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A light- and electron microscopic study of tyrosine hydroxylase-like immunoreactivity in the ciliary ganglia of monkey *(Macaca fascicularis)* and cat

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Summary. The present paper describes tyrosine hydroxylase-like immunoreactivity in the ciliary ganglion of monkey *(Macaca fascicularis)* and cat. Under the light microscope, in the monkey, about 7.6% of neurons were observed to be intensely stained, 27.7% moderately stained and 32.5% lightly stained. In the cat, 1.2% of neurons were intensely stained, 5.4% moderately stained and 10.1% lightly stained. Ultrastructurally, tyrosine hydroxylase-like immunoreactivity was observed in neuronal somata, dendritic profiles and axons in both monkey and cat. Tyrosine hydroxylase-like immunoreactive dendritic profiles were synaptically contacted by tyrosine hydroxylase-negative axon terminals. In the monkey, tyrosine hydroxylase-like immunoreactive fibres were observed to enter the ciliary ganglion via the nasociliary nerve. Such fibres were observed to course among neurons within the ganglion and emerge in the short ciliary nerves. In contrast, tyrosine hydroxylase-like immunoreactive fibres were only occasionally observed in the cat.

Key words: Tyrosine hydroxylase, Ciliary ganglion, Immunocytochemistry, Monkey *(Macaca fascicularis)*, Cat

Introduction

Histochemical and biochemical studies have shown that most of the neurons in the ciliary ganglion of the cat, rat and chick embryo are acetylcholinesterase positive and that the ciliary ganglion is generally considered to be cholinergic in nature (Koelle, 1955; Koelle and Koelle, 1958; Huikuri, 1966; Olivieri-Sangiacomo et al., 1983; Davis et al., 1984; Koelle et al., 1987). Several studies have demonstrated catecholamine (CA) fibres in the ciliary ganglion of birds, rats, guinea-pigs, cats, dogs, and monkeys (Hamberger et al., 1965; Ehinger, 1967; Ehinger and Falck, 1970; Tobari, 1971; Cantino and Mugnaini, 1974; Uemura et al., 1987) and also after treatment with nialamide and L-DOPA or when sacrificed under severe stress (Ehinger and Falck, 1970). Other light microscopic studies have shown the presence of tyrosine hydroxylase (TH)- positive neurons and fibres in the ciliary ganglion of chick embryo (Iacovitti et al., 1985; Teitelman et al., 1985), rat (Landis et al., 1987a,b; Uemura et al., 1987; Hardebo et al., 1992), cat, dog, and monkey (Uemura et al., 1987). However, there is little information on the localization of TH-like immunoreactivity at the electron microscopic level (Landis et al., 1987a). The present study investigated the distribution of TH-like immunoreactivity in the ciliary ganglia of monkey and cat at both light and electron microscopic levels.

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Materials and methods

Animals and preparation of tissue

A total of five monkeys *(Macaca fascicularis)* of either sex weighing 1.8-3.0 Kg, and six cats of either sex weighing 1.6-3.2 kg were used. Three monkeys and four cats were used for light microscopic localization and the rest for electron microscopic localization of TH-like immunoreactivity. Before, sacrifice, all animals were anaesthetized by an intraperitoneal injection of sodium pentobarbitone (30 mg/kg body weight), followed by artificial respiration with an animal ventilator via tracheostomy. Thoracotomy was then performed and a mixture of 1000 units heparin and 1 ml 1% sodium nitrite solution was injected into the left cardiac ventricle. Then the animal was perfused intracardially, initially with 300-500 ml Ringer's solution and subsequently with 2000 ml fixative at room temperature (25 °C). The fixative consisted of 4% paraformaldehyde in 0.1M phosphate buffer for light microscopy and a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for electron microscopy. The tissues were removed after perfusion and postfixed in the same fixative for 4 h before being transferred to 10% sucrose in phosphate

buffer (0.1M, pH 7.4) overnight. In the monkey, the ciliary ganglia of both sides, together with the trigeminal and superior cervical ganglia and midbrain were removed and processed for light microscopy. In the cat, only the midbrain was taken in addition to the ciliary ganglia of both sides, since Grimes and von Sallmann (1960) have shown that the ciliary ganglion, in this species, does not receive any input from the trigeminal and superior cervical ganglia. Only the ciliary ganglia were processed for electron microscopy.

