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Ocular inflammation induced by electroconvulsive treatment: contribution of nitric oxide and neuropeptides mobilized from C-fibres

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1 Electroconvulsive treatment (ECT) of rabbits produced ocular inflammation consisting of conjunctival hyperaemia, miosis and protein extravasation into the aqueous humour, reflected by the so-called aqueous flare response (AFR); the maximal reduction in pupil size was 3.8 ± 0.1 mm (s.e. of mean, $n = 16$) while the maximal AFR was 28.1 ± 2.8 (arbitrary units).

2 ECT also caused release of substance P (SP), pituitary adenylate cyclase-activating peptide (PACAP)-27, -38 and calcitonin gene-related peptide (CGRP). The concentrations of SP and CGRP in the aqueous humour of normal, untreated eyes were 10.6 ± 1.4 and 117.4 ± 12.4 pmol l^{-1}, respectively, while the concentrations of PACAP-27 and -38 were below the detection limit. After ECT the concentrations of SP, PACAP-27, -38 and CGRP were 65.0 ± 9.6, 46.9 ± 8.4, 50.2 ± 5.4 and 1109.9 ± 133.1 pmol l^{-1}, respectively (s.e. of mean, $n = 12$). Conceivably, ECT evoked an antidromic activation of sensory neurones in the trigeminal ganglion with the consequent release of neuropeptides from C-fibres in the uvea and the development of neurogenic inflammation.

3 Rabbits received the nitric oxide (NO) synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 200 mg kg^{-1}, i.v.). This pretreatment inhibited the ECT-evoked conjunctival hyperaemia, miosis and AFR; under these circumstances the maximal reduction in pupil size was 1.9 ± 0.1 mm while the maximal AFR was 2.7 ± 0.9 ($n = 16$). L-NAME also inhibited the ECT-evoked release of SP, PACAP-27, -38 and CGRP into the aqueous humour; the concentrations of SP and CGRP were 13.2 ± 1.5 and 204.8 ± 33.5 pmol l^{-1}, respectively, while PACAP-27 and -38 were below the detection limit ($n = 12$).

4 The ECT-evoked miosis was also inhibited by pretreatment with the tachykinin receptor antagonist D-Pal^9 spantide II (90 nmol, intravitreal injection); under these circumstances the maximal reduction in pupil size was only 0.7 ± 0.03 mm, indicating an important role for SP in the miotic response. Pretreatment of the eye with capsaicin, which is known to cause functional ablation of C-fibres, inhibited the conjunctival hyperaemia, miosis and AFR by 40–50%; the maximal reduction in pupil size being 2.2 ± 0.2 mm and the maximal AFR 13.8 ± 2.1 (arbitrary units) ($n = 8$).

5 The results suggest (1) that ECT evokes ocular inflammation through antidromic C-fibre activation; (2) that SP contributes to the ECT-evoked miosis; and (3) that NO contributes to the antidromic C-fibre activation and possibly to the vascular responses mediated by the C-fibre transmitters.

Keywords: Nitric oxide; C-fibres; substance P; calcitonin-gene related peptide; pituitary adenylate cyclase activating peptide; electroconvulsive treatment; seizure; ocular inflammation; rabbit eye; transmitter release

Introduction

The inflammatory response in the eye consists of miosis, lacrimation, conjunctival hyperaemia and breakdown of the blood-aqueous barrier with consequent leakage of protein into the aqueous humour (for references, see Unger, 1990; Håkanson & Wang, 1996). Ocular C-fibres originate in the trigeminal ganglion (Butler & Hammond, 1980; Butler *et al*., 1980; Tervo *et al*., 1981), and there is much evidence to suggest that C-fibre neurotransmitters, such as substance P (SP) and calcitonin gene-related peptide (CGRP), play a key role in the ocular response to injury (Holmdahl *et al*., 1981; Wahlestedt *et al*., 1986; Stone *et al*., 1987; Unger, 1990; Håkanson & Wang, 1996). Recently, pituitary adenylate cyclase activating peptide (PACAP) has been identified as yet another C-fibre neuropeptide, that takes part in the inflammatory responses of the rabbit eye (Wang *et al*., 1995). As there is no barrier separating the iris and the ciliary body from the anterior chamber, any transmitter that is released from local nerve fibres will diffuse into the anterior chamber, making the eye an excellent model for studies of transmitter release.

