Localization of immunoreactivities for neuropeptides and neurotransmitter-synthesizing enzymes in the pterygopalatine ganglion of the pig

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ABSTRACT: Study on the presence of the selected biologically active substances in nerve structures of the porcine pterygopalatine ganglion was performed with the use of immunofluorescence and RT-PCR. All neurons in the ganglion were ChAT-, VAChT-, NOS- and VIP- positive. However, some neurons displayed strong immunoreactivity, while in other neurons, immunoreactivity was moderate, or weak. Somatostatin (SOM) was present in approx. 11% of neurons. Tyrosine hydroxylase-positive (TH-positive) neurons were not detected, although in single nerve cell bodies, TH antibody revealed very weak staining which could be attributed to some residual TH immunoreactivity. Immunoreactivity to NPY was found in 25% of all neuronal perikarya while PACAP was present only in 2–3% of them. More numerous neurons (6%) contained immunoreactivity to GAL. No neurons stained for SP or CGRP. Numerous ChAT-, VAChT-, NOS-, VIP-, and PACAP-positive, scarce SP and CGRP-positive, single SOM-, NPY- and GAL-positive nerve fibres were observed throughout the ganglion. No TH immunoreactivity was found in the nerve fibers. RT-PCR detected strong signal of the transcripts of ChAT, SOM, NOS, VIP, NPY, PACAP, and GAL. Only very weak signal was observed in case of TH, SP and CGRP. No RT-PCR was performed for VAChT message.

Keywords: pterygopalatine ganglion; pig; immunohistochemistry; RT-PCR

The pterygopalatine ganglion (PPG) is one of the cranial ganglia responsible for the parasympathetic innervation of the structures of the head. The ganglion is associated with the maxillary nerve, the branch of the trigeminal nerve (Gienc and Kuder, 1982; Szczurkowski *et al*., 2002). Postganglionic nerve fibers originating from the pterygopalatine ganglion are responsible for the innervation of the lacrimal gland: monkey (Vanderwerf, 1996), cat (Cheng *et al*., 2000; Kuchiiwa *et al*., 2000), other structures of the eye (Kuchiiwa, 1990; Elsas *et al*., 1994; Simons and Smith, 1994), glands of the nasal cavity (Kondo *et al*., 2000), palatum and vomeronasal organ (Matsuda *et al*., 1996). It is also possible that nerve fibers innervating the pineal gland originate from the PPG (Moller *et al*., 1996). Apart from the involvement of the pterygopalatine ganglion in the regulation of secretory functions, there is growing accumulation of evidence that the ganglion is deeply involved in the regulation of the cerebral blood flow (Talman and Dragon, 2000). The substance involved in the regulation of the tonus of the cerebral arteries is nitric oxide released by nitrergic nerve fibers originating from the PPG (Yoshida *et al*., 1993; Goadsby *et al*., 1996; Toda *et al*., 2000) but involvement of other biologically active substances, like vasoactive intestinal polypeptide (VIP) is also possible (Hara *et al*., 1985). It is possible that pterygopalatine ganglion is somehow involved in the pathogenesis of headache and migraine (Ekbom, 1999; Goadsby, 2000). Two populations of neuronal cells can be distinguished in the pterygopalatine ganglion: secretomotor neurons (large) and vasodilator neurons (small) (cat; Kuchiiwa *et al*., 2000). Varieties of biologically active substances were detected in the neurons of the pterygopalatine ganglion in various mammalian species. These substances include nitric oxide synthase (NOS) detected in the pterygopalatine neurons of humans (Uddman *et al*., 1999), rat (Warn *et al*., 1997) and cat (Goadsby *et al*., 1996), and neuropeptides such as VIP (Elsas *et al*., 1994; Uddman *et al*., 1999), pituitary adenylate cyclase-activating peptide (PACAP) (Elsas *et al*., 1994, Uddman *et al*., 1999) and peptide histidine-isoleucine (PHI) (Elsas *et al*., 1994). It was also suggested that some NPY-positive parasympathetic nerve fib-

