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Immunohistochemical characterisation of neurons in the mandibular ganglion and nerve fibres supplying the porcine mandibular gland

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ABSTRACT: The present study was designed to investigate the chemical coding of neurons in the mandibular ganglion (MGn) and nerve fibres supplying the porcine mandibular gland (MGl) with the use of immunofluorescence and RT-PCR. The cryostat sections from MGn and MGl were processed for double-labelling immunohistochemistry using antisera against vesicular acetylcholine transporter (VAChT), choline acetyltransferase (ChAT), dopamine β-hydroxylase (DβH), neuronal nitric oxide synthase (nNOS), vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY), galanin (GAL), substance P (SP) and calcitonin gene-related peptide (CGRP). The MGl was found to be richly supplied by VAChT-positive nerve fibres that surrounded intra- and interlobular salivary ducts. A large number of VAChT-immunoreactive (VAChT-IR) nerve terminals were also observed around acini. Many periductal and periacinar nerve fibres stained positive for DβH. Immunoreactivity to GAL, NPY or VIP was observed in an intermediate number of nerve terminals which were associated with both salivary ducts and acini. Double-immunostaining revealed that in MGn nearly all neurons stained positive for VAChT/ChAT (98.45 ± 0.59%, mean ± SEM) and nNOS (99.71 ± 0.18%). An intermediate number of the nerve cell bodies displayed immunoreactivity to NPY or VIP (18.67 ± 0.52% and 8.11 ± 0.36%, respectively). Single GAL-IR and CGRP-positive neurons were also observed. RT-PCR revealed the presence of transcripts of ChAT, VAChT, nNOS, NPY, VIP and GAL. For SP and DβH very weak signals were observed. RT-PCR with primers targeting CGRP did not generate any PCR product.

Keywords: mandibular gland; mandibular ganglion; neuropeptides; immunohistochemistry; RT-PCR; swine

It has been found that innervation plays an essential role in regulatory circuits involved in the control of saliva synthesis and excretion (Proctor and Carpenter 2007). Major salivary glands are known to receive sympathetic innervation from neurons in the cranial cervical ganglion (Gibbins 1991; Klimaschewski et al. 1996; Arciszewski and Zacharko 2003). The parasympathetic nerve supply is provided by respective parasympathetic ganglia of the head. The mandibular (MGl; also named submandibular and sublingual glands are innervated by parasympathetic neurons in the MGn, while parasympathetic innervation to the parotid gland originates in the otic ganglion. Sensory innervation to the major salivary glands derives from the trigeminal ganglion (Chibuzo and Cummings 1980; Kobashi et al. 2005).

It has been demonstrated in a variety of species including the rat, mouse, cat, ferret and humans that nerve fibres innervating the MGl may contain biologically active substances, including vesicular acetylcholine transporter (VAChT; cholinergic marker), neuronal nitric oxide synthase (nNOS), vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase activating peptide (PACAP), VIP-related peptide histidine methionine (PHM), neuropeptide Y (NPY), somatostatin (SOM), galanin (GAL), Met5-enkephalin (Met-ENK), substance P (SP) and calcitonin gene-related peptide (CGRP) (Soinila et al. 1989; Tobin et al. 1990; Shida et al. 1991; Hauser-Kronberger et al. 1992; Lohinai et al. 1995; Tobin et al. 1995; Alm et al. 1997; Jia et al. 1997; Kusakabe et al. 1997; Takai et al. 1999;

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Del Fiacco et al. 2014). Although relatively much is known about the innervation of the MGl, it should be emphasised that the studies mentioned above have mostly focused on laboratory mammals and humans. Furthermore, studies on the neurochemical coding of neurons in the MGn are scarce and were performed exclusively on laboratory animals. Neurons in the rat, monkey and ferret MGn have been found to exhibit tyrosine hydroxylase (TH)-, nNOS-, VIP-, NPY-, ENK-, and SP-immunoreactivity (Shida et al. 1991; Alm et al. 1997; Takai et al. 1999). Interestingly, only a very limited amount of information is available on the expression of cholinergic markers in these parasympathetic nerve cells (Schafer et al. 1998).

