Histol Histopath (1990) 5: 275-280

Histology and Histopathology

The autonomic innervation of the liver and gallbladder of *Podarcis hispanica*

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Summary. The innervation of the liver and gallbladder of the lizard *Podarcis hispanica* has been studied by the following methods: a) demonstration of cholinesterase activity; b) FIF method for catecholamines; and c) immunohistochemistry for VIP.

The hepatic parenchyma of the reptile's liver show hepatocytes arranged in regular rows of hepatic cords, the portal triad being typical of higher vertebrates (birds and mammals). Nerve fibers are found in the scarce connective tissue distributed among the hepatocytes. The innervation is restricted to the big branches of blood vessels and biliary ducts. It is represented by cholinergic, noradrenergic and VIPergic fibers.

The gallbladder shows a well developed cholinergic plexus with pyramidal cells in the interconnection points of the fiber network. The noradrenergic and VIPergic plexuses are also more widely distributed in the gallbladder than in the liver.

Key words: Liver, Gallbladder, Autonomic innervation, Lizard

Introduction

In an ultrastructural comparative study Tsuneki and Ichihara (1981) describe relatively abundant nerve fibers in the liver of the turtle *Pseudemys scripta* distributed not only in the hepatic parenchyma connective tissue but also making direct contact with hepatocytes. Although the liver is one of the most extensively studied organs, rather little attention has been paid to nerve fibers in the liver or in the gallbladder of the vertebrate phylum particularly in lower vertebrates. The neurochemical nature of the nerve fibers in mammalian liver has been studied by: i) histochemical methods: acetylcholinesterase method (Sutherland, 1964; Skaaring and Bierring, 1976; Reilly et al., 1978; Amenta et al., 1981; Sanz et al., 1982) and fluorescence method for catecholamines (Ungvary and Donath, 1969; Metz and Forssmann, 1979, 1980; Jarhult et al., 1981; Sanz et al., 1982); ii) immunohistochemistry methods (Garin et al., 1982; Sanz et al., 1982); iii) and at ultrastructural level (Forssmann and Ito, 1977; Nobin et al., 1978; Metz and Forssmann, 1979, 1980; Tsuneki and Ichihara, 1981; Sanz et al., 1982). We have also described the cholinergic, catecholaminergic and VIPergic innervation of the liver and gallbladder of *Rana ridibunda* at optic and electron microscopy level (Azanza et al., 1989). It is a controversial matter whether the nerve fibers enter the hepatic lobules and terminate directly on the hepatocytes. The presence of intralobular nerves has been reported electron microscopically so far only in four species: mouse (Yamada, 1965), tree shrew (Forssmann and Ito, 1977), rhesus monkey (Nobin et al., 1978) and man (Ito and Shibasaki, 1968; Nobin et al., 1978). We have observed with light microscopy that in mammals the fine cholinergic fibers can rise the first row of the hepatic lobule hepatocytes (Sanz et al., 1982). We have also observed that in some mammals (cat, pig, sheep and lamb) the noradrenergic innervation is also extended to the hepatic parenchyma (Sanz et al., 1982). Furthermore, and as an exception, a fiber plexus in the rat hepatic parenchyma seems to exist of nervous nature and vagal origin as has been shown by the cobaltous chloride impregnation method (Azanza, 1987).

The aim of the present work was to carry out the study of the liver and gallbladder of *Podarcis hispanica* in order to establish the cholinergic, catecholaminergic and VIPergic innervation pattern at optic level.

Materials and methods

The liver of 10 specimens of *Podarcis hispanica* have been used in this study. The general histology of the liver and gallbladder was examined after staining with

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haematoxilyn and eosin.

Acetylcholinesterase method (AchE)

Acetylthyocholine (Sigma) was used for demonstrating the acetylcholinesterase activity (AchE) according to the El Badawi and Schenk method (1967). The tissue 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 2-6 hr at room temperature. The 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).

