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Innervation of the palpebral conjunctiva and the superior tarsal muscle in the cynomolgous monkey: a retrograde fluorescent tracing study

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ABSTRACT

Retrograde fluorescent transport of Fast Blue (FB) and Diamidino Yellow (DY) was used to study the localisation of neurons that innervate the palpebral conjunctiva and the superior tarsal muscle in the cynomolgous monkey. Labelled cell bodies of sensory neurons including a few double labelled cell bodies were found in the ophthalmic part of the ipsilateral trigeminal ganglion. Labelled cell bodies of the sympathetic neurons including a few double labelled cell bodies were located in the middle and cranial part of the ipsilateral superior cervical ganglion, with a few in the contralateral ganglion. Labelled cell bodies of the parasympathetic neurons were all found in the ipsilateral pterygopalatine ganglion and randomly distributed. Neurons were disposed in the ophthalmic part of the trigeminal and superior cervical ganglia, whereas parasympathetic neurons were distributed randomly. Cells of the nodose, ciliary, geniculate, otic and first 3 spinal ganglia were unlabelled. Tracing FB and DY from the palpebral conjunctiva and superior tarsal muscle respectively, revealed double labelled neurons in the trigeminal and superior cervical ganglia, probably indicating the presence of collaterals of axons serving both the palpebral conjunctiva and the superior tarsal muscle.

Key words: Trigeminal ganglion; superior cervical ganglion; pterygopalatine ganglion.

INTRODUCTION

Sensitivity of the eyelids and blinking play an important role in the protective system of the eye. The conjunctiva, a richly vascularised membrane, is responsible for the production of mucus, which is essential for tear film stability and has an enormous potential for combatting infection (Srinivason et al. 1982). A plexiform network of nerve fibres is present within the eyelids (Baljet et al. 1989; van der Werf & Baljet, 1989; Chung et al. 1996), composed of ophthalmic nerve branches, with the margin of the palpebral conjunctiva the most richly innervated (Duke-Elder & Wybar, 1961). In addition, the upper eyelid of the primate contains fine-meshed intrinsic nerve plexuses in the superior tarsal muscle (of Müller). A dense meshwork of nerve fibres interconnects the 2 plexuses and contains (histochemically) autonomic as well as sensory nerve fibres (Baljet et al. 1989; van der Werf & Baljet, 1989).

Recently we identified the ganglia involved in the innervation of the superior tarsal muscle of the cynomolgous monkey using the WGA-HRP retrograde tracing method, i.e. labelled neurons were found in the ipsilateral trigeminal, superior cervical and pterygopalatine ganglia (van der Werf et al. 1993). To our knowledge there exist no studies presenting a comparable account of sources of sensory and autonomic nerves innervating the palpebral conjunctiva in primates. In the human palpebral

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conjunctiva the presence of scant nerves has been described in the lamina propria (Munger & Halata, 1984) and sensory corpuscular nerve endings were detected in a narrow zone of the human limbal conjunctiva (Lawrenson & Ruskell, 1991). However, the origin of these nerve endings is unclear, although numerous acetylcholinesterase (AChE)-positive and catecholamine-positive nerves fibres were found in the stroma of the limbal and tarsal conjunctiva of the rabbit, and a few were localised in the fornix (Karjalainen et al. 1978). A preliminary tracing-immunohistochemical study of the conjunctiva in the rat revealed some labelled neuropeptide Y-positive somata in the pterygopalatine and superior cervical ganglia (Luthala et al. 1991). Superior cervical ganglionectomy in the rat caused a decrease of nerve fibres in the superior tarsal muscle (Smith et al. 1987) and, when coupled with pterygopalatine ganglionectomy, AChE-positive innervation of the muscle was virtually eliminated (Sharp & Smith, 1992). Superior cervical and pterygopalatine ganglionectomy in the cynomolgous monkey each revealed a partial degeneration of autonomic nerve fibres in the palpebral conjunctiva (Macintosh, 1974; Ruskell, 1985).

