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Spatial and temporal recruitment of the neurovascular unit during development of the mouse blood-retinal barrier

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ABSTRACT

The inner blood-retinal barrier (BRB) is made up by the neurovascular unit, consisting of endothelial cells, pericytes and glial cells. The BRB maintains homeostasis of the neural retina, but in pathological eye conditions the neurovascular unit is often disrupted, causing BRB loss. Here, we investigated in detail temporal and spatial recruitment of the neurovascular unit in the neonatal mouse retina from postnatal day (P)3 to P25 employing immunohistochemical staining of vascular endothelium (isolectin B4), pericytes (α-SMA and NG2) and astrocytes (GFAP). In addition, we investigated gene expression of polarized astrocytic end-feet markers aquaporin-4 and laminin α2 chain with qPCR. We observed GFAP-positive cells migrating ahead of the retinal vasculature during the first postnatal week, suggesting that the retinal vasculature follows an astrocytic meshwork. From P9 onwards, astrocytes acquired a mature phenotype, with a more stellate shape and increased expression of aquaporin-4. NG2-positive cells and tip cells co-localized at P5 and invaded the retina together as a vascular sprouting front. In summary, these data suggest that recruitment of the cell types of the neurovascular unit is a prerequisite for proper retinal vascularization and BRB formation.

1. Introduction

The neural retina is protected by the inner blood-retinal barrier (BRB), which maintains homeostasis by selectively regulating the entry of molecules into the retina and controlling vascular permeability (Klaassen et al., 2013). The BRB consists of endothelial cells lining the retinal microvessels, pericytes, their basal lamina and glial cells, together forming the neurovascular unit. The presence and crosstalk between these cell types in the neurovascular unit is essential for the maintenance of a tight BRB (Wisniewska-Kruk et al., 2012).

In humans, the retinal vasculature is completed around mid-gestation (Gariano, 2003), but in mice, retinal vascular development starts in the first postnatal week. The retinal vasculature provides oxygen and nutrients to the inner retina, which has several (anatomically tightly-arranged) cellular layers that contain interneurons, ganglion cells and glial cells. Vessels grow from the optic disc into the ganglion cell layer (containing ganglion cells, amacrine cells and (peri-)vascular cells) and radiate outwards to the periphery, that is reached around postnatal day (P) 7. From this superficial vascular plexus, collateral sprouting of capillaries into the inner plexiform layer (where photoreceptor input is processed through bipolar cells, amacrine cells and ganglion cells) and into the inner nuclear layer up to the boundaries of the outer plexiform layer (where photoreceptor cells form connections with bipolar cells and horizontal cells) initiates generation of the interconnected intermediate and deep capillary layers (Fruttiger, 2007). The retinal pigment epithelium and photoreceptors are part of the outer retina and are supplied with oxygen and nutrients by a different vascular bed, the choriocapillaris, which is not discussed here.

Initially, the retinal vasculature lacks a functional barrier during development, as is demonstrated by leakage of plasma proteins and small molecules from the lumen of the vessels (Chow and Gu, 2017; van der Wijk et al., submitted). Recruitment of pericytes and astrocytes to the retina to form the neurovascular unit may be crucial for final maturation of the endothelium and for the formation of a functional BRB.

Previously, it has been shown that astrocytes serve as a meshwork for growing vessels in the retina, thereby guiding endothelial cell migration (Dorrell et al., 2002;O'Sullivan et al., 2017). In addition, pericytes may be involved in the early formation of the BRB by providing a

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suitable microenvironment for retinal endothelial cells to develop barrier characteristics, as was shown in blood-brain barrier development (Daneman et al., 2010). Moreover, permeability of the blood-brain barrier in neonatal mice inversely correlated with pericyte coverage (Armulik et al., 2010; Daneman et al., 2010), confirming the essence of pericytes in barrier integrity. As the pericyte-to-endothelial ratio is relatively high in the retina (1:1), when compared to brain (1:3) and other microvascular beds (1:10) (Stewart and Tuor, 1994) such a function of pericytes may be particularly important in the BRB.

In the present study, we investigated in detail the temporal and spatial composition of the neurovascular unit in the neonatal mouse retina using gene and protein expression of selected markers of retinal pericytes and astrocytic end-feet.

2. Materials and methods

2.1. Animals

Animal experiments were performed with the approval of the Animal Ethics Committee of the University of Amsterdam and in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. To study the development of the BRB, neonatal (wildtype) mice were killed on P3, P5, P7, P9, P11, P13, P15, P17 and P25 with an intracardial injection of ketamine-medetomidine-atropine for young mice (until P13), whereas older mice were euthanized with CO_2 asphyxiation. Eyes were enucleated and either snapfrozen in liquid nitrogen (for qPCR and immunohistochemistry) or processed immediately for retinal wholemount staining.

2.2. RNA isolation and mRNA quantification

Retinas (at least 6 to 8 retinas per group) were treated by hypotonic lysis to enrich for retinal vessels (Kowluru et al., 1998). Each retina was incubated in 1 ml sterile water for 2 h at 4 °C. Next, retinas were spun down and sterile water was replaced with sterile water containing 40 µg DNase I (Life Technology, Breda, The Netherlands) and left for 5 min at room temperature. Retinas were spun down, supernatant was removed and the retinal vessels were resuspended in 500 µl TRIzol reagent (Invitrogen, Bleiswijk, The Netherlands) and stored at −20 °C until further processing. Total RNA was isolated according to the manufacturer's protocol and dissolved in RNAse-free water. RNA yield was measured using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) and 1 µg of RNA was treated with DNAse-I (Invitrogen) and reverse transcribed into first strand cDNA with a Maxima First Strand cDNA Synthesis Kit (ThermoFisher). Real-time quantitative PCR was performed on 20× diluted cDNA samples using a CFX96 system (Bio-Rad, Hercules, CA) as described previously (Klaassen et al., 2009). Specificity of the primers was confirmed by NCBI BLAST. The presence of a single PCR product was verified by both the presence of a single melting temperature peak and detection of a single band of the expected size on 3% agarose gel. Non-template controls were included to verify the method and the specificity of the primers. Relative gene expression was calculated using the equation: $R = E^{-Ct}$, where E is the mean efficiency of all samples for the gene being evaluated and Ct is the cycle threshold for the gene as determined during real-time PCR. Primer efficiencies (E) were determined with LinRegPCR software (Ruijter et al., 2009) and ranged from 1.631 to 1.973. PCR products that did not show a single melting temperature peak were excluded from analysis. Expression data was normalized by the global mean normalization method (Mestdagh et al., 2009) using expression data of 30 genes in total.