The cholinergic plexus characterization of the gallbladder wall was undertaken *in toto* preparations. We have combined the method of El Badawi and Schenk (1967), above described, and that of Quayyum and Fatani (1985). The gallbladder was fixed for 5-30 min in a solution of 2% glyoxylic acid (pH 7.3) at room temperature and then the mucose layer was removed. The tissue was postfixed in 2% formaldehyde in PBS, washed in distilled water and then incubated in the specific AChE medium of El Badawi and Schenk (1967). The cholinesterase reaction was controlled every 15 min under the light microscope. The best results were obtained after incubation periods of 1-2 h. The tissue samples were then dehydrated through a graded series of increasing ethanol concentrations, cleared in xylene and mounted with DPX.

Catecholamines method (FIF)

The noradrenergic fibres were visualized by the glyoxilic-formaldehyde method (Furness and Costa, 1975). The tissue was fixed by immersion in 2% glyoxilic acid in PBS (pH 7.4) for 3 hr. It was then frozen in methylbutane with liquid nitrogen, frozen overnight, exposed to paraformaldehyde vapours (3 hr at 80° C) and vacuum embedded in paraffin wax. Sections of 15-20 μm were examined under a Leitz orthoplan fluorescence microscope. Adrenaline produced an apple green fluorescence, while histamine and serotonine produced a yellow fluorescence.

The characterization of the noradrenergic plexuses in the gallbladder wall was made *in toto* preparations. The tissue samples were processed following the method applied in the *in toto* AChE characterization above described. Once the gallbladder wall was delaminated the tissue sheets were stretched and exposed to paraformaldehyde vapours (1-3 h at 80° C). The preparations were visualized under UV light.

Immunohistochemistry methods

We have applied a modified method of Costa et al. (1980), on sections from the liver and on whole mount preparations from the gallbladder. Liver cryostat blocks were made by immersion in methylbutane frozen with liquid nitrogen. 20 μm sections of liver and the whole mount preparation of the gallbladder were fixed by immersion in a solution of 15% saturated picric acid with 2% formaldehyde in 0.1 M PBS (pH 7.3) for 18 hr at 4° C. After washing in 80% ethanol for 30 min the pieces were dehydrated through a graded series of ethanol and cleared in xylene; 30 min in each solution. The pieces were then rehydrated back to PBS. At this stage the delamination of the gallbladder was made removing the mucous layer. We applied the primary antisera to the liver sections and to the exposed surface of the gallbladder. They were incubated for 16 hr at room temperature in a moist chamber. After 3 washes in PBS, the pieces were incubated for 1 hr with the conjugated FITC (fluorescein isothyocyanate-conjugated antibodies, dilution 1:20, Miles Lab.Ltd.) and then washed in PBS for 15 min and mounted in pure glycerol. The VIP antisera (antirabbit VIP, INC Stilwater, MN), were tested at a dilution of 1:200 in PBS, pH: 7.2-7.4. For control, non immunorabbit serum was used as first layer and the FITC globulin was used alone.

Results

We have observed that the histological organization of the hepatic parenchyma of the lizard *Podarcis hispanica* consists of hepatocytes arranged in regular rows or hepatic cords. The hepatic artery, portal vein and bile ducts are not organized in the portal triad typical of higher vertebrates. The amount of intrahepatic connective tissue was scanty except for that surrounding the large blood vessels.

The acetylcholinesterase-positive fibres are scarce in the liver. They are only seen among the collagen fibres surrounding the large blood vessels (Fig. 1) and bile ducts whose finer branches seem not to be innervated. The connective tissue surrounding these fine ramifications is scanty. We have studied the part of the liver proximal to the hepatic hilus. This portion of the liver shows a rich connective tissue and being the entrance to the big branches of the vagus nerve it is not surprising to observe small ganglia with neuron somas positive for the AChE method which could be representative of the parasympathetic ganglionated nerve cells (Fig. 2A, B).

The AChE method applied to the *in toto* preparations from the lizard gallbladder wall reveals a well developed cholinergic plexus organized as a nerve fibre network, the neurons being located in the points of interconnection of the net (Fig. 3A, B).

