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Morphology and Neurochemistry of Rabbit Iris Innervation

Jiucheng He and Haydee E.P. Bazan

Neuroscience Center of Excellence and the Department of Ophthalmology, Louisiana State University Health Sciences Center, School of Medicine, New Orleans, Louisiana, USA

Abstract

The aim of this study was to map the entire nerve architecture and sensory neuropeptide content of the rabbit iris. Irises from New Zealand rabbits were stained with antibodies against neuronal-class β III-tubulin, calcitonin gene-related peptide (CGRP) and substance P (SP), and whole-mount images were acquired to build a two-dimensional view of the iridal nerve architecture. After taking images in time-lapse mode, we observed thick nerves running in the iris stroma close to the anterior epithelia, forming four to five stromal nerve rings from the iris periphery to the pupillary margin and sub-branches that connected with each other, constituting the stromal nerve plexus. In the anterior side, fine divisions derivated from the stromal nerves, forming a nerve network-like structure to innervate the superficial anterior border layer, with the pupillary margin having the densest innervation. In the posterior side, the nerve bundles ran along with the pupil dilator muscle in a radial pattern. The morphology of the iris nerves on both sides changed with pupil size. To obtain the relative content of the neuropeptides in the iris, the specimens were double stained with β III-tubulin and CGRP or SP antibodies. Relative nerve fiber densities for each fiber population were assessed quantitatively by computer-assisted analysis. On the anterior side, CGRP-positive nerve fibers constituted about 61%, while SP-positive nerves constitute about 30.5%, of the total nerve content, which was expressed as β III tubulin-positive fibers. In addition, in the anterior stroma of the collarette region, there were non-neuronal cells that were positive for SP. On the posterior side, CGRP-positive nerve fibers were about 69% of total nerve content, while SP constituted only up to 20%. Similarly, in the trigeminal ganglia (TG), the number of CGRP-positive neurons significantly outnumbered those that were positive for SP. Also, all the SP-positive neurons were labeled with CGRP. This is the first study to provide a two-dimensional whole mount and a cross-sectional view of the entire iris nerve architecture. Considering the anatomical location, the high expression of CGRP and SP suggests that these neuropeptides may play a role in the pathogenesis of anterior uveitis, glaucoma, cataracts and chronic ocular pain.

Correspondence should be addressed to Jiucheng He or Haydee E.P. Bazan, Neuroscience Center of Excellence, Louisiana State University Health Sciences Center, 2020 Gravier Street, Suite D, New Orleans, LA 70112-2272. jhe@lsuhsc.edu or hbazan1@lsuhsc.edu.

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Keywords

Iris innervation; sensory nerves; neuropeptides; Substance P; calcitonin gene-related peptide; trigeminal ganglia; neurogenic inflammation; anterior uveitis

INTRODUCTION

The iris is the anterior portion of the uveal tract and constitutes the diaphragm localized in front of the lens and the ciliary body, which separates the anterior and posterior chambers. Its main function is to control the amount of light reaching the retina by adjusting the size of the pupil. The iris has three layers: (1) the superficial anterior border layer, which is a modification of the stroma composed of fibroblasts and melanocytes; (2) the stroma, which comprises the bulk of the iris and the sphincter muscle; and (3) pigmented epithelial cells and dilator muscle, which constitute the posterior layers (Rodriguez et al., 1982). The stroma connects to the sphincter muscle (the sphincter pupillae), which contracts the pupil, and to the dilator muscle, which pulls the iris to enlarge the pupil. The collarette is the thickest region where the sphincter and dilator muscles overlap. The outer edge of the iris, known as the root, is attached to the sclera and the ciliary body. The iris muscles are innervated by autonomic nerves, mainly sympathetic and parasympathetic nerves that control pupil size by their antagonist actions. The iris is also supplied with sensory nerve fibers derived from the ophthalmic branch of the trigeminal ganglion (Stone et al., 1982; Kuwayama and Stone, 1987). For many years it was postulated that the function of the sensory nerves was to mediate protective reflexes, but more recently it has been shown, mainly through denervation of the ophthalmic nerve (Fujimara, 1984; Kuwayama and Stone, 1987), that it influences intraocular blood vessels, smooth muscle responses and immune functions through release of various peptides (Neuhuber and Schrod, 2011).

