Expression of neurotransmitters and neurotrophins in neurogenic inflammation of the rat retina

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Antidromic stimulation of the rat trigeminal ganglion triggers the release of substance P (SP) and calcitonin gene-related peptide (CGRP) from sensory nerve terminals of the capsaicin sensitive C-fibers. These pro-inflammatory neuropeptides produce a marked hyperemia in the anterior segment of the eye, accompanied by increased intraocular pressure, breakdown of the blood-aqueous barrier and myosis. To assess the effects of neurogenic inflammation on the retina, specifically on the immunostaining of neurotransmitters and neurotrophins, as well as on the expression of neurotrophin receptors in the retina. RT-PCR was also accomplished in control and stimulated animals to confirm the immunohistochemical results. In the electrically stimulated eyes, immunostaining for SP, CGRP, VIP and nNOS demonstrated a marked increase in the RPE/POS (Retinal Pigment Epithelium/Photoreceptor Outer Segments), in the inner and outer granular layers and in the ganglion cells in comparison to the control eyes. CGRP and SP were found increased in stimulated animals and this result has been confirmed by RT- PCR. Changes in neurotrophin immunostaining and in receptor expression were also observed after electric stimulation of trigeminal ganglia. Decrease of BDNF and NT4 in the outer and inner layers and in ganglion cells was particularly marked. In stimulated rat retinas immunostaining and RT-PCR showed a NGF expression increase. Neurotrophin receptors remained substantially unchanged. These studies demonstrated, for the first time, that antidromic stimulation of the trigeminal ganglion and subsequent neurogenic inflammation affect immunostaining of retinal cell neurotransmitter/neuropeptides and neurotrophins as well as the expression of neurotrophin receptors.

Key words: trigeminal nerve, neurogenic inflammation, neuropeptides, neurotransmitters, neurotrophins, retina.

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Sensory innervation of the eye is supplied by the first division of the trigeminal nerve: sensory neurons have been demonstrated in all layers of the eyeball except the retina (Belmonte *et al.,* 2004). Sensory nerve terminals have a dual function in both normal conditions and in diseases: (i) perception of the thermal, mechanical, and chemical signals which are transmitted to and processed by the relative sensory brain centers; (ii) release of various neuropeptides/neurotransmitters, mainly tachykinins and calcitonin gene-related peptides (CGRP), play an important role in local and systemic biological functions (Szolcsanyi 2004). The synonyms *neuropeptides* and *neurotransmitters* refer to their chemical composition and mode of action respectively. Receptors for SP and CGRP have been demonstrated in conjunctival and corneal epithelium where these neuropeptides function as stimulating or trophic factors for maintaining the normal structure and function of the epithelial cells (Davis & Dohlman 2001). Recent results have shown that electrical stimulation of the trigeminal ganglion elicits tear secretion that is not influenced by ganglionic, muscarinic, or noradrenergic transmission blockade but is abolished by pre-treatment with capsaicin. These findings suggest that neuropeptides released from capsaicin sensitive sensory nerve endings also regulate tear gland, goblet cell and Meibomian gland function (Kovacs *et al.,* 2005). CGRP binding sites, found in the vascular system of the ciliary body and iris, further demonstrate the role of CGRP as a vasoregulatory peptide in the anterior segment of the eye. Although the retina has no sensory innervation, SP and CGRP have been found to contain nerve endings in the choroidal and neuronal cells of the retina in various species, including humans (Casini 2005). The presence of SP has been observed in amacrine cells and in ganglion cells of the rat retina (Caruso *et al.,*

1990). Histological investigations have demonstrated the morphology and connectivity of SP-immunoreactive (SP-IR) amacrine cells. Amacrine and ganglion cells are also immunopositive for neuronal nitric oxide synthase (nNOS), suggesting that a certain synergy exists between these two substances (Cheon *et al.,* 2002). Furthermore, the detection of nNOS immunoreactivity in choroidal ganglion cells implicates their role in the blood supply to the choroids, retinal pigment epithelium (RPE) and photoreceptors (May *et al.,* 2004).