Immunocytochemistry

For the light microscopic study, 30 µm-thick frozen sections of the tissue were cut and mounted on gelatinized glass slides. The sections were treated in 4% normal goat serum for 2 h at room temperature, after which they were washed in 0.1M phosphate-buffered saline (PBS) containing 0.1% Triton-X100 and were subsequently incubated in a serum raised against TH in rabbit (Incstar, Minnesota) for 24-48 h at 4 °C. The TH antiserum was diluted 1:400 with PBS containing 1% normal goat serum and 0.1% Triton-X100 before use. Antibody detection was carried out with the Vectastain ABC-kit (PK-4001, Vector Laboratories, Burlingame, CA) against rabbit IgG with 3,3'-diaminobenzidine as a peroxidase substrate. The sections were then dehydrated and coverslipped with Permount. In both monkey and cat, a random cell count of TH-like immunoreactive (TH-LI) cells was made on the immunocytochemically-reacted sections of the ciliary ganglia. About 600 ganglion cells (Total about 1950 cells in the monkey and 2350 cells in the cat) were randomly selected for each animal and the percentages of intensely-stained, moderately-stained and lightly-stained cells were determined. For negative control immunocytochemical incubation, some sections were incubated in PBS containing 1% normal goat serum and 0.1% Triton X100, without specific antiserum. The superior cervical ganglion of monkey was used as a positive control (Lyon et al., 1992).

For electron microscopy, 50 µm-thick Vibratome sections of the ciliary ganglia were cut. Free sections were incubated in 4% normal goat serum for 1-2 h at room temperature (25 °C). After washing with PBS, the sections were incubated in TH antiserum (1:400) and then in secondary antibody, as for light microscopy, without Triton-X100. The sections were finally reacted with 3,3'-diaminobenzidine plus 0.01% H_2O_2 and 4% nickel ammonium sulphate in 0.1 M TRIS buffer, pH 7.6. The sections were washed in PBS and postfixed in 1% osmium tetroxide before being dehyderted in ethanol and embedded in Araldite. Ultrathin sections were cut and stained with lead citrate for 2 to 3 min and viewed with either a Philips 400T or JEOL 1200 EX electron microscope.

Table 1. The percentages of TH-LI neurons in the ciliary ganglia of monkey and cat.

ANIMAL	INTENSELY STAINED CELLS (%)	MODERATELY STAINED CELLS (%)	SLIGHTLY STAINED CELLS (%)	TOTAL (%)
Monkey	1.6±3.0	27.7±1.6	32.5±8.9	67.8±9.9
Cat	1.0±1.1	4.9±2.2	7.6±4.4	13.4±7.7

Results

Light microscopy for immunocytochemistry

Monkey ciliary ganglion: In the 3 monkeys studied, there was a consistently high percentage of neurons showing TH-LI. Just over two thirds of the neurons showed TH-LI staining which ranged from dark to faint staining (Figs. 1, 2). Most of the TH-LI neurons were lightly stained (32.5%) and just under 10% were darkly stained (Table 1). The TH-LI cells in the ciliary ganglion showed no special distribution within the ganglion, nor were they restricted to any particular cell size. The processes of certain darkly stained cells were also stained (Fig. 1). Numerous TH-LI fibres could be seen entering the ganglion through the nasociliary nerve (Fig. 2). Such fibres could be observed coursing among the TH-LI neurons in bundles within the ganglion (Fig. 3). Some delicate TH-LI fibres could also be observed travelling among the neurons in the ganglion. TH-LI fibres could also be observed in the short ciliary nerves (Fig. 4). In addition, some aberrant neurons within the oculomotor nerve near the ciliary ganglion (Zhang et al., 1993, 1994) were also TH positive.

