Abstract
Retinal hypoxia triggers abnormal vessel growth and microvascular hyper-permeability in ischemic retinopathies. Whereas vascular endothelial growth factor A (VEGF-A) inhibitors significantly hinder disease progression, their benefits to retinal neurons remain poorly understood. Similar to humans, oxygen-induced retinopathy (OIR) mice exhibit severe retinal microvascular malformations and profound neuronal dysfunction. OIR mice are thus a phenocopy of human retinopathy of prematurity, and a proxy for investigating advanced stages of proliferative diabetic retinopathy. Hence, the OIR model offers an excellent platform for assessing morpho-functional responses of the ischemic retina to anti-angiogenic therapies. Using this model, we investigated the retinal responses to VEGF-Trap (Afiblercept), an anti-angiogenic agent recognizing ligands of VEGF receptors 1 and 2 that possesses regulatory approval for the treatment of neovascular age-related macular degeneration, macular edema secondary to retinal vein occlusion and diabetic macular edema. Our results indicate that Afiblercept not only reduces the severity of retinal microvascular aberrations but also significantly improves neuroretinal function. Afiblercept administration significantly enhanced light-responsiveness, as revealed by electroretinographic examinations, and led to increased numbers of dopaminergic amacrine cells. Additionally, retinal transcriptional profiling revealed the concerted regulation of both angiogenic and neuronal targets, including GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; INL, inner nuclear layer; IPL, inner plexiform layer; NG2, neural/glial antigen 2 proteoglycan; NGS, normal goat serum; OIR+AFL, afiblercept-treated OIR mice; OIR, oxygen-induced retinopathy; ONL, outer nuclear layer; OPL, outer plexiform layer; P, post-natal day; RRID, research resource identifier (see scicrunch.org); RT-qPCR, reverse transcription-quantitative polymerase chain reaction; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.
The identification of the vascular endothelial growth factor A (VEGF-A) as a key player in hypoxia-induced pathological angiogenesis (Shweiki et al. 1992; Aiello 2005; Watkins et al. 2013) boosted the development of anti-VEGF-A therapies against retinal neovascularization (Penn et al. 2008; Kim and D’Amore 2012; Caplan and Kesselheim 2016; Miller 2016; Bolinger and Antonetti 2016; Whitehead et al. 2018). Anti-VEGF-A agents reduce vascular leakiness, inhibit neovascularization, and improve visual acuity. Yet, whether the visual benefits of anti-VEGF-A therapy are because of reduced vasopermeability or linked to direct neuroretinal effects is not completely understood. Furthermore, the efficacy of VEGF-A inhibitors in the context of neovascular retinopathies with complex functional deficits such as retinopathy of prematurity (Fulton et al. 2009; Hansen et al. 2017) and proliferative diabetic retinopathy (Han et al. 2004; Antonetti et al. 2006; Cheung et al. 2010; Gardner et al. 2011; Coorey et al. 2012; Aung et al. 2013; Zhang et al. 2013; Pardue et al. 2014; Altmann and Schmidt 2018) remains unclear. Besides extensive vascular abnormalities, both retinopathy of prematurity and proliferative diabetic retinopathy patients exhibit deficits in elementary visual submodalities and reduced electroretinographic responses, which are linked to inner nuclear layer (INL) disturbances and interneuron damage (Akula et al. 2007; Jackson et al. 2012a; Jackson et al. 2012b; Ramsey and Arden 2015). More specifically, dopaminergic deficiency appears causal to visual performance decline both in animal models of hypoxic retinopathies and in early diabetic retinopathy patients (Zhang et al. 2013; Aung et al. 2014; Spix et al. 2016; Kim et al. 2018). Indeed, dopaminergic amacrine cells (DACs) within the INL are highly hypoxia-sensitive (Roufail et al. 1999). These cells are central to the balance between retinal metabolic demand and oxygen consumption (Yu and Cringle 2002; Abbouwer and Gardner 2014; Usui et al. 2016; Usui et al. 2015a). Moreover, INL-resident amacrine cells sense oxygen and fine-tune local VEGF-A levels in response. Consequently, their dysfunction not only results in impaired vision but also induces drastic morphological changes in intraretinal vascular beds (Usui et al. 2016).

The oxygen-induced retinopathy (OIR) mouse model is the most widely used animal model to study ischemic neovascular retinopathies including retinopathy of prematurity and late neovascular proliferative diabetic retinopathy (Smith et al. 1994; Stahl et al. 2009). As in human ischemic retinopathy patients, retinal function is markedly impaired in OIR mice (Stahl et al. 2009; Dorfman et al. 2010; Vessey et al. 2011; Ridano et al. 2017; Matsuda et al. 2017). Moreover these mice also exhibit reduced numbers of dopaminergic amacrine cells (Spix et al. 2016) and delayed intermediate vascular plexus formation (Ritter et al. 2006; Spix et al. 2016). So far, the efficacy of anti-angiogenic therapies in attenuating hypoxic damage to the neuroretina has been reported in relation to improved electroretinographic responses. Yet, the effects of anti-VEGF therapy on DACs remain poorly documented.

The multifactoriality of ocular vasculopathies and adaptive resistance because of compensatory up-regulation of factors other than VEGF-A might represent a significant hurdle for VEGF-A inhibitors that ultimately renders tissues non-responsive to these agents. Such phenomenon is well-described in tumor biology (Croci and Rabinovich 2014; Li et al. 2018) and, in the context of neovascular retinopathies, might result in the persistence of visual deficits (Rakic et al. 2003; Shih et al. 2003; Cheung et al. 2010; Hammes et al. 2011; Gupta et al. 2013; Semeraro et al. 2015; Roy et al. 2017; Ridano et al. 2017) or lead to substantial visual decline in initial responders even several years after anti-VEGF-A treatment (Rofagha et al. 2013). Interestingly, conventional anti-VEGF-A agents fail to restore visual function also in OIR mice, presumably because of compensatory up-regulation of alternative angiogenic factors (Ridano et al. 2017). Thus, the use of broad-spectrum inhibitory agents is warranted in the context of neovascular retinopathies, including diabetic retinopathy (Van Huang et al. 2011; de Veire et al. 2015b; Ridano et al. 2017).

Hence, we investigated the efficacy of Afibercept (VEGF-Trap, Eylea), a recombinant decoy of VEGFR1 and VEGFR2 ligands, in mediating the functional and morphological recovery of the hypoxia-damaged murine OIR retina. Capable of simultaneously neutralizing multiple members of the VEGF family, including VEGF-A and the placental growth factor, Afibercept was developed as a therapeutic alternative to circumvent evasive resistance to anti-VEGF-A treatment (Holas et al. 2002). Compared to other anti-VEGF-A agents, Afibercept binds VEGF-A with higher affinity (Papadopoulos et al. 2012), and exhibits a more

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**Keywords:** afibercept, dopaminergic amacrine cells, endothelial tip cells, neovascularization, oxygen-induced retinopathy, vegf-trap.

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prolonged inhibitory effect (Fauser et al. 2014; Li et al. 2014). The effectiveness of Aflibercept in improving visual acuity and preserving retinal microvascular integrity is documented in neovascular age-related macular degeneration (Fassnacht-Riederle et al. 2014; Sarwar et al. 2016), diabetic macular edema (Diabetic Retinopathy Clinical Research Network et al. 2015; Krick and Bressler 2018), retinopathy of prematurity (Sukgen and Kocluk 2019), and proliferative diabetic retinopathy (Krick and Bressler 2018). Notably, Aflibercept is more effective in patients non-responsive to anti-VEGF-A therapy or in diabetic macular edema patients with worse initial visual acuity (Fassnacht-Riederle et al. 2014; Diabetic Retinopathy Clinical Research Network et al. 2015).