Nitric oxide (NO) is a short-lived molecule displaying numerous bioactivities (Moncada *et al*., 1991; Snyder & Bredt, 1991). It is generated from L-arginine by the enzyme NO synthase (NOS), which can be inhibited by analogues of L-arginine, e.g. N^G-nitro-L-arginine methyl ester (L-NAME) (Moncada *et al*., 1991). Recent observations suggest that NO may be of physiological and/or pathophysiological significance in the control of ocular functions. Thus, NOS activity has been demonstrated in the anterior uvea of the rabbit (Osborne *et al*., 1993) and NOS immunoreactivity has been detected by immunostaining of nerve fibres in the anterior uvea of the rat (Yamamoto *et al*., 1993). Intravenous injection of L-NAME was found to reduce the regional blood flow in the uvea of the rabbit (Seligsohn & Bill, 1993), suggesting a role for NO in the regulation of ocular blood vessels. Interestingly, NO seems to play a role in the activation of ocular C-fibres in response to noxious stimuli (Wang & Håkanson, 1995; Wang *et al*., 1996a). We decided to investigate whether NO plays a role in the ocular response to electrical stimulation of the trigeminal ganglion by electroconvulsive treatment (ECT), with an emphasis on the relationship between NO and sensory nerves (C-fibres) in the uvea.

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Methods

Animals

All experiments were carried out on adult pigmented rabbits (1.5–2.5 kg) of mixed strain. The study was approved by the local Animal Welfare committee.

ECT

ECT was performed by using round steel electrodes of 8 mm diameter, attached bitemporally to shaved areas on the head of the rabbit. Standard conductive electrode jelly was applied on the skin to increase conductivity. Monophasic pulses, with an initial amplitude of 150 V followed by a sinusoidal decline during 5 ms, were applied with a frequency of 50 Hz for 5 s (Konvulsator 2077, Siemens, Germany). The amplitude of stimulation was determined in preliminary experiments in which voltage was increased progressively until reproducible seizures were observed. Pulse shape, pulse duration and frequency were chosen in accordance with those used in clinical therapy (Fink, 1987). The electrical stimulus was delivered twice with an interval of 5 min. Just before the first electrical stimulus was applied, the rabbits were given methohexitone sodium (5 mg kg^{-1}) into an ear vein. The anaesthesia lasted for about 20 min, sufficient to prevent the discomfort associated with the treatment. Each rabbit was killed 1–3 h after ECT.

Measurements of ocular responses

ECT produced symptoms of ocular inflammation. The breakdown of the blood-aqueous barrier was determined by photoelectric measurement of the aqueous flare response (AFR) in the anterior chamber (Anjou & Krakau, 1961). This response is a Tyndall phenomenon in the anterior chamber reflecting protein leakage across the blood-aqueous barrier. Briefly, a narrow beam of light is passed through the anterior chamber. In the presence of large molecules (usually proteins) in the aqueous humour, light scattering (aqueous flare) occurs. A correlation between the AFR and the protein concentration has been established (Anjou & Krakau, 1961; Dyster-Aas & Krakau, 1964). The AFR is expressed in arbitrary units with reference to a standard (Dyster-Aas & Krakau, 1964). Conjunctival hyperaemia was assessed visually. The miosis was monitored by measuring the pupillary diameter with a transparent ruler under constant and uniform illumination.

Pretreatment with L-NAME, capsaicin and tachykinin receptor antagonist

L-NAME (200 mg kg^{-1}, in 3 ml saline) was given by intravenous injection 30 min before the ECT. The rabbits were divided into two groups: one group received L-NAME, the other group received vehicle (0.9% saline). Each group included eight rabbits. The dose of L-NAME was chosen from earlier studies, showing that L-NAME (10–200 mg kg^{-1}) inhibits NOS in a dose-dependent manner and that 30 mg kg^{-1} lowered the regional blood flow in rabbit uvea for a short period of time only (<20 min) (Persson *et al*., 1991; Seligsohn & Bill, 1993; Wang & Håkanson, 1995; Wang *et al*., 1996a).