In the nervous system, including the retina, several neurotrophic factors (neurotrophins) , playing an essential role in the physiology of the neuronal cells (neurogenesis, growth, differentiation and axon regeneration) have been identified (Yip & So 2000). Brain-derived neurotrophic factor (BDNF) and Neurotrophin-4 (NT-4) improved the survival rate and growth of ganglion cells, but these results are not consistent (Bonnet *et al.,* 2004). Nerve growth factor (NGF) has been implicated in microglia-induced programmed cell death (Srinivasan *et al.,* 2004) whereas BDNF, NT-3 and NT4 have been seen to promote motor neuron survival *in vitro* and rescue motor neurons from natural or axotomy-induced cell death (Sendtner *et al.,* 1992). The specific, high-affinity neurotrophin receptor tyrosin-related kinase A, B, C (TrkA, TrkB, TrkC) and low-affinity receptor protein 75 (p75), are widely distributed throughout the retina (Lindqvist *et al.,* 2002), in developing, neonatal and adult rats (Perez & Caminos 1995). In dystrophic rat retina (Sheedo *et al.,* 2002) there is an altered expression following light damage or ischemia (Vecino *et al.,* 1998). Co-localisation of BDNF and Trk-B in green/red sensitive photoreceptor outer segment (POS) has been described (Di Polo *et al.,* 2000). Concomitant alterations in neurotrophins and neurotrophin receptors have been observed during ocular hypertension (Rudzinski *et al.,* 2004).

However, although experimental observations have demonstrated that neurotransmitters and neurotrophins are present in the retina, *in vivo* data concerning the effect of neurogenic inflammation is lacking. The aim of our study was to assess how antidromic stimulation of the trigeminal ganglion affected the rat retina, particularly in terms of immunoreactivity for neuropeptides, neurotrophins and neurotrophin receptors. Our findings suggest that neurogenic inflammation (although considerable differences exist in the irritative response among the various species, i.e. relevant in the anterior segment of rabbit eye, much weaker in higher primates) may influence some physiological aspects of the retina while the changes observed in neurotransmitter and neurotrophin immunoreactivity may indicate their involvement in some common retinal diseases.

Methods

Electrical stimulation of the trigeminal ganglion

The experiments were carried out in the University of Pecs on twenty male Wistar rats weighing 250-350 g , using a previously described technique (Peitl *et al.,* 1999). All the procedures performed in this study were in agreement with the rules of the Ethics Committee on Animal Research of University of Pecs and were in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. The animals were anaesthetized with thiopentone-sodium (Trapanal 100 mg/kg. i.p.), and a fine polyethylene cannula was implanted into the right femoral vein for drug administration. The animals were gently intubated. The endotracheal tube was then connected to a small animal respirator (SAR-830/P, IITC Inc./ Life Science Instrument) for artificial ventilation once skeletal muscle paralysis had been obtained with pipecuronium-bromide (Arduan 0.3 mg/kg i.v.). Rectal temperature was maintained at 37°C by means of a controlled infrared lamp (Experimetria Ltd). The head of each rat was fixed within a stereotactic frame. The skin, periosteum of the skull and the dorsal part of the masseter muscle were removed and a 2 mm diameter hole was drilled on the left side of the top of the skull, 3.2 mm lateral to the sagittal suture and 3.7 mm posterior to the bregma for placement of bipolar stimulating electrodes. The electrode was lowered into the left trigeminal ganglion to a depth of 9.5 mm from the dura mater overlying the dorsal surface of the brain and the ganglion was electrically stimulated (15 V, 2 Hz, for 20 min). At the end of the experiments the animals were sacrificed by an overdose of thiopentone-sodium. In order to assess the eventual effects of any misplacement of electrodes or incidental lesion of the trigeminal ganglion by the stimulating electrode, stimulation was performed with either a false electrode position or at a correct position with no electrical stimulation at all in a

control group of 4 subjects. Following sacrifice, the procedure was identical in all animals: the left eyeball (stimulated side) was used to obtain the immunohistochemical profile of the treated organs; the right eyeball acted as the control specimen. Both groups of organs underwent immunohistochemical analysis that was confined to retinal samples.

Immunohistochemical analysis

The following molecules were investigated: neurotransmitters SP, CGRP, VIP, and nNOS, and neurotrophins NGF, BDNF, NT3, NT4, as well as some high-affinity neurotrophin receptors such as TrkA, TrkB, TrkC and low-affinity receptor p75.