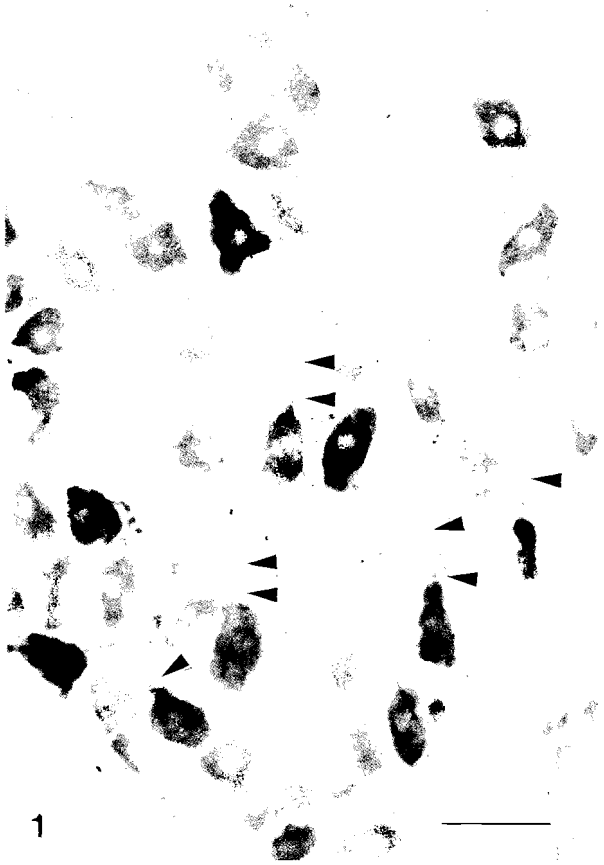
Cat ciliary ganglion: In contrast to the monkey, there was great variability in the number of TH-LI neurons in the ganglion of the 4 cats studied. The number of TH-LI neurons varied from 5.6% to 25% (Mean=13.4%). However, as in the ciliary ganglion of monkey, most of the TH-LI neurons were lightly-stained (Table 1) and

Fig. 1. Photomicrograph of monkey ciliary ganglion cells showing variable intensities of TH-LI staining ranging from faint to moderate. Arrowheads indicate neurons with TH-LI processes. Scale=bar. 100 µm. x 140

Fig. 2. Photomicrograph showing TH-LI fibres (arrowheads) entering the ciliary ganglion of monkey by the nasociliary nerve. Scale bar=200 µm. x 55

Fig. 3. Photomicrograph showing TH-LI fibres (arrowheads) coursing among the TH-LI neurons in the ciliary ganglion. Monkey. Scale=bar x 100 µm. x 110

Fig. 4. Photomicrograph showing TH-LI fibres in the short ciliary nerves (arrowheads). Monkey. Scale=bar 200 µm. x 55



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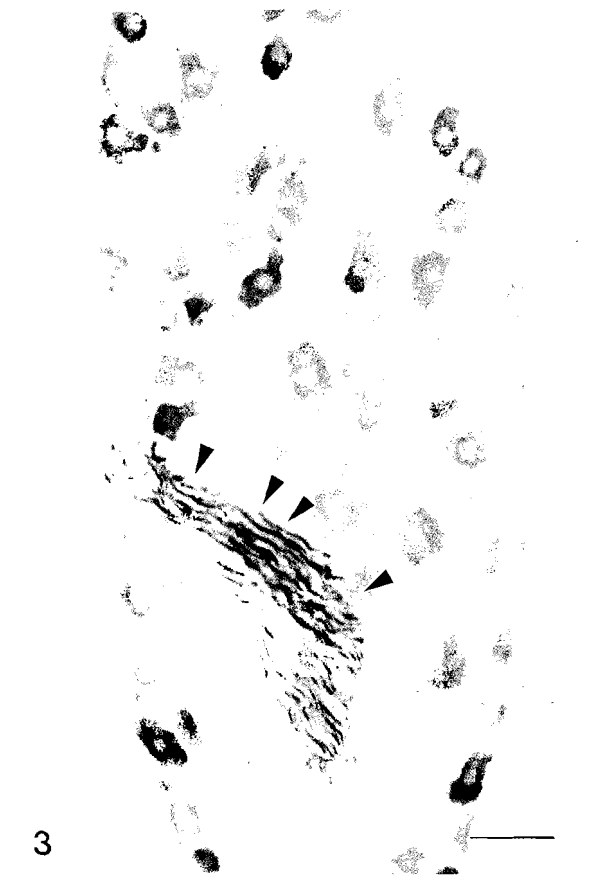

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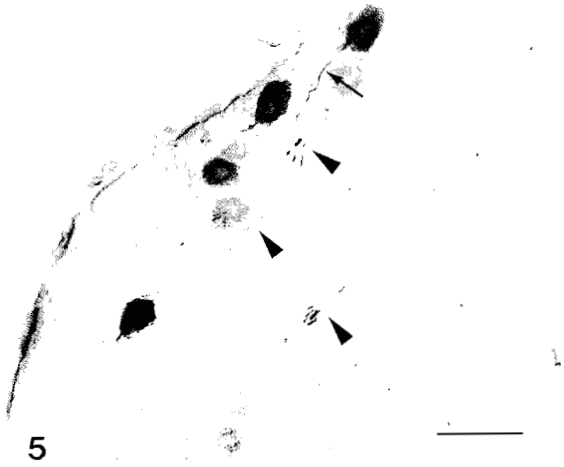