2.3. Retinal wholemount staining

Enucleated mouse eyes were washed in PBS and fixed in 4% paraformaldehyde for 5 min, transferred to 2× PBS for 10 min and retinas were dissected in PBS. Isolated retinas were post-fixed in methanol and stored in −20 °C until further use. For immunofluorescence staining, retinas were briefly washed in 2x PBS and incubated in wholemount-blocking buffer (1% fetal calf serum, 3% TritonX-100, 0.5% Tween20, 0.2% sodium azide in 2x PBS) for 2 h at room temperature. Next, retinas were incubated overnight with the following antibodies: rabbit anti-glial fibrillary acidic protein (diluted 1:400, Cat # Z0334; GFAP; Dako, Heverlee, Belgium), mouse monoclonal anti-alpha-smooth muscle actin (α-SMA) antibody (diluted 1:200, Cat # C6198; Cy3 labeled, Sigma-Aldrich, Zwijndrecht, The Netherlands), isolectin B4 (diluted 1:30, Cat # I21411; Alexa Fluor-488 labeled, Invitrogen) or rabbit polyclonal anti-NG2 antibody (diluted 1:100, kindly provided by prof. W. Stallcup from the Burnham Institute, La Jolla, CA) diluted in wholemount blocking buffer. GFAP was used as a marker for astrocytes, α-SMA as marker for smooth muscle cells and pericytes, isolectin B4 was used to detect endothelial cells and NG2 was used as marker for pericytes. After 3 wash steps (3 times 30 min in wholemount blocking buffer), secondary antibody was added and wholemounts were incubated for 2–3 h (goat-anti-rabbit Alexa Fluor-633 or goat-anti-rabbit Cy3; Invitrogen, diluted 1:100 in wholemount blocking buffer). After overnight washing in wholemount blocking buffer, retinas were mounted on glass and covered in Vectashield (Vector Laboratories, Burlingame, CA). All staining procedures were performed under gentle agitation at room temperature.

For staining of the cryostat sections, samples were fixed for 10 min using 4% paraformaldehyde, permeabilized for 10 min with 0.2% Triton X-100 and blocked for 1 h with 10% normal goat serum. Staining was performed in wholemount blocking buffer with anti-rabbit Apq4 (diluted 1:100, Cat # AB2218; Millipore, Amsterdam, The Netherlands) for 2 h, followed by secondary antibody incubation (goat-anti-rabbit Cy3) for 1 h.

Images of retinal wholemounts were taken at the central retina (at the site of the optic disc), the middle retina and the peripheral retina and were captured using a confocal laser scanning microscope SP8 (Leica Microsystems, Wetzlar, Germany) with a 20x or 63x objective at the Cellular Imaging Core Facility of the Academic Medical Center. Specificity of the staining was checked by absence of fluorescent signal in samples that were stained in the absence of primary antibody.

2.4. Transmission electron microscopy (TEM)

Eyes were harvested and immersion fixed in McDowell fixative in phosphate buffer. To facilitate access of the fixative into the eye, eyes were punctured with a 29 G (which equals a diameter of 0.287 mm) needle and the cornea was dissected. Samples were processed for routine TEM, as described previously (Wisniewska-Kruk et al., 2016). Ultrathin sections of 80 nm were examined with a Technai-12 G2 Spirit Biotwin microscope (FEI, Eindhoven, The Netherlands) and micrographs were captured with a Veleta TEM camera (Emsis; Münster, Germany) using Radius acquisition software (Emsis) at a magnification of 11.000–30.000×, at the Electron Microscopy Center of the Academic Medical Center.

3. Results and discussion

3.1. Ultrastructure of the neurovascular unit

The close association of endothelial cells and pericytes, which share their basal lamina, and glial cells, which envelope the retinal vessels with their end-feet and a second basal lamina, is referred to as the neurovascular unit of the BRB in the mature retina (Fig. 1A–C). The endothelial cells of the BRB have no fenestrations, few pinocytotic vesicles and a continuous array of inter-endothelial tight junctions, which seal the intercellular space between endothelial cells (Klaassen et al., 2013)(Fig. 1B and b).

