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The innervation of rainbow trout (*Oncorhynchus mykiss*) liver: protein gene product 9.5 and neuronal nitric oxide synthase immunoreactivities

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ABSTRACT

We have explored the innervation of the rainbow trout (*O. mykiss*) liver using immunohistochemical procedures and light microscopy to detect in situ protein gene product 9.5 and neuronal nitric oxide synthase immunoreactivities (PGP-IR and NOS-IR). The results showed PGP-IR nerve fibres running with the extralobular biliary duct (EBD), hepatic artery (EHA) and portal vein (EPV) that form the hepatic hilum, as well as following the spatial distribution of the intrahepatic blood vessel and biliary channels. These nerve fibres appear as single varicose processes, thin bundles, or thick bundles depending on their diameter and location in the wall of the blood vessel or biliary duct. No PGP-IR fibres were detected in the liver parenchyma. NOS-IR nerve fibres were located only in the vessels and ducts that form the hepatic hilum (EBD, EHA, EPV); in addition, NOS-IR nerve cell bodies were found isolated or forming ganglionated plexuses in the peribiliary fibromuscular tissue of the EBD. No PGP-IR ganglionated plexuses were detected in the EBD. The location of the general (PGP-IR) and nitrergic (nNOS-IR) intrinsic nerves of the trout liver suggest a conserved evolutionary role of the nervous control of hepatic blood flow and hepatobiliary activity.

Key words: Autonomic nervous system; hepatobiliary system; hepatic artery; portal vein.

INTRODUCTION

The liver has sympathetic and parasympathetic innervation. In mammals, both extrinsic and intrinsic peripheral nerves have been extensively studied (Friedman, 1988; Berthoud et al. 1992, 1995; Lin et al. 1995). Although the extrinsic innervation of fish liver was described long ago (Bertin, 1954), many aspects of the distribution of intrinsic nerve fibres in the liver of this vertebrate group remain to be elucidated.

Protein gene product 9.5 (PGP 9.5) is a soluble cytoplasmic protein that is used as a general marker of central and peripheral nervous tissue as well as of neuroendocrine cells (Thompson et al. 1983; Rode et al. 1985; Gulbenkian et al. 1987). This protein has been highly conserved throughout evolution, being detected from trout liver to the human brain (Jackson et al. 1985). PGP 9.5 is located in the nerve fibres of the liver of different mammalian species, and the location of these fibres depends on the species (Lin et al. 1995).

Currently, a promising field in research on autonomic innervation is the study of nitrergic neuroeffector transmission (Rand & Li, 1995), which involves the location of the neuronal isoform of nitric oxide synthase (nNOS), a constitutive enzyme which catalyses the formation of nitric oxide (NO) and L-citrulline from L-arginine (Bredt et al. 1990). Two other isoforms of this enzyme have been detected in several animal tissues, one constitutive endothelial (eNOS) and another isoform that is inducible under immunological stimuli (iNOS) (Moncada & Higgs, 1993; Griffith & Stuehr, 1995).

Previous studies have demonstrated the presence of nitrergic structures in the enteric nervous systems of

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the dog (Ward et al. 1992), guinea pig (Young et al. 1992; Furness et al. 1994) and rat (Belai et al. 1992, 1995). In addition nNOS immunoreactivity or NADPH-diaphorase (NADPH-d) activity has also been found in the enteric nervous systems of elasmobranch (Olsson & Karila, 1995), teleost (Li & Furness, 1993; Olsson & Karila, 1995), amphibian (Li et al. 1992; Murphy et al. 1994) and avian species (Li et al. 1994). The neuronal elements in the gallbladder are derived from the enteric nervous system (Mawe & Gershon, 1989). Under this assumption, Siou et al. (1994) demonstrated the presence of a rich NADPH-d positive ganglionated plexus in the guinea pig gallbladder. Recently, we demonstrated the presence and distribution of nNOS in the rat and cat liver (Esteban et al. 1997, 1998), where we suggested that nitrergic fibres may be involved in the regulation of hepatic blood flow, hepatobiliary activity and hepatocellular metabolic function. Furthermore, iNOS and eNOS have been shown to be present in the liver (Bandaletova et al. 1993; Geller et al., 1993). In freshly isolated rat hepatocytes (Geller et al. 1993) iNOS has been induced after stimulation with cytokines and endotoxin (lipopolysaccharide; LPS). In the liver, as well as in other tissues, of rats treated with *Propionibacterium acnes* and LPS, the presence of iNOS was detected immunocytochemically in Kupffer cells, endothelial cells and hepatocytes (Bandaletova et al. 1993).

In an effort to ascertain the possible conserved evolutionary distribution both of general and nitrergic nerve fibres, the aim of this study is to demonstrate, at the light microscopic level, the distribution of the general PGP 9.5 immunoreactive (PGP-IR) trout liver innervation, as well as the location of the nNOS immunoreactive (nNOS-IR) nerve fibres pervading the liver vascular supply and the hepatobiliary ducts of this teleost fish.

MATERIALS AND METHODS

Immunohistochemical studies were performed on 7 rainbow trout (450–500 g body weight). The specimens were anaesthetised in water containing 0.3 ml/l ethyleneglycol-monophenylether (Merck) and heparinised through the dorsal aorta (500 I.U., Rovi). The abdominal and heart cavities were exposed, and a blunt 20 gauge cannula (Abbot) was inserted into the major tributary to the hepatic portal vein and tied securely in place. The liver was cleared of blood by an in situ perfusion with 3–4 ml of 0.01 M carbogenated phosphate buffered saline (PBS, pH 7.4), at room temperature (R.T.) and at a flow rate of 5.2 ml/min/kg body weight, using a peristaltic pump (Gilson minipulse). Immediately after the flow began, a small cut was made in the tail kidney to permit blood and perfusate to escape from the portal venous system. Fixation was carried out with a solution (20–50 ml) containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), at the same flow rate.

