

# Acetylcholinesterase-positive and paraformaldehyde-induced-fluorescence-positive innervation in the upper eyelid of the sheep (*Ovis aries*)

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**Summary.** This is the first study which describes the innervation of some eyelid structures, such as the glands of Moll and the glands of Zeiss. It is also the first to investigate the innervation pattern of the eyelid as a whole. We have studied the acetylcholinesterase-positive and paraformaldehyde-induced-fluorescence-positive (FIF+) innervation pattern of the different structures that constitute the upper eyelid of the sheep. There is widespread acetylcholinesterase-positive innervation in the epithelium, but not such an abundant FIF+ innervation. Both types of innervation are represented in the connective tissue by trunks or fibers that are distributed towards the different structures immersed within them. In the glands of Zeiss, cholinesterase-positive innervation is much more widespread than FIF+ innervation. On the contrary, the glands of Moll present denser FIF+ innervation than acetylcholinesterase-positive innervation. The Meibomian glands and the lachrymal glands show a rich acetylcholinesterase-positive and FIF+ innervation. Eyelid muscle innervation is mainly acetylcholinesterase-positive. In the conjunctive membrane there is no acetylcholinesterase-positive innervation, and only scarce FIF+ fibers can be demonstrated.

**Key words:** Innervation, Acetylcholinesterase, Catecholaminergic, S-100 protein, Eyelid

## Introduction

Several descriptions have been published regarding the innervation of specific structures of the eyelid in several species of mammals such as the Guinea pig, cat, rabbit and monkey (Nikkinen et al., 1984; Hiramoto et al., 1995; Chung et al., 1996; Kirch et al., 1996; Seifert and Spitznas, 1996; Van der Werf et al., 1997), although there has been only one in sheep (Yamanohi and

Burnstock, 1967), exclusively focused on the innervation of the lachrymal gland. There are also several electrophysiological studies (Krizsan-Agbas et al., 1998; Beuregard and Smith, 1994). Most of the morphological studies were carried out by means of optic microscopy, using histochemical and immunohistochemical techniques to study catecholaminergic and peptidergic innervation. There are also some reports with electron microscopy (Hiramoto et al., 1995; Chung et al., 1996; Kirch et al., 1996; Seifert and Spitznas, 1996). However, exhaustive knowledge of the innervation pattern of the different components of the upper eyelid in mammals is still needed.

The sympathetic and parasympathetic innervation of the lachrymal gland has been investigated (Arenson and Wilson, 1970, 1971; Botelho et al., 1973). Some electrophysiological and pharmacological studies have also been performed on the neurological regulation of the lachrymal flow (Botelho et al., 1976; Aberg et al., 1979). During the 80s there were more reports on lachrymal gland innervation and physiology (Pholpramood and Tangkrisanaviront, 1983; Tangkrisanaviront, 1984; Bomberg et al., 1989). Mircheff (1989) reported that the rate of lachrymal gland fluid secretion was principally controlled by parasympathetic innervation, and was modulated by sympathetic innervation (Botelho et al., 1966). Walcott et al. (1989) described the presence of an extensive acetylcholinesterase-positive network throughout the lachrymal gland. Powell and Martin (1989) studied the distribution of cholinergic and adrenergic nerve fibers in the lachrymal gland in dogs, finding a loose network of adrenergic nerves throughout the interstitium around acini and blood vessels and vessel walls. Dartt et al. (1985) demonstrated that cholinergic agonists stimulate lachrymal gland protein secretion primarily by mobilizing  $Ca^{2+}$  from intracellular stores.

The relationship among sympathetic, parasympathetic and sensorial innervation of the anterior eye segment has been studied by Ten-Tusscher et al. (1989) by means of ( $^3H$ )leucine injection into the superior cervical ganglion. They found bundles of sympathetic

nerves in the trigeminal ganglion and the pterygopalatine ganglion. Ten-Tusscher et al. (1994) later found that somatic afferents of trigeminal origin synaptically innervate parasympathetic neurons in the pterygopalatine ganglion, and that sympathetic nerve fibers originating in the superior cervical ganglion course through the trigeminal and pterygopalatine ganglion without forming direct synaptic contacts.

Based on previous studies (Manson et al., 1986; Porter et al., 1989) new articles have furthered our knowledge of eyelid muscle innervation (Van der Werf et al., 1993, 1996; Simons and Smith, 1994; Kirch et al., 1996; Seifert and Spitznas, 1996; Van Ham and Yeo, 1996; Krizsan-Agbas et al., 1998). There has also been a study on the upper tarsal muscle and conjunctive membrane of the monkey (Van der Werf et al., 1996). Van der Werf et al. (1993) described the parasympathetic innervation of Muller's muscle by means of retrograde tracing studies. They also studied the localisation of neurons that innervate the superior tarsal muscle in the cynomolgous monkey (Van der Werf et al., 1996) by means of retrograde fluorescent transport of Fast Blue (FB) and Diamino Yellow (DY). Van Ham and Yeo (1996) investigated the rabbit trigeminal inputs to eye-blink motoneurons, including the distribution of motoneurons in the facial nucleus and motoneurons in the oculomotor nucleus by retrograde tracing of wheat germ-agglutinated horseradish peroxidase injected into the appropriate muscles. Later (Kirch et al., 1996), the localization of the motoneurons innervating the levator palpebral superioris muscle was described in the oculomotor nucleus (called the central caudal nucleus) after double fluorescent neuronal retrograde tracing, using FB and DY.

