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Innervation of the chicken esophagus wall: localization and distribution of the acetylcholinesterase-positive nervous elements and ultrastructure

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SUMMARY

We have studied the distribution pattern of the acetylcholinesterase-positive innervation of the four-week-old chicken esophagus. We localized the different innervation plexuses and their interconnections on sections of tissue samples. Later, we observed the three-dimensional structure of the plexuses by means of histochemical methods on whole-mount preparations, after delaminating the esophagus wall. We also studied the components of the nervous plexuses by optic microscopy on semi-thin cuts stained with toluidin blue, and studied their ultrastructure by transmission electron microscopy. In the myenteric plexus we observed compact ganglions between both muscle layers, as well as intra and inter-fascicular neuronal cell bodies in the circular muscle layer. Nervous trunks and fibers distribute along the connective tissue among the muscular bundles. The submucous plexus was found to be less abundant in nervous elements than the myenteric plexus, and its ganglions were smaller. In the lamina propria abundant AChE+ fibers, which innervate the glands at their base, and in the surroundings of the tubules up to the epithelium were seen. On whole-mount preparations we describe that the ganglions of the myenteric plexus are larger and form a denser network than in the submucous plexus. TEM revealed varicosities with cholinergic, adrenergic, peptidergic and mixed vesicles.

Key Words: Acetylcholinesterase – Immunoreactivity – Esophagus – Innervation

INTRODUCTION

In birds, the anterior gut can be divided into four different regions: esophagus, crop, proventriculus and gizzard. Some basic aspects of the innervation of the anterior gut were described in the late 60's, such as Auerbach's plexus (Bennett and Cobb, 1969b), gizzard innervation development (Bennett and Cobb, 1969a) and extrinsic and intrinsic innervation (Bennett, 1969).

The avian esophagus receives sympathetic innervation from the cervical ganglions. By contrast, parasympathetic innervation arrives at the esophagus from the glosopharyngeal nerves, anastomosed with the vagus nerves.

The relationship between the intrinsic and the extrinsic enteric nervous system (ENS) has been demonstrated ultrastructurally (Aisa et al., 1997, 1998) and also by means of electrophysiological studies (Smith and Lunam, 1998). These authors observed the behavior of neurons in Remak's juxta-jejunal ganglia in response to jejunal distension in the domestic fowl.

Most studies on the innervation of the avian digestive tract have been performed on developing embryos (Fontaine-Perus et al., 1981, 1984; Saffrey et al., 1982). Some studies have focused on the cholinergic and adrenergic innervation of

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the gizzard. Our group has previously studied some aspects of anterior gut innervation during postnatal development (Aisa et al. 1987a,b, 1988).

Some ultrastructural studies have been published on the intrinsic innervation of the avian gizzard. Bennett and Cobb (1969) described the synaptic contacts on cell bodies or their processes in the ganglion cells of the gizzard. Young et al. (1983, 1989) discovered catecholaminergic varicosities in the avian intestine and rectum.

Recently, Schiltz et al. (1999) studied the avian ENS on sections and whole-mounts of the embryonic chick gastrointestinal tract. They demonstrated neural crest-derived precursors migrating through the primitive esophagus to colonize the gizzard, where an extensive cellular network is formed.

Salvi et al. (1998) performed ontogenic studies on the neuroendocrine system of the chicken esophagus using immunohistochemical techniques. These authors described that the number of positive nerve elements increased progressively up to some weeks after hatching.

Using double-labeling immunohistochemistry (Lunam et al., 1993), four types of neurons have been identified in both the juxta-jejunal and juxta-rectal ganglia of Remak's nerve in the domestic fowl.

By means of biocitin injections, Lunam and Smith (1996) visualized the morphology and projections of Remak's nerve neurons, suggesting that different neural circuits exist between Remak's nerve and the small and large intestines. In earlier studies by us (Aísa et al., 1997, 1998), we established the connection between extrinsic (Remak's nerve) and intrinsic innervation of the avian rectum and cloaca.

