

The autonomic innervation of the liver and gallbladder of *Rana ridibunda*

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Summary. 1.- The innervation of the liver and gallbladder of *Rana ridibunda* has been studied by the following methods: (a) demonstration of cholinesterase activity; (b) FIF method for catecholamines; (c) immunohistochemistry for VIP and (d) electron microscopy.

2.- The hepatocytes are arranged in regular rows of hepatic cords, very little connective tissue is distributed in the parenchyma, the innervation being restricted to the big branches of blood vessels.

3.- Well defined cholinergic and adrenergic plexuses surround the hepatic arteries, portal veins and biliary ducts. The VIPergic innervation is scarce in the liver but a richly branched plexus spreads in the wall of the gallbladder.

4.- Cholinesterase-positive cells are widely distributed accompanying the nerve trunks of the gallbladder. The innervation distribution is prominent in the portion of the gallbladder next to the hepatic hilus.

5.- A population of melanin-storing cells besides free melanine granules are present in the liver parenchyma and are prominent in the gallbladder where the melanocytes are disposed in close contact with blood vessels and nerve structures. We have observed that the number of these visceral melanocytes considerably increases in winter, particularly in the liver.

Key words: Liver, Gallbladder, Autonomic innervation, Frog, Visceral melanocytes

Introduction

Although the liver is one of the organs most extensively studied, rather little attention has been paid to nerve fibers in the liver or in the gallbladder of the vertebrates phylum. The neurochemical nature of the nerve fibers in

the 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 Forsmann, 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 (Forsmann and Ito, 1977; Nobin et al., 1978; Metz and Forsmann 1979, 1980; Tsuneki and Ichihara, 1981; Sanz et al., 1982).

Very few data describe the non-mammalian vertebrates liver innervation. In an ultrastructural comparative study Tsuneki and Ichihara (1981) report that in amphibians and in lower vertebrates in general the intrahepatic nerves are rare or absent.

The aim of the present work is to carry out the study of the *Rana ridibunda* liver and gallbladder in order to establish the cholinergic, catecholaminergic and VIPergic innervation pattern at optic level as well as to characterize the ultrastructural organization of the nerve elements.

Materials and methods

The liver of 15 specimens of *Rana ridibunda* have been used in this study. The general histology of the liver and gallbladder was examined after staining with haematoxylin and eosin.

Acetylcholinesterase method (AChE)

Acetylthiocholine (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 tetraisopropylpyrophosphoramidate (ISOOMPA, Sigma).

Catecholamines method (FIF)

The adrenergic fibers 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, left frozen overnight, exposed to paraformaldehyde vapours (3 hr at 80° C) and vacuum embedded in paraffin wax. Sections of 15-20 μ m were examined under a Leitz orthoplan fluorescence microscope. Adrenaline produced an apple green fluorescence, while histamine and serotonin produced a yellow fluorescence.

Immunohistochemistry methods

We have applied the method of Costa (Costa et al., 1980), modified, 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 μ m 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 humid chamber. After 3 washes in PBS, the pieces were incubated for 1 hr with the conjugated FITC (fluorescein isothiocyanate-conjugated antibodies, dilution 1:20, Miles Lab. Ltd.) and then washed in PBS for 15 min and mounted in pure glycerol. We tested the VIP antisera (antirabbit VIP, INC Stilwater, MN) 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.

Electron microscopy method

Following the methods described by Tsuneki and Ichihara (1981) the liver was fixed by immersion in 1.5% glutaraldehyde buffered to pH 7.3 by cacodylate, postfixed in 2% OsO₄, stained with 70% uranyl acetate, dehydrated and embedded in Araldite.

Results

The liver of amphibians 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 is scanty except for that surrounding the large blood vessels. Melanin granules as well as melanin storing cells were localized in the liver (Fig. 1) and gallbladder where thick axon bundles were seen below the epithelium (Fig. 2).

The acetylcholinesterase positive fibers were scarce in the liver. They were only seen among the collagen fibers surrounding the large blood vessels and bile ducts whose finer branches seem not be innervated. The connective tissue surrounding these fine ramifications was scanty. The gallbladder at the level of the hepatic hilus was more richly innervated. The AChE positive fibers spread in the adventitia surrounding the veins and arteries. Thick axon bundles were very often seen accompanied by cells positive for the AChE method (Fig. 3a). The ultrastructure confirmed the optic microscopy observations (Fig. 3b).

The adrenergic innervation was also scarce in the liver. Fine fiber meshes surrounded the portal vein, hepatic artery (Fig. 4) and bile ducts. In the gallbladder adventitia thick adrenergic fibers were disposed in the connective tissue. Varicosities with adrenergic vesicles were observed in the axon bundles (Fig. 5).