Krizsan-Agbas et al. (1998) studied the presynaptic adrenergic facilitation of parasympathetic neurotransmission in sympathectomized rat smooth muscle.

This study examined the relative contributions of cholinergic and adrenergic mechanisms mediating these contractions by means of electrophysiology, which occurs prior to the neuroeffector junction formation as determined previously by electron microscopy. Smith and Marzban (1998) described that intact eyelid smooth muscle varicosities are predominantly sympathetic, but a small number of parasympathetic varicosities are present, some of which may form pre-junctional synapses with sympathetic nerves. In addition, Beauregard and Smith (1996), described that acetylcholinesterase inhibition results in conversion of orbital parasympathetic nerve function from inhibition of sympathetic neurotransmission to smooth muscle excitation, as a result of cholinergic activation of excitatory nicotinic receptors on sympathetic varicosities, which elicit the release of noradrenaline.

In addition to some interesting articles on the Meibomian gland Chung et al. (1996) studied the peptidergic innervation of the Meibomian gland. Fan and Smith, (1993) described the VIP immunoreactivity of parasympathetic neurons following long-term

sympathectomy. Other authors observed the sensory and autonomic innervation of the Meibomian gland in rats (Simons and Smith, 1994).

We have not found any study on the innervation of other eyelid structures such as hair follicles, the glands of Zeiss or the glands of Moll. These glands are of functional importance. The glands of Zeiss are considered sebaceous and the glands of Moll are apocrine sweat glands, with a role that is associated to the hair follicle. There are no studies in the literature describing the global innervation pattern of upper eyelid structures in any species.

The aim of the present study was to study the innervation pattern of the AChE-positive and paraformaldehyde-induced-fluorescence-positive (FIF) nervous elements that constitute the upper eyelid (epithelium, hair follicle, connective tissue, muscles, the lachrymal gland, the Meibomian gland, the glands of Zeiss, the glands of Moll and conjunctive membrane) of the sheep (*Ovis aries*).

## Materials and methods

We used 30 upper eyelids of adult *Ovis aries*. From each upper eyelid, two samples of the internal, medium and external zones were obtained. For the acetylcholinesterase and the catecholamine methods, the samples were placed on cork slices and frozen in methylbutane by immersion in liquid nitrogen. Later, 15- $\mu$ m sections were cut with a SLEE cryostat (model 4R). The sections were placed on slides and air-dried at room temperature for 30 min. These preparations were treated in parallel by means of the AChE and catecholamine methods.

### Acetylcholinesterase (AChE) method

Method described by El Badawi and Schenk (1967) but slightly modified (Aisa et al., 1997). The frozen samples were incubated for a 2-hour period at 37 °C in the medium described by El Badawi and Schenk (1967), with acetylthiocholine iodide as a substrate which reacts before acetylcholine. The cholinesterase activity sites were recognized as dark-brown precipitates. Two controls were made by incubating samples in a substrate-free medium, and by incubating them in a medium with tetraisopropylpyrophosphoramidate (ISOOMPA, Sigma) to control specificity.

### Catecholamine method

To determine the catecholaminergic innervation of the eyelid we used a method which combines the glyoxilic acid method described by Furness and Costa (1975) and the Falck-Owman paraformaldehyde method (Falck and Owman, 1965). Frozen sections of tissue were immersed in 2% glyoxilic acid in PBS (pH 7.1) for 1 hour at 4 °C (Furness and Costa, 1975). They were dried on slides at room temperature and were later

exposed to humidified paraformaldehyde vapours in a hermetic vessel at 80 °C for 1 hour. Noradrenaline produces a characteristic apple-green fluorescence under an ultraviolet light optic microscope when the slide has been exposed for 1 hour to the paraformaldehyde vapours.

#### *Peroxidase anti-peroxidase method*

Sections from paraffin blocks were immersed for 5 min in xylene and later rehydrated by immersion in decreasing concentrations of ethanol, from 100% to 0% in distilled water. To block the endogenous peroxidase, the sections were kept in 0.3% hydrogen peroxide in PBS for 30 min, and later washed in PBS. To perform the background blocking, they were kept for 30 min in humid chambers, and drops of normal goat serum 1/30 were added to the sections. Serum surplus was drawn off with a pipette, and drops of the primary antiserum solution were added: S-100 1/50 rabbit polyclonal antibody (DAKO Z0311) and were kept for 1 hour at room temperature. After rinsing in PBS three times for 5 min each rinse, the sections were incubated with the secondary antiserum: conjugated goat anti-rabbit 1/10 (VECTOR, ABC kit peroxidase IgG rabbit) for 30 min, and later rinsed three times in PBS for 5 min each rinse. This extra layer is necessary, as the PAP is a rabbit globulin. The sections were exposed for 30 min to PAP 1/50, rinsed in PBS two times for 5 min each rinse, and finally rinsed once in Tris buffer 0.05M pH 7.6. Later, the sections were incubated in DAB solution: 50 mg DAB/100 cm<sup>3</sup> (DAKO DAB-K3468). Finally, the sections were incubated in 60 µL/100 ml of Tris buffer of 30 vols hydrogen peroxide. The reaction was controlled by microscopy, as background could be too low to be seen macroscopically. Negative controls for these sections were created by omitting the primary antibody.

