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Frog stomach enteric plexuses in culture: isolation, morphological characterization and bioelectrical recordings

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Summary. We have succeeded in the isolation, culture and morphological characterization of *Rana ridibunda* stomach enteric plexuses. We have furthermore obtained intra and extracellular bioelectric recordings from the explants in culture. The culture medium used (Eagle MEM), the collagenase digestion and the general culture conditions followed are similar to those applied to mammal enteric plexus explant cultures. The most striking difference is that the solutions were diluted to 70% in order to maintain the osmolar conditions required by the amphibian cells. Acetylcholinesterase, osmium tetroxide-zinc iodide- and para-formaldehyde-induced fluorescence methods reveal similar morphological images from the perivascular fibre plexuses. The different cell types observed by phase contrast light microscopy from the myenteric explants in culture have been identified by comparison with those revealed by the acetylcholinesterase method. The prevailing neurons show piramidal somas; other neurons are bipolar with oval somas and a third type shows oval somas tightly aligned, following sinusoidal courses. The intra and extracellular bioelectric recordings from the explants in culture show that the culture conditions we have applied preserve the electrophysiological properties of the neuronal membranes. These preliminary recordings will allow us to undertake the synaptic characterization of the gastrointestinal neurotransmitters in frogs.

Key words: Enteric plexuses cultures, *Rana ridibunda,* Stomach innervation, Cholinergic innervation, Myenteric plexus

Introduction

We have described elsewhere the cholinergic, adrenergic, and peptidergic (VIP, SOM and SP) innervation of the enteric nervous system (ENS) of *Rana ridibunda* stomach (Junquera et al., 1986, 1987a), intestine (Junquera et al., 1987b) and esophagus (Junquera et al., 1988). We have shown that the different plexus innervation patterns follow the general organization exhibited by mammals (Gershon, 1981). Myenteric ganglia are nonetheless very simple in their organization with only 3 to 6 neurons. The ENS shows a neurochemical complexity in its organization unique among the neuron networks outside the central nervous system (CNS). Since it is very widely distributed in the gastrointestinal wall, from the esophagus to the anus, it contains, at least in mammals, as many neurons as the spinal cord itself (Furness and Costa, 1987). The nerve cells are disposed in an intricate system of circuits which contains the different elements mediating the local reflexes: «sensory neurons», recording factors such as the mechanical tension of the gastrointestinal wall or the chemical content of the lumen; «interneurons» and «motor neurons» which are able to control the muscle coat contractions blood vessel lumen, secretory mechanisms, etc. It is very well established that the ENS is very independent from a functional point of view, in spite of the connections with the CNS and with autonomic ganglia outside the walls of the gastrointestinal tract.

The electrophysiological interelationships among the neuronal components of the gut wall have been undertaken almost exclusively in mammals (Nishi and North, 1973; Hirst et al., 1974; North, 1982; Bornstein et al., 1989). The aim of the present work is the isolation and culture of the *Rana ridibunda* stomach plexuses in order to morphologically characterize the neurons observed by phase-contrast light microscopy and to obtain intracellular and extracellular bioelectric recordings as a test of the preservation of the electrophysiological properties of the neuronal membrane.

Materials and methods

Tissues were obtained from 10 adult frogs *(Rana*

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ridibunda). Segments of the entire stomach wall, measuring approximately 2.5mm^2 were cut out and processed as follows:

Myenteric plexuses culture

We have mostly followed the method of Jessen et al. (1983). The mucose layer was removed from the stomach wall. The tissue was washed in Hank's Balanced Salt Solution (HBSS) to remove all tissue traces and then rinsed twice in HBSS containing antibiotics and antimycotic (antibiotics/antimycotic mixture, Sigma A7292) for a total of 30min. The tissue was immersed in a 0.5% collagenase solution in culture medium, placed overnight at 4° C and then incubated in tissue culture medium, with bovine foetal serum and the antibiotics/ antimycotic mixture, at room temperature for 1h. After the enzyme treatment, the myenteric plexus was dissected from the circular muscle coat leaving part of the longitudinal musculature. The explants were transferred with a Pasteur pipette onto polylysine-coated coverslips and were kept in Petri dishes at room temperature. The tissue culture medium was changed every couple of days. Plexus explants were grown, after the first change, in Eagle's MEM medium (Sigma M1018), containing foetal bovine serum (10%), glucose (0.5%), HEPES (10mM), bicarbonate (0.35g/L) and penicillin (100u/ml). Both HBSS and the culture medium were diluted up to 70% in distilled water in order to maintain the osmolar requirements of the amphibian cells (Wolf and Quimbly, 1964).

