

A COMPARATIVE STUDY OF CHOROIDAL INNERVATION IN THE HUMAN AND THE RABBIT (*ORYCTOLAGUS CUNICULUS*)

ESTUDIO COMPARATIVO DE LA INERVACIÓN COROIDEA EN EL HOMBRE Y EN EL CONEJO (*ORYCTOLAGUS CUNICULUS*)

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ABSTRACT

Objective: To analyze morphological differences between the choroidal innervation of the human and the rabbit, the latter being a species frequently used as an experimental model of human ocular diseases.

Methods: Twelve human and 12 rabbit choroidal whole mounts were processed using an indirect immunohistochemical technique, peroxidase-anti-peroxidase and antibodies against 200 kD neurofilament.

Results: Choroidal nerve fibers were perivascular and intervascular. Perivascular fibers surrounded all arteries forming a network that was more developed in the rabbit. In humans, intervascular fibers were mainly concentrated at the posterior pole where they formed a denser and more highly organized plexus than in the rabbit, which did not exhibit a preferential location for these fibers. Human choroidal ganglion cells were far more numerous than in the rabbit and were concentrated in a circumferential area corresponding to the entrance of the short posterior ciliary arteries of the submacular area. In the rabbit, these cells were restricted to the peripheral choroid.

Conclusions: Some differences were observed between human and rabbit choroidal innervation. The

RESUMEN

Objetivo: Analizar las diferencias morfológicas entre la inervación coroidea del hombre y el conejo, especie frecuentemente utilizada como modelo experimental de enfermedades oculares.

Método: Se estudiaron montajes planos de coroides (12 humanas y 12 de conejo albino) con la técnica de inmunohistoquímica indirecta de la peroxidasa-antiperoxidasa, utilizando un anticuerpo frente al neurofilamento de 200 kD.

Resultados: Las fibras nerviosas coroideas pueden ser perivasculares e intervasculares. Las perivasculares rodeaban las arterias formando una red que estaba más desarrollada en la coroides del conejo. En el humano, las fibras intervasculares se concentraban principalmente en el polo posterior donde formaban un plexo más denso y organizado que en el conejo, el cual no tenía una localización preferencial. Las células ganglionares eran más numerosas en el humano, concentrándose en un área circunferencial correspondiente a la entrada de las arterias ciliares cortas posteriores y en el área submacular. En el conejo estas células se situaban sólo en la periferia.

Conclusiones: Existen diferencias entre la inervación coroidea humana y del conejo. En el humano,

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abundance of ganglion cells and their preferential distribution could be necessary to maintain a constant blood flow in the central area of the human choroid. The lack of organization of rabbit choroidal innervation at the posterior pole could be associated with an absence of the macula. These differences, along with peculiarities of retinal vascularization, should be taken into consideration when using the rabbit as an experimental model to study human eye diseases in which regulation of choroidal blood flow is involved (*Arch Soc Esp Oftalmol* 2006; 81: 463-470).

Key words: nerve fibers, ganglion cells, eye, immunohistochemistry, anti-NF.

la abundancia de células ganglionares y su distribución, podrían ser necesarias para mantener un flujo sanguíneo constante en el área central de la coroides. La falta de organización nerviosa en el polo posterior del conejo podría estar asociada a la ausencia de mácula. Estas diferencias, junto a las diferencias anatómicas de la vascularización retiniana, deberían ser tenidas en cuenta al utilizar el conejo como modelo experimental para estudiar enfermedades oculares en las que esté implicada la regulación del flujo sanguíneo coroideo.

Palabras clave: fibras nerviosas, células ganglionares, ojo, inmunohistoquímica, anti-NFs.

INTRODUCTION

Choroidal innervation is a complex system of nerve fibers and ganglion cells. These nerve structures have considerable impact on the physiology of the eye globe by exerting control over the vascular caliber.

