Peptidergic Innervation of the Retinal Vasculature and Optic Nerve Head
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Using immunocytochemistry, the authors studied the peptidergic innervation to the vasculature of the optic nerve and retina in the rhesus monkey and rat. In the monkey, beaded nerve fibers immunoreactive to the vasoactive peptides, calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY), substance P (SP), and vasoactive intestinal peptide (VIP), are present in the adventitia and perivascular space along the course of the central retinal artery within the optic nerve. The CGRP and SP immunoreactivities fully co-localize. Perivascular peptidergic nerve fibers terminate as the blood vessel enters the globe and do not follow the branches of the central retinal artery inside the eye. Within the substance of the optic nerve behind the lamina cribrosa, small blood vessels occasionally are supplied with CGRP-, SP-, and sometimes NPY- or VIP-immunoreactive nerve fibers. Of special note, fine nerve fibers not clearly related to blood vessels are seen within the lamina cribrosa; their simultaneous immunoreactivity to CGRP and SP suggests a sensory function. In the rat as in the monkey, the retinal arterioles beyond the surface of the optic disc lack evident peptidergic innervation. Perhaps an explanation for the known physiologic reactivity of the retinal circulation to neurohumors in the absence of recognizable peripheral innervation can be based on comparison to the brain where intraparenchymal blood vessels may be regulated by local neurons. Since the inner plexiform layer has abundant amacrine-derived nerve processes containing classical neurotransmitters and/or neuropeptides, a local mechanism coupled to intrinsic retinal activity might contribute to the regulation of the circulation.

Whether or not peripheral nerve fibers innervate the intraocular branches of the central retinal artery (CRA) remains controversial. Claims for such innervation originally made on the basis of silver stains have not been supported by electron microscopic or histochemical studies, except in the rabbit. As a result, the retinal vascular tree in most species is now considered devoid of extrinsic nerve fibers. Yet a paradox exists: evidence is mounting that the retinal blood vessels have receptors for and sensitivity to neurotransmitters and neurohormones, including biologically active peptides. In peripheral ocular nerves, CGRP and SP nerve fibers are sensory, and VIP nerve fibers are parasympathetic. Generally associated with the sympathetics, the assignment of NPY is problematic; it also is found in some parasympathetic neurons supplying the eye, indicating a mixed autonomic derivation.

Materials and Methods

Eight rhesus monkey eyes were obtained immediately after death from animals killed under deep pentobarbital anesthesia as part of the polio-vaccine testing program of the Bureau of Biologies of the Food and Drug Administration; the eyes were fixed by immersion in Zamboni's solution at 4°C for 48 hr. Twelve male Wistar rats also under deep pentobarbital anesthesia were perfused through the left ventricle first with 0.85% NaCl and then with Zamboni's solution. The enucleated eyes were postfixed at 4°C for 48 hr. All tissues were washed overnight at 4°C in 0.1 M phosphate-buffered saline (PBS), pH 7.4, with 30% sucrose. Then 16–20 μm thick cryostat tissue sections were cut in a horizontal plane to include the optic nerve head. Tissue sections were thaw mounted on gelatin-coated slides, dried at room temperature, and...
stored at −20°C until stained with the indirect immunohistochemical technique.

For primary antisera, we used polyclonal antisera generated in rabbits against CGRP (Lot # V054/5517; Cambridge Research Biochemical, Valley Stream, NY), NPY (Lot # 0031/1098; Cambridge), SP (Lot # 8706025; INCStar, Stillwater, MN), VIP (Lot # 8726017; INCStar), and monoclonal antibodies raised in the rat against SP (Lot # B3K35; Pel-Freez, Rogers, AR). All primary and secondary antisera were diluted in 0.05 M PBS, pH 7.2, containing 0.3% Triton X-100, and all rinses were done with 0.05 M PBS, pH 7.2, containing 0.2% Triton X-100.

Tissue sections from both species were incubated overnight at room temperature with the primary antibody (anti-VIP diluted 1:500; anti-SP, 1:500; anti-NPY, 1:800; and anti-CGRP, 1:800). After rinsing, the tissue sections were incubated for 1 hr at 37°C with the biotinylated F(ab')2 fragment of donkey anti-rabbit IgG (Pierce) diluted 1:300, and then for 1 hr at 37°C with fluorescein-labeled avidin diluted 1:300 (Amersham International, Arlington Heights, IL). The stained tissue sections were mounted in a TRIS-glycerin mixture and were examined with an epi-illumination system.

For the co-localization of CGRP and SP in the monkey eye, tissue sections were incubated overnight at room temperature with a mixture of rabbit CGRP antiserum and rat SP monoclonal antibodies, each diluted 1:300, and then for 1 hr at 37°C with a mixture of goat anti-rabbit IgG conjugated to rhodamine isothiocyanate (RITC; Cappel Laboratories, West Chester, PA) and goat anti-rat IgG conjugated to fluorescein isothiocyanate (FITC; Cappel), respectively diluted 1:400 and 1:300. The secondary antisera were diluted in 0.05 M PBS, pH 7.2, containing 0.2% Triton X-100.