In the present study fluorescent retrograde tracers were used to identify the loci of neurons distributing fibres towards the injected eyelid structures. Moreover, in order to determine whether or not the palpebral conjunctiva and the superior tarsal muscle were served by collaterals of the same neuron, a Fast Blue and Diamidino Yellow double labelling tracing method was used.

MATERIALS AND METHODS

Five adult cynomolgous monkeys (*Macaca fascicularis*) of both sexes weighing between 3.2 and 8.0 kg, served as experimental subjects. Each animal was anaesthetised with a mixture of ketamine, xylazine (Rompun) and atropine in the proportion 10:1:0.1 mg/kg, respectively. In 1 monkey a small piece of Willospon soaked in a 2% Diamidino Yellow (DY) phosphate buffer solution, pH 7.4, implanted surgically into the superior tarsal muscle was performed and in 1 monkey 4 microinjections of a 0.5% phosphate buffer solution (pH 7.4) of Fast Blue (FB) were made (0.5 μl each) into the marginal part of the palpebral conjunctiva (ranging 2 mm from the lid margin) between the tarsal plate and the epithelial layer of the palpebral conjunctiva of the right orbit. In 3 monkeys 4 microinjections were made (0.5 μl each) of a 0.5% FB-solution into the marginal part of the palpebral conjunctiva, in a similar manner and a small piece of Willospon soaked in a 2% DY phosphate buffer solution implanted surgically into the superior tarsal muscle. The retrograde tracers FB and DY fluoresce at the same wavelength (340–380 nm), but label with contrasting colours different features of the cell. In neuronal somata FB is predictably located in the cytoplasm whereas DY is restricted to the nucleus of the neuron (Skirboll et al. 1989).

The animals were anaesthetised with ketamine (0.4 ml/kg) 3–4 d after surgery followed by 5000 units of thromboliquine/kg. Next the animals were killed by an overdose of pentobarbital and perfused directly through the internal carotid artery with 2 l phosphate-buffered saline, pH 7.4, at 37 °C, followed by 2 l 4% paraformaldehyde in 0.1 M phosphate buffer solution, pH 7.4. Immediately after perfusion the trigeminal, superior cervical, pterygopalatine, nodose, ciliary, geniculate, otic, and the first 3 spinal ganglia on both sides were dissected. In addition 25 μm serial cryosections of the ganglia, upper eyelid and cornea from both sides were mounted directly on chrome-alum-gelatin-coated slides and directly examined under a Leitz–Ploemopak fluorescent microscope with epi-illumination (excitation range 340–380 nm, excitation filter RKP 510).

Where appropriate camera lucida drawings were made of every 4th 25 μm cryosection of the ipsilateral trigeminal, superior cervical and pterygopalatine ganglia of monkeys 15, 211, 212, 735 and 1E in order to count labelled neurons, to measure the diameter of the labelled somata and to estimate the number and percentages of labelled somata in the ganglia innervating the palpebral conjunctiva and/or superior tarsal muscle. Only those neurons with a nucleus were counted and their diameter measured. Using this method, double counting of neurons can be avoided, although some neurons will be missed (van der Werf et al. 1996).

Cryosections of the upper eyelid were cut and examined to determine whether or not FB or DY had leaked from the implantation sites, and parts of the brainstem and the cornea were inspected to verify that superficial damage had not occurred.

The animal experiments were carried out with the approval of the ethical committee of The Royal Netherlands Academy of Arts and Sciences acting in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

RESULTS

After application of FB in the palpebral conjunctiva retrograde labelled neurons were found in the ipsilateral trigeminal, superior cervical and pterygopalatine ganglia and in the contralateral superior cervical ganglion. DY application into the superior tarsal muscle revealed retrograde labelled neurons in the same ganglia. The double labelling experiments revealed FB/DY-labelled neurons in the ipsilateral trigeminal and superior cervical ganglion (Table 1). In all experiments the ipsilateral nodose, ciliary, geniculate, otic, and 1st 3 spinal ganglia were devoid of labelled somata.

In cryosections of the upper eyelid small amounts of FB were found around some meibomian glands adjacent to the implants. Otherwise, spread of the tracers was not observed.