ers originate from pterygopalatine ganglion in the rat (Elsas *et al*., 1994). However, systematic studies on the neurochemical coding of the neurons in the PPG are scarce and done exclusively on laboratory animals. Thus we decided to study the presence of some biologically active substances in the neurons of the porcine PPG using immunofluorescence and RT-PCR. The substances under study were: nitric oxide synthase (NOS) a marker of nitrergic neurons, choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT), markers of cholinergic neurons, tyrosine hydroxylase (TH), a marker of catecholaminergic neurons, as well as peptides: vasoactive intestinal polypeptide (VIP), PACAP, galanin (GAL), somatostatin (SOM), neuropeptide Y (NPY), substance P (SP) and calcitonin gene-related peptide (CGRP).

MATERIAL AND METHODS

6 female, sexually immature (body weight approx. 20 kg) pigs were used. 4 animals were deeply anaesthetized with pentobarbital (Vetbutal, Biowet, Poland; 30 mg/kg b.w. *i.v.*) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Pterygopalatine ganglia were dissected out, postfixed in the same fixative for 30 min, placed in 18% sucrose in 0.1 M phosphate buffer and stored at +4°C until they sank to the bottom of the container. 10 μm cryostat sections were put on chrome alum-gelatine-coated slides, allowed to dry and stored desiccated at –70°C until processed.

The slides were rehydrated in phosphate buffered saline (PBS, pH 7.4) and processed for double-immunofluorescence as described previously (Kaleczyc *et al*., 1999). Antibodies data are shown in Table 1.

Two animals were deeply anaesthetized as described previously and exsanguinated. The pterygopalatine ganglia were dissected out, snap-frozen in liquid nitrogen and stored at –70°C until RNA extraction. RNA isolation was done with Chomczynski and Sacchi method (Chomczynski and Sacchi, 1987). 2 μg of total RNA were reversely transcribed with $(dT)_{12}$ primer (Sigma USA) and Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT; Fermentas, LT) in 30 μl at 42°C for 1 h according to enzyme manufacturer's instructions. 0.5 μl of this reaction was used directly for PCR performed with primer pairs designed specifically to detect tran-

Table 1.Antisera used in the study

Antigen	Species	Code	Dilution	Supplier
Primary antibodies				
TH	mouse	2/40/15	1 : 120	Boehringer, Mannheim,GER
GAL	rabbit	Rin-7153	1 : 1 600	Penisula, SanCarlos, USA
SP	rat	RPN 1572	1 : 700	Amersham, Buckinghamshire, UK
CGRP	rabbit	RPN 1612	1 : 3 000	Amersham, Buckinghamshire, UK
VIP	mouse	MaVIP	1 : 1 500	East Acres, Southbridge,USA
NPY	rabbit	RNP1702	1 : 5 000	Amersham, Buckinghamshire, UK
SOM	rat	YC7	1 : 100	Serva, Heidelberg, GER
NOS	rabbit	210-504	1 : 5 000	Alexis, Lausen, Switzerland
PACAP	rabbit	IHC 8922	1 : 20 000	Penisula, SanCarlos, USA
VaChT	rabbit	G4481	1 : 5 000	Promega, Medison, USA
ChAT	rabbit	AB143	1 : 5 000	Chemicon, Temacula, USA
Secondary reagents				
FITC-coniug. goat anti-mouse IgG		55493	1 : 400	Cappel, Durham, USA
FITC-coniug. goat anti-rat IgG		55745	1 : 400	Cappel, Durham, USA
Biotinyl. goat anti-rabbit IgG		E 0432	1 : 400	Dako, Glostrup, DK
Streptavidin-CY3		016-160-084	1 : 4 000	Jackson Immunoresearch Lab. Inc.,USA

