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Substance P and calcitonin gene-related peptide intrinsic choroidal neurons in human choroidal whole-mounts

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Summary. To determine the presence in the human choroid of substance P (SP)-and calcitonin gene-related peptide (CGRP) positive intrinsic choroidal neurons (ICNs), choroidal whole-mounts were processed for indirect immunofluorescence. An antibody to a component of the neuronal cytoskeleton, neurofilament 200 kDa (NF-200), was combined with antibodies to SP and to CGRP (neuropeptides proper to the sensory nervous system). The human choroid possesses numerous SP(+) and CGRP(+) ICNs. These neurons were observed in the suprachoroid, both in isolation and forming microganglia. For both types of ICNs studied, neurons were more numerous in the temporal than in the nasal regions. In both locations, SP(+) and CGRP(+) ICNs were more abundant in the central choroid (the choroid underneath the macular area of the retina), with cell density diminishing outwards to the choroidal periphery. There were no appreciable differences between the two populations of ICNs studied in terms of size, morphology or immunostaining characteristics. In conclusion, given that peripheral sensory innervation could be involved in the regulation of both choroidal blood flow and vascular architecture, the SP(+) and CGRP(+) ICNs described for the first time in the present work may be involved in these mechanisms of vascular regulation.

Key words: Innervation, Ganglion cells, Choroid, CGRP, SP

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Introduction

It is generally accepted that choroidal innervation in most species is from the sympathetic system (from the superior cervical ganglion), the parasympathetic system (from the ciliary and sphenopalatine ganglia), and the sensory system (from the trigeminal ganglion). This innervation could perform a vasoregulatory function, since it has been observed in association with the choroidal blood vessels (Miller et al., 1983; Stone and Kuwayama, 1985; Stone, 1986a,b; Stone and Mcglinn, 1988; Triviño et al., 2002). Innervation of the choroidal blood vessels, by primary afferent fibres co-expressing substance P (SP) and calcitonin gene-related peptide (CGRP), is well established in mammals and birds (Terenghi et al., 1982, 1983, 1985, 1986; Lee et al., 1985; Stone and Kuwayama, 1985; Reiner, 1987; Stone and Mcglinn, 1988; Schrödl et al., 2001). Abundant evidence indicates that nerves which are immunoreactive for SP and CGRP are sensory in nature (Shimizu, 1982; Terenghi et al., 1982, 1983, 1985, 1986; Stone and Kuwayama, 1985; Stone and Mcglinn, 1988) and that SP and CGRP immunoreactivities co-exist in trigeminal neurons (Lee et al., 1985; Terenghi et al., 1985; Kuwayama et al., 1987).

In experimental animals, following the lesion of the trigeminal ganglion or capsaicin treatment (a potent neurotoxic substance which acts selectively on sensory fibres), there is a considerable decrease, but not disappearance, of SP and CGRP immunoreactivity in the choroidal fibres (Terenghi et al., 1982, 1985; Tervo et al., 1982). This suggests that some fibres might have an origin other than the trigeminal ganglion, possibly intrinsic neuronal cell bodies.

Additionally, there is growing evidence that peripheral nerves are key elements in regulating not only vasomotor function, but also vascular structure. It has

been shown that, after chronic sympathectomy, choroidal vascularity increases (choroids are thicker and more vascular with increased numbers of venules and arterioles, as well as the capillaries), requiring intact sensory innervation (Steinle and Smith, 2003). In contrast to the effect of sympathetic denervation, neither sensory-nerve destruction with capsaicin nor parasympathetic-nerve surgical ablation prompted significant changes in choroidal vascularity. This could be due to a choroidal compensatory mechanism to attenuate or alter the effects of denervation (Steinle and Smith, 2003). Intrinsic choroidal neurons (ICNs) could be involved, at least in part, in this mechanism. The existence of parasympathetic ICNs has been demonstrated in human choroid (Flügel et al., 1994). However, to the best of our knowledge, SP(+) and CGRP(+) ICNs have not been reported in humans to date.

The purpose of the present work was to study the presence in the human choroid of SP(+) and CGRP(+) ICNs, using antibodies to these neuropeptides, which are characteristic of the sensory nervous system (Shimizu, 1982; Terenghi et al., 1982, 1986; Stone and Kuwayama, 1985).

Material and methods

Twenty eyes from adult humans (age range 30-58) with no ocular disease, enucleated about 2-4 h post mortem for corneal transplantation, were obtained from the Spanish Eye Bank and studied in accordance with the Helsinki Declaration and local regulations for the use of human tissue in research. All donors had given permission to use their tissues for research.