Here, we provide evidence that Aflibercept administration in OIR mice potently accelerates the retinal vasoregenerative response by reducing aberrant vasoproliferation and enforcing tip cell selection. Moreover our results indicate that Aflibercept significantly enhances light-responsiveness as a long-term effect while reducing the hypoxia-induced damage to retinal dopaminergic cellular networks. Altogether, our findings support that Aflibercept possesses unexplored translationally-relevant neuroretinal effects in the context of progressive neovascular retinopathy.

Materials and methods

Study approval

Animal experiments were performed in strict accordance with German Animal Welfare legislation. Mouse experiments were approved by the authorities of the Saxonian Government (Landesdirektion Sachsen). All efforts were made to minimize animal suffering. Anesthesia overdosing prior to eye collection was verified by the absence of interdigital withdrawal reflexes. The maintenance of eye lubrication during electroretinographic recordings was ensured by application of eye lubricant (Vidisic, Bausch and Lomb GmbH, Berlin, Germany). After recordings, mice were sacrificed by cervical dislocation. All mice were acquired from Charles River Laboratories (Sulzfeld, Germany), either to breed in-house or as pregnant females. Newborns were allocated to experimental groups irrespective of their sex. All experimental protocols (TVV26/2017) were checked and approved by the Landesdirektion Dresden with the approval number DD24.1-5131/394/29. The study was not pre-registered.

OIR protocol, Aflibercept administration, and tissue collection

Retinal neovascularization was induced by the OIR protocol. Briefly, C57BL/6J (IMSR Cat# JAX:000664, RRID:IMSR_ JAX:000664) mouse pups and their mothers were exposed to 75% oxygen from P6 to P11 (P0 = birthdate); before and afterward, all mice were housed in ambient air conditions in cages with wood bedding and using a 12 h light/dark cycle. Temperature control and water quality was continuously monitored and followed the regulations of the TU Dresden Medical Faculty Animal House. Aflibercept (Bayer Vital GmbH, Leverkusen, Germany) diluted in sterile PBS (Sigma-Aldrich, Munich, Germany) was administered intra-peritoneally to half the pups in each litter at 25 mg/kg BW as follows: on P13 for examination at P14; on P13 and P15 for examination at P17, P19 or P50; on P13, P15 and P17 for examination at P19 or P50 (Fig. 1a). Non-injected OIR littermates were kept as controls. Half the animals in each litter were arbitrarily allocated by the experimenter to the Aflibercept-treated group and the remaining half to the non-injected OIR control group. Alternatively, Aflibercept was administered intra-peritoneally to P4 mice for evaluation at P7 (P7 + Aflibercept; Fig. 1b). Retinas of age-matched (P7) mice were used as reference. Experimental outcomes were assessed in an arbitrary order with no blinding. No exclusion criteria were enforced and all animals were considered. Samples were only omitted in the case of procedural/imaging artifacts hindering an accurate morphometric characterization. In total, 327 mice were used in the present study. The number of animals required per experimental group was calculated in conjunction with the TU Dresden Institute for Medical Informatics and Biometrics based on an exploratory study in P17 OIR mice. An alpha = 0.05 and a power of 0.8 were used. Calculations were performed in R (https://www.r-project.org/). For experiments for the revision, we aimed at having at least three retinas or three eyes per experimental group. In certain cases, when the number of newborn animals was odd, the additional animal was arbitrarily allocated to either the Aflibercept-treated group or to the non-injected OIR control group. All mice were sacrificed between 13 : 00 and 17 : 00 h by an overdose of ketamine (300 mg/kg BW; Ratiopharm, Ulm, Germany) and xylazine (30 mg/kg BW; Ratiopharm). Pharmacological euthanasia was followed by decapitation for all animals younger than the age of P21. Mice aged P50 were sacrificed by cervical dislocation. Thereafter, eyes were promptly removed and fixed in 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer pH 7.4 (Merck Millipore, Darmstadt, Germany) for 4 h at 4°C.

Histology

PFA-fixed eyes were embedded in paraffin and sections (4 µm) were prepared. Periodic acid – Schiff staining and quantification of intravitreal cell nuclei at > 8 µm intervals (OIR, n = 5 eyes; OIR + AFL, n = 4 eyes) were performed as previously described (Troullinaki et al. 2019a; Troullinaki et al. 2019b).

Immunolabeling of paraffin sections

For immunofluorescence, deparaffinized sections were boiled 2 × 5 min in citrate buffer pH 6 for antigen unmasking. Slides were subsequently washed in PBS, blocked 1 h in PBS 10% Normal Goat Serum (NGS 10%; Vector Laboratories, Burlingame, CA, USA), and incubated overnight in a humidified chamber at 4°C with primary Abs in 10% NGS (Table S1). After 3xPBS washes, fluorophore-coupled secondary Abs were added (10% NGS, 1 h RT) (Table S1). DAPI (1 µg/mL; Molecular Probes, Eugene, OR, USA) was added with secondary Abs. After 3xPBS washes, cross-sections were mounted and coverslipped using Mowiol (Sigma-Aldrich, Cat. 81381). CD31+, Ki67+ and secretagogin+ cells were counted in 6–10 cross-sections in ImageJ (NIH, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/index. html). For quantifications of CD31+, Ki67+ and CD31+Ki67- cells, three regions were considered: vitreous, ganglion cell layer (GCL) vicinity and deep retina. The number of intraretinal blood vessels with diameters > 10 µm were counted in > 7 CD31-immunolabeled cross-sections per eye. The field of view adjacent to
Fig. 1 Experimental pipeline. (a) Newborn pups and their nursing mothers were subjected from P6 to P11 to hyperoxia (O₂ = 75%) and subsequently treated with Afibercept (purple stars) and analyzed (green circles) at distinct time-points. (b) Alternatively, P4 mice were injected with Afibercept and retinas collected and examined at P7.

### Retinal whole-mount immunolabeling

Retinal cups were isolated from PFA-fixed eyes, washed with ice-cold PBS, and twice with PBS 0.1% Triton X-100 (PBSTX-0.1%). After 2 × 15 min permeabilization with PBSTX-1%, retinas were washed 3 × 10 min with PBSTX-0.1% and blocked 1 h with PBSTX-0.1% NGS 10%. Subsequently, primary Abs were added in PBSTX-0.1% NGS 10% (overnight, 4°C) (Table S1). After 3x10 min PBSTX-0.1% washes, retinas were incubated 1 h with fluorochrome-conjugated Abs in NGS 10% in PBSTX-0.1% (Table S1). After 3 × 10 min PBSTX-0.1% washes, retinas were flattened by four radial incisions, transferred onto microscopic slides, flat-mounted in PBS-glycerol (2 : 1) with the GCL upwards and coverslipped, except in the case of photoreceptor analysis with opsin blue- and opsin red/green-immunolabeled samples which were mounted with photoreceptor outer segments upwards.