Iridal innervations have been studied by electron microscopy and histochemical methods in a wide range of animal species including rats, guinea pigs, rabbits, cats, monkeys and humans (Ayer-Le Lievre et al., 1984; Beckers et al., 1993; Ehinger, 1967; Fujimara et al., 1984; Gibbins and Morris, 1987; Hirai et al., 1994; Jones and Marfurt, 1998; Seiger et al., 1985; Selbach et al., 2000; Stone et al., 1982; Terenghi et al., 1985; Tervo et al., 1981); however, the detailed architecture of these innervations remains unclear. Recently, our laboratory developed a modified method of immunofluorescence and imaging that could provide a map of the entire corneal nerve architecture in both humans and experimental animals (Cortina et al., 2010; He et al., 2010; He and Bazan, 2012; He and Bazan, 2013). In the current study, this technique was used to investigate the whole nerve architecture and the distribution of sensory neuropeptides in the rabbit iris. The reasons for using the rabbit model are as follows: 1) rabbits are among the most common animal models available for investigating eye diseases; 2) the iris sizes are similar to those of humans; and 3) most importantly, all the antibodies used (β III-tubulin, CGRP and SP) have been well tested in studies of corneal nerve regeneration in our laboratory (Cortina et al., 2010; Cortina et al., 2012; Cortina et al., 2013; Esquenazi et al., 2005) and have proven to be specific to the corneal nerve structures (which share the same sensory nerve origin as the iris from the trigeminal ganglia).

2. Materials and Methods

2.1 Animals

New Zealand white rabbits of both sexes weighing between 2.5 and 3.5 kg were housed in the Neuroscience Center of Excellence at the Louisiana State University Health Sciences Center, New Orleans, and treated in compliance with the guidelines of the ARVO Resolution on the Use of Animals in Ophthalmic Research. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center, New Orleans. Rabbits were euthanized with an overdose of sodium pentobarbital via ear vein injection.

2.2 Antibodies

Mouse monoclonal anti- β III-tubulin (Tuj1, MMS-435p) antibody was purchased from Covance Antibody Services Inc., (Berkeley, CA); rat monoclonal anti-SP (NMM1679661) was from Millipore (Temecula, CA). Mouse monoclonal CGRP and goat polyclonal anti-CGRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies, including Alexa fluor® 488 goat anti-mouse IgG (H+L), Alexa fluor® 488 donkey anti-rat IgG (H+L), Alexa fluor® 594 donkey anti-goat IgG (H+L) and Texas red®-X goat anti-rat IgG, were purchased from Invitrogen (Carlsbad, CA).

2.3 Immunofluorescence Staining and Imaging

Rabbit eyes were enucleated immediately after death and opened by a 2–3 mm incision dorsal to the limbus, followed by excision of the iris from the ciliary margin. The time between sacrifice and fixation was between 10–15 seconds for each eye. In this experiment, in which eight rabbits were sacrificed in standard laboratory lighting, the pupil size was about 2–3 mm in diameter. In order to observe the shape of the iris nerves in the state of mydriasis, two rabbits were killed at the time when the interior lights were dimmed for 5 minutes. Irises were fixed in freshly-prepared 4% paraformaldehyde in 0.01M phosphate buffer (pH 7.4) for 2 hours at room temperature. After gradient dehydration in 10%, 15%, and 20% sucrose in 0.01M phosphate-buffered saline (PBS), each for 2 hours, the whole irises were kept in a 24-well plate (one iris per well) and incubated with 10% normal goat serum plus 0.3% Triton X-100 solution in PBS for 1 hour at room temperature to block non-specific binding. This was followed by incubation with primary antibodies against β -III tubulin (1: 1000), CGRP (1:500) and SP (1:500) in 0.1M PBS containing 1.5% normal goat serum plus 0.1% Triton X-100 for 72 hours at 4°C. After thorough washing with PBS-bovine serum albumin (BSA, 4 × 15 minutes), the irises were incubated with the secondary antibodies for 24 hours at 4°C and washed again with PBS-BSA (4 × 15 minutes). The tissue was kept in its natural shape by soaking it in a proper volume of 0.1 M PBS (to allow the iris to flatten on the bottom of the well and not move or fold). Consecutive images, from the pupillary margin to the periphery as well as from the anterior to the posterior iris, were acquired in a time-lapse mode with a fluorescence microscope (Nikon Eclipse TE200) equipped with a Photometrics digital camera (CoolSNAP™ HQ) using MetaVue imaging software. To avoid differences in contrast between the anterior and posterior sides of the iris, some of the images were also taken with a fluorescent microscope (Olympus IX71) with capability to adjust the contrast automatically. The images, recorded on the same plane at

