Lack of netrin-4 alters vascular remodeling in the retina

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Abstract

Purpose Netrin-4 (NTN4) is a protein that plays an important role in the regulation of angiogenesis in the pathological retina. Some evidences show that it can also have a role in inflammation and vascular stability. We will explore these questions in vivo in the mature mouse retina.

Methods We created a NTN4 knockout that expresses EGFP in mononuclear phagocytes (CSFR1-positive cells) to track inflammation in vivo in the retina by scanning laser ophthalmoscopy (SLO). Fundus angiography permitted to study blood vessels. Retinal function was assessed with electroretinography (ERG).

Results Lack of NTN4 leads to an increased amount of amoeboid mononuclear phagocytes in the adult retina, and blood vessels displayed increased tortuosity when compared with the wildtype. Inner retina function also seemed affected in NTN4 null. Lack of NTN4 resulted in a higher persistence of hyaloid artery and spontaneous leakage in the adult retina. No differences were found regarding vessel bifurcation, vessel width, or vein/artery ratio.

Conclusions These in vivo data show for the first time that lack of NTN4 induces changes in the retinal vascular phenotype in a non-pathological scenario. This evidence widens the role of NTN4 as a guidance cue in vascular remodeling.

Keywords Netrin-4 · Vascular remodeling · Basal membrane · Hyaloid vessel · Mononuclear phagocyte · In vivo imaging

Introduction

Netrins represent a family of secreted extracellular matrix molecules that were originally described for their role in neuron migration and axon guidance, but they are also involved in organogenesis, tumorigenesis, angiogenesis, and inflammation.

The first identified and most explored netrin member is Netrin-1 (NTN1), which was found crucial in angiogenesis having a dual pro- and anti-angiogenic effect controlling endothelial cell migration and proliferation [1]. It wields these diverse functions through different receptors such as deleted in colorectal carcinoma (DCC), neogenin, or Unc5B. Despite the existent homology with NTN1, netrin-4 (NTN4) does not bind directly to any of these receptors although there is evidence of interaction with them [2–4].

Complete deletion of NTN1 is lethal during development [5]. However, in a NTN4-null mouse, retinal development was not altered [6], and the lack of NTN4 manifests pathological features only under distress conditions such as hypoxia or laser-induced choroidal neovascularization [7].

NTN1 was found to exert anti-inflammatory effects in different disease settings such as experimental autoimmune encephalomyelitis [8], multiple sclerosis [9], obesity [10], colitis [11], lung injury [12], peritonitis [13], acute kidney injury [14], or diabetes [15]. Furthermore, netrin-1 has been proved to inhibit leukocyte migration [16–18]. On the other hand, NTN4 has not been studied in a context of inflammation in the retina and only one study proved anti-inflammatory effects in a corneal wound healing model [19].

Contrarily to NTN1, NTN4 is present in the vascular basal membrane (BM). Reuten and colleagues [4] showed that
NTN4 disrupts laminin networks and basement membranes (BMs) through high-affinity binding to the laminin γ1 chain. They propose that this laminin-related function is essential for its known effects on axon growth promotion and angiogenesis. NTN4, however, has been reported to influence angiogenesis in a context of disease rather than in development [3, 7].

In accordance with these findings, we hypothesize that lack of NTN4 in the retina contributes to higher instability of blood vessels, together with alterations in leukocyte activity and inflammation. To study this phenomenon, we explored the retina in vivo due to its advantage to visualize non-invasively vasculature and myeloid cells. To do so, we used a genetically modified mouse model to track myeloid cells based on autofluorescence detection. The vascular phenotype was studied by means of fundus angiography (FAG) and analyzed using multiple algorithms. Retinal function was assessed by electroretinography (ERG).

**Methods**

**Animal model**

MacGreen (B6N.Cg-Tg(Csf1r-EGFP)1Hume/J, in this paper referred to as NTN4WT) mice [20] allow the evaluation of myeloid cells in the retina in vivo and have been proved an effective inflammation assessment tool [21, 22]. Ntn4−/− (C57Bl/6-129SvJ-Netrin4tm) mice [6, 7] were crossed with MacGreen mice (NTN4WT). Offspring littermates were crossed for three generations to generate a stable mouse model lacking NTN4 and expressing EGFP in the myeloid lineage (MacGreen.Ntn4−/−, from this point referred to as NTN4KO). Animals had free access to water and food and were kept in 12–12 h light-darkness cycles. All animal experiments adhered to the ARRIVAL guidelines, to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local ethical authority.

**Experimental design and statistical analysis**

Ten-week-old males (N = 25 NTN4WT and N = 28 NTN4KO) were subjected to retinal evaluation in vivo. All experiments were performed at least three times, and results are expressed as mean ± SEM. Statistical analysis was done using Student’s t test, and values were considered statistically significant when p < 0.05.