Balaskas et al. (1995) studied the role of nitric oxide in the neural control of the gut musculature in the gut of newly hatched chicks, and observed neurons expressing NADPH-diaphorase activity, NOS-ir and VIP-ir in both the myenteric and submucous plexuses.

In the 90s some phylogenetic studies on the vertebrate ENS were published (D'Este et al., 1994; Gabriel et al., 1990). These authors studied the distribution of GABA-like immunoreactivity in the myenteric plexus of the carp, frog and chicken. In the chicken they described extensive immunoreactive plexuses for GABA and also found GABA-positive fibers distributed within the circular muscle layer.

This study aim at gaining further insight into the acetylcholinesterase (AChE)-positive innervating pattern of the chicken esophagus. Once its nerve structures had been localized, transmission electron microscopy demonstrated the different vesicles of the neurotransmitters involved.

Materials and Methods

Twenty four-week-old chickens were used. All birds were anesthetized with ether vapours before sacrifice. Once the samples had been extracted, they were divided into three portions: one of them for the acetylcholinesterase technique for frozen samples, another one for whole-mount preparations, and the third portion for transmission electron microscopy.

Acetylcholinesterase technique for frozen samples

Using the acetylcholinesterase technique we obtained clear images to study the nervous plexuses. Acetylcholine iodine (Sigma) was used to demonstrate acetylcholinesterase (AChE) activity according to the method of El Badawi and Schenk (1967). Esophagus samples were frozen in methylbutane and 30 µm sections were cut, air-dried at room temperature and fixed for 15 min at 4°C in a solution of 10% formaldehyde in PBS (pH 7.0). After washing in distilled water they were incubated for periods of 10-18 h at 37°C. Cholinesterase activity sites were recognized as dark-brown precipitates. Controls were made by (i) incubating in a substrate-free medium, and (ii) incubating in a medium with tetraisopropylpyrophosphoramide (ISOOMPA, Sigma).

Acetylcholinesterase technique on whole-mount preparations

We performed this technique in order to study the three-dimensional distribution of the nervous plexuses.

Pieces of tissue were stretched and pinned onto fine cork sheets. Fixation was accomplished by immersion in a solution of 15% saturated picric acid with 2% formaldehyde in 0.1M PBS (pH 7.3) for 18h at 4°C. After washing in 80% ethanol for 30 minutes, the pieces were dehydrated through a graded series of ethanol and cleared in xylene (30 minutes in each solution). The pieces were then rehydrated back to PBS. At this stage, delamination was carried out. Under a light microscope, using cold light and microsurgical instruments, we removed the mucosa, submucosa and inner muscular layers, uncovering the contact surfaces between the two external muscle coats: circular and longitudinal. The myenteric plexus was observed on the longitudinal muscle layer, whereas the submucous plexus was studied on the circular muscle layer. Once the laminar portions of tissue had been obtained, the acetylcholinesterase technique was applied as previously described for the tissue sections.

Transmission electron microscopy

The standard method for electron microscopy was applied: fixation in 2.5% glutaraldehyde in

Milloning buffer (pH 7.3); post-fixation in 2% OsO_4; staining with 70% uranyl-acetate; dehydration and embedding in araldite. To localize the nervous structures constituting the plexuses, semi-thin cuts were made and stained with toluidin blue. The ultra-thin sections were contrast-stained following conventional methods.

Results

The ENS of the avian esophagus is very well developed at four weeks after hatching. In sections of the esophagus wall both the AChE-positive plexuses, myenteric and submucous, could be observed.

Myenteric plexus (Fig. 1)

AChE in frozen samples.- In the myenteric plexus, compact ganglia were found, constituted by AChE+ cells, which were located in the connective tissue of both muscle layers. These muscle layers were strongly innervated by nervous trunks distributed along the connective tissue among the muscle bundles, as well as fibers from these ganglia and nervous trunks. AChE+ cell bodies, either inter- or intrafascicularly, were also found (Fig. 1A).

