Species Differences in Choroidal Vasodilative Innervation: Evidence for Specific Intrinsic Nitrergic and VIP-Positive Neurons in the Human Eye

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Purpose. There is evidence that vasodilation of choroidal vessels results from facial nerve stimulation. To obtain more information about the role of this innervation, the authors examined the presence and spatial organization of nitrergic and vasoactive intestinal peptide (VIP) immunoreactive nerves in the human choroid. For comparison, the choroid of rabbit and rat eyes, with different types of retinal vascularization and no fovea, were studied.

Methods. Whole mounts of five human, nine rat, and two rabbit choroids were stained for NADPH-diaphorase. In addition, immunocytochemical staining was carried out on tangential frozen sections of two human choroids using antibodies against nitric oxide synthase (NOS), synaptophysin, and VIP.

Results. In all species, a perivascular network of diaphorase-positive nerve fibers with varicose terminals accompanied the arteries and arterioles of the choroidal stroma. A striking difference to rat and rabbit choroids was the presence of numerous positively stained ganglion cells in human choroids. Positively stained axons connected the neurons with each other and with the perivascular network. Most of the ganglion cells were concentrated in the temporal-central region, adjacent to the fovea. Immunocytochemically, the choroidal ganglion cells were immunoreactive for NOS. Some ganglion cells stained for VIP. Staining for synaptophysin demonstrated varicose terminals innervating the perikarya of the ganglion cells. Many of these terminals stained for NOS and VIP.

Conclusions. The presence of an intrinsic nerve cell plexus that is specifically localized in human eyes in the temporal-central portion of the choroid indicates a functional significance of the nitrergic choroidal innervation for the fovea. Invest Ophthalmol Vis Sci. 1994;35: 592-599.

$\mathbf{P}$erivascular nerve fibers showing positive immunoreaction for nitric oxide synthase (NOS) have recently been described in the rat choroid.[1] There is considerable evidence that neurons containing NOS use nitric oxide (NO) as a neurotransmitter.[2,3] NO, a free radical gas, has been identified as a mediator of endothelium-derived vascular relaxation.[4] In addition, neuronal

NO, released by perivascular nerves in various organs throughout the body, is able to mediate vasodilation.[5,6] A similar vasodilative role of NO in the regulation of choroidal blood flow in cat eyes has recently been shown by Mann et al.[7] In several organs, neuronal NOS seems to be colocalized with vasoactive intestinal peptide (VIP).[8-10] The same might be true of choroidal NOS immunoreactive nerves because the choroidal vessels are also supplied by numerous axons staining for VIP,[11-13] another potent dilator of choroidal vessels.[14] VIP immunoreactive axons most probably derive from neurons that are localized in the pterygopalatine ganglion[11,15] and are innervated from the facial or greater petrosal nerve.[16,17] Also in the rat choroid, NOS-positive axons presumably originate from neurons in the pterygopalatine ganglion, which stain both for NOS and VIP.[1] Facial nerve stimulation is known to cause a marked increase in choroidal blood

flow that cannot be abolished by cholinergic inhibition.[18,19] VIP has been discussed as the causative transmitter.[20] Most likely, neuronal NO contributes to the increase in choroidal blood flow after facial nerve stimulation.

In monkeys, about 65% of the oxygen consumed by the retina is delivered from the choroid.[21] Moreover, the avascular fovea is nourished nearly exclusively from the choroid. To obtain more information about the role of the vasodilative innervation of the choroid, we investigated the spatial organization and the architecture of the NOS-containing nerves in the human choroid using whole mount preparations. The results were compared with similar preparations from the eyes of two species with marked differences in the functional anatomy of retina and choroid, e.g., rat eyes with a vascular retina but no fovea and rabbit eyes with a predominantly nonvascularized retina and no fovea. For visualization of NOS-containing nerves, antibodies against NOS as well as histochemical staining for NADPH-diaphorase were used. The colocalization of NOS-immunoreactivity and NADPH-diaphorase stain in brain and peripheral nervous system is amply documented.[2,22,23]