After perfusion, fixed livers were removed, cut into 4–5 mm blocks, submerged in additional primary fixative, and fixed at R.T. for an additional 3 h. The blocks of livers were then rinsed by immersion overnight at 4 °C in 0.1 M PB, containing 30% sucrose. After this, the blocks were covered with O.C.T. compound and frozen 2-methylbutane at liquid nitrogen temperature. Serial sections (40 μm) were prepared using a cryostat (2800 Frigocut E, Reichert-Jung). Endogenous peroxidase was inhibited on free-floating sections with 0.03% H_2O_2 in PBS for 30 min, and afterwards the free-floating sections were incubated overnight with PGP 9.5 (1:400; Ultraclone Ltd, Isle of Wight, UK) or nNOS (1:2000; Springall et al. 1992; Riveros-Moreno et al. 1993) antisera diluted in PBS containing 0.2% Triton X-100 at 4 °C. After several washes in PBS, the sections were incubated with biotinylated goat antirabbit IgG (Vector Laboratories Ltd) followed by peroxidase-linked ABC. The peroxidase activity was demonstrated following the nickel-enhanced diaminobenzidine procedure (Shu et al. 1988). Sections were then mounted on slides, counterstained with toluidine blue, dehydrated in graded ascending ethanol series, and covered using DPX.

Control procedures were carried out on some sections. No immunolabelling was observed when the primary antibody was either omitted or replaced with an equivalent concentration either of preimmune serum or normal rabbit serum. A similar result was obtained when the sections were incubated overnight at 4 °C, with preadsorbed primary antiserum either with antigen extracted from rat brain or with 2 nmol/ml of full-size pure recombinant rat nNOS (Riveros-Moreno et al. 1993). For confirmation of antiserum specificity, an aliquot was preabsorbed with 2 nmol/ml of rat eNOS, which did not alter the staining of neuronal structures.

RESULTS

PGP-9.5

Extralobular (hepatic hilum) innervation

In general, the extralobular biliary duct, hepatic artery and portal vein (EBD, EHA, EPV) showed

PGP-IR fibres (Fig. 1*a*, *b*). The EBD presented PGP-IR in thick bundles of fibres distributed to the fibromuscular and adventitial layers, forming a rich plexus (Fig. 1*c*). In addition, single varicose processes and thin bundles of fibres were seen at the base of the peribiliary connective tissue, running in the lamina propria and beneath the epithelium (Fig. 1*d*). In the EHA, PGP-IR was detected in single varicose nerve fibres that emerged from thick bundles of nerve fibres of the surrounding connective tissue; these varicose fibres ran across the smooth muscular layer, reaching the lamina propria under the endothelium (Fig. 1*e*, *f*). The EPV showed single varicose nerve fibres preferentially in a transverse disposition along its wall (Fig. 1*g*, *h*); these fibres crossed through the adventitia and muscular layers, reaching the zone under the endothelium. In addition, thin bundles of PGP-IR nerve fibres were detected in the arteriolar vasa vasorum surrounding the EPV (Fig. 1*h*).

Intralobular innervation

Recently, a systematic spatial distribution of the blood vessels and biliary channels was described in trout liver (Rocha et al. 1995). Because we have detected the intrinsic innervation associated with the vascular supply and biliary ducts, we have used the nomenclature of Rocha et al. (1995).

Venous-biliary-arteriolar tracts (*VBAT*). These tracts are associations of portal veins (PV), arterioles (HA) and bile ductules (BD), the large tracts connecting to the hepatic hilum. Figure 2*a* shows a VBAT with one type of each of the 3 components, in which PGP-IR nerve fibres appeared not only as single varicose elements, but also forming thick bundles. The general disposition of the nerves in VBAT was almost the same than in the corresponding vessels or ducts of the hepatic hilum; in fact, the BD showed single nerve fibres just beneath the epithelium, although in the trout liver these ductules have no basal lamina between the epithelium and the hepatocytes.

Venous-arteriolar tracts (*VAT*), *biliary-arteriolar tracts* (*BAT*), *and venous-biliary tracts* (*VBT*). In addition to VBAT, the trout liver also has strict associations between 2 of these structural components (Fig. 2*b*). In the VAT, PGP-IR nerve fibres are located mainly around the HA; in fact, in PV, only single varicose nerves were detected (Fig. 2*c*, *d*). BAT associations showed bundles of immunoreactive fibres in the connective tissue near the HA and the BD, and single PGP-IR nerves running along the walls of both structures (Fig. 2*e*); some thick bundles of nerves were located in the adjacent connective tissue. VBT had single PGP-IR nerve fibres in a longitudinal and transverse disposition in the PV wall, the richer innervation belonging to the BD (Fig. 2*f*).