As already mentioned, the vast majority of data dealing with the chemical coding of neurons and nerve fibres contributing to the innervation of mammalian salivary glands including the MGl have been gained in studies performed in laboratory animals. However, corresponding information regarding other mammalian species including domestic animals as well as the domestic pig, are scarce and fragmentary. In the domestic pig, especially in males, the MGl is an interesting structure which has been found to be involved not only in digestive processes but also in endocrine circuits associated with sexual behaviour (Booth 1980). Moreover, it should also be mentioned that not only is the pig an important animal for veterinary medicine and agriculture; it has also become a critically important experimental animal in biomedical research (Swindle et al. 2012). Recently, it has been postulated that many structures of the porcine head, including salivary glands, should be considered as better models for research of pathological processes affecting corresponding areas in humans than those of small laboratory animals (rodents; Stembirek et al. 2012).

Therefore, the present study was designed to investigate the expression and co-occurrence of the cholinergic marker VAChT and other biologically active substances including adrenergic marker dopamine β-hydroxylase (DβH) and nNOS, neuropeptides previously found in porcine adrenergic and cholinergic peripheral autonomic neurons, NPY, VIP, and GAL, and neuropeptides involved in sensory transmission, CGRP and SP, in nerve terminals supplying the porcine MGl and neurons in the MGn using single- and double-labelling immunofluorescence and RT-PCR. In addition, the distribution of the immunoreactive nerve fibres in the MGl was compared between serous and mucous acini, because some differences in the innervation of the human gland associated with the acinar structure have been reported.

MATERIAL AND METHODS

The experiments were performed on ten juvenile (five male and five female, 12–15 kg body weight) pigs of the Large White Polish race obtained from a commercial fattening farm in Purda (Poland). All the animals were housed and treated in accordance with the rules approved by the local Ethics Commission (affiliated to the National Ethics Commission for Animal Experimentation, Polish Ministry of Science and Higher Education).

Removal, fixation and sectioning of mandibular glands. Prior to perfusion, the pigs (three males and three females) were deeply anaesthetised. All the animals were pre-treated with atropine (Polfa, Warsaw, Poland; 0.01 mg/kg body weight, *s.c.*) and azaperone (Stresnil, Janssen, Beerse, Belgium; 8 mg/kg body weight, *i.m.*). The main anaesthetic, pentobarbital (Vetbutal, Biowet, Pulawy, Poland: 25mg/kg body weight) was given *i.v.* Then, the animals were transcardially perfused with 1 l of preperfusion solution containing 0.9% sodium chloride (Chemia, Gliwice, Poland), 2.5% polyvinylpyrrolidone (Sigma, Deisenhofen, Germany), 0.5 procaine hydrochloride (Polfa, Warsaw, Poland) and 20 000 IU of heparin (Heparinum; Polfa; Warsaw, Poland, added *ex tempore*), followed by 4 l of 4% paraformaldehyde in phosphate buffer (PB, pH 7.4).

Samples of mandibular glands with salivary ducts and whole mandibular ganglia were dissected out and postfixed by immersion in the buffered paraformaldehyde solution as described above for 2 h, then rinsed with phosphate buffer (pH 7.4) and transferred to and stored in 18% buffered sucrose solution (pH 7.4) at 4 °C until further processing. (The mandibular ganglion is located in the hilum of the gland, nevertheless, it should be mentioned that some solitary small ganglia consisting of a few to several neurons embedded in the glandular tissue were also encountered while analysing the sections for the distribution and immunohistochemical characteristics of intraglandular nerve fibres.) 10 µm cryostat sections from the salivary glands were mounted on chrome alum-gelatine-coated glass slides.