The morphology and distribution of choroidal innervation was initially studied using silver impregnation techniques, scarcely selective due to silver's affinity with non-neuronal structures (1,2). Later on, thanks to the development of immunohistochemical techniques which allowed more specific markings for nerve structures, it was possible to study choroidal innervation from a functional perspective using Neuropeptide-Y (NPY), vasoactive intestinal peptide (VIP) and substance P (SP), which determine the sympathetic, parasympathetic and sensitive origins for such structures respectively (3-11). Use of NADPH-diaphorase and nitric oxide synthase (NOS) allowed for a more physiological study of choroidal innervation by determining which choroidal nerve fibers use nitric oxide as neurotransmitter (9-10). Nevertheless, there are very few morphological studies on choroidal innervation that use these techniques. When monoclonal antibodies are faced with neurofilaments (NFs), they exclusively mark nerve fibers found in both the central and peripheral nervous system (12,13). NFs are structural proteins of the axonal cytoskeleton, so that their visualization allows for the analysis of neuronal morphology (8). We recently used antibodies with NFs in order to study the morphological

traits and distribution of choroidal innervation in different species (11,14-18). Furthermore, by comparing the different antibodies against NFs (anti-NF 68, -160, -200) we proved that anti-NF 200 is best for performing a morphological study of nerve fibers and ganglion cells (11,14,17,18).

Rabbits were used as model animals in the study of human ocular diseases. Nonetheless, studies using immunohistochemical techniques with NADPH-diaphorase and NOS revealed differences between both species both in the distribution of nerve fibers and number of ganglion cells (9,10).

For the present paper, we used anti-NF 200 in flat mounts of human and rabbit choroids in order to analyze morphological differences in innervation for both species and thus consider whether rabbits may be an adequate experimental model in the study of eye diseases whose physiopathological mechanisms involve choroidal nervous regulation.

SUBJECTS, MATERIAL AND METHODOLOGY

Humans

Twelve normal adult eyes (age range of 1.858 years) coming from corneal transplant donations and enucleated 2-4 hours after death, fixed thru immersion with 4% paraformaldehyde (PFA) (Sigma, USA) in 0.1M phosphate buffered saline (PBS) at pH 7.4 for 24 hours. In order to segregate the choroid from the remaining ocular coats, we performed a cut

around the sclero-corneal limb, removing at once cornea, iris and lens. We are thus left with a front cap holding the retina, choroid-ciliary body and sclera. Finally, we proceeded to segregate these tissues with tweezers and a fine brush (000). First of all, we dissected the retina and then segregated the choroid attached to the ciliary body. Subsequently, choroids were washed in TFS for several hours at 4°C. We then incubated them for 10-15 days in 2% hydrogen peroxide at 4°C in order to block endogenous peroxidase. The incubation time, longer than the one commonly used for blocking purposes, is necessary in order to achieve depigmentation of the tissue (11). Immunostaining was performed following the technique described above (14). The primary monoclonal antibody against 200 kDa NF (clon NE14, Sigma, USA) was used in a 1:200 dilution.

Rabbits

Six adult albino New Zealand rabbits were sacrificed with an overdose of sodium pentobarbital following ARVO standards and infused perimortem with 4% PFA (Sigma, USA) in 0.1M TFS at pH 7.4. The eyes were enucleated and then postfixed by immersion during 4 hours at 4°C in 4% PFA in 0.1M TFS, pH 7.4. We proceeded to segregate the choroids and wash them in TFS for several hours at 4°C. The immunostaining protocol applied was the same used in human eyes except for the procedure to block peroxidase, which lasted 30 minutes at room temperature and included dilution of primary antibodies at (1:100).

Images were obtained using the Jenalumar Light optical microscope (Carl Zeiss, Jena) and Ektachrome 100 ASA film (Kodak).

In both humans and rabbits, all antibody dilutions were performed in .2% Triton X-100, .1% regular goat serum in 0.1M TFS at pH 7.4. Negative controls were applied by suppressing primary antibody incubation. In order to proceed to counting ganglion cells, we selected those choroids exhibiting a uniform stain across the preparation. Countings were magnified at x12.5.

Ganglion Cell Quantification

Ganglion cells on human choroids were quantified following the above technique (11).