Biliary tracts (*BT*), *arteriolar tracts* (*AT*) *and venous tracts* (*VT*). These tracts are formed by individual structural components. BT are bile ductules, quite numerous and with different diameters, all with a well-defined epithelium; they show single PGP-IR nerve fibres located along the epithelial layer and just under it, as was described for the biliary duct of the BAT and VBT elements. AT proved scarce, with only a few fine single varicose immunoreactive fibres running along the vessel wall (Fig. 2*g*). VT are branches of the portal vein and, as for the BT, were numerous. The PGP-IR found in these vessels had the same characteristics as did the other branches of the portal veins described above (Fig. 2*g*, *h*).

nNOS

We detected neuronal nitric oxide positive immunoreactive (nNOS-IR) nerve fibres only in the vessels and ducts that form the hepatic hilum. Thick bundles of nNOS-IR nerves were located in the fibromuscular layer of the EBD and in the connective tissue that surrounds the afferent blood vessels (EHA and EPV) (Fig. 3*a*). The peribiliary fibromuscular layer showed single varicose nerve fibres, these fibres also being located in the adventitial level of the EHA and EPV, close to the arterial wall (Fig. 3*a*). No immunoreactivity appeared in the intrahepatic branches of the EBD, EHA or EPV described above as showing PGP-IR (Fig. 3*f*).

In the EBD, the peribiliary fibromuscular tissue contained single varicose branches of the nerve bundles as well as nNOS-IR large and rounded cell bodies that were found singly or forming ganglia (Fig. 3*b–e*). In addition, thick and thin immunoreactive nerve bundles, as well as single varicose nNOS-IR nerve fibres were observed in the neuronal plexuses found in each of the 3 main layers of the EBD: adventitia, muscularis and lamina propria (Fig. 3*b–e*).

nNOS-IR ganglia were compact structures containing more than one neuron arranged in a monolayer with thick nNOS-IR fibre bundles (Fig. 3*b*, *c*). These ganglia were located in the fibromuscularis layer (Fig. 3*b*), or in the adventitial layer just outside the muscularis (Fig. 3*b*, *c*), although we did not find nNOS-IR ganglia in the plexus located at the lamina propria level. The single nNOS-IR neurons were