The liver VIPergic innervation was very scarce. Isolated varicose fibers were seen near the vein wall but a richer plexus of interconnected VIPergic fibers spread in the gallbladder wall (Fig. 6).

It is worth noting the high number of melanin granules freely spread among the hepatocytes and that of melanin storing cells more frequently seen at the level of the gallbladder. They were very often disposed in close contact with the blood vessel walls (Fig. 7) and nerve structures (Fig. 3b). Their number increased in winter particularly in the liver parenchyma.

Fig. 1. Melanin-storing cells in the liver parenchyma among collagen fibers. N = nucleus. M = melanosomes. $\times 4,500$

Fig. 2. Axons trunk with accompanying glia cells below the epithelia of the gallbladder. NG = glial cell nucleus. $\times 9,100$

Fig. 3. a. Thick axon bundles accompanied by AChE positive cells and melanocytes are very often seen in the gallbladder. MY = melanocyte. NT = nervous trunk. C = AChE positive cell. $\times 40$. **b.** A parallel structures association is shown with EM. Gallbladder lamina propria near a blood vessel. N = neuron. NT = nervous trunk. MY = melanocyte. $\times 4,500$

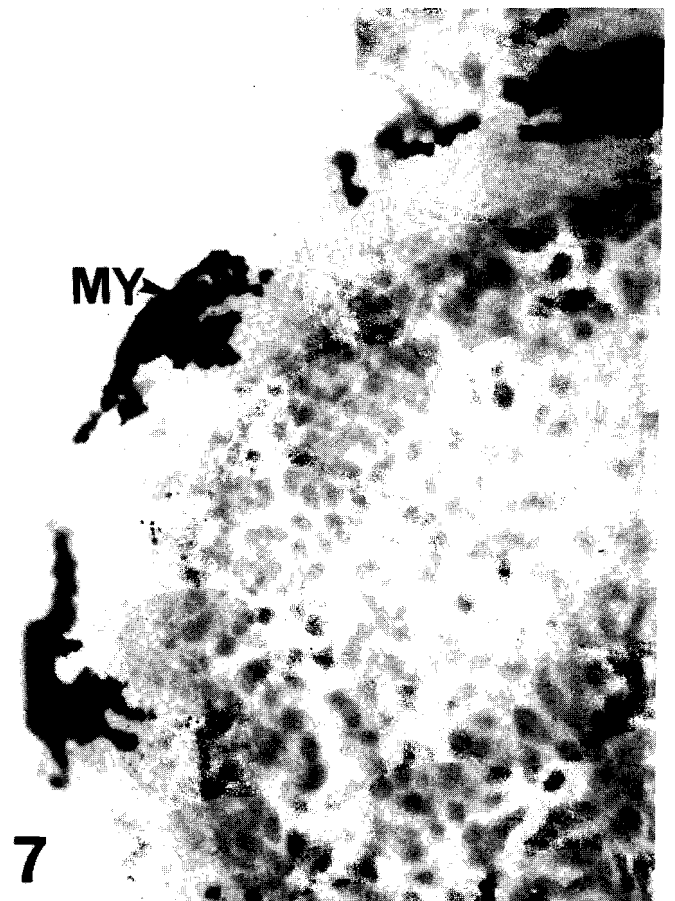
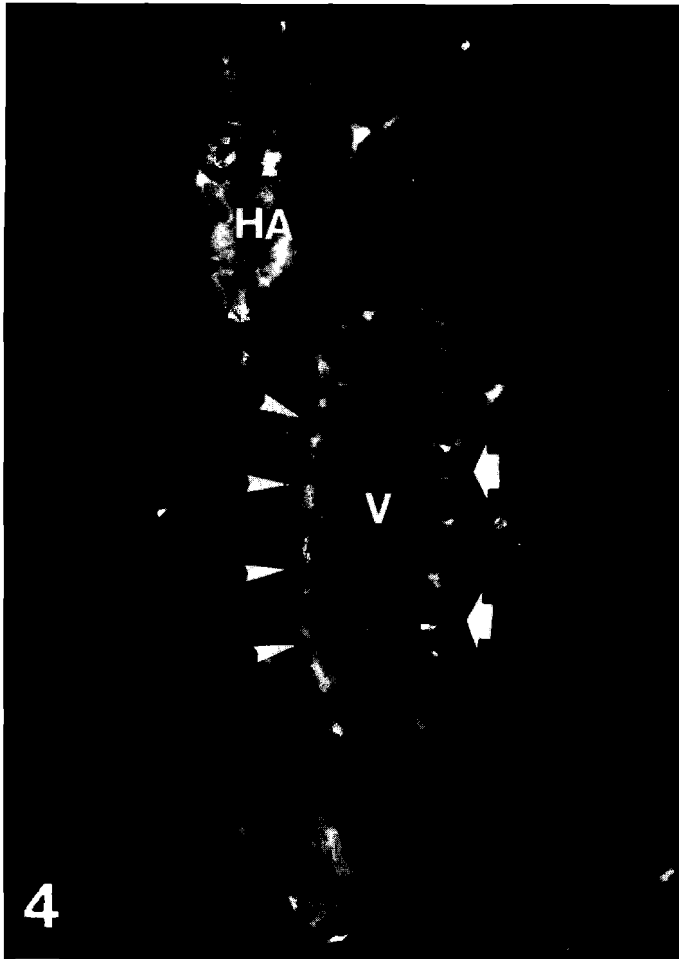
Fig. 4. A fine adrenergic fiber mesh surrounds the portal vein and hepatic artery in the liver. V = portal vein. HA = hepatic artery. $\times 25$