The lizard liver noradrenergic innervation is also scarce. Fine fibre meshes surround the portal vein, hepatic artery and bile ducts. In the gallbladder adventitia fine varicose noradrenergic fibres are organized in a plexus (Fig. 4A,B). *In toto* preparations allow the observation of a wider extension of these plexuses in comparison with the results from the liver sections.

The liver VIPergic innervation is also very scarce.

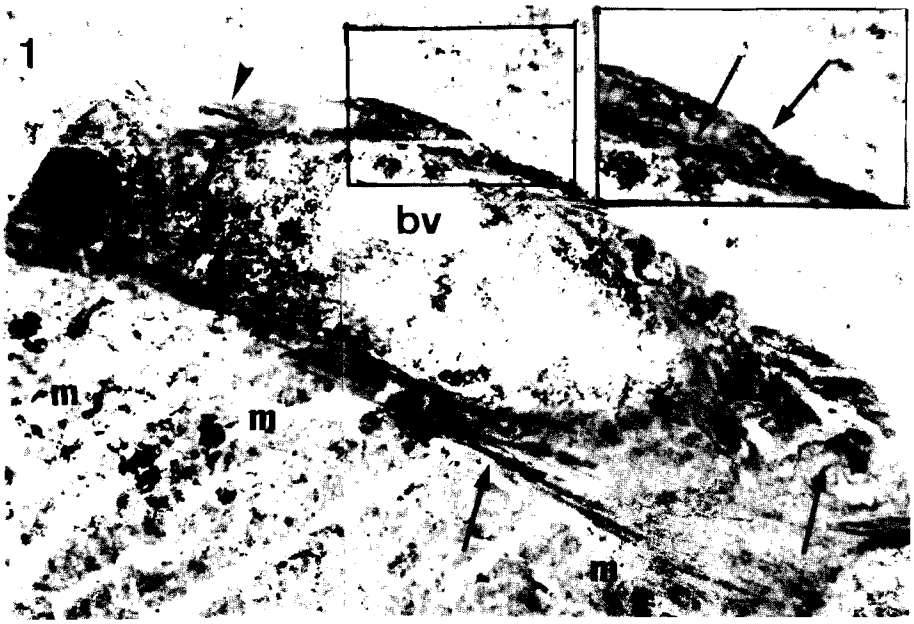

Fig. 1. Lizard liver. Nerve fibres positive for the AChE method are located in the connective tissue surrounding the large blood vessels. (m: melanine granules, bv: blood vessels). × 15