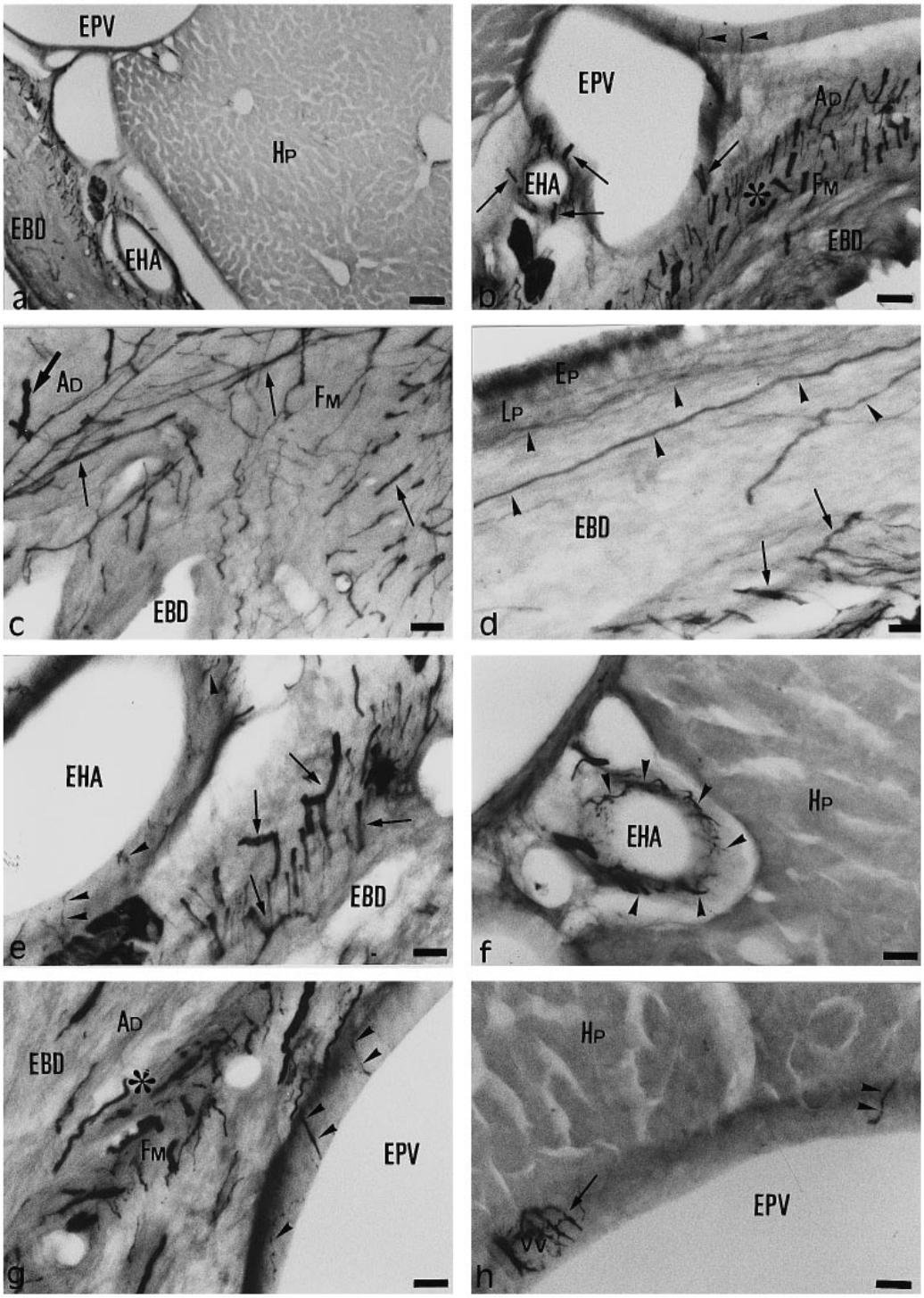

Fig. 1. Micrographs of PGP 9.5 immunoreactivity in sections of trout hepatic hilum counterstained with toluidine blue. (*a, b*) Extralobular biliary duct (EBD), hepatic artery (EHA), and portal vein (EPV); arrowheads: fine single PGP-IR varicose processes at the EPV wall; arrows: thin bundles of PGP-IR fibres at the EPV and EHA wall; asterisk: thin and thick bundles of PGP-IR fibres at the EBD adventitia (AD) and fibromuscular (FM) layers; HP: hepatic parenchyma. (*c*) EBD section showing the rich plexus of fibre bundles distributed among the adventitial (AD) and fibromuscular (FM) layers; bold arrow: thick PGP-IR bundles of fibres; small arrows: thin PGP-IR bundles of fibres. (*d*) EBD section showing the epithelium (EP) and lamina propria (LP); arrowheads: fine single PGP-IR varicose processes in the lamina propria and beneath the epithelium; arrows: thin bundles of PGP-IR fibres in peribiliary connective tissue. (*e*) Section of the hepatic hilum including the EHA and the EBD; arrowheads: fine single PGP-IR varicose processes at the EHA wall; arrows: thick PGP-IR bundles of fibres in the EBD connective tissue. (*f*) EHA branch section; arrowheads: PGP-IR varicose fibres forming a plexus around the EHA; HP: hepatic parenchyma. (*g*) EPV and EBD section; arrowheads: fine single PGP-IR varicose processes at the EPV wall; asterisk: thin and thick bundles of PGP-IV fibres at the EBD adventitial (AD) and fibromuscular (FM) layers. (*h*) Detail of the EPV near to the hepatic parenchyma (HP); arrowheads: fine single PGP-IR varicose processes at the EPV wall; arrow: PGP-IR nerve fibres in the arteriolar vasa vasorum (VV) of the EPV. Bar in (*a*) 130 μm, (*b, c*) 50 μm, (*d, e, f, g*) 30 μm, (*h*) 12 μm.

