Intersublamina Vascular Plexus: The Correlation of Retinal Blood Vessels With Functional Sublaminae of the Inner Plexiform Layer

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PURPOSE. Interactions between vasculature and neurons provide important insight into the function of the nervous system, as well as into neurological diseases wherein these interactions are disrupted. This study characterizes a previously unreported retinal vascular plexus and examines potential sites of neurovascular interaction.

METHODS. Vascular, neuronal, and glial elements were visualized using immunohistochemical markers. The distribution of vascular layers was measured and compared across eccentricities. Intensity profiles were calculated from confocal image reconstructions to reveal the proximity of vasculature to neuronal and glial processes.

RESULTS. Retinal vasculature forms a plexus that coincides with the dendritic processes of OFF cholinergic amacrine cells within the inner plexiform layer. Across eccentricities, this plexus comprises approximately 8% of the total length of horizontally running blood vessels in the retina. Processes of Müller glia and OFF cholinergic amacrine cells colocalize with the blood vessels that form the intersublamina plexus.

CONCLUSIONS. In the retina, vasculature lacks autonomic control, but shows efficient local regulation. Although the source of this regulation is unclear, these results suggest that cholinergic amacrine cells and Müller glia may interact with the intersublamina plexus to influence vasomotor activity. This may indicate a key role in modulating reciprocal interactions between neuronal activity and blood flow.

Keywords: vasculature, glia, retina, neurovascular unit, inner plexiform layer

Neurons, glia, and vasculature form an interdependent physical and biochemical association that sustains homeostatic function within the central nervous system. This relationship is known as the neurovascular unit.¹ The retina has the highest energy demand of any tissue in the body,² exceeding the oxygen consumption of the higher brain,³ making it an ideal candidate for studying neurovascular interactions. Additionally, new insights into retinal physiology suggest that the dysfunction associated with diseases such as retinal degeneration and diabetic retinopathy may be interpreted as an alteration of the retinal neurovascular unit.¹

In contrast to other brain regions, retinal blood vessels (BVs) lack autonomic regulation.^{4,5} Growing evidence suggests that retinal blood flow may be affected in an activity-dependent manner by neurons.⁶ For example, flicker stimulation of the eye evokes increases in retinal vessel diameter and retinal blood flow.⁷ When the retina is stimulated with a small flickering spot, blood flow increase is limited to the stimulated region, suggesting tight local control of blood flow.⁸ The source of this local regulation is unclear, although it may be coupled with neuronal and glial activity. Thus, the link among vasculature, glia, and neurons becomes the key component to understanding retinal physiology in normal and pathological conditions.

The aim of the present study was to investigate the retinal neurovascular unit. In the retina, a variety of neurons,⁹ glia,

and vascular cells have unique properties and occupy precise locations. Thus, it was expected that the composition and functional properties of the neurovascular unit will vary between distinct retinal regions. To test this, BVs, neurons, and glia were labeled with immunohistochemical markers and visualized using confocal microscopy. This revealed a previously unreported vascular plexus that coincides with the processes of OFF-cholinergic amacrine cells, which stratify in a narrow band within the inner plexiform layer (IPL), and may be interpreted as a border between sustained and transient ganglion cells.^{10–13} Due to its location within the IPL, this is referred to as the intersublamina plexus. Because of its location at a site of mixed neuronal input, it is possible that neurovascular interactions occur at this plexus. Indeed, the BVs of the intersublamina plexus receive extensive contacts from Müller glial cells, which coincide with cholinergic amacrine cell processes, suggesting that these cells have a role in mediating neurovascular interactions.

MATERIALS AND METHODS

Animals

In all experimental procedures, animals were treated according to the regulations in the ARVO Statement for the Use of Animals

in Ophthalmic and Vision Research, in compliance with protocols approved by the Institutional Animal Care and Use Committee Weill Cornell Medical College, and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice homozygous for the Thy1-YFP allele (B6.Cg-Tg(thy1-YFP)/J) and wild-type mice (C57BL/6J) of either sex were obtained from the Jackson Laboratory (Bar Harbor, ME).