adjoining points, were merged using Photoshop imaging software (Adobe, Mountain View, CA) and then pasted onto a Microsoft Office PowerPoint template to build the whole view of the iris nerve architecture. For double immunofluorescence, after labeling with the first set of antibodies (β III tubulin and correspondent secondary antibodies), the tissue was once more fixed in 2% paraformaldehyde for 30 minutes, followed by three washings (15 minutes each), and incubated with a second primary antibody (CGRP or SP) for 72 hours, followed by a corresponding FITC- or TRITC- conjugated secondary antibody; washings were performed in the same manner as described above. To exclude non-specific labeling, the primary antibodies were replaced by serum IgG of the same host species as the primary antibody. In controls without primary antibodies, there was no staining (data not shown). For transected images of iridal nerves, 15 μ m cryostat sagittal sections were prepared from the samples after finishing the whole mount examination using the same method as described previously (Cortina et al., 2010; He et al., 2010; He and Bazan, 2012).

Briefly, the whole iris was cut into two halves and embedded into optimal cutting temperature (OCT) compound. Serial 15- μ m cryostat sections were cut in a sagittal direction, air-dried, and stored in the dark. The sections were used directly or kept at -80°C . When in use, the sections were washed in 0.1 M PBS to remove OCT, and 4–6-diamidino-2-phenylindole (DAPI) was added to stain the nuclei, which were then covered with a mounting medium (Aqua-mount; Lerner Laboratories, Pittsburgh, PA); images taken with the Olympus IX71 microscope. For further hematoxylin and eosin staining, after fluorescence images were finished, the same sections were washed in distilled water for 10 minutes. Then routine procedures were performed, and images were taken.

To test the origin of the sensory neuropeptides (CGRP and SP), four adult rabbits were euthanized as explained above. The crania were opened, and both left and right trigeminal ganglia (TG) were removed and immediately fixed in freshly-prepared 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 2 hours at room temperature. After washing with 10%, 15%, and 20% sucrose in 0.1M PBS, each for 2 hours, the whole TG was embedded in OCT compound. Serial 10- μ m cryostat sections were cut, dried at room temperature for 2 hours, and stored at -20°C until use.

For double immunofluorescence, the sections were washed in 0.1 M PBS to remove OCT, and then incubated with 10% normal goat serum plus 0.3% Triton X-100 solution in PBS for 30 minutes at room temperature to block non-specific binding, followed by incubation with primary antibodies against β -III tubulin (1: 1000) plus CGRP (1:500), β III tubulin plus SP (1:500), or CGRP plus SP in 0.1M PBS containing 1.5% normal goat serum overnight at 4°C . After a thorough wash with PBS-BSA (3×5 minutes), the sections were incubated with corresponding FITC- or TRITC-conjugated secondary antibodies for 1 hour at room temperature. Images were taken in the same way as described above.

2.4 Data analysis

To obtain the relative contents of neuropeptides in the rabbit irises, 12 irises were double-stained with β III tubulin combined with CGRP or SP. For each neuropeptide, a total of 32 whole-mount images per iris were taken at an objective lens with 20 X magnification from the collarette part of two sides of the four irises, with each side having four images per iris.

Afterwards, the same number of images for β III tubulin (which represents the total nerve content) was taken at the same plane (or visual field) in the same iris. To acquire better contrast, some of the images were changed to grayscale mode and placed against a white background.

The nerve fibers in each image were carefully drawn with 4-pixel lines following the course of each fiber by using the brush tool in Photoshop imaging software. The percentage of nerve area was quantified for each image. In the same visual field, β III tubulin equaled the percentage of total nerve area, and the average ratio of the peptide-positive nerve area against β III tubulin represented the relative content.

