Published in final edited form as:

Curr Eye Res. 2005 December ; 30(12): 1089–1095. doi:10.1080/02713680500371532.

Serotonergic Retinopetal Axons in the Monkey Retina

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Abstract

Purpose—To describe serotonergic retinopetal axons in monkeys.

Methods—Whole macaque and baboon retinas, fixed in 4% paraformaldehyde, were labeled
with antisera raised against serotonin (5-HT).

Results—Several large-diameter 5-HT-immunoreactive (IR) axons emerged from the optic disk.
Most axons ran to the peripheral retina, where they branched extensively. Most terminated in the
ganglion cell layer, but a few 5-HT-IR axons terminated in distal inner plexiform or within inner
nuclear layer. Some axons branched extensively near the fovea, and a dense plexus of 5-HT-IR
axons was also found around the optic disk. Varicose 5-HT-IR axons were also associated with
blood vessels, especially in the central retina.

Conclusions—Immunoreactive serotonin is present in a distinct population of retinopetal axons
in the monkey retina. Receptors for serotonin are present in the primate retinas, and based on
physiological studies in other mammals, these retinopetal axons are expected to modulate neuronal
activity and regulate blood flow.

Keywords

blood vessel; centrifugal; primate; retinopetal; serotonin

INTRODUCTION

Mammalian retinas receive input from neurons in the brain stem and hypothalamus. One
population of retinopetal axons uses histamine as its neuro-transmitter,^{1,2} and there is
evidence for a second population of these retinopetal axons containing serotonin. Neurons in
the dorsal raphe of various rodents are labeled after intraocular injections of retrograde

tracers and therefore are candidates for cells of origin of the retinopetal axons.³⁻⁷ Serotonergic axons are found in mouse optic nerves,⁸ and after preincubation of rat retinas in serotonin, axons are labeled with antibodies against serotonin.^{6,9} Furthermore, after electrolytic lesions³ or injections of an indoleamine that destroys serotonergic neurons¹⁰ in the dorsal raphe of rats, retinal serotonin levels are reduced.

Serotonergic neurons in the dorsal raphe of green monkeys are retrogradely labeled after intraocular injections of cholera toxin subunit B.¹¹ This finding suggests that monkey retinas also receive serotonergic input from the dorsal raphe. To test this hypothesis, whole baboon and macaque retinas were labeled with antisera raised against serotonin. The serotonin immunoreactive (5-HT-IR) axons emerged from the optic disk, ran in the optic fiber layer (OFL), and ramified in the ganglion cell layer (GCL) or, less frequently, in the inner plexiform layer (IPL) or inner nuclear layer (INL). Some labeled axons were associated with retinal blood vessels.

MATERIALS AND METHODS

Adult baboon (*Papio anubis*) eyes were obtained through the Biological Materials Distribution Program at the Southwest Foundation for Biomedical Research (San Antonio, TX, USA). Most of the adult macaque (*Macaca mulatta*) eyes were purchased from Covance Research Products (Alice, TX, USA). The monkeys were sedated with ketamine (15 mg/kg), exsanguinated via the femoral vein, and euthanized with an overdose of sodium pentobarbital (100 mg/kg), according to institutionally approved protocols and following NIH guidelines. The eyes were removed, hemisected, and the posterior halves were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hr at either 20°C or room temperature. The retinas were isolated and rinsed several times in phosphate-buffered saline (PBS) after this and all succeeding steps. The retinas were placed on membrane filter paper, ganglion cell side up, and the vitreous was removed with a fine brush.