Flat-Mount Retinal Preparation

After the animal was euthanized, its eyes were enucleated and placed in bicarbonate-buffered Ames medium (Sigma, St. Louis, MO) continuously equilibrated with O₂/CO₂. The retina was dissected and attached photoreceptor surface down to a modified hydrophilic Biopore Insert (Millipore, Bedford, MA), as described in detail elsewhere.¹⁴ The preparation was then transferred to a chamber and bathed (1 mL/min) with bicarbonate-buffered Ames medium at 32°C.

Immunohistochemistry

Mice at 1 to 2 months of age were deeply anesthetized with CO₂ and killed by cervical dislocation. Retinas were fixed in the eyecups with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH = 7.3) for 15 minutes. For retinal vertical sections, the retinas were cryoprotected in a sucrose gradient (10%, 20%, and 30% wt/vol in PB), and 20-μm-thick cryostat sections were cut.

For immunostaining, retinal whole mounts were blocked for 1 hour in a solution containing 5% Chemiblocker (membrane-blocking agent, Chemicon, Temecula, CA), 0.5% Triton X-100, and 0.05% sodium azide (Sigma). Primary antibodies were diluted in the same solution and applied for 48 hours, followed by incubation for 24 hours in the appropriate secondary antibody, conjugated to AMCA (1:300, blue fluorescence; Jackson Immuno, West Grove, PA), Alexa 568 (1:800, red fluorescence; Molecular Probes, Eugene, OR), Alexa 488 (1:800, green fluorescence; Molecular Probes), Alexa 647 (1:100, far red fluorescence; Molecular Probes), or Cy5 (1:400, far red fluorescence; Jackson). In multilabeling experiments, sections were incubated in a mixture of primary antibodies, followed by a mixture of secondary antibodies. All steps were carried out at room temperature. After staining, the retina was flat mounted on a slide, ganglion cell layer up, and coverslipped using Vectashield mounting medium (H-1000; Vector Laboratories, Burlingame, CA). The coverslip was sealed in place with nail polish. Small pieces of a broken coverslip glass (Number 1 size) were placed in between the slide and the coverslip glasses to avoid extensive squeezing and damage to the retina. The YFP and Alexa 568 fluorescence were sufficient to visualize the YFP-expressing or Alexa 568-filled neurons, without antibody enhancement. For staining on the retinal sections, the primary antibodies were applied overnight, followed by incubation for 1 hour in the appropriate secondary antibody. All primary antibodies used in this study are listed in the Table.

Stock solution was made using 500 μg isolectin Alexa 488 powder (I21411; Invitrogen, Carlsbad, CA) dissolved in 500 μL PBS-calcium solution (in millimoles): 8.1 Na₂HPO₄ dibasic, 1.9 NaH₂PO₄ monobasic, 154 NaCl, 1 mM CaCl₂. Isolectin was diluted 1:150 in the solution used for immunostaining and applied with the secondary antibodies. In some cases, to improve the labeling, 1 mM calcium chloride was added to the incubation solution. In addition, BVs were visualized in fixed retinal tissue based on the unspecific labeling produced by secondary anti-mouse antibodies.

Data Acquisition and Analysis

Confocal Z-stacks were taken from the areas of 212 × 212 μm² with a ×60 oil objective. All images were made using Nikon Eclipse Ti-U (Morrell Instruments, Melville, NY) and Zeiss (Thornwood, NY) confocal microscopes. Z-stack images of 0.5 μm step size were captured. Images were processed using ImageJ software. For choline acetyl transferase (ChAT) staining, the image was filtered and threshold of intensity was established. Horizontal BVs were manually traced; the vertical vessels were ignored. The profiles of processed ChAT images and the traces of BVs were achieved using the z-axis profile function (ImageJ; National Institutes of Health, Bethesda, MD).