AChE on whole-mount preparations.- Using light microscopy, in whole-mount preparations we observed the organization of the myenteric plexus (Fig. 1B). We found a three-dimensional network of nervous elements, constituted by compact ganglia, where AChE+ cells were seen with their typical large nucleus and prominent nucleolus. From these ganglia thick nervous trunks interconnected with other ganglions, or sometimes with cell bodies, and branched into thinner nervous fibers.

Semi-thin sections.- In semi-thin sections stained with toluidin blue, we observed the detailed structure of the ganglia of the myenteric plexus (Fig. 1C). They were compact and contained abundant large cell bodies, which were not stained by toluidin blue, and had a large nucleus and prominent nucleolus which was stained with toluidin blue.

TEM.- TEM preparations revealed the detailed ultrastructure of the nervous trunks, neurons, Schwann cells, and varicosities containing different types of vesicles, according to each type of neurotransmitters.

In the myenteric plexus, we observed the presence of abundant interneurons along the circular muscle layer; these were inter- and intrafascicular. We found thick nervous trunks in the interfascicular connective tissue (Fig. 1D).

All types of varicosities present on trunks were related to the smooth muscle cells next to them, although the space between them was variable (Fig. 1D).

The nervous trunks forming the myenteric plexus (Fig. 1E) were accompanied by some cell bodies. These cell bodies had an elongated irregular nucleus, with abundant marginal chromatin (Fig. 1E) and sometimes displayed a prominent nucleolus (Fig. 1F). Sometimes, contacts between nervous trunk varicosities and neuronal bodies are observed (Fig. 1F). These varicosities were frequently mixed: cholinergic and peptidergic. Most vesicles were cholinergic. They were electron lucent and small in size (40-60 nm) (Fig. 1F). Some interfascicular neurons close to a nervous trunk with some adrenergic varicosities were found (Fig. 1G). These were of medium size (45-70 nm), and displayed an electron-dense nucleus with a light halo. These adrenergic varicosities were found to be the less abundant of all. We also observed peptidergic vesicles, which were electron-dense, quite large (60-130 nm) and round- or oval-shaped (Fig. 1H).

Submucous plexus

The submucous plexus (Fig. 2) is less dense in nervous elements than the myenteric plexus. The ganglia that form part of the submucous plexus are smaller than the myenteric ones. Both plexuses are interconnected in the circular muscle layer.

AChE in frozen samples (Fig. 2A).- The submucous plexus is located in the connective tissue that constitutes the mucous, where it branches. It also innervates the muscularis mucosae and the lamina propria, reaching up to the epithelium base (Fig. 2A). In the lamina propria, abundant cholinergic fibers can be observed innervating the glands, involving their base and the area around the tubules of the glands up to their drainage site on top of the esophageal epithelium.

AChE on whole-mount preparations.- The three-dimensional network of the nervous components of the submucous plexus was also studied on whole-mount preparations (Fig. 2B). From the ganglia, which are small, nervous trunks depart and branch into thin fibers coursing in all three spatial dimensions. These fibers are distributed towards the circular muscle layer and also towards the muscularis mucosae.

Semi-thin sections.- In semi-thin sections the submucous ganglia were seen to be smaller and less compact than the myenteric one, although the morphology of their constituent neurons is similar.

TEM.- On studying the submucous plexus by TEM, we observed medium-sized nervous trunks, whose axons were accompanied by