Fig. 5. Varicosities with adrenergic-type vesicles (arrows) are shown in axon bundles in the gallbladder. $\times 34,000$

Fig. 6. VIP-like varicose fibers (arrows) make a thin plexus in the gallbladder wall. $\times 40$

Fig. 7. Visceral melanocytes are very often seen in close contact with the blood vessel walls. MY = melanocytes. $\times 20$





Discussion

The structure of the liver in vertebrates seems to follow an evolutive law of increasing complexity. In lower vertebrates like amphibians the hepatic cells are arranged in cords with scarce connective tissue while in mammals the liver is organized in hepatic lobules with well defined portal triads and well developed interlobular connective tissue.

The location of cholinergic and adrenergic plexuses in amphibian liver suggests a preferential role in the control of the vascular bed tone. The regulatory action of neurotransmitters would directly affect the main branches of the vasculature. The higher organization of the mammal liver might enable it to regulate liver function more finely. We have observed for instance that in mammals the fine cholinergic fibers can raise the first row of the hepatic lobules hepatocytes (Sanz et al., 1982) furthermore and as an exception there exists a fiber plexus in the rat hepatic parenchyma of nervous nature and vagal origin (Azanza, 1987). In some mammals (cat, pig, sheep and lamb) the noradrenergic innervation is also extended to the parenchyma rising 2/3 of the distance to the central veins (Sanz et al., 1982) and in man (Nobin et al., 1978) noradrenergic innervation is also extended through the parenchyma suggesting a possible direct effect on the hepatocytes.

The VIPergic innervation in *Rana* is as in mammals, very scarce (Garin et al., 1982). It is restricted to the vasculature. Nonetheless the amphibian gallbladder wall is covered by a richly branched VIPergic varicose plexus.

A population of catecholamine-containing cells of the SIF type (small intensely fluorescence cells), has been described in the ganglionated plexus and around blood vessels of the guinea pig gallbladder (Cai and Gabella, 1984). In the frog gallbladder we have found cells positive for the AChE method. We have not found catecholaminergic cells at any level.

We have described the distribution and morphological characteristics of the visceral melanocytes in the different portions of the gut of *Rana ridibunda* (Junquera et al., 1987). A rich population of visceral melanocytes is also present in the liver and gallbladder. The connection with blood vessels and nervous structures as well as the exceptional increment of free granules in the liver parenchyma in cold periods suggests again a metabolic function. Sichel et al. (1981) have reported that the melanins isolated from the liver and skin of frogs undergo structural modifications between the cold months and the warm ones, correlated probably with metabolic changes. Sauerbier (1977) considers that catecholamines probably play a role in the initiation of temperature acclimation in the frog since he found high levels of noradrenaline and adrenaline at the end of winter, just before spawning, and even higher levels in the autumn before hibernation. Carlson (1966) has shown that noradrenaline has a calorogenic effect exposed to cold stimulating the oxygen consumption and the release of free fatty acids from the brown fat of ground squirrels. On the other hand, diminished catecholamine synthesis is achieved by diverting L-tyrosine

metabolism towards the formation of neuromelanin or melanin in brain cells (Marsden, 1965). The appearance of neuromelanin suggests in this way a diminution in catecholamine synthesis in pigmented nerve cells so that the neuromelanin could indicate diminishing catecholamine requirements. The distribution and seasonal variations of visceral melanocytes observed in frog seems to confirm those assumptions. If high levels of catecholamines are recorded in autumn and, as we have seen a notable decrease in the adrenergic gut innervation in winter (unpublished observations), it is evident that there is a diminution in the metabolism of catecholamines during the cold period. In this way visceral melanocytes could play a role at peripheral sites that pigmented nerve cells seem to play at the CNS level. The visceral melanocytes could also be the peripheral indicative cells of the seasonal catecholamine requirements related to the hibernation process.

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