For controls, the primary antisera were preabsorbed by overnight incubation at 4°C of each diluted primary antiserum with 1.0 μM of the respective peptide (Peninsula Laboratories, San Carlos, CA); the preabsorbed antisera then were substituted in the immunohistochemical procedure. As another control, the primary antiserum was omitted entirely. To evaluate specificity for the co-localization studies, the primary antibody mixture was preabsorbed with 1.0 μM CGRP or SP. For additional specificity controls, tissue sections stained with rabbit primary antiserum were reacted with the anti-rat secondary antiserum; those stained with the rat monoclonal antibodies were reacted with the anti-rabbit secondary antiserum. All control studies demonstrated appropriate immunohistochemical specificities.

For the histologic sections of rat CRA, enucleated rat eyes were dehydrated and embedded in Historesin (Microscopic Optical Consulting, Valley Cottage, NY). Then 4-μm tissue sections were stained with a 0.5% azure II, 0.5% methylene blue, 1% sodium borate solution. All studies conformed with the ARVO Resolution on the Use of Animals in Research.

Results

Peptidergic Innervation of Central Retinal Artery and Optic Disc

In the monkey orbit, the CRA and central retinal vein together enter the optic nerve some 3–8 mm behind the lamina cribrosa, surrounded by a sheath of glia and connective tissue. They rapidly gain a central position, travel forward, and finally pass through the lamina cribrosa to divide at the face of the optic nerve head. Immunoreactive nerve fibers of a beaded appearance and meandering course, typical of peripheral nerve fibers, were seen in the vascular adventitia of the artery during its intraorbital course. Frequently they extended inwards to the adventitia-media border. In separate preparations, nerve fibers immunoreactive for each of the four neuropeptides under study were visualized (Fig. 1). The NPY-like immunoreactive (−LI) nerve fibers were consistently more numerous than those of CGRP, SP, or VIP. As nerve fibers traveled forward with the blood vessels in the optic nerve, no significant change of innervation density was detectable behind the lamina cribrosa. Between the lamina cribrosa and the optic disc surface, a rapid and profound attenuation occurred: no CRA branches emanating from the optic disc had an observable peptidergic nerve supply (Fig. 2). When specifically studied, CGRP and SP fully co-localized (Fig. 1). In comparison with the artery, the nerve supply to the central retinal vein was consistently far lower in density and less predictable; in many instances, it was absent.

In the intracanicular segment of the monkey optic nerve, individual fine CGRP- and SP-LI nerve fibers also were seen in the posterior part of lamina cribrosa (Fig. 3). They were immediately adjacent to laminar beams oriented perpendicular to the optic nerve axons. CGRP and SP co-localized fully in these nerve fibers as in other locations. Even though small intrascleral blood vessels adjacent to the optic nerve head in the circle of Zinn-Haller and others passing between the sclera and lamina cribrosa were occasionally surrounded by nerve fibers similarly immunoreactive to CGRP and SP, we could not identify...
clear vascular associations of the fine nerve fibers in the lamina cribrosa. No NPY- or VIP-LI nerve fibers of similar distribution were observed within the lamina region.

In contrast to its course in the monkey, the CRA in the rat passes inferolaterally to the optic nerve and enters the optic nerve head obliquely between the sclera and optic nerve. CGRP-, NPY-, SP-, and VIP-LI nerve fibers were present in the adventitia of the CRA. The CGRP-LI fibers terminated just before the CRA entered the globe. The other three types of peptidergic nerve fibers underwent a small decrease in...
density as the blood vessel passed forward to the optic disc and then also ended abruptly (Figs. 4, 5). As in the monkey, very few peptidergic nerve fibers were observed around the central retinal vein.

Neurovascular Relationships Within the Retina

The arteriolar branches of the CRA repeatedly bifurcate in the superficial nerve fiber layer to distribute into layered capillary beds, the deepest of which reach the outer plexiform layer. As capillaries penetrate the inner plexiform layer, they are in immediate proximity to amacrine cell processes. The monkey and rat retina contained VIP- and SP-LI amacrine cell bodies with inner plexiform layer processes. In both species, alternating-phase and fluorescence microscopy clearly demonstrated that capillaries penetrate the inner plexiform layer in close proximity to peptidergic nerve fibers (Fig. 6).

Discussion

The existence of a rich and complex nerve fiber plexus to the CRA in its course through the optic nerve has been well known for many years. In fact, the sympathetic contribution was described by Tiedemann and others in the early 19th century. As specific histochemical methods for the identification of neurotransmitters and/or their enzyme systems supplemented nonspecific silver staining, a cholinergic component was ascribed to parasympathetic sources. In the rat and monkey, the two species we studied, only a few adrenergic or cholinergic fibers were observed to surround the branch retinal blood vessels at the optic nerve head, and none served the retinal blood vessels.