FINDINGS

1. Nerve Fiber Morphology and Distribution

Depending on their location, nerve fibers were divided into perivascular and intervascular (figure 1A, 1B).

1.1. Rabbit

Perivascular Fibers

Thin caliber nerve fibers surrounding the arteries walls were observed. Those from larger vessels exhibited a circular pattern. When vessels branched out, they did so as a network of transversal and longitudinal fibers covering the vessel's surface (fig.2A).

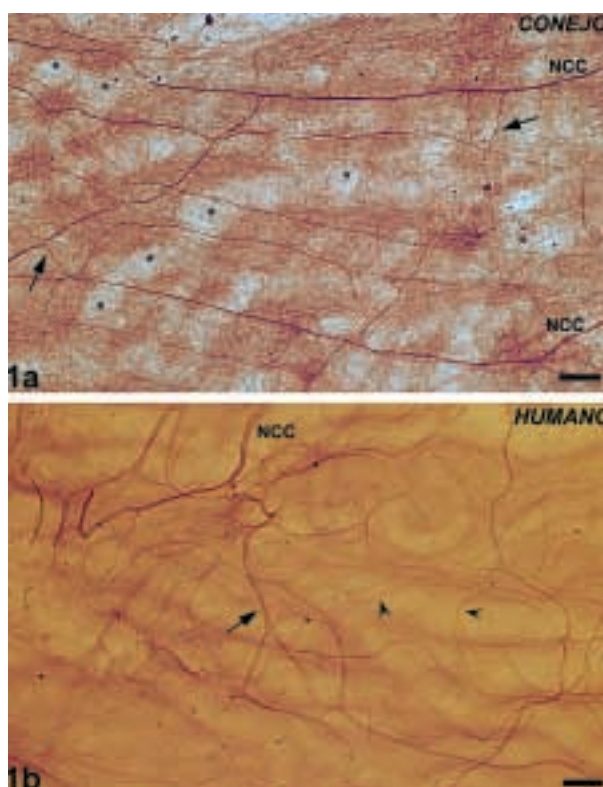


Fig. 1: Choroidal intervascular nerve fibers. 1A: The rabbit's intervascular nerve fibers: branches (arrows) of the short ciliary nerves (SCN). Blood vessels (*). 100 μ m bar. 1B: Human intervascular nerve fibers: branches (arrow) of the short ciliary nerves (SCN) divided into small caliber branches (arrow heads). 250 μ m bars.

Intervascular Fibers

We observed two bundles of flattened fibers (50-70 fibers each) on the suprachoroid, in the equator between the nasal and temporal regions of the choroid, which may correspond to long ciliary nerves (LCN).

Additionally, on the suprachoroid and vascular layers, bundles made of 5-20 fibers (fig.3A) branched out into medium sized bundles consisting of 3-4 fibers. Similarly, the latter branched out into extremely fine fibers which contacted the vessels walls. On certain occasions, intervascular fibers ran parallel to the vessels (paravascular fibers) (fig.2A). Intervascular fibers may be branches of both LCNs and short ciliary nerves (SCNs) (fig.1A, 3A). We also observed fibers traversing the suprachoroid from the front pole up to the ciliary body, long-distance fibers (LDF). On their way to the peripheral choroid, LDFs increased their numbers of fibers up to 15-20 in the vicinity of the ciliary body, making

contact with blood vessels at several spots. On the ciliary body, bundles formed a large network and could be terminal branches of LCNs and SCNs.

1.2. Human

Perivascular Fibers

On large vessels, they exhibited a circular pattern. As the vessels branched out and decreased in caliber, they formed a net consisting of longitudinal and transversal fibers (fig.2B).