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Immunohistochemical procedure. MGl sections were processed for single- or double-immunofluorescence. They were washed and pre-incubated in phosphate buffered saline (PBS) containing 0.3% Triton X-100 (pH 7.4), 0.1% sodium azide, and 4% normal goat serum or normal horse serum (NGS or NHS) for 45 min at room temperature (RT). Then, the sections were incubated with the primary antibodies diluted in the preincubation solution for 14–24 h at RT. Afterwards, they were washed thoroughly with PBS and incubated with the secondary antibodies. After extensive washing with PBS the sections were mounted in 70% glycerol in PBS. The primary antisera and secondary reagents are listed in Table 1. The labelled sections were examined with a fluorescence microscope and a LSM 700 confocal laser scanning microscope (Zeiss, Oberkochen, Germany). Stacks of images were compiled to produce maximum intensity projection images with ZEN 2009 software (Zeiss, Oberkochen, Germany).

The relative frequency of nerve fibres (– = not found, ± = single fibres, + = few fibres, ++ = intermediate number of fibres, +++ = many fibres) was assessed using a semi-quantitative method matching that applied in our previous paper (Kaleczyc et al. 1999). The evaluation of the structures in the same preparations was independently performed by two investigators. To determine percentages of particular neuronal populations, at least 200 neuronal profiles investigated for the expression of one of the biologically active substances were counted. The sections stained for the same combination of the antigens assigned to quantitative investigations were separated by at least 100 µm to avoid double-analysis of neuronal somata. All results are expressed as means ± SEM. Statistical analysis dealing with potential differences in the chemical coding of the neurons between male and female animals was carried out in columns with Student's *t*-test (GraphPad Prism v. 2.0, GraphPad Software Inc., San Diego, USA). The differences were considered as statistically significant at $P \leq 0.05$.

To differentiate between the serous and mucous acini, some immunostained sections were counterstained with haematoxylin and eosin.

Controls. Standard controls, i.e. pre-absorption for the neuropeptide antisera (20 µg of appropriate antigen per 1 ml of corresponding antibody at working dilution; all antigens purchased from Peninsula, Sigma or Dianova), as well as omission and replacement of the respective primary antiserum with the corresponding non-immune sera completely abolished immunofluorescence and eliminated specific staining.

RT-PCR. RT-PCR methods are much more sensitive than immunohistochemistry. It allows

Table 1. List of primary antisera and secondary reagents used for immunohistochemistry

Antigen	Host	Code	Dilution	Supplier
Primary antisera				
DβH	mouse, monoclonal	MAB308	1 : 500	Milipore, Temecula, CA, USA
ChAT	goat, polyclonal	AB144P	1 : 50	Milipore, Temecula, CA, USA
VAChT	rabbit polyclonal	V5387	1 : 4000	Sigma, Saint Louis, MI, USA
CGRP	rabbit polyclonal	11535	1 : 2000	Cappel, Aurora, OH, USA
SP	rat monoclonal	8450-0505	1 : 250	ABD Serotec, Oxford, UK
GAL	rabbit polyclonal	RIN 7153	1 : 2000	Peninsula, San Carlos, CA, USA
VIP	mouse monoclonal	MaVIP	1 : 500	East Acres Biologicals, Southbridge, MA, USA
NPY	rabbit polyclonal	NA 1233	1 : 400	Biomol, Exeter, UK
NPY	rat polyclonal	NZ 1115	1 : 200	Biomol, Exeter, UK
nNOS	mouse monoclonal	N2280	1 : 100	Sigma, Saint Louis, MI, USA
Secondary reagents				
Alexa Fluor 488-donkey anti-rabbit IgG			1 : 500	Invitrogen, Paisley, UK
Alexa Fluor 488-donkey anti-mouse IgG			1 : 500	Invitrogen, Paisley, UK
Alexa Fluor 488-donkey anti-rat IgG			1 : 500	Invitrogen, Paisley, UK
Alexa Fluor 555-donkey anti-rabbit IgG			1 : 500	Invitrogen Paisley, UK
Alexa Fluor 555-donkey anti-mouse IgG			1 : 500	Invitrogen, Paisley, UK
Alexa Fluor 555-donkey anti-goat IgG			1 : 500	Invitrogen, Paisley, UK

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detection of neurotransmitters or neurotransmitter-synthesising enzymes of interest that are expressed at very low levels. To confirm or exclude the presence of the biologically active substances of interest in the MGn at the mRNA level an RT-PCR method was applied. In the present study, the term MGn was related to a large ganglion located in the hilum of the gland which was observed in all pigs which underwent immunohistochemical investigations.