The data from several eyes were summarized and data are presented as mean ± SE. For the vertical views, the rotation of the stacks was adjusted based on the cholinergic bands that formed thin, well-separated bands with the perfectly vertical orientation of the stack.

To create side views and investigate the stratification of the BVs the z-stacks were rotated 90 degrees along the x-axis and the projections through approximately 50–70 μm of retinal thickness were achieved (Fig. 1C, dorsal direction). Supplementary Movie S1 was generated using bioView3D software (Center for Bio-Image Informatics, Santa Barbara, CA).

RESULTS

A Retinal Vascular Plexus Coincides With Cholinergic Processes Within the Inner Plexiform Layer

The aim of this study was to evaluate retinal BV distribution and localization relative to neuronal components within the inner retina. First, we analyzed the distribution of BVs relative to neurons in mouse retinal whole mounts, at different eccentricities (marked by squares, Fig. 1A) and retinal poles (labeled by letters, Fig. 1A). BVs were labeled with either isolectin (Fig. 1B, green) or nonspecific secondary anti-mouse antibody (Fig. 1B, red). Isolectin labeled endothelial cells, pericytes, and microglia.¹⁵ Secondary anti-mouse antibody labeled the inner surface of BVs but not the membranes of the vascular cells. Both markers stained the entire retinal vasculature in all retinal layers and therefore were used interchangeably. Neurons were labeled for ChAT and calbindin to visualize cholinergic amacrine cells and horizontal cells, respectively. Cholinergic amacrine cells stratify in two distinct bands in the IPL and are established landmarks within the inner retina.¹⁶ Horizontal cells stratify in the outer plexiform layer (OPL) and serve as markers for the outer retina.

It has previously been shown that retinal BVs form three major plexuses: superficial, intermediate, and deep.¹⁷ Accordingly, we observed the superficial plexus at the nerve fiber layer (NFL), the intermediate plexus near the proximal border of the inner nuclear layer (INL), and the deep plexus proximal to the dendrites of horizontal cells in the OPL, but distal to their soma (Fig. 1C). In contrast to the superficial and deep plexuses, which narrowly stratify, the intermediate plexus had a broad distribution. Surprisingly, we found that the intermediate plexus was not composed of a single band, but rather consisted of two distinct plexuses. A dense layer was located at the border of the IPL and the INL, and a sparse layer coincided with the OFF-ChAT band within the IPL (see Supplementary Movie S1). From this point, these will be referred to as the intermediate plexus (canonical intermediate dense layer) and the intersublamellar plexus (newly described sparse layer). The name “intersublamellar” was derived from the proximity of this plexus to the OFF-ChAT band, which is a border between the

FIGURE 1. Blood vessels form an intersublamina vascular plexus within the IPL. (A) Retinal vasculature visualized by isolectin Alexa 488. Retinal poles are marked by Temporal, Ventral, Nasal, and Dorsal. Retinal eccentricities are marked by squares (c, central; p, peripheral retina). (B) Blood vessels (BV) were double labeled by isolectin (green) and Dms568 (donkey anti-mouse Alexa 568, red). (C) Thin vertical sections ($\sim 50 \times 212 \mu\text{m}$) of confocal z-stacks, taken at the eccentricities shown in (A), were rotated 90 degrees along the x-axis to obtain a vertical view. Retinal neurons were labeled for ChAT and calbindin (both green) and BVs labeled with Dms568 (red). Numbers indicate the borders of the IPL (1, GCL; 4, INL) and positions of the cholinergic bands (2, ON-ChAT band; 3, OFF-ChAT band). (D) Locations of BVs in the IPL (red) and the cholinergic bands (green). The relative densities of BVs were calculated based on the length of the vessels at the eccentricities shown in (C) ($212 \times 212 \mu\text{m}$). The plexuses in the IPL marked by the numbers (D, top) were identified based on the staining against ChAT (see Methods) and correspond to the numbers from (C). Scale bar: 1 mm in (A), 50 μm in (B) and (C).