Trigeminal ganglion

FB-labelled cell bodies were found in the ipsilateral proximal ophthalmic part of the trigeminal ganglion (Table 1; Figs 1, 3*A*). The majority of the FB-labelled cell bodies (90%) were located in the ganglionic portion, the rest in the nerve. 50% of the FB-labelled cell bodies had a diameter < 40 µm, 40% were 40–60 µm in size and 10% had a diameter > 60 µm.

After application of DY in the superior tarsal muscle labelled cell bodies were distributed in the ophthalmic portion and to a greater extent in the

Table 1. *Counts of retrogradely labelled neurons, counted by nuclear profiles, after target injections with a fluorescent dye*

Monkey no.	Target	Dye	Trigeminal	Double labelled*	Superior cervical	Double labelled*	Pterygopalatine
212	Conjunctiva	FB	388	—	302	—	154
15	Conjunctiva	FB	364	18	253	36	137
	Superior tarsal	DY	132		108		18
735	Conjunctiva	FB	406	3	252	48	102
	Superior tarsal	DY	115		150		72
1E	Conjunctiva	FB	420	22	314	28	105
	Superior tarsal	DY	64		36		45
211	Superior tarsal	DY	157	—	189	—	32

* Number of cells FB/DY-labelled.

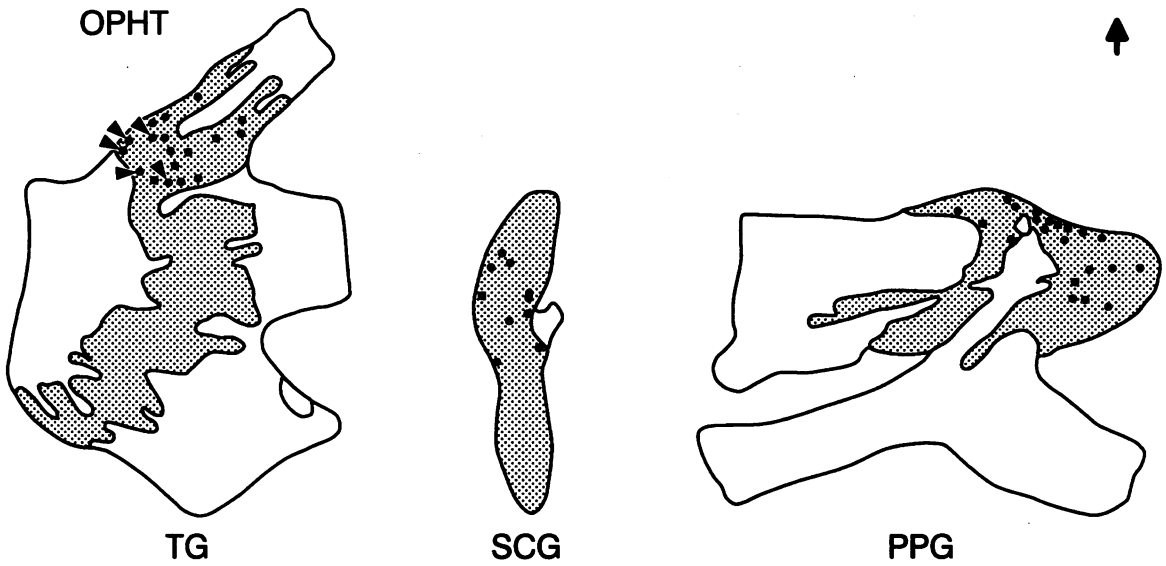

Fig. 1. Schematic drawings of sections illustrating the distribution of FB-labelled somata (dots) in the trigeminal (TG), the superior cervical (SCG) and the pterygopalatine ganglia (PPG). In TG, labelled somata are all located in the ophthalmic part (OPHT), with a concentration in the ganglionic part (arrowheads). In SCG, labelled somata are found in the cranial part of the ganglion. Most of the labelled somata in PPG are located in the superficial layer of the ganglion. (↑, upper.)