Four animals (two males and two females) were deeply anaesthetised as described previously and exsanguinated. Samples of the tissues from the hilum region were dissected out, snap-frozen in liquid nitrogen and stored at –70 °C until RNA extraction. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse transcribed to cDNA using RevertAid™ Reverse Transcriptase (Fermentas, LT) and oligo (dT). PCR was conducted on cDNA, using the primers listed in Table 2. The PCR protocol included initial denaturation (at 95 °C for 2 min), followed by 34 cycles comprising denaturation (at 95 °C for 15 s), annealing (temperature according to Table 2, for 20 s) and polymerisation (at 72 °C for 30 s), and final extension (at 72 °C for 5 min). cDNA from the porcine superior cervical ganglion and dorsal root ganglion were used as positive controls. PCR products were run on agarose gels with the GeneRuler™ 100 bp DNA Ladder (Fermentas, LT) and stained with ethidium bromide.

RESULTS

Because no distinct differences were observed in the innervation of the MGl and neurochemical coding of MGn neurons between male and female pigs, the results are described together. Further, since no apparent variations were found in the innervation of the serous and mucous glandular acini, the relevant information is provided without differentiation between these two types of glandular structures.

The mandibular ganglion

Intraganglionic VAChT-positive varicose nerve fibres were very numerous and supplied nearly all neuronal profiles in the MGn, often forming dense basket-like formations surrounding particular nerve cell bodies (Figures 1A, 2A, 4A and 6B). Smooth VAChT-immunoreactive nerve terminals were intermediate in number and were observed within thick nerve bundles both traversing as well as emanating

Table 2. Primer sets for RT-PCR

Gene	Oligonucleotide sequence	Annealing temperature (°C)	Size of PCR product (bp)
DβH	forward 5' CCACTACAGCCCACACTT3' reverse 5' GCAGGTGCAGACTTCCTC 3'	63	533
ChAT	forward 5' TGTCTGAGTACTGGCTGAAC3' reverse 5' AGATGCACCGCTCGATCATA 3'	56	576
VAChT	forward 5' GTTTCGCTGCTCGACGCCCT 3' reverse 5' ATCACTGCCAGCCCGAACGC 3'	63	350
nNOS	forward 5' TTCGTGCGTCTCCACACCAA 3' reverse 5'AGTACTTGAAGGCCTGGAAGAT 3'	60	250
NPY	forward 5' GAGGACTTGGCCAGATACTA 3' reverse 5' AGAAGGGTCTTCGAGCCTA 3'	52	159
VIP	forward 5' CTTTTTGGAGCACCTTCTGC 3' reverse 5' TCGCTTCTCCTTCTCCTTCA 3'	58	492
GAL	forward 5' GGTCACCGGTGAAGGAAAAG 3' reverse 5' GCTCAAACCTACTCCCAAAG 3'	52	450
SP	forward 5' AACATGAAAATCATGGAGGC 3' reverse 5' CATCCCGTTTGCCCATYAAT 3'	57	220
CGRP	forward 5' CTGCCCAGAAGAGAGCCTGC 3' reverse 5' TGAAGGTCCCTGCGGCGGCG 3'	66	150