Morphological characterization of plexuses

We have applied the following methods:

Acetycholinesterase method (AChE)

We have combined the methods of El Badawi and Shenck (1967) and Qayyum and Fatani (1985). The stomach pieces were fixed for 5-30 min in a solution of 2% glyoxylic acid (pH 7.3) at room temperature and then delaminated. The mucose, submucose and inner muscular layer was removed, uncovering the contact surfaces between the two external muscle coats: the circular and the longitudinal ones. Both tissue sheets were postfixed in 2% formaldehyde in PBS, washed in distilled water and then incubated in the specific AChE medium of El Badawi and Schenck. The cholinesterase reaction was controlled every 15 min under a light microscope. We have obtained the best results after incubating periods of 1-2h. The tissue samples were then dehydrated through a graded series of ethanol, cleared in xylene and mounted with DPX.

Catecholamines method (FIF)

We have modified the method of Furness and Costa (1975). The tissue samples were processed as previously described in the AChE method. Once the stomach wall was delaminated both tissue sheets were stretched out on a slide and exposed to paraformaldehyde vapours (1-3 h at 80° C). The preparations were visualized under U.V. light.

Osmium tetroxide - zinc iodide method (OTZI)

We have followed the method described by Jabonero et al. (1961). Once the mucose layer had been removed, the stomach wall portions were stretched out on small pieces of cardboard. The tissue was then fixed in a solution made by a mixture of one part 2% osmium tetroxide plus three parts 3% zinc iodide in distilled water. If an excess of staining was observed, the tissue samples were decolourized by immersion during 1/2 h in a solution of 3% potassium permanganate followed by 5 min immersion in an aqueous solution of oxalic acid dehydrated and DPX mounted.

Bioelectric recordings

The explants were transferred into a perspex chamber and were visualized under an inverted microscope (Olympus CK2). We used glass microelectrodes with inner filament. For the intracellular recordings we filled the microelectrodes with a solution of 1M potassium acetate (tip resistance 2-20M Ω) and for the extracellular recordings we used tip agar microelectrodes filled with a 3M NaCl solution (tip resistance 40-50M Ω). The microelectrode was connected to a preamplifier (microelectrode probe 5176, Palmer BioScience), amplifier (Mod 6362 Palmer Bioscience) which in turn was connected to an oscilloscope (HAMEG HM 205-2).

Results

We have undertaken the morphological characterization of the *Rana ridibunda* stomach plexuses by appling the methods of acetylcholinesterase-(AChE), osmium tetroxide-zinc iodide- (OTZI) and indirect paraformaldehyde-induced fluorescence (FIF). The OTZI method inspecifically stains all the nerve fibres, the AChE method reveals the cholinergic fibre population and the FIF method shows the catecholaminergic fibres, which in amphibians are adrenergic (Junquera, 1986). The three methods above mentioned have revealed perivascular nervous fibre plexuses quite similar in their distribution (Fig. 1). The OTZI method shows a nervous fibre myenteric network similar to the one observed with AChE method and under phase-contrast light microscopy from the cultured explants (Fig. 2). In the submucous plexuses the AChE and OTZI methods also show a parallel distribution of the nervous fibres (Fig. 3). The networks are narrower than those shown at the level of the myenteric plexuses since the fibres are disposed surrounding the mucous glands.

The cholinergic fibre networks spread between ganglia containing 3 to 5 neurons. Pyramidal soma neurons are the predominant nerve cells in the myenteric plexuses. We have found their homologous with the OTZI method and they have also been observed by phase-contrast light microscopy in the culture explants