areas within the IPL, wherein OFF-transient and OFF-sustained ganglion cells stratify.^{12,13}

Next, we measured the extent of the intersublamina plexus at different eccentricities. Normalized intensity profiles (see Methods) were created for the wavelengths corresponding to BVs and ChAT bands, respectively, between the ganglion cell layer (GCL, 0 μm) and the INL (50 μm , Fig. 1D). At each eccentricity, the intersublamina plexus was present and coincided with the OFF-ChAT band. It was clearly separated from the intermediate plexus in some areas, exhibiting distinct peaks on the intensity profile (Fig. 1D, eccentricities 3D and 5D). In other areas, it was less distinguished from the intermediate plexus, with no distinctive peak at the OFF-ChAT band, indicative of BVs traversing between the intermediate and intersublamina plexuses (Fig. 1D, eccentricities 1D, 2D, and 4D). Occasionally, we noted a small blood vessel that appeared shortly before the OFF-ChAT band (Fig. 1D, eccentricity 2D).

How prominent was the intersublamina plexus relative to the other BVs in the retina? To determine the relative weight of this plexus, we obtained a series of confocal horizontal plane reconstructions of the IPL. This is illustrated in representative single images acquired at the level of each plexus (Fig. 2A). The deep plexus was the densest, followed by the superficial and

intermediate plexuses, whereas the intersublamina plexus was relatively sparse. A subset of BVs within the intersublamina plexus did not stratify there, but instead ascended to the intermediate plexus. Most intersublamina BVs smoothly ascended to the intermediate plexus at an oblique angle (Fig. 2B, top). In most cases, however, intersublamina BVs were well separated from vertically running BVs that connect the superficial plexus and the deep plexus (Fig. 2B, middle). Rarely was there an absence of BVs stratifying at the OFF-ChAT band (Fig. 2B, bottom).

We quantified these observations across all eccentricities at each pole in both retinas of three different adult mice with age ranging from P30 to P60. Horizontally running BVs were manually traced through each plexus within a $212 \times 212 \mu\text{m}^2$ area at five eccentricities. The proportional length was calculated by dividing the length of horizontally running BVs within a given area of a given plexus by the cumulative length of horizontally running BVs across all plexuses within the same area (Fig. 3A). Vertically running BVs at the ON-ChAT band served as a control measurement, comprising approximately 1% of the total length (red sector), and were consistent across all retinas and eccentricities (ANOVA, $P > 0.7$, $n = 3$ retinas, 20 eccentricities per retina across four poles). Overall, the intersublamina plexus comprised more than 7% (green sector)

FIGURE 2. The intersublamellar vascular plexus is located at the OFF-ChAT band. (A) Distributions of four vascular layers shown in distinct confocal planes. The locations of vascular layers (stained with Dms568, red) coincided with neuronal markers (ChAT and calbindin, both green). (B) Thin slits ($\sim 50 \times 212 \mu$ m) of confocal z-stacks, taken at the middle eccentricity, were rotated 90 degrees along the x-axis to obtain a vertical view. BVs are labeled with isolectin (green), cholinergic cells are labeled indirectly with the antibodies against ChAT (red). The intersublamellar plexus (arrow) may merge smoothly with the intermediate layer of BVs at the OFF-ChAT band (top); or represent a separate layer (middle); and can be absent in certain areas (bottom). Scale bars: 50 μ m. V, ventral; T, temporal; D, dorsal. The lines in the IPL are similar to those shown in Figure 1C.

FIGURE 3. Blood vessel distribution across retinal eccentricities and poles. (A) Distribution of BVs between vascular plexuses was similar across eccentricities from Fig. 1A. (B) Proportion of the intersublamellar plexus did not differ between retinal poles across all tested eccentricities. The data are shown as average $\pm$ SEM.