Table 2. Design of primers

Substance	PCR primer	Size of PCR product	Sequence of origin	Nucleotides	Supplier
TH	Sense 5′ TGCACCCAGTAYATCCGCCAYGC 3′ Antisense 5′ TAGYTCCTGAGCTTGTCCTT 3′	423 bp	Bovtha	950–1 372	Genset, France
GAL	Sense 5′ GGTCACCGGTGAAGGAAAAG 3′ Antisense 5′ GCTCAAACCTACTCCCAAAG 3′	450 bp	M13826	301–750	IDT, Coralville, USA
SP	Sense 5′ AACATGAAAATCATGGAGGC 3′ Antisense 5′ CATCCCGTTTGCCCATYAAT 3′	220 bp	Btta01	141–360	IDT, Coralville, USA
CGRP	Sense 5′ CTGCCCAGAAGAGAGCCTGC 3′ Antisense 5′ TGAAGGTCCCTGCGGCGGCG 3′	150 bp	Ratcal5	71–220	IDT, Coralville, USA
VIP	Sense 5′ GAGCAGTGAGGGAGAATCTC 3′ Antisense 5′ GTTCTGCTCTGTTGAATAG 3′	479 bp	Humviph	581–1 059	Genset, France
NPY	Sense 5′ GAGGACTTGGCCAGATACTA 3′ Antisense 5′ AGAAGGGTCTTCGAGCCTA 3′	130 bp	Q9N0M5	71–200	Genset, France
SOM	Sense 5′ GCCAAGTACTTCTTGGCG 3′ Antisense 5′ TGCAGCCCGCTTTGCGTT 3′	172 bp	SSU36385	176–347	IDT, Coralville, USA
NOS	Sense 5′ CGCATACGCACCCAGAGCTT 3′ Antisense5′ CCGGTCCAGTCTATTACGGT 3′	490 bp	SSU59924	3 466–3 955	Genset, France
PACAP	Sense 5′AATACTGCAGACGCTCATGG 3′ Antisense 5′ CTGAAGTAGCGGAACTGA 3′	218 bp	ADCYAP1	1–218	Genset, France
ChAT	Sense 5′ TGTCTGAGTACTGGCTGAAC 3′ Antisense 5′ AGATGCACCGCTCGATCATA 3′	576 bp	PIGCHAT	384–959	Genset, France

scripts of the studied biologically active substances except VAChT. The primer design and PCR parameters used for detection of the particular transcript are shown in Table 2. PCR products were analyzed on agarose gel stained with ethidium bromide along with molecular size marker M1 (DNA-Gdansk II Poland) and documented with 35 mm high speed black and white film.

In the experiments, the principles of laboratory care as well as the specific national laws on the protection of animals were followed.

RESULTS

All neurons in the ganglion studied displayed immunoreactivity to ChAT. Very numerous, evenly distributed ChAT-positive nerve fibers were also present in the ganglion under study. Small number of the ChAT-positive neurons contained simultaneously immunoreactivity to SOM (Figure 1a,b). SOM was present in ca. 11% of neurons of the PPG (only in the neurons of small diameter) and only single nerve fibers containing immunoreactivity to this peptide were observed in the ganglion. All neurons in the PPG displayed weak to moderate immunoreactivity to VAChT. VAChT-positive nerve fibers were unevenly distributed throughout the ganglion. Some areas of the PPG contained dense plexuses of VAChT-positive nerve fibers, which were sometimes forming basket-like structures around neurons, while some other areas of the ganglion were devoid of VAChT-positive nerve fibers. The relation of immunoreactivities to VAChT and SOM in the neurons was the same as in case of ChAT and SOM (Figure 1c,d). All neurons in the PPG were NOS-positive, however some nerve cell bodies displayed markedly by stronger immunoreactivity to this substance, while in other neurons, NOS immunoreactivity was moderate. This was