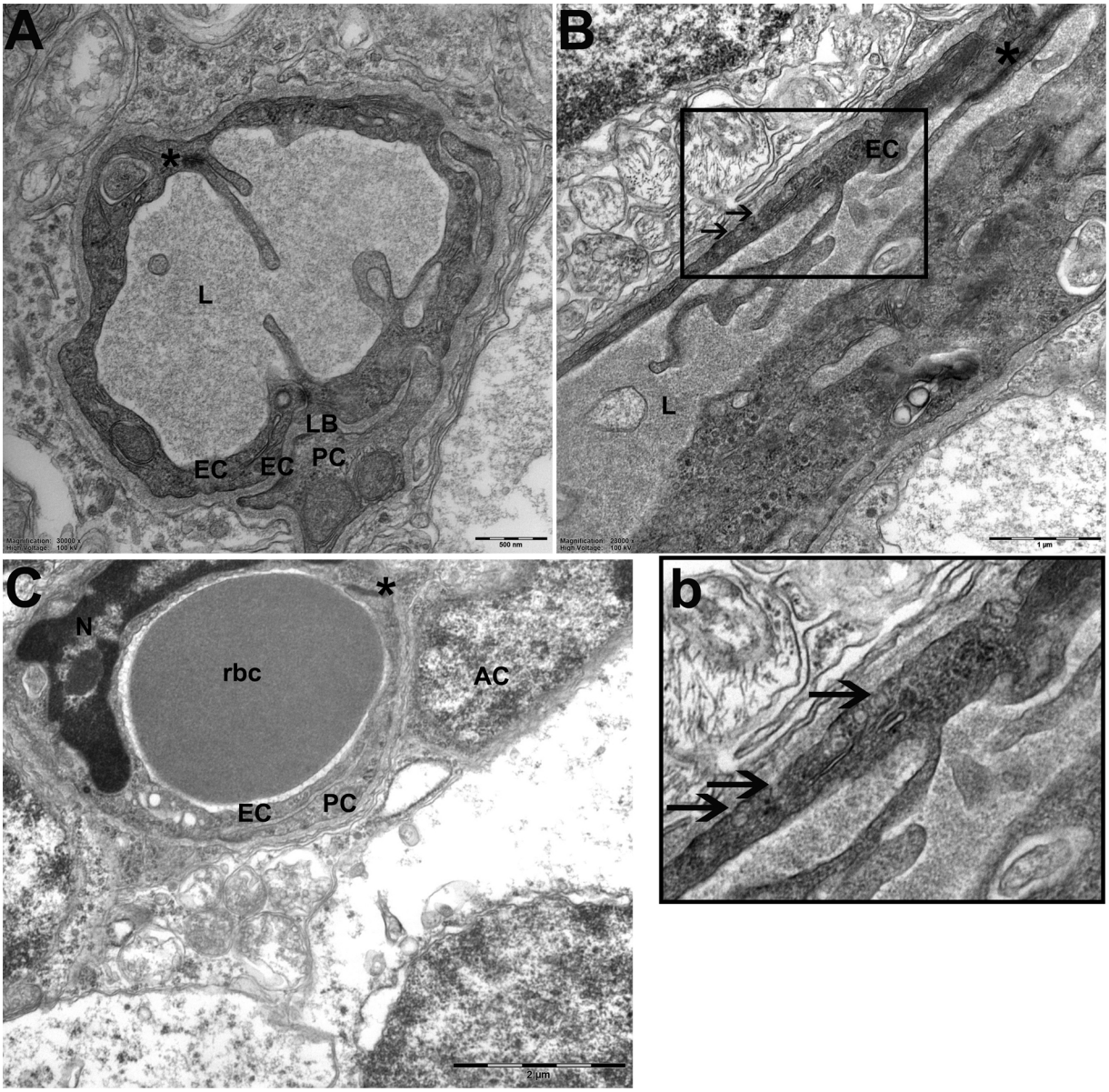

Fig. 1. Ultrastructure of the neurovascular unit of the BRB of a mouse at P30. (A) Endothelial cells and pericytes share a basal lamina and are in close association. (B) Endothelial cells of the BRB have few pinocytotic vesicles and tight junctions seal the intercellular space in between endothelial cells, (b) is a magnification of the boxed region in (B). (C) Astrocytic end-feet envelope the retinal vessels and are in contact with pericytes and endothelial cells. EC = endothelial cell, PC = pericyte, AC = astrocyte, LB = basal lamina, L = vessel lumen, N = nucleus, rbc = red blood cell, * = tight junction, black arrow = abluminal caveola. Scale bar = 500 nm (A), 1 µm (B), 2 µm (C).

3.2. An astrocytic meshwork precedes the vascular sprouting front during retinal development

Astrocytes have been shown to induce barrier properties of blood-brain barrier and BRB cells *in vitro* (Nakagawa et al., 2009; Wisniewska-Kruk et al., 2012), and they are a major source of VEGF during pathological retinal vascularization (West et al., 2005). The retinal vasculature appeared to follow an astrocytic meshwork when forming the superficial vascular plexus during development (Fig. 2A), which confirmes previous studies (Dorrell et al., 2002; Gariano and Gardner, 2005; O'Sullivan et al., 2017), as was detected by GFAP staining in retinal wholemounts. GFAP-positive cells emerged from the optic disc and were found in close contact with retinal vessels in the ganglion cell layer at all time points (Fig. 2A–I), but not in the intermediate and deep capillary layers (data not shown). The time of appearance of glial cells during vascular development is different between retina and brain. In the rat cerebral cortex, angiogenesis begins as early as E12, whereas astrocytes are first generated directly after birth, and physical contact with vessels is made during the first postnatal week (Daneman et al., 2010). In the retina, astrocytes appear before vascularization, and both ablation of astrocytes altogether, or shortly after birth during early postnatal development, resulted in severely impaired retinal vascularization and mispatterning of growing vessels (O'Sullivan et al., 2017), suggesting that the role of astrocytes in vascularization of the retina is more critical than in the brain. In the retina, astrocytes are tightly wrapped around arteries and even more so around veins (Fig. 2C–I), and GFAP expression is stronger in the central retina as compared to the middle and peripheral retina (Fig. 2C). From P9 onwards, the astrocytes had a more stellate shape (Fig. 2D–I), indicating a more mature astrocyte phenotype (West et al., 2005).