To calculate neurons positive for CGRP and SP in the TG, 16 images randomly selected from 16 sections of 4 rabbits (2 sections/ganglion) were recorded with a 10 X objective lens and counted in a blind fashion.

Data analysis was performed using SPSS software, and data were expressed as means $\pm$ SD.

3. Results

3.1 Iris nerve architecture

To show the entire iris nerve architecture, the irises were stained with mouse monoclonal anti- β III-tubulin antibody, a pan-neuronal marker that stains all the nerves in the tissue. In Fig. 1A, a portion of rabbit iris whole mount shows the stromal nerve architecture when the pupil size was 2–3mm in diameter. Nerve fiber bundles branching out from the stromal nerve rings connect with each other and form the stromal nerve plexus. Subdivisions originating from the plexus innervate the anterior and posterior border layers. Images in Fig. 1B represent stacks obtained from videos acquired in time-lapse mode showing the topographic images at the planes of superficial anterior border layer, mid-stroma, and posterior layer in the iris parts of pupillary margin, collarette and root. Each stack represents a focus plane with a 10–15 μ m difference in depths between the adjacent stacks. There is a fine network of nerve fibers in the superficial anterior and posterior border layers and thicker nerve trunks in the mid-stroma. In the stroma and posterior layers of the pupillary region (where the sphincter muscle is located), there are many fine nerve fibers running in a circular pattern around the pupil.

Around the iris root, there was an average of $15.1 \pm 2.6\%$ ($n = 8$ irises) thick nerve trunks (diameter $41.03 \pm 5.3 \mu$ m) that were equally distributed between the four quadrants. These nerves ran in the middle of the stroma, forming four to five stromal nerve rings from the iris periphery to the pupillary margin (as marked by circles in Fig. 2A).

Iris innervation of the anterior surface when the pupil size was 2–3 mm in diameter is shown in Fig. 2A. Fine divisions, deriving from the stromal nerves, form a network-like nerve structure that innervates the superficial anterior border layer (Fig. 2B). Regardless of the state of dilation, the pupillary margin presents the densest innervation (Fig. 2C). The iris nerve architecture, viewed from the posterior surface, shows a network of thinner nerves (diameter, $5.5 \pm 1.2 \mu$ m, $n=4$ irises) that run along the pupil dilator muscle in a radial pattern that converge on the pupil (Fig 2D). In comparison with the nerves from the anterior surface,

which are beaded and form loops, the posterior nerve fibers are smoother and straighter, as seen in more detail in the highlighted image of the collarette area in Fig. 2E.

The fiber course of the iris nerves changes in diameter with pupil size. A comparison of nerves in the anterior and posterior sides shows that when the pupil is 2–3 mm in diameter (Fig. 2C and E), the nerve fibers along the dilator are straight; however, when the pupil is dilated 5–6 mm in diameter, the nerve fibers on both sides become more loose and curled (Fig. 2F).

After whole-mount examination, the samples were processed for cross section. In Fig. 3A, the montage of the iridal sagittal sections, which was demonstrated with β III-tubulin labeling (green), illustrate a cross-sectional view of the innervation of the entire iris. Highlighted images (double-stained with DAPI) reveal the detailed nerve structures in the regions of the iris periphery (root), collarette, and pupillary margin (Fig. 3B, C and D). The periphery and the collarette sections show a higher nerve density in the posterior side than in the anterior, while the pupillary margin was more highly innervated in the anterior area of the iris. An entire cross-section stained with H & E shows the sphincter in the pupil region and the lumen of a blood vessel in the root (Fig. 3E). The overall fiber density calculated from the β III-tubulin-positive nerve fibers based on whole mount images was about 1.46 times higher in the posterior side ($21.01 \pm 1.32\%$) than in the anterior side ($14.51 \pm 1.51\%$) ($n=4$ irises).