## **Results**

### *Acetylcholinesterase-positive cells*

In the flat stratified epithelium of the eyelid edge we found a widespread acetylcholinesterase-positive cells. We observed acetylcholinesterase-positive nervous fibers (Fig. 1A) as well as acetylcholinesterase-positive cells (Fig. 1B). These cells showed prolongations in a variety of numbers. Some were bipolar and locate in the basal layer of the epithelium (Fig. 1B). Others were multipolar and occupied the intermediate layers of the epithelium.

In the epithelium, the results obtained from the AChE were compared with those obtained by means of S-100 protein immunoreactivity. Initially, we expected that all of the acetylcholinesterase-positive dendritic cells found in the epithelium would be Langerhans' cells or melanocytes. However, we found that only a small number of these cells were S-100 protein immunoreactive (Fig. 1C, 1D).

### *Acetylcholinesterase-positive innervation*

In the connective tissue we observed prevalent acetylcholinesterase-positive nervous fibers (Fig. 2A). These fibers reached hair follicles, surrounded the dermis layer (Fig. 2B) and established close contact with the peripheral cells (Fig. 2C). Blood vessels in the connective tissue were also acetylcholinesterase-positive. We observed arterioles innervated by thin acetylcholinesterase-positive fibers (Fig. 2D) and venules with an extensive net of thin acetylcholinesterase-positive nervous fibers (Fig. 2E). As well as hair follicles, the glands of Zeiss and the glands of Moll could be seen (Fig. 2F).

The sebaceous glands of Zeiss, which open into the hair follicles, showed a rich cholinesterase-positive innervation, which was constituted by fibers distributed through the connective tissue between the alveoli walls (Fig. 2B,C,F,G), with a network-like distribution. The sweat glands of Moll draining into the follicles, were apocrine type, and appeared with almost no acetylcholinesterase-positive innervation (Fig. 2F). In the panoramic cuts (Fig. 2F), where the glands of Zeiss and the glands of Moll can be seen at the same time, the rich acetylcholinesterase-positive innervation of the glands of Zeiss contrasted with the scarce or non-existent acetylcholinesterase-positive innervation in the glands of Moll, despite acetylcholinesterase-positive nervous trunks distributed near them.

The Meibomian glands appeared with a rich acetylcholinesterase-positive innervation that was composed of thick nervous trunks distributed along the connective tissue among the acini to branch into other thinner trunks among the gland cells (Fig. 2H). The appearance of these networks was similar to that observed in the sebaceous glands of Zeiss.

The lachrymal gland showed a rich acetylcholinesterase-positive innervation constituted by widespread thick nervous fibers in the connective tissue that separated the gland acini (Fig. 2I) and were distributed among tubules (Fig. 2J). Acetylcholinesterase-positive innervation of this gland, in comparison to the glands of Zeiss or the Meibomian glands, was more concentrated and less widespread. However, acetylcholinesterase-positive cell bodies, whose prolongations connected with the tubule walls could be seen in the connective tissue beside the nervous trunks.

Acetylcholinesterase-positive nervous fibers were well represented in the bundles of striated muscle that constitute the palpebral portion of the orbicular muscle of the eye, parallel to the muscle fibers (Fig. 2K). At greater microscopic detail, motor plaques could be seen in some muscle bundles (Fig. 2L,M). We did not find any acetylcholinesterase-positive reaction in the conjunctive mucous (Fig. 2N). Neither were acetylcholinesterase-positive fibers observed reaching the basal layer of the caliciform secretory cells.