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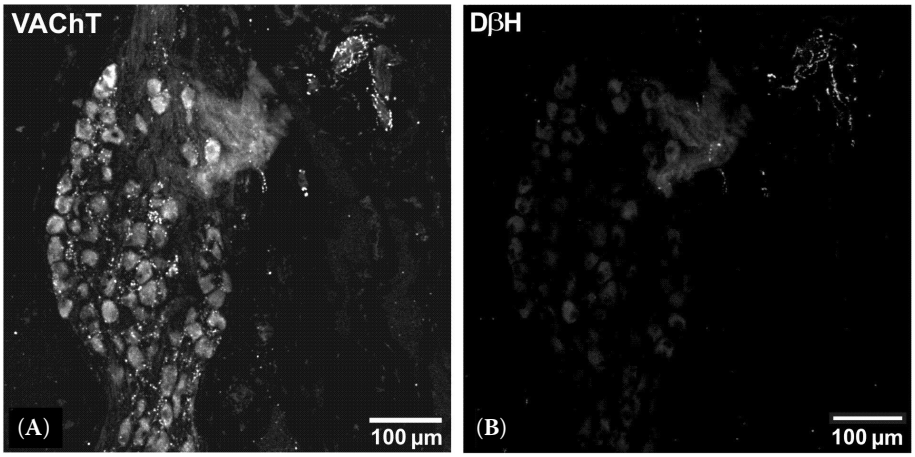

Figure 1. Photomicrographs showing a section from the porcine mandibular ganglion (MGn) double-stained with antibodies against vesicular acetylcholine transporter (VAChT) and dopamine β-hydroxylase (DβH). Numerous VAChT-positive MGn neurons (**A**) were DβH negative (**B**)

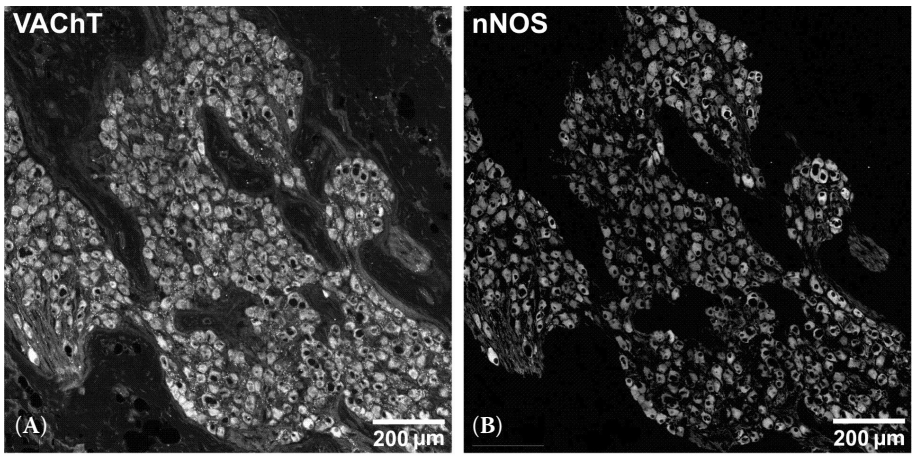

Figure 2. Photomicrographs showing a section from the porcine mandibular ganglion (MGn) double-stained with antibodies against vesicular acetylcholine transporter (VAChT) and neuronal nitric oxide synthase (nNOS). All the neurons simultaneously exhibited immunoreactivity to VAChT (**A**) and nNOS (**B**)

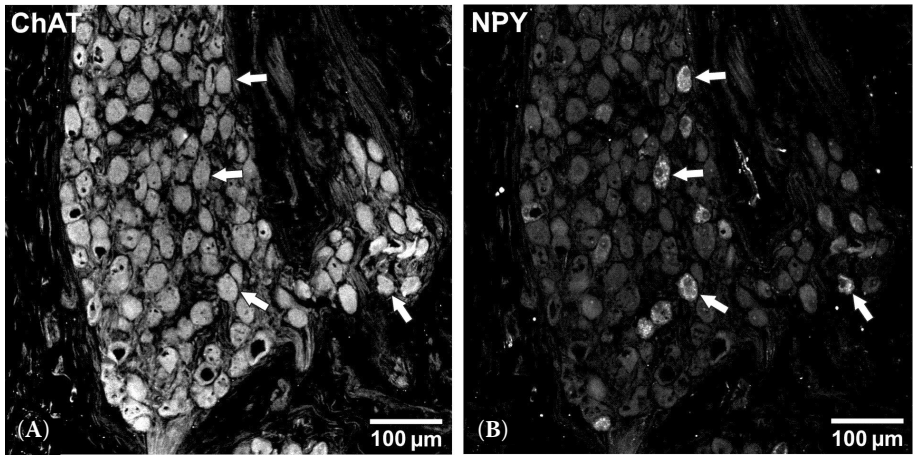

Figure 3. Photomicrographs showing a section from the porcine mandibular ganglion (MGn) double-stained with antibodies against choline acetyltransferase (ChAT) and neuropeptide Y (NPY). All the neurons were CHAT-positive (**A**) but only some of them were simultaneously NPY-positive (**B;** arrows)