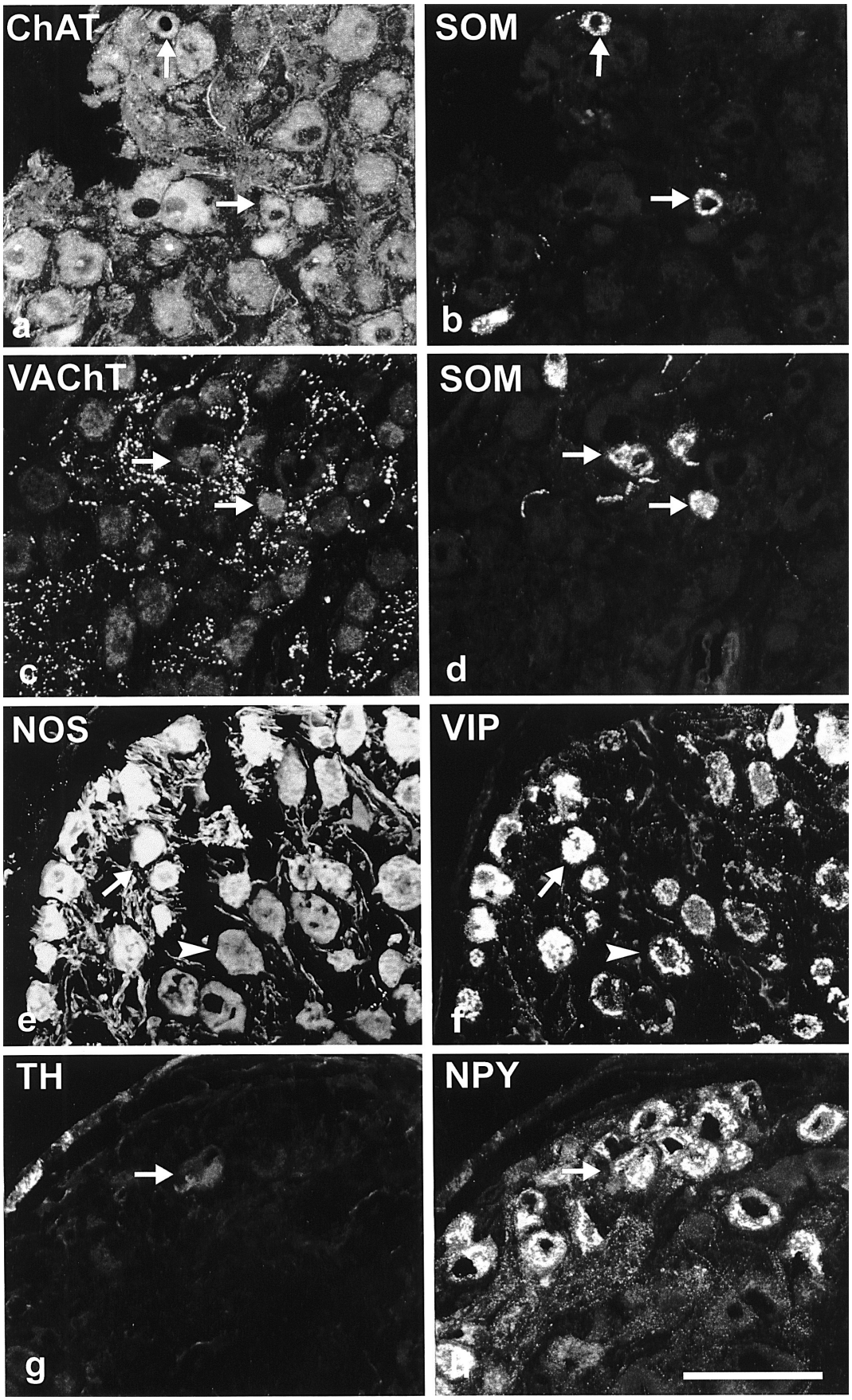

Figure 1. Double-labeling of sections from the porcine PPG. Scale bar 100 μm

true both for small and large neurons. In addition, numerous NOS-positive, non-varicose nerve fibers were observed throughout the PPG. These fibers were evenly distributed throughout the ganglion forming sometimes plexuses around nerve cell bodies. The presence of immunoreactivity VIP was detected in all ChAT-positive neurons of the PPG (Figure 1e,f), however the intensity of staining was different from neuron to neuron. In some nerve cell bodies strong VIP-positive staining was observed, whereas in other neurons only moderate, or weak immunostaining was detected. Numerous VIP-positive nerve fibers were distributed throughout in the PPG. They were usually traversing the PPG as non-varicose fibres, but sometimes varicose VIP-positive nerve terminals were also found. Contrary to the situation with NOS, ChAT and VAChT, no clearly TH-positive neurons were observed in the PPG, although in single neurons very weak TH immunostaining was detected which could be attributed to some residual TH immunoreactivity. No TH-positive nerve fibers were found in the PPG, although TH immunoreactivity was observed in some nerve fibers of nerve trunks running outside the ganglion (Figure 1g,h). NPY was present in 25% of neurons (mainly of large diameter) of the porcine PPG while NPY-positive nerve fibers (non-varicose) were scarce. All NPY-positive neurons in the ganglion under study contained immunoreactivity to VIP (Figure 2a,b).

The immunoreactivity to PACAP was revealed only in a relatively small subpopulation of neurons in the PPG (2–3% of all neuronal cell bodies – these neurons belonged exclusively to the subpopulation of large neurons), while numerous, usually varicose PACAP-positive nerve fibers formed basket-like structures around nerve cell bodies. All PACAP-positive neurons in the porcine PPG contained immunoreactivity to VIP (Figure 2c,d). More numerous neurons (ca. 6%) and single nerve fibers contained immunoreactivity