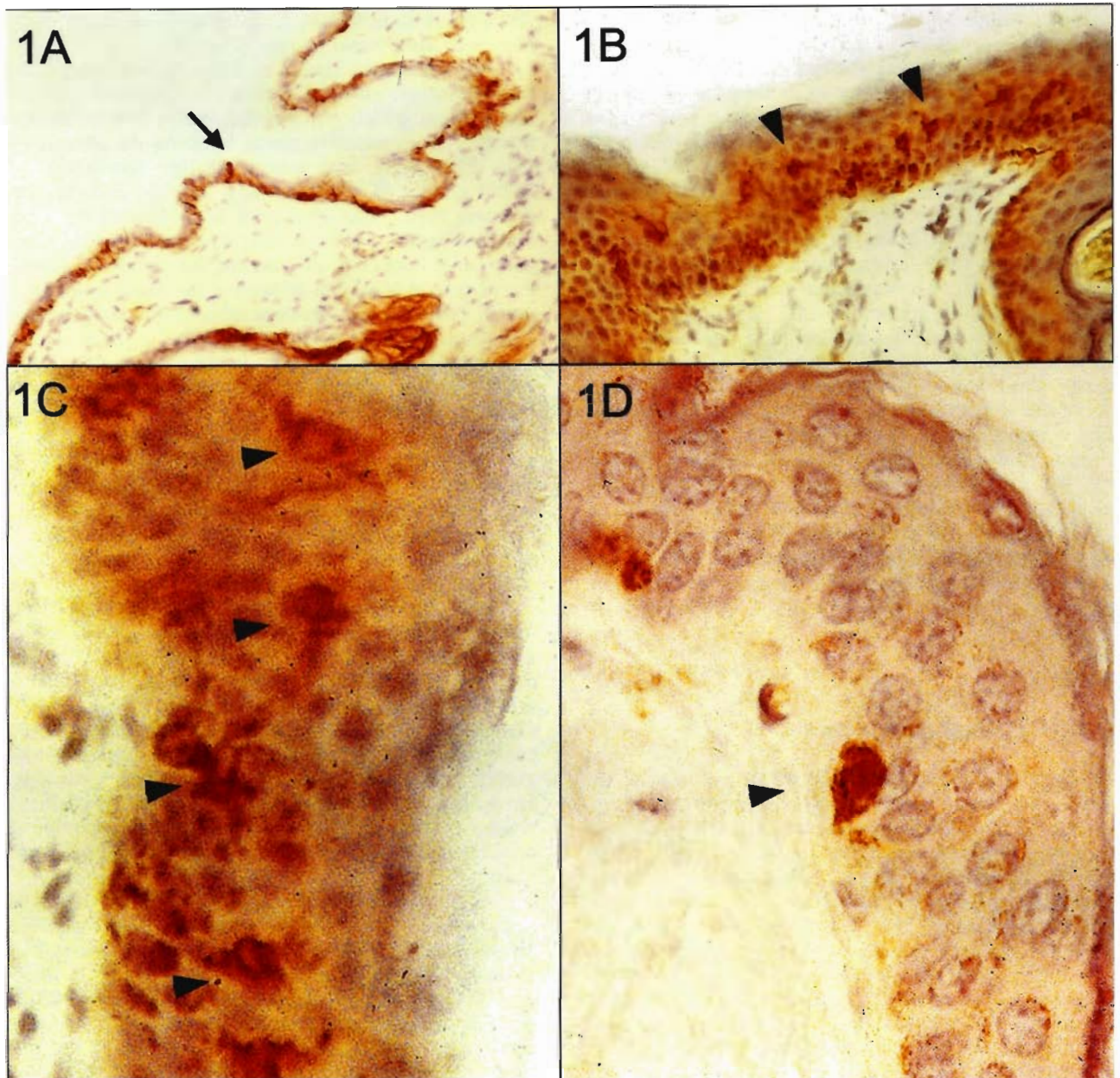
### Catecholaminergic innervation

Fluorescent noradrenergic nervous fibers (FIF+) innervated the plane stratified epithelium of the eyelid edge, predominantly in the basal lamina and were not so well distributed as the acetylcholinesterase-positive fibers (Fig. 3A).

In the connective tissue, some thick noradrenergic nervous trunks (Fig. 3B,C) could be observed, as well as thin scattered FIF+ nervous fibers, sometimes

surrounding the gland ducts (Fig. 3C). These fibers were also found around the base of hair follicles and reached the inner root sheath (Fig. 3D-F). FIF+ fibers were distributed throughout the arteriolar adventitia but did not enter the muscle wall (Fig. 3G).

In the glands of Zeiss, thin and scarce FIF+ fibers were observed, distributed in the connective septum around the glandular alveoli (Fig. 3H). This scarce innervation contrasts with the abundance of acetylcholinesterase-positive nervous fibers in these



**Fig. 1.** Acetylcholinesterase-positive cells. **A.** Epithelium of the eyelid edge. Nervous fibers (arrow). x 60. **B.** Plane stratified epithelium of the eyelid edge. Cell bodies (arrowhead). x 240. **C.** Acetylcholinesterase-positive cells in the epithelium base (arrowhead) x 780. **D.** S-100-positive cell in the epithelium base (arrowhead). x 780