Capsaicin acts on C-fibres to release C-fibre transmitters (Holzer, 1991). Retrobulbar injection of 0.5 ml of 1% capsaicin solution to one eye was given under pentobarbitone anaesthesia (Bynke, 1983; Wang *et al*., 1996b). The contralateral eye received vehicle. ECT was applied 4 days after treatment with capsaicin when the capsaicin-induced responses had subsided.

The tachykinin receptor antagonist, D-Pal^9 spantide II, was given by intravitreal injection (90 nmol in 30 μl) into the corpus vitreum, 3–4 mm posteriorly to the limbus; the contralateral eye received the same volume of saline (Holmdahl *et al*., 1981). D-Pal^9 spantide II is a specific and potent tachykinin receptor antagonist with pA_2 values of 7.5 for NK_1 receptors and 6.5 for NK_2 receptors (Wang *et al*., 1994a). ECT was applied 3 h after the injection.

Radioimmunoassay (RIA) of neuropeptides in the aqueous humour

Samples of aqueous humour were collected from the anterior chamber of both eyes 1 h after ECT (usually the AFR had reached maximum at this time). Each group included six rabbits. The samples were frozen on dry ice and stored at −80°C until assayed.

SP-, PACAP-27- and PACAP-38-like immunoreactivity (LI) were measured by RIA kits from Peninsula (Merseyside, St. Helens, U.K.). Briefly, aqueous humour samples or standard solution of each peptide in RIA buffer (19 mM NaH_2PO_4, 81 mM Na_2HPO_4, 50 mM NaCl, 0.1% Triton X-100, 0.01% sodium azide, pH 7.4) were incubated with their respective antiserum at a final dilution of 1 : 120,000 at 4°C for 24 h. Then iodinated tracer (≈10,000 c.p.m.) in RIA buffer was added. The mixture was incubated for another 24 h at 4°C. Antibody-bound tracer was separated from free tracer by addition of goat anti-rabbit IgG serum and of normal rabbit serum. After incubation at room temperature for 90 min, 500 μl of cold RIA buffer was added, and the samples were centrifuged at $1700 \times g$ for 30 min. The tubes were gently aspirated and the radioactivity of the precipitates was measured. The IC_{50} values are about 27, 4 and 4 pmol l^{-1} for SP, PACAP-27 and PACAP-38, respectively. The detection limits for all three peptides are about 1 pmol l^{-1}. The PACAP-27 antiserum cross-reacts 1% with PACAP-38 and does not recognize vasoactive intestinal peptide (VIP), SP, neurokinin A (NKA) and CGRP. The PACAP-38 antiserum cross-reacts 0.01% with PACAP-27 and does not recognize VIP, SP, NKA and CGRP. The SP antiserum does not cross-react with either NKA or neurokinin B (Wang & Håkanson, 1995; Wang *et al*., 1995; 1996a, b).

(Tyr^0) CGRP (rat) (Peninsula) was radioiodinated by conventional chloramine-T oxidation and used as a tracer after purification by high performance liquid chromatography. The specific radioactivity of the tracer was 1000–2000 μCi $nmol^{-1}$. Aqueous humour samples or standard solutions of rat CGRP were incubated with 200 μl antiserum (at a final dilution of 1 : 40,000) for 24 h at 4°C. Then, tracer (≈10,000 c.p.m.) was added and the mixture was incubated for another 24 h. Free tracer was separated from bound by adding 100 μl solid phase second antibody coated cellulose suspension. After incubation at room temperature for 30 min, 500 μl cold redistilled water was added and the mixture was centrifuged at $1700 \times g$ for 20 min. The radioactivity of the precipitates was measured. The detection limit is about 100 pmol l^{-1} and the interassay variation is less than 10% in the 100–1250 pmol l^{-1} range. CGRP variants (human CGRP I and II) cross-react with the antiserum better than 100% on a molar basis, but there is no cross-reaction with calcitonin, katacalcin, C-terminal adjacent peptide, tachykinins, neuropeptide Y (NPY) and VIP (Grunditz *et al*., 1986; Wahlestedt *et al*., 1986; Wang *et al*., 1995). Concentrations are expressed as pmol rat CGRP equivalents l^{-1}.