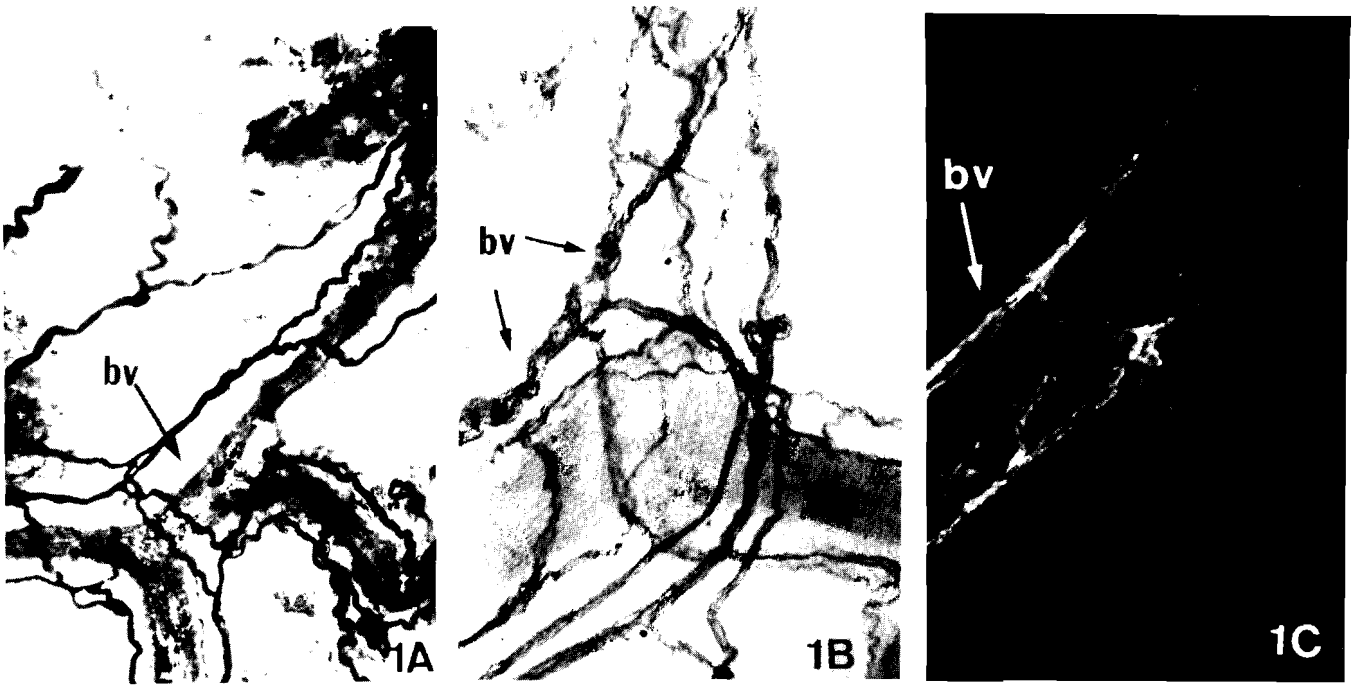


Fig. 1. Perivascular nerve plexuses in the stomach of *Rana ridibunda*. **A)** Osmium tetroxide-zinc iodide (OTZI) method. x40; **B)** AChE method. x50; **C)** FIF method (bv: blood vessel). x40