Immunofluorescence method

One to two hours after enucleation and corneal processing, the eyes were fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline, pH 7.4, at 4°C. The lens, vitreous humour, and retina were removed after 15 min of fixation. The choroids were then removed from the resulting eyecup and placed in the fixing solution for 24 h at 4°C. Nineteen choroids were then incubated free-floating for up to 10-15 days in 2% hydrogen peroxide at 4°C for depigmentation. As a means of checking whether the depigmentation process affected the antigenicity of the tissues, one choroid was not depigmented and was therefore directly processed by immunofluorescence. Double labelling was performed to demonstrate the co-localization of neurofilament 200 kDa (NF-200) with SP (NF-200/SP) (9 eyes) or CGRP (NF-200/CGRP) (5 eyes), as described elsewhere (Triviño et al., 2002). In addition, five human choroids were used to perform triple immunolabelling to demonstrate co-localization of SP and CGRP with NF-200. Choroidal whole-mounts were incubated for five days with the following primary antibodies: NF-200 (clone NE14, Sigma Saint Louis, MO, USA) in a dilution 1/150 plus SP (raised in rat, Chemicon Temecula, CA, USA) in a dilution 1/50, or CGRP (raised in rabbit, Chemicon Temecula, CA, USA) in a dilution 1/250. Binding sites of the primary antibodies were visualized after five days of incubation with the corresponding secondary antibodies: goat antimouse conjugated to fluorescein isothiocyanate (Chemicon Temecula, CA, USA) diluted 1/100, goat antirat conjugated to Texas-red (Vector Burlingame, CA, USA) diluted 1/50, or goat antirabbit conjugated to Texas-red (Vector Burlingame, CA, USA) diluted 1/50. For triple immunostaining, goat antirat conjugated to AMCA (Chemicon Temecula, CA, USA) diluted 1/50 and goat antirabbit conjugated to Texas-red (Vector Burlingame, CA, USA) diluted 1/50 were used as secondary antibodies. Negative controls included preabsorption and the replacement of primary and secondary antibodies by normal serum from those species in which the primary antibodies were raised. For preabsorption we used one choroid that was cut into two pieces, each one containing nasal and temporal regions. One of these pieces was used to study the peptide corresponding to the antibody against SP and the other one to that corresponding for anti-CGRP.

Documentation

Human choroidal whole-mounts were examined with a fluorescence microscope (Zeiss, Axioplan 2 Imaging Microscope) equipped with appropriate filters for fluorescence emission spectra of fluorescein isothiocyanate (Filter set 10, Zeiss), Texas-red (Filter set 15, Zeiss) and AMCA (Filter set 01, Zeiss). This microscope is motorized on the x, y, and z axes to allow spatial correlation of the choroidal nerve structures. Each of the nerve structures at the same choroidal location was digitally photographed with the microscope's own automatic system. The relative correlation of the choroidal structures in the z-axis, or the depth, was estimated by extended focus imaging from up to 60 sections at z-increments of 1-2 microns (z-series stacks). Images were processed using the colour-combination tool provided by the Metamorph Imaging System computer program version 4.5 (© Universal Imaging Corps) associated with the Axioplan 2 Imaging Microscope (Zeiss).

For the preparation of multi-panel plates, some of the tools of the program Adobe Photoshop cs2 were used, specifically the bright/contrast tool and the illumination one (tone-balance tool).

Assessing ICNs density and size

Quantitative evaluation of SP- and CGRP-positive ICNs was performed on whole-mounts. ICNs were counted throughout the choroidal whole-mounts using 40x objective and oil immersion. For accurate quantification, all choroidal ICNs observed were digitally photographed with the adquire tool included in

the Metamorph Imaging System version 4.5 computer program (© Universal Imaging Corps) in association with an Axioplan 2 Imaging Microscope (Zeiss). Only choroids exhibiting good staining quality in the whole sample were selected. As a result of this selection, three and a half (the temporal region) choroids were considered for quantitative study of SP(+) ICNs and three and two half (two temporal regions) choroids for quantitative study of CGRP(+) ICNs. Using the same inclusion criteria, three choroids were suitable for quantification of SP/CGRP co-localization. ICN size was calculated with the measuring tool included in the Metamorph Imaging System version 4.5 computer program (© Universal Imaging Corps) in association with an Axioplan 2 Imaging Microscope (Zeiss). The diameter used to estimate ganglion-cell size was the longest distance between opposing cell boundaries when passing through the centre of the cell.

Statistical analysis

The data from ICN size analysis were uploaded to SPSS 12. The Kolmogorov-Smirnov test for one sample showed that the distribution data for CGRP and SP cell size were normal when isolated and for cells grouped in ganglia. The ANOVA test and the Bonferroni test were run to compare the four study groups. Differences were considered significant at $p \leq 0.05$.

Negative controls

The analysis of the tissues used as negative controls with filters for fluorescein isothiocyanate and Texas-red revealed a background fluorescence that corresponded to the autofluorescence of the retinal pigment epithelium, as evidenced by the fact that it was not detected in those areas where the retinal pigment epithelium was not present. Additionally, they showed that with the filter sets used, there was no "bleed through" fluorescence between the fluorochromes employed (green, red and blue). The specificity of CGRP and SP antibodies was confirmed by preabsorption with CGRP and SP peptides (10 nmol/ml dilution for both peptides) (Anaspec, Inc San José, Ca). The antibodies did not show any cross-reactivity with either CGRP, SP or with other peptides known to be present in the eye. The only signal found was that corresponding to the autofluorescence of the retinal pigment epithelium.

