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Enteric plexus and interstitial cells of Cajal: Interrelationship in the stomach of *Podarcis hispanica* (Reptilia). An ultrastructural study

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Summary. The ultrastructure organization of the stomach enteric plexus was examined in the lizard *Podarcis hispanica*. The ganglions of the myenteric plexus present a low number of nerve cell bodies with a peculiar nucleus, which occasionally establish direct contacts with cells of the circular muscle layer. Glial cells are smaller than the neurones, and their nucleus is very electron-dense. They surround the axons that constitute the fibres of the myenteric plexus. Four main types of axon profile are described in a morphological consideration of the vesicle population. In the interstice of the circular muscle layer we describe two types of interstitial cells that, due to their ultrastructural characteristics, may be equivalent to the interstitial cells of Cajal which have been described in mammalians. These cells shows parallel distribution to the stomach nerve plexuses, establishing close contacts with them through their long cytoplasmic prolongations. By means of small gap-like unions, they contact both each other and the smooth muscle cells near them. We describe a submucous plexus, where neuronal bodies are scattered among bundles of nervous fibres, some of which are myelinated. A mucous plexus with isolated neurones is located in the lamina propria. Axonal varicosities containing vesicles contact with the cells of the mucous. Interconnected interstitial cells may also be found in this plexus.

Key words: Ultrastructure, Enteric nervous system, Interstitial cells of Cajal, Reptilia

Introduction

Phylogenetic studies of the Enteric Nervous System (ENS) are of great interest, not only for discovering evolutionary aspects leading to the establishment of a higher structural complexity as we rise in the philogenetic scale of vertebrates (Burnstock, 1969; Nilsson, 1983; Jensen and Holmgren, 1994), but also because they have provided unique functional models for each species.

Some ultrastructural studies have been published on the myenteric and submucosal plexuses of different phylum: fishes (Wong and Tan, 1978; Ezeasor, 1979, 1981; Watson, 1981; Kiliaan et al., 1997); amphibians (Wong, 1973; Junquera et al., 1986, 1987a, 1988; Torihashy, 1990; Gabriel et al., 1992); birds (Young, 1983; Aisa et al., 1987, 1990); and mammals (Baungarten et al., 1970; Cook and Burnstock, 1976; Gabella, 1979, 1981, 1982; Komuro et al., 1982). However, studies in reptiles are scarce (Junquera et al., 1998; Martinez-Ciriano et al., 2000).

Some previous ultrastructural studies were limited to the enteric neuronal plexus organization but no research examined the inter-relationship between the enteric nerve structures and the elements of the enteric connective tissue. Especially in non-mammalian vertebrates, a cellular type with an increasing functional role in the gastrointestinal tract is often missed: the Interstitial Cell of Cajal (ICC). Although Cajal discovered them in 1893 in the amphibian and mammalian intestine (Cajal, 1893, 1911), they have not been studied until recent years in amphibians (Prosser, 1995; Williams and Parson, 1997), reptiles (Junquera et al., 1998; Martinez-Ciriano et al., 2000), and birds (Imaizumi and Hama, 1969; Lecoin et al., 1996; Reynhout and Duke, 1999).

The aim of this study was to investigate the ultrastructure of the stomach enteric plexus of *Podarcis hispanica*. We also analyse the relationships of these plexuses with two cellular types of interstitial cells (ICC-like and fibroblast-like cells) in the reptilian stomach, which have not been described before.

Materials and methods

We used twenty adult lizards *Podarcis hispanica* (Reptilia). The animals (10 males and 10 females) were collected in spring, summer, and autumn. They were sacrificed by cervical dislocation, under ether anaesthesia. Tissue samples from the anterior and

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posterior portions of the stomach (2 mm size) were fixed for 4 hours in glutaraldehyde at 25% in Milloning buffer (pH 7.3). Later they were washed three times for 20 minutes, with the same buffer. Post-fixation was performed in 1% osmium tetroxide. The tissue was then washed in Palade tampon, dehydrated in graded acetone (30%, 50%, 70% with 2% uranyl-acetate, 90%, 100%) cleared in propylene oxide and embedded in Durcupan. We studied the basic histology of the stomach in semi-thin cross sections stained with methylene blue. Ultra-thin sections were contrasted with lead citrate and examined on a Phillips 301 electron microscope.

Results

In the upper portion of the stomach, the ganglia of the myenteric plexuses were only covered by the adventitia (Fig. 1a). In cross-section, they were displayed sequentially (in tandem), this structural organisation being repeated in all the section. Each structural group was made up of one or two central neurones, surrounded by nervous trunks that contained approximately thirty axons, grouped together by a glial cell (Fig. 1b).

The nucleus of these neurones had three to four times the volume of the glial cell nucleus. Their typical aspect was irregular with small indentations and finely granular chromatin with small condensations and a spotted appearance. They often had a thin frame of marginal chromatin and prominent nucleolus. The nucleus of glial cells was smaller and much more irregular, very electron-dense and with a compact distribution of heterochromatin (Fig. 1c). The space between ganglia and the underlying circular muscle layer was so tiny, that sometimes direct contact could be observed between the membranes of ganglion neurones and of muscle cells (Fig. 1d).

The nervous trunks were located laterally to the neurones. In their axoplasm, neurofilaments, neurotubules and some mitochondria could be observed, as well as axonal varicosities containing different types of vesicles. These axons may be located beside the neuronal membranes or be separated from them by thin collagen frames (Fig. 2).

Nervous trunks with fewer axons than in the myenteric plexus were distributed among muscle cells in the circular muscle layer (Fig. 3a). These nervous trunks contained predominantly four types of axonal varicosities. The most common contained electron-lucent, 40-60 nm sized vesicles (SAV), which could be considered as cholinergic. Peptidergic type varicosities were also abundant, and contained 90-140 nm electron-dense vesicles (LGV). Small granular vesicles (SGV), which were characteristic of adrenergic nerves, were observed less frequently. There were also widespread varicosities with a mixed population of small electron-lucent vesicles and large electron-dense vesicles (Fig. 3a-c).

The distance from these varicosities to the muscle cells was variable. However, when there was direct contact between a varicosity and a muscle cell, the clear definition of the varicosity membrane disappeared and abundant caveolaes appeared in the membrane of the muscle cell. This may suggest a direct liberation of the neurotransmitter towards the muscle cell (Fig. 3a). These varicosity-muscle cell contacts could also be seen in the longitudinal muscle cells, with a characteristic adaptation of the muscle cell to the round-shaped profile of the varicosities. The hook-like structures that could be observed with a higher magnification in the membrane of the vesicles could be interpreted as fusion proteins that take part in the exocytosis of neurotransmitters (Fig. 3c). Metabolic coupling was demonstrated by the presence of gap junctions between cell membranes of adjacent muscle cells, with a characteristic appearance of small electron-dense shadowing (Fig. 3a).