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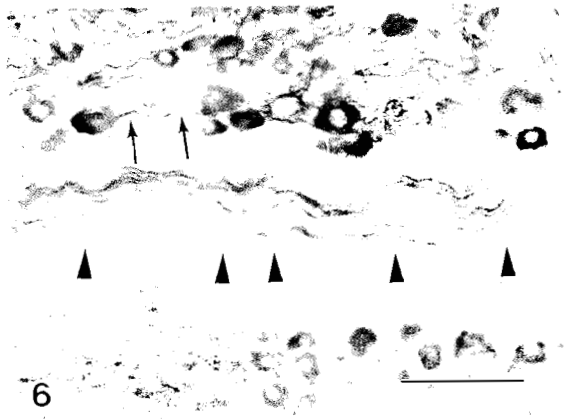

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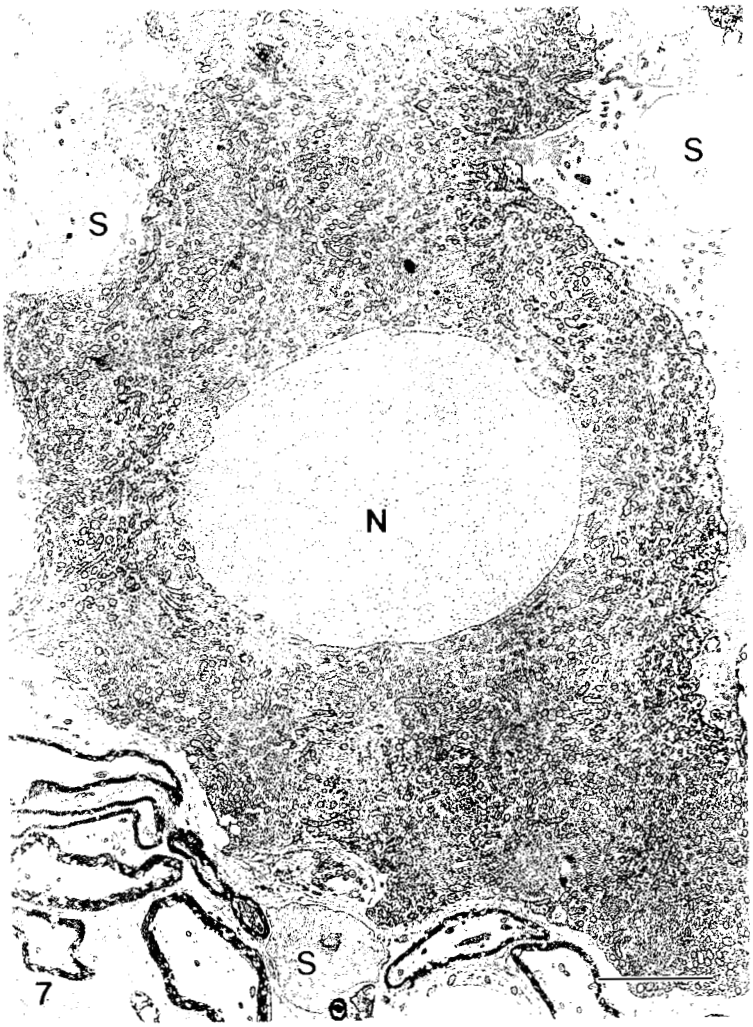

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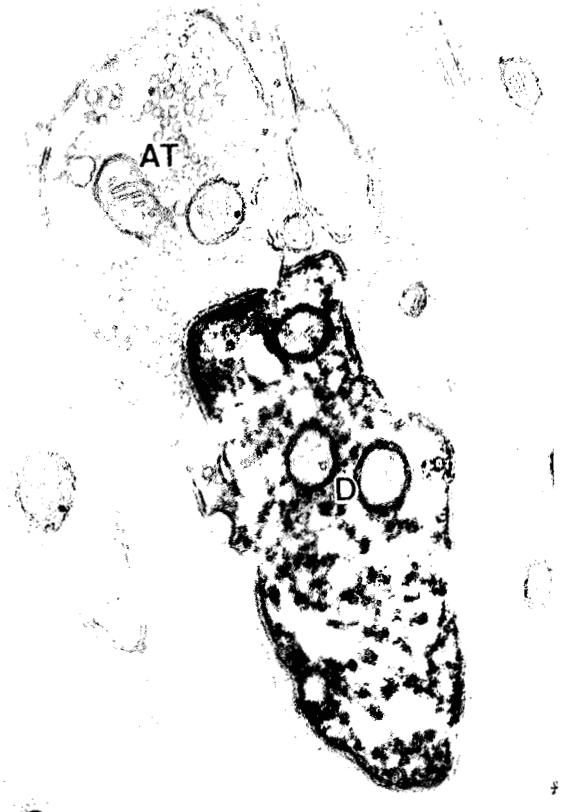