Astrocytes make contact with blood vessels through their astrocytic end-feet, which are polarized structures that firmly attach to the

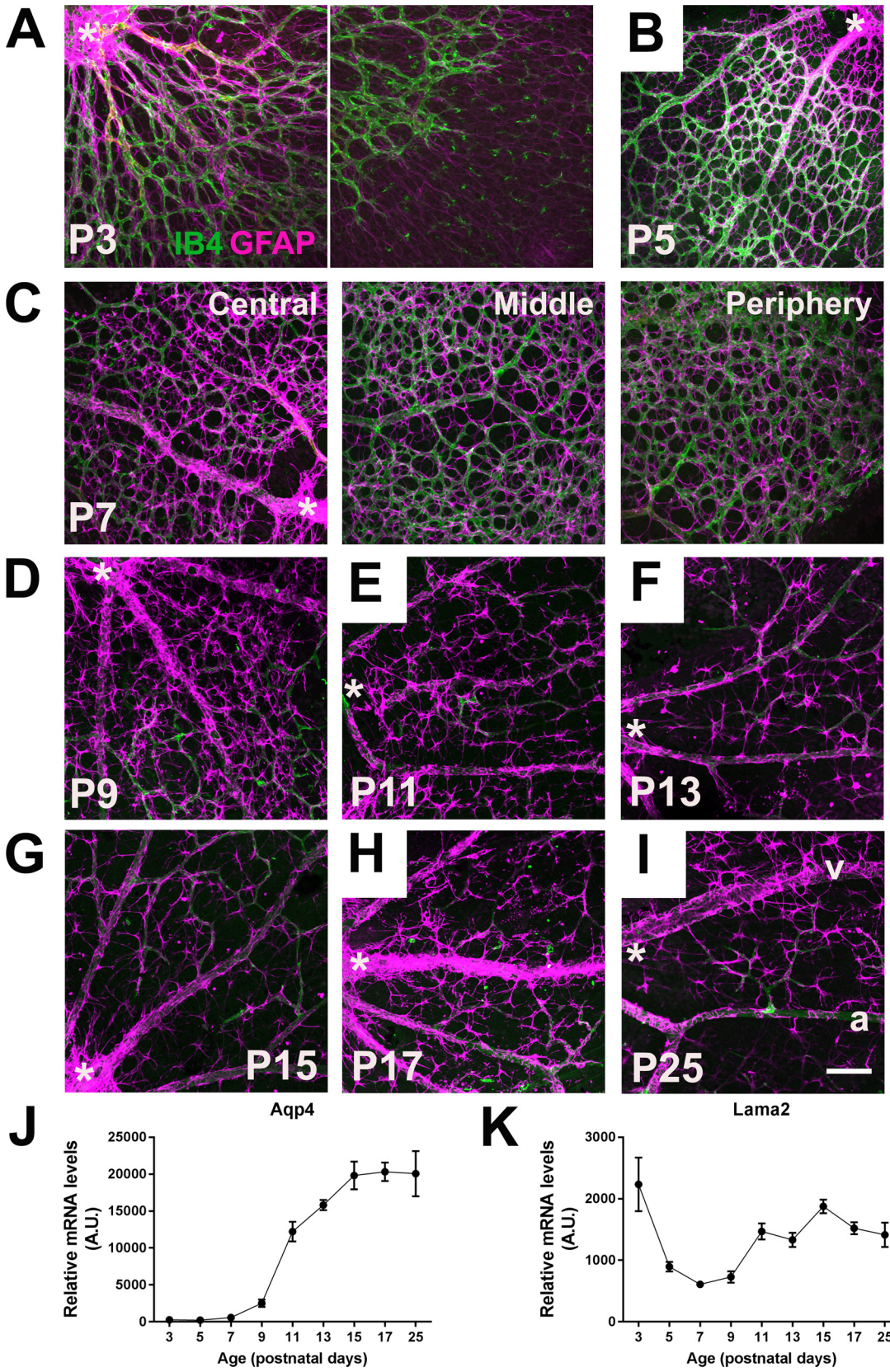

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Fig. 2. Astrocytes precede the retinal vasculature during development. (A) At P3, astrocytes emerge from the optic disc (indicated by an asterisk) and an astrocytic meshwork was preceding the retinal vasculature, as shown with immunostaining of retinal wholemounts for GFAP (purple). Vasculature is stained by using isolectin B4 (green). GFAP-positive cells are found in close association with retinal vessels in the superficial vascular plexus at (B) P5, (C) P7, (D) P9, (E) P11, (F) P13, (G) P15, (H) P17 and (I) P25. mRNA expression of the marker for polarized astrocytic end-feet Aqp4 (J) increases during development of the retina, whereas (K) Lama2 mRNA levels decrease from P3 to P7-9 and stabilize from P9 onwards. n = 7–11 for all time points. Data are depicted as mean ± s.d. At the protein level, we observed a few Aqp4-positive cells in the ganglion cell layer at P9 (L), as shown in retinal cryostat sections. At P13 (M) and P25 (N), there was strong Aqp4 expression around retinal vessels in all the 3 vascular layers. (O) Higher magnification of a retinal vessel which is enveloped by Aqp4-expressing astrocytic end-feet. In (L–O), brightness of the IB4 and Aqp4 signal was enhanced to the same extent at all ages, to improve clarity. IB4 = isolectin B4, GFAP = glial fibrillary acidic protein, Aqp4 = aquaporin 4, a = artery, v = vein, * = optic disc. Scale bar (A–I) = 100 µm, (L–N) = 50 µm, (O) = 15 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