In the connective tissue that separated the bundles of muscle cells in the circular muscle layer, we could find three types of cells regarding their characteristic morphology (Fig. 4a):

- Isolated intramuscular neurones, with voluminous nucleus, narrow frame of marginal chromatin and prominent nucleolus. These cells were surrounded by a well developed neuropil. Axonal varicosities established contact with the neuronal membrane (Fig. 4b).
- One type of interstitial cells (IC-1) were smaller than neurones, present with a round or oval shaped nucleus, and their cytoplasm around the nucleus was small and of similar electron-density to the surrounding smooth muscle cells (Fig. 4a,c). The cytoplasm was poorly differentiated with several mitochondria, little rough endoplasmic reticulum (RER) and abundant cisternaes of smooth endoplasmic reticulum (SER) with a widespread system of vesicles. These cells established contacts with smooth muscle cells by means of finger-like protusions reinforced by small type gap-like unions (Fig. 4d). Their cytoplasmic prolongations established several contacts with the prolongations of other cells near them (Fig. 4e).
- A second type of interstitial cell (IC-2) was located in the intramuscular tissue (Fig. 4a, 5a,b). They presented an elongated nucleus with compact heterocromatin. They were characterized by electron-dense cytoplasm that constituted a narrow perinuclear frame and extended through two long primary processes, following the longitudinal axis of the cell. Shorter secondary processes projected from the cell body or from the primary prolongations. Each cell could have from two to four cytoplasmic projections which were generally separated from the axons by a collagen frame, although sometimes the separation between both membranes was as small as 20 nm (Fig. 5a). In the cytoplasm there were abundant mitochondria, particularly in the cytoplamic processes. Golgi apparatus, rough (RER) and smooth endoplasmic reticulum (SER) were also observed. Basal bodies were frequent and lipid droplets were occasionally seen. No basal lamina could be clearly identified whereas numerous caveolae were observed on the cell membranes (Fig. 5b1,b2). These cells interconnected

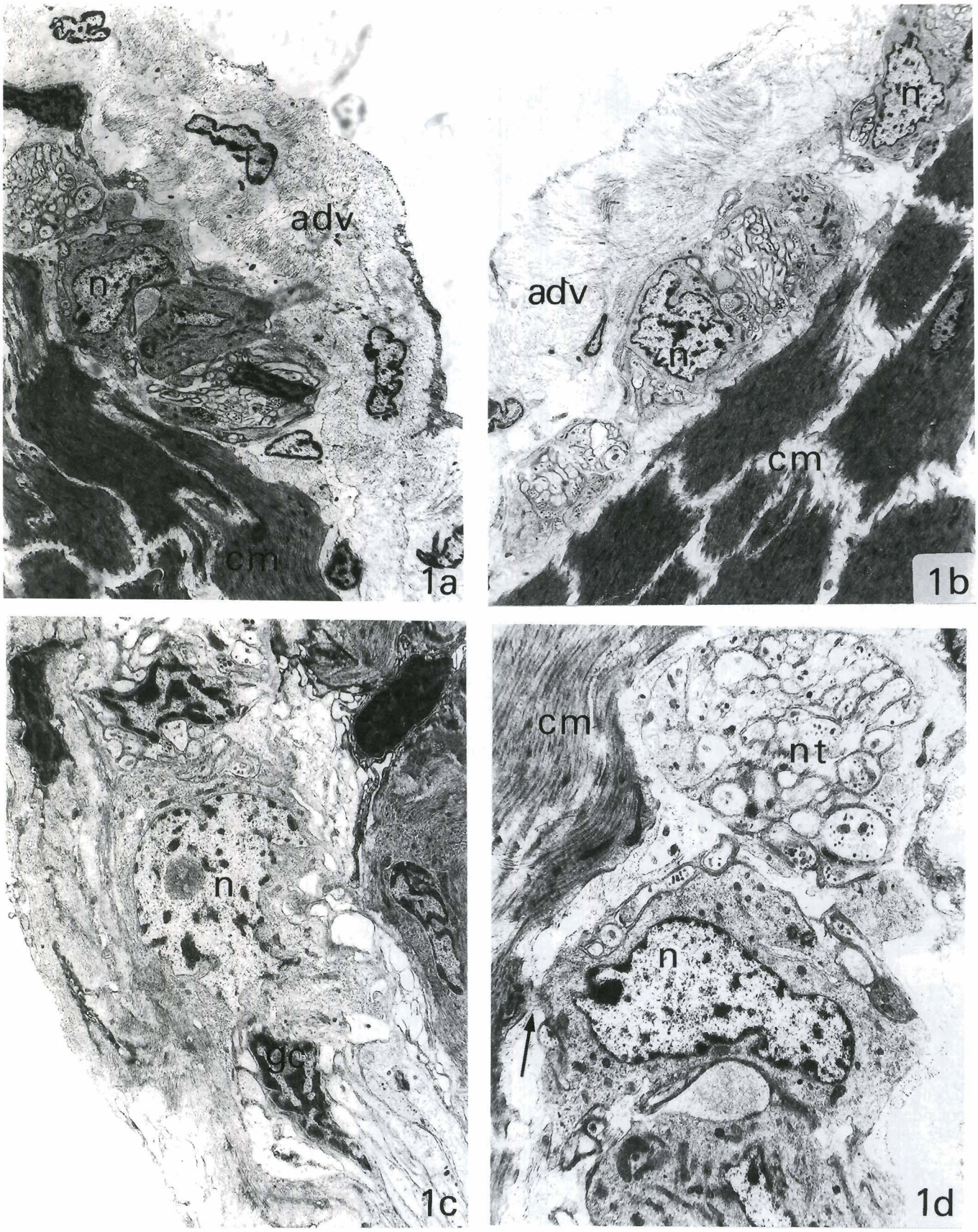


Fig. 1. a. Ganglion in the myenteric plexus of the upper portion of the stomach. x 1,900. **b.** Ganglia displayed in tandem, in the myenteric plexus. x 1,900. **c.** Characteristic view of neurones and glial cells of a ganglion in the myenteric plexus. x 3,400. **d.** Direct contact between a neurone in the myenteric plexus and a muscle cell (arrow). x 4,500. adv: adventitia; cm: circular muscle layer; n: neurone; gc: glial cell; nt: nervous trunk.