Eyeballs were removed, washed in PBS, fixed in formalin and embedded in paraffin. Serial 10 μm thick sections were obtained using a rotatory microtome, mounted on gelatin-coated slides and processed for immunohistochemistry as described elsewhere (Bronzetti *et al.,* 1995). For immunohistochemical detection of NTs, NT receptors and neurotransmitters, the following antibodies were used: a) rabbit anti-NGF polyclonal antibody (Santa Cruz); this displayed less than 1% cross-reactivity against recombinant human NT-3, NT-4 and BDNF; b) rabbit polyclonal antibody anti-BDNF (Santa Cruz); this did not cross-react with NT-3 or NGF; c) rabbit polyclonal antibody anti NT-3 (Santa Cruz); this did not cross-react with BDNF or NGF; d) rabbit polyclonal antibody anti NT-4 (Santa Cruz); this was raised against a peptide mapping within an internal region of NT-4 of human origin and it did not cross react with NGF, BDNF and NT-3; e) goat polyclonal TrkA immunoglobulin (Santa Cruz, CA, USA); this recognized an epitope corresponding to amino-acids 763-777, mapping adjacent to the carboxy terminus of human TrkA p140, non cross-reactive with TrkB or TrkC; f) rabbit polyclonal TrkB immunoglobulin (Santa Cruz); this recognized an epitope corresponding to amino-acids 794-808 of mouse TrkB p145, non cross-reactive with TrkA or TrkC; g) rabbit polyclonal TrkC immunoglobulin (Santa Cruz); this recognized an epitope corresponding to aminoacids 798-812 of porcine TrkC p140 non cross-reactive with TrkA or TrkB; h) goat polyclonal antibody p75 NT receptor (Santa Cruz); this recognized the amino-acid sequence mapping in the carboxy terminus of the p75 NT receptor. Specific recognition of rat p75 receptor, non cross-reactive with other growth factor receptors; i) rabbit anti-substance P polyclonal antibody (anti SP) (Chemicon International); l) rabbit anti-vasoactive intestinal peptide (anti VIP) polyclonal antibody (Chemicon International); m) goat anti-calcitonin related peptide (anti CGRP) polyclonal antibody, recommended by Santa Cruz as highly specific and not reacting with other amino- acids; this is directed against the terminal fragment of CGRP; n) rabbit anti-nNOS polyclonal antibody (Chemicon International). The specificity of antibodies for corresponding peptides was assessed by Western blotting (data not shown) using homogenates of rat brain as NT and NT receptor source. Briefly, from each paraffin block, consecutive sections were exposed to anti-VIP (diluted 1:500); anti-SP (diluted 1:5000); anti-TrKA, anti-TrKB, anti-TrKC, anti-NT3 (diluted 1:100); anti-p75 (diluted 1:10); anti-nNOS (diluted 1:2500) antibodies alone or in the presence of the antibodies pre-absorbed with corresponding peptides (10 μg/mL) and to anti-NGF, anti-BDNF and anti-NT-3 antibodies (diluted 1:1000) and to the antibodies pre-absorbed with human NGF (10 μg/mL), human BDNF blocking peptide (10 μg/mL), human NT-3 (10 μg/mL). Optimal antisera dilutions and incubation times were assessed in a series of preliminary experiments. After incubation, slides were rinsed twice in phosphate buffer and exposed for 90′ at room temperature, to IgG peroxidase-conjugated secondary antibodies raised against rabbit (anti rabbit for TrK and NT immunohistochemistry) (Boehringer Mannheim GmbH, Mannheim, Germany), against mouse (anti mouse for p75 receptor and CGRP immunohistochemistry) (Sigma Chemicals Co, St Louis, MO, USA). The product of immune reaction was revealed using 0.05% 3,3-diaminobenzidine in 0.1% H_2O_2 as a chromogen. Sections were then washed, dehydrated in ethanol, mounted in a synthetic mounting medium and observed using a light microscope. Endogenous peroxidase activity was blocked by H_2O_2, while the non-specific binding of immunoglobulin to glass and tissue was prevented by 3% fetal calf serum added to the incubation medium. In a series of preliminary experiments, immunohistochemistry was performed using both paraffin-embedded and frozen sections (*data not shown*).There were no difference in the intensity or distribution of immunostaining using the 2

types of sections, although microanatomical details were better preserved in paraffin-embedded material. In view of this, as in other reported studies (Ricci *et al.,* 2000), we used paraffin-embedded material in standard immunohistochemistry experiments.

Sections of retina obtained from the treated and untreated eyeballs were exposed to the primary/secondary antibodies and developed a dark-brown (intense=++), yellow-brown (slight=+) or questionable (±) immunostaining or no staining at all (-). No immunostaining developed in sections incubated with antibodies previously adsorbed with peptides used for raising them or with a pre-immune serum.