MATERIALS AND METHODS

Seven eyes of six human donors, enucleated 5 to 12 hours post mortem (donor ages, 48, 51, 68, 71, 75, and 82 years) were investigated. The 75-year-old donor contributed both eyes. Except for typical age-related morphologic alterations (e.g., peripheral cystoid retinal degeneration in the very old eyes), none of the eyes showed major abnormalities of the posterior sector. The eyes were bisected along the ora serrata, and the posterior halves of four of the donors were fixed for 4 hours in neutral buffered formalin (4%). The posterior halves of two donors (51 and 68 years) were fixed overnight in Zamboni's solution.[24] In addition, the eyes of nine Sprague-Dawley rats and the eyes of two New Zealand albino rabbits were studied. The animals were sacrificed in conjunction with other experimental protocols not involving the eye. Rats were anesthetized with thiopental (0.55 mg/100 g body weight) and rabbits with a mixture of xylazine (3.5 mg/kg body weight) and ketamine (30 mg/kg body weight).[25] The deeply anesthetized animals were perfusion fixed via the heart with neutral buffered formalin after perfusion with heparinized NaCl. After 1 hour, the eyes were enucleated and incubated in the same fixative for another hour. After fixation, the eyes were opened, and the choroid was removed from retina and sclera and washed in Tris-buffered saline (TBS), pH 7.4, for 24 hours. In the authors' opinion, methods for securing animal and human tissues were humane, included proper consent and approval, and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the Declaration of Helsinki.

NADPH-Diaphorase Staining

Whole mounts of the human choroids fixed in neutral buffered formalin, and those of the animals were incubated free floating in a moist chamber at 37°C using the following medium: nicotinamide adenine dinucleotide phosphate/tetrasodium salt (reduced NADPH; Biomol, Hamburg, Germany), 1 mg/ml; nitroblue tetrazolium chloride (Serva, Heidelberg, Germany), 0.1 mg/ml; 0.3% Triton X-100 in 0.1 M phosphate buffered saline, pH 7.4. The incubation time was 2 hours. The reaction was stopped by rinsing the specimens in phosphate buffered saline. Finally, the choroid was mounted in Kaiser's glycerin gelatine (Merck, Darmstadt, Germany).

The number of positively stained neurons was quantified. The whole mounts were viewed with a light microscope using a magnification of X20. Each quadrant of the choroids was evaluated separately.

Immunohistochemistry

Immunohistochemical staining was carried out on 10 to 20 μm thick frozen sections cut tangentially through the human choroids fixed in Zamboni's solution. The sections were mounted on poly-L-lysine coated glass slides and initially incubated with Blotto's dry milk solution for 20 minutes at room temperature to reduce nonspecific background staining. Incubation with the primary antibody was performed in a moist chamber for a period of 1 to 12 hours (overnight) at room temperature. For demonstration of synaptophysin, a monoclonal mouse antibody against synaptophysin isolated from presynaptic vesicles of bovine brain[26] (Dakopatts, Hamburg, Germany) was used at a dilution of 1:10. The presence of NOS was shown with polyclonal rabbit antibodies raised against NOS purified from porcine cerebellum (established by B. Mayer, Graz, Austria, dilution 1:500). This antiserum previously has been successfully used for Western blot analysis[27] and immunohistochemistry.[8,28] For demonstration of VIP, polyclonal rabbit antibodies (1:500, Medscand Diagnostics, Lund, Sweden) were used. All antibodies were diluted in TBS containing 2% bovine serum albumin (BSA) and 0.2% Triton-X-100. After incubation with the primary antibody and rinsing in TBS (3 times for 10 minutes each), the sections were incubated for 1 hour with biotinylated anti-mouse or anti-rabbit immunoglobulins (1:200, Dakopatts). After rinsing in TBS, the sections were incubated with FITC-conjugated streptavidin (1:50, Dakopatts), rinsed and mounted in Entellan (Merck, Darmstadt, Germany) containing 2.5 % 1,4-diazabicyclo [2,2,2]-octane (DABCO, Merck). Controls were performed by

replacing the primary antibody with TBS or with a preimmune serum, or by omitting the biotinylated immunoglobulins.