### Morphometric characterization

Total surface, neurovascular area size, vaso-obliterated area size, intervascular area size and branching points were quantified in micrographs of cluster of differentiation 146 (melanoma cell adhesion molecule, MCAM) (CD146)-immunolabeled flat-mounted retinas using ImageJ. Neovascular tufts were visually identified by their abnormally aggregated “glomeruloid” morphology in 8-bit images using ImageJ, subsequently isolated using the cut tool, transformed into a mask using the threshold tool, and overlaid in white on the original micrograph – a method based on SWIFT_NV (Stahl et al. 2010). At least 40 adjacent intervascular areas were measured in ImageJ (except in cases of vaso-obliteration) in three different concentric regions defined relative to their distance from the optic nerve entry-point: 0.1-0.8 mm, central; 0.9-1.6 mm, mid-peripheral; 1.7-2.4 mm, peripheral (OIR, n = 11 retinas; OIR + AFL, n = 12 retinas; normoxia, n = 4 retinas). Micrographs with low visibility of the vascular network because of imaging artifacts were not possible to characterize and were thus not considered. Branching points were counted considering the entire retinal surface (OIR, n = 13 retinas; OIR + AFL, n = 13 retinas; normoxia, n = 7 retinas). Tip cells were identified by their protruding filopodia and counted along the perimeter of the vaso-obliterated area. Optical sections (Z-stacks) of arbitrarily selected tip cells (OIR, 3 mice, 4 retinas, 10 tip cells, 54 filopodia; OIR + AFL, 6 mice, 7 retinas, 28 tip cells, 143 filopodia) were stored in the LSM format and used for the generation of 3-dimensional reconstructions and volume renders in Imaris 8.2 (Bitplane AG, Zurich, Switzerland; RRID:SCR_007370). Filopodia were manually segmented with the scissors tool of Imaris in volume renders, counted and measured. No filtering operations were used and a 0.25 µm smoothing factor was applied to all images. Filopodial length was determined in Z-Stack maximum intensity projections. Micrographs of tyrosine hydroxylase (TH)-immunolabeled retinal flat-mounts were used to quantify the number of DACs in the entire retinal surface. TH-immunoreactive fibers were evaluated in 400 × 400 µm regions in each retinal flat-mount quadrant by counting the number of pixels with intensities within the top 5–10% in ImageJ. Micrographs of P4 (n = 7 retinas), P7 (n = 7 retinas) and P7 + Afibercept (n = 9 retinas) retinas were used to assess the number of branching points, the spreading distance of the vascular front, and the vascularized fraction of the retinal surface. Opsi n blue- (S-opsin) and opsin red/green- outer segments were semi-automatically counted in four arbitrarily chosen 500 × 500 µm regions within micrographs of flat-mounted retinas. Briefly, regions were isolated using the square selection tool of ImageJ, copied into a new image, transformed into an 8-bit image, and the threshold tool used to generate a mask of high fluorescence intensity pixels. Masks were subsequently subjected to the ‘watershed’ operation of ImageJ to separate objects likely to represent two or more distinct outer segments, and the “analyze particles” tool was finally utilized to count the number of outer segments. The thicknesses of the retina and its layers were manually assessed in DAPI-labeled cross-sections using the line tool of ImageJ at 250 µm intervals starting from the optic nerve entry-point and moving towards the periphery.

### Microscopy

PAS/hematoxylin-stained cross-sections were imaged using a Nikon Optiphot-2 microscope and software supplied by the manufacturer (NIS Elements 7, Nikon, Düsseldorf, Germany). Overviews of
immunolabeled flat-mounted retinas and cross-sections were generated by acquiring adjacent images of the regions of interest in an Axio Observer.Z1 microscope (Carl Zeiss Microscopy, Jena, Germany) and merging them with the Mosaix stitching tool of AxioVision Rel. 4.9.1 or ZEN Software. Z-Stacks of tip cell filopodia were acquired with a 300 nm step-size in a laser scanning confocal microscope (Zeiss LSM 510, Carl Zeiss Microscopy) with a 63x Plan-Apochromat, 1.4 NA, oil immersion DIC objective and manipulated in Imaris. All additional imaging was performed in a Zeiss LSM780 laser scanning confocal microscope using a LD LCI Plan-Apochromat 25x0.8 NA oil immersion DIC and a Plan-Apochromat 63x/1.4 NA oil immersion DIC objective.

**Evans blue leakage assay**

Evans blue (Sigma-Aldrich) was used to assess the blood–retinal barrier stability (Xu et al. 2001). P17 OIR and OIR + AFL mice were injected intra-peritoneally with 200 μL Evans blue (10 mg/mL in sterile PBS). After 2 h, mice were sacrificed, their eyes were collected and fixed, and retinas were isolated, flat-mounted and imaged. Alternatively, mice were anesthetized following PELASA recommendations with ketamine (100 mg/kg BW) and xylazine (10 mg/kg BW) and transcardially perfused with 20 mL 1% PFA in citrate buffer (50 mM, pH 3.5) at 37°C via the left ventricle. Thereafter, eyes were collected, retinas were isolated, dried in a centrifugal evaporator (Speed-Vac; Thermo Fisher Scientific; Waltham, MA, USA) for 5 h, weighted, and Evans blue was extracted by incubating retinas 30 h at 70°C in 60 μL formamide (Sigma-Aldrich). Extracts were centrifuged 20 min at 10 000 g and supernatants were collected for triplicate measurements of absorbance at 620 nm in a SunriseTM microplate reader (Tecan, Männedorf, Switzerland). Background absorbance at 740 nm was subtracted. Formamide was used as blank and a standard curve from 0 to 1000 ng Evans blue/mL was used as reference for calculating concentrations.

**FITC-dextran administration**

Two hours prior to sacrifice, P17 AFL-treated OIR mice were injected with 50 μL of a 0.2 μm-filtered FITC-labeled dextran solution (MW 150 kDa, 50 mg/mL sterile PBS, Sigma-Aldrich, FD150S). Subsequently, mice were either sacrificed by an overdose of ketamine/xylazine or transcardially perfused (as before) and eyes were removed and fixed in 4% PFA.

**Electroretinography**

P50 mice were dark-adapted (> 12 h) and anesthetized (as before) under dim red light conditions. Pupils were dilated with 2.5% phenylephrine and 0.5% tropicamide and mice were placed on the recording stage of a Ganzfeld Q450SC system (Roland Consult, Brandenburg, Germany). A reference, a ground gold-ring and two recording gold-ring electrodes were placed under the skin, on the tongue, and in close contact with each cornea, respectively. Eye lubricant (Vidisic, Bausch and Lomb GmbH, Berlin, Germany) was applied on each eye to ensure efficient electrical transmission and to avoid eye drying. Body temperature was kept constant with a homeothermic control unit (Cat. 70-5020; Harvard Apparatus, Holliston, MA, USA). Light stimuli were presented as described previously (Santos-Ferreira et al. 2015). Briefly, at 5 s intervals for light-intensities of 0.0003, 0.001, 0.003, 0.01, 0.03, and 0.1 cd s/m², at 10 s intervals for 0.3 cd s/m², and at 17 s intervals for 1.0, 3.0, and 10.0 cd s/m². Responses to single-flashes were recorded for 400 ms at 512 evenly distributed time-points. Recordings were processed offline for each light-intensity in the manufacturer’s software using a 75 Hz filter. A-wave and b-wave amplitudes and average recordings at each time-point were extracted as .csv files and processed in Microsoft Excel.

**RNA isolation**

Retinas of 2 mice per condition were dissected in PBS at 37°C and pooled together. Warm PBS was replaced by 750 μL TRIzol (Thermo Fisher Scientific), and retinal tissues were disrupted by 10 rounds of aspiration/ejection through 18G and 23G needles and incubated at RT for 5 min. Thereafter, 150 μL chloroform (Merck, Darmstadt, Hesse, Germany) were added and samples were vortexed, incubated 15 min at RT, and centrifuged 20 min at 12 000 g and 4°C. The aqueous phase was subsequently collected and mixed with 1.5 μL RNA-grade glycogen (Thermo Fisher Scientific) and 375 μL isopropanol (Merck). Samples were subsequently vortexed and centrifuged 20 min at 12 000 g and 4°C. Pellets were washed twice in 1 ml 75% ethanol, air-dried 10–20 min, and re-suspended in 20 μL RNase-free water.