Whole retinas were labeled with a polyclonal goat anti-serotonin antiserum (1:2000; Immunostar, Hudson, WI, USA)¹² with 0.3% Triton X-100 in 0.3% sodium azide in PBS at 4°C for 7–12 days. The retinas were incubated at 4°C in affinity-purified biotinylated donkey anti-goat IgG (1:100) for 1 or 2 days and overnight in streptavidin-conjugated Cy3 (1:100) (Jackson ImmunoResearch, West Grove, PA, USA), both at 4°C. They were mounted ganglion cell side up on slides and coverslipped with VectaShield (Vector Laboratories, Burlingame, CA, USA). Images were acquired with a Zeiss confocal laser-scanning microscope (LSM 410; Carl Zeiss, Thornwood, NY, USA) with a krypton-argon laser using a laser emission filter of 590–610 nm for Cy3 with a 40× oil immersion objective. Short stacks of optical sections were projected along the *x*-axis, and measurements were made using the Zeiss LSM image browser. Images and photomontages were processed in Adobe Photoshop (Adobe Systems, San Jose, CA, USA) to enhance brightness and contrast. To visualize nuclei, one flat-mount was incubated in the dark with 1.4 nM 4',6-diamidino-2-phenylindole, di-hydrochloride (DAPI) (Molecular Probes, Eugene, OR, USA) for 5 min at 4 °C. Serial optical sections (0.5-μm step size) were acquired using a Zeiss 510 LSM Meta confocal microscope with a Plan-Neofluar 40× oil objective. The bandpass filter parameters were set to 420–480 nm for DAPI and 561–582 nm for Cy3. The image stacks were then projected along the *y*- or *z*-axis.

One adult female *Macaca mulatta* was sacrificed at the conclusion of experiments not affecting the eyes and following protocols approved by the University of Texas Health Science Center Animal Care and Use Committee. The animal was overdosed with ketamine HCl (10 mg/kg, IM), followed by sodium pentobarbital (25–30 mg/kg i.v.). It was perfused transcardially with PBS followed by 4% paraformaldehyde (pH 7.4) in 0.9% PBS, followed

by 5% glycerol in 4% paraformaldehyde and 10% glycerol in 4% paraformaldehyde. The eyes were removed under room illumination and immediately immersion fixed in 4% paraformaldehyde, 0.1 M PB (pH 7.4) overnight at 4°C. The tissue was processed as described above except that it was incubated in 1:2000 rabbit anti-5-HT (generously provided by Dr. David Pow, The University of Queensland, Queensland, Australia). This antiserum has been used to study the retina previously, and it recognizes 5-HT but not the closely related compounds 5-hydroxytryptophan, melatonin, or 5-hydroxyindoleacetic acid.¹³ Labeled axons were drawn using Neurolucida (v. 4.36 MicroBrightField, Williston, VT, USA) software and a Zeiss Axioskop upright microscope with epifluorescence and a 20× objective. The area occupied by the axon was calculated in Neurolucida by drawing four rectangular boxes to enclose all axons beyond the first branching point.

RESULTS

Serotonin immunoreactive axons were labeled in five baboon and three macaque retinas; no perikarya were labeled. Both antisera yielded the same results, and there were no apparent species differences. In each retina, 3 to 10 5-HT-IR axons emerged from the optic nerve head and ran in all directions. Each axon covered a large area of the retina; for example, one axon occupied an area of 23.14 mm² (Fig. 1). However, the terminals never completely covered the retina. The secondary and higher order branches were studded with varicosities, ranging in size from 1 to 2.5 μ m in diameter (Figs. 1 and 2A).

In both the central and the peripheral retina, the majority of 5-HT-IR axons branched repeatedly and terminated in a sparse, irregular plexus in the GCL (Fig. 3). Most terminals ended with small swellings approximately 1 to 2 μ m in diameter, and a few ended in larger swellings (Fig. 2B). Occasionally, these axons in the GCL made orthogonal branches to the distal IPL or the INL (Figs. 2A and 3A). These branches only ran for short distances and typically ended in small swellings (Figs. 2C and 3B); in some cases, the axons returned to the GCL. None of the axon terminals made pericellular baskets around amacrine cell bodies.