**Fundus angiography**

Scanning laser ophthalmoscopy (SLO) was performed using a Heidelberg Spectralis HRA-OCT (Heidelberg Engineering, Heidelberg, Germany) as described in Crespo-Garcia et al. [21]. FAG was employed to screen the retinal vasculature in vivo in both NTN4KO and WT controls. Vessels were imaged with FAG after subcutaneous injection of fluorescein (Alcon, Berlin, Germany) using the SLO. All images were processed using the software Heidelberg Eye Explorer (Heidelberg Engineering).

**Blood vessel segmentation and analysis**

Vessel segmentation and analysis were performed using MATLAB (MathWorks Inc., Natick, MA, USA). First, a template matching procedure was used to detect the optic disc in each image. The main arteries and veins were then separated from the background (mesh vessel network) through median filtering and morphological operations. The detected optic disc region was then removed from the image, leaving just the detected vessel segments. These segments were then skeletonized, and each segment was manually marked as either an artery or vein.

From the vessel segments, width measurements were taken normal to the vessel centerlines and averaged across the length of the vessel. All artery and vein segments were then averaged to give an artery/vein ratio. Tortuosity was measured following the method developed by Grisan et al. [23] and averaged across all arteries and veins in an image.

**Retina flat mount**

Eyes were fixated in 4% PFA for 15 min, and retina was carefully dissected. Tissue was incubated with *Griffonia simplicifolia* isoelectin B4 (IB4; Invitrogen, Cat# l21411; 1:200 in TBS) to label the blood vessels. Flat mounts were mounted onto glass slides and imaged using a Zeiss Axio Imager microscope with Apotome (Zeiss, Jena, Germany). Images were processed using Zen Lite 2010 software (Zeiss).

**Fundus autofluorescence**

Auto-fluorescence mode in the ophthalmoscope (AF, 488 nm) permitted the visualization in vivo of CSFR1-positive cells in the retina via EGFP signal. Mononuclear phagocyte (MP; CSFR1-positive cells) quantification was performed manually in a masked fashion as described in Crespo-Garcia et al. [22]. For doing so, we considered (1) round-like CSFR1-positive described as amoeboid MPs and (2) CSFR1-positive cells sitting on blood vessels and described as perivascular MPs.

**ERG**

Scotopic Ganzfeld ERG was performed in dark-adapted animals in a Ganzfeld bowl (Roland Consult, Brandenburg, Germany) according to previously published methods [24].
Results

**NTN4KO has increased retinal vascular tortuosity and hyaloid artery persistence**

Vein/artery ratio and fractal dimension (vessel bifurcation degree; data not shown), as well as mean vessel width, were unaltered (Fig. 1a–b), although the knockout showed a significant increase in vessel tortuosity compared with wild-type controls (Fig. 1c–d). Unexpectedly, FAG also revealed a high persistence of hyaloid artery in adult NTN4KO (76.9 ± 12.2% of the screened animals) when compared with NTN4WT (Fig. 1e–d, g). This finding was verified ex vivo (Fig. 1f). In 46.2 ± 14.4% of the NTN4KO animals (N = 13) screened, focal areas of leakage were detected (Fig. 1e).

![Fig. 1 Vascular phenotype in the retina.](image)

**Fig. 1** Vascular phenotype in the retina. a Representative FAG images (inverted colors) show in red the segmentation for vessel width detection. b Bar chart represents the mean vessel width artery-vein (A/V) ratio (A.U.). c Representative FAG images show in a color scale the segmentation for vessel tortuosity degree (red = high, yellow = intermediate, and green = mild). d Bar chart represents mean vessel tortuosity artery-vein (A/V) ratio (A.U.). e Representative FAG sequence of the different vascular plexus in the retina and vitreous. Hyaloid artery (red arrowhead) is observed in the NTN4KO adult eye. f Representative image of a retinal flat mount. Vessels are stained with IB4 (yellow). Hyaloid vessels are indicated with red arrows. Scale bar represents 500 μm. g Bar chart represents the persistence of the presence of hyaloid vessels in the adult retina in the presence and absence of NTN4. h Representative FAG image showing vascular leakage (red arrowheads) in NTN4KO retina. For all panels, numbers at the base of the bars represent N.
Fig. 2. Mononuclear phagocytes in the retina and retinal function. 

a Representative AF fundus image of the retina in a NTN4WT mouse. CSFR1 is detected by means of EGFP signal in the transgenic animals. In the micro-capture, perivascular MPs are indicated with blue arrowheads.

b Representative AF fundus images of the retina in a NTN4KO mouse. CSFR1 is detected by means of EGFP signal in the transgenic animals. In the micro-capture, amoeboid (red arrowheads) and perivascular (blue arrowheads) MPs are indicated.