**Gene expression profiling**

For microarray analysis, total RNA of 4 pooled retinas from 2 mice per experimental group (Normoxia, OIR, OIR + AFL) was extracted by incubating retinas 30 h at 70°C in 60 μL formamide (Sigma-Aldrich). Extracts were centrifuged 20 min at 10 000 g and supernatants were collected for triplicate measurements of absorbance at 260 nm and 280 nm in a SunriseTM microplate reader (Tecan, Männedorf, Switzerland). Background absorbance at 740 nm was subtracted. Formamide was used as blank and a standard curve from 0 to 1000 ng Evans blue/mL was used as reference for calculating concentrations.

**Fig. 2** (a) Normoxic (left), OIR (middle) and OIR + AFL (right) flat-mounted CD146-immunolabeled P17 retinas. Insets display magnified views of enclosed regions; scale: 1 mm; insets, 100 μm. (b) Neovascularization (white) in OIR mice decreases upon AFL administration. n = 13 retinas per group; 5 litters. ****P < 0.0001, unpaired two-tailed Student’s t-test with Welch’s correction. (c and e) P17 OIR (left) retinal cross-sections stained with periodic acid Schiff/haematoxylin exhibit a higher number of pre-retinal endothelial cell nuclei than OIR + AFL (right) retinas. *P < 0.05, unpaired two-tailed Mann–Whitney U test; each dot in (e) represents the average value of 8–10 cross-sections from one eye (OIR, n = 5 eyes from 5 mice; OIR + AFL, n = 4 eyes from 4 mice). Black arrows, pathologic pre-retinal vessels; red arrowheads, pre-retinal endothelial cell nuclei. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale: 100 μm. (d) Representative micrographs of cross-sections of Normoxic, OIR and OIR + AFL retinas immunofluorescently labeled with CDS1 (green, endothelial cells) and Ki-67 (red, proliferating cells). White arrowheads, Ki-67+ proliferating cells; dashed line, inner limiting membrane. Scale bar: 50 μm. (f–i) Endothelial cells (CDS1; f–h) and proliferating endothelial cells (CDS1+ Ki-67+; i–k) were quantified in cross-sections within the vitreous (f and i), along the GCL (g and j) and in the deep retina (h and k). Additionally, microvessels with a diameter > 10 μm were quantified in full retinal cross-sections (l). Normoxia, n = 3 eyes from 3 mice; OIR, n = 6 eyes from 6 mice; OIR + AFL, n = 5 eyes from 5 mice. *P < 0.05, **P < 0.01, one-way ANOVA with Tukey’s post hoc test. Data are shown as mean ± SEM. © 2019 The Authors. Journal of Neurochemistry published by John Wiley & Sons Ltd on behalf of International Society for Neurochemistry, J. Neurochem. (2020) 153, 390–412.
collected in triplicate at each time point (P14 or P17), i.e. 3 sets of 4 pooled retinas from 2 mice for each P14 Normoxia, P14 OIR, P14 OIR + AFL, P17 Normoxia, P17 OIR and P17 OIR + AFL. RNA was then sent to the Gene Expression Facility of MPI-CBG (Dresden, Saxony, Germany; details: NCBI GEO accession number GSE124956). In our analysis, pairwise comparisons of the expression level of each transcript were performed at each time-point (P14 and P17) between OIR and OIR + AFL. Data were exported as .csv files for further analysis.

Quantitative RT-PCR
1 μg total RNA treated with DNase I (DNase I kit, Thermo Fisher Scientific; Waltham, MA, USA) was used for cDNA synthesis with random primers using the Affinity Script Multiple Temperature cDNA Synthesis kit (Agilent). cDNA was treated 20 min at 47°C with RNase H (M0297; New England BioLabs, Ipswich, MA, USA) to remove RNA templates from heteroduplexes. The GoTaq qPCR Master Mix (M7123; Promega, Madison, WI, USA) was used for qPCRs in a LightCycler 480 (Roche Diagnostics, Mannheim, Germany). Primers designed to amplify products between 70 and 300 bp were acquired from Biospring GmbH (Mannheim, Germany; Table 1). Data were manipulated in the LightCycler 480 software version 1.5 (Roche Diagnostics) and analyzed in Microsoft Excel. Rpl19 was used as normalizer. Quantification was performed by the 2^(-ddCt) method using total RNA of 4 pooled retinas from 2 mice per experimental group (Normoxia, OIR, OIR + AFL) collected in sextuplicate (Normoxia) or quintuplicate (OIR and OIR + AFL) at each time point (P14 or P17), i.e. 6 sets (n) of 4 pooled retinas from 2 mice for each P14 Normoxia and P17 Normoxia, and 5 sets (n) of 4 pooled retinas from 2 mice for each, P14 OIR, P14 OIR + AFL, P17 OIR, and P17 OIR + AFL.

Statistical analysis
Statistical analyses were performed in GraphPad Prism (GraphPad, RRID:SCR_002798). For comparisons of two treatments, a two-tailed unequal Student’s t test was used for normally distributed data. Normality was confirmed by the Shapiro–Wilk test for each individual dataset. The Welch’s correction was applied if datasets exhibited distinct variances. Alternatively, Mann–Whitney U tests were performed for non-normally distributed datasets. Comparisons between 3 or more treatments were performed using one-way ANOVA with post hoc tests for pairwise comparisons and are indicated in figure legends. Differences were considered significant when p < 0.05. No tests for outliers were conducted and no data points were excluded from the statistical analyses.

Results
Aflibercept efficiently reduces neovascular tuft formation, inhibits endothelial cell over-proliferation, and normalizes the intra-retinal vascular architecture
C57BL6/J mice subjected to the OIR protocol (Smith et al. 1994) exhibited an extensive neovascular area relative to retinal surface area at P17 (Fig. 2a and b). The neovascular area was significantly reduced in OIR retinas upon intraperitoneal administration of Aflibercept on P13 and P15 (Fig. 2a and b; see Experimental Scheme in Materials and Methods). This agent was detected in the retinal microvasculature as early as 2 h after injection at P13 (Fig. S1). Next, epiretinal nuclei were quantified in eye cross-sections from P17 mice. As expected from decreased neovascular tuft formation, the number of epiretinal nuclei was significantly lower in Aflibercept-treated mice relative to non-injected OIR littermates (Fig. 2c and e). The endothelial identity and proliferative status of cells invading the vitreous was determined by immuno-detection of CD31 (PECAM-1, platelet endothelial cell adhesion molecule) and Ki-67, respectively (Fig. 2d). Significantly fewer CD31+ cells invading the vitreous body were detected in Aflibercept-treated retinas compared to OIR controls, although no significant changes were found along the GCL or within the deep retina (Fig. 2f–h). Similarly, the number of proliferating endothelial cells, that is, CD31+Ki-67 cells, was significantly lower within the vitreous and in the proximity of the GCL after Aflibercept treatment, but remained unaltered within the deep retina (Fig. 2i–k). Interestingly, while vessels in normoxic samples were homogeneously distributed along the superficial, intermediate and deep vascular beds, that is, along the GCL and above...
and below the inner nuclear layer (INL), vessels in OIR mice were highly disorganized and dilated, forming even within the INL. In this context, the number of microvessels with a diameter above 10 µm per cross-section was significantly higher in retinas of OIR mice compared to normoxic controls and was normalized by Afibercept administration (Fig. 2f). Whereas normoxic and OIR + AFL retinas exhibited such vessels forming mainly in far peripheral regions, they were found along the entire retina in OIR cross-sections, suggesting that Afibercept normalizes retinal blood vessel diameter and distribution in OIR mice.