to GAL. The vast majority of GAL-positive nerve cell bodies belonged to the subpopulation of large neurons. All GAL-positive neuronal somata contained simultaneously immunoreactivity to VIP (Figure 2e,f). No neurons of the PPG stained for SP or CGRP, while immunoreactivity to these peptides was found in a small population of nerve fibers. All SP-positive nerve fibers contained simultaneously immunoreactivity to CGRP, but only a fraction of CGRP-positive nerve fibers contained immunoreactivity to SP (Figure 2g,h). Sometimes CGRP-immunoreactivity was present in nerve fibers forming clusters in discrete regions of the PPG.

RT-PCR detected strong signal of the transcripts of NOS, ChAT, VIP, PACAP, GAL, SOM and NPY. In cases of TH, SP and CGRP only very weak bands of the PCR products were observed in the gel (Figure 3). No RT-PCR was performed for VAChT.

DISCUSSION

The data dealing with the immunohistochemical coding of the neurons in the mammalian pterygopalatine ganglion are relatively scarce. In humans, the presence of VIP, PACAP, NOS and CGRP was studied with immunohistochemistry (Uddman *et al*., 1999). It was found that the great majority of neurons in this ganglion contained VIP (>90%) and NOS (ca. 80%) while 60% of these neurons contained PACAP. On the contrary, NOS-positive nerve cell bodies in the pterygopalatine ganglion of the rat made only 40% of the neuronal

Figures 1a,b present the same section double-stained for ChAT (a) and SOM (b). All neurons were ChAT- positive and some of them (of small diameter) contained simultaneously immunoreactivity to SOM. Arrows mark some of the double-labeled neurons

Figures 1c,d show the same section double-stained for VAChT (c) and SOM (d). Neurons in Fig. 1c displayed different intensity of VAChT immunostaining. Moderately dense plexuses of VAChT-positive nerve fibers were also observed in the section

Figure 1d presents a small population of neurons containing immunoreactivity to SOM. Arrows show some of the neurons, which were immunoreactive simultaneously for VAChT and SOM