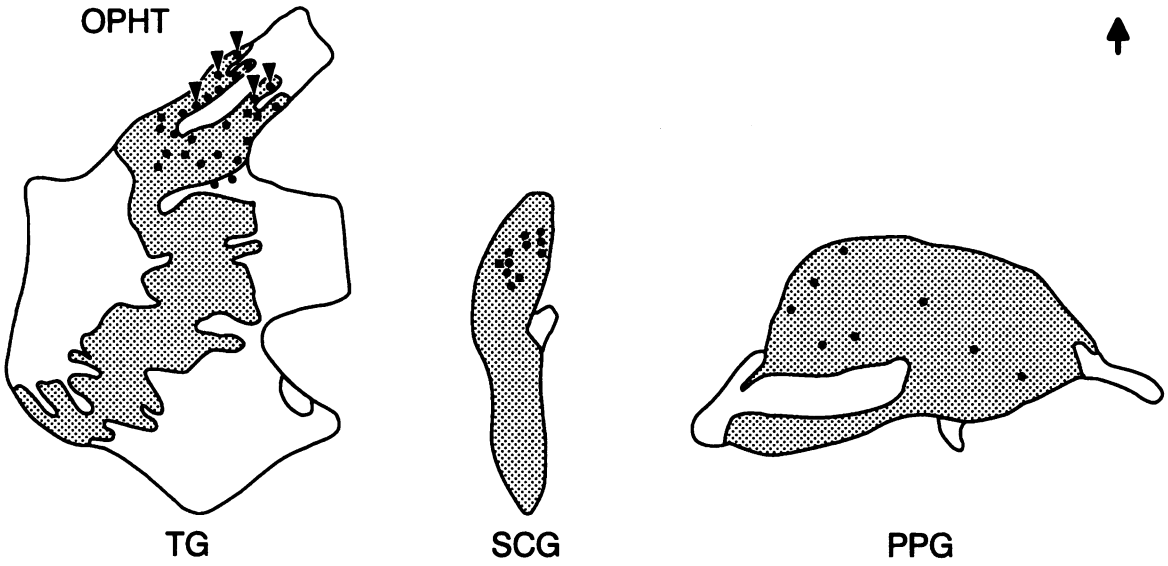

Fig. 2. Schematic drawings of sections illustrating the distribution of DY-labelled somata (dots) in the trigeminal (TG), superior cervical (SCG), and the pterygopalatine ganglia (PPG). In TG-labelled somata are all located in the ophthalmic portion (OPHT), with a concentration in the nerve (arrowheads). In SCG labelled somata are found in the upper cranial part of the ganglion. In PPG labelled somata are distributed randomly over the ganglion. (↑, upper.)