The 5-HT-IR axons that supplied the central retina followed the same trajectories as the ganglion cell axons. Several of the 5-HT-IR axons branched and terminated in a dense plexus nasal to the fovea (Fig. 4). In some retinas, a similar plexus of 5-HT-IR axons was found on the temporal side of the fovea. Because these axons terminated in an avascular zone of the retina, they cannot be contacting blood vessels there. The axon terminal morphology was the same in the central retina as in the peripheral retina, but the density of terminals near the fovea was higher than in the peripheral retina.

5-HT-IR axons were also associated with a subset of retinal blood vessels, particularly in the central retina (Figs. 4B and 5). Sometimes axons ran alongside blood vessels for several hundred micrometers (Fig. 5A). Other 5-HT-IR axon branches appeared to loop and branch extensively around the blood vessels (Fig. 5B). Without double labeling experiments using a blood vessel marker, we cannot rule out the possibility that other 5-HT-IR axons end on blood vessels. This is unlikely, however, because the axons typically did not follow the same trajectories as blood vessels and avascular areas were innervated. Furthermore, there were many clearly visible blood vessels that were apparently not contacted, and the blood vessels were typically innervated for relatively short distances.

Numerous smaller diameter 5-HT-IR axons were also found in the OFL near the optic disk (Fig. 6). These axons had numerous varicosities, but due to the high density of the axons, they could not be followed for long distances. Some of the axons that supplied other regions of the retina originated from this plexus.

DISCUSSION

This is the first description of 5-HT-IR axons in monkey retinas. They were clearly retinopetal in morphology because (1) they emerged from the optic disk, (2) after running away from the optic disk they made varicose terminal branches, and (3) there were no labeled cell bodies in the retina. The 5-HT-IR axons were similar to retinopetal axons described previously in primate retinas. The axons labeled using the Golgi method in macaque and chimpanzee retinas by Polyak¹⁴ resemble the ones labeled with antibodies to serotonin in this study. Retinopetal axons like these have also been described by several groups using silver stains in primates¹⁵⁻²⁰. Two other lines of evidence suggest that retinopetal axons are present in macaques. Terminals in the macaque IPL degenerate after lesions of the optic nerve,²¹ and retinopetal axons are labeled anterogradely when horseradish peroxidase is applied to the cut optic nerve.²²

One population of retinopetal axons in primates is labeled with antibodies to the neurotransmitter histamine and originates from neurons in the posterior hypothalamus.² Although the 5-HT-IR axons were similar in many respects to the histamine-IR axons, there were several morphological differences. (1) The 5-HT-IR primary axons were narrower in diameter than the primary histamine-IR axons. (2) The histamine-IR axons encircled the fovea, and many of them returned to the optic disk. Although the 5-HT-IR axons also supplied the central retina, they did not surround the fovea or return to the optic disk. (3) The 5-HT-IR axons sometimes extended into the INL, but the majority of the histamine-IR axons terminated in the IPL. (4) The 5-HT-IR axons formed a plexus near the optic disk, but the histamine-IR axons did not. (5) Although both types of retinopetal axons ran alongside and contacted retinal blood vessels, the terminals of 5-HT-IR made more frequent contact with blood vessels. We concluded that there are at least two types of retinopetal axons in primate retinas.

Rabbit²³ and rat²⁴ retinas contain populations of indoleamine-accumulating amacrine cells. These amacrine cells do not contain detectable concentrations of endogenous serotonin, but contamination with blood platelets that release serotonin is sufficient to produce detectable labeling with antibodies to serotonin in rabbits.²⁵ It is unlikely that the blood was the source of the immunoreactive serotonin in our experiments, however, because the monkeys were exsanguinated or perfused before the eyes were removed, and no blood was detected in the retinal vessels.