Afbibercept reduces the complexity of the retinal microvasculature but does not interfere with early physiological retinal vascular development

During development, the density of the retinal microvascular network correlates with its maturation status (Korn and Augustin 2015). OIR + AFL tissues exhibited a significantly reduced number of branching points per unit area relative to OIR retinas (Fig. 3a and b). Nonetheless, normoxic age-matched retinas displayed an even lower number of branching points than either OIR or OIR + AFL retinas. Conversely, intervascular areas were significantly larger in the mid-peripheral region of OIR + AFL retinas than in OIR specimens (Fig. 3c and d). Still, intervascular areas remained smaller in OIR and OIR + AFL retinas compared to normoxic controls in all retinal regions. As VEGF-A-mediated signaling is essential during early postnatal development, we assessed the effects of administering Afibercept in the first postnatal week. Compared to retinas of non-injected P7 littermates, retinas of P7 mice that received Afibercept at P4 (P7 + AFL) showed no differences in vascular front migration capacity, as revealed by measurements of microvasculature spreading distance and vascularized area (Fig. 3e–g). Yet, P7 + AFL retinas displayed a reduced number of branching points than P7 specimens (Fig. 3h), suggesting an accelerated pruning process or reduced sprouting angiogenesis as a consequence of Afibercept administration. Labeling of pericytes with an antibody against NG2 (neural/glial antigen 2) revealed no overt changes in their coverage of the retinal microvascular network.

Afbibercept accelerates retinal revascularization

The central murine OIR retina is vaso-obliterted at P17 (Fig. 4a). Vaso-oblitertion was significantly reduced upon AFL administration (Fig. 4b). Moreover, a higher number of endothelial tip cells was observed along the vaso-oblitertated perimeter of OIR + AFL retinas than in OIR control tissues (Fig. 4c and d). Furthermore, NG2⁻/-pericytes were located in closer proximity to tip cells and their filopodial protrusions in OIR + AFL than in OIR retinas (Fig. 4e). To determine if tip cell phenotypes differed between OIR and OIR + AFL retinas, their morphologies were characterized in 3D reconstructions generated from high-resolution optical sections (Fig. 4f and Video S1 and S2). Although no significant differences were detected in the number of filopodia per tip cell nor in the angle between filopodia, the average filopodium length and volume, as well as the accumulated filopodia volume per tip cell were significantly higher in OIR + AFL retinas than in OIR specimens (Fig. 4g–k). Interestingly, filopodia-like structures were also observed protruding from neovascular tufts in both OIR and OIR + AFL retinas, although their length was higher in samples from Afibercept-treated mice (Fig. 4l). Moreover although differing in size, neovascular tufts found in both OIR and OIR + AFL retinas expressed not only the endothelial marker CD146, but were also highly immunoreactive to markers of astrocytes (glial fibrillary acidic protein) and pericytes (NG2; Fig. 4m). Yet, the morphology of these neovascular tuft-forming cells was uncommon for these lineages and their definitive cellular identity is unclear.
Vascular hyperpermeability is reduced by Aflibercept treatment

Vascular hyperpermeability because of destabilization of the blood-retinal barrier is a typical complication of VEGF-related ischemic retinopathies, including diabetic retinopathy (Qaum et al. 2001). Similarly, the retinal microvasculature of OIR mice is unstable and leaky (Qaum et al. 2001; Fischer et al. 2002). We assessed the effect of Aflibercept on vascular leakiness by Evans blue, a dye that remains bound to albumin (69 kDa in mice) leaking from the circulation (Xu et al. 2001). OIR + AFL retinas displayed fewer and smaller regions of Evans blue extravasation compared to non-treated littermates (Fig. 4n). The Aflibercept-mediated reduction of vascular hyperpermeability was quantified by extracting and spectrophotometrically measuring albumin-bound Evans blue from P17 retinas (Fig. 4o). The latter suggested that Aflibercept stabilizes the contacts between endothelial cells. Thus, we decided to check for the expression of the tight junction-associated proteins Claudin-5 and ZO-1 (Zonula Occludens 1), which are essential components of the blood-retinal barrier (Fig. S2). As expected, the expression of these molecules was restricted to the vascular network in both normoxic, OIR and OIR + AFL retinas, albeit at higher levels in neovascular tufts. Interestingly, a mosaic expression of ZO-1 was observed in OIR NV tufts (Fig. S2b). Moreover Claudin-5 exhibited an apparently higher and more homogeneous expression in the microvasculature of Aflibercept-treated mice (Fig. S2a). We also aimed at detecting the plasmalemma vesicle-associated protein (PLVAP), whose up-regulation has been previously linked to increased permeability of retinal capillaries in OIR mice (Wisniewska-Kruk et al. 2016). However, the expression of this factor remained below detection level in our samples (Fig. S2b).

In order to assess if molecules with a molecular mass higher than Aflibercept could cross the blood-retinal barrier, 150 kDa FITC-labeled dextran was injected (Fig. S3a). After removing the circulating fraction of FITC-dextran by perfusion, the presence of a punctate signal was evident in retinal flat-mounts (Fig. S3b). Similarly, Aflibercept was detected in a dotted pattern within blood vessels, primarily in a compartmentalized manner and more strongly along endothelial cell membranes. In agreement with our observations, Aflibercept was recently shown to be transported by endothelial cells in vitro by transcytosis (Deissler et al. 2017).

Neovascularization reinitiates upon Aflibercept discontinuation but is inhibited by an additional Aflibercept dose

The peak of neovascularization in the OIR retina is reached at P17 (Stahl et al. 2009). Indeed, analysis of the retinal OIR microvasculature at P19 revealed no significant differences in neovascular area compared to P17 OIR animals (Fig. 5a and b). Furthermore, as in P17 samples, P19 Aflibercept-treated animals continued to exhibit less retinal neovascularization than their P19 OIR littermates. However, retinal neovascularization in OIR + AFL mice was significantly higher at P19 than at P17. We hypothesized this was a consequence of increased levels of angiogenic factors in the absence of their Aflibercept-mediated inhibition. Thus, an additional dose of Aflibercept was administered to OIR + AFL mice on P17 (OIR + AFLx3). Retinal neovascularization was significantly reduced in P19 OIR + AFLx3 retinas compared to retinas of individuals that received only 2 Aflibercept injections, that is, P17 OIR + AFL and P19 OIR + AFL. Unexpectedly, the vaso-obliterated areas of P19 OIR and OIR + AFLx3 retinas exhibited similar proportions (Fig. 5c). The latter might be a consequence of inhibiting pro-angiogenic cues to sub-physiological levels, potentially compromising endothelial cell survival and revascularization capacity.