3.2 Sensory neuropeptide iris innervation

The entire view of the CGRP-positive nerve architecture, with a contracted pupil at the two sides of a rabbit iris, is shown in Fig. 4A. The pattern of CGRP-positive nerve fibers was similar to the one obtained with β III-tubulin staining, with the pupillary zone having the densest innervation consisting of thin fibers and abundant CGRP nerve terminals (Fig. 4B). High expression of CGRP-positive nerves was also found around the blood vessels in the peripheral ciliary zone (Fig. 4A, arrows and inlet). The overall CGRP-positive fiber density, calculated as the percentage of the total area, was $6.24 \pm 0.89\%$ ($n=4$ irises) in the anterior side and $14.44 \pm 1.5\%$ in the posterior side within the collarette region. Double staining with antibodies directed against β III-tubulin and CGRP showed that in the collarette region CGRP-positive nerve fibers constitute $61.1 \pm 4\%$ ($n=4$ irises) of the total nerve content in the anterior side, and about $68.8 \pm 3.4\%$ in the posterior side (Fig. 4C). This demonstrates a higher density in CGRP-positive nerve fibers ($P<0.05$) in the posterior side. Figure 4D are images of double-stained cross sections of the pupillary region, collarette and iris root. In the pupillary region, there are many cross sections of the nerve fibers that run along the sphincter muscle. In the iris root, there are abundant CGRP-positive nerve fibers surrounding the vessel wall (Fig. 4D, arrow).

In Fig. 5A, the entire view of the SP-positive nerves is shown for the two sides of a rabbit iris with a pupil size of 2–3 mm in diameter. In comparison with the CGRP, there is less density of SP-positive nerves in both sides of the iris, with fewer thick nerves forming the stromal rings and fewer nerves in the sphincter area (Fig. 5B). Although there were no significant differences in the total SP-positive nerve fiber densities between the anterior ($4.42 \pm 0.52\%$) and posterior ($4.18 \pm 0.38\%$) ($n=4$ irises) sides, double immunostaining with

antibodies against β III-tubulin demonstrated that SP-positive nerve fibers in the collarette area constitute $30.5 \pm 3\%$ of the total nerve content in the anterior side, and about $19.8 \pm 1.8\%$ in the posterior side (Fig. 5C). This demonstrates that there are significantly less SP-positive nerves in the anterior side with respect to total innervation. Double immunohistochemistry (Fig. 5D) with antibodies directed against CGRP (green) and SP (red) showed that all SP-positive nerves were also CGRP positive. A detailed distribution of SP-positive fibers in the pupillary margin and collarette is seen in the cross section in Fig. 5E. In the pupillary region, many SP-positive nerve fibers innervate the sphincter muscle. In the collarette, in addition to nerves, SP-positive scattered cell-like structures, located in the anterior stroma, were also observed. The close-up image counterstained with DAPI shows that the cells are oval-shaped and small in size. They have no dendrites and are negatively stained with β III-tubulin.

3.2 Sensory neuropeptide neurons in the trigeminal ganglion

To investigate the origin and relative content of the sensory neuropeptides, CGRP and SP double immunofluorescence with β III-tubulin was performed in rabbit TG cross sections. As shown in Fig. 6A–C, $29.12 \pm 6.4\%$ ($n=4$ rabbits) of the total neurons were CGRP-positive and $21.98 \pm 4.75\%$ were SP-positive neurons, indicating that in the rabbit TG the number of neurons positive for CGRP significantly outnumbered those that were positive for SP. Double immunofluorescence with CGRP and SP antibodies demonstrates that SP-positive neurons were also CGRP-positive (Fig. 6D).

4. Discussion

In the present study, we used a modified method of immunofluorescence staining and imaging that previously produced a map of the entire human cornea nerve architecture (He et al., 2010; He and Bazan, 2012; He and Bazan, 2013). The advantages of this approach allow us to show, for the first time, not only a two-dimensional whole mount view, but also a detailed cross-sectional view of the nerves in the entire rabbit iris. It also allows us to investigate the total distribution of the sensory neuropeptides CGRP and SP. Most of the previous studies in iris neurobiology have been conducted on tissue sections. An earlier study tested the technique of tissue whole mounts stained with silver cyanate on rat iris nerves (Ayer-Le Lievre et al., 1984).

In the current study, we first investigated the entire iris nerve architecture by labeling the nerve fibers with a pan neuronal marker β III-tubulin, which immunostains all the nerve fibers regardless of their origins or phenotypes. Our results show that the rabbit iris is densely innervated and displays a different pattern of nerve distribution in the anterior and posterior sides. In the anterior side, the nerve fibers originate from the stromal nerve rings and constitute a network-like structure that innervates the superficial anterior border layer, which contains fibroblasts and melanocytes. The highest nerve fiber density occurs around the pupillary region, which allows for innervation of the pupil sphincter muscle. In the posterior side, the nerve fibers run along the pupil dilator muscle in a radial pattern. The nerve fiber courses change with pupil size.