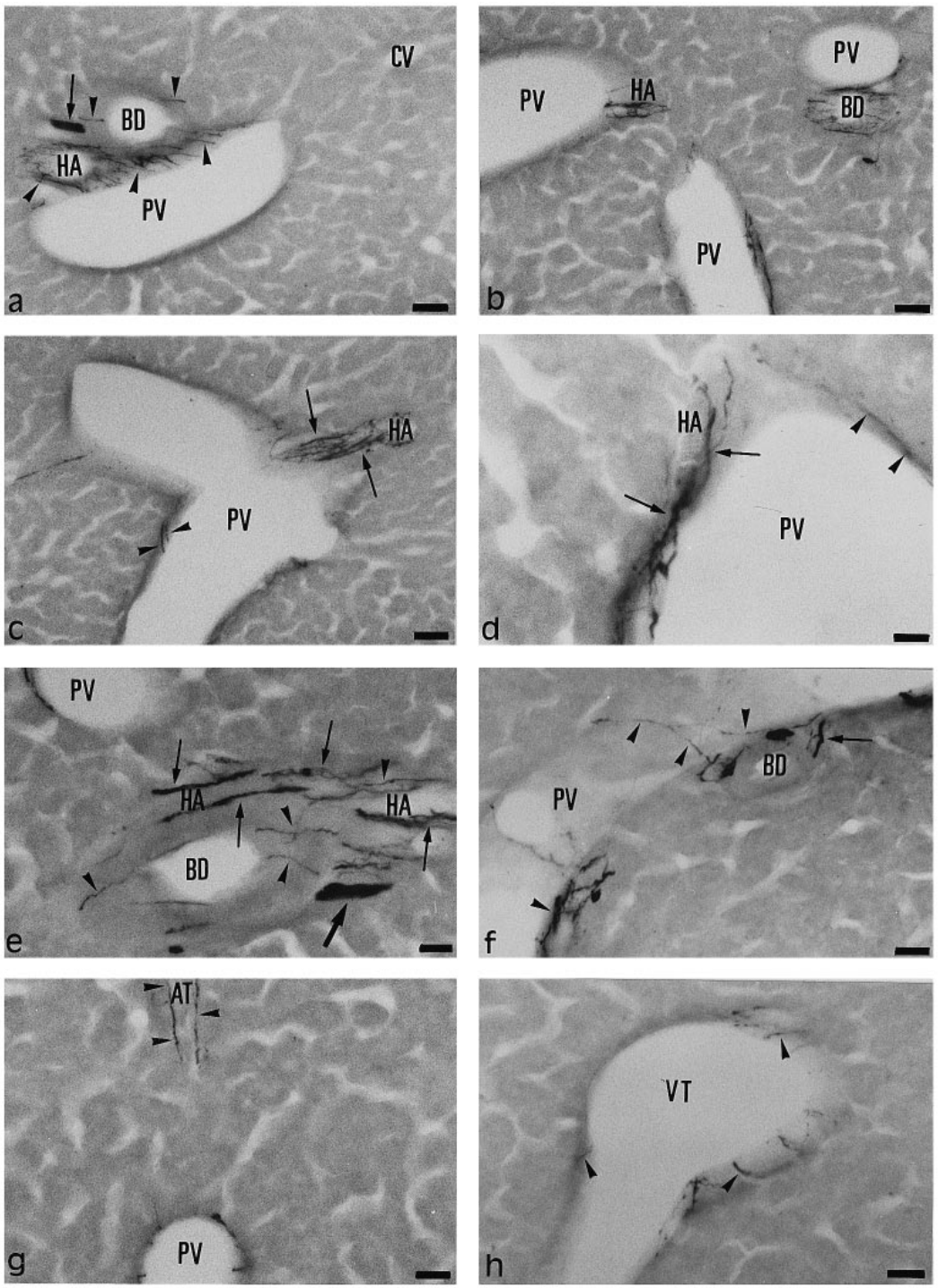

Fig. 2. Micrographs of intralobular PGP 9.5 immunoreactivity in sections of trout liver counterstained with toluidine blue. (*a*) Venous-biliary-arteriolar tract; arrowheads: single PGP-IR nerve fibres; arrow: thick bundle of PGP-IR nerves; HA: hepatic artery; BD: biliary duct; PV: portal vein; CV: central vein. (*b, c, d, e, f*) Associations of 2 types of component. (*c, d*) Venous-arteriolar tracts; arrowheads: single PGP-IR nerve fibres in the portal vein (PV) wall; arrows: a rich plexus of PGP-IR nerves around the hepatic artery (HA). (*e*) biliary-arteriolar tracts; arrowheads: single varicose PGP-IR nerves; arrows: thin bundles of immunoreactive nerve fibres; bold arrow: thick bundle of PGP-IR nerves. (*f*) venous-biliary tract; arrowheads: single PGP-IR nerve fibres; arrow: thin bundle of immunoreactive fibres. (*g*) arteriolar tract (AT); arrowheads: fine single varicose PGP-IR fibres. (*h*) venous tract (VT); arrowheads: single PGP-IR fibres. Bars: (*a, b*) 50 μm, (*c, e, f, g, h*) 30 μm, (*d*) 12 μm.