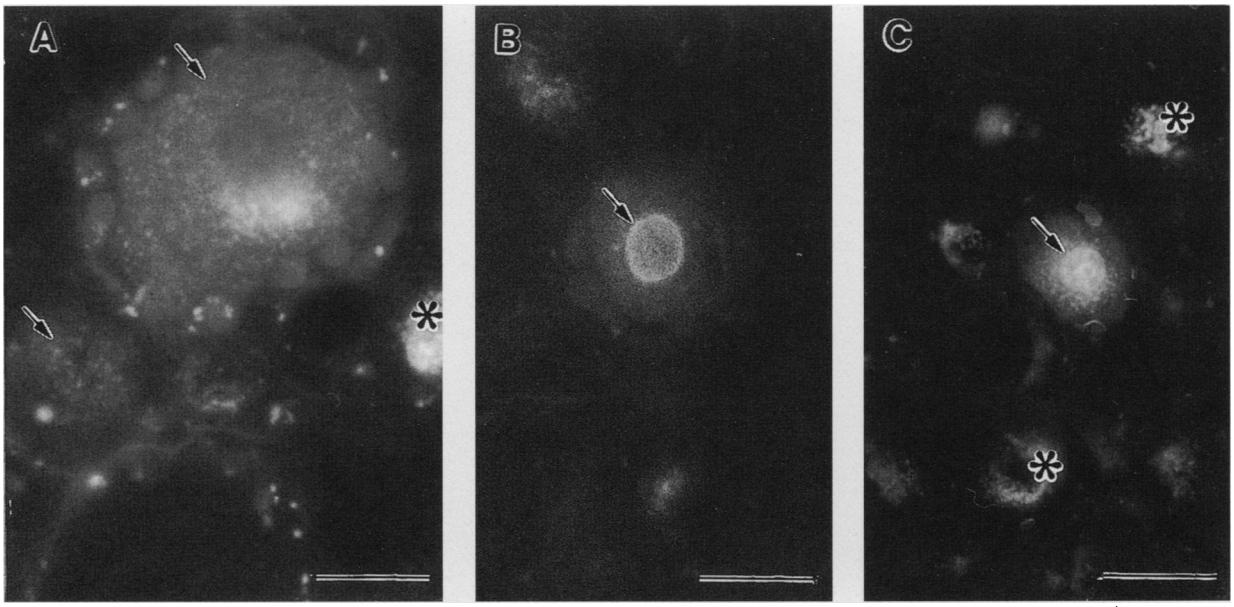

Fig. 3. Micrographs of the ophthalmic part of the trigeminal ganglion the cynomolgous monkey revealing fluorescent labelled somata after FB-injection into the palpebral conjunctiva and DY-implantation into the superior tarsal muscle. *A*, FB-labelled somata (arrows). *B*, DY-labelled nucleus (arrow) of a soma. *C*, FB/DY-labelled soma; DY is restricted to the nucleus (arrow). Autofluorescence of lipofuchsin is present in some somata (asterisks). Bar, 50 μm.

ophthalmic nerve part of the trigeminal ganglion (Table 1; Figs 2, 3*B*). 55% of the DY-labelled cell bodies had a diameter of < 40 μm, 40% were 40–60 μm in size and 5% had a diameter of 60 μm.

In animals in which FB was applied to the palpebral conjunctiva and DY to the superior tarsal muscle a small amount of the labelled cell bodies, all of them located in the ophthalmic part of the ganglion, were positive for FB as well as for DY (Table 1; Fig. 3*C*). The diameter of the cell bodies was < 40 μm.

Superior cervical ganglion

In the sympathetic superior cervical ganglion FB-labelled cell bodies were found distributed randomly in the cranial and middle parts of the ganglion (Table