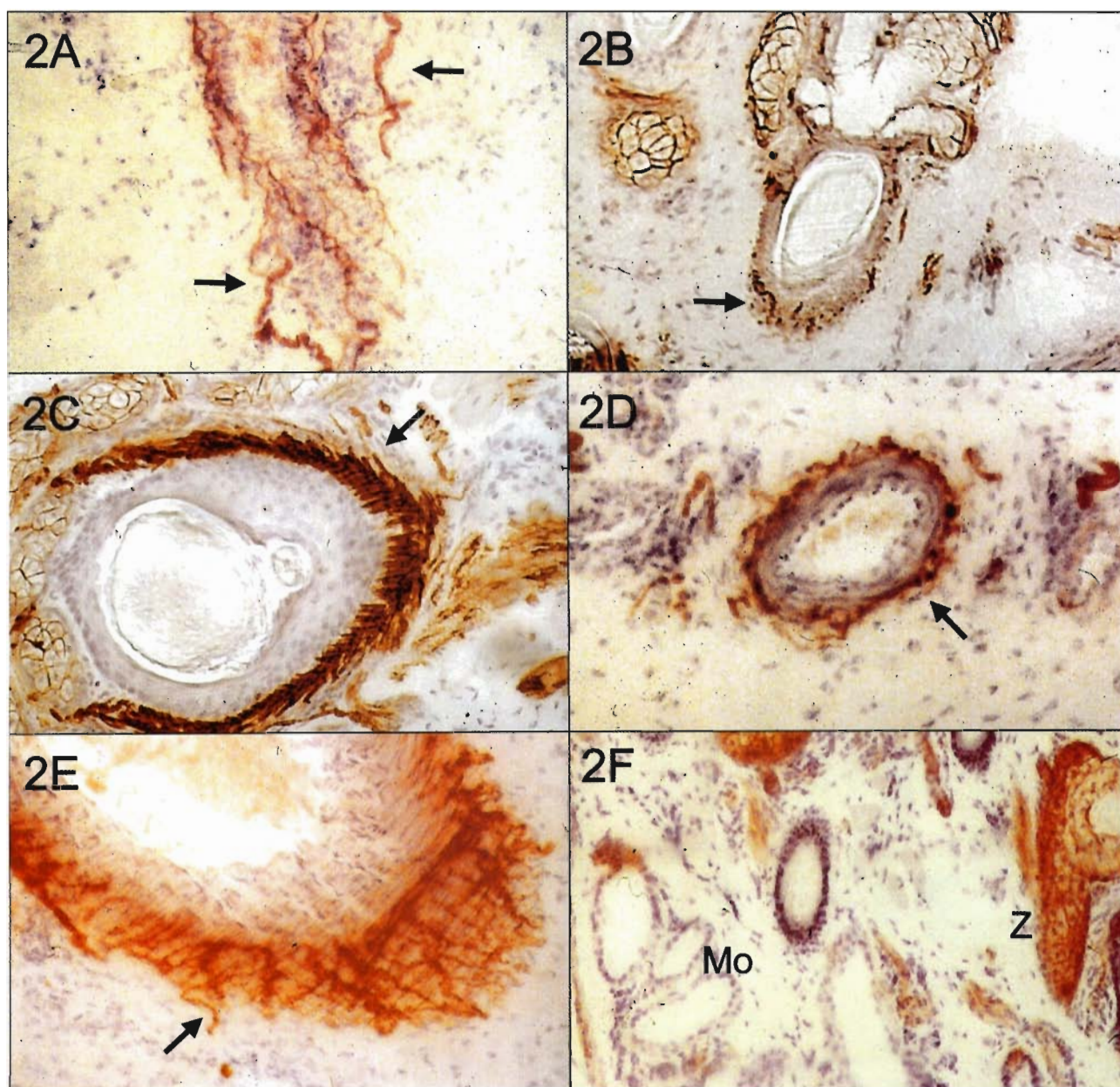
## Innervation in the eyelid

glands (Fig. 2B,F,2G). The glands of Moll, which were located beside hair follicles, showed a widespread noradrenergic innervation (Fig. 3I,J), in contrast with the scarce, if present, acetylcholinesterase-positive innervation (Fig. 2F). In the Meibomian glands noradrenergic innervation (Fig. 3K) was as abundant as acetylcholinesterase-positive innervation (Fig. 2H).

In the lachrymal glands thick FIF+ nervous trunks

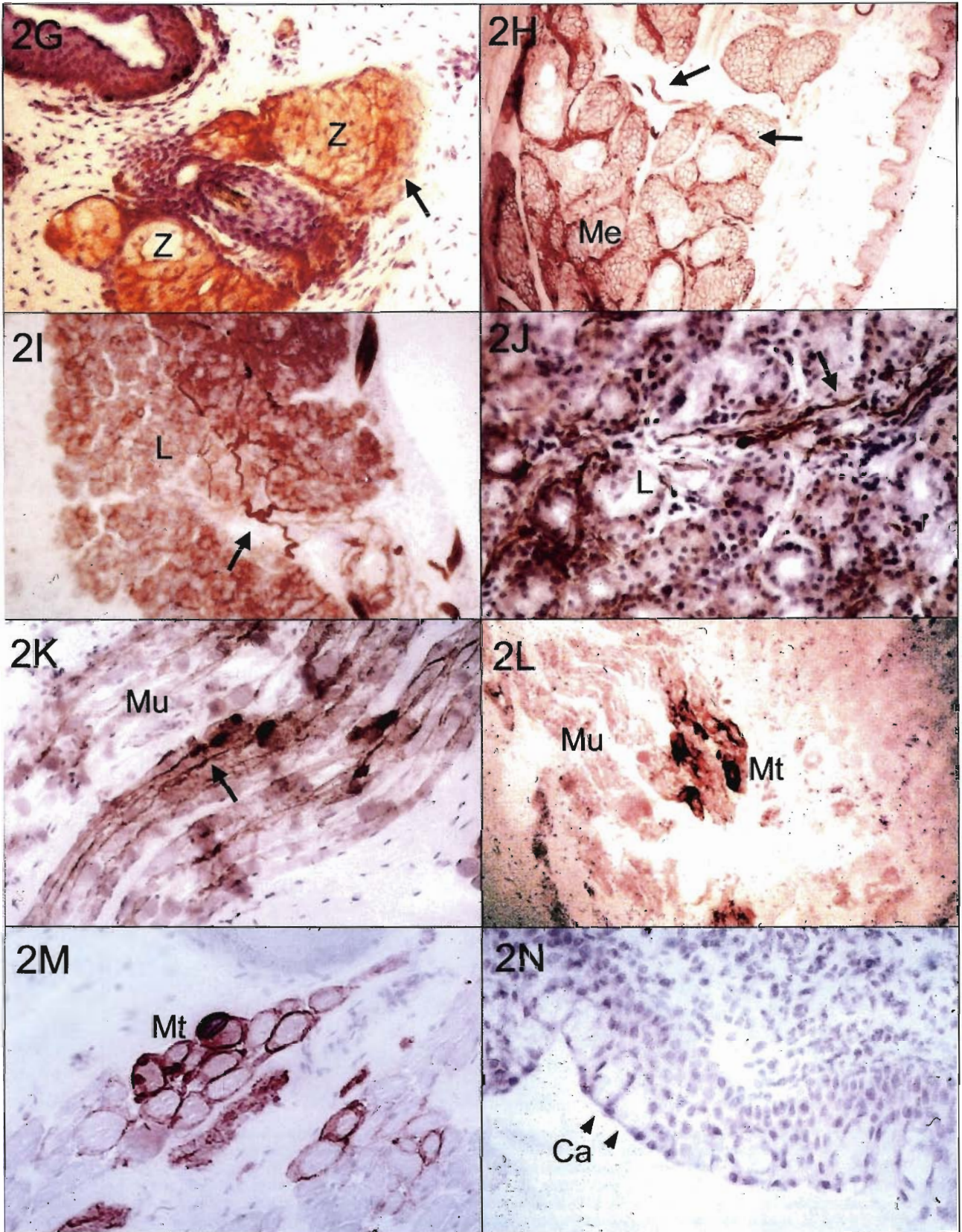
were observed in the nearby connective tissue, surrounding the gland duct (Fig. 3L) and around blood vessels (Fig. 3M). These nervous trunks branch into thinner fibers that surround thick gland ducts (Fig. 3N) and distribute along the tubules (Fig. 3O).