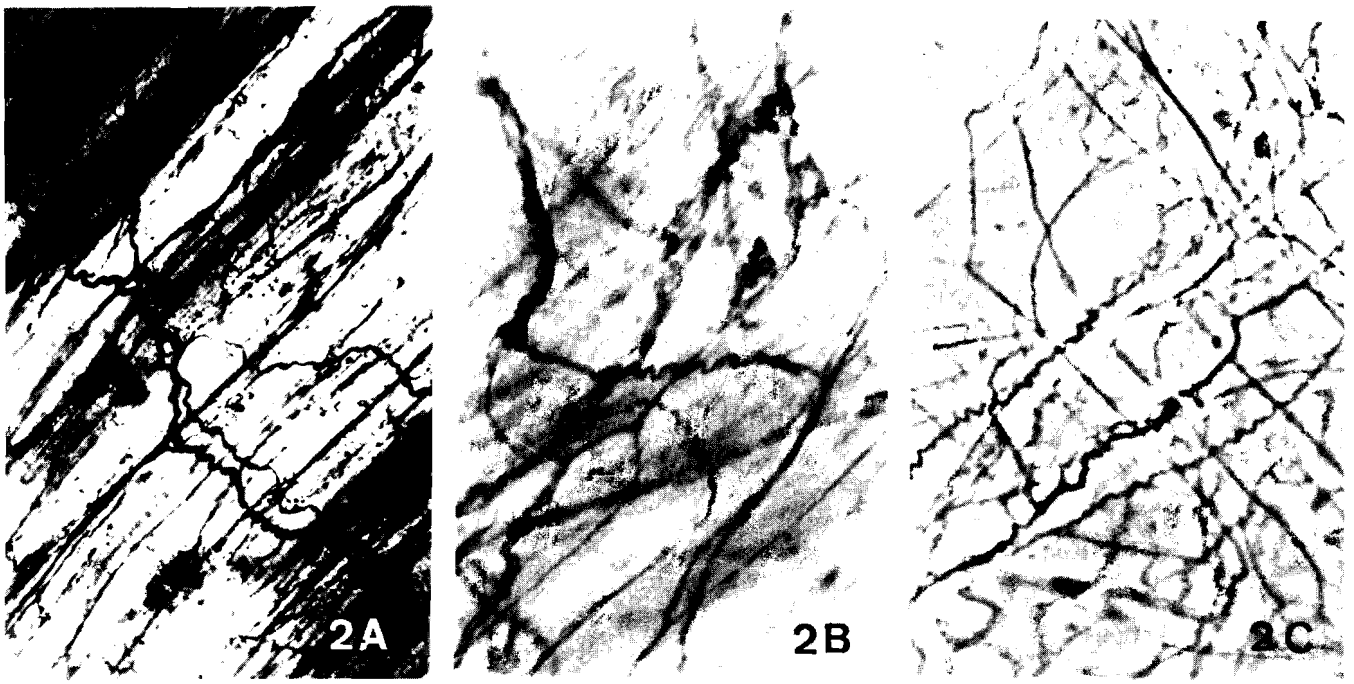


Fig. 2. The OTZI method **(A)** reveals a myenteric nervous fibre network similar to the one observed with the AChE method **(B)** and to the cultured explants **(C)** with phase-contrast light microscopy (A: x25; B: x30; C: x30)

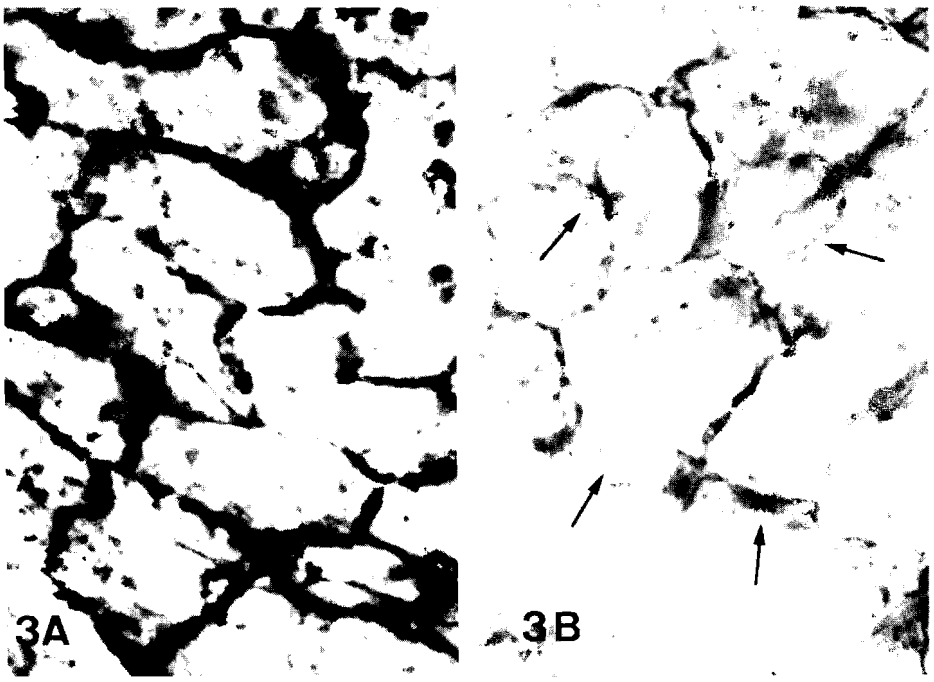


Fig. 3. The submucous fibre plexuses observed with the OTZI method **(A)** and with the AChE method **(B)** are quite parallel, the networks being narrower than those observed between the outer muscle coats (A: x25; B: x20)