Immunostaining was evaluated in four areas of the retina. The outmost layer was the RPE and POS complex. It was justified by the fact that their structural and functional correlation is well established *in vivo* and the separation of these structures is impossible in these sections. The other evaluation sites were the outer and inner granular layers and ganglion cell layer.

RT-PCR

On the basis of immunohistochemical results RT-PCR experiments were carried out for neurotrophins and for neurotransmitters which were all investigated, but the results have been reported only for those found more significant. Total RNA was isolated from rat retinal tissue by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to customer's instructions. cDNA was synthezised from 1 μg total RNA in a final reaction volume of 20 μL. Briefly, a mixture of total RNA, oligo (dT), dNTP mix and DEPC-treated distilled water was preicubated for 5 min at 65° C; then SuperScript III reverse transcriptase (200U), RNase Ribonuclease Inhibitor, DTT and buffer (250 mM Tris pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) were added to the mixture and incubation was continued for 45 min at 50° C. Finally, Superscript III was inactivated by heating for 15 min at 70°C.

All reagents were from Invitrogen. Three to five μL of the resulting cDNA were amplified by polymerase chain reaction (PCR). Each PCR tube contained the following reagents: 0.2 μM of both sense and antisense primers, 3 to 5 μL template cDNA, 0.2 mM 4-dNTP mix (Invitrogen), 2.5 U Platinum Taq DNA polymerase (Invitrogen) and 1X reaction buffer (Invitrogen). $MgCl_2$ was added at a final concentration of 1 mM. The final volume was 50 μL.

RT-PCR for neurotrophins

The PCR primers used for amplifying neurothrophins and their receptors (M-Medical, Florence, Italy) were: for NGF forward TCATCATCCCATCCCATCTT, reverse CTTGACAAAGGTGTGAGTCG; for BDNF forward AGCCTCCTCTGCTCTTTCTGCTGGA, reverse CTTTTGTCTATGCCCCTGCAGCCTT; for NT-3 forward TTTCTCGCTTATCTCCGTGGCATCC, reverse GGCAGGGTGCTCTGGTAATTTTCCT; for TrkA forward TCTTCACTGAGTTCCTGGAG, reverse TTCTCCACCGGGTCTCCAGA; for TrkB forward AAGACCCTGAAGGATGCCAG, reverse AGTAG TCAGTGCTGTACACG; for TrkC forward GGAA AGGTCTTCCTGGCCGAGTGC, reverse GCTTTCCATAGGTGAAGATCTCCC; for p75 forward TGGACAGCGTGACGTTCTCC, reverse GATCTCCTCGCACTCGGCGT.

The specificity of the primers was verified by searching in NCBI data base every possible homology to cDNAs of unrelated known proteins. PCR reaction consisted of incubation for 2 min at 94° C followed by 30-35 cycles of incubations at 94° C for 30 sec, 56° C (for NGF, TrkA and TrkB) or 62° C (for BDNF, NT-3, p75 and TrkC) for 30 sec and 72° C for 1 min. PCR products were separated by agarose gel electrophoresis (Submarine Agarose Gel Unit, Hoefer, San Francisco, CA, USA) and visualized using a digital gel documentation system (GelDoc 2000 System/Quantity One Software; Bio-Rad Laboratories, Hercules, CA, USA).

RT-PCR for neurotransmitters

RT-PCR for CGRP

Total RNA was isolated from animals and prepared for reverse transcription-polymerase chain reaction (RT-PCR). The PCR reactions for β-actin were cycled 35 times at 94°C (denaturation) for 1 min, 60°C (annealing) for 1 min, and 72°C (extension) for 1 min. The PCR reactions for CGRP were cycled 33 times at 94°C (denaturation) for 1 min, 58°C (annealing) for 1 min, and 72°C (extension) for 1 min. Samples were incubated at 72°C for an additional 7 min after the last cycle was completed. All nucleotide primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). The primer pairs were chosen from the published cDNA sequences of rat CGRP and human β-actin as follows: CGRP (sense) 5′-TCCTGCAACACCGCCACCTG-3′; CGRP (antisense) 5′-GGTGGGCACAAAGTTGTCCT-3′; β-actin (sense) 5′-AACCGC-

GAGAAGATGACCCGATCATGTTT-3′; β-actin (antisense) 5′-AGCAGCCGTGGCCATCTCTTG CTCGAAGTC-3′. The PCR products were separated on a 2% (w/v) agarose gel in 0.5x Tris-borate-EDTA buffer, stained with ethidium bromide (0.5 μg/mL), visualized under ultraviolet light, and quantitated by densitometric analysis.