RESULTS

NADPH-Diaphorase

Rat. Numerous strongly labeled nerve fibers were found in the stroma of the choroid. These axons emerged at the optic nerve head, where they formed thick bundles associated with the two long posterior ciliary arteries and their principal branches. Toward the periphery, the bundles followed the arborization of the arterial vessels. The axon bundles, which were running in parallel with the arteries and arterioles, continuously gave rise to smaller axons that left the bundles and joined a delicate and dense network surrounding the vascular wall of all arteries and arterioles (Fig. 1). In six of the nine animals, one to four stained ganglion cells were observed. The axon of the

FIGURE 1. Whole mount of a rat chorid stained for NADPH-diaphorase. The temproal region in the vicinity of the optic nerve head is shown. Bundles of positively stained nerve fibers follow the course of the arterial vessels (double arrows). Small axons originate from these bundles forming a delicate network around the vascular wall (arrowhead, × 80).
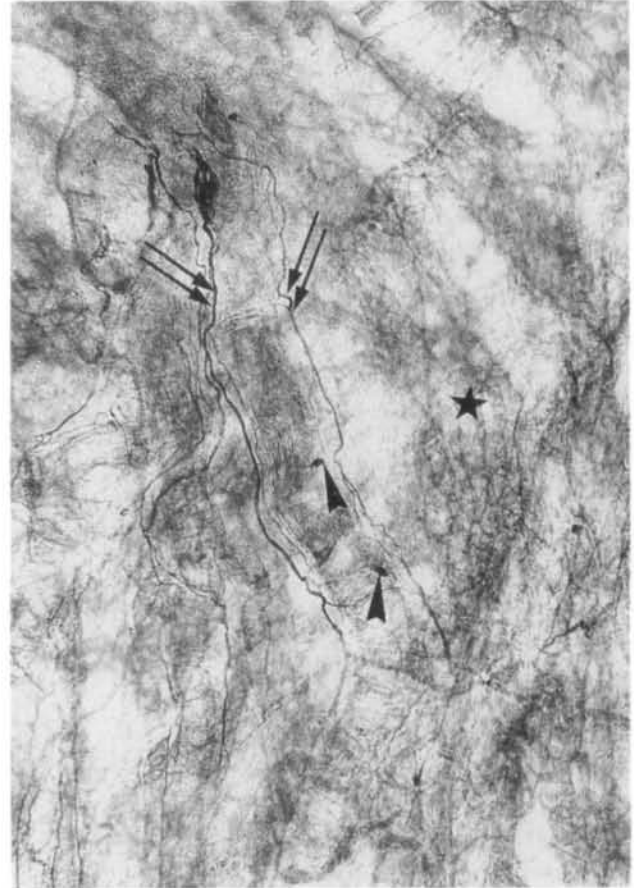

FIGURE 2. Whole mount of a rabbit chorid stained for NADPH-diaphorase. A sector of the temporal region is shown. Stained nerve fiber bundles (double arrows) are running parallel to the arterial vessel. From these nerves, smaller axons branch off and attach close to the vascular wall where they form varicose terminals (arrowheads). Between the straight-running larger arteries, additional nerve fibers form a coarse and irregular network (asterisk, × 80).

ganglion cells joined the positively stained axon bundles. The cells were located in the vicinity of the optic nerve head and were adjacent to the long posterior ciliary arteries. They were often round or oval with diameters of 5 to 8 μm. In the other three animals, no stained cells were observed. In the choriocapillaris no stained nerve fibers were seen.

Rabbit. In the rabbit eye, a similar but less dense and less regular nerve fiber network with varicose terminals was visible after incubation for diaphorase (Fig. 2). As in the rat, stained axon bundles lined the ciliary arteries. However, the network of perivascular axons surrounding the arterial wall was wider and less dense than in the rat choroidal stroma. In addition, positively stained ganglion cells, similar in size and shape to those found in the rat were observed. The number amounted to 20 cells per choroid. Some of these ganglion cells were associated with the long posterior ciliary arteries. In contrast to the rat, some ganglion cells

were also found close to some of the peripheral choroidal vessels. The vessels of the choriocapillaris were not surrounded by a diaphorase-positive nerve fiber plexus.

Human. In the human choroid, bundles of diaphorase-positive nerve fibers ran parallel to the arteries in the stroma vascularis and gave rise to a delicate network of fine axons and varicose terminals surrounding the vascular wall of arteries and arterioles. This perivascular network was less dense and more irregular than that of rat and rabbit eyes. The distribution of the stained nerve fibers showed significant regional differences. Most stained nerve fiber bundles were found in the central region of the choroid underlying the macula. In contrast, there were nearly no stained nerve fibers in the periphery of the choroid.

There were also staining differences within the central part where some arterioles were surrounded by a dense and others by a loosely arranged network.