FIGURE 4. Inputs to ganglion cells correspond to the intersublamellar plexus. (A) Depth-coded z-stack projection of images through retinal whole mount labeled with isolectin. The intersublamellar plexus is encoded by *green*. Stratification of a bistratified GFP-positive ganglion cell is also depth-coded. (B) Rotated view of the area marked by solid lines in (A). ChAT-positive amacrine cells are shown in *blue*; Type 2 bipolar cells were stained for synaptotagmin II. (C) Magnified area taken from (A) (*dashed box*). Scale bars: 50 μ m. In all panels, *arrows* mark the intersublamellar layer.

of BVs at all eccentricities. In 6 of 60 evaluated areas, the proportion of the intersublamellar plexus was between 2% and 4%. Regardless, all 60 areas were included in further analysis. There was no significant difference in the proportional length of the intersublamellar plexus across eccentricities or poles (2-way ANOVA, $P = 0.19$ and $P = 0.98$, respectively). The proportional length of other plexuses also did not vary across locations ($P > 0.5$).

Neuronal Interactions Relative to the Intersublamellar Plexus

Processes of bipolar, amacrine, and ganglion cells interact within the IPL. We have already shown that the intersublamellar plexus coincides with the OFF-ChAT band; next, we investigated its location relative to the processes of other cell classes within the IPL. In the next set of experiments, we used immunohistochemical markers for distinct physiological neuronal cell classes to further dissect the relationship of the intersublamellar plexus with various cell classes within the IPL.

To determine whether the intersublamellar plexus coincides with bipolar cell input to OFF ganglion cells, we stained for OFF cone bipolar cells. The location of the structures of a bistratified ganglion cell relative to vascular depth is illustrated in a z-stack projection with a depth-coded color gradient (Fig. 4A). The soma is close to the superficial plexus (red), while the OFF dendrites coincide with the intersublamellar plexus (green, marked by arrows). Dispositions and densities of the vascular layers are clearly seen. Type 2 bipolar cells, which have axon terminals that occupy the distal portion of the IPL, were labeled for synaptotagmin II.¹⁸ The intersublamellar

vascular plexus was located below these axon terminals (Fig. 4B). A vertical view of a single confocal slice of the same retina (Fig. 4C) shows the same ganglion cell and BVs (both green) counter-labeled for ChAT (blue) and synaptotagmin II (red). The magnified image of a single confocal section at the OFF-cholinergic band shows that the dendrites of the bistratified ganglion cell (green), the axon terminal of bipolar cells (red), and the cholinergic processes (blue) occur at the same level.

Anatomy of the Neurovascular Unit at the Intersublamellar Plexus

Finally, we aimed to characterize potential neurovascular interactions at this newly identified intersublamellar plexus. In the brain, the neurovascular unit consists of astrocytes, neurons, and vascular cells.^{19,20} In the retina, two types of macroglia, astrocytes and Müller cells, have been found to interact with BVs.^{21–23} Thus, we aimed to investigate which type of glial cells may contribute to the neurovascular unit at the intersublamellar plexus.

First, we determined whether astrocyte processes, which extend from the NFL to the IPL, contact the intersublamellar plexus. Astrocytes, labeled for glial fibrillary acidic protein (GFAP), were concentrated at the NFL (Figs. 5A–D), where processes tightly enveloped BVs of the superficial plexus (Figs. 5E–G). While astrocytic processes extended toward the IPL and sometimes reached the ON-ChAT band, none went beyond to reach the OFF-ChAT band or the intersublamellar plexus (Figs. 5H–J). Thus, astrocytes are not likely to be directly involved in neurovascular interactions at the intersublamellar plexus.

FIGURE 5. Astrocyte processes do not reach OFF-cholinergic band. (A–D) Triple labeling of a retinal whole mount for GFAP (astrocytes, *blue*), isolectin (BVs, *green*), and ChAT (cholinergic amacrine cells, *red*). The images are projections of five confocal sections (2.6 μm total thickness). (E–G) Projection of two confocal sections (0.65 μm apart) of areas boxed in (A) and (B). (H–J) Dotted line area of the confocal image stack from (A–C) rotated 90 degrees along the *x*-axis of that shown in (D). Astrocytes (H, *blue*) were found around BVs (I, *green*) in the NFL with some processes extending into the IPL but not reaching the intersublamina plexus (arrows) stratifying at the OFF-ChAT band (J, *red*). Scale bars: 25 μm .