The expression of the sensory neuropeptides CGRP and SP in the anterior uvea has been studied previously by both biochemical and histochemical methods (Ayer-Le Lievre et al., 1984; Beckers et al., 1993; Ehinger, 1967; Fujimara et al., 1984; Gibbins and Morris, 1987; Hirai et al., 1994; Jones and Marfurt, 1998; Seiger et al., 1985; Selbach et al., 2000; Stone et al., 1982; Terenghi et al., 1985; Tervo et al., 1981), but their distribution in the entire iris and their relative contents remained unclear. Our results show that the iris contains a large amount of CGRP-positive nerve fibers, with higher density in the posterior side. A whole mount view of the iris nerve architecture indicates that CGRP-positive nerves share the same nerve pattern as β III-tubulin-stained nerves.

CGRP is a potent peptide vasodilator produced in both central and peripheral neurons (Rosenfeld et al., 1983). The release of CGRP from afferent terminals is believed to cause vasodilation and neurogenic inflammation, and efferent release is supposed to be part of the nociceptive relay to the central nervous system (CNS) (Brain et al., 1985; McCulloch et al., 1986). In the eye, mechanical or chemical stimulation of sensory nerves or intracameral injection of CGRP induces an increase in intraocular pressure, breakdown of the blood- aqueous barrier, and elevated levels of cAMP in the aqueous humor associated with ocular neurogenic inflammatory response (Krootila, 1988; Krootila et al., 1988; Unger et al., 1985; Wahlestedt et al., 1986). CGRP acts through G protein-coupled receptors and requires the CGRP-receptor component protein (RCP) for signal transduction. RCP expression was detected in rabbit eyes by immunohistochemistry in the epithelial cells and the blood vessels of the ciliary processes as well as the iris (Rosenblatt et al., 2000). In the current study, the rich density of CGRP nerve fibers in the vessels and pupillary region close to the sphincter muscle suggest that this neuropeptide may play an important role in regulating iris blood flow and pupil reflexes. In addition, the release of this neuropeptide into the aqueous may have important physiological and pathological effects on the surrounding tissues. Early studies have shown that CGRP is constitutively expressed in the rabbit aqueous humor and exerts anti-inflammatory activity (Taylor, et al., 1998). Levels of CGRP in the aqueous humor are significantly higher in patients with proliferative vitreoretinopathy but lower in patients with cataracts and uncomplicated rhegmatogenous retinal detachment (Troger, et al., 2000). Therefore, it is possible that under normal physiological conditions, CGRP may serve as a trophic factor to support the homeostatic activity of the lens epithelium and corneal endothelium, which are normally avascular tissues. When CGRP flows into the trabecular meshwork and aqueous channels, it may act as a vasodilator to ensure the flow of the aqueous humor to maintain normal intraocular pressure. In pathological conditions, CGRP may serve as a neurogenic inflammatory mediator to exacerbate intraocular inflammation, leading to lens opacity and endothelial defects. Excessive release of CGRP through the aqueous channel into the blood circulation may be an important cause of ocular migraines (Durham, et al., 2006; Edvinsson, et al., 2010; Eftekhari, et al., 2010).

The proportion of SP positive nerve fibers was less than half of CGRP in the iris, but much higher than the content in rabbit cornea (7.8% of total nerve area) (Cortina et al., 2012). Several studies have shown that this neuropeptide serves as a trophic factor to promote corneal wound healing by modulating the various aspects of corneal epithelial cell behavior, such as proliferation, adhesion, stratification and migration (Ko et al., 2014; Nishida et al., 1996; Reid et al., 1993). Substance P is also an important neurotransmitter in pain

perception (De Felipe et al., 1998; Zubrzycka and Janecka, 2000). It has been proposed that its release is also involved in neurogenic inflammation, a local inflammatory response to certain types of infection or injury (Donkin et al., 2007; Fujishima et al., 1997; Lambiase et al., 1997; Motterle et al., 2006). In the eye, SP-positive fibers innervate several tissues of the anterior segment, including the cornea, conjunctiva, episclera, trabecular meshwork and iris. Studies have shown that upon stimulation of the trigeminal nerve, SP can be released into the aqueous humor, inducing miosis, breakdown of the blood aqueous barrier and an increase in intraocular pressure (Seiger et al., 1985). SP also has a contracting effect in the isolated iris sphincter muscle (Fujimara et al., 1984). In the current study, the higher density of SP-positive fibers was detected around the pupillary area. It is conceivable that the release of this neuropeptide could be involved in intraocular inflammation and ocular pain.