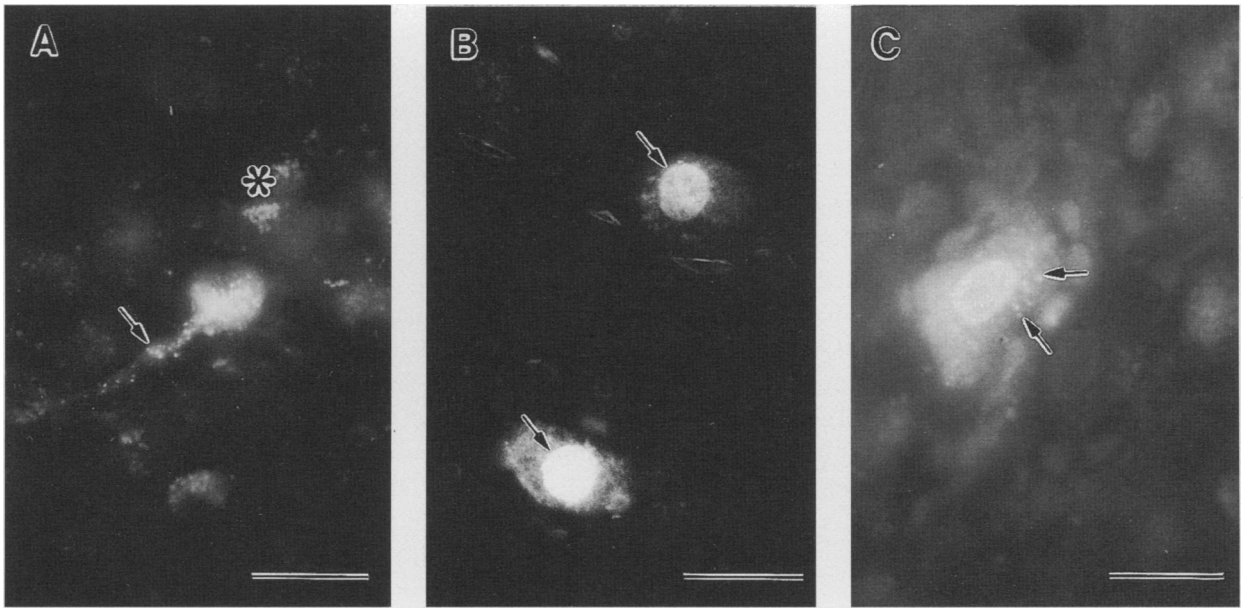

Fig. 4. Micrographs of the superior cervical ganglion of the cynomolgous monkey revealing fluorescent labelled somata after FB injection into the palpebral conjunctiva and DY-implantation into the superior tarsal or orbicularis oculi muscles. *A*, FB-labelled soma with an extension (arrow). *B*, Two DY-labelled nuclei of somata (arrows). *C*, FB/DY-labelled soma; DY is restricted to the nucleus (arrowhead) and FB is located in the cytoplasm, precipitated as fluorescent granules (arrows). Autofluorescence of lipofuchsin (asterisk). Bar, 50 μm.

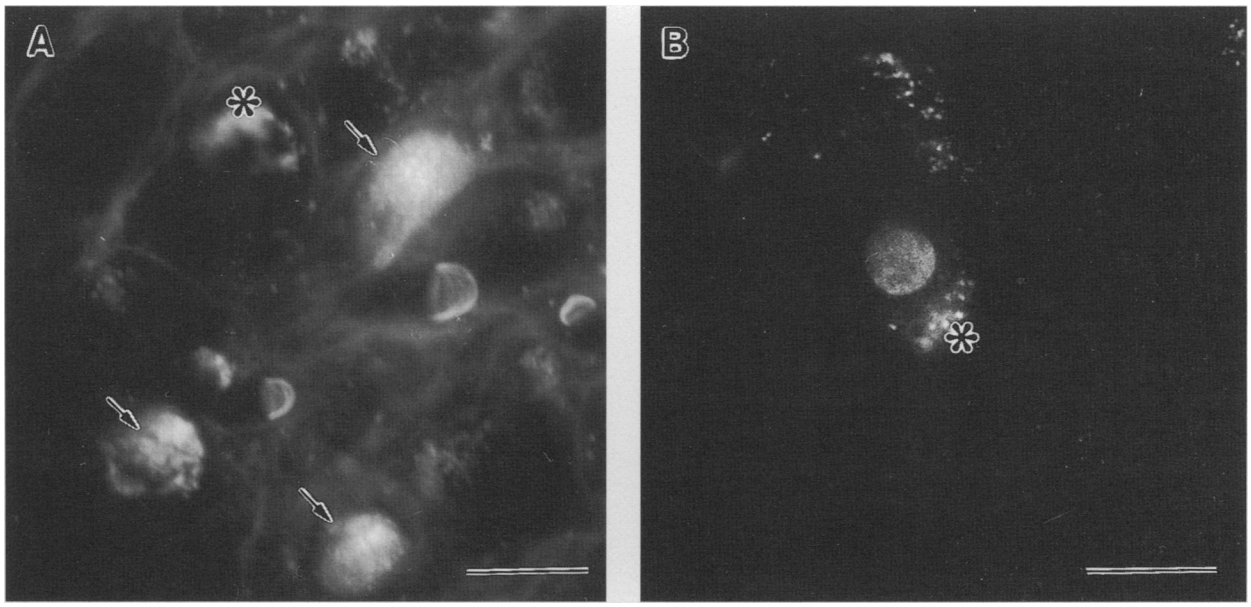

Fig. 5. Micrographs of sections of the pterygopalatine ganglion of the cynomolgous monkey revealing fluorescent labelled somata after FB-injection into the palpebral conjunctiva and DY-implantation into the superior tarsal muscle. *A*, FB-labelled somata (arrows). *B*, DY-labelled nucleus of a soma, autofluorescence of lipofuchsin (asterisks). Bar, 50 μm.