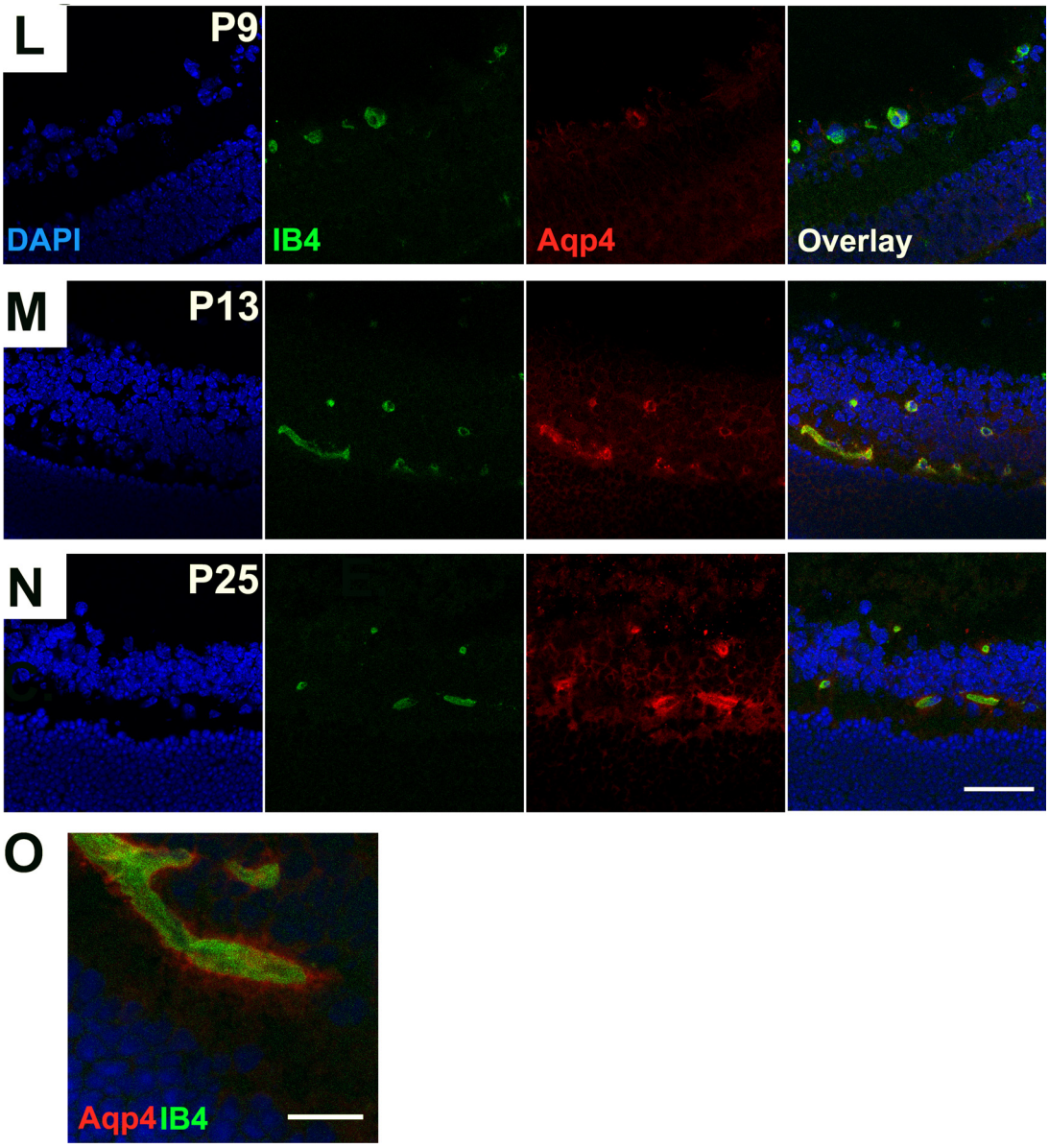

Fig. 2. (*continued*)

abluminal surface of capillaries (Abbott et al., 2006). We investigated the developmental expression pattern of 2 markers of polarized astrocytic end-feet, aquaporin 4 (Aqp4) and laminin α2 chain (Lama2). Expression of Aqp4, a water channel protein involved in the control of ion concentration and volume regulation (Nagelhus et al., 1998), increased significantly during development. There was very low mRNA expression during the first postnatal week, followed by a steep increase from P9 to P15 (Fig. 2J). This was confirmed at the protein level in retinal cryostat sections, where there was no Aqp4 expression at P5 (data not shown) and only few Apq4-positive cells around vessels in the ganglion cell layer at P9 (Fig. 2L). At P13 and P25, strong Aqp4 expression was observed around retinal vessels in all the 3 vascular layers (Fig. 2M–O). This pattern may be consistent with astrocyte end-feet, as it coincided with the time point that astrocytes acquire a stellate shape (Fig. 2D), a phenotype which suggests that the cells have become mature and polarized. Aqp4 is also expressed by horizontal cells and

Müller cells (Bosco et al., 2005; Hamann et al., 1998), but the staining pattern we observed was not consistent with the localization of these cell types. It has been suggested that the specific Aqp4 localization at the end-feet membrane of astrocytes allows transcellular water redistribution and the maintenance of extracellular osmolality during neuronal activity, without inducing inappropriate volume changes in the extracellular space (Hamann et al., 1998; Nagelhus et al., 1998). If this is the case, the marked increase in retinal perivascular Aqp4 expression between P9 and P15, in either astrocytes and/or other glial cells, may be related to the opening of the mouse eye (around P14), which is preceded by increased spontaneous retinal waves (i.e., bursts of action potentials among neighboring retinal ganglion cells)(Ackman et al., 2012; Rochefort et al., 2009; Thompson et al., 2017).