RT-PCR for SP

A1-I3 primers were designed to amplify 231 bp fragment between nucleotides 3223–3453 of mRNA (upstream primer: 5'CTCAAGTCTTTTGC-CCAAGC3'; downstream primer: 5'GGAGA-CAACGGGATCTGTGT3'). This region spans exons 27–29 of the 58,910 bp A1-I3 gene located on chromosome 4 (GeneBank). PCR generated fragments were separated and detected using 2% agarose gels (E-Gel, Invitrogen). Controls for RT-PCR reactions included: 1) separate amplification of GAPDH (primers obtained from Ambion, Austin, TX) as a positive control for total RNA concentrations and reverse transcription reactions, 2) A1-I3 PCR amplification of liver cDNA as a positive control for A1-I3 expression and 3) total RNA without reverse transcriptase in PCR reaction as a negative control.

Results

Neurotransmitters (Table 1)

In the control eyes questionable or very mild immunostaining for SP, VIP and nNOS was found in the outer and inner granular layers and, for SP, in the ganglion cell layer too: for CGRP it was negative in all layers.

In the electrically stimulated rats, immunostaining for SP, CGRP, VIP and nNOS in all four layers was markedly increased in comparison to the control eyes (Figures 1, 3).

Table 1. Comparison of immunostaining for neurotransmitters.

		control	*stimulated*
SP	RPE/POS	-	++
	outer granular layer	±	++
	inner granular layer	±	++
	ganglion cell layer	±	++
CGRP	RPE/POS	-	++
	outer granular layer	-	++
	inner granular layer	-	++
	ganglion cell layer.	-	++
VIP	RPE/POS	-	++
	outer granular layer	+	++
	inner granular layer	+	++
	ganglion cell layer	-	++
nNOS	RPE/POS	-	++
	outer granular layer	±	++
	inner granular layer	±	++
	ganglion cell layer	-	++

Table 2. Comparison of immunostaining for neurotrophins.

		control	*stimulated*
NGF	RPE/POS	++	++
	outer granular layer	+	++
	inner granular layer	-	++
	ganglion cell layer	++	++
BDNF	RPE/POS	++	++
	outer granular layer	++	-
	inner granular layer	++	-
	ganglion cell layer.	++	-
NT3	RPE/POS	++	++
	outer granular layer	++	+
	inner granular layer	+	+
	ganglion cell layer	++	++
NT4	RPE/POS	++	++
	outer granular layer	++	-
	inner granular layer	++	-
	ganglion cell layer	++	-

Neurotrophins (Table 2)

The localization of immunostaining for NTs was similar to that of neurotransmitters, namely the POS/RPE, outer and inner granular layers, and the ganglion cells.

In the control retinas, immunostaining for NGF was well-expressed in the RPE/POS and ganglion layers of control rats and presented a marked increase in the corresponding tissues of the electrically stimulated animals. Furthermore, in the stimulated eyes, immunostaining was also observed in the outer and inner granular layers which were negative in the control specimens (Figure 2).

In control retinas, immunostaining for BDNF was found in all four layers of the retina as compared to the POS alone of the stimulated retinas, although they showed more intense staining as compared to the controls.

Immunostaining for NT3 occurred in all four localizations and had a similar intensity in both control and stimulated eyes.

In the control retinas, all four sites presented immunostaining for NT4 , while in the stimulated retinas this was only present though very marked in the RPE/POS.

Neurotrophin receptors (Table 3)

Immunostaining for neurotrophin receptors in the retina showed a similar distribution to neurotrophins.

TrKA was well-expressed in the retina of both control and stimulated eyes, and no significant differences were detected between them (Figures 2, 4).

TrKB expression was greater in all four layers of the retina of stimulated eyes.

TrKC expression was detected in all four localizations and it was very similar in both control and stimulated eyes.

Immunostaining for expression of p75 was more marked in the stimulated rats in comparison to the control ones, in which it was slightly less.

RT-PCR

RT-PCR analysis revealed the occurrence in control and stimulated rat retinas of specific transcripts for neurotrophins (NGF, BDNF, NT-3) and their high-affinity receptors (TrkA, TrkB, TrkC). Data not significant (BDNF, NT-3, TrKB, TrKC) have been not shown.