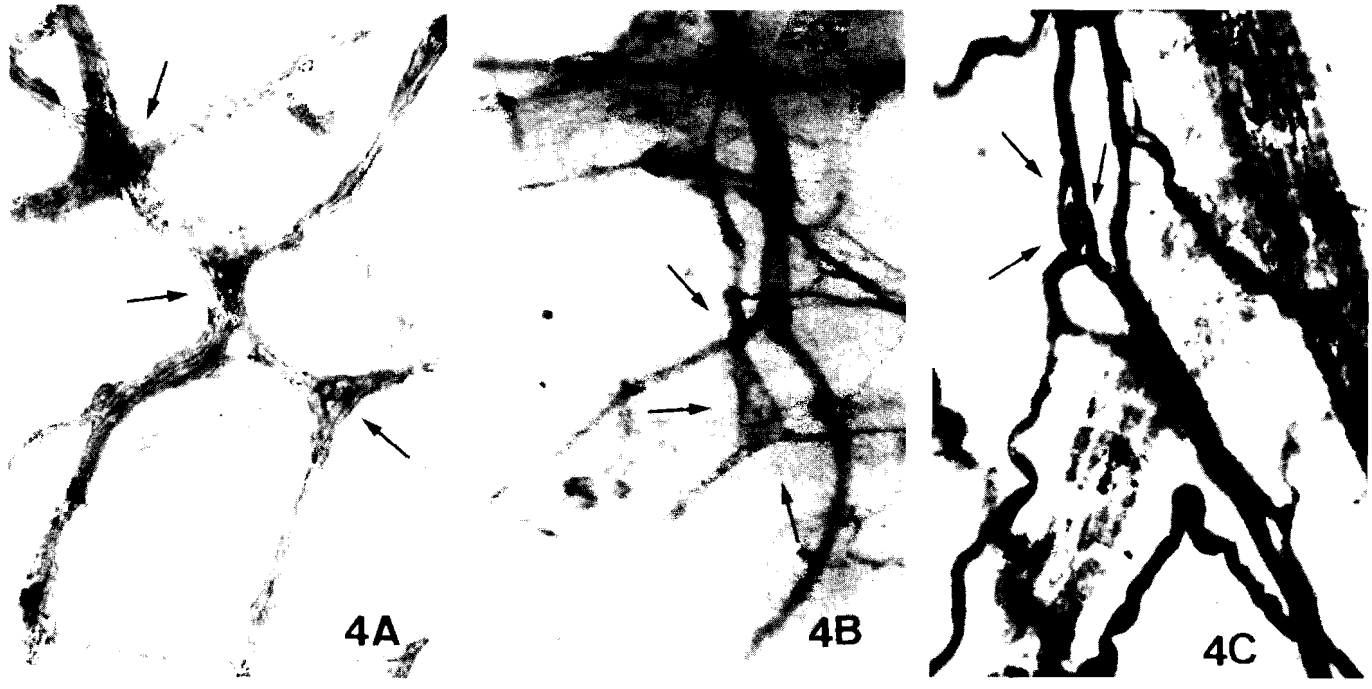
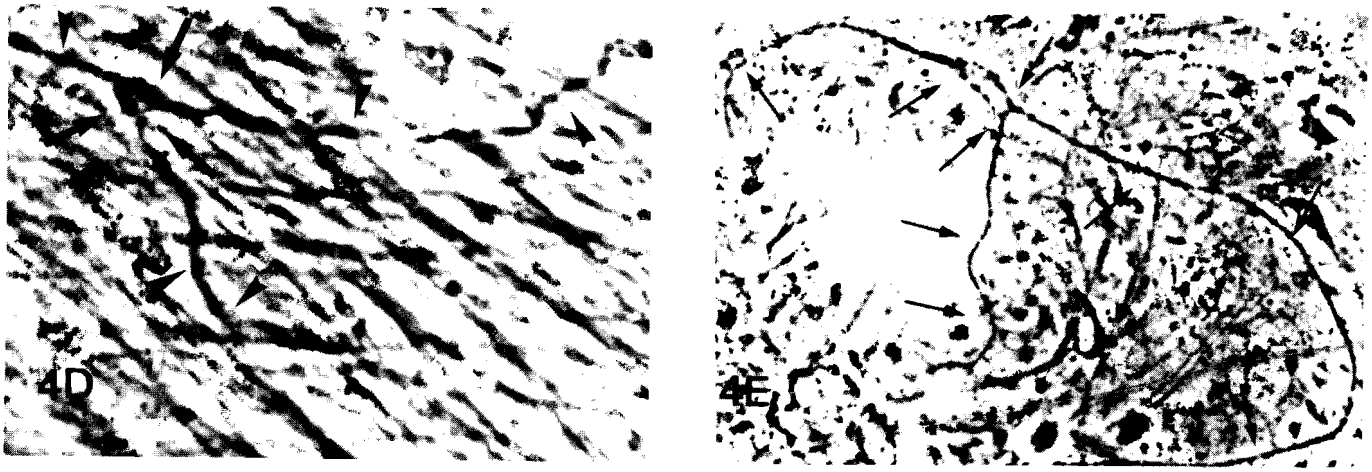
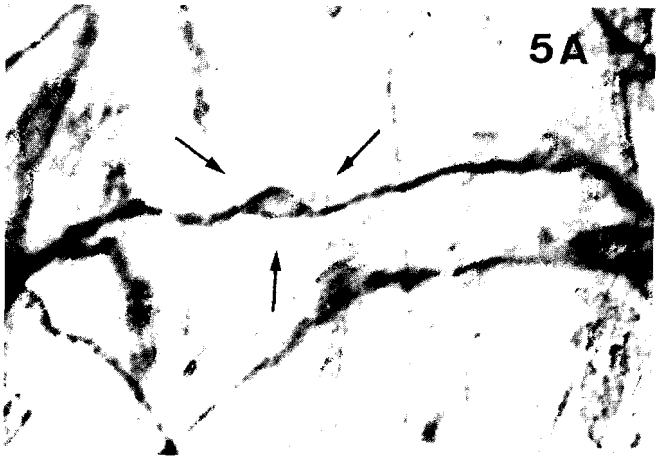