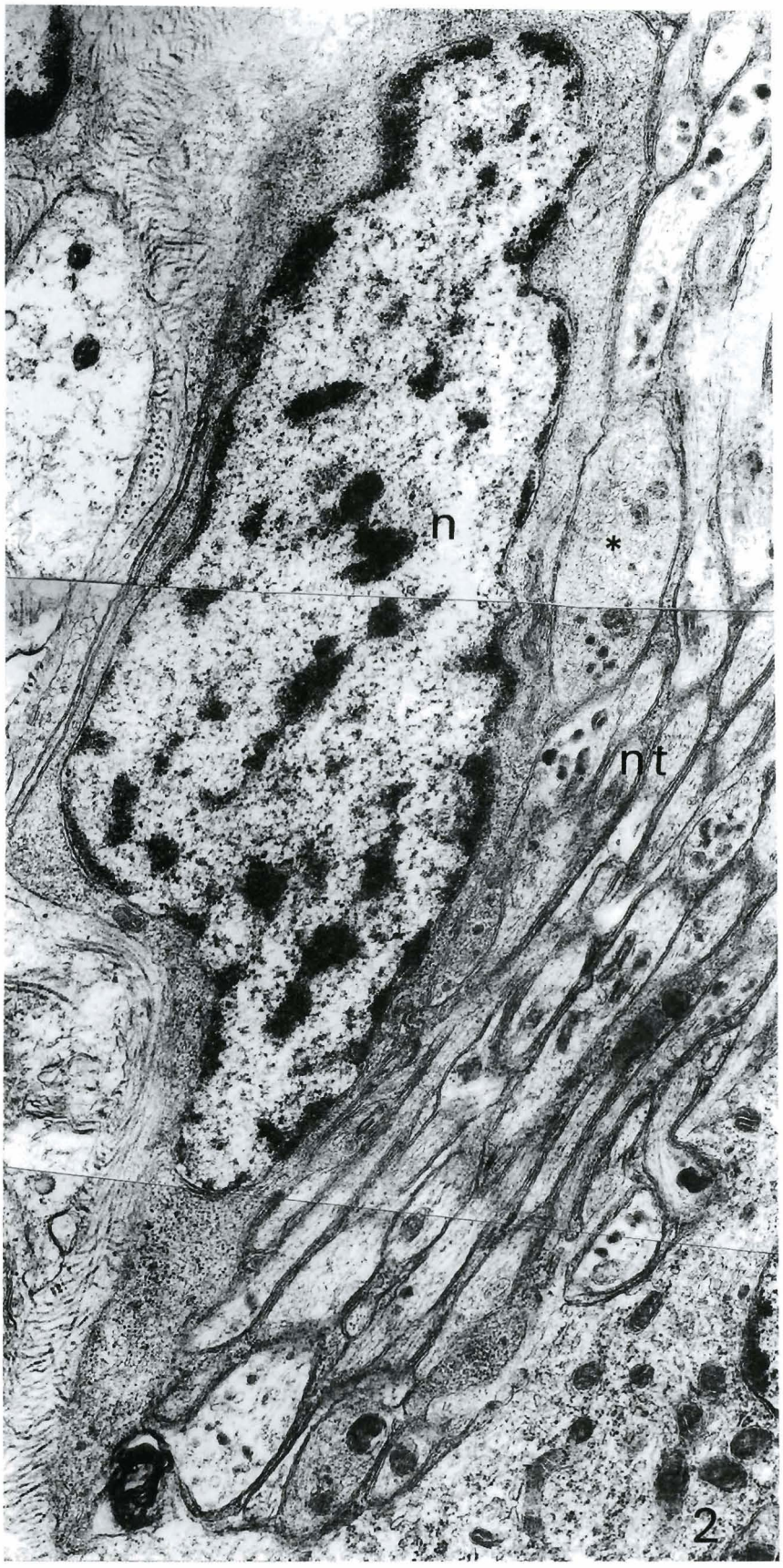


Fig. 2. Axonal varicosities in contact with a neurone in the myenteric plexus. Asterisk: varicosities with vesicles SAV; n: nucleus; nt: nervous trunk. x 15,000