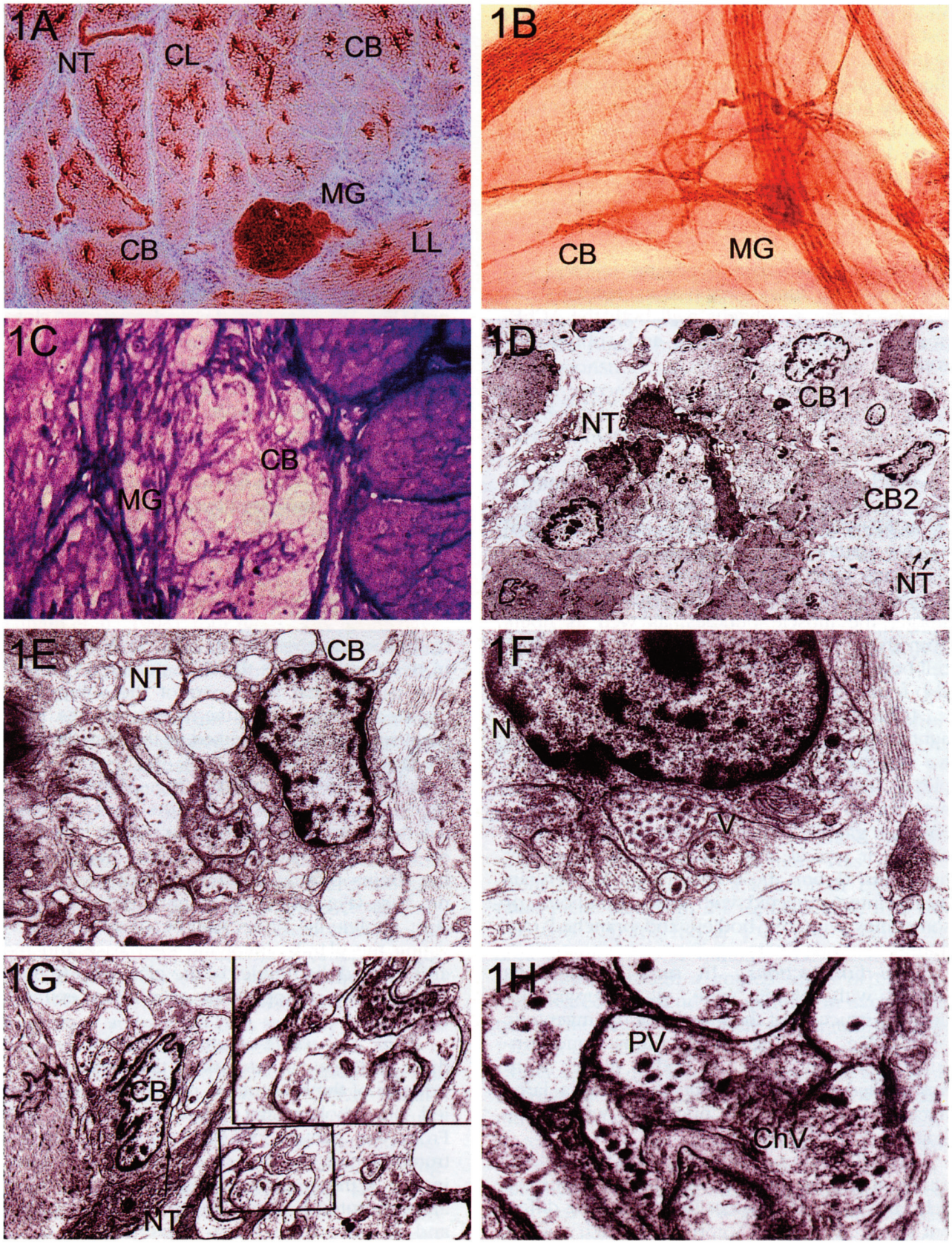

Fig. 1.- Myenteric plexus. **1A**.- Longitudinal section of the esophagus. AChE. x10. MG: Myenteric ganglion. CB: Cell body. CL: Circular muscle layer. LL: Longitudinal muscle layer. NT: Nervous trunk. **1B**.- Whole-mount preparation. AChE. x10. MG: Myenteric ganglion. CB: Cell body. **1C.-** Semi-thin section. Toluidin blue. x100. MG: Myenteric ganglion. CB: Cell body. **1D.-** Transversal section of the circular muscular layer. TEM. x3,500. CB-1: Intrafascicular cell body. CB-2: Interfascicular cell body. NT: Nervous trunk. **1E.-** Interfascicular neuron. TEM. x9,100. CB: Cell body. NT: Nervous trunk. **1F.-** Interfascicular cell body in contact with axonal varicosities. TEM. x15,000. N: Neuron. V: Mixed (cholinergic and peptidergic) varocisity. **1G**.- Transversal section of the longitudinal muscle layer. TEM. NT: Nervous trunk. CB: Cell body. x3,400 and x11,000. **1H**.- Mixed varicosities. TEM. x25,000. PV: Peptidergic vesicles. ChV: Cholinergic vesicles.