The presence of SP-positive cell-like structures in the both sides of the iris is a new finding. In addition to SP be recognized as a neuropeptide in sensory neurons, SP gene expression has been reported in non-neural cells, such as human fibroblasts, keratinocytes, endothelial cells, lymphocytes and platelets (Bae, et al., 2002; Jones, et al., 2008; Lai, et al., 1998; Linnik, et al., 1989). Our previous study showed that rabbit corneal epithelial cells express SP after experimental surgery (Cortina, et al., 2012). In the current study, those cells do not look like neurons because they have no dendrites and are negatively stained with the neuronal marker (β III-tubulin). Their character and function in this tissue deserve further study.

Another interesting finding is that the proportion of SP-positive nerves on both sides of the iris is in inverse proportion to that of the CGRP, with a significantly higher percentage of SP in the anterior side of the iris. Although the reason for these differences has not been investigated, it is tempting to speculate that the functions of the two neuropeptides are mainly directed to the tissues that they face, e.g., the corneal endothelium in the anterior side and the lens in the posterior side, both of which lack vascularization and innervation. Therefore, it is conceivable that the release of those neuropeptides into the aqueous may have important physiological and pathological effects on the activities of those tissues.

Trigeminal nerves that innervate the ocular tissue have been considered to be primary sensory nerves. Early studies by immunohistochemistry and retrograde tracing have shown that in the TG, SP co-localizes with CGRP in rats (Gibbins, et al., 1985; Terenghi et al., 1985; Skofitsch et al., 1985; Lee et al. 1985) and guinea pigs (Kuwayama et al., 1987; Ivanusic et al., 2013). In the rabbit, trigeminal denervation studies have suggested that the TG is the origin of SP (Ueda et al. 1982). In the current study, we used a double-labeling immunofluorescent technique to study the relative content of the neuropeptides in the rabbit TG. Our results are in agreement with the previous studies of other animal models (e.g., rat and guinea pig), suggesting that those species may share a similar expression pattern of sensory neuropeptides in the TG. The finding that the amount of neurons positive for CGRP significantly outnumbers those positive for SP may also explain the higher density of CGRP-positive nerve fibers in the iris and cornea (Cortina et al., 2012) in comparison with that of SP-positive nerve fibers.

In summary, using a modified technique of immunofluorescence and imaging we showed, for the first time, an entire view of the rabbit iris nerves. The high expression of the sensory neuropeptides CGRP and SP leads us to speculate that they may play important roles in the pathogenesis of anterior uveitis, glaucoma, cataracts and chronic ocular pain (ocular migraine).

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Abbreviations

SP	Substance P
CGRP	calcitonin gene-related peptide
TG	trigeminal ganglia
PBS	phosphate-buffered saline
cAMP	Cyclic adenosine monophosphate
RCP	receptor component protein
OCT	optimal cutting temperature

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Highlights

- We used a modified technique of immunofluorescence and imaging, for the first time, to provide a complete map of the nerve architecture in rabbit iris;
- The rabbit iris displays a different pattern of nerve architecture in the anterior and posterior sides;
- The morphology of iris nerves changes with the pupil movements;
- The high expression of CGRP and SP in this tissue suggests that these neuropeptides may play a role in maintaining the homeostasis of intraocular tissues and/or cells such as corneal endothelia, lens epithelia, and trabecular meshwork, etc.

Figure 1.