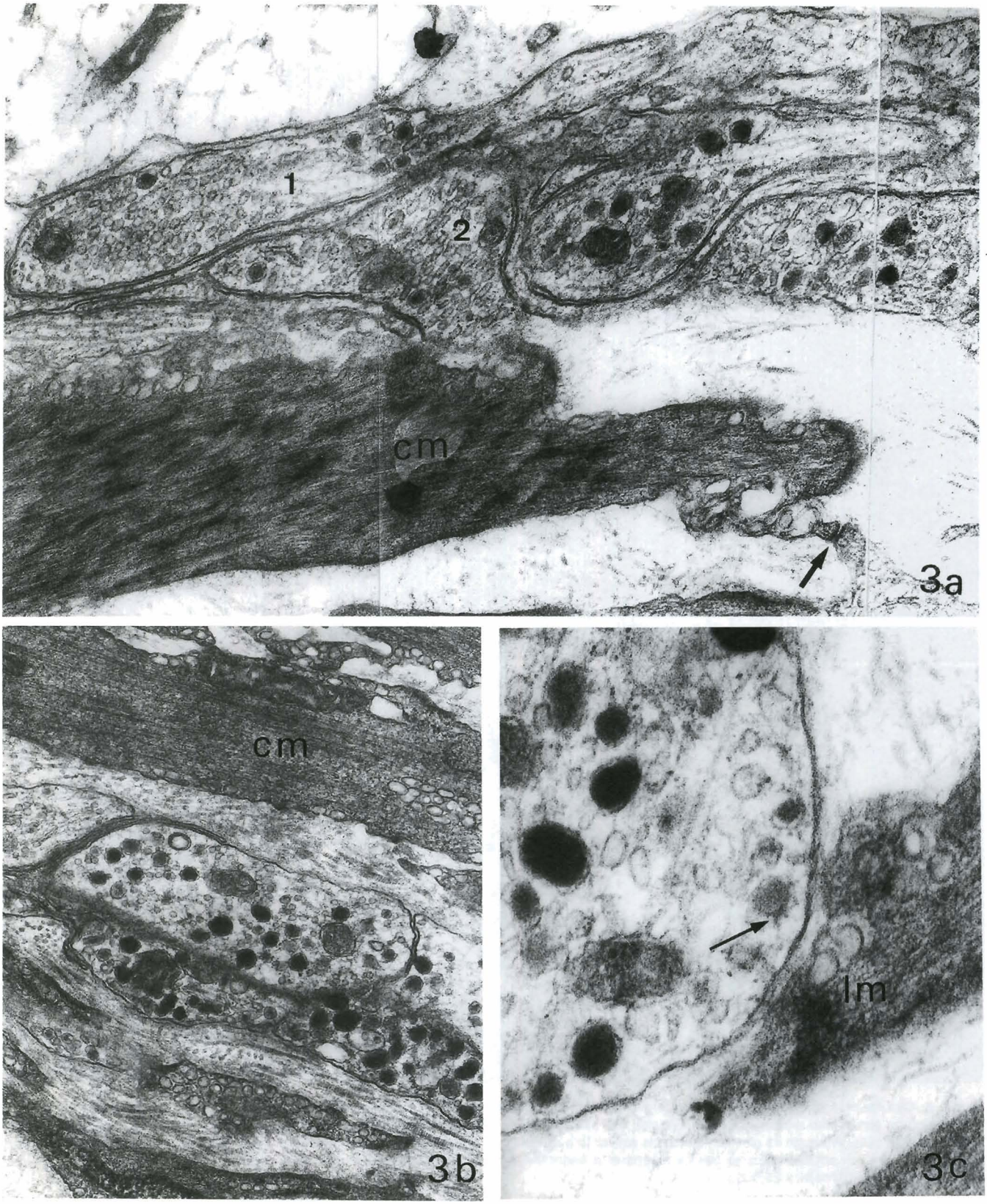


Fig. 3. Axonal varicosities among muscle cells. **a.** Mixed varicosity with electron-lucent vesicles (SAV) and some vesicles with electron-dense nucleus (1). Mixed varicosity in direct contact with a smooth muscle cell of the circular muscle layer (2). Arrowhead: gap junction between two muscle cells. x 25,000. **b.** LGV and SGV vesicles can be seen in the interior of varicosities near a muscle cell of the circular muscle layer. x 19,000. **c.** Mixed varicosities adapting their membrane profile to the surface of a muscle cell in the longitudinal muscle layer. Electron-dense enhancements in a vesicle disposed to exocytosis (arrow). x 57,000. cm: circular muscular layer; lm: longitudinal muscular layer.

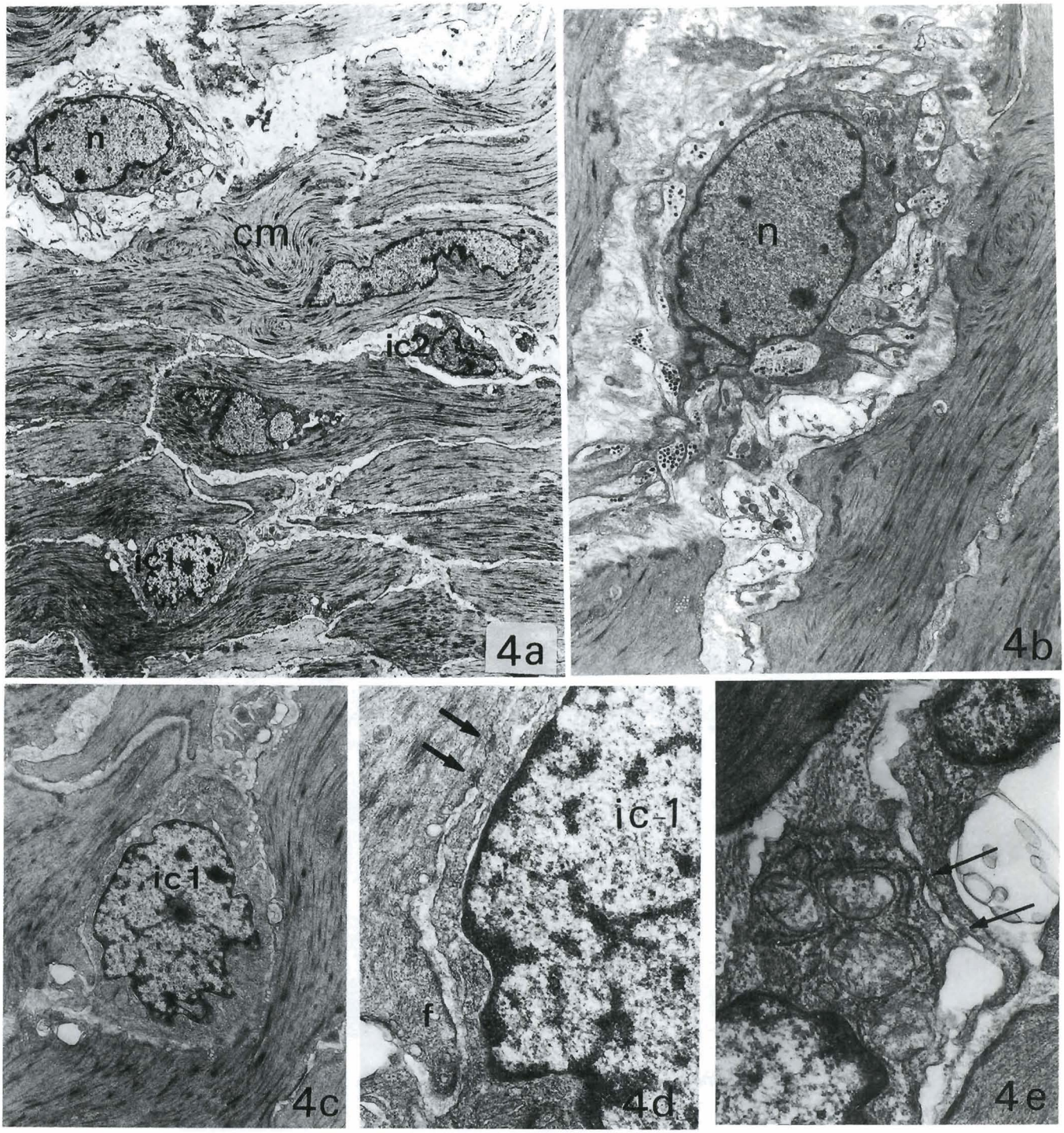
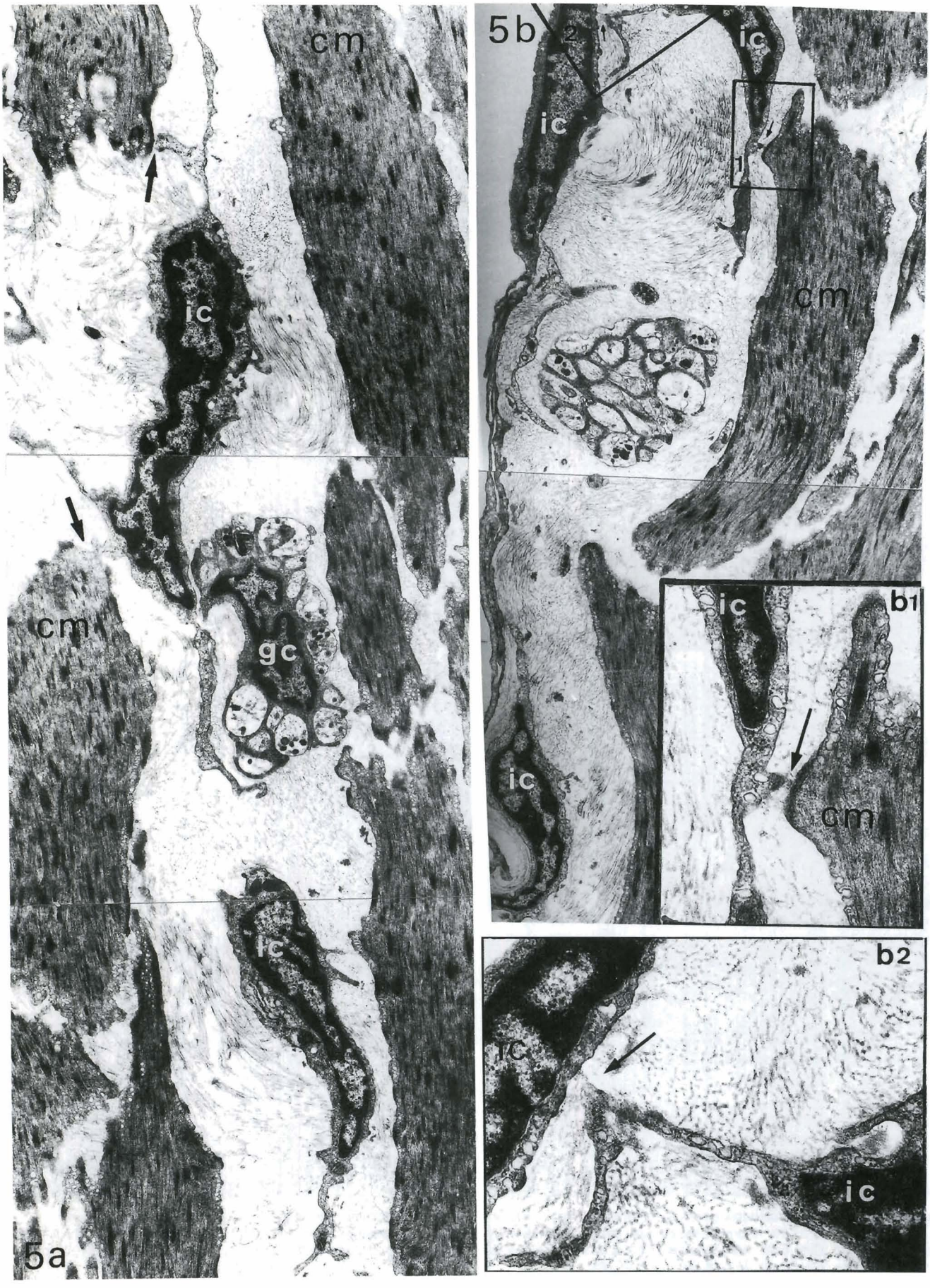