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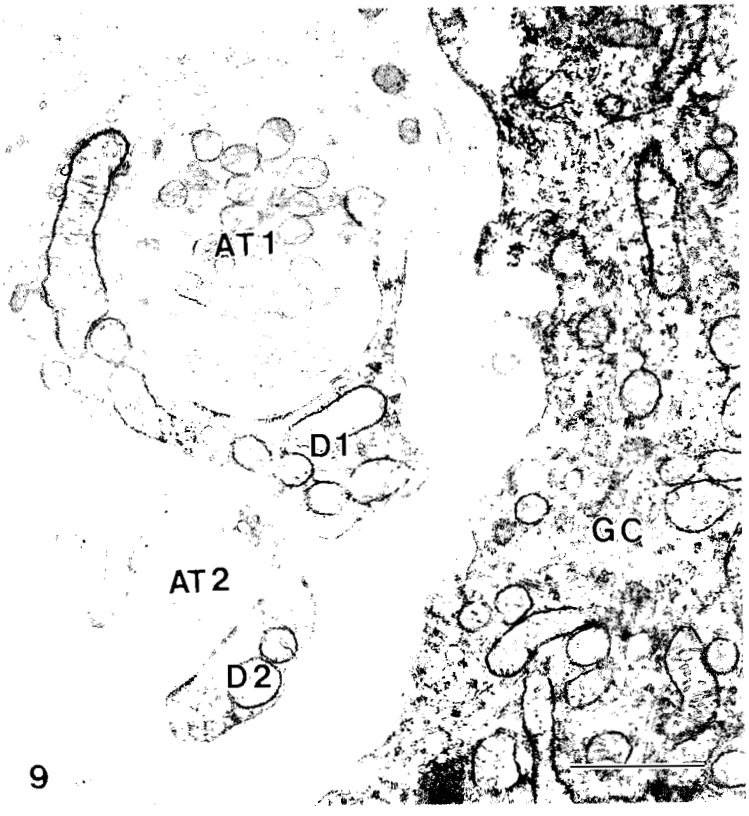

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Fig. 5. Photomicrograph showing TH-LI neurons in the ciliary ganglion. Arrowheads indicate the TH-LI fibres in the ganglion. Note a TH-LI fibre (arrow) arising from a neuronal soma. Cat. Scale=bar 100 μm. x 110

Fig. 6. Photomicrograph showing TH-LI neurons in the superior cervical ganglion of monkey. Numerous TH-LI fibres (arrowheads) can be observed in the ganglion. Note some TH-LI fibres (arrows) arising from TH-LI cells. Scale=bar 100 μm. x 160

Fig. 7. Electron micrograph showing a TH-LI neuron in the ciliary ganglion. The nucleus (N) of the neuron and the surrounding satellite cells (asterisks) are not stained. Monkey. Scale=bar 5 μm. x 2,975

Fig. 8. Electron micrograph showing a TH-negative axon terminal (AT) synapsing with TH-LI dendritic profile (D). Monkey. Scale=bar 0.25 μm. x 38,500

Fig. 9. Two TH-negative axon terminals (AT1, AT2) making synaptic contacts with two TH-LI dendritic profiles (D1, D2) close to a TH-LI ganglion cell soma (GC). Monkey. Scale=bar 1 μm. x 17,500

few were heavily-stained (Fig. 5). As in the monkey, these cells showed no special distribution within the ganglion either, nor were they restricted to any particular cell size. Unlike the monkey, only very occasionally could TH-LI fibres be observed within the ciliary ganglion of the cat (Fig. 5).

Monkey superior cervical ganglion: Most, if not all, of the neurons in the superior cervical ganglion showed intense TH-LI staining (Fig. 6). Numerous TH-LI fibres could be seen within the ganglion and the pre- and post-ganglionic nerves (Fig. 6).

Monkey trigeminal ganglion: No TH-LI neurons could be observed in the trigeminal ganglion. No TH-LI fibres were observed in the ganglion nor in its three divisions; namely, ophthalmic, maxillary and mandibular.

Midbrain of cat and monkey: No TH-LI neurons nor fibres were observed in the Edinger-Westphal (EW) nucleus.