Intervascular Fibers

These were mainly found on the suprachoroid. In the most outward area we observed several bundles of flattened fibers. There were two bundles of greater thickness (125 fibers each) penetrating the nasal

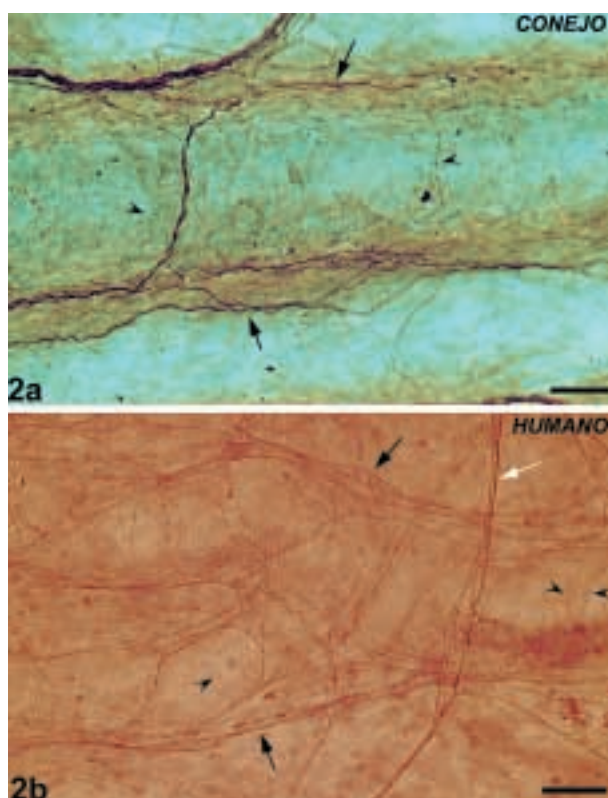


Fig. 2: Choroidal perivascular nerve fibers. 2A: The rabbit's perivascular nerve fibers (arrow head). Paravascular fibers (arrows). 50 µm bar. 2B: Parivascular nerve fibers (arrow heads). Paravascular fibers (black arrow). Intervascular fibers (white arrow). 50 µm bar.

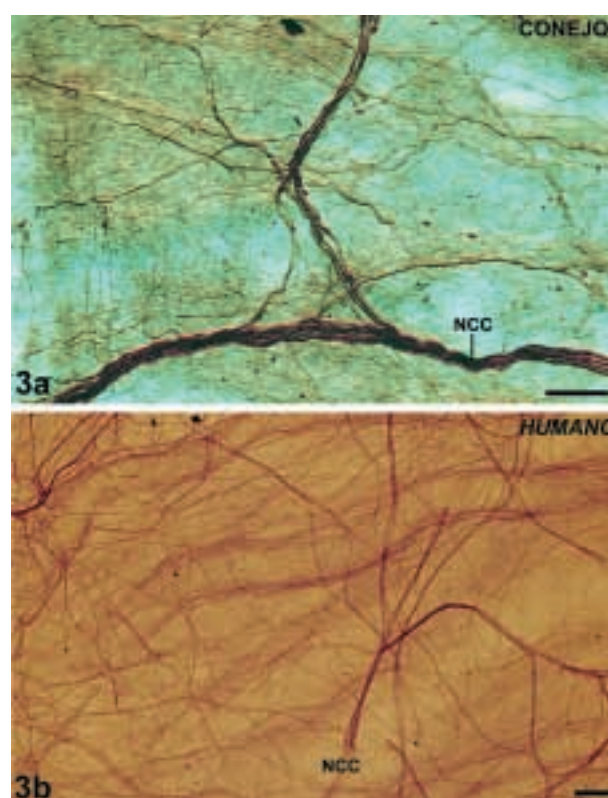


Fig. 3: Suprachoroidal intervascular plexus. 3A: The rabbit's SCN branches divided into multiple fascicles of variable caliber. 50 µm bar. 3B: Human SCN branches forming a grid on the front pole. 250 µm bar.

and temporal suprachoroid at the equator. Based on their location, these could be LCNs. We also observed eight to ten thinner bundles (75-100 fibers each) between the front pole and the equator which reached the ciliary body, where they turned into anastomotic branches of the neighboring fascicles to then form a plexus with many fibers. Based on their location, these fine flattened bundles may have been SCNs (figures 1B, 3B). SCN primary branches (40 fibers) were found on the central choroid and gave way to secondary branches of variable caliber (2, 3 or more fibers). In turn, secondary branches divided (at times they were formed by a single axon) and formed a vast plexus (fig.3B). The plexus density was far greater on the front pole and increased considerably in size once it had reached the submacular area, where it formed a real grid (figures 1B, 3B).