Fig. 4. a. Circular muscle layer (cm). Three different types of cells can be observed: neurone (n), ic-1, and ic-2. x 2,000. **b.** Intramuscular neurone surrounded by axons with different types of vesicles (n), showing finely granular chromatin and prominent nucleolus. x 4,000 **c.** Interstitial cell ic-1 among muscle cells. Although its electron-density is similar to a muscle cell, these ic-1 cells do not present dense bodies. x 4,000 **d.** Detail of a union between ic-1 and an adjacent muscle cell by means of a finger-like protrusion. Arrowhead shows small gap-like junctions. x 25,000. **e.** Contacts between two interstitial cells. x 25,000

Fig. 5. Interstitial cells between smooth muscle cells in the circular muscle layer. Long prolongations surround axons and establish gap-like junctions with smooth muscle cells (arrow). The same cell establishes contact with two muscle cells at the same time. x 4,000. **b.** Interstitial cells interconnected by their long prolongations, near a nervous trunk. x 3,700. **b1.** Contact between an ic-2 and a muscle cell. Note the presence of caveolae in the surface of the membrane. x 19,000. **b2.** Contact between two ic-2. x 19,000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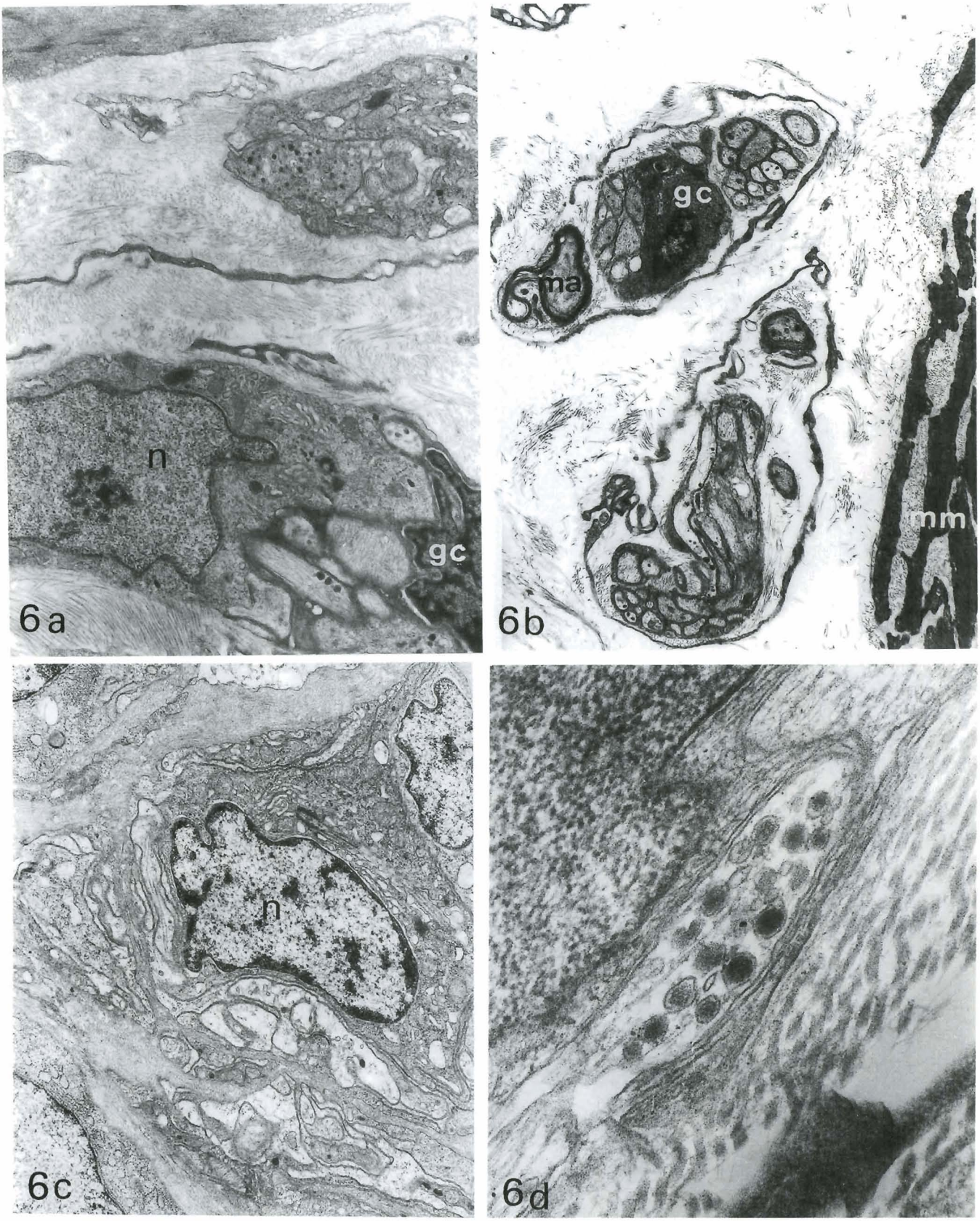