Results

SP(+) and CGRP(+) immunostaining of nerve fibers in human choroids

Suprachoroid

The short and long ciliary nerves presented both SP and CGRP immunoreactive fibers. SP(+) fibers were more abundant than CGRP(+) fibers in the ciliary nerves. In the primary branches of the long ciliary nerves, the SP(+) fibers were bunched together, making it impossible to see details of their morphology (Fig. 1A). When the fibers left the primary branches to form bundles of smaller fibers, they showed up as thin, varicose-like fibers. The ciliary nerve branches contained CGRP(+) fibers that presented a linear morphology at low magnification (10x) and a varicose appearance at higher magnification (20x and 40x).

Vessel layers

In the large and medium vessel layers paravascular

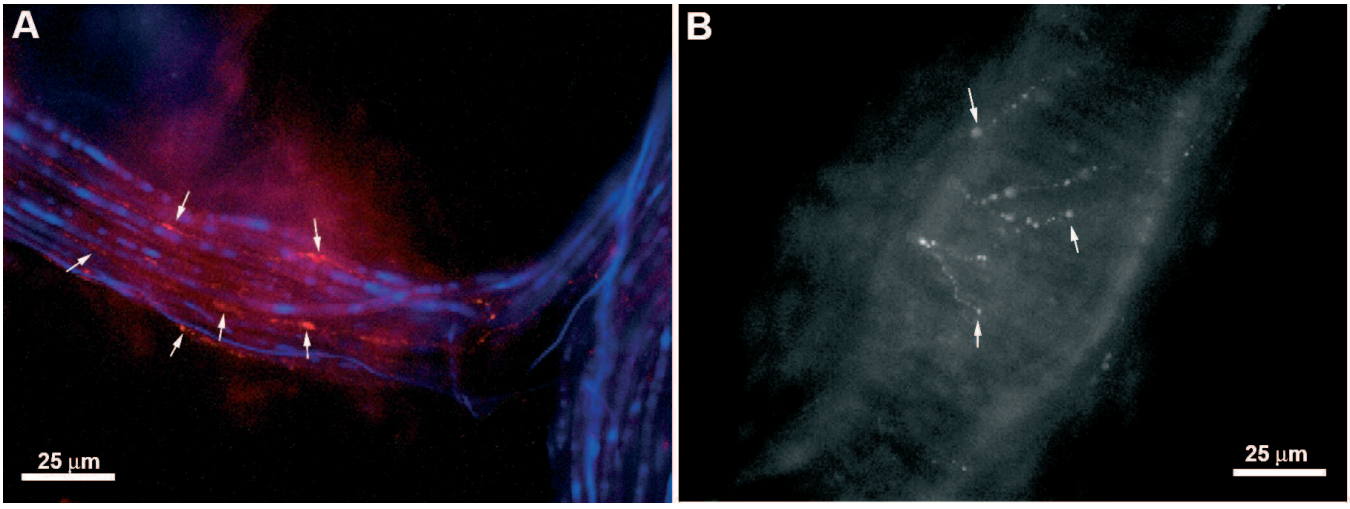

Fig. 1. Immunofluorescence of human choroidal whole-mounts. Triple immunostaining for NF-200, calcitonin gene-related peptide (CGRP) and substance P (SP) (NF-200 staining is not shown). **A.** Branch of a long ciliary nerve. The proportions of neuropeptides were much higher for SP (blue) than for CGRP (red) (white arrow). B. CGRP(+) paravascular and perivascular axons. Terminal dilations (white arrow).