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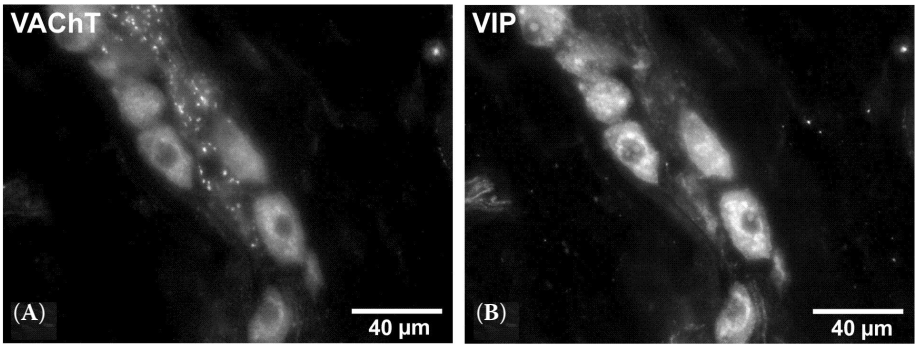

Figure 4. Photomicrographs showing a section from the small intraglandular ganglia double-stained with antibodies against vesicular acetylcholine transporter (VAChT) and vasoactive intestinal polypeptide (VIP). All visible neurons simultaneously exhibited immunoreactivity to VAChT (**A**) and VIP (**B**)

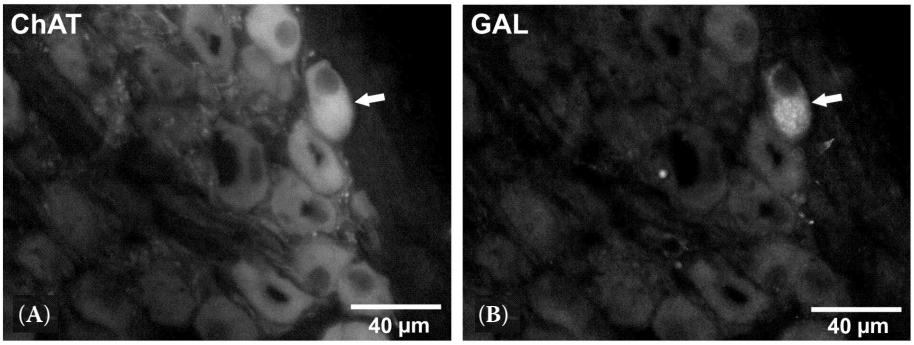

Figure 5. Photomicrographs showing a section from the porcine mandibular ganglion (MGn) double-stained with antibodies against choline acetyltransferase (ChAT) and galanin (GAL). The arrow indicates a ChAT-positive neuron (**A**) which also stained positive for GAL (**B**)