Fig. 6. a. Neurone of the submucous plexus (n), showing finely granular chromatin and prominent nucleolus. Glial cell surrounding axons (gc). x 7,100. **b.** Groups of axons in the submucosal plexus, located immediately under the muscularis mucosae (mm), surrounded by thin fibroblast-like prolongations. gc: Glial cells; ma: myelinated axon. x 3,400. **c.** Neurone in the lamina propria, with a typical spotting nucleus. x 6,400. **d.** Mixed varicosity in close contact with an epithelial cell. x 45,000

with each other and with neighbouring smoth muscle cells. Each cell could contact with several smooth muscle cells (Fig. 5a).

A net of fibre bundles constituted a submucosal plexus located under the circular muscle layer, with disperse isolated neuronal bodies among bundles of fibers. As in the myenteric plexus, glial cells surrounded the axons. These neurones presented homogeneously and finely distributed granular nuclear chromatin, and prominent nucleolus (Fig. 6a).

Bundles of nerve fibres constituted by groups of axons surrounding a glial cell could be seen in the connective tissue near the muscularis mucosae. Occasionally, some myelinated axons could appear in the peripheral part of the trunks, which were always surrounded by fine fibroblast-like prolongations (Fig. 6b).

A mucous plexus was located mainly in the triangular notches (in cross-sections) delineated by the lamina propria, surrounding the glandular crypts. Among the numerous groups of axons reaching this zone, we could also find isolated neurones, well differentiated from the glial cells due to their size and the tinctorial characteristics of the nucleus (Fig. 6c). Direct contacts between varicosities and glandular mucous cells could be observed (Fig. 6d).

Stellate interstitial cells with elaborate branching patterns were found in relationship with the nervous trunks of the lamina propria (Fig. 7a). These cells were characterized by a highly electron-dense cytoplasm, in which it was easy to see frequent basal bodies (Fig. 7b). The prolongations of these cells contacted each other by