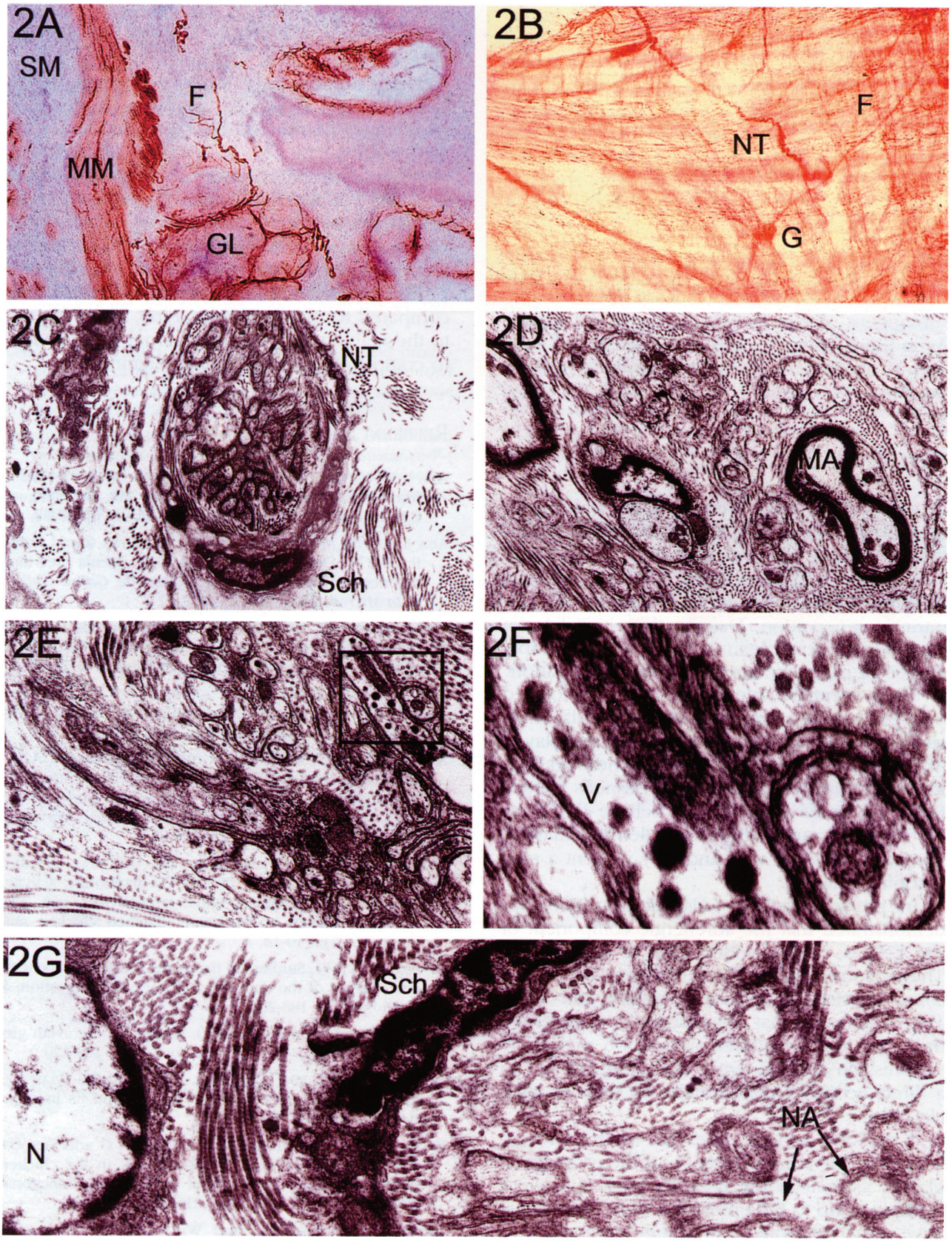

Fig. 2.- Submucous plexus. **2A**.- Longitudinal section of the esophagus. AChE. x10. SM: Submucous. MM: Muscularis mucosae. F: Nervous fibers. GL: Gland. **2B**.- Whole-mount preparation. AChE. x10. G: Ganglion. NT: Nervous trunk. F: Nervous fibers. **2C.-** Nervous trunk in the submucous, surrounded by a Schwann cell. TEM. x7,100. Sch: Schwann cell. NT: Nervous trunk. **2D.-** Nervous trunk with some myelinated axons in the submucosal layer. TEM. x11,000. MA: Myelinated axon. **2E.-** Nervous trunks in the submucosal layer. TEM. x11,000. **2F.-** Detail of the previous image. TEM. x34,000. V: Varicosity containing peptidergic vesicles. **2G**.- Neuron close to a Schwann cell surrounding non-myelinated nervous trunks in the submucosal layer. TEM. x11,000. N: Neuron. Sch: Schwann cell. NA: Non-myelinated axons.

Schwann cells (Fig. 2C). Most of these trunks were non-myelinic (Fig. 2C), although some myelinic axons were seen (Fig. 2D). The nervous trunks of the submucous had varicosities (Fig. 2E and 2F), with vesicles of the same types that were found in the myenteric plexus. The neurons of the submucous plexus had the same morphology as the neurons of the myenteric plexus, but were less abundant. Sometimes, a single neuron accompanied a nervous trunk (Fig. 2G).

In the lamina propria we have found nervous trunks that were similar to those found in the submucous plexus, mainly at the glandular base. As in the submucous plexus, myelin fibers were seen to accompany the nervous trunks up to the mucous layer.

Discussion

In the present work we obtained clear results using AChE methods to define the AChE+ innervating pattern. According to our results, the esophagus of four-week-old chickens shows a well-developed and widespread cholinergic intrinsic innervation. Despite this, it is known that the myenteric plexus receives a considerable degree of parasympathetic extrinsic innervation.