Control: In all cases, the negative control sections for immunostaining failed to showed any TH-LI staining.

Electron microscopy for immunocytochemistry

TH-like immunoreactivity was demonstrated as electron-dense immunoreactive products deposited on the outer membrane of cytoplasmic organelles in the TH-labelled profiles.

In the ciliary ganglion of monkey, the TH-LI product was observed to be evenly distributed in the cytoplasm of the neuronal somata; the nuclei being unstained (Fig. 7). Dendritic profiles some of which were observed to arise from the TH-LI neuronal soma were also labelled with TH-LI product (Figs. 8, 9). Satellite cells were also unstained. Numerous myelinated and unmyelinated axons showed TH-LI reaction, but the surrounding Schwann cells were unlabelled (Fig. 10). The axon terminals which formed synapses with TH-positive dendritic profiles were observed to be unstained (Figs. 8, 9). The synaptic vesicles within such axon terminals were spherical; some containing flattened vesicles were also observed.

In the cat, the distribution of electrondense TH-LI product in the neuronal somata and the dendritic profiles was similar to that observed in the monkey (Fig. 11). The satellite cells did not contain any TH-LI product. Both myelinated and unmyelinated axons showed TH-like immunoreactivity, but the surrounding Schwann cells were unlabelled (Figs. 12, 13). Axon terminals forming synapses with TH-positive dendritic profiles were not labelled (Figs. 11, 14) and they contained spherical vesicles; no axon terminal containing flattened vesicles was observed.

Discussion

In the ciliary ganglion, TH-like immunoreactivity has previously been demonstrated in the chick embryo and adult animals of other species (Iacovitti et al., 1985; Teitelman et al., 1985; Uemura et al., 1987; Landis et al., 1987a,b). The present study has confirmed the presence of TH-like immunoreactivity in the ciliary ganglia of monkey and cat. Ultrastructurally, TH-like immuno-

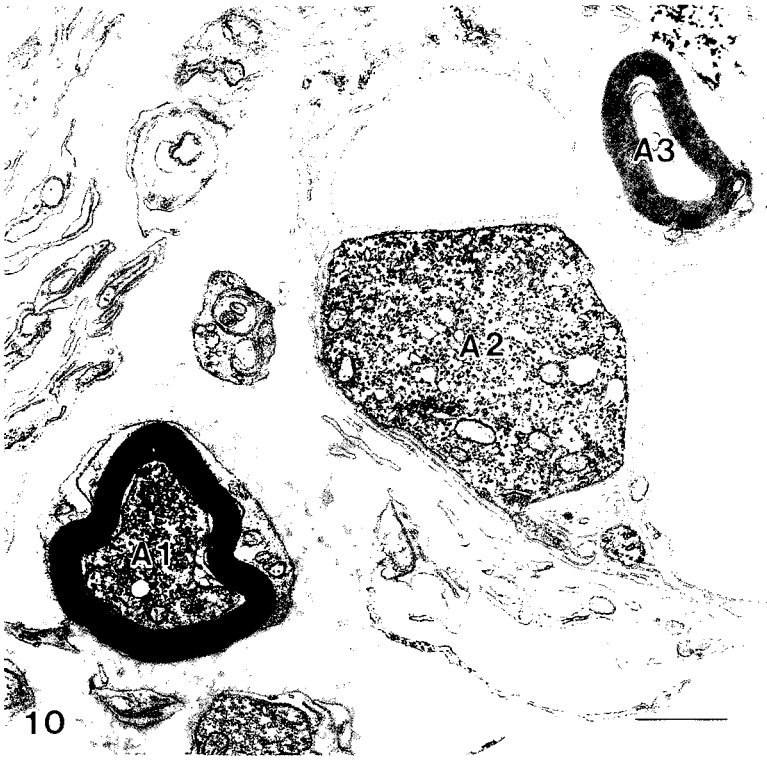
Fig. 10. Electron micrograph showing a TH-LI myelinated (A1) and unmyelinated axons (A2) lying beside a TH-negative myelinated axon (A3). Monkey. Scale=bar 1 μm. x 12,000

Fig. 11. Electron micrograph showing a TH-negative axon terminal (AT) synapsing with a TH-LI dendritic profile (D) beside a TH-LI neuronal soma (GC). Cat. Scale=bar 0.5 μm. x 29,750

Fig. 12. Electron micrograph showing a TH-LI myelinated axon (A1) lying close to a TH-negative myelinated axon (A2). Note that the Schwann cell is not stained. Cat. Scale=bar 1 μm. x 14,000