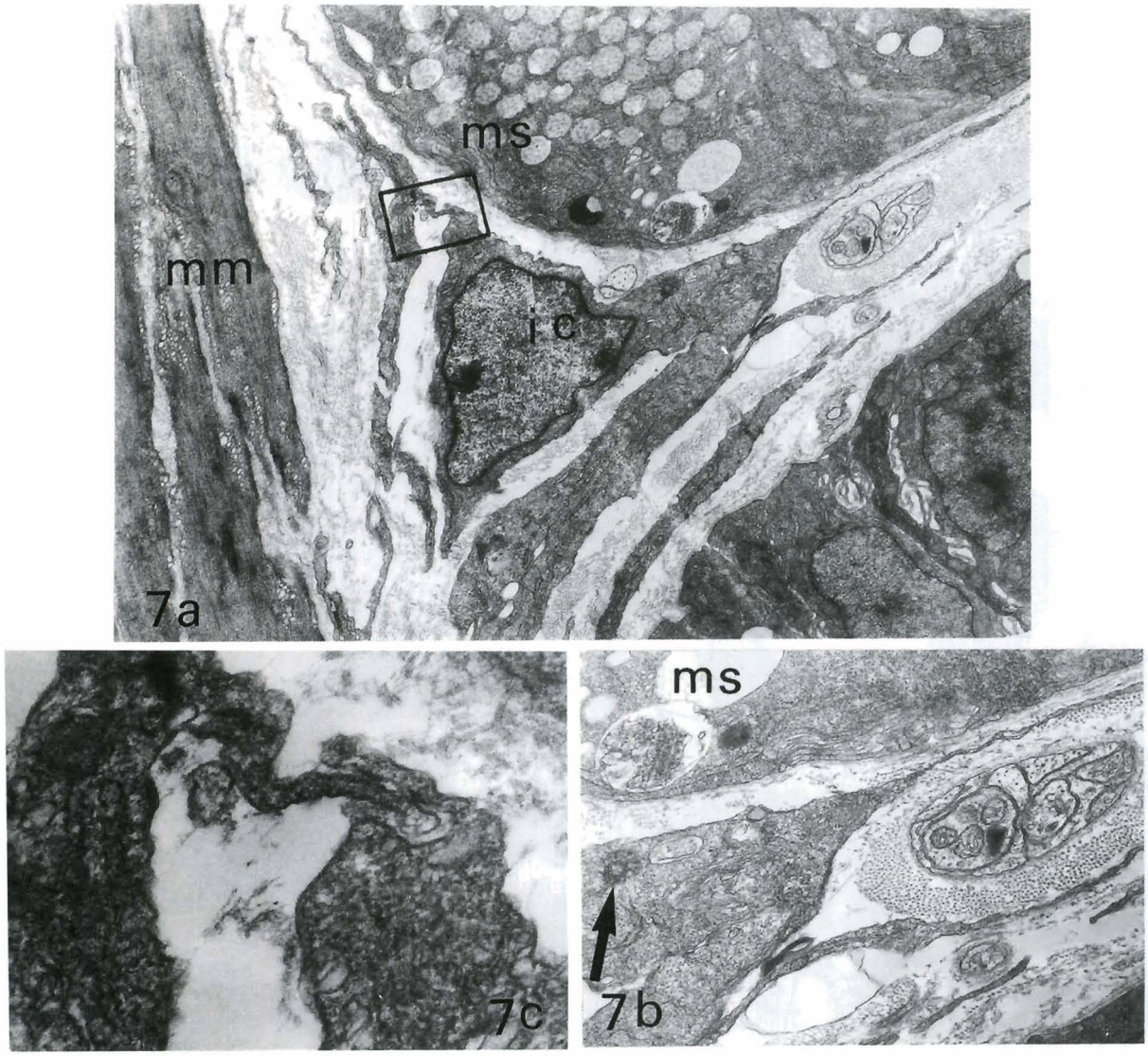

Fig. 7. a. Interstitial cell in the lamina propria. x 6,000. **b.** Detail of the previous image. We observe a basal body in the cytoplasm (arrow). Two prolongations distribute towards a small group of axons near the epithelium. x 20,000. **c.** Detail of Fig. 7a. Peg-and-socket junctions between two IC. x 60,000

means of peg-and-socket junctions (Fig. 7c). Some of these prolongations located between the epithelium base and the small axonal bundles reaching it (Fig. 7b).

In this area, there were also small nervous trunks innervating blood vessel walls (Fig. 8a). Some vesicle-containing varicosities established close contact with the endothelial cells, although we did not seen any synaptic specialisation between membranes of varicosities and endothelial cells (Fig. 8b). The previously described blood vessel innervation was the same throughout the stomach wall.

Discussion

The presence of ganglia protected only by the adventitia in the proximal part of the stomach, close to the oesophagus, may be interpreted as a morphological adaptation to the lack of a well-defined longitudinal muscle layer in this region. However, the posterior localisation of nodose ganglion in reptiles (Gaskell, 1886) may turn the vagus nerve into a ganglionated nerve, containing the neuronal bodies observed in this nervous trunk. The number of neurones per ganglion (two to six) is higher than in amphibians, and the ultrastructural characteristics of neurones and glial cells are very similar in both classes of vertebrates (Junquera et al., 1986).

The presence of a well-defined submucosal plexus in the stomach of *Lacerta hispanica* is evidence of increasing complexity in the ENS the higher we rise in the phylogenetic scale. Thus, in amphibians, the submucosal plexus is made up of a low number of scattered neurones (Wong et al., 1971; Wong, 1973; Junquera et al., 1987b). However, the dispersion of neuronal bodies among axonal bundles represents a structural organisation still far from the ganglion-organised mammalian innervating models. These results agree with the presence of neuronal cell bodies in the gut submucosal plexus of *Pseudemys scripta elegans* (Timmermans et al.,1991) and in the oesophagus of

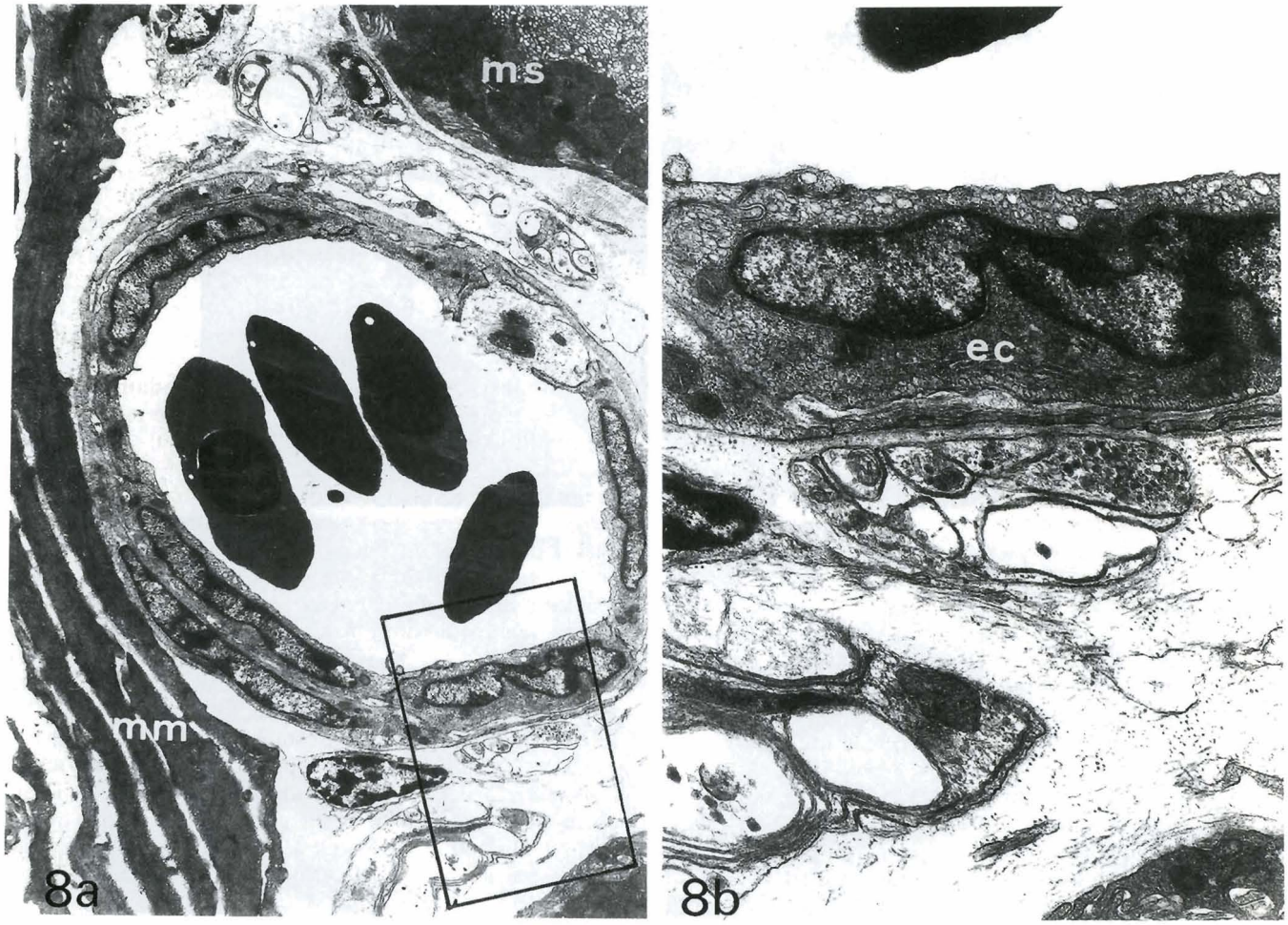

Fig. 8. Blood vessel in the stomach lamina propria. **a.** Innervation of a vessel, showing nucleated red blood cells, characteristic of reptiles. ms: mucosa; mm: muscularis mucosae. x 2,500. **b.** Detail of the previous image. Axonal varicosities in direct contact with the membrane of an endothelial cell (ec). x 11,000

Podarcis hispanica (Junquera et al., 1998).

