesis proceeds normally in netrin-4 null mice, suggesting that endothelial-astrocyte interactions are not altered in the absence of netrin-4.

Second, the branching of deeper retinal microvessels may be increased in the absence of netrin-4, suggesting that the patterning of superficial and deeper capillary plexus may be genetically and mechanistically separable. The sprouting and plexus formation of capillary beds in the inner and outer retina are driven by Müller cell interactions, certainly by growth factors and, possibly, by cell-cell interactions. Our *in vivo* data demonstrate that both middle and deep plexuses may be altered by the *Ntn4* deletion, though subtly. Because netrin-4 inhibits endothelial cell proliferation *in vitro* (Lejmi et al., 2008), the possible increase in branching in the *Ntn4*<sup>-/-</sup> mouse may be due to a direct effect of the loss of netrin-4.

Mutations of several ECM genes, including collagens XV and XVIII (from which endostatin is derived), produce persistent hyaloid vessels (Hurskainen et al., 2005). We did not observe persistent hyaloid vessels in netrin-4 null mice. Local production of netrin-4 has been reported to promote proangiogenic remodeling (Wilson et al., 2006). In this regard, it will be of interest to determine whether remodeling events in retinal vasculature, such as hypoxia-driven remodeling, are altered in the *Ntn4* null mouse. In addition, neovascularization at the vitreal border leads to the blinding disorder, proliferative vitreoretinopathy (PVR), many ECM molecules are up-regulated in PVR (Hiscott, et al., 1999; Hiscott et al., 2002), and matrix metalloproteinases are implicated in complications of diabetic retinopathy (Yang et al., 2007). In this regard, the netrin-4 null mouse will be an important tool for studying the role of netrins in neovascularization.

#### 4.4 Netrin-4 in the Cornea and Epithelial Proliferation

One striking spatial and temporal pattern of netrin-4 expression is its corneal expression. Netrin-4 is expressed first in the corneal epithelial BM (Bowman's) and later in the stroma and endothelial basement membrane (Descemet's membrane). There is an abrupt transition from high level of expression and deposition in these regions to completely negative expression in the adjacent sclera, with the limbus acting as the boundary. The cornea is avascular, transparent and richly innervated. However, we discerned no primary defect in corneal organization in the netrin-4 null mice, suggesting that netrin-4 is not required for epithelial differentiation or guidance of the neural crest cells into the corneal endothelium or stroma. However, the finding that corneal epithelium proliferation was considerably increased in these mice suggests that netrin-4 may be involved in the regulation of epithelial turnover and renewal. Thus, it will be of interest to investigate whether corneal wound healing is altered in netrin-4-null animals.

#### 4.5 Netrin-4 and RGC axon guidance and fasciculation

In the developing mouse retina, RGCs differentiate between E11 and P3 (Young, 1985). RGC axons follow complex paths to several targets in the brain, each of which serves a distinct visual function including perception and reflex coordination. Along the path, RGC axons traverse many extracellular cues and guidance molecules (Stuermer and Bastmeyer et al., 2000). The first, and perhaps most critical, guidance event occurs within the eye, where axons are directed down the optic fissure into the developing optic nerve. Within the eye, RGC axons emerge from the cell soma, then grow toward the vitreal border and the ILM, where they make a sharp perpendicular turn to run parallel to the retinal surface in a narrow zone beneath the ILM and Müller cell endfeet. They are guided along the retinal surface towards the optic fissure and the later optic nerve head, where they turn again to enter the optic nerve.

Many molecular components have been suggested to be involved in RGC axon guidance to the optic nerve head, such as chondroitin sulfate (CS) (Brittis et al., 1992), slit 1 (Jin et al., 2003), Eph/Ephrins (Birgbauer et al., 2000), and netrin-1/DCC (Deiner et al., 1997). In the *Ntn4*<sup>-/-</sup> mouse retina, we detected no ectopic axons, suggesting either that netrin-4 is not a critical molecule for RGC axon guidance or there is redundancy in the netrin family such that another netrin, such as netrin-1, might have substituted for netrin-4. The critical test for redundancy would be to substitute *Ntn4* in the *Ntn1* locus and the reverse thereby dissecting the individual functions of these family members.

#### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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### Highlights

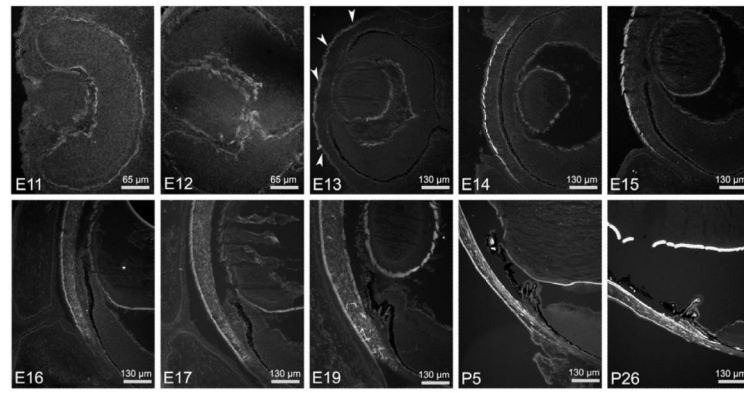
Netrin-4 is expressed in corneal stroma and basement membranes

Netrin-4 knockout has increased corneal epithelial proliferation

Netrin-4 is expressed in retinal vascular basement membrane

Netrin-4 knockout has increased deep vascular branching

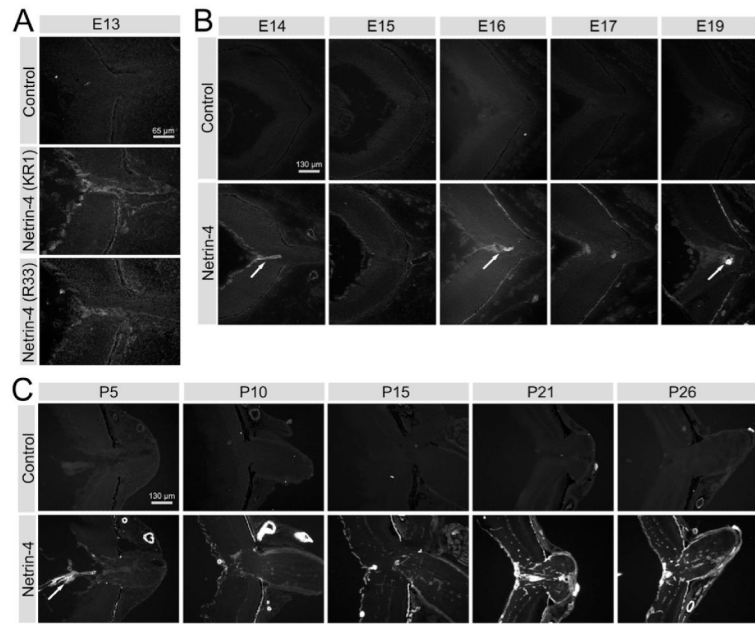
Netrin-4 knockout retina is normal



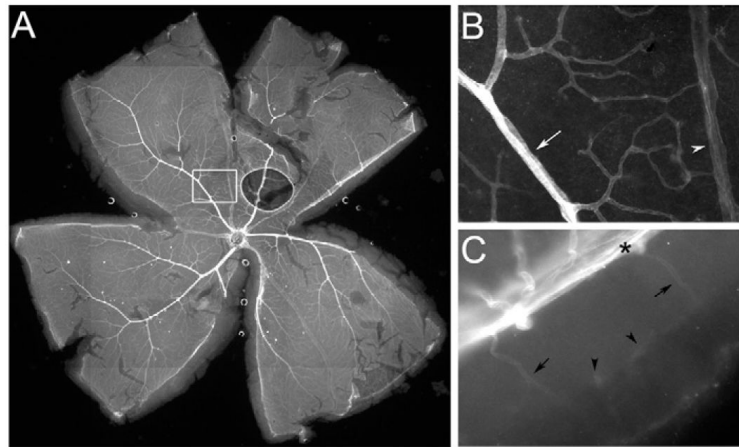
**Figure 1.**

Developmental expression of netrin-4 in cornea and lens capsule. Netrin-4 is detected in cornea epithelium from E13 (arrowheads) and persists into adulthood. Starting at E16, netrin-4 is deposited in the corneal stroma, especially at the peripheral cornea, resulting in a clear boundary between the cornea and the sclera. Netrin-4 IR in Decemet's membrane, the discontinuous corneal endothelium basement membrane, was first detected at E17. The netrin-4 IR in Decemet's membrane persists to adulthood. Netrin-4 is also detected in lens capsule as early as E11; the netrin-4 IR is stronger in the posterior lens capsule than that in the anterior capsule.