The most striking difference between the human and the other two species, however, was the presence of numerous positively stained ganglion cells in the human choroid (Figs. 3A, 3B). The majority of these cells were polygonal, with diameters ranging from 10 to 40 μm. The cells were either solitary or were clustered in groups of 2 to 10 (Fig. 3B). Many of the ganglion cells were located adjacent to the wall of the larger choroidal arteries but were never observed in the layer of the choriocapillaris. Individual groups of ganglion cells were connected with each other by diaphorase-positive axons. These connecting axons showed no apparent association with the vasculature. In places, thin axons originated from the connecting bundles and joined the network of perivascular nerve fibers (Fig. 3B).

The positively stained ganglion cells were not evenly distributed throughout the human choroid but showed significant regional differences. The highest number of cells (approximately 1000) and the largest cells (20 to 40 μm) were found in the central-temporal area of the choroid (below the fovea) (Table 1). In other parts of the eye, fewer cells were found (approximately 1000 in all remaining quadrants together) (Table 1). Toward the periphery, the number of stained cells decreased, as did the size of the cells. Thus, in the periphery, few and slightly smaller (diameter <10 μm) ganglion cells were seen.

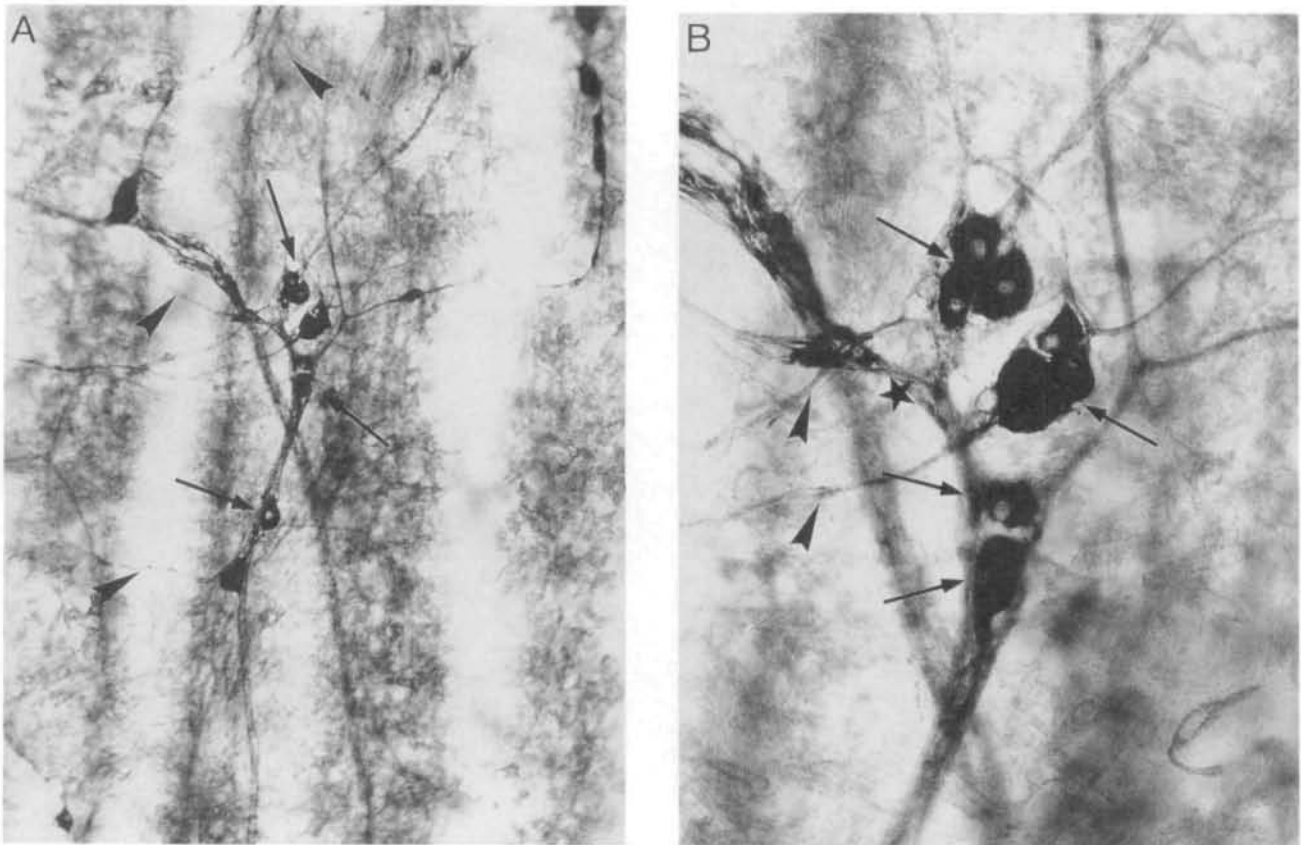The differences in size of the ganglion cells were

FIGURE 3. (A) Whole mount of a human choroid (temporal region) stained for NADPH-diaphorase (B: higher magnification). Positively stained ganglion cells are shown (arrows). These cells are connected to each other by stained axons (asterisk). Additional nerve fibers lead to the perivascular fiber network (arrowheads) (A: × 51; B: × 160).