Figures 1e,f present the same section double-stained for NOS (e) and VIP (f). Figure 1e shows the neurons displaying strong (arrow) and moderate (arrowhead) immunoreactivity to NOS. Fig. 1f shows different intensity of staining VIP immunoreactivity in nerve cell bodies. Arrows and arrowheads mark some of the double-labeled neurons

Figures 1g,h present the same section double-stained for TH (g) and NPY (h). In Figure 1g, arrow shows the neuron displaying very week immunoreactivity to TH. Figure 1h presents neurons containing immunoreactivity to NPY. Neurons, which stained for weakly TH in Figure 1g, were simultaneously NPY-positive (arrow, Figure 1h)

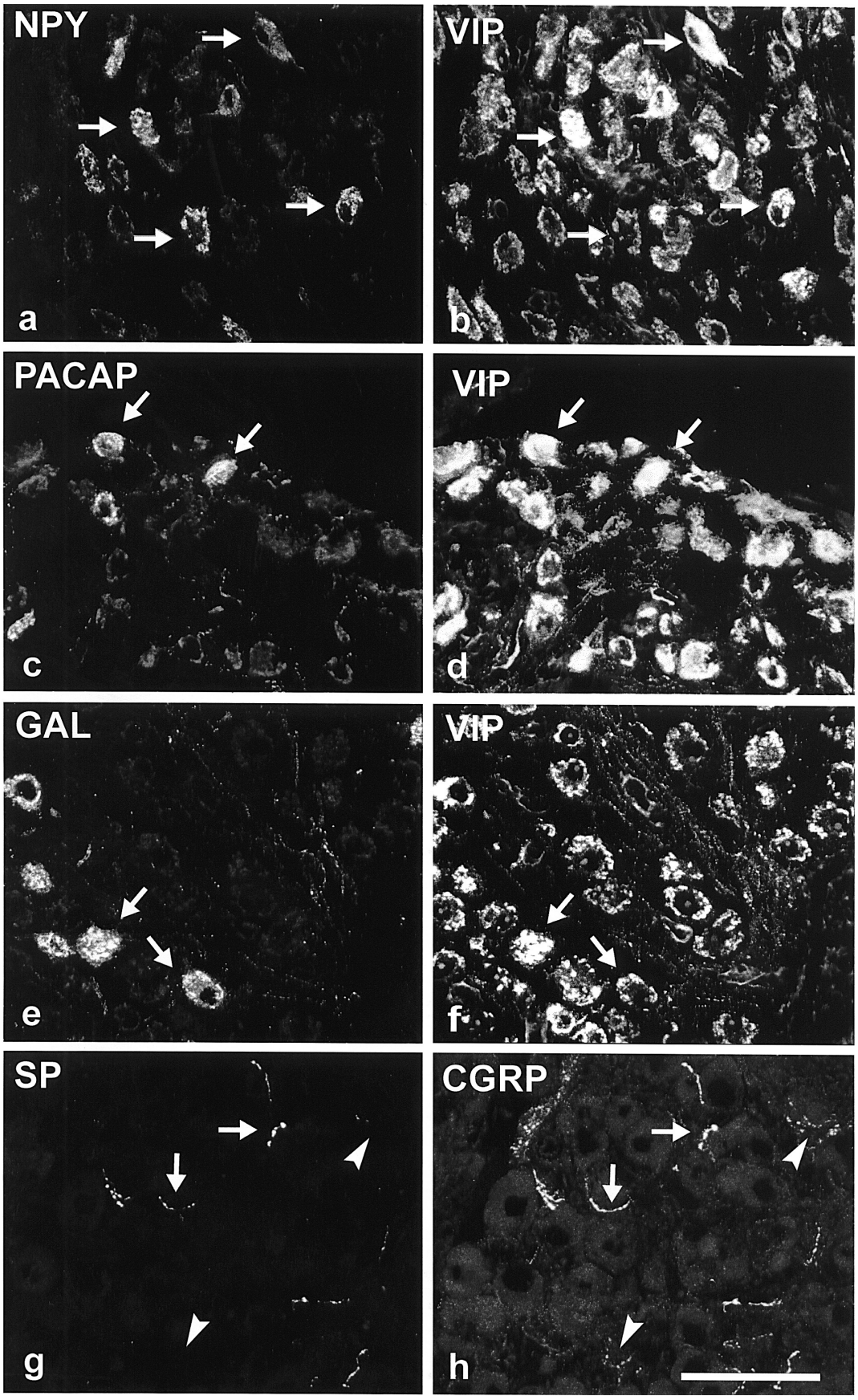

Figure 2. Double-labeling of a section from the porcine PPG. Scale bar 100 μm

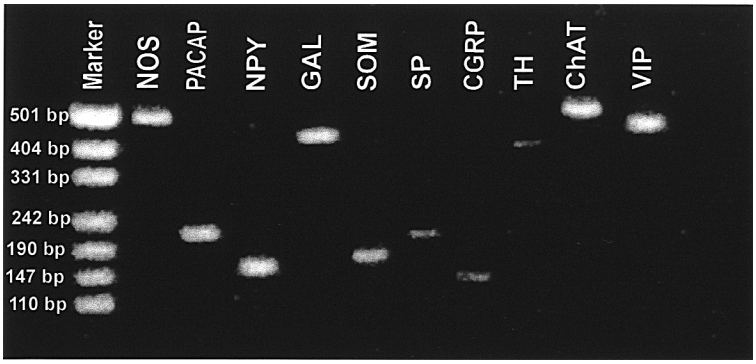

Figure 3. Gel electrophoresis of RT-PCR products corresponding to mRNA species encoding the porcine neuropeptides and neurotransmitter-synthesizing enzymes in the PPG. Strong signal was coming from the transcripts of ChAT, SOM, NOS, VIP, NPY, PACAP, and GAL. In cases of mRNAs for TH, SP and CGRP, only very weak signal was observed

population (Warn *et al*., 1997). Since we claim the presence of VIP, and NOS in all neurons of the porcine pterygopalatine ganglion, it seems that there are some species related differences in the number of the neurons displaying immunoreactivity to VIP and NOS. The amazing finding is also a very low number of PACAP-immunoreactive neurons in the porcine PPG as compared to that found in the human ganglion.