Some SCN branches penetrated the layer of larger vessels, where they adapted to the vascular morphology by forming two groups: 1) parallel fibers nearing the walls of the larger arteries and their primary branches in the form of «vascular sheaths» (vascular fibers) (fig.3B); 2) fascicles near the vascular wall which adapted to its contour forming a highly specialized polygonal network with frequent anastomoses (fig.4). Both groups were observed mainly on the front pole, decreasing in density, adapting to the surrounding vessels and acquiring a network appearance when they reached the periphery. Near the ciliary plexus, we could only observe a few and thin fascicles.

Branches derived from the two groups described above reached the vascular wall without specific terminations. Sometimes, these fibers penetrated the layer of medium-sized vessels forming a network running parallel to the wall of the arterioles in the macular region.

We observed LDFs on the suprachoroid forming a dense network on the ciliary body.

2. Ganglion Cell Morphology and Distribution

2.1. Rabbit

Choroidal ganglion cells (CGC) were present in the intervascular fiber networks of the peripheral choroid. CGCs were very scarce, approximately 10 per choroid. Bipolar morphology prevailed over multipolar morphology. In general, they clustered

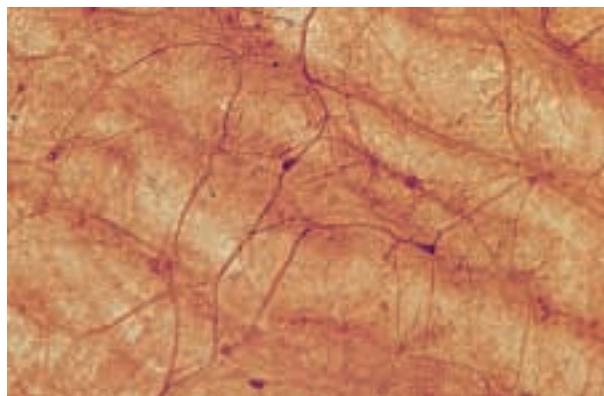


Fig. 4: Human intervascular fibers adapted to the choroidal vascular morphology. Fibers (arrow head) near the wall of vessels forming a polygonal network. Ganglion cells (arrows). 100 μ m bar.

in microganglions of 2 or 3 cells, though at times they could be isolated (fig.5A).

2.2. Human

Human choroid contains many ganglion cells (figures 5B, D, C), approximately 1,300 to 1,500 CGCs irregularly distributed, concentrating more in the temporal area (approximately 1,000) than in the nasal region (around 500). In both cases, the number of cells was larger on the front pole, particularly in the circumference corresponding to the entrance of the front short ciliary arteries (FSCA) and around the submacular area (fig.4). The number of CGCs decreases gradually from the front pole to the peripheral choroid (11).

Human CGCs were basically located on the suprachoroid in relation to the primary and secondary branches of ciliary nerves (figures 5C, D). Most CGCs were found near the wall of larger vessels (figures 5B, C, D) as part of the polygonal plexus found along the vascular contours (fig. 4). We observed them both in isolation (fig.5B) and forming groups of 2-10 cells making up microganglions (figures 5C, D), multipolar morphology (figures 5C, D) prevailing over bipolar morphology (fig. 5B).