TABLE 1. NADPH-Diaphorase Positive Cells Counted in the Various Quadrants of Human Choroid

	48 Years Left Eye	71 Years Left Eye	75 Years Right Eye	75 Years Left Eye	82 Years Left Eye
Temporal + central	1160 (45%)	905 (58%)	1132 (64%)	1549 (70%)	1116 (70%)
Nasal	287 (11%)	421 (27%)	83 (5%)	42 (2%)	89 (5%)
Superior	146 (6%)	49 (3%)	210 (12%)	556 (25%)	31 (2%)
Inferior	986 (38%)	180 (12%)	336 (19%)	63 (3%)	375 (23%)
Total	2579	1555	1761	2210	1661

less pronounced in the 48-year-old eye than in the older ones. In the 48-year-old donor eye, even centrally located cells had diameters of 20 to 30 μm.

Immunocytochemical Staining

In tangential sections through the human choroid, the ganglion cells could be identified, even unstained, by their characteristic size, their large nuclei, and their numerous cytoplasmic granules expressing a strong autofluorescence. Antibodies against the enzyme NOS showed strong positive staining in the cytoplasm of all of the ganglion cells (Fig. 4). Staining for synaptophysin did not label the cytoplasm of the ganglion cells (Fig. 5). Positive immunoreactivity, however, was found in the axons of the cells and in numerous varicose terminals closely associated with the perikarya of the ganglion cells (Fig. 5). Staining for VIP showed that the ganglion cells were embedded in a thin network of varicose axons expressing VIP-like immunore-
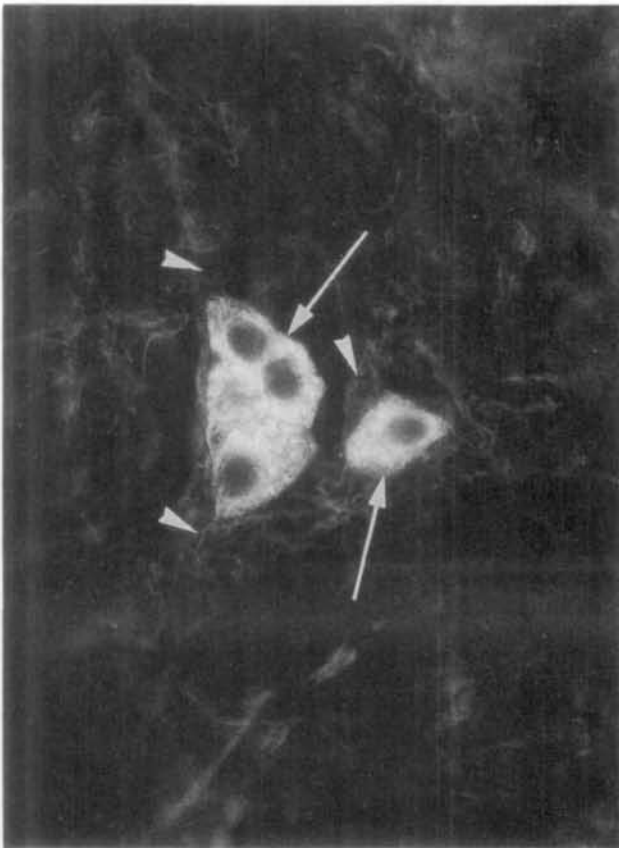