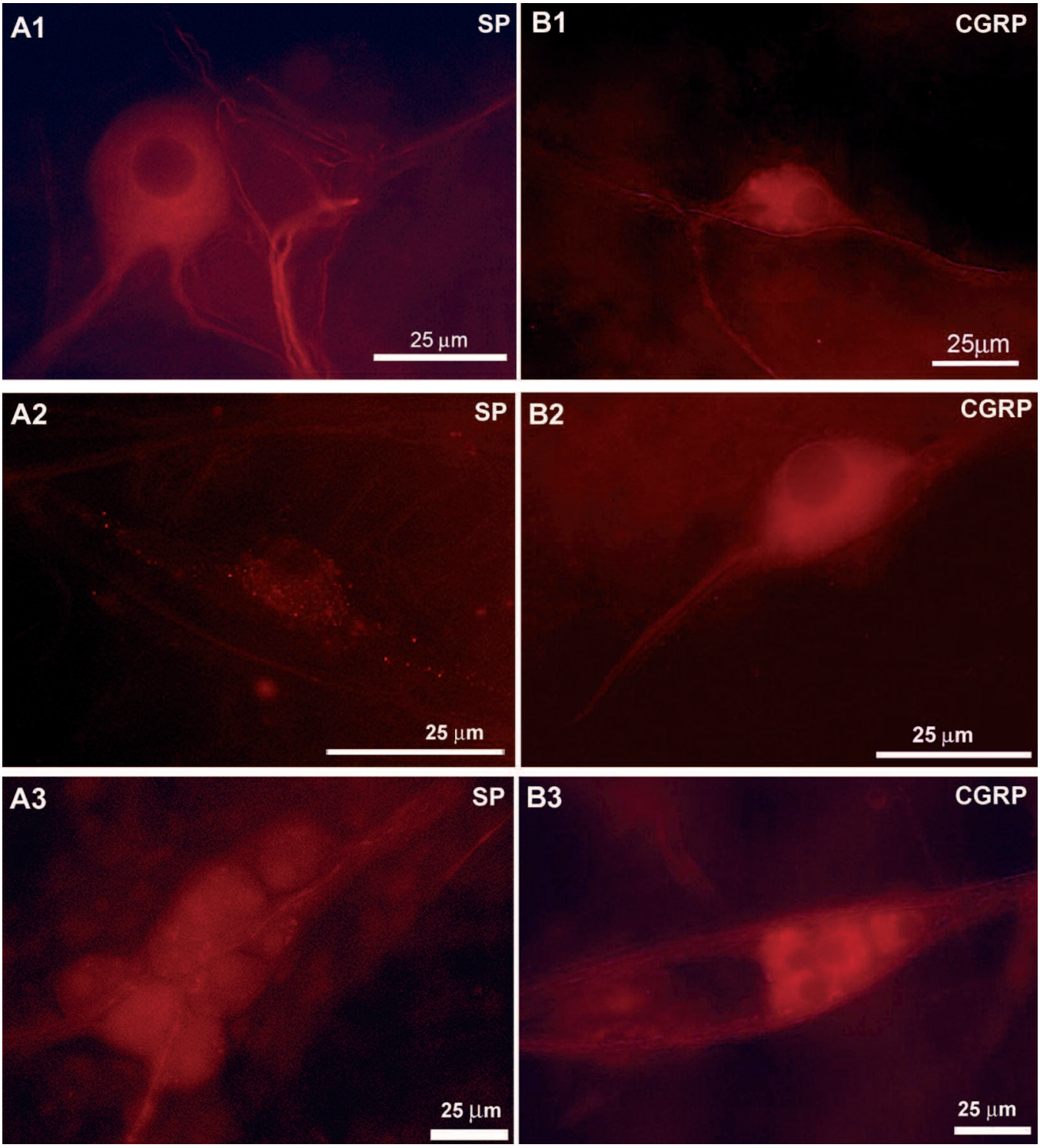

Fig. 2. Immunofluorescence of human choroidal whole-mounts. ICNs in human suprachoroid with intense homogeneous immunoreactivity. **A.** Substance P (SP). **B.** Calcitonin gene-related peptide (CGRP) A1. Multipolar SP(+) ICN related with branches of the short ciliary nerves. A2. Bipolar SP(+) ICN. A3. Microganglia of eight SP(+) ICNs. B1. Multipolar CGRP(+) ICN. B2. Bipolar CGRP(+) ICN. B3. Microganglia of four CGRP(+) ICNs.

and perivascular axons were immunoreactive for SP and CGRP. On reaching the vessel wall, SP and CGRP(+) perivascular axons exhibited small terminal dilations (Fig. 1B).

At a deeper level of the medium vessel layer, thin varicose fibers that stained for both SP and CGRP were visible. This innermost plexus exhibited no NF-200 immunostaining.

Distribution of SP(+) and CGRP(+) ICNs

The human choroids studied contained SP(+) (Figs. 2A, 3B3) and CGRP(+) (Figs. 2B, 3 A2, B2) ICNs. All ICNs observed were also NF-200(+) (Fig. 3A). Location was restricted to the suprachoroid along with the primary and secondary branches of the ciliary nerves. In most cases, the neurons were associated with the short ciliary nerve branches (Fig. 2 A1, A3, B3 and Fig. 3A). Overall, SP(+) and CGRP(+) ICNs occurred more frequently in the central suprachoroidal area (the choroid extending from the optic nerve head to the equator of the eye) than in the equator-periphery. In both temporal and nasal regions, SP(+) and CGRP(+) ICNs were more abundant in the central choroid, with cell density diminishing outwards to the choroidal periphery. For both types of ICNs studied, neurons were more numerous in the temporal than in the nasal regions, showing a preference for location in the central area of the temporal region (the choroid underneath the macular area of the retina) (Table 1,2).

Characteristics of SP(+) ICNs

Cell counting and organization

The number of SP(+) ICNs counted in the three and a half choroids appropriate for cuantification are shown in Table 1. SP(+) ICNs were observed both in isolation (Figs. 2 A1, A2, 3 B3) and in groups of 2-8 cells forming microganglia (Fig. 2 A3) (Table 1). In some instances, SP(+) ICNs formed part of mixed ganglia (only some of the NF-200(+) ICNs were positive for SP). Most of the microganglia were isolated and in some cases were apparently interconnected.

Cell morphology and size

The morphology of most SP(+) ICNs seemed to be multipolar (Fig. 2 A1), with a few bipolar cells (Fig. 2 A2). In most ICNs, SP immunopositivity of the cytoplasm was homogeneous (Fig. 2 A1, A3 and Fig. 3 B3) and the intensity of the staining was variable among ICNs (Fig. 2A).