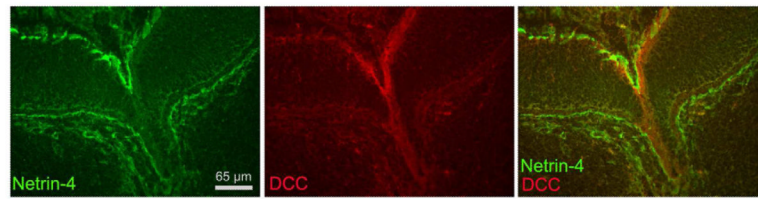




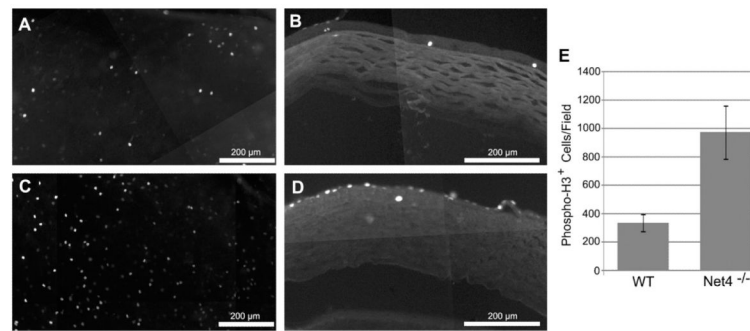
**Figure 2.** Developmental expression of netrin-4 in mouse retina and optic nerve. (A) Cross sections of E13 mouse eye through optic nerve head. Netrin-4 IR is present at the ILM, Bruch's membrane and the BM along the optic fissure associated with the hyaloid vessels. In cross sections of E14 to E19 (B) and P5 to P26 (C) retinas, in addition to netrin-4 IR in the ILM and Bruch's membrane, netrin-4 IR is present in hyaloid vessels (arrows) and, at later times, on retinal vasculature BMs. Netrin-4 IR is also present postnatally in the epineurium of the optic nerve.



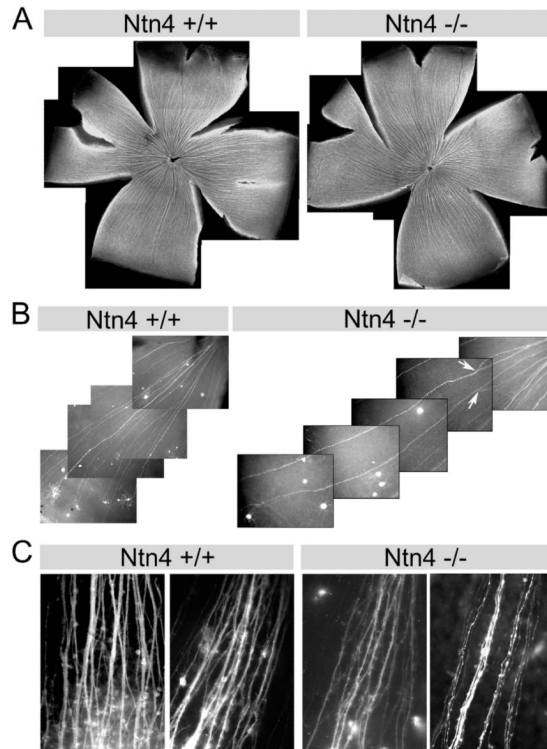
**Figure 3.** Netrin-4 IR in the retinal vasculature. (A) Netrin-4 IR is stronger in arteries than that in veins. (B) Higher magnification of the boxed area in (A), demonstrating stronger netrin-4 IR in an artery (arrow) than a vein (arrowhead). (C) From the cut edge of a retina whole-mount, netrin-4 IR can be detected at the ILM (\*) and in vascular branches (arrows) and capillaries (arrowheads).