FIGURE 4. Frozen tangential section of human choroid (temporal quadrant) stained immunocytochemically with antibodies against nitric oxide (NO)-synthase. Strong positive labeling is seen in the cytoplasm of the ganglion cells (arrows). Very weak staining for NO-synthase is also found for the axons of these nerve cells (arrowheads, × 220).
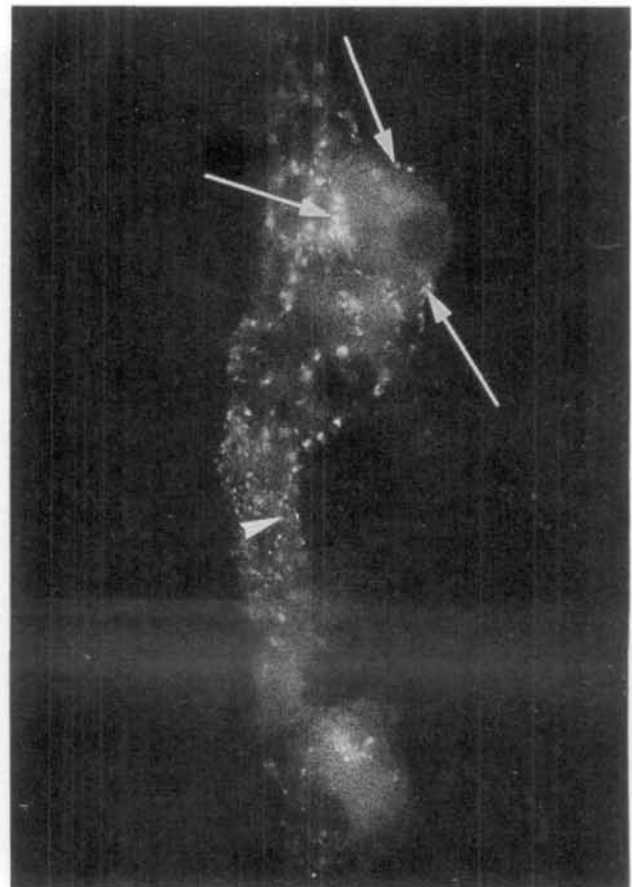

FIGURE 5. Frozen tangential section through the temporal quadrant of a human choroid after incubation for anti-synaptophysin. Two ganglion cells are surrounded by positively labeled varicosities (arrows). In addition, positive staining is also present in small vesicles lying in the cytoplasm of axons (arrowhead, × 270).
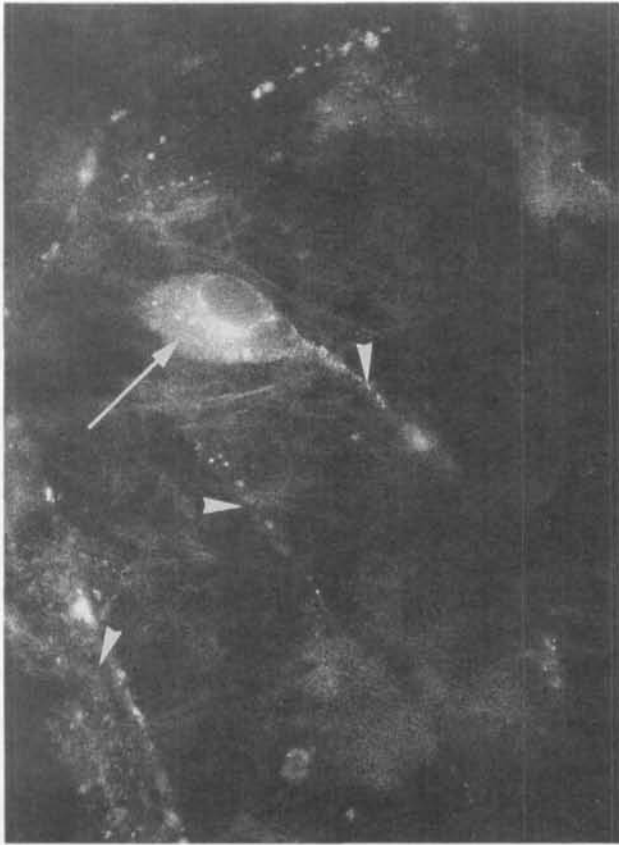
FIGURE 6. Frozen tangential section through human choroid (temporal quadrant) stained immunocytochemically with antibodies against vasoactive intestinal polypeptide (VIP). A ganglion cell is seen showing a positive VIP-like immunoreactivity in the cytoplasm (arrow) and the axon (arrowhead). Additional labeling is seen in numerous nerve fibers passing by (arrowheads, 220).

activity. Positive labeling was also seen in the cytoplasm of many ganglion cells (Fig. 6).