Fig. 13. Electron micrograph showing a TH-LI unmyelinated axon (A). The Schwann cell is not stained. Cat. Scale=bar 1 μm. x 17,500

Fig. 14. Electron micrograph showing a TH-negative axon terminal (AT) synapsing with a TH-LI dendritic profile (D1) beside another TH-LI dendiritc profile (D2). Cat. Scale=bar 0.5 μm. x 29,750



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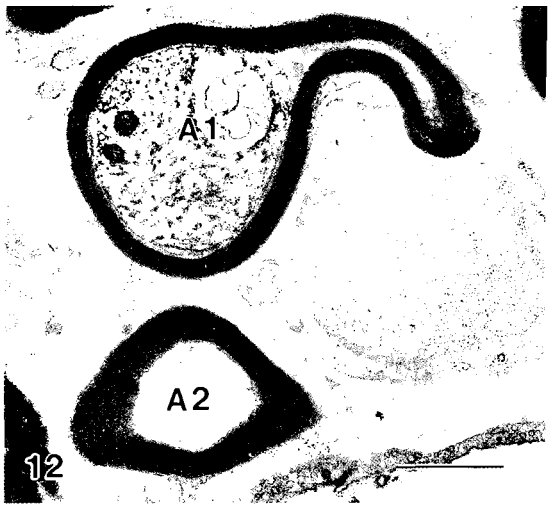

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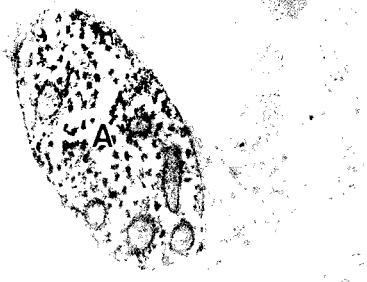

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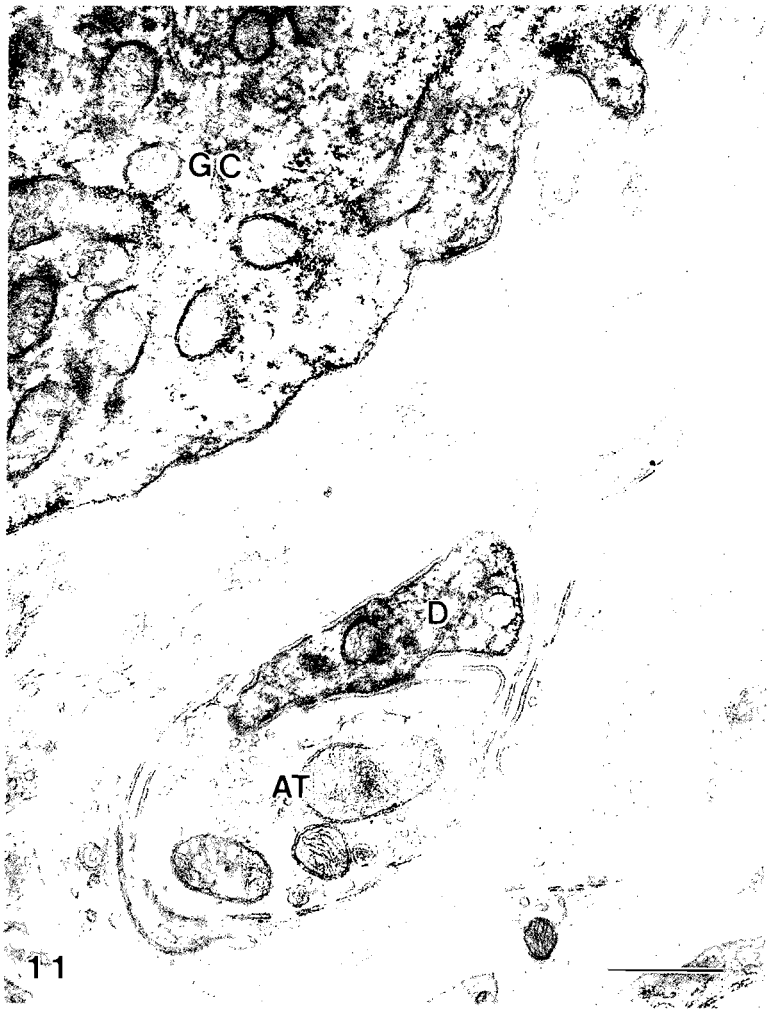