Next, we determined whether Müller cells come into contact with the intersublamina plexus. Vertical retinal sections were quadruple-labeled for BVs (isolectin), cholinergic cells (ChAT), Müller cells (glutamine synthetase), and astrocytes (GFAP). As before, astrocytic processes were located in the NFL (Fig. 6A) and did not reach the intersublamina plexus at the OFF-ChAT band (Fig. 6B). In contrast, the processes of Müller cells tightly enveloped BVs of the intersublamina plexus (Figs. 6C, 6D). In fact, the location of BVs could be easily identified even without isolectin labeling, based on the prominent staining against glutamine synthetase around BVs. At high magnification, Müller cell processes were visibly intercalated between cholinergic dendrites and vascular cell membranes (Figs. 6E–G). An intensity profile along the line marked by arrows reflects this arrangement (Fig. 6H). The close proximity of Müller cell processes to the BVs suggests that they are a component of the neurovascular unit in the IPL and may perform similar functions as the astrocytes in the NFL. Thus, the composition of the neurovascular unit in the retina depends on retinal location and the presence of the particular types of vascular cells, neurons, and glia.

DISCUSSION

In this study, we characterized the distribution of vasculature relative to neurons in the mouse retina. Similar to other investigators¹⁷ we found three major plexuses of BVs: superficial at the NFL, intermediate between the INL and the IPL, and deep at the OPL. An additional layer of BVs was found to coincide with the OFF-ChAT band, and thus was termed the intersublamina plexus. This plexus was present at all eccentricities and was equally prominent at all retinal poles and eccentricities, comprising approximately 7% to 8% of the total length of horizontally running BVs. Finally, we show that Müller cells may putatively mediate neurovascular interactions at the intersublamina plexus.

The Retinal Neurovascular Unit and Its Components

The “classic” neurovascular unit consists of neurons, glia, and vascular cells. In reality, the specific players of each group will be different; therefore, the interaction and vulnerability of the system to disease will depend on the precise architecture. In the retina, neurons have been shown to stratify in specific layers or laminae.²⁴ Astrocytes, the glial component of the neurovascular unit in the brain, are present only at the superficial plexus of the BVs and are excluded from deeper layers. In contrast, radial glia (Müller cells) are present both at the superficial and deeper layers. Radial glia are a common feature of the developing brain. These are the first cells to develop from neural progenitors. After maturation, radial glia transform into stellate astrocytes, with the only exceptional tissue of our body, the retina. Retinal tissue consists of neurons, macroglia (including astrocytes and Müller cells), microglia, and vascular cells (including endothelial cells, pericytes, and smooth muscle cells). The neurovascular unit refers to the interactions between these cell types, which fosters an interdependence that is essential for homeostasis.¹ In the brain, astrocytes are the major type of glial cells that ensheath BVs. In the retina, this depends on the particular vascular plexus. At the superficial plexus, BVs are ensheathed by astrocytes (Fig. 5),²⁵ while at the intersublamina and deep plexuses, BVs contact Müller cells (Fig. 6).²⁶ This suggests that Müller cells and astrocytes have similar roles with regard to vasculature. Indeed, it has been shown that the processes of Müller cells ensheath the vessels of the deep plexus and play a major role in the formation of the blood retinal barrier.²⁶ Moreover, Müller cells can express VEGF in response to hypoxia and induce the formation of deep retinal layers.²⁷ The neuronal composition around each retinal vascular plexus is also different. Therefore, depending on their location, vascular cells may be regulated by different neurotransmitters released