Aflibercept modulates the expression level of multiple angiogenic genes

To identify Aflibercept-responsive genetic targets, we performed global gene expression profiling using total RNA isolated from whole retinas of P14 and P17 mice. These time-points represent the peaks of hypoxia and neovascularization, respectively. Retinas from normoxic age-matched controls. Multiple differentially regulated transcripts (absolute fold-change > 1.2 and P < 0.05) were identified at each time-point in OIR + AFL retinas compared to OIR controls (Fig. 6a and Tables S3–S6). A subset of these transcripts comprising 13 genes, many of them with recognized roles in angiogenesis, was differentially regulated at both P14 and P17 (Fig. 6b and Table S7). The expression level of 11 of these genes increased in OIR retinas relative to normoxic samples and was subsequently normalized upon Aflibercept administration (Fig. 6c). Two hits (Plcb1 and A119744) showed reduced expression levels in OIR retinas relative to normoxic conditions, and were either normalized (Plcb1) or further down-regulated (A119744) in response to Aflibercept. We validated the differential expression of 9 targets by real time RT-qPCR at least at one time-point, that is, P14 or P17 (Fig. 6d). Additionally, the expression levels of Kdr (VEGFR-2) and Ptprb (Vascular endothelial-phosphotyrosine phosphatase, VE-PTP) were significantly reduced in OIR + AFL retinas at specific time-points (Fig. 6e). Hence, Aflibercept likely inhibits neovascularization and promotes microvascular stability by simultaneously normalizing the expression levels of multiple angiogenic factors.

Aflibercept modulates the expression of genes involved in neurotransmission and enhances retinal light responses

Unexpectedly, beyond regulating the expression of angiogenic factors, Aflibercept also enhanced the expression of
Fig. 5 Microvascular characterization of OIR retinas at P19 in response to two or three AFL doses. (a) Representative images of P19 flat-mounted CD146-immunolabeled OIR, OIR + AFLx2 and OIR + AFLx3 retinas. White, neovascular tufts; red, vaso-obliterated area; dashed line, retinal contour. Scale bar: 1 mm. (b and c) Neovascularization (b) and vaso-obliteration (c) were quantified at P19 relative to total retinal surface area and are shown in comparison to P17 samples. In (b), OIR P17, n = 13 retinas from 11 mice; OIR + AFL P17, n = 13 retinas from 12 mice; OIR P19, n = 19 retinas from 10 mice; OIR + AFLx2 P19, n = 13 retinas from 8 mice; OIR + AFLx3 P19, n = 9 retinas from 6 mice. In (c), OIR P17, n = 15 retinas from 13 mice; OIR + AFL P17, n = 15 retinas from 14 mice; OIR + AFLx2 P19, n = 13 retinas from 9 mice; OIR + AFLx3 P19, n = 9 retinas from 6 mice. Data are shown as mean ± SEM. NS, non-significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, unpaired two-tailed Student’s t tests.
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(a) Venn diagram showing the overlap between P14 and P17 datasets.

(b) Venn diagram comparing genes unique to P14 and P17.

(c) Log2-normalized intensity plots for various genes across different conditions.

(d) mRNA levels of various genes normalized to Rpl19.

(e) mRNA levels of various genes normalized to Rpl19.

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genes involved in synaptic transmission. The Aflibercept-mediated up-regulation (and normalization) of P2rx2 and Gabra4 expression levels was confirmed by real-time RT-qPCR (Fig. 6e). Notably, the expression of these genes was dramatically reduced (> 50%) in OIR retinas relative to normoxic controls at P17. Suggesting a beneficial effect for neurotransmission, we assessed the potential impact of these molecular changes on light responsiveness in young adult mice (P50). In agreement with previous findings (Vessey et al. 2011; Zhou et al. 2016), we detected significantly reduced a-wave and b-wave amplitudes in OIR mice relative to normoxic controls (Fig. 7a and b). Remarkably, Aflibercept-treated animals exhibited increased a-wave amplitudes relative to OIR littersmates after either 2 or 3 injections at light intensities above log -1.0 and log -1.5 cd s/m², respectively. In comparison, OIR mice exhibited dramatic reductions in b-wave amplitudes at all evaluated light-intensities relative to normoxic controls (Fig. 7b). A significant recovery in b-wave amplitudes was detected in OIR + AFLx3 mice, but not in OIR + AFLx2 animals, at certain light intensities, namely log -2.0, -1.5, 0.5, and 1.0 cd s/m², suggesting a dose-dependent effect of Aflibercept treatment on postreceptorial function. Strikingly, oscillatory potentials were detected in Aflibercept-treated mice but not in OIR non-injected littersmates (Fig. 7c). Oscillatory potentials represent a sensitive indicator of hypoxic deficits in ischemic retinopathies (Drasdo et al. 2002; Dorfman et al. 2010), and their recovery suggests an improved functionality of amacrine cells, which are widely recognized as responsible for the emergence of these wavelets in electroretinograms (Spix et al. 2016). Notably, post-electroretinography immunohistochemical examinations of P50 OIR and OIR + AFL retinas revealed no gross morphological differences in retinal vascular architecture, astrocytic scaffolding, or pericytic coverage (Fig. S4a and S4b). As previously reported (Tokunaga et al. 2014), however, the inner and outer nuclear layers visibly merged in the retinal region adjacent to the optic nerve head in OIR and OIR + AFL mice (Fig. S4c).

Aflibercept prevents the hypoxia-induced loss of inner-retina resident dopaminergic amacrine cells

The outcome of our electroretinography measurements suggested that Aflibercept reduced hypoxia-induced neuroretinal damage. Indeed, extensive death of amacrine cells has been reported during the phase of relative hypoxia in the OIR model (Du et al. 2013; Spix et al. 2016). Thus, we decided to assess the impact of Aflibercept administration on hypoxia-sensitive tyrosine hydroxylase-positive (TH⁺) DACs. The number and density of TH⁺ DACs in P17 OIR retinas was significantly lower than in normoxic age-matched controls (Fig. 8a and b). Compared to OIR littersmates, P17 OIR + AFL retinas exhibited a modest increase in these parameters. By P19, however, a significantly higher total count and density of TH⁺ DACs was observed in OIR + AFLx2 retinas relative to non-injected controls (Fig. 8a and c). No further gain in TH⁺ DAC numbers was detected in OIR + AFLx3 retinas. Similarly, comparison of P17 and P19 OIR retinas revealed no significant differences in the number or densities of DACs, suggesting the impaired maturation of this cell population in the absence of antiangiogenic intervention. Furthermore, as we detected altered DAC morphologies in OIR mice relative to normoxic controls, we evaluated DAC fiber network occupancy as a proxy for neuronal maturation (Fig. 8d). At P17, no differences were detected in DAC network occupancy between normoxic, OIR and OIR + AFL retinas (Fig. 8e). Meanwhile, at P19, DAC network occupancy was significantly higher in OIR + AFLx3 retinas, but only moderately and non-significantly in OIR + AFLx2 tissues, compared to OIR controls (Fig. 8f). At this time-point, no significant differences in DAC network occupancy were observed between Aflibercept-treated OIR mice and normoxic controls, whereas tissues of non-injected OIR mice exhibited a significant reduction in this parameter relative to normoxic samples. The Aflibercept-mediated enhancement in DAC maturation and functionality was further evidenced by the presence of TH-immunoreactive fibers in the retinal vaso-obliterated region of Aflibercept-treated mice, but not of non-injected OIR littersmates, at both
P17 and P19 (Fig. 8g). Remarkably, the effect of Aflibercept on the this cell population was long-lasting, with increased numbers of DACs being also detected at P50 in retinas of OIR + AFL mice compared to those of non-injected mice (Fig. S5). Nonetheless, all mice subjected to the OIR protocol exhibited dramatically lower DAC counts irrespective of Aflibercept administration. Interestingly, no changes in the number of rod bipolar cells, whose cell bodies also lie within the inner nuclear layer, were detected in secretagogin-labeled cross-sections (Fig. S6a). Yet, secretagogin+ bipolar cell bodies exhibited a scattered localization within the inner nuclear layer of OIR retinas, which was largely restored upon Aflibercept administration and resembled the distribution patterns observed in normoxic animals. The functional relevance of such changes in the OIR model, however, is presently unclear.