Besides the shared basal lamina of endothelial cells and pericytes, astrocytes provide a second basal lamina that is in contact with the retinal endothelial cells (Fig. 1C). Lama2 is an astrocyte-derived basal lamina component, and also a marker of polarized astrocytic end-feet. In the blood-brain barrier, Lama2 expression is critically involved in gliovascular-pericyte interactions (Armulik et al., 2010; Menezes et al., 2014). Moreover, *Lama2*$^{-/-}$ mice had significant vascular abnormalities and a defective (leaky) blood-brain barrier (Menezes et al., 2014). During development, we observed a ~2-fold decrease in Lama2 mRNA expression from P3 to P7-9, and stable Lama2 mRNA expression from P11 onwards (Fig. 2K).

3.3. Expression of alpha-smooth muscle actin (α-SMA) increases during retinal development

The interaction of smooth muscle cells and pericytes with endothelial cells is crucial for vessel stabilization. Unlike pericytes in other parts of the body, pericytes of the central nervous system are considered to originate from the neural crest (reviewed in Armulik et al., 2011). α-SMA is the predominant actin isoform found in smooth muscle cells, and is also expressed by a subset of mature pericytes (Nehls and Drenckhahn, 1991). In the retinal vasculature, expression of α-SMA is associated with smooth muscle cell and pericyte maturation and vessel stability (Benjamin et al., 1998). Smooth muscle cells and pericytes express growth factors that regulate whether vessels are stable or undergo remodeling. Therefore, a role has been suggested for these cells in pruning of retinal vessels during the formation of a highly organized vasculature (Uemura et al., 2002). Here, we observed a relative decrease in α-SMA mRNA expression during development (Fig. 3A). However, at P3, α-SMA protein expression was weak and only visible in the most proximal part of vessels of the central retina (Fig. 3B), indicating that there is a proximal-to-distal gradient of maturation in the retinal vessels, as has been reported previously (Fruttiger, 2007; Hughes and Chan-Ling, 2004). α-SMA protein expression increased during maturation of the retinal vasculature (Fig. 3B–J) and this allowed for the identification of arteries and veins, since α-SMA is expressed in higher amounts around arteries (characterized by tightly packed actin filaments) than veins (with a more diffuse and patchy staining, Fig. 3K; (Hughes and Chan-Ling, 2004)), and is not expressed around capillaries (data not shown). Of course, we cannot infer from these data whether α-SMA expression had any effect on the vascular remodeling processes that occur in the 2 weeks after birth. However, in α-SMA null mice, differences in retinal vascular patterning were not observed when compared to wildtype mice, and all vessels were covered by smooth muscle cells and pericytes, suggesting that α-SMA expression is not necessary for the development of a normal retinal vascular pattern (Tomasek et al., 2006). Nevertheless, lack of α-SMA expression resulted in altered BRB function, including increased BRB permeability and decreased retinal function (Tomasek et al., 2006). Therefore, the presence of α-SMA appears to be crucial for a fully functional BRB.

3.4. NG2-positive pericytes invade the retina together with the vascular sprouting front during retinal development

Pericytes are required for the formation of the blood-brain barrier and are essential for stabilization of mature vessels (Armulik et al., 2010; Daneman et al., 2010). To date, a marker that is entirely pericyte-specific is not known, and the markers that are currently used may differ in expression during different developmental or pathological stages (Armulik et al., 2011). Expression of CD13 mRNA, a type II membrane metalloprotease, that is used to identify brain pericytes (Kunz et al., 1994), increased during development of the retina, and stabilized around P11 (Fig. 4A). NG2 mRNA expression, a chondroitin sulfate proteoglycan that is considered to be one of the most reliable pericyte markers (Hughes and Chan-Ling, 2004; Schlingemann et al., 1990), was relatively stable during development (Fig. 4B). NG2 protein expression was found at all time points and stained pericytes ensheathing arteries, capillaries and veins (Fig. 4C–F). We observed NG2-positive cells in close proximity to the vascular tip cells at P5 (Fig. 4D). Perivascular NG2 staining was observed in the superficial vascular plexus, as well as in the descending intermediate and deep capillary layers (Fig. 4E and F), and was present around all vessel types in the retina (Fig. 4G). mRNA expression of N-cadherin, a cell-adhesion molecule which is involved in endothelial cell – pericyte interactions (Armulik et al., 2011), initially decreased from P3 to P11, the period associated with high angiogenic activity, and stabilized from P11 onwards (Fig. 4H). Together, these data suggest that pericytes are present around endothelial tubes during early vessel formation, and that they localize in the vicinity of tip cells. This is not completely in line with an earlier report by Fruttiger (2002), who showed by in situ hybridization of PDGFRβ, in combination with immunofluorescence of collagen type IV in retinal wholemounts, that pericyte recruitment lagged slightly behind the leading edge of the sprouting network (Fruttiger, 2002). However, this discrepancy can be explained by the use of different markers, since PDGFRβ-positive cells are co-recruited with angiogenic sprouts when PDGF-B is released by angiogenic endothelial cells (Armulik et al., 2011), whereas NG2-positive cells have been shown to be activated, extramural pericytes (Schlingemann et al., 1990) which may play a role in early stages of angiogenesis (Ruiter et al., 1993). High molecular weight-melanoma associated antigen (HMW-MAA), the previous name of human NG2, has been shown to be a marker of pericytes associated with vascular sprouts and capillaries during angiogenesis (Schlingemann et al., 1990; Ruiter et al., 1993).