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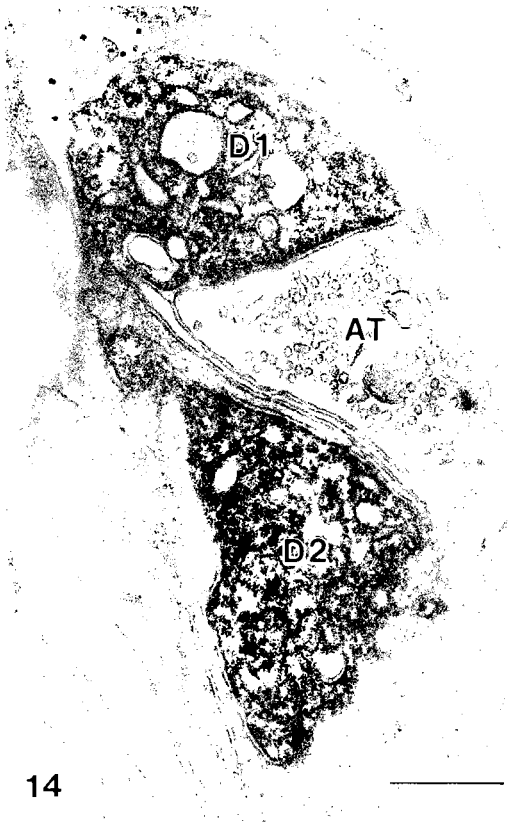

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reactivity was detected in neuronal somata, dendritic profiles, and both myelinated and unmyelinated axons, but not in the axon terminals. The functional implication of the cholinergic neurons containing TH-LI is unknown. Tyrrell et al. (1992), however, have reported that the expression of TH in the ciliary ganglion could be increased in rats in which sympathectomy was done at birth. This suggests that TH in the neurons of ciliary ganglion may be regulated by alterations in their environment.

Grimes and von Sallmann (1960) have reported that the ciliary ganglion of monkey receives not only preganglionic input from the nucleus of Edinger-Westphal (EW), but also fibres from the trigeminal and superior cervical ganglia. It is generally accepted that the fibres from the trigeminal and superior cervical ganglia only pass through the ganglion without interruption (Grimes and von Sallmann, 1960). The present study has also confirmed that the TH-LI fibres only passed through the ciliary ganglion without synapsing. In contrast to the present study, Landis et al. (1987a) have reported the presence of TH-LI axon terminals making synaptic contact with TH-LI dendritic profiles in adult rat ciliary ganglion.

In the present study, no TH-LI neurons nor fibres were found in the trigeminal ganglion, although a large number of TH-LI neurons and fibres were observed in the superior cervical ganglion of the monkey. The present findings thus suggest that most, if not all, of the TH-LI fibres in the ciliary ganglion of the monkey arise from the superior cervical ganglion. A sympathetic root to the ciliary ganglion of monkey such as that described by Grimes and von Sallmann (1960) and Kuntz (1961) was not found in the present or a previous study (Zhang et al., 1994). The present study also suggests that TH-LI fibres entered the ganglion along the nasociliary nerve. Lyon et al. (1992) have also reported that, in cynomolgus monkey, the sympathetic nerve fibres do not directly join the ciliary ganglion but join the nasociliary nerve before the latter enters the ganglion. In contrast, an independent sympathetic root to the ciliary ganglion has been reported in the human by Sinnreich and Nathan (1981).

In the case of the cat, the ciliary ganglion does not receive any input from the superior cervical and trigeminal ganglia (Grimes and von Sallmann, 1960). Thus it suggests that the few TH-LI fibres observed in this species in the present study might not be of sympathetic origin but could possibly arise from the TH-LI neurons within the ganglion itself. This was suggested by the observation of some TH-LI fibres arising from the neuronal somata within the ganglion itself. Considerable variation was observed in the cat. However, since immunostaining was carried out under the same experimental conditions as for the monkey, methodological reasons for such species variations could be ruled out. Whether such variations reflect the differences in functional status, age factor or other physiological conditions between each animal remains to be established.

In the present study, TH-LI fibres were also observed in the short ciliary nerves of the monkey. Such fibres innervate the intrinsic muscle of the eye ball. Björklund et al. (1985) had shown that many TH-LI fibres were present in the iris and ciliary body of the eye; they concluded that such fibres were sympathetic in origin. They have also shown that, in long-term sympathectomized animals, the TH-LI fibres reappeared in the iris, and that such fibres could be reduced drastically after removal of the ciliary ganglion. This might indicate that the neurons in the ciliary ganglion do not normally contribute to TH-LI innervation but have a compensatory ability to express TH innervation to the eye under certain conditions.

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