**The effect of Aflibercept on photoreceptors**

As a-wave deficits are considered a consequence of impaired photoreceptor functionality, the number of both cone populations was determined by detecting expression of short wavelength (S opsin+) and middle wavelength (M opsin+) opsins in cone photoreceptors (Fig. S7). The expression of cone opsins is a late post-mitotic event that occurs several days after the birthdate of prospective cone photoreceptors (Szél et al. 1993). S opsin expression sets on earlier (at around P4) than M opsin (at around P11). In contrast to the ventral retina, the dorsal retina displays an extremely low S opsin+ cone density (Szél et al. 1993). More, S opsin-positive cone photoreceptors have been described as hypoxia-sensitivities (Greenstein et al. 1989). We found that a minor fraction of S opsin+ cells were lost in P19 OIR retinas within the ventral region, but such fraction was partially recovered in response to Aflibercept treatment (Fig. S7a–e). In contrast, beyond a phenotypic lag (delay) at P17, no changes were detected in the numbers (Fig. S7f–h) or morphology (Fig. S6c) of M opsin-expressing cones between OIR or OIR + AFL. Whereas the morphology of S opsin+ cone outer segments remained unaltered (Fig. S6b), the length of rod outer segments was significantly reduced in OIR mice compared to normoxic controls. Aflibercept administration did not counteract this morphological alteration. In contrast, rhodopsin-labeled OIR + AFL retinal cross-sections exhibited a slight but non-significant reduction in outer segment length (Fig. S6d and f).

In agreement with previous reports, we observed a significant reduction in retinal thickness in P17 OIR and OIR + AFL mice relative to age-matched normoxic controls (Fig. S8a and b). Whereas this thickness reduction was particularly prominent within the INL (Fig. S8c), differences were minimal within the outer nuclear layer (ONL) (Fig. S8d). Compared to non-injected OIR controls, Aflibercept treatment only led to a further reduction in the ONL approximately 250 µm apart from the optic nerve head (Fig. S8d).

**Discussion**

We investigated the response of the hypoxic retina to Aflibercept using the murine OIR model with special focus on microvascular architecture and light responsiveness, while also addressing the cellular and molecular effects of the treatment.

Systemic Aflibercept administration led to a strong reduction in neovascular tuft formation and intravitreal endothelial cell proliferation in OIR retinas, suggesting that Aflibercept efficiently counteracts localized endothelial cell hyperproliferation underlying impaired microvascular patterning (Jakobsson et al. 2010; Ubezio et al. 2019b). Whereas treatment discontinuation results in the re-emergence of microvascular aberrations, an additional Aflibercept dose inhibits neovascular tuft formation almost completely. Notably, Aflibercept effects were not limited to the superficial vascular bed, as the treatment deterred intraretinal microvessels from forming at non-canonical locations and confined them to stereotypical locations, that is, intermediate and deep vascular beds. As disorganized or dilated intraretinal vessels might compromise blood supply to interneurons, induce mechanical damage to neighboring cell populations, and lead to long-lasting functional deficits, this represents a key finding of our study. In contrast to intravitreal delivery (Tokunaga et al. 2014), systemic Aflibercept treatment promotes revascularization, as suggested by a reduced vaso-obliterted area in P17 OIR retinas. Accordingly, previous studies indicate that administration routes are pivotal to the therapeutic index of mAbs in preclinical settings (Barcelona et al. 2018). Compared to intravitreal injections, systemic application routes are less invasive and might result in improved safety profiles and long-term therapeutic efficacy in preclinical models.

While the mechanisms driving revascularization, that is, ordered vessel regrowth, remain poorly understood, we detected an Aflibercept-mediated increase in tip cell numbers and tip cell filopodia length and volume along the retinal...
revascularization. Tip cell and tip cell filopodia formation are both dependent on VEGF-A levels, which are tightly modulated by the neuroretinal metabolic demand (Chan-Ling et al. 1995; Gardel et al. 2010). During development, VEGF-A availability is fine-tuned in the angiogenic front microenvironment by the local action of soluble VEGFR-1 (sVEGFR-1), a stalk cell-secreted decoy receptor (Chappell et al. 2009). Considering the structural similarities between Aflibercept and sVEGFR-1, the positive effect of AFL treatment on tip cell selection might resemble the action of sVEGFR1, but this notion needs further experimental clarification. Such a mechanism would explain the higher number of tip cells detected along the vaso-obliterated perimeter of OIR + AFL retinas. Nevertheless, determining the optimal Aflibercept dose is a critical factor, since prolonged Aflibercept administration, as observed in P19 OIR + AFlx3 retinas, might slow down revascularization possibly as a result of inhibiting VEGF-A below physiological levels.

The activation of compensatory angiogenic programs is known to undermine the anti-angiogenic efficacy of VEGF-A inhibitors (Croci et al. 2014). Profiling the gene expression patterns of OIR and OIR + AFL retinas revealed Aflibercept-responsive targets with recognized roles in angiogenesis. Moreover, a number of inhibitors for these factors are presently under clinical investigation because of their influence on neovascularization, vascular permeability, endothelial cell migration and pericyte behavior. Ang2 inhibition, for instance, leads to reductions in retinal and tumor neovascularization in mice (Das et al. 2003). Furthermore, Ang2 acts synergistically with VEGF-A in mediating vascular permeability and regeneration (Hammer et al. 2004; Scharpfenecker et al. 2005; Peters et al. 2007; Reiss et al. 2007; Felcht et al. 2012), and is up-regulated in the vitreous of diabetic individuals (Watanabe et al. 2005). Consistent with our results, the RUBY Study (ID: NCT02712008) reported no differences in visual acuity between diabetic macular edema patients treated intravitreally with Aflibercept or the Ang2-inhibitor REGN910-3 (Regeneron). Moreover a phase I study of the Ang2/VEGF-A bipspecific inhibitor RG7116 (Roche) revealed gains in visual acuity in patients with neovascular age-related macular degeneration (Chakravarthy et al. 2017), pointing at the high efficacy of simultaneously inhibiting these factors and suggesting that the modulation of Ang2 by Aflibercept might underlie its remarkable anti-angiogenic properties in our setting. Besides down-regulating Ang2, the Aflibercept-mediated reduction in Cdh3 levels in OIR retinas might represent a central molecular mechanism by which Aflibercept inhibits neovascularization, as this adhesion molecule mediates endothelial cell-pericyte interactions (Orlandini et al. 2014; Galvagni et al. 2017), which are known to force endothelial cells to return to their non-proliferating phalanx cell phenotypes and to shut down their tip cell proteolytic status (Chung et al. 2010). Interestingly, Aflibercept also modulates VEGF-R2 (Kdr) expression, thereby potentially contributing importantly to reducing neovascularization and vascular permeability (Hudson et al. 2014; Wisniewska-Kruk et al. 2016). In general, our transcriptomic analysis identified druggable targets relevant for multiple angiogenic processes, including: regulating VEGF-A bioavailability and tip cell behaviour (Esm1) (Stenzel et al. 2011; Rocha et al. 2014), modulating endothelial cell-pericyte interactions (Cd93) (Orlandini et al. 2014; Galvagni et al. 2017), mediating Ang2 effects in vessel destabilization (Piprb) (Shen et al. 2014), and controlling neovessel formation (Apnhr, Cd34, Eng) (Kidoya et al. 2008; Nielsen and McNagny 2008; Wittig et al. 2013; Barnett et al. 2014; Siemerink et al. 2016). Altogether, our results suggest that Aflibercept is endowed with features that combination therapies aim at achieving in the treatment of retinal vasopathies.