In this report, we extended prior histochemical results on adrenergic and cholinergic innervation to neuropeptides and found that peptidergic nerve fibers disappear as the CRA passes forward into the globe. The attenuation is such that, although a few peptidergic nerve fibers can be traced as far forward as the vitreal surface of the optic nerve head, none follow the blood vessels in their course over the retina. The entire series of histochemical and immunocytochemical observations is supported by electron microscopic findings which also demonstrate no nerve terminals on branch retinal blood vessels in rats, monkeys, and humans. The rabbit is a limited exception to this generalization. Although SP-LI nerve fibers surrounding its CRA do not extend into the globe, a definite adrenergic innervation to retinal blood vessels has been observed on several occasions. In this respect, however, the rabbit and hare are distinct among mammals: their retinal blood vessels are restricted in distribution to the horizontal region of the vascular stripe, an area more or less coextensive with myelinated ganglion cell axons. An observation that retinal blood vessels in the dog and in pathologic human eyes are innervated has not been replicated.

Based on these considerations, the regulatory potential for the vascular innervation would seem to depend on the location of blood vessels in relation to the optic nerve head. Regarding the extraocular com-
Fig. 3. Presumed sensory nerve fibers at the lamina cribrosa of the monkey. (A, B) A small nerve fiber (arrow) at the lamina cribrosa stains simultaneously for CGRP in (A) and SP in (B) evidence for a sensory origin. (C) The location of this nerve fiber is indicated on the phase micrograph of the identical field (arrow) seen in (A) and (B). Magnification bar, 50 μm.

Fig. 4. Vasoactive intestinal peptide and innervation of the rat central retinal artery. (A) The central retinal artery (CRA) enters the eye inferior to the optic nerve. Its course through the optic nerve is oblique and short. (B) Vasoactive intestinal peptide-like immunoreactive (VIP-LI) nerve fibers (arrow) accompany the artery as it enters the eye. Note that branch blood vessels (asterisks) within eye are devoid of VIP nerves. A few VIP-LI nerve fibers are visible in the inner plexiform layer (arrowhead). Magnification bar, 50 μm.
Neuropeptide Y and the innervation of the rat central retinal artery. Neuropeptide Y-like immunoreactive (NPY-LI) nerve fibers (arrow) supply the central retinal artery (CRA) as it travels to the surface of the optic nerve (ON). Note also the NPY-LI nerve fibers in the choroid (arrowheads). R = retina; magnification bar, 50 μm.

ponent, an active innervation probably influences the head of pressure reaching the retina at the optic disc. Evidence for this comes from several sources. For example, superior cervical ganglion stimulation in the cat is followed by a widespread drop in retinal oxygen tension independent of the choroidal circulation. More directly, the isolated perfused ophthalmic artery of the Japanese monkey demonstrates graded reactivity to vasoactive amines, such as noradrenaline and tyramine.

Less certain is what occurs to the blood vessels in the retina. Although such blood vessels lack extrinsic innervation, autonomic receptors are present on their walls. Also altered vascular diameter and/or retinal blood flow in response to change in the partial pressure of blood gases indicates control of vessel caliber. In most vascular beds such functions are attributed to precapillary arterioles, but in the retina a site for regulatory control remains uncertain. Although precapillary endothelial specializations have been observed in several species, there are none in monkeys or humans, and thus there is no accepted locus of control for circulation through the retinal microvascular bed. In this respect, direct innervation of capillaries by retinal neurons can be proposed as a candidate mechanism for local circulatory control. Anatomic support of this hypothesis, we repeatedly visualized neuropeptide-containing amacrine processes immediately adjacent to retinal capillaries in the inner plexiform layer both in the monkey and rat retina. Our observations complement the recent finding in the same species of dopamine-containing amacrine processes to retinal capillaries. In fact, we...
found dopamine-containing amacrine processes immediately adjacent to capillaries as far scleral as the outer plexiform layer in the spider monkey retina (data not shown). Support for local neural control also comes by comparison with the brain, where neuronal processes associated with intraparenchymal blood vessels are suggested to regulate blood flow at the capillary level.29 Unfortunately, confirmation of a hypothesis of intrinsic neuronal regulation of retinal blood flow requires a physiologic understanding of its microcirculation that at present is lacking. Clearly for retinal amacrine cell processes to alter retinal blood flow, retinal capillaries would need to contract, a function that remains to be established.

Regarding the fine nerve fibers in the lamina cribrosa, the coexistence of CGRP and SP immunoreactivities leads at once to speculation of a specialized sensory function. Possibly relevant to intraocular pressure regulation, this presumption is reinforced by the lack of visceral motor fibers in the same location. However, the difficulty of discriminating a free-running nerve fiber from one serving a small but unseen blood vessel by light microscopy makes it impossible to state firmly that these nerves truly serve the lamina cribrosa and not the local circulation. Yet, the observation is sufficiently provocative to justify further inquiry by methods better suited to define local tissue relationships, such as electron microscopy.

Key words: central retinal artery, innervation, neuropeptides, monkey, rat

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References