c Bar chart represents the quantification of amoeboid MPs (CSFR1-positive cells/field) in the retina. ***p < 0.001.

d Bar chart represents the quantification of perivascular MPs (CSFR1-positive cells/field) in the retina.

e Bar chart represents the statistical analysis for scotopic a- and b-wave in the retina. *p < 0.05.

f Mean response curve to scotopic single flashes in the retina. For all panels, numbers at the base of the bars represent N.
**NTN4KO has increased amount of amoeboid mononuclear phagocytes in the retina**

Lack of NTN4 increases significantly the number of amoeboid MPs in the retina of healthy adult animals (Fig. 2a–c). Regarding perivascular MPs, no significant differences were observed (Fig. 2a–b, d). Visualization of MPs in the hyaloid vasculature was not possible due to image resolution and focus.

**Discussion**

We reported for the first time that lack of NTN4 results in alterations in the mature retina. In vivo data show that retinal function is altered in the NTN4KO in the inner retina. Amoeboid mononuclear phagocytes are more abundant in the NTN4KO retina. Furthermore, we have multiple evidences showing the role of NTN4 in vascular remodeling such as persistence of the hyaloid artery in adult mice, an increased retinal blood vessel tortuosity, and spontaneous focal retinal leakage. Previous studies have always showed that the absence of NTN4 had only deleterious effects under pathological conditions in the eye [3, 7, 25], but this is the first study with an in vivo approach in adult, otherwise normal animals.

Not much is known about NTN4 in the eye under physiological development. Li and colleagues previously characterized ex vivo the distribution of NTN4 expression in the developing eye structures, and NTN4 was found predominantly in the majority of BMs in the retina and in the Bruch’s membrane. Lack of Ntm4 does not seem to affect the BM integrity, as it has been shown in the null mouse previously [6, 7]. During development, NTN4 is associated with the hyaloid vessels from E13 to E19, and, interestingly, with the posterior capsule of the lens. This suggests that it can play a role in angiogenesis via grading through guidance cues as its expression corresponds with the tunica vasculosa lentis. Contrary to our data, Li and colleagues never found persistence of the hyaloid vessels; data concerning this issue, however, are not shown in the paper [6]. Despite little is known about hyaloid vasculature regression mechanisms, literature seems to agree that they are mediated by apoptosis triggered by macrophages [26–29]. Since macrophages contribute actively in BM remodeling, our data suggests that the presence of NTN4 could be critical in modulating macrophage physiological activity.

The mechanism of action of NTN4 and NTN1 regarding immune cells might, however, differ since they have different receptors. In the absence of NTN4, MPs in the retina show a distinct phenotype that could be correlated with the BM composition and NTN4 signaling. Retinal vasculature also seemed to have a higher perivascular MP coverage, but still numbers were not significant.

Reuten and colleagues [4] showed that NTN4 has a high binding affinity for the matrix protein laminin \(\gamma\)I and, when overexpressed, leads to BM disruption by interfering with the laminin network. This non-enzymatic mechanism of action helps to understand our findings related to vascular remodeling and, perhaps, the disturbance of hyaloid vessel regression in the adult eye. Notch signaling is a key regulator in angiogenesis during the tip and stalk endothelial cell interplay [30]. There exist evidences that NTN4 participates from Notch signaling in endothelial cells [31]. In vivo, deletion of Notch was associated with microphthalmia and hyaloid vasculature defects [32]. Altogether, these evidences support a possible mechanism of interaction by which NTN4 can modulate BM and consequently vascular development.

Li and colleagues characterized the microvasculature in the NTN4 null having subtle but important vascular differences in terms of vessel architecture [6]. Our in vivo data could not support this evidence due to lack of resolution, but we could track focal edema in several cases. The decrease in the a-wave in the knockout could be justified by a compromise in rod differentiation since NTN4 is highly expressed during embryonic development. As other netrins, NTN4 could contribute to neuron maturation providing a proper environment [33, 34]. All and all, we presented the first evidence in the literature showing that the lack of NTN4 can lead to loss of visual function affecting the inner retina, e.g., affecting rods.

Further studies are necessary to unravel the mechanism of how NTN4 influences vascular remodeling. Our in vivo data, however, highlight the importance of widening or redefining the role of NTN4 as a crucial BM protein both in physiological and pathological conditions.

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**Compliance with ethical standards**

**Conflict of interest** AMJ has received honorarium for advisory services from Bayer, Novartis, and Merck. SCG, NR, JW, SS, NK, and OS declare no conflict of interest.

**Ethical approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Local authorities (Landesamt für Gesundheit und Soziales) approved this study under the protocols G0081-15 and G0216-15.
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