Other reports of serotonin-like immunoreactivity in primate retinas have been published. In the developing human retina, photoreceptors contain immunoreactive serotonin, presumably as a precursor for melatonin.²⁶ There have been two previous reports of indoleamine-accumulating neurons in retinas of New World Monkeys. A subset of horizontal cells in the squirrel monkeys accumulates serotonin,²⁷ and a subset of amacrine cells in capuchin monkeys accumulates 5,6 dihydroxytryptamine.²⁸ However, because these cells do not contain detectable amounts of endogenous serotonin under the conditions in which the retinas were prepared, they are thought to use other molecules as their neurotransmitters. There are no such indoleamine-accumulating retinal neurons in Old World Monkeys.²⁹ In the studies cited above, it is likely that serotonergic retinopetal axons also took up indoleamines, but they were not described. It would have been difficult to detect the sparse 5-HT-IR retinopetal axons in the vertical sections of the retina used in the earlier studies.

Serotonin might act via at least two types of receptors to modulate retinal function in primates. 5-HT₂ receptors are present in the IPL of macaque and human retinas,³⁰ and 5-HT_{3A} receptors are found on human rod photoreceptor terminals,³¹ a finding suggesting that at least some of the actions of 5-HT are mediated by volume transmission. The effects of

serotonin on primate retinal neurons have not been studied, but results in other mammalian retinas suggest that serotonin acts via multiple pathways. Changes in the activity of retinal ganglion cells in rats during sleep have been attributed to serotonergic retinopetal axons.³² Acting through 5-HT₂ receptors, serotonin reduces the current through GABA_C receptors of rod bipolar cells in rats³³ and also inhibits the release of thyrotropin releasing hormone from rat retinas.³⁴ In cat retinas, 5-HT decreases the maintained firing rates of ON center ganglion cells and increases the rates of OFF center cells.³⁵ The effects of serotonin have been studied most extensively in rabbits. Serotonin increases the maintained firing rate of all retinal ganglion cell types^{36,37}; this increase is mediated, in large part, by 5-HT₂ receptors.³⁶⁻³⁸ Acting via 5-HT₂ receptors, 5-HT stimulates inositol phosphate formation.³⁹ Serotonin acting at 5-HT₃ receptors decreases the light responses of dark-adapted OFF center cells and enhances responses of ON center cells⁴⁰; these responses may be mediated by inhibitory actions of 5-HT₃ receptors on rod terminals.³¹ A 5-HT_{1A} agonist decreases the surround responses of both ON and OFF center retinal ganglion cells because it blocks the effects of horizontal cells.³⁸ Acting via 5-HT₇ receptors, serotonin also inhibits dopamine release.⁴¹

We also observed 5-HT-IR axons associated with retinal blood vessels, particularly in central retina. One possible function of serotonin released from retinopetal axons is to constrict retinal blood vessels.⁴² In rats, serotonin applied intravenously or systemically produces retinal ischemia; the vessels supplying the optic disk are particularly sensitive.⁴³ In monkeys, however, intravenous serotonin produces ischemia only in animals with atherosclerosis.⁴⁴ Another possibility is that the terminals on retinal blood vessels have a chemosensory function. In rats, serotonergic neurons in the dorsal raphe are stimulated by increases in arterial CO₂ concentration.⁴⁵ According to this hypothesis, the 5-HT-IR axons contacting blood vessels would convey a signal to the brain reflecting the activity of neurons in the central retina. If the neurons that supply the retina are typical midbrain serotonergic neurons, they would also project throughout the forebrain, where it has been proposed that they mediate the nonrespiratory responses to acidosis such as arousal, anxiety, and changes in cerebral blood flow.⁴⁶

Blood vessels are not the only targets of these retinopetal axons, however. The axons typically do not follow the same trajectories as the blood vessels, and they extend into avascular areas of the retina. Although it is possible that some of the effects of serotonin on mammalian retinal neurons *in vivo* are mediated by changes in retinal blood flow or capillary permeability, these actions on the vasculature cannot account for the physiological effects of serotonin on mammalian retinal neurons *in vitro*. These studies indicate that retinal neurons are also major targets, findings supported by the localization of 5-HT receptors on neurons. To understand how serotonergic retinopetal axons contribute to retinal information processing in primates, it will be necessary to localize additional types of serotonin receptors and to study the effects of serotonin at each set of receptors.