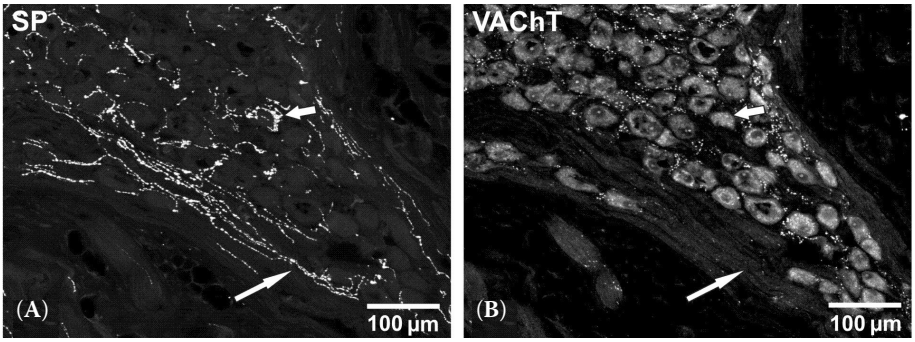

Figure 6. Photomicrographs showing a section from the porcine mandibular ganglion (MGn) double-stained with antibodies against substance P (SP) (**A**) and vesicular acetylcholine transporter (VAChT) (**B**). Intraganglionic SP-positive nerve fibres (long arrow); some fibres surrounded VAChT-positive neurons (short arrow)

from the ganglion. Another abundant population of intraganglionic varicose nerve fibres was that consisting of nerve terminals which stained for SP (Figure 6A). These fibres intensely supplied many neurons frequently forming loose basket-like formations surrounding particular neuronal somata. Intermediate numbers of intraganglionic nerve fibres expressed immunoreactivity to CGRP and solitary nerve terminals stained for GAL. Smooth CGRP- or GAL-positive fibres were observed within nerve bundles passing across the ganglion. Varicose axons were sometimes observed in a close association with the perikarya.

Double-immunostaining revealed that all neurons in the MGn expressed VAChT/ChAT (98.45 ± 0.59%; Figures 1A, 2A, 3A, 4A, 5A and 6B) and

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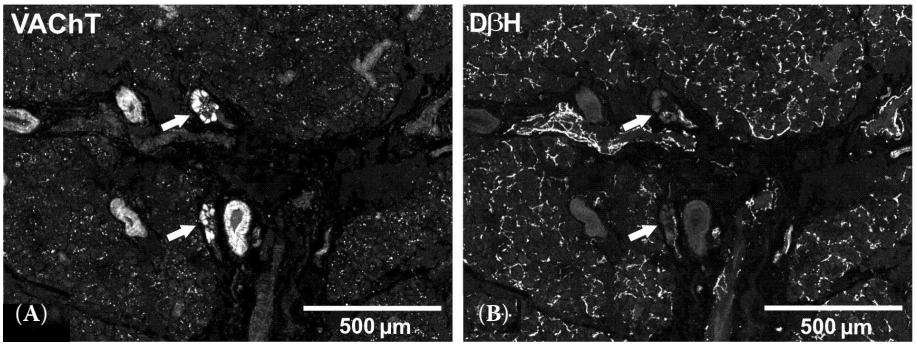

Figure 7. Low magnification photomicrograph of a section from the mandibular gland (MGl) double-stained with antibodies against vesicular acetylcholine transporter (VAChT) and dopamine β-hydroxylase (DβH) showing VAChT-positive (**A**) and DβH-positive nerve fibres (**B**) supplying glandular acini. Note the presence of small ganglia containing VAChT-positive neurons which were DβH-negative (arrows)

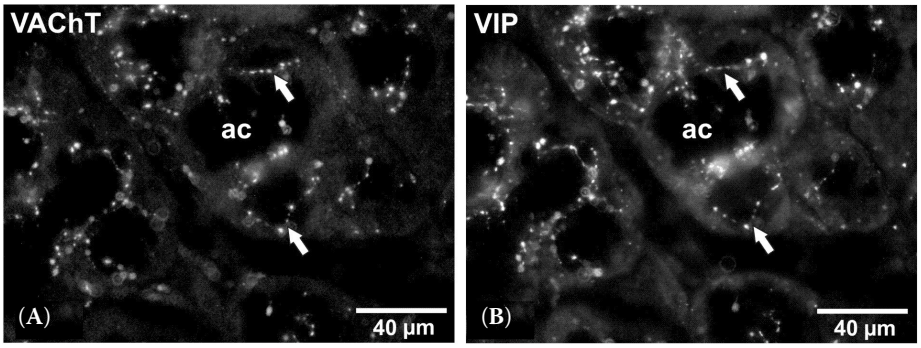

Figure 8. High magnification photomicrograph of a section from the mandibular gland (MGl) double-stained with antibodies against vesicular acetylcholine transporter (VAChT) (**A**) and vasoactive intestinal polypeptide (VIP) (**B**) showing nerve fibres supplying glandular acini (ac) which simultaneously stained positive for both substances (arrows)