DISCUSSION

In all species studied, we observed a dense nitrergic innervation of the choroidal arteries and arterioles, corroborating earlier findings in the rat eye.[1] The density of the nerve meshwork around the individual vessels appears to be somewhat less pronounced in rabbits and humans than in rats. In all these species, the perivascular NADPH-diaphorase or NOS-positive axons are more abundant in the central parts of the choroid. Species differences in the spatial organization of the vascular nitrergic innervation seem only related to the known species differences in the architecture of the arterial supply of the choroid, which were reviewed recently by Funk et al.[29] The regional staining differences in the central parts of the human choroids might reflect either functional differences or might be due to the larger postmortem time in these

eyes, which could have caused some loss of enzyme activity.

In addition to axons, some scattered neurons in the choroid of rat and rabbits are also stained for NOS. Surprisingly, the number of NOS-positive nerve cells is more pronounced in the human choroid. Here, the NOS-positive neurons form a network of numerous intrinsic ganglia connected with each other by bundles of axons. An elaborate system of intrinsic ganglia in the human choroid, which resembles in structure the intrinsic ganglia of the enteric nervous system, was discovered more than a hundred years ago.[30,31] Although this "ganglion choroideae" was originally subject to a considerable number of histologic studies in recent decades,[32-35] nearly nothing is known about the function of the ganglion cells. Our results show that most, if not all, the neurons in the choroidal ganglia stain for NOS and most probably use NO as a neurotransmitter. As already shown by Miller et al,[12] some of these choroidal ganglion cells are also immunoreactive for VIP. Staining for synaptophysin, a transmembranous glycoprotein specifically localized to the membranes of synaptic vesicles,[36] indicates that the choroidal neurons are innervated by varicose terminals that form contacts with the perikarya of the cells. Most of the terminals stain for NOS and seem to derive from other choroidal neurons, as the choroidal ganglia are connected to each other by numerous NOS-positive axons. Some of the terminals also stain for VIP. It seems likely that the choroidal neurons have also an extrinsic preganglionic innervation from outside the eye. The nature and origin of such an innervation remains to be clarified. In addition, our study does not show whether in the human eye all perivascular NOS-positive axons derive from choroidal ganglion cells or whether an additional source of NOS innervation (e.g., from the pterygopalatine ganglion as in the rat choroid[1]) is involved.

During the last 2 years, similar intrinsic NOS-positive ganglion cells have been described in numerous organs such as the intestine,[9,37,38] gallbladder,[10] and trachea.[28] In general, NO causes relaxation of smooth muscle cells.[2,3] In the enteric nervous system, NO is one of the transmitters of intrinsic autonomic neurons that mediate nonadrenergic-noncholinergic relaxation and descending inhibitory reflexes, major components of peristalsis.[3] NO released by perivascular nerves relaxes vascular smooth muscle and serves a vasodilative function. It seems reasonable to assume that the same is true of the choroid. The physiological role of the dense vasodilative innervation of the choroid is not clear. Physiological studies demonstrate that blood flow through the choroid is extremely high.[39] Still, tissue demands seem not to correspond with this high flow. The oxygen extraction from the choroidal vessels is low; the arteriovenous difference

in oxygen concentration measures only 2% to 3%.[39,40] In addition, the capillaries of the choroid are fenestrated and offer a high permeability for glucose or larger macromolecules.[41,42] Studies on the choroidal vasculature in cats and pigs indicate that net extraction of oxygen and glucose is maintained at a normal level, even after large alterations in choroidal blood flow.[40,43] Thus, it has been suggested that the physiological role of the high rate of choroidal blood flow is merely to prevent thermal damage of ocular structures.[39,44] The dense vasodilative innervation of the choroid might be important to increase choroidal blood flow under certain conditions, such as high light intensity or reduction in arterial blood pressure.[45,46] Interestingly, most of the ganglion cells in the human choroid are located in the central-temporal region of the choroid, adjacent to the fovea. The presence of autonomic intrinsic neurons that are able to mediate fast vasodilative reflexes might be most important in those central parts of the retina where the light is focused. Similarly, the absence of such a dense network of ganglion cells in rat and rabbits might be related to the absence of a more vulnerable area of central vision, such as the fovea in primates.

The diameter of the choroidal ganglion cells seems to increase with age, a finding that has already been reported by earlier authors.[31,32] This increase in size might be due to an accumulation of lipofuszin granules, a typical event in the aging process of neurons. That this enlargement appears to occur earlier in the central regions of the choroid might indicate that light intensity is a factor that promotes aging in the choroidal neurons.

Acknowledgments

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