DISCUSSION

In both humans and animals, we found the choroid to be innervated by nerve fibers which relate to

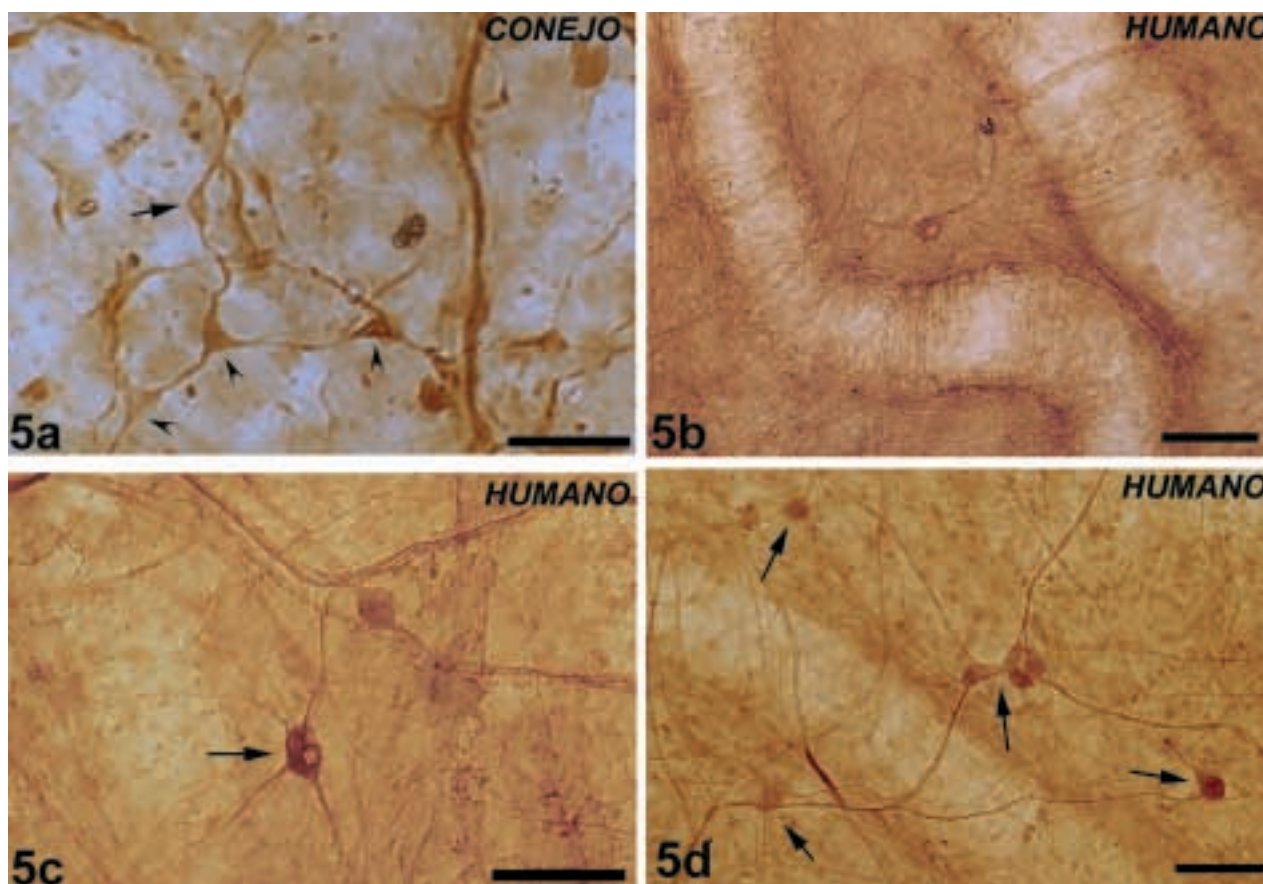


Fig. 5: Choroidal ganglion cells. 5A: The rabbit's choroidal ganglion cells as part of the intervascular plexus. Bipolar cell (arrow). Multipolar cell (arrow heads). 25 μ m bar. 5BCD: Human choroidal ganglion cells (100 μ m bar). Bipolar ganglion cell between two large vessels (5B); multipolar ganglion cells forming microganglions (arrows) (5C); multipolar ganglion cells forming a network on the intervascular plexus (5D).