**Figure 4.** Spatial relationship of netrin-4 IR and DCC IR at the E13 optic nerve head. Netrin-4 IR (left) is concentrated in the ILM and DCC IR (center) is concentrated in RGC axons. When superimposed (right), DCC-expressing RGC axons can be discerned running under the netrin-4 rich ILM at the embryonic optic nerve head.

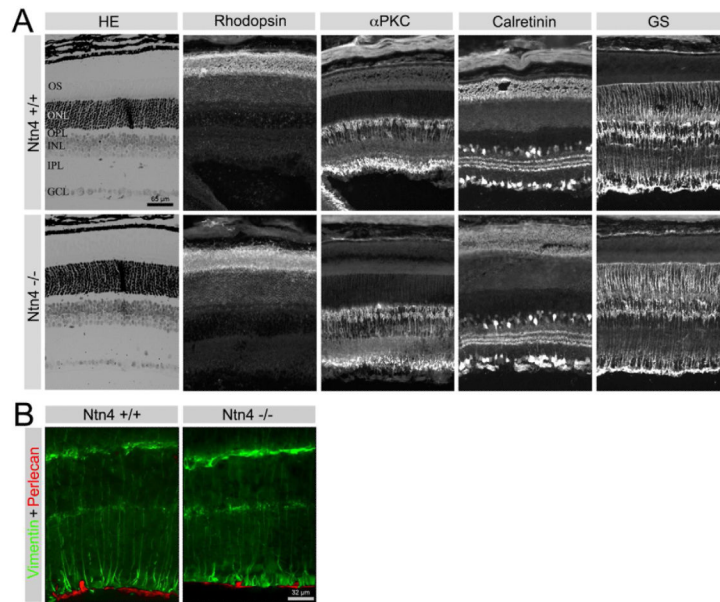


**Figure 5.** Phospho-Histone H3-expressing cells in the cornea of *Ntn4*<sup>+/+</sup> and *Ntn4*<sup>-/-</sup> mice. (A, B) *Ntn4*<sup>+/+</sup> cornea; (C, D) *Ntn4*<sup>-/-</sup> cornea. (A, C) cornea whole-mounts; (B, D) cross-sections of the cornea. (E) Cells expressing Phospho-Histone H3 were captured using Velocity and Phospho-Histone H3-expressing cell numbers were averaged. The number of cells expressing Phospho-Histone H3 is increased in the absence of netrin-4. Mean ± SEM for P33-34 animals (n = 4).