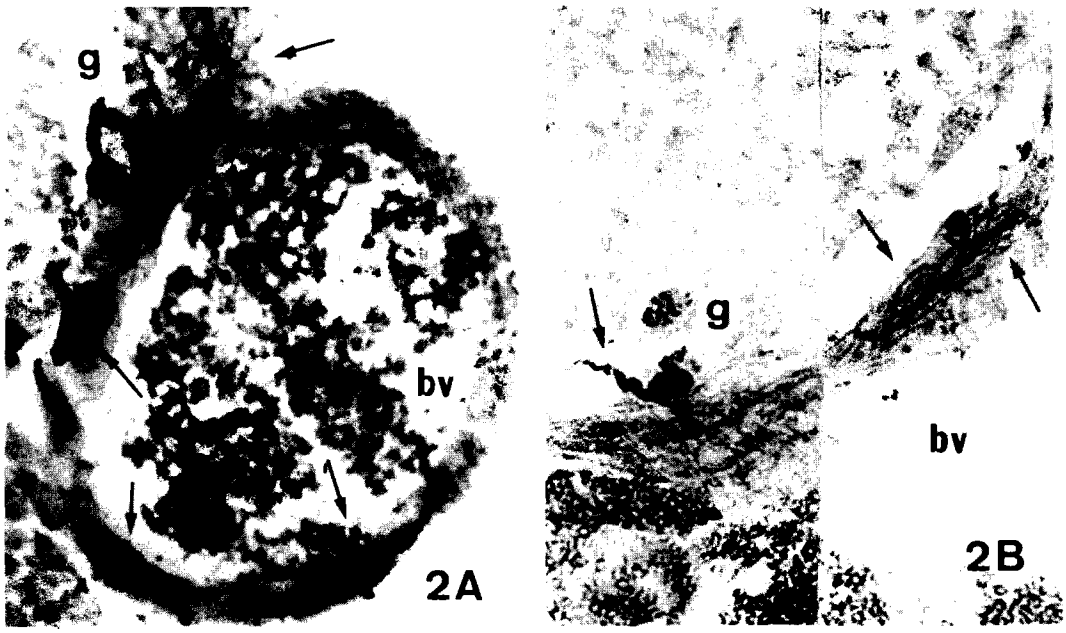

Fig. 2. Lizard liver. Small ganglions (g) with cells positive for the AChE method are located on the large blood vessel walls (bv). Fine fibres positive for the AChE method spread in the connective tissue. A: × 25, B: × 25

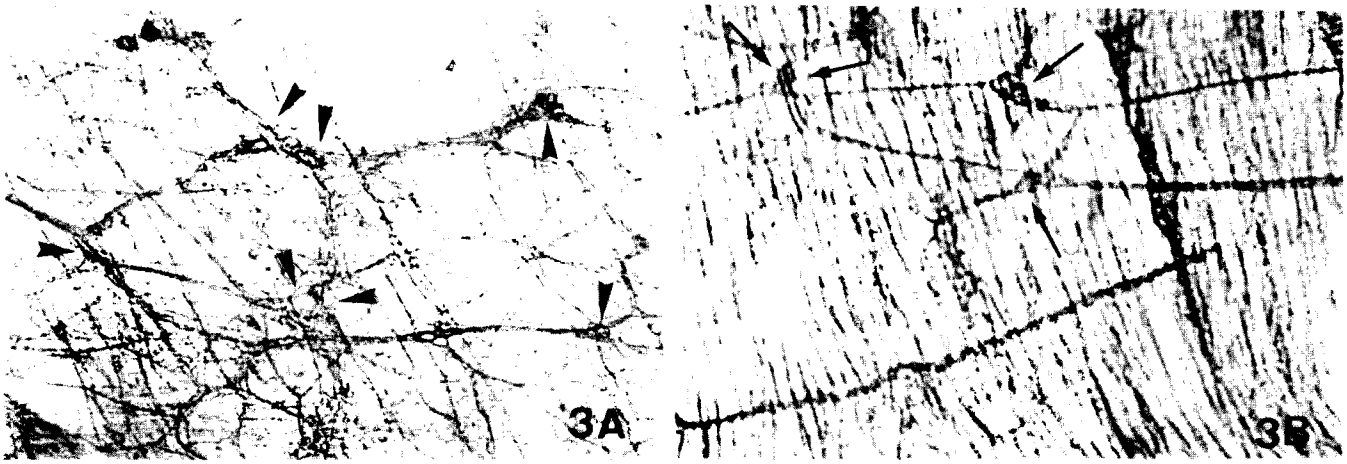

Fig. 3. Lizard gallbladder *in toto* preparation. The AChE method reveals a fibre network with positive cells in the points of interconnection of the net. A: × 15; B: × 25