The essential role of pericytes in maintaining the integrity of the blood-brain barrier during development has been well established. Two studies showed that absolute pericyte coverage of brain vessels controls relative blood-brain barrier permeability (Armulik et al., 2010; Daneman et al., 2010), and that recruitment of pericytes to the vessels temporally correlated with the onset of barrier properties (Daneman et al., 2010). This is not the case in the BRB, where pericytes are already associated with retinal vessels long before the BRB is completely functional, which is, in the mice that we investigated, around P15 (van der Wijk et al., submitted). In addition, a recent study showed that there was no apparent vascular remodeling and leakage after pericyte ablation in the adult retina, whereas during postnatal development, pericyte ablation caused severe vascular defects and BRB disruption (Park et al., 2017). Nevertheless, in pathological conditions such as diabetic retinopathy, early hallmarks are pericyte loss and increased BRB permeability (Hammes, 2005). Thus, in the retina, pericyte recruitment may not be directly and temporally related to barrier genesis, but close

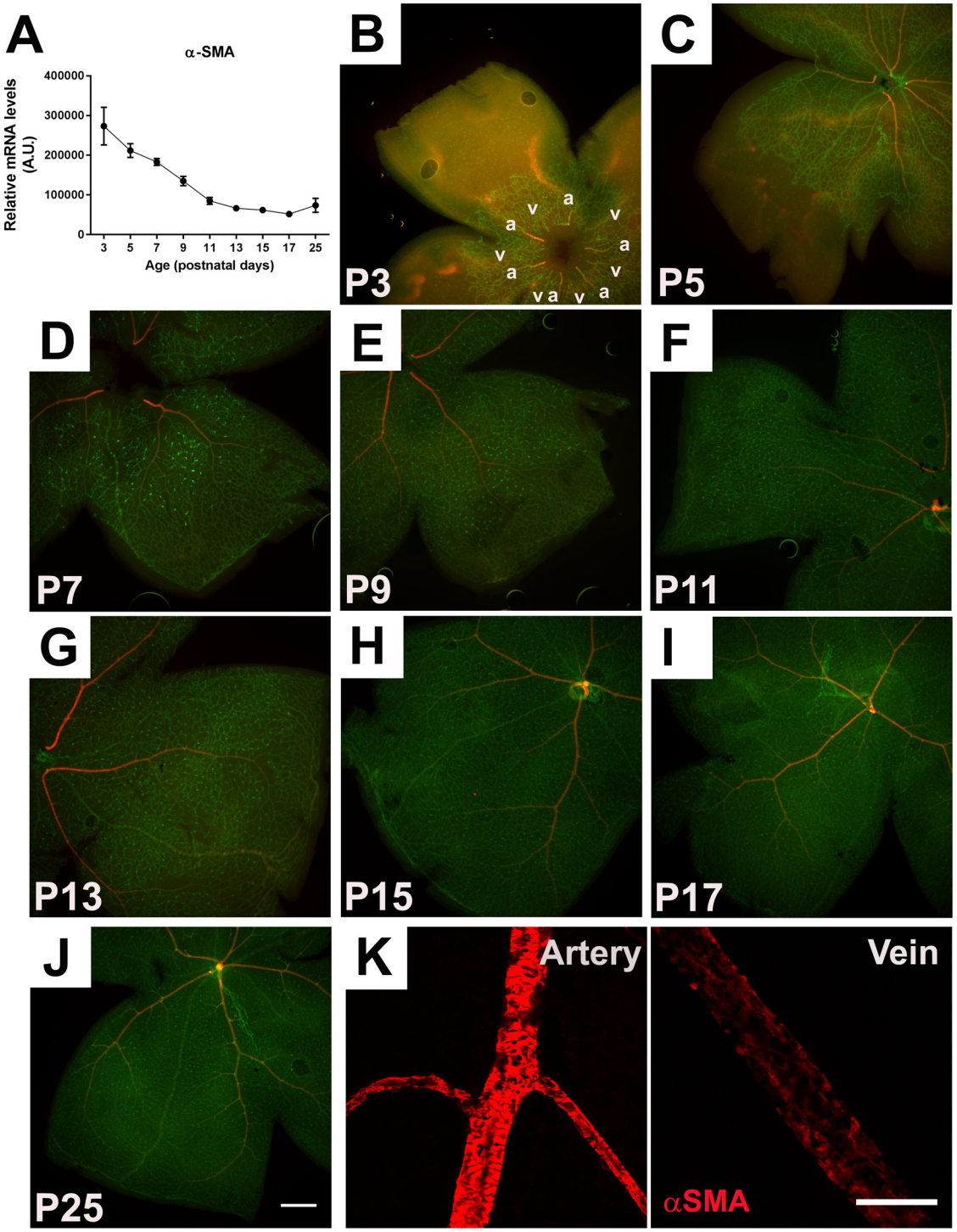

Fig. 3. Expression of α-SMA increases during development. (A) mRNA of α-SMA decreases during development. n = 7–11 for all time points. Data are depicted as mean ± s.d. (B) At P3, α-SMA expression (red) is weak and only expressed at the most proximal part of vessels in retinal wholemounts. Retinal vessels are stained with isolectin B4 (green). Expression of α-SMA protein increases during development in retinal wholemounts at (C) P5, (D) P7, (E) P9, (F) P11, (G) P13, (H) P15, (I) P17 and (J) P25. In (A–I), green and red levels were adjusted to improve clarity. (K) In arteries, α-SMA expression is strong and characterized by tightly packed actin filaments, whereas α-SMA staining in veins is more diffuse and patchy. IB4 = isolectin B4, α-SMA = alpha-smooth muscle actin, a = artery, v = vein. Scale bar (B–J) ~450 μm. Scale bar (K) = 50 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

association of pericytes with retinal vessels may be essential for BRB formation and function.