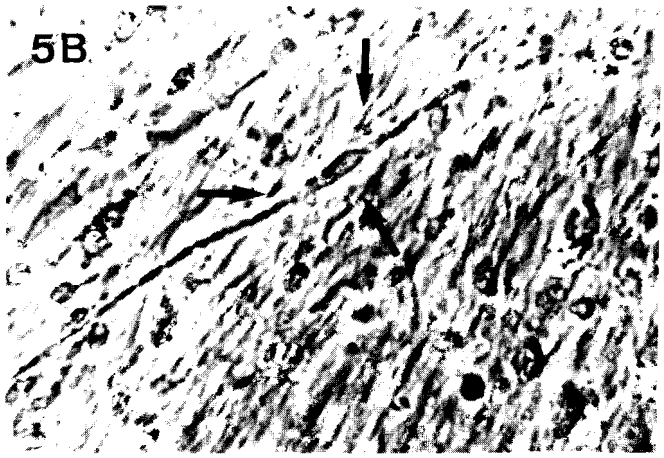
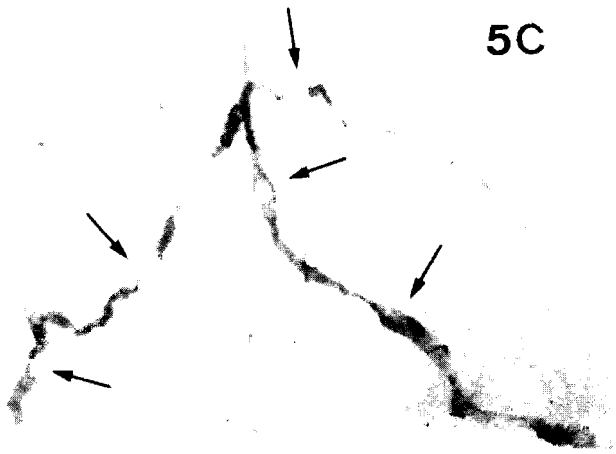