SP(+) ICNs varied in size from 13.98-46.05 μm diameter. Cells were larger when isolated (31.35±6.04 m) (Figs. 2 A1, A2, 3B3) than when grouped in microganglia (25.58±6.51 μm) (Fig. 2 A3).

Characteristics of CGRP(+) ICNs

Cell counting and organization

The number of CGRP(+) ICNs counted in the three and a two half choroids appropriate for quantification are shown in Table 2. These CGRP(+) ICNs were observed either isolated (Fig. 2 B1, B2 and Fig. 3 A2, B2) or forming microganglia of up to 2-7 cells (Fig. 2 B3 and Fig. 3 A2) (Table 2).

Cell morphology and size

Multipolar CGRP(+) ICNs (Fig. 2 B1 and Fig. 3 A2,

Table 1. Distribution of the Substance P (SP) positive intrinsic choroidal ganglion cells (ICNs)

CHOROIDAL SAMPLE	TOTAL ICNs	ISOLATED ICNs	ICNs in MICROGANGLIA	CENTRAL CHOROID	EQUATORIAL PERIPHERAL CHOROID	TEMPORAL CHOROID	NASAL CHOROID
Whole choroid	142	47 (33.10%)	95 (66.90%)	107 (75.35%)	35 (24.65%)	108 (76.06%)	34 (23.94%)
Whole choroid	135	105 (77.78%)	30 (22.23%)	118 (87.40%)	17 (12.60%)	69 (51.12%)	66 (48.89%)
Whole choroid	405	235 (58.02%)	170 (41.97%)	348 (85.92%)	57 (14.07%)	234 (57.77%)	171 (42.22%)
Temporal choroid	59	41	18	43	16	59	

Table 2. Distribution of the Calcitonin Gene-Related Peptide (CGRP) positive intrinsic choroidal ganglion cells (ICNs).

CHOROIDAL SAMPLE	TOTAL ICNs	ISOLATED ICNs	ICNs in MICROGANGLIA	CENTRAL CHOROID	EQUATORIAL PERIPHERAL CHOROID	TEMPORAL CHOROID	NASAL CHOROID
Whole choroid	377	125 (33.16%)	252 (66.84%)	247 (65.52%)	130 (34.48%)	264 (70.03%)	113 (29.97%)
Whole choroid	145	115 (79.31%)	30 (20.68%)	123 (84.82%)	22 (15.17%)	73 (50.34%)	72 (49.65%)
Whole choroid	472	319 (67.58%)	153 (32.41%)	407 (86.22%)	65 (13.77%)	278 (58.89%)	194 (41.10%)
Temporal choroid	243	148	95	232	11	243	
Temporal choroid	152	35	117	114	38	152	

B2) predominated over a few bipolar cells (Fig. 2 B2). CGRP immunostaining was intense and homogeneous (Fig. 2B and Fig. 3 A2, B2). Most of the microganglia were isolated (Fig. 2 B3) but in some cases were interconnected (Fig. 3 A2).

The diameter of CGRP (+) ICNs varied from 15.11-46.05 μm. Cells were larger when isolated (Fig. 2 B1, B2 and Fig. 3 A2, B2) (average30.20±5.94 m) than when grouped in microganglia (Fig. 2 B3 and Fig. 3 A2) (average 25.28±5.28 m).

The ANOVA test showed a statistically significant difference among the four study groups ($p<0.001$). The post hoc (Bonferroni) tests demonstrated that the size differences were independent of the antibody used, depending exclusively on whether the cells were isolated or situated in ganglia ($p<0.001$) (Fig. 4).

Co-localization of SP and CGRP immunostaining

When triple immunostaining was carried out (NF-200/ SP/CGRP) we detected co-localization of CGRP and SP. In the choroids suitable for quantification, all SP(+) ICNs also showed positive immunoreactivity for CGRP, although only a proportion of CGRP(+) ICNs (93.10%, 83.68%, 72.43%) displayed positive immunostaining for SP. The co-localization of both antibodies was detected thoroughout the choroid, both when ICNs were isolated (Fig. 3B) and when forming microganglia.