During early non-proliferative diabetic retinopathy, even patients with good visual acuity exhibit diabetes-induced retinal dysfunctions detectable through functional tests (van der Torren and van Lith 2010; Simo et al. 2014). Thus, electroretinography aberrations, including oscillatory potential abnormalities, are useful tools for early diabetic retinopathy diagnosis (Barse et al. 2006; Simo et al. 2014). In OIR models, which largely recapitulate the pathomorphological alterations of proliferative diabetic retinopathy, visual perturbations are also detectable by electroretinography even after neovascular tufts resolve (Dorfman et al. 2010; Vessey et al. 2011; Zhou et al. 2016). Such perturbations include reduced a-wave and b-wave amplitudes, as well as altered oscillatory potentials. In our study, diminished b-wave amplitudes,
representative of post-receptoral activity, might reflect a hypoxia-induced loss of interneurons and/or damage to synaptic contacts within the inner retina (Pardue and Peachey 2014). Whereas therapeutic agents aimed at preventing pathological angiogenesis are commonly incapable of preventing or repairing retinal functional disturbances (Hatzopoulos et al. 2014; Xu et al. 2017; Ridano et al. 2017), we show that Aflibercept improves visual circuit functionality. In this context, oscillatory potential disturbances are considered to arise, at least partially, because of impaired dopaminergic transmission (Wachtmeister 1998), as affected TH⁺ DACs and their synapses fail to generate intraretinal feedback loops (Spix et al. 2016). As OIR + AFL mice exhibited not only increased a- and b-wave amplitudes but also a dose-dependent re-appearance of oscillatory potentials, we hypothesized that Aflibercept protects DACs.

Consistent with previous reports (Du et al. 2013; Spix et al. 2016), approximately 50% of all DACs were obliterated in P17, P19 and P50 OIR retinas relative to age-matched normoxic controls. Higher DAC counts and more complex dendritic arbors were detected at P19 in OIR + AFL mice relative to non-injected OIR littermates. As the maturation and differentiation of retinal DACs occurs concomitantly with postnatal retinal vascular development, delays in retinal vascularization might underlie this phenotype. Since only ~50% of all retinal DACs are present by P12 (Wulle and Schnitzer 1989), and intermediate vascular plexus formation during the third postnatal week is required for their last wave, ischemia in this time-window dramatically reduces DAC numbers (Gastinger et al. 2006; Spix et al. 2016). Our data suggest that Aflibercept supports the formation of the intraretinal dopaminergic neuronal cell network by stabilizing intraretinal vascular beds, a prerequisite for interneuron nourishment and differentiation. The neuroretinal effects of Aflibercept are further emphasized by the up-regulated expression of genes encoding post-synaptic receptor subunits, that is, Gabra4 and P2rx2, components of ion-channels transmitting the effects of amacrine cells using γ-aminobutyric acid and purines as neurotransmitters. Of particular note, DACs represent a subpopulation of amacrines releasing not only γ-aminobutyric acid but also adenosine triphosphate (ATP) as co-transmitter (Ho et al. 2015). Furthermore, functional ERG disturbances suggest that photoreceptors might be affected, although damage to the choroidal vasculature is not a hallmark of OIR (Vessey et al. 2011). However, no significant thickness reductions of the ONL were detected. Yet, outer segment damage or molecular cascade dysfunction within these cells cannot be discarded. Recently, involution of the choroidal vasculature was linked to long-lasting photoreceptor dysfunction (Zhou et al. 2016). Whereas changes to cone outer segments were reported in the central retina of OIR mice (Vessey et al. 2011), little is known about how the two cone populations of the murine retina react to Aflibercept. Our findings indicate that a minor fraction of S opsin-expressing cones is lost in OIR mice and partially recovered upon Aflibercept administration. In the case of M opsin-expressing cones, no obvious changes were detected in their morphology, although a delay in phenotypic differentiation was observed in flat-mounted retinas. The mechanisms that might render a certain cone subpopulation more sensitive to hypoxia are unknown, although S opsin⁺ cones were previously described as particularly hypoxia sensitive (Greenstein et al. 1989; Yamamoto et al. 1996; Schatz et al. 2014). Alternatively, the differences we detected might be attributable to the expression of S- and M-opsin exhibiting distinct developmental onset time-points (Szél et al. 1993). In general, our observations indicate that Aflibercept administration promotes the phenotypic differentiation of hypoxia sensitive S opsin-positive cones in the OIR retina, whereas other investigated cell populations as rod photoreceptors and bipolar cells were not evidently affected by the treatment. Altogether, our results indicate that Aflibercept possesses a remarkable capacity to simultaneously regenerate the retinal microvasculature and mitigate neuronal deficits, an atypical feature for an anti-angiogenic agent. So far, however, there is no evidence for the general neuroprotective properties of this agent.

Acknowledgments and conflict of interest
disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

Author contribution

JERA and JJ designed research. JERA, DAJL, KHAY, VE, and MM acquired and analyzed data. JERA, ME, DAJL, AK, KHAY, MS, and JJ performed research, ME provided advice on the experimental model. JERA and JJ wrote the paper. JERA, ME, HM, and RHWF contributed new reagents or analytical tools. JJ coordinated the study.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Aflibercept reaches the retinal microcirculation after intra-peritoneal administration.
Figure S2. Assessment of tight junction-associated proteins in retinal flat-mounts.

Figure S3. Localization of Aflibercept and 150 kDa FITC-labeled dextran within the retinal vasculature.

Figure S4. Immunofluorescent and histological examination of P50 retinas.

Figure S5. Dopaminergic amacrine cell (DAC) responses to Aflibercept treatment in OIR mice at P50.

Figure S6. Bipolar cells and cone and rod photoreceptor outer segments in retinal cross-sections.

Figure S7. Cone photoreceptor numbers in retinal flat-mounts.

Figure S8. Aflibercept does not alter the thickness of the retina or its nuclear layers.

Table S1. List of primary and secondary antibodies used in this study to immunofluorescently label cross-sections and flat-mounted retinas.

Table S2. Primers used for real time RT-qPCR.

Table S3. List of differentially up-regulated transcripts at P14 sorted by the Affymetrix Transcript Cluster Id.

Table S4. List of differentially down-regulated transcripts at P14 sorted by the Affymetrix Transcript Cluster Id.

Table S5. List of differentially up-regulated transcripts at P17 sorted by the Affymetrix Transcript Cluster Id.

Table S6. List of differentially down-regulated transcripts at P17 sorted by the Affymetrix Transcript Cluster Id.

Table S7. Differentially regulated genes (DRGs) in retinas from OIR+AFL mice relative to those of OIR animals at both P14 and P17 sorted by the affymetrix Transcript Cluster Id.

Video S1. Representative three-dimensional reconstruction and volume render of a tip cell along the VO perimeter of a P17 OIR retina. CD146, green; NG2, red; filopodia, yellow. Scale: 10 μm.

Video S2. Representative three-dimensional reconstruction and volume render of a tip cell along the VO perimeter of a P17 OIR+AFL retina. CD146, green; NG2, red; filopodia, yellow. Scale: 10 μm.

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