Fig. 4. The AChE method reveals neuron ganglia in the interconnection points of the network **(A)** and pyramidal soma neurons **(B).** The OTZI method reveals similar neuron types **(C).** We have found their homologous in the cultured plexuses **(D-E)** (A: x20; B: x35; C: x50; D: x32; E: x30)









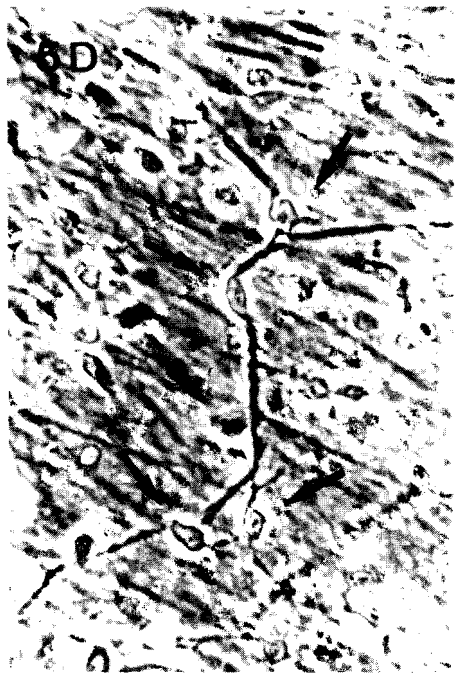
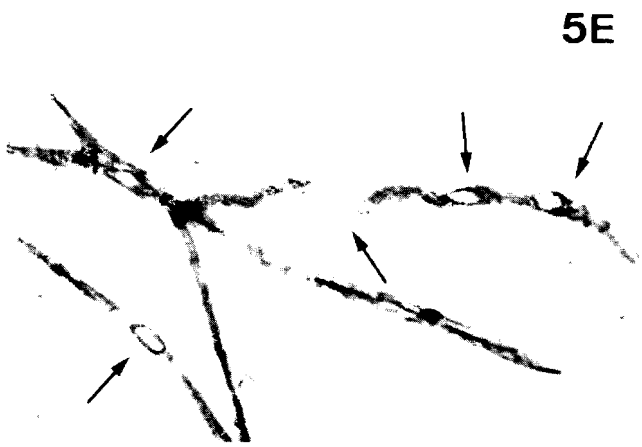
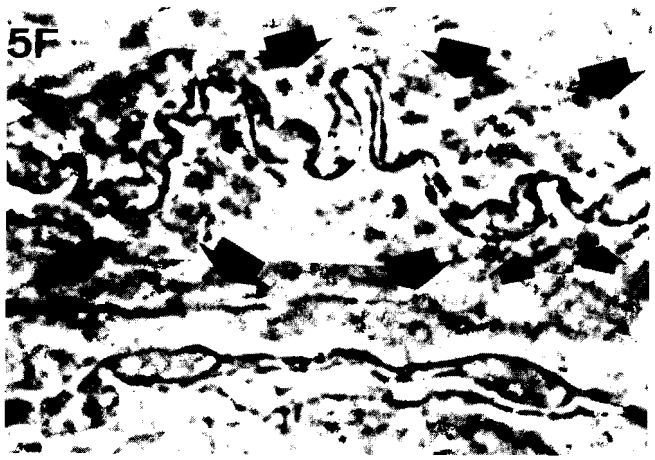
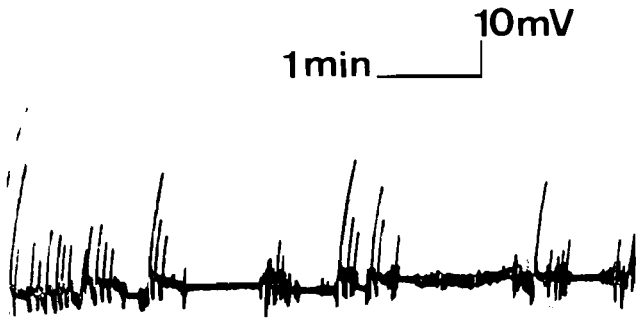


Fig. 5. The AChE method has revealed several other neuron types with: oval somas **(A)** and an oval shape with somas aligned following sinusoidal tracings **(C, E).** Similar shaped neurons have been found in the cultured explants **(A-B, C-D, E-F)** (A: x35; B: x32; C: x32; D: x32; E: x32; F: x60)