In stimulated rats NGF appeared strongly positive, whereas TrKA was mildly detectable. Furthermore, in line with our immunohistochemical results, mRNA for p75 was clearly detectable (Figure 6).

Table 3. Comparison of immunostaining for neurotrophin receptors.

		control	*stimulated*
TrKA	RPE/POS	+	+
	outer granular layer	+	+
	inner granular layer	+	+
	ganglion cell layer	+	+
TrKB	RPE/POS	+	++
	outer granular layer	+	++
	inner granular layer	-	+
	ganglion cell layer	+	+
TrKC	RPE/POS	+	+
	outer granular layer	+	+
	inner granular layer	+	+
	ganglion cell layer	+	+
P75	RPE/POS	+	+
	outer granular layer	+	++
	inner granular layer	+	++
	ganglion cell layer	-	+

The neurotransmitters (SP, CGRP) whose increase after stimulation has been found significant in immunohistochemical experiments, were evaluated even by means of RT-PCR that confirmed the obtained results (Figure 5).

Discussion

Our study showed that antidromic stimulation of the trigeminal ganglion causes increased immunoreactivity for SP, CGRP, VIP and nNOS in the RPE/POS layer, in the outer and inner granular layers and in the ganglion cell layer of the rat retina. These changes were associated with a decrease of immunoreactivity for BDNF, NT3 and NT4. As far as immunoreactivity for TrkA, TrkB, TrkC and

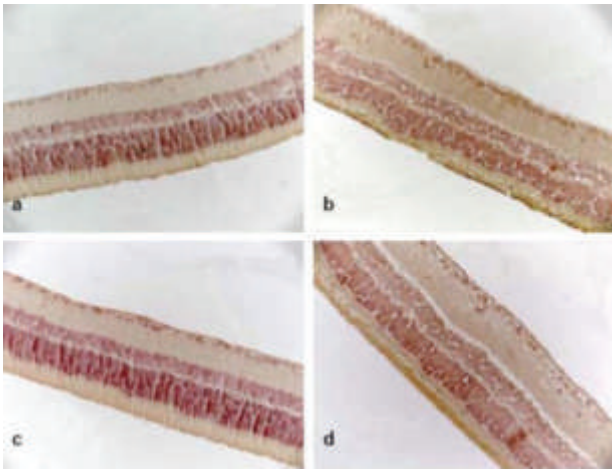

Figure 1. Immunostaining for SP, CGRP. 40x. a, c: control eye; b, d:stimulated eye.

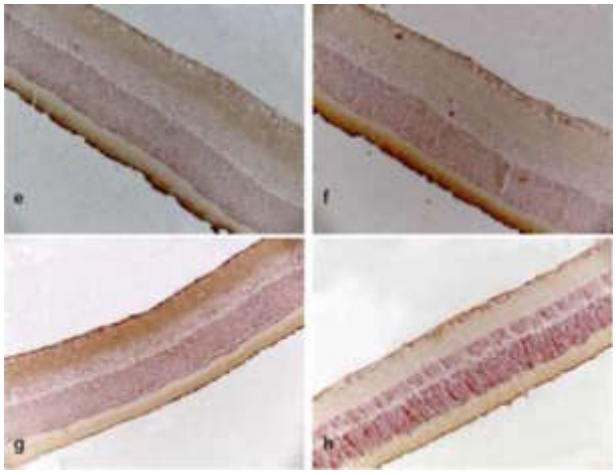

Figure 2. IImmunostaining for NGF, TrKA. 40x. e, g: control eye; f, h: stimulated eye.

p75 was concerned, the values registered after stimulation demonstrate that the immunohistochemical profile of TrkA and TrkC remains unchanged; on the other hand, immunoreactivity for TrkB and p75 appears to be increased. These findings clearly indicate that release of SP and CGRP from sensory nerve endings in the iris, ciliary body and choroid, as well as the neurogenic inflammation caused in the anterior segment of the eye, influence the expression of neuropeptide/neurotransmitter, neurotrophin immunoreactivity in the retina. In addition to its role as neurotransmitter, SP also participates in immune and inflammatory responses (Castagliuolo *et al.,* 1997). In fact, mast cells, polymorphonuclear leukocytes, T lymphocytes, and macrophages can respond to SP, indicating that the effects of SP during inflammation may be mediated by direct activation of these cells (Shanahan *et al.,* 1985; Perianin *et al.,* 1989; Calvo *et al.,* 1992; Wozniak *et al.,* 1989; Hartung *et al.,* 1986). Finding an explanation for these data is certainly the most intriguing aspect of our study. The retina has no direct sensory innervation; on the other hand, the iris, ciliary body and choroids are abundantly innervated by sensory nerves. The influence of sensory nerve stimulation might affect the retina via different possible pathways and it is feasible that SP and CGRP come from the iris and ciliary body through the aqueous humor or from the choroid through the RPE. CGRP is considered as a constitutive neuropeptide in the aqueous humor. Mechanical or electrical stimulation of the trigem-