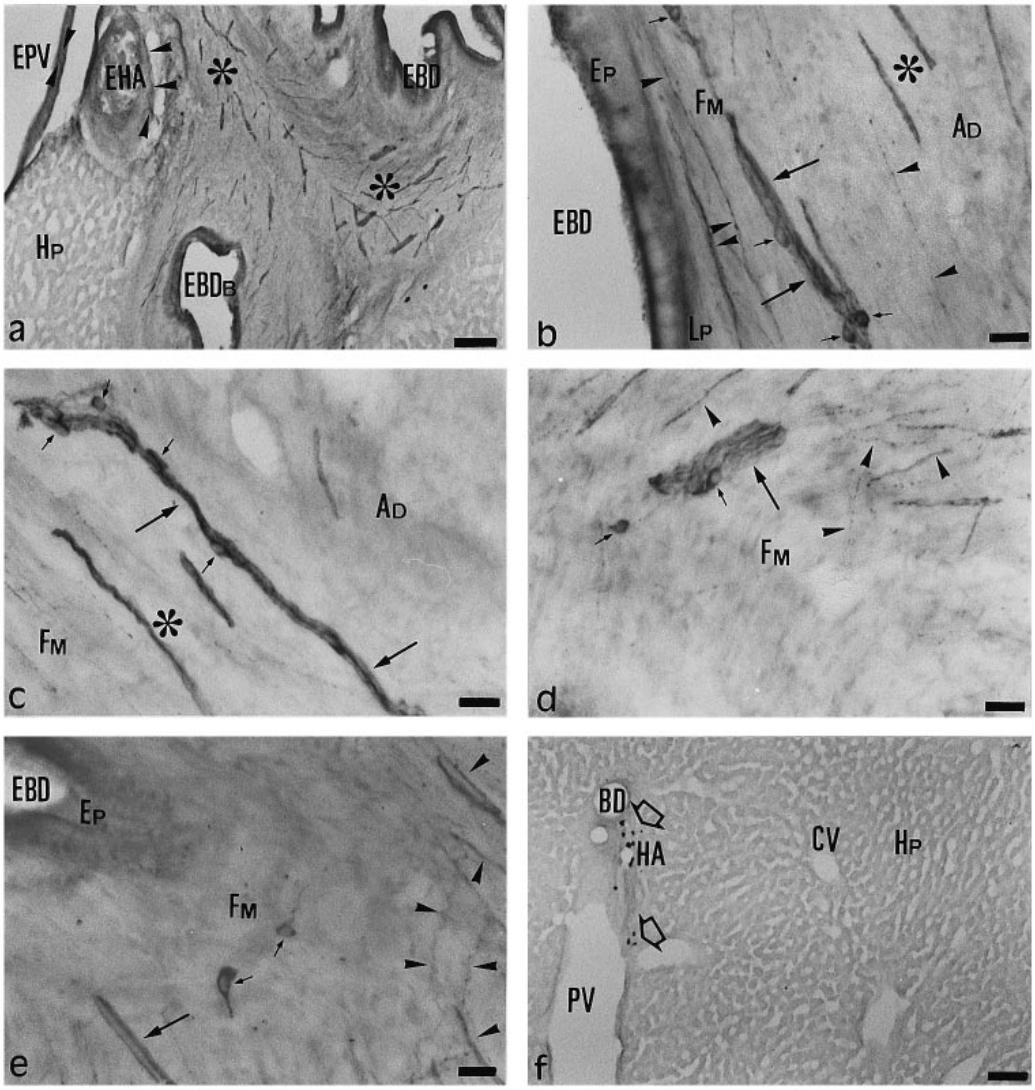

Fig. 3. Micrographs of nNOS immunoreactivity in sections of trout hepatic hilum counterstained with toluidine blue. (*a*) Extralobular biliary duct (EBD), hepatic artery (EHA), and portal vein (EPV); arrowheads: fine single nNOS-IR varicose processes at the EPV and EHA walls; asterisks: thin and thick bundles of nNOS-IR fibres in the peribiliary and perivascular tissues; EBD_B: EBI branch; HP: hepatic parenchyma. (*b, c, d*) EBD sections showing the epithelium (EP), lamina propria (LP), and/or fibromuscular (FM) and adventitial (AD) layers; arrowheads: fine single nNOS-IR varicose processes; arrows: bundles of nNOS-IR fibres forming nitrergic ganglia; small arrows: immunoreactive rounded nerve-cell bodies; asterisk: thin bundles of nNOS-IR nerves in the advential (AD) or fibromuscular (FM) layers. (*e*) Individual nNOS-IR neurons in the EBD fibromuscular layer (FM); arrowheads: fine single nNOS-IR varicose processes; arrow: bundle of nNOS-IR fibres; small arrows: immunoreactive individual rounded nerve-cell bodies. (*f*) Nonlabelled intralobular vessels and ducts; BD: biliary duct; HA: hepatic artery; PV: portal vein; CV: central vein; HP: hepatic parenchyma; open arrows: melanomacrophages. Toluidine blue stained. Bars: (*a, f*) 130 μm, (*b, c, d, e*) 30 μm.

detected in the fibromuscular layer close to nNOS-IR ganglia (Fig. 3*d*) or to other single immunoreactive neurons (Fig. 3*e*).

DISCUSSION

In general, the autonomic hepatic nerves reach the liver through the hepatic hilum, running along the hepatic artery, the portal vein, and the biliary duct (Shimazu, 1996). Our results indicate that the PGP-IR nerve fibres of the trout liver run along the hepatic artery, portal vein and biliary duct. In accord with the nomenclature given by Rocha et al. (1995), PGP-IR nerves were detected in the portal triad forming the hepatic hilum, as well as in association with all the spatially organised stromal elements described in the trout liver: venous-biliary-arteriolar tracts (VBAT), venous-arteriolar tracts (VAT), biliary-arteriolar tracts (BAT), venous-biliary tracts (VBT), biliary tracts (BT), arteriolar tracts (AT), and individual

veins. These results suggest that the hepatic nerves are involved in the regulation of hepatic blood flow and in the regulation of hepatobiliary activity in this teleost fish. Lin et al. (1995) have studied the presence of PGP-IR nerve fibres in the liver of 8 different mammals (mouse, rat, guinea pig, cat, dog, pig, monkey and human), in which they observed immunoreactive bundles in the connective tissue of the interlobular region in all these species, and in close contact with the hepatic arteries, portal veins and biliary ducts of the 8 mammals studied; no PGP 9.-IR fibres were detected in the parenchyma nor in the central vein of the mouse or rat liver, although these authors observed intraparenchymal and centrolobular immunoreactive nerves in the other species. Given that we have detected neither intraparenchymal nor central-vein-associated PGP-IR nerves in the trout liver, and that the innervation observed is closely associated with the afferent vessels and biliary channels, as has been previously shown in the rat and mouse livers, it seems that the general innervation of the trout liver resembles that of the lower mammals.

In the present work, we detected immunoreactive nitrergic nerves (nNOS-IR) in the blood vessels and biliary duct of the hepatic hilum, as well as nNOS-IR ganglionated plexuses in the hepatic branch of the biliary duct. On the other hand, we have not observed nNOS-IR nerve fibres in the intralobular hepatic arteries, portal veins or biliary ducts, nor in the parenchyma or centrolobular veins. Few nNOS-IR fibres were detected in relation to the overall number of PGP-IR nerves. These results partly agree with those recently obtained by us in the rat (Esteban et al. 1997), where we demonstrated the presence of nitrergic fibres running along the hepatic artery, portal vein and biliary duct of the hepatic hilum, together with some nNOS-IR nerves located in the wall of the hepatic arteries near the hepatic hilum; however, we did not detect nNOS-IR neurons in the nerve plexus of the biliary hepatic branch of the rat. On the other hand, we detected nitrergic nerves in the vessels and ducts of the hepatic hilum, in the intralobular portal triads near the hilum, and some scarce varicose intraparenchymal nNOS-IR fibres in cat liver (Esteban et al. 1998); in addition, we have detected nitrergic ganglionated plexuses in the liver of this higher mammal. As can be seen, the nitrergic neuroeffector transmission in the trout liver is related to that of the lower mammals, compared to higher ones, in the same way as the general liver innervation. The most remarkable difference between the trout and rat innervation is the presence of ganglionated plexuses in the hepatic branch of the biliary duct of the trout. This difference may exist because fish possess a gallbladder, as do higher mammals, while the rat does not. According to this, Goehler & Sternini (1996) did not detect ganglionated plexuses in the hepatobiliary tract of the rat, although the presence of intrinsic neurons has been reported in the hepatobiliary duct of the guinea pig (Goehler et al. 1988), a species having a gallbladder.