No data on the presence of ChAT-positive neurons in the pterygopalatine ganglion of other species are available. On the other hand, neurons positive for ChAT were detected in the quail ciliary ganglion. However, in the avian ciliary ganglion, less than 50% of neurons express immunoreactivity to ChAT (Coulombe and Bronner, 1990) what stays in contrast to our findings from the porcine ganglion. Data exist suggesting that pterygopalatine ganglion neurons of the rat are cholinergic (Hara *et al*., 1985). In these neurons, the presence of acetylcholinesterase (AChE), the enzyme of the acetylcholine break-down (believed to be also a marker of cholinergic nerve fibers), was detected. In the rat, AChE co-localized with immunoreactivity for VIP (the peptide being co-transmitter in cholinergic nerve fibers) and these neurons were found to contribute to the innervation of the cerebral arteries (Hara *et al*., 1985).

The presence of ChAT immunoreactivity in all neurons of the porcine PPG corresponds with the absence of TH-immunoreactivity in these neurons. There are no reports of the presence of immunoreactivity to TH in PPG neurons in other species. This proves the exclusively cholinergic character of the neurons in the porcine PPG, and TH-positive nerve fibers present in the nerve trunks located in the vicinity of the PPG must originate from superior cervical ganglion, sympathetic ganglion of the head region. Residual TH-immunoreactivity in some neurons of the porcine PPG concords with reports on the existence of very restricted population of TH-positive neurons present in the ciliary