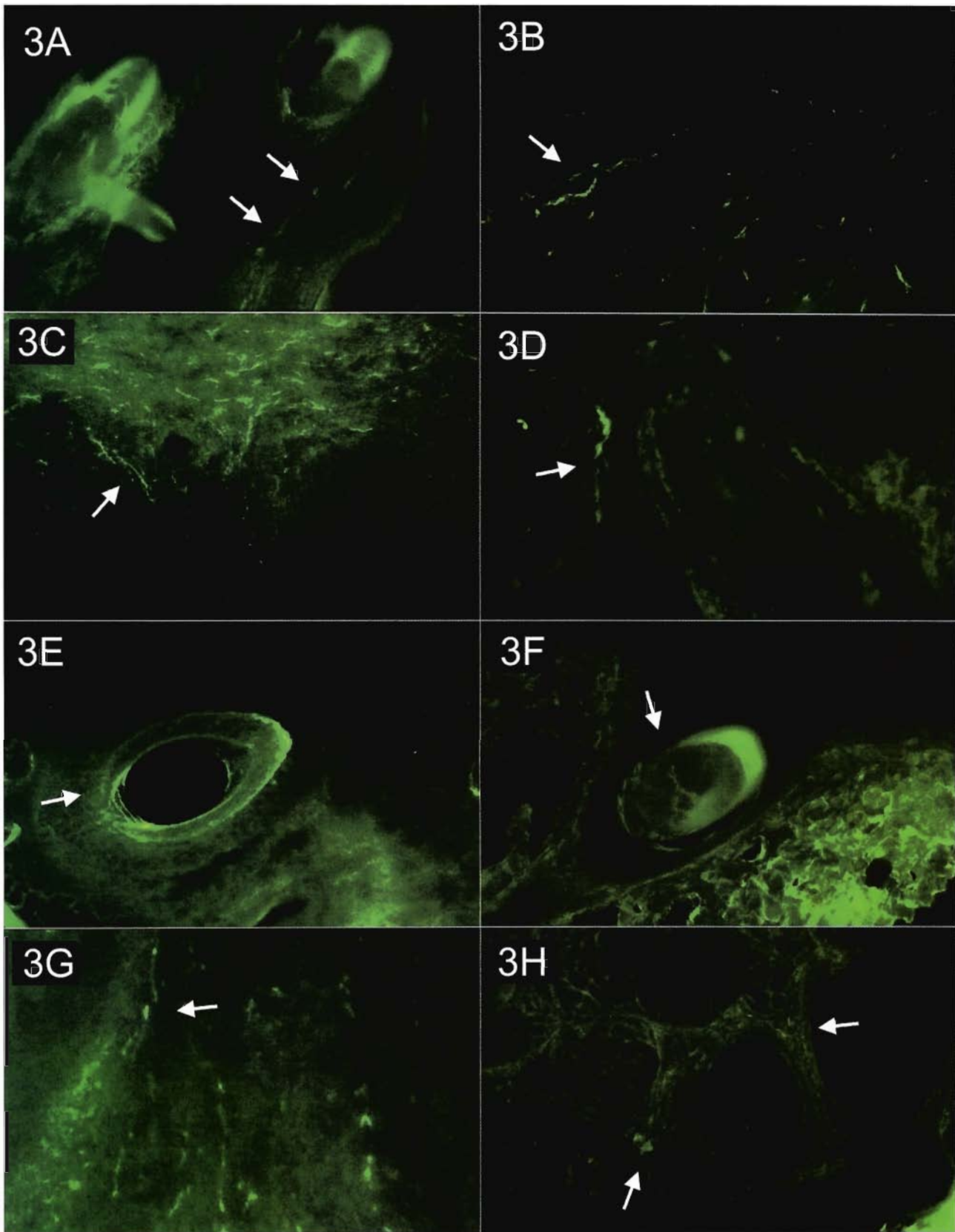
In the conjunctive mucosa some thin catecholaminergic nervous fibers can be seen in the base of the calciform cells (Fig. 3P).