We have not detected PGP-IR neurons in the hepatic branch of the trout biliary duct, a fact of no known significance. Siou et al. (1994) have found NADPH-stained ganglionic neurons devoid of PGP immunoreactivity in the gallbladder of the adult guinea pig, although the functional implications underlying this lack of immunoreactivity have not been determined.

The results presented in this work suggest the possibility that, as in mammals, the nitrergic nerves in the trout liver may be involved in the control of the afferent hepatic blood flow and in the autonomic regulation of hepatobiliary functions. It is widely accepted that nitrergic fibres contribute to the regulation of peripheral vascular tone by mechanisms which include the release of NO (Rand, 1992; Rand & Li, 1995). NO and ATP act as inhibitor neurotransmitters in the rabbit portal vein, and the presence of NADPH-nerves have been detected in the wall of this blood vessel (Brizzarola et al. 1993).

It has been suggested that NO acts as an inhibitor neurotransmitter in the gallbladder (Talmage & Mawe, 1993) where, as mentioned above, NADPH-d neuronal bodies have been detected. Li & Furness (1993) studied the distribution of nNOS-IR and NADPH-d positive nerves in the gastrointestinal tract of the trout; they suggested that NO is an important enteric inhibitor neurotransmitter in this teleost fish, but they included neither the gallbladder nor the liver in that study. Later, Karila & Holmgren (1995) demonstrated the presence of a cholinergic-serotonegic ascending and a nitrergic inhibitory descending peristaltic reflex in the intestine of teleost fish, suggesting that the peristaltic mechanisms were highly conserved throughout vertebrate evolution. Our recent results on the distribution of nNOS in the oesophagus of the cat and monkey (Rodrigo et al. 1998), are also consistent with the above reports.

In conclusion, all these facts taken together support the idea that there is a high degree of evolutionary conservation in the structure-function relationship of general liver innervation and in hepatic nitrergic transmission.

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REFERENCES

Bandaletova T., Brouet I, Bartsch H, Sugimura T, Esumi H, Ohshima H (1993) Immunohistochemical localization of an inducible form of nitric oxide synthase in various organs of rats treated with *Propionibacterium acnes* and lipopolysaccharide. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* **101**, 330–336.

Belai A, Schmidt HHHW, Hoyle CHV, Hassall CJS, Saffrey MJ, Moss J et al. (1992) Co-localization of nitric oxide synthase and NADPH-diaphorase in the myenteric plexus of the rat gut. *Neuroscience Letters* **143**, 60–64.

Belai A, Cooper S, Burnstock G (1995) Effect of age on NADPH-diaphorase-containing myenteric neurons of rat ileum and proximal colon. *Cell and Tissue Research* **279**, 379–383.

Berthoud HR, Kressel M, Neuhuber WL (1992) An anterograde tracing study of the vagal innervation of rat liver, portal vein and biliary system. *Anatomy and Embryology* **186**, 431–442.

Berthoud HR, Kressel M, Neuhuber WL (1995) Vagal afferent innervation of rat abdominal paraganglia as revealed by anterograde DiI-tracing and confocal microscopy. *Acta Anatomica* **152**, 127–132.

Bertin L (1954) Système nerveux. In *Traité de Zoologie*: *Anatomie. Systématique. Biologie. Agnathes et Poissons*. (ed Grassé P) Tome XIII, Fascicule I, pp. 854–923. Paris: Masson.

Bredt DS, Hwang PM, Snyder SH (1990) Localization of nitric oxide synthase indicating a neuronal role for nitric oxide. *Nature* **347**, 768–770.

Brizzolara AL, Rahima C, Burnstock G (1993) Evidence for the involvement of both ATP and nitric oxide in non-adrenergic, non-cholinergic inhibitory neurotransmission in the rabbit portal vein. *British Journal of Pharmacology* **109**, 606–608.

Esteban FJ, Pedrosa JA, Jimenez A, Fernandez AP, Bentura ML, Martinez-Murillo R et al. (1997) Distribution of neuronal nitric oxide synthase in the rat liver. *Neuroscience Letters* **226**, 99–102.

Esteban FJ, Pedrosa JA, Jimenez A, del Moral ML, Rodrigo J, Peinado MA (1998) Nitrergic innervation of the cat liver. *Neuroscience Letters* **243**, 45–48.

Friedman MI (1988) Hepatic nerve function. In *The Liver: Biology and Pathobiology*, 2nd edn, (ed. Arias IM, Jacoby WB, Popper H, Schachter D, Shafritz DA) pp. 949–959. New York: Raven Press.

Furness JB, Li ZS, Young HM, Förstermann U (1994) Nitric oxide synthase in the enteric nervous system of the guinea-pig: a quantitative description. *Cell and Tissue Research* **277**, 139–149.

Geller DA, Nussler AK, Di Silvio M, Lowenstein CJ, Shapiro RA, Wang SC et al. (1993) Cytokines, endotoxin, and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. *Proceedings of the National Academy of Sciences of the USA* **90**, 522–526.

Goehler LE, Sternini C, Brecha NC (1988) Calcitonin gene-related peptide immunoreactivity in the biliary pathway and liver of the guinea-pig: distribution and colocalization with substance P. *Cell and Tissue Research* **253**, 145–150.

Goehler LE, Sternini C (1996) Calcitonin gene-related peptide innervation of the rat hepatobiliary system. *Peptides* **17**, 209–217.

Griffith OW, Stuehr D (1995) Nitric oxide synthases: properties and catalytic mechanism. *Annual Review of Physiology* **57**, 707–736.