The myenteric plexus is typically located between both the longitudinal and circular muscle layers. Within the circular muscle layer, AChE+ cell bodies form a nervous network that interconnects this plexus with Meissner's plexus. Based on these findings, we believe that both the myenteric and submucous plexuses may be integrated in some way and may present a synchronized activity.

In previous studies (Aisa et al., 1988) we demonstrated that the adrenergic component of the ENS is not as widespread as the AChE+. These morphological data may help to explain some physiologic studies (Gershon and Erde, 1981) indicating that relaxation of the digestive tract due to sympathetic stimulation occurs by suppression of cholinergic excitation, but not to a direct action of noradrenalin on smooth muscle fibers.

A striking aspect observed in the present study was the rich innervation of the esophageal glands, as well as the nervous fibers that were distributed up to the epithelium base. Both must have some functional role at their endings.

Chubb et al. (1980) observed that SP-immunoreactive fibers may also display an AchE-positive reaction. This is consistent with our own results; thus, TEM revealed the presence of mixed varicosities containing both cholinergic and peptidergic vesicles at the same places. In addition, Greenfield (1984) suggested that the AChE enzyme is not only related to the cholinergic system but also demonstrated that AChE is associated with neurons of different neurochemical types.

Other authors have found peptidergic innervation in the ENS using immunohistochemical methods (Uddman et al., 1978; Brodin et al., 1981; Saffrey et al., 1982). We also found VIP and SP immunoreactivity in the esophagus (Aisa et al., 1988). Our TEM images agree with those results, in the sense that they show abundant varicosities or axon terminals that contain cholinergic and peptidergic vesicles at the same sites.

The lower amounts of peptidergic vesicles as compared to the cholinergic type, might be due to the inability of the conventional TEM method to completely preserve catecholamines.

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