Chemicals

Capsaicin and L-NAME were purchased from Sigma (St. Louis, MO, U.S.A.). D-Pal^{9}spantide II, a specific tachykinin receptor antagonist (Wang *et al*., 1994a), was a kind gift from Dr Karl Folkers (Austin, TX, U.S.A.). The capsaicin solution (1%) was prepared by dissolving 100 mg capsaicin in 150 μl of 99.5% ethanol; then 850 μl of Tween 80 was added, followed by 9 ml of 0.9% saline (Bynke, 1983). D-Pal^{9}spantide II was dissolved in 0.1 M acetic acid (Wang *et al*., 1994a). L-NAME was dissolved in 0.9% saline.

Analysis of results

Data are expressed as mean ± s.e. of mean. Student's *t* test (two-tailed) for unpaired groups were used for statistical analysis and difference between samples was considered significant when $P<0.05$.

Results

All rabbits exposed to ECT responded with seizures, lasting for 10–12 s, manifested in an initial rigid extensor spasm followed by a series of jerks and occasionally defaecation and micturition. Upon awakening they appeared normal; 46 rabbits were exposed to ECT, 2 of them died, probably due to an over-dose of methohexitone sodium and not from ECT.

Ocular responses induced by ECT

ECT induced a prompt miosis which lasted about 1 h (Figure 1a). A moderate AFR was noted after 15–30 min, reaching maximum after 1 h (Figure 1b). The conjunctiva displayed moderate hyperaemia which lasted for 2–3 h.

Effects of L-NAME on ECT-induced ocular responses

Pretreatment with L-NAME (200 mg kg^{-1}, i.v.) inhibited the ECT-evoked miosis (Figure 1a). The AFR (Figure 1b) and the conjunctival hyperaemia (not shown) were virtually abolished by L-NAME.

Effects of tachykinin receptor antagonist on ECT-induced ocular responses

Pretreatment with D-Pal^9 spantide II (90 nmol, intravitreal injection) inhibited the miosis induced by ECT (Figure 2a), while leaving the AFR and the conjunctival hyperaemia unaffected (Figure 2b).

Effects of capsaicin on ECT-induced ocular responses

Capsaicin (0.5 ml of 1% solution, retrobulbar injection) itself produced a prompt inflammatory response with miosis, AFR and conjunctival hyperaemia and swelling. These responses subsided gradually over a period of 1–3 days. In the contralateral eye, injected with the vehicle, conjunctival hyperaemia, a small miosis and a slight AFR were noted (see also Bynke, 1983).

Capsaicin pretreatment inhibited the miosis, AFR and conjunctival hyperaemia induced by ECT, applied 4 days after capsaicin (Figure 3a and b).

Release of neuropeptides into the aqueous humour

SP and CGRP occurred in measurable amounts in the aqueous humour of the untreated rabbit eye, while PACAP-27 and -38 were below the level of detection.

ECT raised the concentrations of the neuropeptides in the aqueous humour (Table 1). Pretreatment with L-NAME (200 mg kg^{-1}, i.v.) before ECT inhibited the concentration increase (Table 1).

In another series of experiments, the rabbits were treated with L-NAME and the aqueous humour samples were collected after 1 h. Treatment with L-NAME did not affect the concentrations of CGRP and SP in the aqueous humour and PACAP-27 and -38 remained undetectable (data not shown).