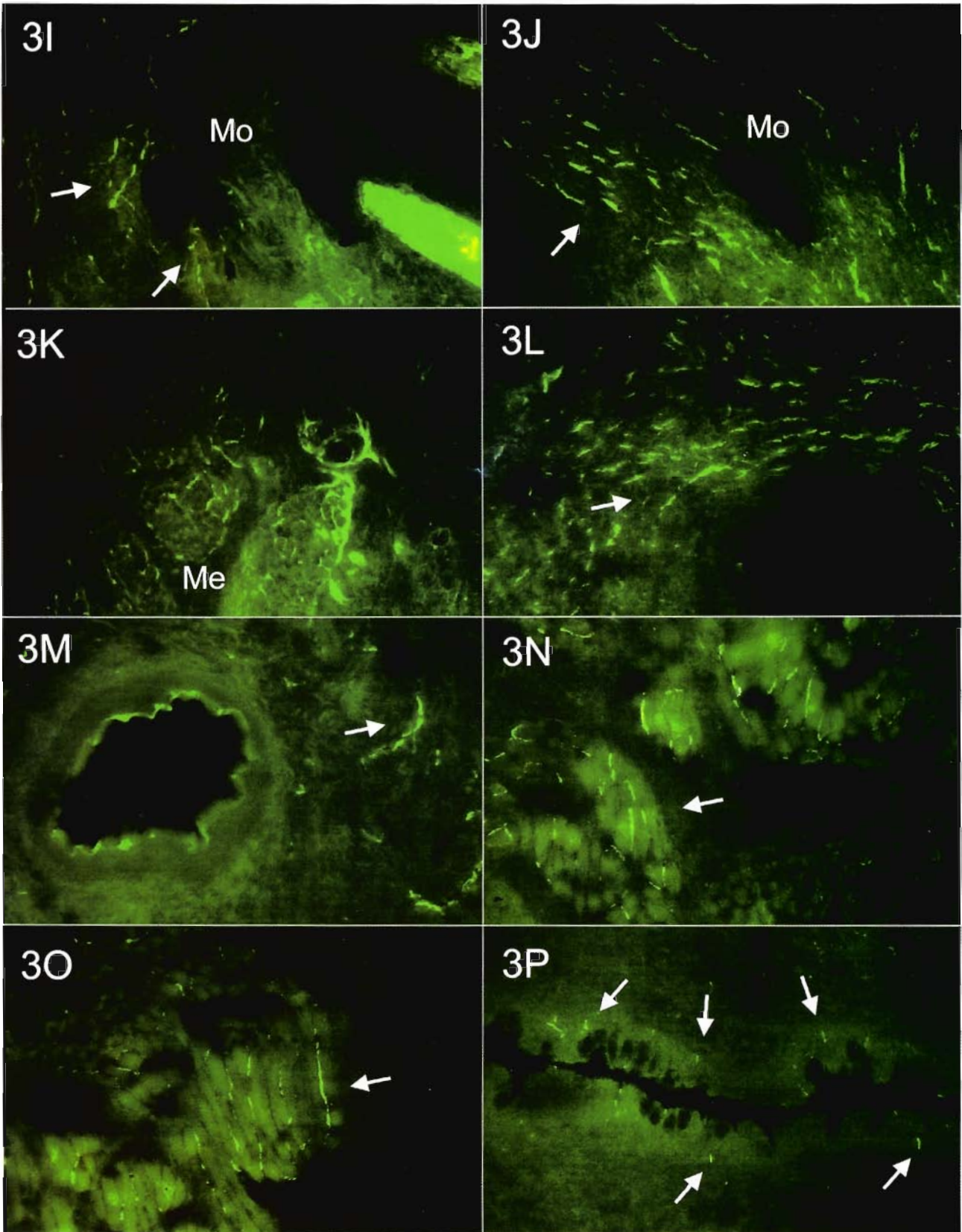
**Fig. 2.** Acetylcholinesterase-positive innervating pattern. **A.** Connective tissue of the eyelid. Nervous fibers (arrow). x 240. **B.** Hair follicle in the eyelid edge. Nervous fibers (arrow) surrounding the sheath. x 150. **C.** Hair follicle in the eyelid edge. Nervous fibers (arrow) in close contact with the peripheral cells of the sheath. x 240. **D.** Arteriola of the eyelid edge, innervated by fine fibers (arrow). x 60. **E.** Venula in the eyelid edge. Nervous fibers in the wall (arrow). x 240. **F.** Glands of Zeiss (Z) and glands of Moll (Mo). x 60. **G.** Glands of Zeiss (Z) and nervous fibers around them (arrow). x 150. **H.** Meibomian gland (Me). Nervous fibers (arrow). x 60. **I.** Lachrymal gland (L). Nervous fibers (arrow). x 60. **J.** Detail of the lachrymal gland (L). Nervous fibers (F). x 240. **K.** Muscle (Mu). Motor plaques (Mt). x 60. **L.** Muscle (Mu). Motor plaques (Mt). x 60. **M.** Detail of motor plaques (Mt). x 150. **N.** Conjunctive membrane (C). Ca: calciform cells. x 150