FIGURE 6. Neurovascular interactions in the IPL. A vertical retinal section quadruple labeled for ChAT (green), GFAP (blue), GS (glutamine synthetase, white), and isolectin (red). IPL borders are marked by lines. (A) The intersublamina plexus (arrows) at the OFF-cholinergic band. (B) Müller cell processes surround blood vessels. (C) Merged image shows BVs surrounded by Müller cells and ChAT processes. (D) Astrocytes do not reach the OFF-cholinergic band and the intersublamina plexus. (E–G) Magnified view of dotted line area in (A–C). Müller cell processes are intercalated between the cholinergic processes and a BV. (H) Intensity profiles across the plane marked by arrowheads in (E) and (G). Scale bars: 25 μ m in (A–D), 5 μ m in (E–G).

from neighboring neurons. The diversity of the neurovascular unit within the retina may account for a tight control of blood flow in a tissue that lacks autonomic regulation.^{4,5} This tight “on-demand” regulation may be especially important in the retina, one of the most oxygen-consuming tissues of the body.³

Functional Significance of Neurovascular Interactions at the IPL

ON and OFF bipolar cell inputs within the IPL generally occur at proximal and distal areas relative to retinal ganglion cells (RGCs), respectively. In the classical view, these have been referred to as sublamina *a* (OFF) and sublamina *b* (ON),^{28–30} although a strict border does not exist between the two, as ON input occurs in the OFF sublamina.^{13,31–35} The intersublamina plexus lies in a transitional area between the sublaminae that receives mixed ON and OFF input.³⁵ Its proximity to the OFF ChAT band may be interpreted as a border between sustained and transient ganglion cells and their input, as this separates OFF-transient from OFF-sustained RGCs.^{12,13} Likewise, bipolar

cells with more transient light responses stratify at an intermediate IPL depth, between the two ChAT bands.^{10,11} Importantly, the location of this newly described vascular plexus at this physiological division of the IPL could serve as an effective way to shape the information flowing along both visual streams. Increased capillary density in the distal portion of the IPL may also be important to match oxygen demands of this area. Similar to photoreceptors, OFF-bipolar cells are depolarized and release glutamate during the dark adaptation. The blood flow is greatly increased in the dark to satisfy high oxygen demands in the OPL for the photoreceptors³⁶ and probably in the distal IPL for the OFF-cone bipolar cells.

As retinal vasculature lacks autonomic control, and shows an efficient local regulation,^{4,5} the function of vascular cells may be modulated by neurons in this region. Pericytes are the contractile cells of the vasculature,⁶ and are more dense in retinal BVs than anywhere in the brain.³⁷ They express functional receptors for a variety of synaptic transmitters, which can cause contraction or relaxation.^{6,38,39} For example, application of acetylcholine alters the membrane potential of

TABLE. Primary Antibodies

Antigen	Immunogen	Manufacturer, Species Antibody Was Raised In, and Type, Catalog No.	Dilution
ChAT	Purified human placental ChAT	Chemicon, goat polyclonal, #AB144P	1:1000
GS	Purified GS from sheep brain	Millipore, mouse monoclonal, clone GS-6, #MAB302	1:2000
GFAP	Synthetic peptide corresponding to residues surrounding Asp395 of human GFAP protein	Cell Signaling, rabbit monoclonal, #12389	1:3000
Synaptotagmin II	1–5-d zebrafish embryos	Zebrafish International Resource Center, mouse polyclonal, #znp-1	1:1000
Calbindin	Recombinant rat calbindin D-28k (CB)	Swant, rabbit polyclonal, #CB38	1:2000

pericytes, leading to cell contraction and constriction of the BV.^{38,40} Thus, it is possible that the cholinergic cells may modulate the BV diameter via pericytes to modulate blood flow, although further investigation is needed to confirm this. Several recent studies also indicate that glial cells can act as intermediaries in signaling from neurons to BVs.^{41,42} These interactions can be disrupted during retinal diseases, which provides a strong impetus for future studies to characterize the retinal neurovascular unit.

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