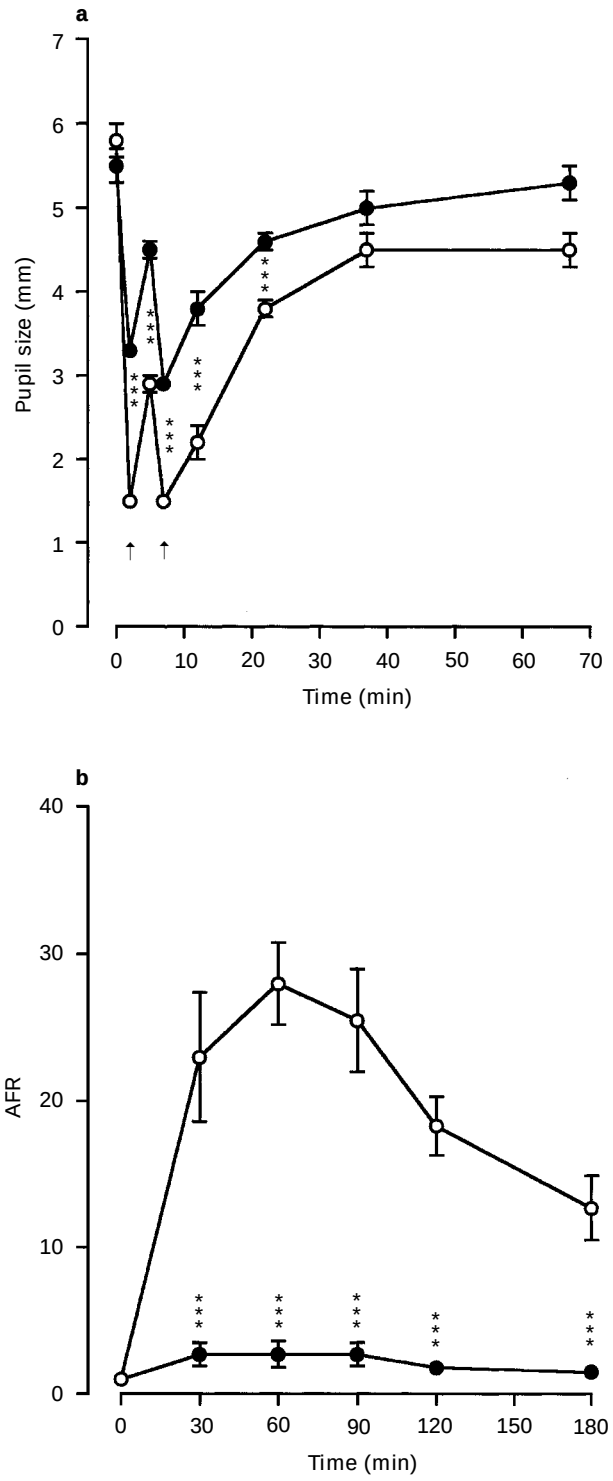

Figure 1 ECT (applied twice, at arrows) induced miosis and aqueous flare (AFR, see text). The NOS inhibitor, L-NAME (200 mg kg^{-1}), inhibited the ECT-evoked miosis (a) and the AFR (b) (●). (○) Rabbits not pretreated with L-NAME (controls). Means ± s.e. of mean (vertical lines) of eight rabbits in each group, statistical difference when compared to controls (ECT alone) indicated by ***$P<0.001$ at corresponding time points.

Discussion

ECT is used to treat severe depression and other affective disorders. Although the mechanisms behind the beneficial effects of ECT are poorly understood, recent studies have revealed a dramatic increase in neuropeptide expression in several regions of the brain. Thus, ECT increases the expression of mRNA coding for NPY in the pyriform cortex and the dentate gyrus (Mikkelsen *et al.*, 1994) and of mRNA coding for tachykinins and cholecystokinin in the ventral periaqueductal gray (Lindefors *et al.*, 1991). In addition, the concentrations of NPY and NKA were increased in the

hippocampus (Stenfors *et al*., 1992). Interestingly, the expression of peptide-processing enzymes was also increased (Bhat *et al*., 1993). These findings favour the view that electrical stimulation causes release of neuropeptides; the accelerated expression of mRNA for various neuropeptides may be viewed as a means to replenish the stores of these transmitters.

Neuropeptides from C-fibres play an important role in inflammation. C-fibres in the anterior uvea derive from neurones in the trigeminal ganglion (for review, see Håkanson & Wang, 1996). Our results suggest that these neurones are activated by ECT, leading to the release of transmitters from their terminals. In support of this view, ECT was found to be associated with a rise in the concentrations of SP, CGRP and PACAP in the aqueous humour. The ECT-evoked miosis was blocked by a tachykinin receptor antagonist, while all ECT-evoked ocular responses were inhibited by pretreatment with capsaicin, which