6A

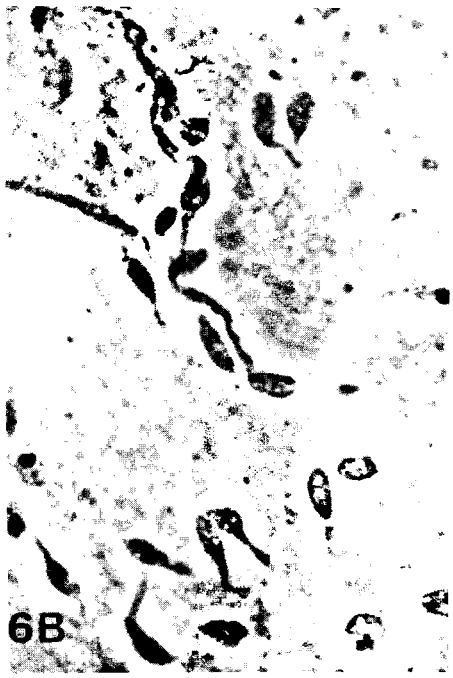
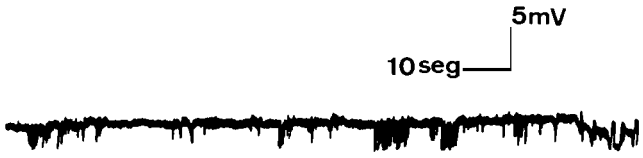


Fig. 6. Intracellular recordings **(A)** from isolated bipolar neurons **(B)** after three days in culture. Em –25mV. (B:x35)



7A

7B

Fig. 7. Extracellular recordings **(A, B)** from neurons disposed in rather compact plexuses **(C)** after 4 weeks in culture (C: x35)

(Fig. 4). The AChE method has also revealed several other neuron types: neurons with oval shaped soma and oval shaped neurons with somas tightly aligned following a sinuosal tracing. We have found these two cell types in the cultured explants (Fig. 5).

The cultures have been maintained for 4 weeks. Intracellular bioelectric activity (Fig. 6) has been recorded from bipolar neurons with oval somas (Fig. 6) after three days in culture. Extracellular activity (Fig. 7) has been recorded from neurons disposed in quite compact networks (Fig. 7) after four weeks in culture.

Discussion

The isolation and culture of enteric plexus explants from the *Rana ridibunda* stomach has been by no means an easy work to do. The small neuronal soma number in the myenteric ganglia makes these plexuses extremely slender, delicate and not very consistent. These characteristics render their extraction and handling difficult. The collagenase treatment favours the separation of the plexuses from the longitudinal and circular muscle coats, but at the same time it affects the ganglia structure. The ganglionic neurons dissociates making it difficult to ascertain ganglionic structures from the explants in culture under phase-contrast microscopy. In order to increase the plexus consistency we have preserved a part of the longitudinal muscle coat in a special situation since the muscle coat can interfere with the plexus observation under the inverted microscope.

The guinea pig ileum myenteric plexuses are mostly the only ones which have so far been used to undertake the electrophysiological properties of the ENS (Nishi and North, 1973; Hirst et al., 1974; North, 1982; Bornstein et al., 1989). More recently new born guinea pigs have been chosen, since a cell proliferation and migration to the neighbouring regions is produced from the ileum explants in culture (Banerman et al., 1987, 1988). In our experiments the explants have been extracted from adult frogs and we have observed neither cell proliferation nor cell migration from the enteric ganglia. In order to impale the microelectrodes we have had to choose those parts of the plexuses which by chance were free from the underlying muscle coat.

Recent works have revealed that the number of neurons in the myenteric plexus of the jejunum, ileum, colon and rectum decreases in aged rats. It has been considered conceivable that changes with age in the distensibility of the gut could affect the neuron count (Santer and Baker, 1988). Gabella (1971) has observed ultrastructural degenerating neurons in adult rat small intestine. Taken as a whole, the evidence suggests that both extrinsic and intrinsic innervation of the rat intestinal tract is greatly reduced in amount in old age. According to these data it seems convenient to choose young animals and better new born ones to undertake these types of experiments.

Once overcome the diverse difficulties which have arisen throughout the experiments, we have verified that the neurons from the enteric plexuses of *Rana ridibunda* stomach are very similar in size to the mamalian ones (10-20 μm). It is well known that conditioned culturing media can alter electrophysiological neuron properties (Nismi and Willard, 1988; Romijn, 1988). The intra and extracellular recordings we have made from the explants show that the electrophysiological properties of the neurons are preserved in our culture conditions. We have shown in previous studies that in frogs, according to their phylogenetic position in the vertebrate scale, the peptidergic nervous system (at least for VIP, SP and SOM) is more developed than the sympathetic nervous system (Junquera et al., 1987a,b). We consider that this experimental model can be of interest to carry out the study of synaptic properties of peptides in the amphibian gut wall.

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