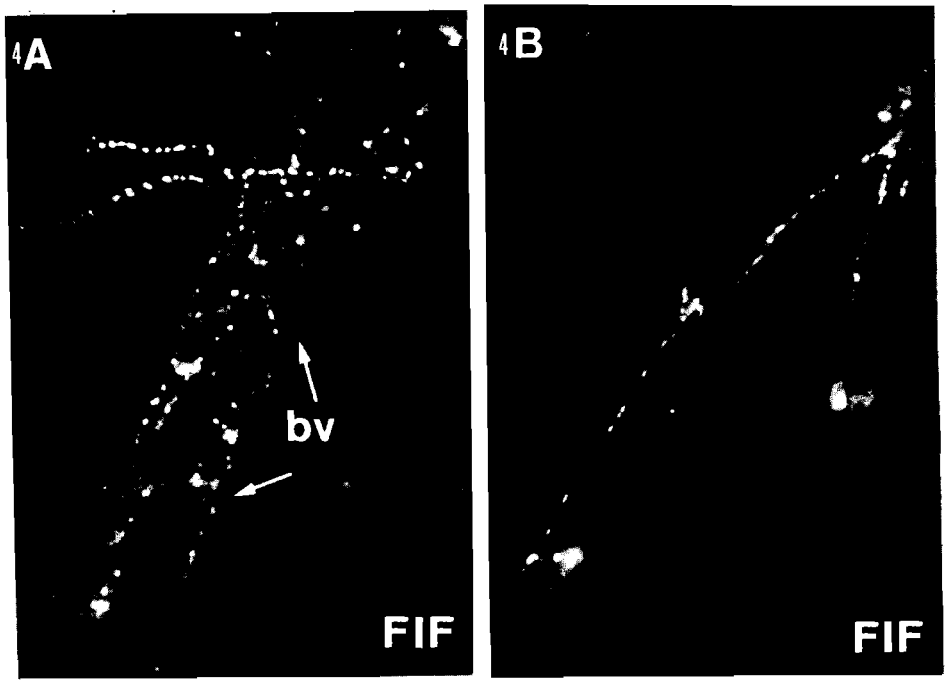

Fig. 4. Lizard gallbladder *in toto* preparation. Network of varicose fibres visualized with the FIF method: A: perivascular plexus × 20. (bv: blood vessel) B: × 20

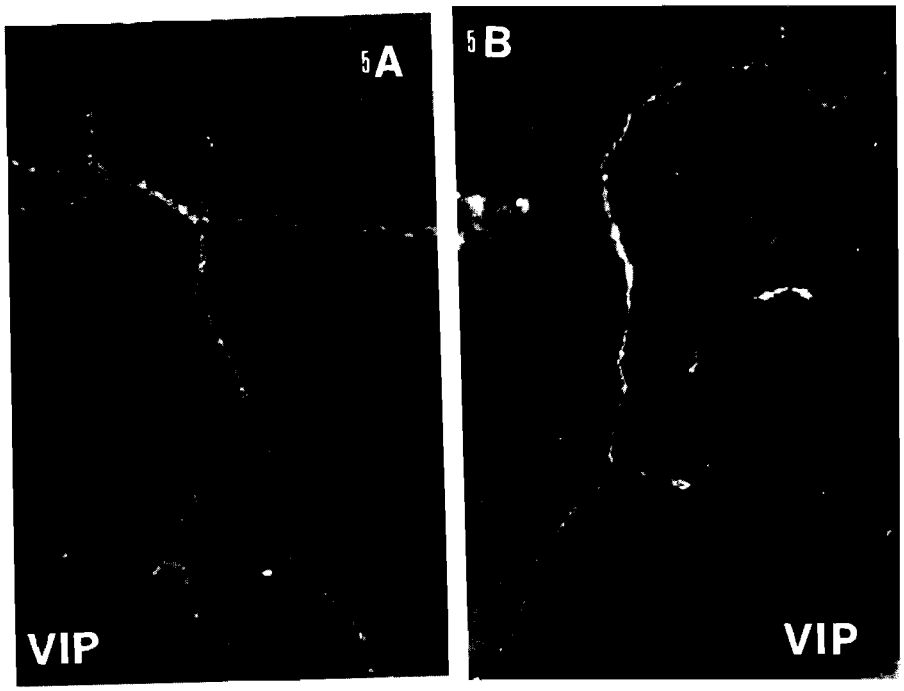

Fig. 5. Lizard gallbladder *in toto* preparation. A rich plexus of fine varicose-interconnected VIPergic fibres spreads in the gallbladder wall. A: × 25, B: × 25

Isolated varicose fibres are seen near the blood vessel walls but a richer plexus of interconnected VIPergic fibres is seen spread widely in the gallbladder wall (Fig. 5A, B).

We have also observed some melanine granules spread in the liver parenchyma as well as isolated melanocytes in close contact with the blood vessel walls and nerve structures (Fig. 6A, B). A similar distribution is observed in the gallbladder wall.

Discussion

The organization of the lizard liver parenchyma is similar to that shown in the frog (Azanza et al., 1989) and fishes (unpublished observations). The portal triads seem to be a characteristic restricted to higher mammals. The amount of intrahepatic connective tissue is higher than in fishes and amphibians.