A singularity of the organisation of the enteric plexuses in reptiles is the presence of occasional contacts between neuronal bodies and smooth muscle cells. In mammals, ganglia are surrounded by glial cells or, when this layer is not complete, by a collagen frame that isolates them from the underlying muscle cells (Gabella, 1982).

Classical studies of electron microscopy have assimilated certain characteristics of the axonal varicosities with their neurotransmitter content. For instance, varicosities that contain small electron-lucent vesicles are interpreted as cholinergic-type and large electron-dense vesicles are considered peptidergic (Baumgarten et al., 1970). However, the ultrastructural image only represents an instant of a dynamic process, such as the production of neurotransmitters. In addition, in some hibernating animals, seasonal variations have been described in the types of neurotransmitters produced (Singh, 1964; Toole et al., 1999). In the reptile stomach there is a predominating presence of mixed varicosities throughout the continuous net of the three plexuses previously described, with a quantitative predominance of the peptidergic component.

Previous immunohistochemical studies have established the presence of up to eleven different neuropeptides in the reptilian enteric nervous system (Reinecke et al., 1981; Buchan et al., 1983; Böttcher et al., 1985; Masini, 1986; Ohtani et al., 1989; Junquera et al., 1998; Martinez-Ciriano et al., 2000). This would explain the abundant varicosities with large electron-dense vesicles. The localisation of different neurotransmitters in the same nervous fibres of the myenteric plexus in turtles (Scheuermann et al., 1991), small intestine (Lezaun, 1997), and stomach (Lamanna et al., 1999) of lizards has been demonstrated by double-labelling techniques, which explains the presence of mixed varicosities. This is further evidence of neurochemical complexity of the ENS in reptiles.

Nerve-muscle contacts have frequently been detected in certain developmental stages of different species: in the chicken midgut (Boros and Fekete, 1993); in the guinea pig (Gershon et al., 1981); in the rabbit small intestine (Daikokv et al., 1975); and in the small intestine of the developing human fetus (Fekete et al., 1996). It has been established that changing microenvironmental conditions cause sequential patterns of nerve-muscle contacts, and that these contacts disappear as embrionary development evolves – i.e. in humans these contacts disappear by the 26th week of gestation (Fekete et al., 1996). We have observed direct contacts between vesicle-containing varicosities and smooth muscle cells in both longitudinal and circular muscle layers in adult lacertas. Our findings should be interpreted as a reminiscence that agrees with the concept of ontogenia summarising phylogenia. However, in 1988, Komuro described direct contacts between the muscle processes and axon varicosities in the ganglia only in the longitudinal layer in the small intestine of the adult rat, considering that they might play a role as synaptic sites between ganglion and longitudinal muscle cells.

The existence of interstitial cells especially related to nerves and smooth muscle in the gut of amphibian and mammalian was first reported by Cajal (1893, 1911). Due to their location in the gut, and in specific species, the appearence of ICC is markedly heterogeneous, ranging from cells closely resembling smooth muscle cells to those similar to fibroblasts. Both cellular types that we describe in this study share their close relationship with nerves and smooth muscle cells. We consider that these characteristics are unequivocal for their consideration as interstitial cells of Cajal. However, their different morphology leads us to consider them as two different types of cells. The ones that we defined as IC-1 present similar ultrastructural features to those described in mammals by Komuro (Komuro et al. 1999) as more muscle-like ICC. Those defined as IC-2 have a high cytoplasmic electron-density and contoured nucleus with dense heterochromatin and have a greater resemblance to the fibroblast-like cells, although the presence of caveolae in their membranes are typical of muscle-like ICC. Fibroblast-like cells in the gut musculature generally form small gap junctions with surrounding smooth muscle cells. The contact regions between the IC-2 cells and muscle cells were often punctate in profile but were extensive enough to reveal that their intercellular clefts were of less than 15-20 nm. These types of contact were also described by Horiguchi in the mouse small intestine (Horiguchi and Komuro, 2000). He proposes that these gap-like unions play a role as routes for intercellular communication. A remarkable feature of the fibroblast-like cells is that their ultrastructure is rather uniform irrespective of the tissue layer, organ, or species in which they are found. This fact suggests that they may have a fundamental and possibly more general role than ICC in the regulation of gut motility (Komuro et al.,1999).

ICCs of various morphologies have been described in the gastrointestinal tract of mammals (Ishikawa et al., 1997; Komuro et al., 1999). Burns et al. (1997) described at least 6 types of ICC in the guinea-pig gut, according to their location. Three of their locations are the same in *Lacerta hispanica*: 1.- ICCs in the myenteric plexus region; 2.- intramuscular ICCs, lying within the muscle layers; and 3.- ICCs at the submucosal surface of the circular muscle layer. However, we have not found any references of ICCs in the lamina propria. The interstitial cells that we describe in this location are interconnected by means of specialized peg-and-socket-type unions between their long prolongations. Their close relationship with small axonal bundles that reach the mucosa and the proximity to the muscularis mucosae in this portion of the posterior stomach of Lacerta may suggest that these interstitial cells play a role in mechanoreception.

Either ICC-like or fibroblast-like cells participate in the structural organisation of the nervous plexuses of

Podarcis hispanica stomach, limiting ganglia and progressing across the circular muscle layer around the nervous trunks.

Physiological studies of ICC have led to a general agreement that the motility of the mammalian digestive tract is mostly regulated by these cells (Daniel and Berezin, 1992). ICCs are pacemaker cells (Thuneberg, 1982) and intermediaries in enteric neurotransmission in the gastrointestinal tract (Cajal, 1911; Publicover et al., 1993; Wang et al., 1999). A role of ICC in mechanoreception seems likely but is still uncertain (Faussone-Pellegrini et al., 1997). We think that such a special type of cell that has been constant throughout the whole phylogenetic scale of vertebrates should carry out similar functional roles in different phylum. New electrophysiological studies or studies with specific markers, such as c-kit, should improve our knowledge of their functional activities in reptiles.

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