blood vessels in different ways. Nerve fibers located on the vessel's wall and in direct contact with it (perivascular) and fibers located among vessels, found nearby and making occasional contact (intervascular). According to Tsai et al (19), whenever perivascular nerve fibers exhibit a network pattern it means they have reached maturity. This network layout has been observed in the internal carotid artery with antibodies against VIP and NPY (19) and on FSCAs through staining with NADPH-diaphorase (9,10). In our works with anti-NFs we found that perivascular nerve fibers on FSCAs exhibited the same pattern both in humans and rabbits (11,14-18). Several authors have reported the presence of NPY- and VIP-positive perivascular fibers in FSCAs and its branches (3-11). Based on these findings, the observed NFs(+) perivascular fibers may

be sympathetic or parasympathetic fibers and thus may be involved in regulating the choroidal blood flow. This adjustment could prove to be different from one species to another, as the choroidal perivascular nerve fiber network is much more evolved in rabbits than in humans.

Perivascular nerve fibers, intervascular fibers and CGCs could also aid in regulating the choroidal blood flow. The present study found that humans and rabbits differ significantly regarding the number and distribution of the above nerve structures; this was proven by use of NADPH-diaphorase (9,10). In human choroids, the largest concentration of NF(+) fibers, coming from long and short ciliary nerves, are intervascular fibers forming a wide plexus of highly-organized fibers, particularly in the front pole and areas adjacent to the macula. On the

other hand, this suprachoroidal intervascular plexus derives in branches that adapt to the vascular contours on the main ACCP branches, thus forming «vascular sheaths» which could play a role in regulating blood flow coming into the choroid. Unlike humans, perivascular innervation prevails in rabbits; furthermore, NF(+) intervascular fibers are scarce and although running parallel to the vessels they do not adapt to their contour nor do they form a plexus organized around the front pole, as in the human choroid. These findings suggest that the mechanisms controlling the blood flow may be different in both species.

As for ganglion cells, comparison between humans and rabbits ought to be made with extreme caution, since there are differences reported in the literature regarding both the number and distribution of these neurons. Therefore, with antibodies versus classic neurotransmitters (3), the rabbit's CGCs do not exhibit a preferential distribution, whereas using NADPH-diaforase we spotted them near the front long ciliary arteries (FLCA) on the front pole and close to the vessels on the peripheral choroid (9). With anti NF-200, the rabbit's CGCs are few and may only be found in the peripheral choroid (14,17).

There are also differences in the number and distribution of human CGCs depending on the marker used to study them. Thus, Miller et al. (4) described how human choroidal VIP(+) CGCs were scarce (approximately 10 per choroid) and apparently did not concentrate in any particular region. Later studies using NADPH-diaforase (9,10) and anti-NF (11) revealed there is a large number of human CGCs (around 1,500-2,000) and these cells are mainly located in the central temporal region adjacent to the macula. The presence of a large number of CGCs in the central area of the human choroid may be linked to the existence of macula (10,11). Such hypothesis is based on the scarcity of NF-200(+) CGCs observed in the central region of the rabbit's choroid. This species lacks a macula and NF-200(+) CGCs are mainly located in the peripheral choroid (14,17).

In human choroids, the macular area exhibits a greater arteriolar density (20) in order to provide blood flow to the cones. The role of a large number of CGCs and the high density of nerve fibers observed in the submacular choroid may be to regulate the arteriolar flow in this area. Some authors have stated that these neurons may be in charge of trig-

gering quick vasodilator reflexes (9) in order to manage blood flow in extreme conditions such as sudden changes in blood pressure or increased temperatures (21,22). High concentration of NF(+) CGCs in the vicinity of the entrance to FSCAs as well as the presence of abundant intervascular innervation in the human central choroid may be necessary in order to maintain a constant blood flow in this area.

Regulation of the choroidal blood flow, where innervation is involved, plays an important role in ocular physiology. Choroidal innervation in humans and rabbits differ in the following aspects: 1) the relationship between nerve fibers and blood vessels; 2) the organization and distribution of nerve fibers; and 3) the number and distribution of CGCs. Differences in innervation of the observed choroids, together with the different retinal vascularization exhibited by both species (23-29) should be taken into account when interpreting the findings obtained from using rabbits as experimental models in the study of human ocular pathologies where choroidal vascular regulation is involved.

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