1; Figs 1, 4*A*). Approximately 70% of the FB-labelled cell bodies had a diameter < 33 μm, 30% had a diameter > 33 μm. Ten to 15 FB-labelled cell bodies are detected in the contralateral superior cervical ganglion, all with a diameter < 33 μm. After application of DY in the superior tarsal muscle most of the labelled cell bodies were disposed over the cranial part of the superior cervical ganglion, the rest over the middle part (Table 1; Figs 2, 4*B*). Fifty per cent of the DY-labelled cell bodies have a diameter < 33 μm, 50% have a diameter > 33 μm.

In animals in which FB was applied to the palpebral conjunctiva and DY to the superior tarsal muscle a substantial number of the labelled cell bodies, all

located in the cranial part of the ganglion, were positive for FB as well as the DY (Table 1; Figs. 4*C*) with a diameter > 33 μm.

Pterygopalatine ganglion

In the parasympathetic pterygopalatine ganglion FB-labelled cell bodies were distributed over the ganglion with concentration in its superficial part (Table 1; Figs 1, 5*A*) all having a diameter < 40 μm. After application of DY in the superior tarsal muscle, labelled cell bodies with a diameter < 40 μm were disposed randomly throughout the ganglion (Table 1; Fig. 5*B*). Contralateral or double labelled cell bodies were not found in the pterygopalatine ganglion.

DISCUSSION

The results of this study provide information on the localisation of neurons that innervate the palpebral conjunctiva and the superior tarsal muscle. Sensory (afferent), sympathetic (efferent) and parasympathetic (efferent) innervation of the palpebral conjunctiva and superior tarsal muscle was determined ipsilaterally. In both experiments afferent innervation was restricted to the ophthalmic part of the trigeminal ganglion; efferent innervation was restricted to the superior cervical and pterygopalatine ganglia.

The identification of double-labelled neurons in the ipsilateral trigeminal and superior cervical ganglia suggests that the 2 structures are probably served by collaterals of single neurons. Although microleakage of the tracer can never be excluded, examination of cryosections of the upper eyelid including the injection/implantation area did not reveal the presence of the tracers in adjacent structures. Moreover, double-labelled somata were absent in the pterygopalatine ganglion and in the brainstem FB or DY-labelled motoneurons of the nucleus of the 7th nerve subserving the orbicularis oculi muscle were not observed. Furthermore, the tracers FB and DY have the same survival time, their fluorescent properties are different (Kuypers et al. 1980; Payne, 1987). The transport of FB over long distances is more effective than DY. This might explain the presence of more FB than DY-labelled neurons in the ganglia registered.

Neuroanatomical studies of human eyelids showed that afferent nerve fibres in the upper eyelid have their origin in the ophthalmic part of the trigeminal ganglion and that they partly pass through the superior tarsal muscle, or close to the surface of this muscle (Munger & Halata, 1984). This indicates that uptake of the tracer by 'en passage' nerve fibres is possible and consequently, the nerve counts for the superior tarsal muscle may be too high. With regard to the conjunctival injections, the possibility of 'en passage' labelling appears improbable, because the location of these injections was remote from the paths of the cutaneous ophthalmic branches.

Trigeminal ganglion

In the ophthalmic part of the trigeminal ganglion the number of FB-labelled neurons appeared high, bearing in mind the low touch-sensitivity of the palpebral conjunctiva (McGowan et al. 1994) and the relative infrequency of sensory terminals in this structure (Macintosh, 1974). In experiments in monkeys following injections of the central area of the highly sensitive cornea, substantially fewer labelled neurons were found in the trigeminal ganglion (Marfurt & Echtenkamp, 1988). However, application of a retrograde tracer in the cornea is a potentially poor method for optimal labelling.

