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Morphology and neurochemistry of rabbit iris innervation

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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 βIII-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 βIII-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 βIII 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.

1. 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 (Rodriguse 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

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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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 Schrodl, 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, 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 (βIII- tubulin, CGRP and SP) have been well tested in studies of corneal nerve regeneration in our laboratory (Cortina et al., 2010, 2012, 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 vain injection.

2.2. Antibodies

Mouse monoclonal anti-βIII-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 and 15 s 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 min. Irises were fixed in freshly-prepared 4% paraformaldehyde in 0.01 M phosphate buffer (pH 7.4) for 2 h at room temperature. After gradient dehydration in 10%, 15%, and 20% sucrose in 0.01 M phosphate-buffered saline (PBS), each for 2 h, 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 h 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 0.1 M PBS containing 1.5% normal goat serum plus 0.1% Triton X-100 for 72 h at 4 °C. After thorough washing with PBS-bovine serum albumin (BSA, 4 × 15 min), the irises were incubated with the secondary antibodies for 24 h at 4 °C and washed again with PBS-BSA (4 × 15 min). 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 (βIII tubulin and correspondent secondary antibodies), the tissue was once more fixed in 2% paraformaldehyde for 30 min, followed by three washings (15 min each), and incubated with a second primary antibody (CGRP or SP) for 72 h, 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 μm 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-μm 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 min. 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 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature. After washing with 10%, 15%, and 20% sucrose in 0.1 M PBS, each for 2 h, the whole TG was embedded in OCT compound. Serial 10-μm cryostat sections were cut, dried at room temperature for 2 h, and stored at −20 °C until use.

For double immunofluorescence, the sections were washed in