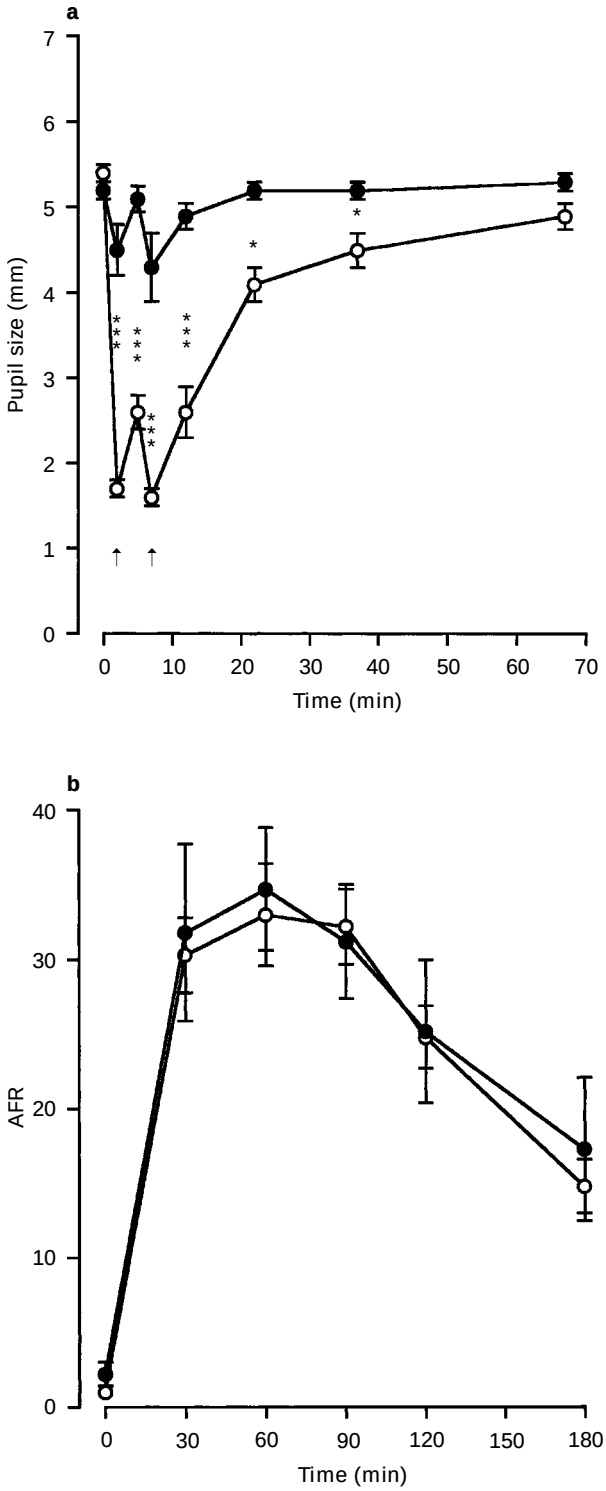

Figure 2 D-Pal^9 spantide II (90 nmol, intravitreal injection), a specific and potent tachykinin receptor antagonist, inhibited the miosis (a) induced by ECT (applied twice, at arrows), while leaving the AFR unaffected (b) (●). (○) Represent untreated eyes (controls). Means±s.e. of mean (vertical lines) of eight rabbits in each group, statistical difference when compared to controls (ECT alone) indicated by $*P<0.05$, and $***P<0.001$ at corresponding time points.