4. Concluding remarks

Here, we provide a spatio-temporal overview of recruitment and maturation of the neurovascular unit during development of the mouse BRB. Although the BRB closely resembles the blood-brain barrier in terms of function and molecular and cellular composition, there are some differences in vascular and barrier development. In the retina, astrocytes migrate ahead of the vasculature and even provide a template along which the retinal vasculature can grow, which is not the

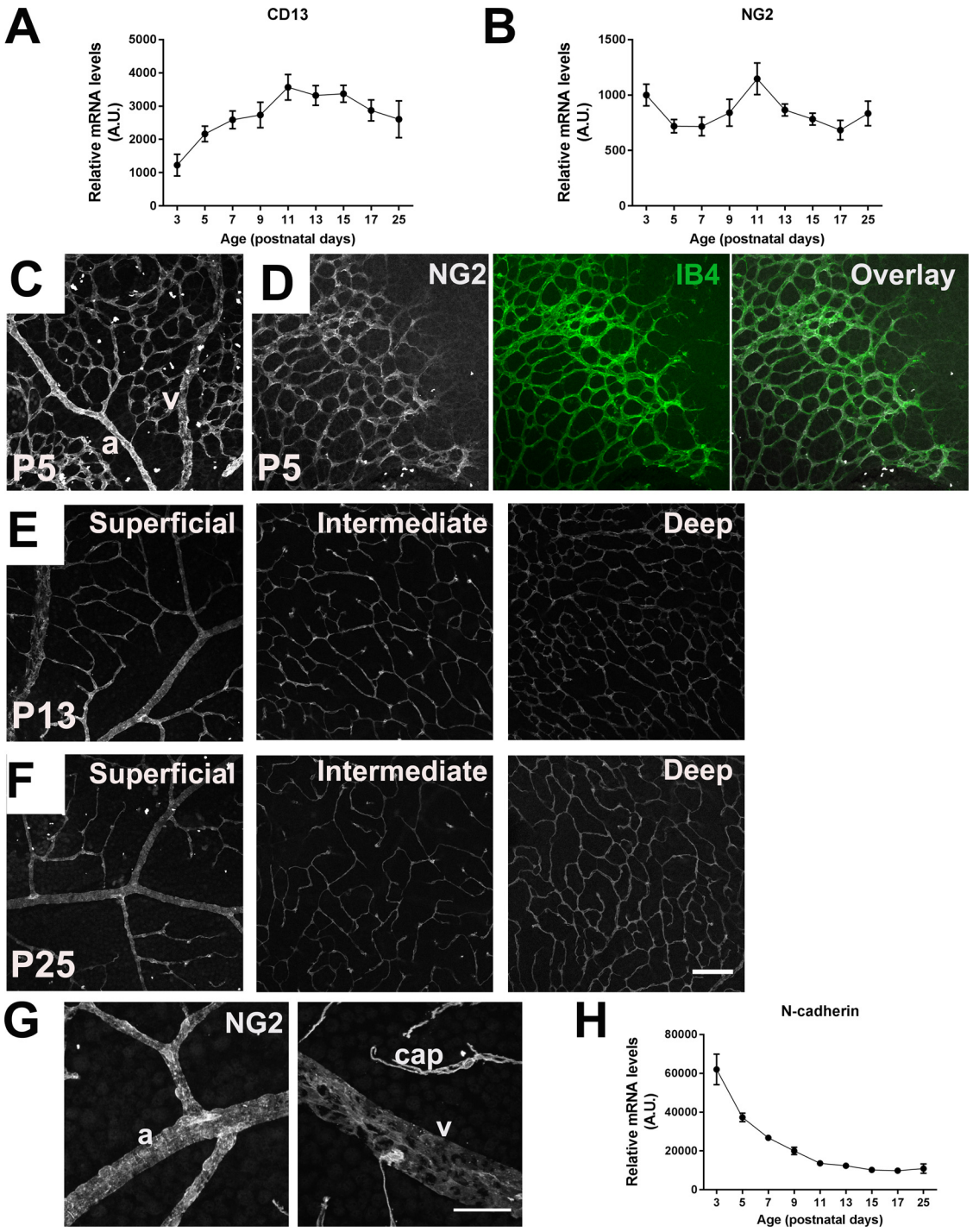

Fig. 4. Pericytes precede the vascular sprouting front during retinal development. (A) Expression of CD13 mRNA in the retina increases during development, and stabilizes around P11. (B) Expression of NG2 mRNA is relatively stable during retinal development. n = 7–11 for all time points. Data are depicted as mean ± s.d. (C) At P5, NG2 protein expression (white) is visualized in retinal wholemounts and show pericytes ensheathing arteries, capillaries and veins. (D) At P5, NG2-positive cells are in close proximity of tip cells in the retinal sprouting front stained with isolectin B4 (green) in the ganglion cell layer. Perivascular NG2 staining is observed in the superficial vascular plexus, and the intermediate and deep capillary layers at P13 (E) and P25 (F). (G) NG2 expression in a retinal artery and vein at higher magnification. (H) mRNA expression of N-cadherin decreased during development. IB4 = isolectin B4, a = artery, cap = capillary, v = vein. Scale bar = 100 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

case in the brain. In addition, we observed NG2-positive extramural pericytes in close proximity to vascular tip cells. Moreover, pericytes are associated with retinal vessels before the BRB is functional, whereas the timing of barrier genesis in the brain has been temporally correlated with pericyte recruitment. Considering the astrocytic template, and the fact that the pericyte-to-endothelial ratio is even higher in the retina than it is in the brain (Stewart and Tuor, 1994), we suggest that perivascular cells may be of greater importance in the retina, when compared to the brain. Together, our data indicate that recruitment of all cell types of the neurovascular unit is a prerequisite for proper retinal vascularization, and that a functional BRB is formed after maturation of the neurovascular unit. In the mouse, a functional BRB is completed

around the time of eye opening and may be required for the increased sensory input which accompanies vision.

Author contributions

AEvdW designed and performed experiments, analyzed data and wrote the manuscript; IMCV performed experiments; HAvV performed the EM analysis; IK analyzed data, contributed to the study design, discussion, and editing of the manuscript, CJFvN and ROS contributed to the study design, discussion, and editing of the manuscript. All authors have read and approved the final manuscript.

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