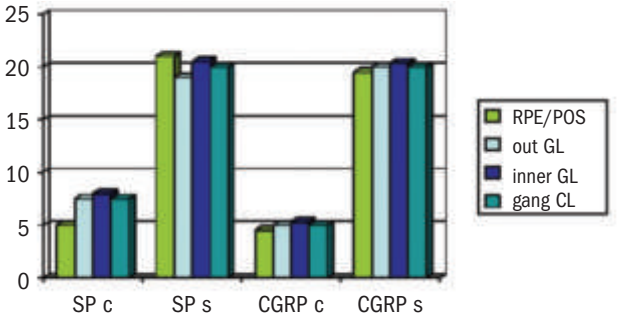

Figure 3. The intensity of the immunostaining for SP and CGRP developed within retinal pigment epithelium/photoreceptor outer segment (RPE/POS), outer granular layer (out GL), inner granular layer (inner GL) and ganglion cell layer (gang CL) was assessed microdensitometrically in control and stimulated rats (SP c/s; CGRP c/s). The intensity of immune staining was obtained by a program of the image analyzer and it was expressed in arbitrary units.

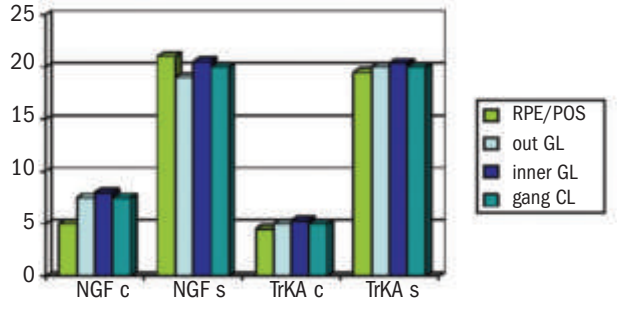

Figure 4. The intensity of immunostaining for NGF and TrKA expressed in the retinal pigment epithelium/photoreceptor outer segment (RPE/POS), outer granular layer (out GL), inner granular layer (inner GL) and ganglion cell layer (gang CL) was assessed microdensitometrically in control and stimulated rats (NGF c/s; TrKA c/s). The intensity of immune staining was obtained by a program of image analyzer and it was expressed in arbitrary units.

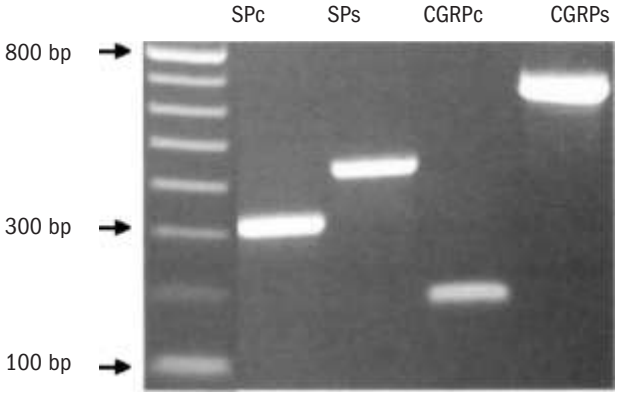

Figure 5. RT-PCR analysis of neurotransmitters (SP, CGRP) in control and stimulated rat retina.

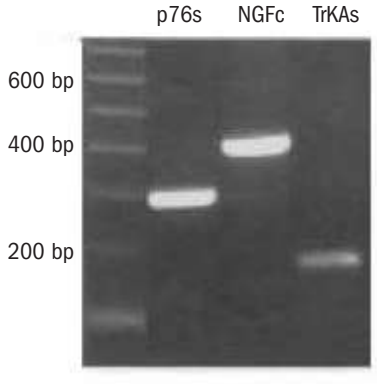

Figure 6. RT-PCR analysis of NGF and related receptors (TrkA, p75) in stimulated rat retina.

inal nerve/ganglion in rabbits was, in fact, followed by an increase in CGRP-immunoreactivity in the aqueous humor accompanied by marked hyperemia in the anterior segment of the eye, increased intraocular pressure, breakdown of the blood-aqueous barrier and myosis (Wahlestedt *et al.,* 1986). Our findings show that this neurogenic inflammation is also associated with an increase in some neuropeptide levels (SP, CGRP) in the retina.