Discussion

It is well established that the choroid possesses a

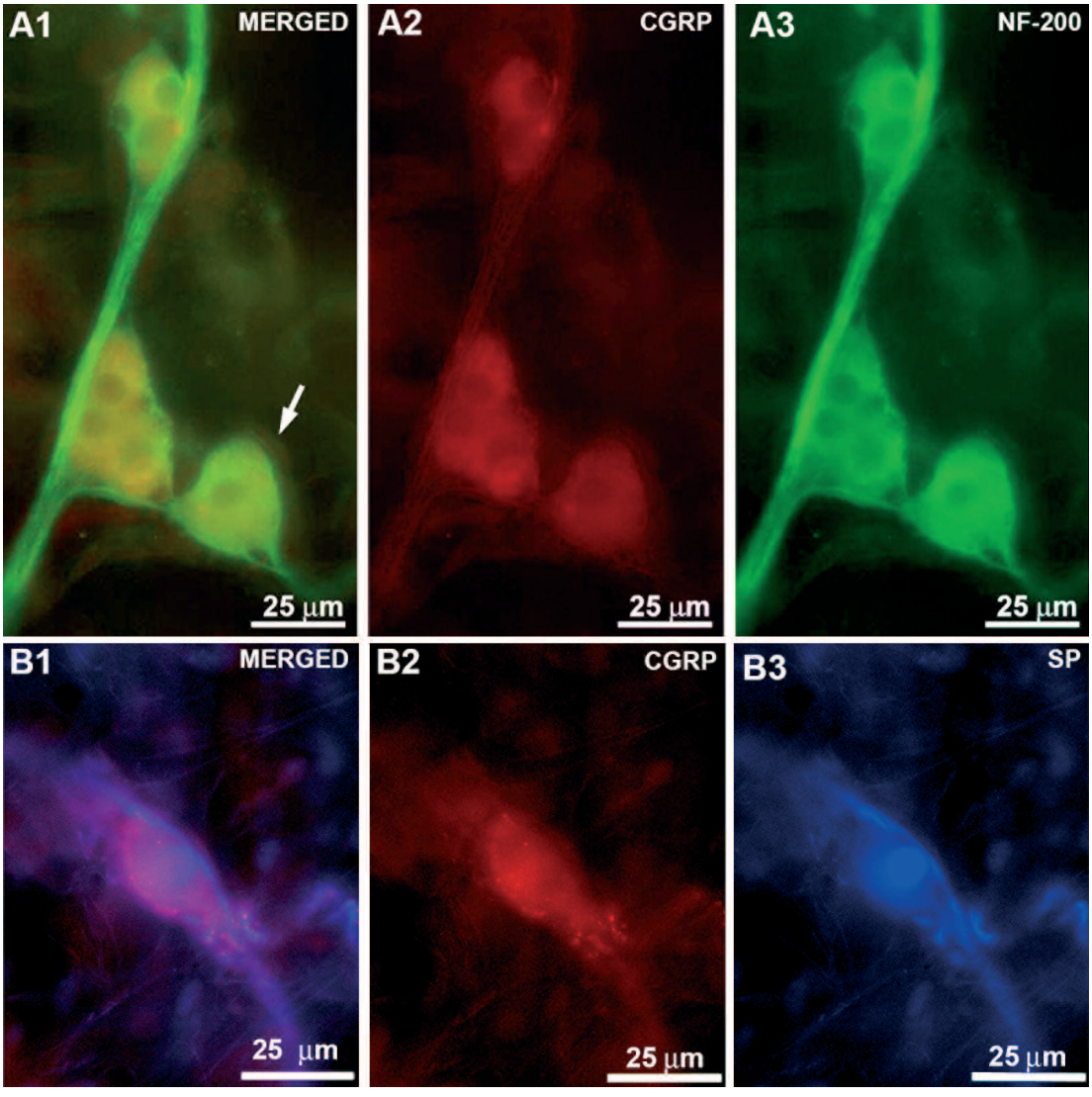

Fig. 3.
Immunofluorescence of human choroidal whole-mounts. **A.** Double immunostaining for neurofilament (NF-200) (green) and calcitonin gene-related peptide (CGRP) (red). **B.** Triple immunostaining for calcitonin gene-related peptide (CGRP) (red) and substance P (SP) (blue) (NF-200 staining is not shown). Figures A1 and B1 combine double immunostaining. For better visualization of the structures, the same area marked with each antibody is shown (A2-3; B2-3). A: Intense homogeneous CGRP(+) immunoreactivity in two microganglia formed by three and two CGRP(+) ICNs and an isolated CGRP(+) ICN (arrow). B: Co-localization of CGRP and SP immunostaining in an ICN.

rich sensory innervation projecting to the trigeminal ganglion. These fibres stain with antibodies against SP and CGRP, and are known to be sensory from the observation that trigeminal ganglion injury or capsaicin treatment (a selective denervator of sensory neurons) induces an appreciable decrease of SP(+) and CGRP(+) fibres (Lee et al., 1985; Terenghi et al., 1985; Kuwayama et al., 1987). Their location around choroidal vessels has prompted the idea of a possible role in choroidal blood-flow (ChBF) regulation (Lee et al., 1985; Terenghi et al., 1983, 1985, 1986; Reiner, 1987; Stone and Kuwayama, 1985; Kuwayama et al., 1987; Stone and Mcglinn, 1988; Schrödl et al., 2001; Triviño et al., 2002).

In the choroid, there are not only numerous nerve fibres but also intrinsic choroidal neurons (ICNs). In humans, these ganglion cells (approximately 1300-1500 cells per choroid) form a delicate network located mainly in the suprachoroid. Human ganglion cells stain for parasympathetic (Nitric oxide, VIP) (Bergua et al., 1993; Flügel et al., 1994; Flügel-Koch et al., 1994; Triviño et al., 2002) and sympathetic neurotransmitters (NPY and TH) (May et al., 2004; Triviño et al., 2005).

With immunohistochemical techniques using different markers (neuronal nitric oxide synthase, galanin, CGRP, and TH) Schrödl's group demonstrated that 40% of ICNs in the duck are closely associated with CGRP(+) nerve fibres, most probably originating in the trigeminal ganglion (Schrödl et al., 2001). Years later, CGRP-positive fibers forming boutons were found closely associated with human ICNs (Schrödl et al., 2003). However, no CGRP(+) neurons were observed in either of the two species (Schrödl et al., 2001, 2003). In contrast, May et al. (2004) found neither boutons close to the ICNs nor immunoreactive ICNs when using antibodies to SP and CGRP in human choroids.