The location of cholinergic and adrenergic plexuses in

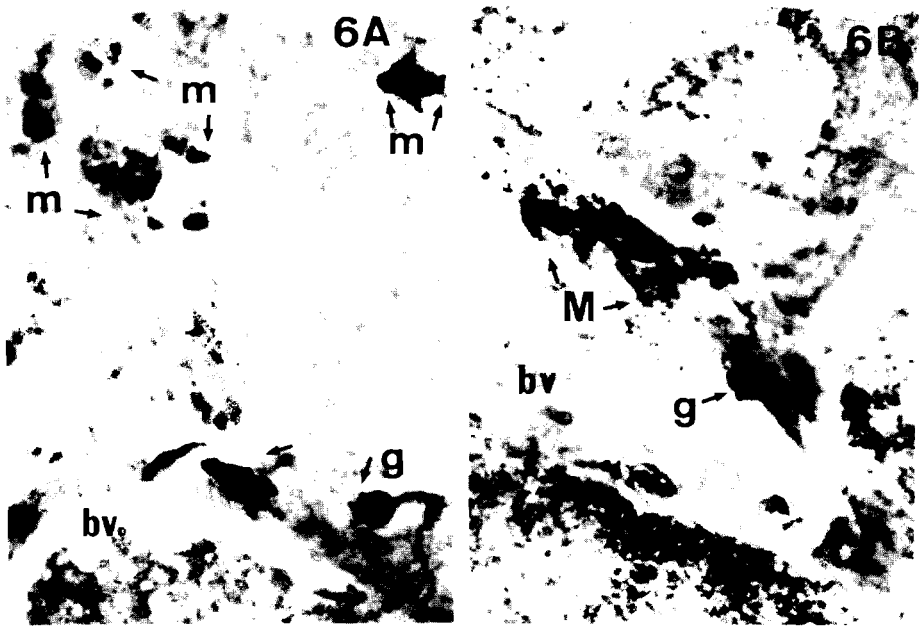

Fig. 6. Lizard liver. A) Melanine granules (m) spread in the liver parenchyma. × 20. B) Isolated melanocytes (M) in close contact with blood vessel walls (bv) and nerve structures (g). × 20

reptilian livers suggests a preferential role in the control of the vascular bed tone. The regulatory action of neurotransmitters could directly affect the main branches of the vasculature. The higher organization of the mammal liver might enable it to regulate liver function more finely.

The VIPergic innervation in *Podarcis* and *Rana* (Azanza et al., 1989) liver is, like in mammals, very scarce (Garin et al., 1982). It is restricted to the vasculature.

In toto preparations from the gallbladder wall allow the observation of a wider surface than the tissue sections from the liver. In this way the cholinergic, catecholaminergic and VIPergic nervous plexuses are visualized as wide nerve fibre networks with small varicosities in both catecholaminergic and VIPergic fibres. The organization fo these plexuses is similar to that observed in amphibian gallbladder (Azanza et al., 1989).

Tsuneki and Ichihara (1981) have described direct nervous contact with hepatocyte membranes under electron microscopy in reptilian liver. Nonetheless, we have not observed at optic level nervous fibres spreading inside the hepatic parenchyma nor being in contact with the first row of hepatocytes in the connective tissue distributed among the hepatocytes. We have studied the vertebrate liver characteristics through the phylogenetic scale and we have only observed this distribution in some mammals (Sanz et al., 1982). The increased number of intrahepatic nerves appears to be correlated to the development of a higher organization of liver structures and a concomitant increase in the amount of connective tissue.

A population of catecholamine-containing cells of the SIF type (small intensely fluorescent cells), has been described in the ganglionated plexus and around blood vessels of the guinea pig gallbladder (Cai and Gabella, 1984). We have found catecholaminergic cells at any level.

Although we have observed melanin-storing cells (visceral melanocytes) related to nervous and blood vessel structures in the liver and gallbladder of *Podarcis* they are not as widely distributed as shown in the frog (Azanza et al., 1989).

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Accepted January 9, 1990.