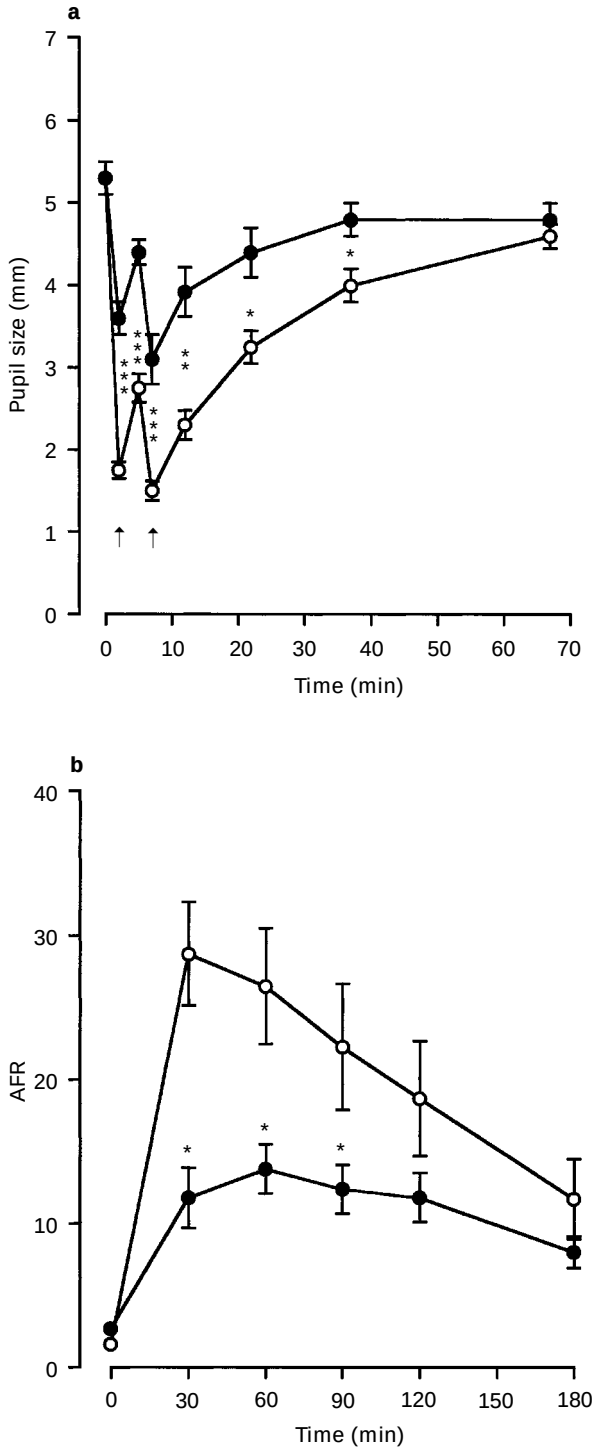

Figure 3 Capsaicin (0.5 ml of 1% solution, retrobulbar injection) pretreatment inhibited the ECT-evoked miosis (a) and AFR (b) (●). (○) Represent untreated eyes (controls). Means±s.e. of mean (vertical lines) of eight rabbits in each group, statistical difference when compared to control group indicated by $*P<0.05$, $**P<0.01$ and $***P<0.001$.

Table 1 Inhibitory effect of L-NAME on ECT-evoked release of neuropeptides into aqueous humour ($pmol\,l^{-1}$)

	Normal	*ECT*	*ECT + L-NAME*
SP	10.6 ± 1.4	65.0 ± 9.6[a]	13.2 ± 1.5[b]
CGRP	117.4 ± 12.4	1109.9 ± 133.1[a]	204.8 ± 33.5[b]
PACAP-27	ND	46.9 ± 8.4	ND
PACAP-38	ND	50.2 ± 5.4	ND

Means ± s.e. of mean of 12 samples. ECT: electroconvulsive treatment. ND: Not detectable. Normal means that the aqueous humour samples were collected from the eye of untreated control animals. [a]Significant difference ($P < 0.001$) when compared to normal and L-NAME-treated rabbits, respectively. [b]Significant difference ($P < 0.05$) when compared to normal rabbits.

is known to cause functional ablation of C-fibres (Holzer, 1991). Thus, ECT generates prompt inflammation in the eye (miosis occurs within seconds), probably through antidromic stimulation of ocular C-fibres (neurogenic inflammation). An alternative, but less likely, interpretation is that ECT causes release of mediators into the systemic circulation which evoke release of neuropeptides from C-fibres in the eye.