To date, no SP(+) or CGRP(+) ICNs have been reported either in humans or in experimental animals. In this study, combining anti-SP and anti-CGRP (neuropeptides of the sensory nervous system: Shimizu et al., 1982; Terenghi et al., 1982, 1986; Stone and Kuwayama, 1985) with anti-NF 200 (a useful neuron marker for the study of choroidal innervation: Ramírez et al., 1999; Triviño et al., 2002) we have demonstrated the presence of SP(+) and CGRP (+) ICNs in the human choroid. SP(+) and CGRP(+) neurons have also been identified innervating the hearts of humans, rats and guinea pigs (Wimalawansa, 1996; Vaishnava and Wang, 2003) and in the submucous plexus of the horse intestine (Domeneghini et al., 2004).

In a previous study, we quantified and analysed the distribution of the intrinsic neurons in the human choroid (Triviño et al., 2002). We found that there are 1300-1500 neurons in the human choroid, and therefore from this study we find that SP(+) ICNs would account for 14.93-17.23% of such cells, and CGRP(+) ICNs for 20.08-25.48%.

The selection of some choroids for the analysis of each antibody (SP and CGRP) may appear unrepresentative. Nevertheless, it should be taken into account that the aim of the present work is not only to demonstrate the existence of SP and CGRP neurons in the human choroid but also to quantify and describe their distribution. To obtain a whole-mount of human choroid in which both aims can be achieved (furthermore simultaneously) is very difficult for several reasons. First, a tissue without the fluorescent background of the RPE must be obtained from the entire choroidal whole-mount. Also, a double immunostaining must be found, one which presents adequate quality for the count and to establish the distribution of the SP(+) and CGRP(+) ICNs throughout the entire expansion of the choroid. This explains why, of the 19 choroids used, only three entire choroids and three temporal samples were considered appropriate for the count and distributions of the ICNs, and two samples for quantification of CGRP/SP co-localization. In the choroids not selected we could make only partial estimations and therefore these were inadequate for our purposes. Given that SP(+) and CGRP(+) ICNs have not previously been described in the human choroid, we considered that a preliminary approximation of the quantification and distribution would be worthwhile.

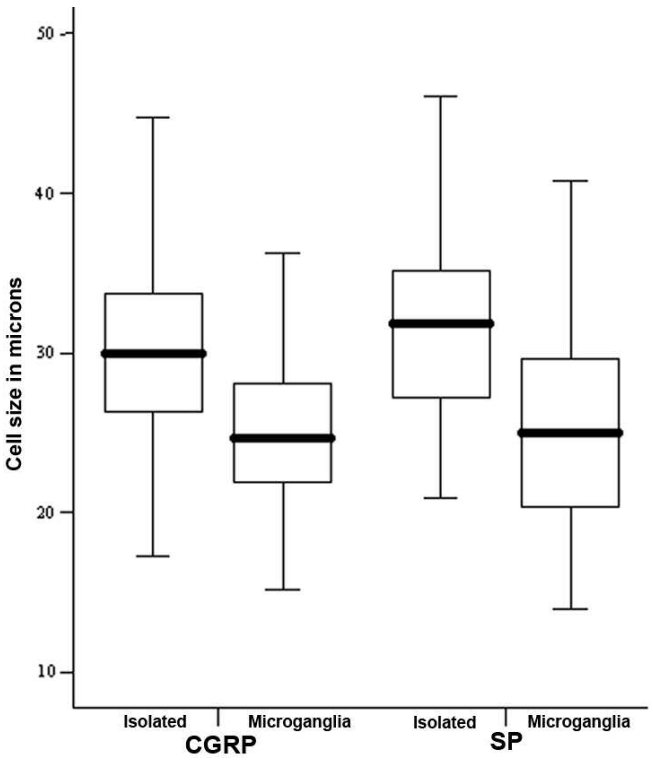

Fig. 4. Size comparison of intrinsic choroidal ganglion cells (ICNs) when isolated or grouped in microganglia. Both substance P (SP) and calcitonin gene-related peptide (CGRP) ICNs are larger when isolated. Each bar shows the mean (± SEM).

As most ICNs of the human choroid (Triviño et al., 2002, 2005), both SP- and CGRP-positive ICNs were more frequently observed in the choroidal central area, also exhibiting preferential distribution in the central area of the temporal region (the choroid underneath the macular area of the retina). There were no observable differences between the two populations of ICNs in terms of size, morphology or immunostaining characteristics. Co-localization of these neuropeptides has been demonstrated in the trigeminal neurons (Terenghi et al., 1985; Lee et al., 1985; Kuwayama et al., 1987) and in a subpopulation of capsaicin-sensory primary afferent neurons innervating the hearts of humans, rats and guinea pigs (Wimalawansa, 1996; Horackova et al., 2002; Vaishnava and Wang, 2003). In the present work, we demonstrate that CGRP and SP co-localize in human ICNs. As in the trigeminal ganglion (Terenghi et al., 1985), all SP(+) ICNs also showed positive immunostaining for CGRP, although only a proportion of CGRP(+) ICNs were immunoreactive for SP.