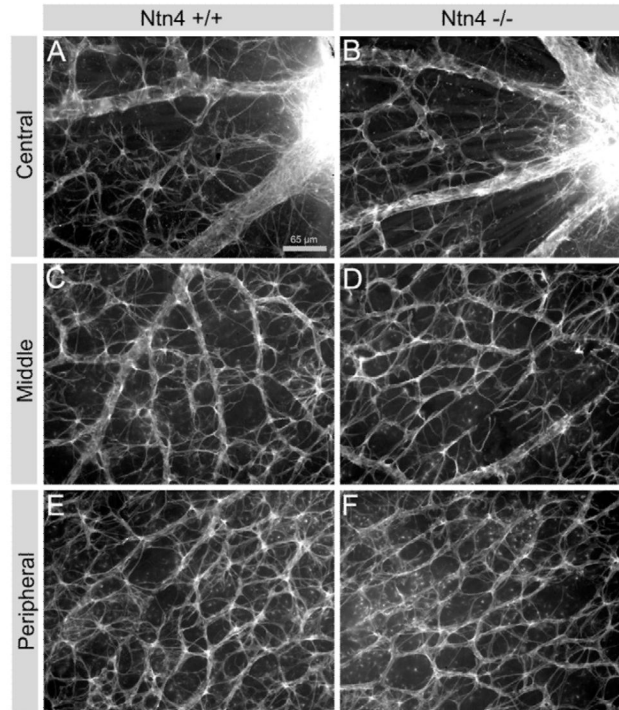


**Figure 6.**

Axon pathfinding and fasciculation in the *Ntn4* $-/-$  retina. (A) RGC axon fascicles, revealed with anti-neurotubulin, reach the optic nerve head. No ectopic axon fascicles were observed in the *Ntn4* $-/-$  retina. (B) In retinæ expressing EYFP in a subset of RGCs, some of the EYFP (+) RGCs fasciculate with each other in *EYFP* $+/Ntn4$  $+/+$  as well as in *EYFP* $+/Ntn4$  $-/-$  retinas. However, the fascicles appear to be less stable in the *EYFP* $+/Ntn4$  $-/-$  retina, occasionally defasciculating to form 'fork-like' structures (*Ntn4* $-/-$ , arrows). (C) DiI labeling of *Ntn4* $+/+$  and *Ntn4* $-/-$  retinas. The fascicles in the *Ntn4* $-/-$  retina appear narrower, and more individual axons are present.

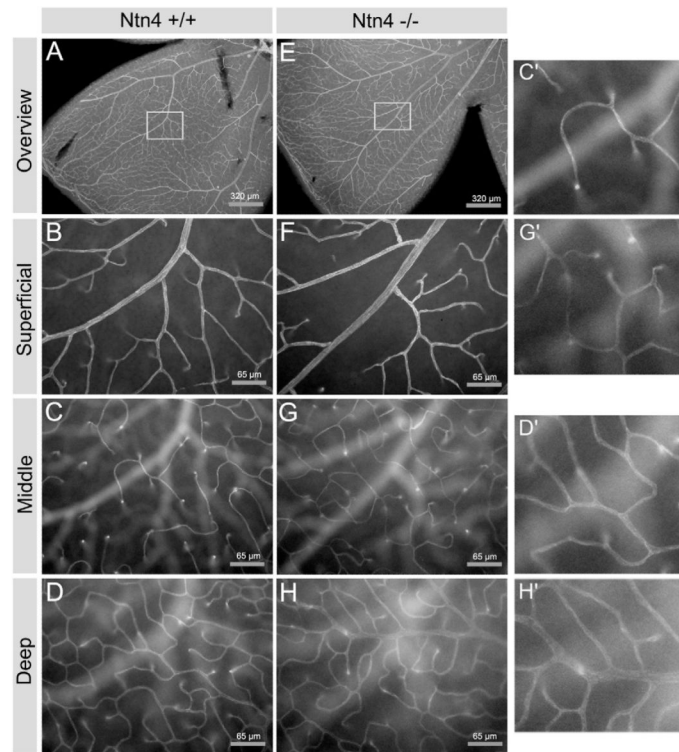


**Figure 7.** Deletion of netrin 4 leads to no obvious change of cell organization in the retina. (A) In the *Ntn4*<sup>-/-</sup> retina, there are no lamination defects or ectopic ganglion cells (HE); there are no obvious morphological changes in photoreceptors (Rhodopsin), bipolar cells ( $\alpha$ PKC), amacrine cells (Calretinin) and Müller cells (GS). (B) The relationship between Müller cell endfeet (visualized with anti-vimentin, green) and the ILM (visualized with anti-perlecan, red) is not disturbed in the *Ntn4*<sup>-/-</sup> retina.



**Figure 8.**

Deletion of netrin 4 leads to no change in astrocyte morphology and density in the retina. Astrocytes were visualized with GFAP immunoreactivity. (A, C, and E), *Ntn4*<sup>+/+</sup>; (B, D, and F), *Ntn4*<sup>-/-</sup>. (A, B) near the optic nerve; (C, D) near the fundus; (E, F) at the peripheral retina.



**Figure 9.**

Comparison of vascular branching in the *Ntn4*<sup>+/+</sup> and *Ntn4*<sup>-/-</sup> retina. Retinal vasculature was visualized using an antibody to an endothelial marker, PECAM-1. (A, B, C, and D), *Ntn4*<sup>+/+</sup>; (E, F, G, and H), *Ntn4*<sup>-/-</sup>. (A, E) low power view of a retina quadrant; (B, F) higher magnification of the boxed area from the fundus with the focus at the vitreal surface; (C, G) the same area and magnification as (A, E), with the focus at INL-IPL border; (D, H) the same area and magnification as (A, E), with the focus at the OPL. In the absence of netrin-4, there is some increased branching in the middle and deep levels of the microvessels. Portions of Figs. 9 C, D, G and H are shown at higher magnification in C', D', G' and H'.