The somatotopic arrangement of neurons innervating the palpebral conjunctiva in monkeys is in agreement with results in cats; retrograde tracing of the supraorbital nerve in the cat revealed labelled afferent somata in the anteromedial region of the ophthalmic ganglionic part of the trigeminal ganglion (Marfurt, 1981). The distribution of cell bodies in the trigeminal ganglion innervating the superior tarsal muscle confirms the results of previous studies in monkeys (Van der Werf et al. 1993) and rats (Smith et al. 1987; Luthala et al. 1991; Luthala & Uusitalo, 1991).

Superior cervical ganglion

The present study confirms the numerous existing accounts of a sympathetic innervation of the conjunctiva (Macintosh, 1974; Ruskell, 1985; Luthala et al. 1992). Others have identified adrenergic nerve fibres in the palpebral conjunctiva of the rabbit using the Falck–Hillarp technique (Karjalainen et al. 1978). The present study shows that neurons innervating the palpebral conjunctiva are distributed over the cranial–middle region of the ipsilateral superior cervical ganglion. A remarkable 70% of FB-labelled neurons innervating the palpebral conjunctiva were small in size (< 33 μm). Neurons < 33 μm should provide amongst others vasomotor fibres as is indirectly supported by electrophysiological evidence (Eccles, 1935).

The finding that the superior tarsal muscle is innervated by the superior cervical ganglion is in

accordance with previous studies in the monkey (Van der Werf et al. 1993) and the rat (Flett & Bell, 1991; Sharp & Smith, 1992; Smith et al. 1987).

Pterygopalatine ganglion

Detection of labelled cell bodies in the pterygopalatine ganglion after FB injections into the palpebral conjunctiva is indicative of a parasympathetic innervation and supports the results of earlier studies employing different techniques in monkeys. In cynomolgous monkeys, nerve fibres and endings of pterygopalatine origin were found in vessel walls and the subepithelium of the palpebral conjunctiva (Macintosh, 1974; Ruskell, 1985). In rabbits, unilateral damage to the pterygopalatine ganglion reduced vasoactive intestinal polypeptide-like immunoreactivity in the conjunctiva (Butler et al. 1984). In a preliminary study a parasympathetic innervation of the rat conjunctiva of pterygopalatine ganglion origin was demonstrated with combined fluorescent tracing and immunochemical techniques (Luthala et al. 1992). Some doubts attend the conclusion that the pterygopalatine ganglion serves the palpebral conjunctiva because of the slight leakage of RF to the adjacent meibomian gland which might be a recipient of pterygopalatine ganglion axons. A large parasympathetic innervation of the gland in monkeys has been suggested (Chung et al. 1996).

Regarding the innervation of the superior tarsal muscle by pterygopalatine neurons, the present results confirm our earlier work (Van der Werf et al. 1993) and is consistent with those obtained in rats (Luthala et al. 1992: Sharp & Smith, 1992). Presumably, the parasympathetic innervation of the tarsal muscle is opposed to the sympathetic supply and serves to relax the muscle.

Double labelling

The identification of double-labelled neurons is of particular interest, as it represents evidence of a broad distribution of axon collaterals. For double-labelled neurons in the trigeminal and superior cervical ganglia, the possibility of DY-uptake by FB-labelled axons from the palpebral conjunctiva injection site cannot be excluded, as tracer uptake 'en passage' can occur if axons are damaged at the injection site (Payne, 1987). However, similar results were obtained in rats where collaterals serving the masseter muscle and periodontal ligaments were identified (Zhang et al. 1992). Subsequently, autonomic double-labelled neurons indicating the presence of collaterals of axons were described in the enteric system in the rat (Groen et al. 1985). The failure of Flett & Bell (1991) to demonstrate double labelling of superior cervical neurons in rats, using the anterior chamber and the superior tarsal muscle or the nictitating membrane as targets, is probably attributable to the functional independence of these structures. In other studies a decreasing number of double-labelled neurons was found when comparing neonatal and adult animals, which suggests double labelling to be a transient feature associated with development (Vidovic & Hill, 1988). Our results indicate that double-labelling persists in the adult animals, indicating a functional link of the 2 structures in the eyelid.

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