The effectiveness of capsaicin in depleting neuropeptides from C-fibres depends on age, strain and species (Holzer, 1991). The rabbit is less sensitive to capsaicin than the rat. Also, adult animals are more resistant to capsaicin than young (Holzer, 1991). Systemic treatment of adult rats reduces, but does not eliminate, the SP-immunoreactive nerve fibres in the eye (Terenghi *et al*., 1985; Moller *et al*., 1993). Thus, although capsaicin was found to mobilize neuropeptides from ocular C-fibres of the adult rabbit, it has not been possible to deplete neuropeptides from the uvea in this way (Wang & Håkanson, unpublished observations). Our previous studies have shown that exposure to capsaicin inhibits the contractions induced by other C-fibre excitants, such as bradykinin and resiniferatoxin, on the rabbit iris (Wang & Håkanson, 1992b), it could be shown by radioimmunoassay that approximately 70% SP and CGRP remained in the iris after exposure to capsaicin (100 μM) *in vitro* (Håkanson *et al*., 1987). Also, pretreatment with capsaicin reduced the ocular response to noxious stimulation (Bynke, 1983). It appears reasonable to assume that the reduced ocular response to ECT following capsaicin pretreatment reflects the partial depletion of neuropeptides from ocular C-fibres.

It is reasonable to assume that different C-fibre neuropeptides may play different roles in the ocular responses to noxious stimuli. Tachykinins are likely to be responsible for the ECT-evoked miosis. Since the ECT-evoked AFR was unaffected by treatment with the tachykinin receptor antagonist, tachykinins are less likely to play an important role in the breakdown of the blood-aqueous barrier. CGRP and PACAP are known to be quite powerful in this respect (Wahlestedt *et al*., 1986; Wang *et al*., 1995).

The inhibition of the ECT-evoked miosis by a tachykinin receptor antagonist is in line with our view that tachykinins are responsible for the miotic response to noxious stimuli (Håkanson & Wang, 1996). The rabbit iris is known to possess NK_1 and NK_3 but not NK_2 receptors (Hall *et al*., 1991; 1993; Wang & Håkanson, 1992a; 1993; Wang *et al*., 1994a, b). While both SP and NKA have been demonstrated in the rabbit iris, there is no convincing evidence for the presence of neurokinin B (Beding-Barnekow *et al*., 1988). Since the electrically evoked, noncholinergic and nonadrenergic contractions of the rabbit iris sphincter can be abolished by selective NK_1 receptor antagonists (Hall *et al*., 1993; Wang & Håkanson, 1992a; 1993; Wang *et al*., 1994a, b), it is unlikely that NK_3 receptors are involved in the C-fibre-evoked, tacahykinin-mediated contractions. Hence, we suggest that the receptor involved in ECT-evoked miosis belongs to the NK_1 type (Hall *et al*., 1991; 1993; Wang & Håkanson, 1992a; 1993; Wang *et al*., 1994a, b).

The demonstration of NOS-like immunoreactivity in neurones and fibres of the dorsal root ganglia (Morris *et al*., 1992) has stimulated the idea that NO may have a signalling function within the sensory nervous system. The present study demonstrates that L-NAME inhibits ocular responses to ECT, partly by inhibiting the release of neuropeptides from the C-fibres. This is in line with our previous findings, indicating that NO plays an important role in the ocular responses to a minor injury (infrared irradiation of iris) (Wang & Håkanson, 1995) and to intravitreal injection of endotoxin (Wang *et al*., 1996a) by activating C-fibres, causing release of C-fibre neuropeptides into the aqueous humour. NO is claimed to increase guanosine 3′:5′-cyclic monophosphate (cyclic GMP) formation in its target cells (Moncada *et al*., 1991). Whether the stimulating effect of NO on sensory neurotransmitter release reflects increased cyclic GMP levels remains to be clarified.

In summary, ECT evoked ocular responses that probably reflect the excitation of C-fibres in the anterior uvea. SP (and NKA) seem to contribute to ECT-evoked miosis but not to ECT-evoked aqueous flare and conjunctival hyperaemia. NO probably plays an important role in the ECT-evoked ocular inflammation, since ECT-evoked ocular responses (miosis, AFR, conjunctival hyperaemia) were inhibited by pretreatment with the NOS inhibitor L-NAME. From our previous findings, it seems that NO stimulates the release of C-fibre neuropeptides (Wang & Håkanson, 1995; Wang *et al*., 1996a). In addition, NO mediates the vascular effects of CGRP and partly, those of PACAP (Wang & Håkanson, 1995; Wang *et al*., 1996a).

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