In the light of these findings, we can hypothesize the existence of physiological levels of SP and CGRP in the eye and that these neuropeptides are under sensory nerve control. Stimulation of sensory nerves brings about an increase in the levels of these neuropeptides in the aqueous humor together with inflammation-related phenomena, simultaneously increasing their levels in the retina, while depletion of sensory nerve activity may be associated with decreasing levels in both the anterior and posterior segments of the eye. Despite the simultaneous increase of neuropeptide levels in both segments of the eye, the mechanism responsible for the appearance of these substances in the retina has still to be established.

Since previous studies showed that extrinsic SP and CGRP act as excitatory neurotransmitters in the retina, the effects of human CGRP on rabbit electroretinogram were evaluated in a dose-related study. Intracameral administration of CGRP significantly increased the amplitudes of both A and B waves. These results indicate that CGRP may play a functional (excitatory) role in modulating retinal responses to light stimulation (Cao *et al.,* 1993). Moreover, SP mediates positive effects on dopamine release (Boelen *et al.,* 1998), which is related to light intensity, thus indicating that the functional role of SP is also probably related to light adaptation (Casini *et al.,* 2000).

Another crucial point of our study hinges on the influence of neurogenic inflammation on the expression of retinal neurotrophins and their receptors.

In our experimental model, electric stimulation of trigeminal ganglia modified immunostaining of neurotrophins and the expression of some of their receptors, as well as for SP and CGRP. Immunostaining for NGF markedly increased in the outer and inner granular layers after stimulation, but remained the same or even decreased for other localizations and for all other neurotrophins. There was a particularly marked decrease for BDNF and NT4 in the inner and outer layers and in ganglion cells. On the other hand, no substantial changes occurred in the expression of neurotrophin receptors, apart from that of TrKB in RPE/POS, in the outer and inner granular layers and of p75 in the outer and inner granular layers and in the ganglion cell layer where it increased. The results obtained by immunohistochemical experiments have been confirmed by the RT-PCR (Figure 5).

The relationships between the levels of neurotrasmitters and neurotrophins appear to be interesting in terms of their possible implications for a variety of diseases. A true correlation between neurotransmitters and neurotrophins in the retinal compartment cannot be postulated although our results have shown that trigeminal electrical stimulation simultaneously exerts an inverse effect on the levels of both groups of substances in an animal model. Diminished levels of neurotrophins presumably favour apoptotic retinal cell death during neurogenic inflammation.

Previous *in vitro* studies have described a stimulating effect of SP and CGRP on RPE cells: this seems to suggest that RPE cells are under neural control and the low effective concentration of peptides may have a physiological effect on these cells (Troger *et al.,* 2003). Age-related macular degeneration (AMD) is known to be dependent on RPE changes. The fact that CGRP was not detected in the aqueous humor in primary open-angle glaucoma, exfoliative glaucoma or cataract (Vesaluoma *et al.,* 1998) seems to be of particular clinical relevance.

By means of immunocytochemistry, CGRP and SP peptidergic innervation to the vasculature of the optic nerve and retina were identified in the rhesus monkey and rat. Perivascular peptidergic nerve fibers terminate as the blood vessel enters the eyeball, but do not follow the branches of the central retinal artery inside the eye (Bergua *et al.,* 2003). Alterations in the expression of neurotrophins and their receptors have been observed in ocular hypertension (Rudzinski *et al.,* 2004). In the light of these findings, it seems likely that neuropeptides are involved in the regulation of blood flow in both the anterior and posterior segments.

To conclude, by taking into consideration all the findings described above, it seems safe to say that neuropeptides SP and CGRP, which are present in the aqueous humor, may be increased in the retina after sensory nerve stimulation. Both SP and CGRP may influence retinal physiology by modify-

ing the expression of neurotrophins and their receptors. Increased levels (i.e. via mechanisms related to neurogenic inflammation) may play a role in the pathogenesis of inflammatory or vasoproliferative diseases of the retina, while decreased levels may be related to age-related degenerative diseases.

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