Acknowledgments

Support for this study was provided by National Eye Institute grants EY06472 and EY12610, a grant from the Juvenile Diabetes Foundation (to D.W.M.) and Core Grant EY10608. We are grateful to Mrs. Lillemor Krosby and Mr. John Healy for excellent technical assistance, Dr. David V. Pow for providing antiserum, and Dr. Tamas Horvath for valuable discussions. We also wish to thank Drs. Karen Rice, Jerilyn Pecotte, and Gene Hubbard at the Southwest Foundation for Biomedical Research in San Antonio for providing baboon eyes and Dr. Jocelyne Bachevalier of the University of Texas Medical School at Houston for providing macaque eyes.

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FIGURE 1.

Neurolucida sdrawing at low magnification of the major branches of a 5-HT-IR retinopetal axon in a macaque retina labeled with rabbit antiserum against serotonin. The axon diameters are not drawn to scale. Beginning from the first branch point, the axonal arbor occupies an area of 23 mm². F, fovea; OD, optic disk. Scale bar = 1 mm.

FIGURE 2.

(A) A photomontage of 5-HT-IR axons in the nasal region of the baboon retina. A typical axon emerges from the optic disk and ran approximately 2 mm before it made its first branches (arrows). Some axons also make orthogonal branches to the distal border of the IPL (asterisks). Scale bar = 200 μm . (B) Many of the branches in the ganglion cell layer overlap and terminate in swellings (arrowhead). (C) Terminals of the 5-HT-IR axons in the distal IPL (arrowhead) that originate from an orthogonal branch (asterisk) in the GCL. Scale bars = 50 μm in (B) and (C)

FIGURE 3.

(A) A photomontage of 5-HT-IR axons in the central baboon retina. Two axons (arrows) branch repeatedly nasal to the foveas (F). One of these axons branches and contacts blood vessels (boxed area). In this retina, 10 contacts similar to this one are observed on other blood vessels surrounding the fovea. Scale bar = 200 μm . (B) A high-magnification image of the blood vessel in the central retina. The axon makes an orthogonal branch to the inner plexiform layer and encircles a small region of the blood vessel. (C) High-magnification image of the axons near the fovea and some that terminate along the foveal rim (arrowhead) that originate from an orthogonal branch (asterisk) in the GCL. Scale bar = 50 μm in (B) and (C).

FIGURE 4.

5-HT-IR axons contacting a blood vessel in the baboon retina. (A) This is a stack of optical sections through the entire blood vessel. Two axons (arrows) supply varicose branches that contacted the surface of the blood vessel. The axons branch repeatedly and run along the surface toward a bifurcation in the blood vessel (boxed area). (B) A single optical section through the center of the blood vessel. The axons loop several times around the vessels and have several large swellings (arrowhead). Scale bars = 50 μm .

FIGURE 5.

Three-dimensional reconstruction of a stack of optical sections through a macaque retina with 5-HT-IR axons (red) and DAPI-labeled nuclei (blue). (A, B) The 5-HT-IR axons branch primarily in the optic fiber layer (OFL) and ganglion cell layer (GCL). The 5-HT-IR axons sometimes make orthogonal branches (asterisk, panel A) through the inner plexiform layer (IPL), terminating in the most distal part. Some 5-HT-IR axons also terminate in the inner nuclear layer (INL) (arrow). Scale bars = 20 μm .

FIGURE 6.

A photomontage of 5-HT-IR axons surrounding the optic disk (OD) in the baboon retina. Some of these axons are co-fasciculated (arrows). Some axons emerge from this plexus and run away from the optic disk in the optic fiber layer. A dashed line indicates the border between the retina and the optic disk. Scale bar = 200 μm .