Gulbenkian S, Wharton J, Polak JM (1987) The visualization of cardiovascular innervation in the guinea-pig using an antiserum to protein gene product 9.5 (PGP 9.5). *Journal of the Autonomic Nervous System* **18**, 235–247.

Jackson P, Thomson VM, Thompson RJ (1985) A comparison evolutionary distribution of the two neuroendocrine markers, neurone-specific enolase and protein gene product 9.5. *Journal of Neurochemistry* **45**, 185–190.

Karila P, Holmgren S (1995) Enteric reflexes and nitric oxide in the fish intestine. *Journal of Experimental Biology* **198**, 2405–2411.

Li ZS, Furness JB, Young HM, Campbell G (1992) Nitric oxide synthase immunoreactivity and NADPH-diaphorase enzyme activity in neurons of the gastrointestinal tract of the toad, *Bufo marinus*. *Archives of Histology and Cytology* **55**, 333–350.

Li ZS, Furness JB (1993) Nitric oxide synthase in the enteric nervous system of the rainbow trout, *Salmo gairdneri*. *Archives of Histology and Cytology* **36**, 185–193.

Li ZS, Young HM, Furness JB (1994) Nitric oxide synthase in neurons of the gastrointestinal tract of an avian species, *Coturnix coturnix*. *Journal of Anatomy* **184**, 261–272.

Lin Y-S, Nokada S, Amakata Y, Maeda T (1995) Comparative study of the mammalian liver innervation: an immunohistochemical study of protein gene product 9.5, dopamine β-hydroxylase and tyrosine hydroxylase. *Comparative Biochemistry and Physiology* **110**, 289–298.

Mawe GM, Gershon MD (1989) Structure, afferent innervation, and transmitter content of ganglia of the guinea-pig gallbladder: relationship to the enteric nervous system. *Journal of Comparative Neurology* **283**, 374–390.

Moncada S, Higgs A (1993) The L-arginine-nitric oxide pathway. *New England Journal of Medicine* **329**, 2002–2012.

Murphy S, Li ZS, Furness JB, Campbell G (1994) Projections of nitric oxide synthase- and peptide-containing neurons in the small and large intestines of the toad (*Bufo marinus*). *Journal of the Autonomic Nervous System* **46**, 75–92.

Olsson C, Karila P (1995) Coexistence of NADPH-diaphorase and vasoactive intestinal polypeptide in the enteric nervous system of the Atlantic cod (*Gadus morhua*) and the spiny dogfish (*Squalus acanthias*). *Cell and Tissue Research* **280**, 297–305.

Rand MJ (1992) Nitrergic transmission: nitric oxide as a mediator of non-adrenergic, non-cholinergic neuroeffector transmission. *Clinical and Experimental Pharmacology and Physiology* **19**, 147–169.

Rand MJ, Li CG (1995) Nitric oxide as a neurotransmitter in peripheral nerves: nature of transmitter and mechanism of transmission. *Annual Review of Physiology* **57**, 659–682.

Riveros-Moreno V, Beddell C, Moncada S (1993) Nitric oxide synthase. Structural studies using autopeptide antibodies. *European Journal of Biochemistry* **215**, 801–808.

Rocha E, Monteiro RAF, Pereira CA (1995) Microanatomical organization of hepatic estroma of the brown trout *Salmo trutta fario* (Teleostei, Salmonidae): a qualitative and quantitative approach. *Journal of Morphology* **223**, 1–11.

Rode J, Dhillon AP, Doran JF, Jackson P, Thompson RJ (1985) PGP 9.5, a new marker for human neuroendocrine tumors. *Histopathology* **9**, 147–158.

Rodrigo J, Uttenthall LO, Peinado MA, Esteban FJ, Fernandez AP, Serrano J et al. (1998) Distribution of nitric oxide synthase in the esophagus of the cat and monkey. *Journal of the Autonomic Nervous System*, in press.

Siou GPS, Belai A, Burnstock G (1994) A developmental study of the localization of NADPH-diaphorase in the ganglionated plexus of the guinea-pig gallbladder. *Cell and Tissue Research* **276**, 61–68.

Shimazu T (1996) Progress and perspective of neuro-hepatology. In *Liver Innervation and the Neural Control of Hepatic Function* (ed. Shimazu T), pp. 3–13. London: John Libbey.

Springall DR, Riveros-Moreno V, Buttery L, Suburo A, Bishop AE, Merrett M et al. (1992) Immunological detection of nitric oxides synthase(s) in human tissues using heterologous

antibodies suggesting different isoforms. *Histochemistry* **98**, 259–266.

Talmage EK, Mawe GM (1993) NADPH-diaphorase and VIP are co-localized in neurons of gallbladder ganglia. *Journal of the Autonomic Nervous System* **43**, 83–89.

Thompson RJ, Doran JF, Jackson P, Dhillon AP, Rode J (1983) PGP 9.5, a new marker for vertebrate neurons and neuroendocrine cells. *Brain Research* **278**, 224–228.

Ward SM, Xue C, Shuttleworth CWR, Bredt DS, Snyder SH, Sanders KM (1992) NADPH-diaphorase and nitric oxide colocalization in enteric neurons of canine proximal colon. *American Journal of Physiology* **263**, G277–G248.

Young HM, Furness JB, Shuttleworth CWR, Bredt DS, Snyder SH (1992) Co-localization of nitric oxide synthase immunoreactivity and NADPH-diaphorase staining in neurons of the guinea-pig intestine. *Histochemistry* **97**, 375–378.