Figures 2a,b present the same section double-stained for NPY (a) and VIP (b). Figure 2a shows NPY immunoreactivity in PPG neurons. Figure 2b shows different intensity of immunostaining for VIP in nerve cell bodies. Arrows mark some of the neurons double-labeled for NPY and VIP

Figure 2c,d present the same section double-stained for PACAP (c) and VIP (d). Neurons which displayed PACAP immunoreactivity (Figure 2c) intensely stained also for VIP (Figure 2d). Arrows mark some of the neurons double-labeled for PACAP and VIP

Figure 2e,f present the same section double-stained for GAL (e) and VIP (f). Figure 2e shows not numerous, GAL-positive neurons and scarce GAL-positive nerve fibers. Figure 2f demonstrates numerous, intensely VIP-positive neurons. Arrows mark some of the neurons double-labeled for GAL and VIP

Figure 2g,h present the same section double-stained for SP (g) and CGRP (h). Nerve fibers contained simultaneously SP and CGRP (arrows) or CGRP only (arrowheads). No neurons stained for these peptides.

ganglion of species such as monkey, dog, cat and rat (Uemura *et al*., 1987). The lack of immunoreactivity to dopamine hydroxylase (DBH) and phenylethanolamine-N-methyltransferase (PNMT) in these neurons suggests their dopaminergic character (Uemura *et al*., 1987). However, the presence of DBH and PNMT in TH-positive neurons of the porcine PPG must still be elucidated.

Relatively low number of neurons (ca. 6%) in the porcine PPG contained immunoreactivity for GAL, another peptide considered as closely associated with cholinergic neurons. In the cat, ca. 80% of neurons in the PPG contained immunoreactivity to GAL (Grimes *et al*., 1994), thus the low number of GAL-positive neurons in the porcine PPG is quite surprising. No data exist on the presence existence of SOM in neurons of the PPG in other species. SOM-positive neurons, which make approx. 11% of total neuronal population of the porcine PPG, belonged exclusively to the subpopulation of small nerve cells. This suggests that SOM is associated with vasomotor functions exerted by neurons of the PPG, in contrast to neuropeptides like GAL, PACAP associated preferably with large nerve cells (regarded to be secretomotor neurons), or VIP present in the porcine PPG in both types of neurons.

The surprisingly high number of PPG neurons shows immunoreactivity to NPY, the peptide regarded to be the co-transmitter of adrenergic nerve fibers. The lack of TH-immunoreactivity in these neurons suggests that NPY is involved in non-adrenergic regulation of the peripheral tissues function. In the rat, PPG neurons expressing NPY were found to contain also VIP and/or ChAT immunoreactivity, and did not contain catecholamines and TH (Leblanc *et al*., 1987).

RT-PCR used to detect transcripts of the precursors of the substances studied confirmed results of the immunohistochemical staining. It detected transcripts of NOS, ChAT, VIP, PACAP, GAL, NPY and SOM, thus substances immunoreactivities for which were found in the neurons of the porcine PPG. In case of TH, SP and CGRP, immunoreactivities for which were absent in neuronal cell bodies of the porcine PPG, very weak signals were detected with RT-PCR. This may be due to the presence of the mRNAs for TH, SP and CGRP in other, non-neuronal, tissues like leukocytes, but it cannot be excluded that a very low level of transcription of genes for these substances may occur in neurons of the porcine PPG. It may correspond to the very low level of immunostaining for TH displayed by certain nerve cell bodies in the porcine PPG. Very low signal of mRNA for TH confirms cholinergic character of neurons in this ganglion. No data exist on the expression of the neuropeptides and NOS, ChAT and TH at mRNA level in the PPG of other species.

The present results suggest the existence of profound inter-species differences regarding the immunohistochemical characteristics of PPG nerve structures. This suggests, also profound differences in the organization of the cranial parasympathetic system in higher vertebrates. Further studies are necessary to elucidate the morphological peculiarities of the PPG in different species and their physiological significance.

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