*Innervation in the eyelid*





**Fig. 3.** Catecholaminergic innervating pattern. **A.** Nervous fibers (arrow) in the base of the eyelid epithelium. x 150. **B.** Nervous trunks (arrow) in the connective tissue. x 240. **C.** Nervous fibers (arrow) in the connective tissue around the glandular ducts. x 150. **D.** Hair follicle. Nervous trunk (arrow) reaching the sheath. x 240. **E.** Hair follicle. Nervous fibers (arrow) in the sheath. x 240. **F.** Hair root. Nervous fibers (arrow) of the follicle. x 240. **G.** Fine nervous fibers (arrow) in the adventitia of an arteriola. x 150. **H.** Fine nervous fibers (arrow) in a gland of Zeiss. x 240. **I.** Glands of Moll (Mo) beside hair follicles. Nervous trunks (arrow) surrounding these glands. x 150. **J.** Abundant innervation (arrow) of a gland of Moll (Mo). x 150. **K.** Meibomian gland (Me). Abundant innervation (arrow). x 150. **L.** Nervous trunks (arrow) in the connective tissue of a lachrymal gland. x 150. **M.** Innervation around an arteriola near a lachrymal gland. x 150. **N.** Nervous fibers (arrow) around the ducts of a lachrymal gland (LG). x 150. **O.** Innervation of the lachrymal gland. x 150. **P.** Nerve endings in the base of the caliciform cells (arrow) in the conjunctive membrane. x 150





## Discussion

Although the acetylcholinesterase enzyme does not appear only in the nervous structures, but also appears in others such as blood or muscular cells, the acetylcholinesterase method lets us clearly distinguish the nervous components from the rest of structures, once the reaction times are strictly controlled (Aisa et al., 1997, 1998).

To further investigate the nature of the acetylcholinesterase-positive cells that we had found in the eyelid epithelium, we performed S-100 protein immunoreactivity to detect either Langerhans cells or melanocytes. We thought that all acetylcholinesterase-positive cells would be S-100 protein immunoreactive, but we only found a small number of S-100 protein immunoreactive cells in the epithelium. As is well known, both Langerhans cells and melanocytes are S-100 protein immunoreactive (Takahashi et al., 1984).

Simons and Smith (1994) found widespread VIP-ir innervation in the Meibomian gland, coming from the parasympathetic pterigopalatine ganglion, as well as a certain calcitonin gene-related peptide immunoreactive innervation from the trigeminal ganglion, which was sympathetic and sensorial. In addition, Chung et al. (1996), by means of immunohistochemical techniques for neuropeptides reported a rich innervation by different types of nervous fibers in the Meibomian gland of primates, suggesting a predominantly parasympathetic origin for this innervation. Perra et al. (1996) found a strong acetylcholinesterase-positive reaction in the Meibomian glands, suggesting that the cholinergic system is involved in the regulation of the Meibomian gland secretory function. Our results agree with these authors regarding the Meibomian glands. Moreover, after sympathectomy, Fan and Smith (1993) found a decrease in VIP immunoreactivity in the parasympathetic pterigopalatine ganglion and in the Meibomian gland.

Mircheff (1989) reported that the lachrymal gland had a predominantly parasympathetic innervation, with sympathetic modulation. In the same way, Walcott et al. (1989) demonstrated widespread acetylcholinesterase-positive innervation in the lachrymal gland in avians. Our results agree with the above-mentioned data, confirming a rich acetylcholinesterase-positive and FIF+ innervation in the lachrymal and the Meibomian glands.

Powell and Martin (1989) found a loose net of adrenergic fibers in the interstitium around acini, blood vessels and vessel walls in dogs. They also indicated that the cholinergic innervating pattern was denser than the adrenergic one, and described the presence of both types of fibers around acini, suggesting a sympathetic-parasympathetic relationship in the lachrymal gland of dogs. From our findings in the sheep eyelid, we can achieve the same conclusions regarding the presence of a sympathetic-parasympathetic modulation in the function of the lachrymal glands of sheep.

As far as we know, this study is the first to describe the glands of Zeiss innervation, with a rich acetyl-

cholinesterase-positive and not so rich FIF+ innervation. It is also the first time that the glands of Moll innervation have been observed, with the finding of scarce acetylcholinesterase-positive innervation and more widespread noradrenergic innervation.

Simons and Smith (1994) found a rich sympathetic innervation in the tarsal muscle, as well as a moderate parasympathetic and sensorial CGRP-ir innervation. The relationship between cholinergic and adrenergic innervation was confirmed by Kirzsan-Agbas et al. (1998), by means of electrophysiological methods, in the smooth muscle in the rat. Our findings in sheep confirm the rich acetylcholinesterase-positive innervation of the eyelid smooth muscle, where abundant acetylcholinesterase-positive motor plaques can be demonstrated.

Ten-Tusscher et al. (1988) studied the conjunctive of the anterior eye segment using horseradish peroxidase-wheatgerm agglutinin as a tracer. They found a consistent retrograde labelling of neurons in the trigeminal (sensory) superior cervical (sympathetic) and ciliary (parasympathetic) ganglion. Although we have observed some thin sympathetic fibers at the base of the calciform cells, we have not found any acetylcholinesterase-positive innervation in the conjunctive membrane.

Ruskell and Van der Werf (1997) using electron microscopy found many terminals identified as autonomic on morphological grounds in the conjunctive. We have only found a mild noradrenergic innervation, mostly in the marginal zone of the conjunctive, which agrees with McGowan et al. (1994) who described higher touch sensitivity in the marginal zone of the eyelid conjunctive.

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