Pattern of rabbit iris innervation by β III-tubulin immunofluorescent labeling. A) Part of the rabbit iris whole mount showing details of the stromal nerve architecture. The main stromal nerve trunks enter the iris at the mid-stromal level and run in a circular pattern, forming 4–5 rings from the root to the pupil region. Sub-branches of the stromal nerve trunks connect with each other to constitute the stromal nerve plexus. B) Image stacks recorded from videos showing details of the nerve architecture at the planes of the superficial anterior border layer, stroma and posterior layer in the pupillary margin, collarette and periphery (root) region. In the stroma and posterior layer of the pupillary region, there are many fine nerve fibers visible (arrows) that run in a circular pattern around the pupil.

Figure 2.

Whole mount view of the complete iris nerve architecture in the anterior and posterior sides when the pupil is 2–3 mm in diameter. The entire iris was stained with β III-tubulin, a pan-neuronal marker, and images were acquired in a time-lapse mode with an Olympus IX71 microscope and with a 5x objective lens maintaining the natural shape of the iris. On each side, more than 100 images were merged together. A) Iris total innervation on the anterior side. The circles indicate the stroma nerve rings. Highlighted images show the detailed nerve architecture in the papillary margin (B) and collarette region (C). D) Total innervation of the iris on the posterior side. E) A highlighted image of the nerve architecture in the iris collarette. Images in F) show the tortuous courses of the nerve fibers in both sides of iris collarette area when the pupil size was 5–6 mm in diameter.

Figure 3.

(A) Entire cross-sectional view of rabbit iris innervation stained with β III-tubulin antibody (green). Highlighted images counterstained with DAPI show the nerve distribution in detail at the root (B), collarette (C), and pupillary margin regions (D). White arrows in B, C and D indicate the fine nerve fibers in the superficial anterior border layer. Double red arrows in C illustrate the thicker stromal nerves in the middle stroma at the collarette region. Dashed red arrows in the pupil margin image show the dot-like cross sections of the nerve fibers running around the pupil. E) The same section stained with hematoxylin and eosin displays the anatomical location of the sphincter in the pupil region, and a cross section view of a blood vessel in the iris root.

Figure 4.

Whole mount view of CGRP-positive nerves in the anterior and posterior sides of the rabbit iris during pupil contraction. A) The iris was stained with guinea pig polyclonal anti-CGRP antibody (red). The arrows indicate many CGRP-positive nerves innervating the blood vessel in the iris periphery. The magnified inlet image shows a blood vessel surrounded by CGRP-positive nerve fibers. B) Highlighted images illustrate the intense CGRP-positive labeling of nerve fibers in the pupil area. C) Representative images of double staining with βIII -tubulin antibody (green) in the collarette region on both sides of the iris. D) Representative cross-section images double stained with CGRP and βIII -tubulin show the distribution of CGRP-positive nerve fibers in the pupillary margin, collarette and the root.

Arrow in the root indicates a blood vessel surrounded by several CGRP-positive nerve fibers.

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Figure 5.

Whole mount view of SP-positive nerves in the anterior and posterior sides of the rabbit iris when the pupil size was contracted. A) The entire iris was stained with rat monoclonal anti-SP antibody. Highlighted images (B) show the SP-positive nerve distribution around the pupil area. C) Representative images illustrate the relative contents of SP positive nerve fibers in the collarette of the rabbit iris double-stained with βIII -tubulin. Arrows indicate SP-positive cell-like stains in the posterior layer. D) Representative images double stained with SP plus CGRP demonstrate that the density of CGRP-positive nerve fibers is higher than that of SP-positive nerve fibers in both sides of the rabbit iris. E) Cross-section images double stained with SP plus βIII -tubulin show the distribution of SP-positive nerve fibers in the pupillary margin and collarette. A highlighted image counterstained with DAPI, as framed in the collarette image, shows the SP-positive cells (arrows) in the anterior stroma.

Figure 6. Sensory neuropeptides in rabbit trigeminal ganglion. A) Relative content of CGRP- or SP-positive cells. For each neuropeptide, 16 sections randomly selected from 4 rabbits were counted in a blind fashion. Data represent the average ratios (Mean $\pm$ SD) of CGRP- or SP-positive cells vs. β III-tubulin neurons, * $p < 0.01$. B and C show neurons in the TG labeled with β III-tubulin, SP and CGRP (representative images). D) Co-localization of CGRP- and SP-positive neurons. Arrows indicate dendrites or nerve fibers.