In the trigeminal ganglia, CGRP(+) and SP(+) neurons are predominantly small- (20-30 μm) and medium-sized (30-50 μm) cells (Ma et al., 2001; Lazarov, 2002). In the rat intrinsic cardiac nervous system, the SP(+) neuron size is 25-50 μm (Horackova et al., 2002). The size of SP(+) and CGRP(+) ICNs demonstrated in the present work is consistent with the neuron sizes mentioned above.

Traditionally, sensory nerves were defined as purely afferent neurons that monitor changes in their chemical and physical environment and convey this information to the central nervous system. However, they also have the capacity to act in an efferent manner. This efferent function is mediated by the release of neuropeptides including SP and CGRP from the peripheral terminals, thus regulating vasodilation and other tissue activities independently of sensation (Holzer and Maggi, 1998; Supowit et al., 2005). CGRP and SP are potent vasodilator neuropeptides that are reportedly involved in the regulation of regional organ blood flow and blood pressure, both under normal physiological conditions as well as under hypertension (Wimalawansa, 1996; Brain et al., 1985; Brain and Grant, 2004; Supowit et al., 2005). ChBF augments slightly with increasing blood pressure (Polak et al., 2003). Additionally, there is evidence that in healthy subjects ChBF is self-regulated over a wide range of perfusion pressures (Riva et al., 1997a,b), indicating that ChBF is almost constant (Michelson et al., 1994; Polak et al., 2003). For the present, a logical explanation for the need to regulate ChBF is the preservation of the function of the different neurons that constitute the delicate three-dimensional cytoarchitecture of the human retina. Restricting the degree of ChBF while intraocular pressure is high could be a protective mechanism for the retina, which would otherwise undergo considerable compressive forces exerted inwards on the outer retina by the retinal pigment epithelium through its displacement by the engorged choroid, as well as an outwardly increased force on the inner retina by the vitreous because fluids are non-compressible. (Lovasick et al., 2003).

It is thought that ChBF is regulated by the autonomic nervous system (Bill, 1975; Koss, 1994), and there is growing evidence that sensory peripheral nerves also play an important role in regulating ChBF (Nakanome et al., 1995; Shimura et al., 2000). Additionally, it has been postulated that SP may be involved in the regulation of blood flow during ocular irritation (Tervo et al., 1982). There is ample evidence that trigeminal primary afferents in the eye can mediate vasodilation and plasma extravasation by locally releasing SP and CGRP upon mechanical, chemical and thermal stimulation (Bill et al., 1979; Holzer, 1988; Bill, 1991; Schrödl et al., 2001). This local effector function makes peptidergic trigeminal afferents an important factor for the regulation of ocular blood supply and inflammatory processes (Bill et al., 1979; Uusitalo et al., 1989; Bill, 1991; Kahl and Reid, 1995; Maggi, 1995). In addition, a vasodilator role as cholinergic co-mediator has recently been attributed to SP and CGRP (Domeneghini et al., 2004). All this evidence could support the possibility that the SP(+) and CGRP(+) ICNs and nerve fibers found in the present work could be implicated in ChBF regulation under physiological and pathological conditions.

Another important function recently attributed to the peripheral nerves is regulation of the vascular bed. In this respect, it has been reported that vascular changes noted in choroidal rat after sympatectomy could be due to increased numbers of sensory peptides acting directly on vessels and promoting their remodelling (Kessler et al., 1983; Zhang et al., 1984; Hill et al., 1988; Aberdeen et al., 1992; Steinle et al., 2002; Steinle and Smith, 2003). Thus, it is tempting to speculate that SP(+) and CGRP(+) ICNs and nerve fibers found in the human choroid could participate in vascular remodelling.

Peripheral innervation is highly susceptible to damage under a variety of conditions, such as aging, high blood pressure, intraocular hypertension, or diabetes (Ernest et al., 1988; Steinle and Smith, 2003). It has been postulated that chronic loss of sympathetic activity may contribute to abnormal vascular regulation in diseases such as age-related macular degeneration (Smoliakova and Radivoz, 1988; Schmidt et al., 1997) and diabetic macular oedema retinopathy (Ishikawa et al., 1985; Fulk et al., 1991). Additionally, the diabetes-like conditions induced by streptozotocin reduce the content of CGRP in the sensory nerves and exogenous CGRP-mediated vasodilatation. CGRP is likely an important regulator of vascular tone, and compromising its function could contribute to nerve ischemia and diabetic neuropathy (Yorek et al., 2004). The dysfunction of SP(+) and/or CGRP(+) ICNs could be involved in the physiopathology of ocular diseases associated with peripheral innervation damage.

We demonstrate for the first time the existence of SP and CGRP positive intrinsic ganglion cells in the human

choroid. This should serve to encourage physiological studies to elucidate their role in sensory regulation and their possible implication in ocular diseases.

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