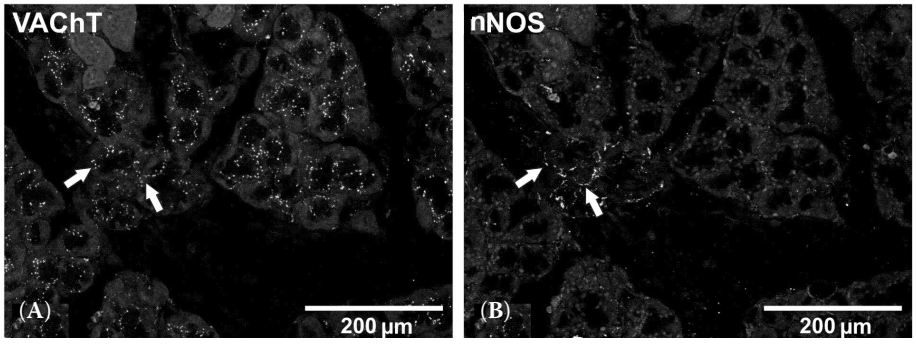

Figure 9. High magnification photomicrograph of a section from the mandibular gland (MGl) double-stained with antibodies against vesicular acetylcholine transporter (VAChT) (**A**) and neuronal nitric oxide synthase (nNOS) (**B**) showing nerve fibres supplying glandular acini which simultaneously stained positive for both substances (arrows)

nNOS (99.71 ± 0.18%; Figure 2B). An intermediate number of nerve cell bodies displayed immunoreactivity to NPY (Figure 3B) or VIP (Figure 4B) (18.67 ± 0.52% and 8.11 ± 0.36%, respectively). Single perikarya stained positive for GAL (Figure 5B). No neurons exhibited immunoreactivity to DβH (Figure 1B).

The mandibular gland

Single-labelling immunofluorescence disclosed that the MGl was richly supplied by VAChT-positive nerve fibres that surrounded intra- and interlobular salivary ducts (Figures 7A, 8A and 9A).

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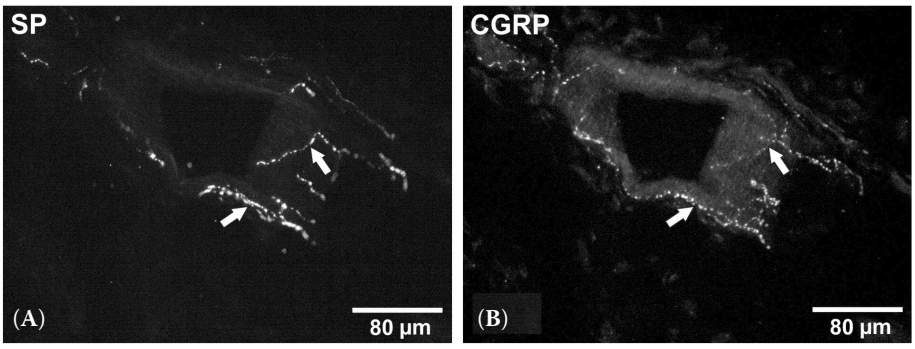

Figure 10. Photomicrograph from the mandibular gland (MGl) double-stained with antibodies against substance P (SP) and calcitonin gene-related peptide (CGRP). Note the presence of numerous SP-positive (**A**) nerve fibres supplying a salivary duct which were simultaneously CGRP-positive (**B**; arrows)

A large number of these nerve terminals were also observed around the acini. A small number of VAChT-immunoreactive axons were associated with intraglandular arteries and the solitary fibres were found to supply veins.

Many axons associated with both the salivary ducts and acini, and intraglandular arteries exhibited immunoreactivity to DβH (Figure 7B). Some DβH-positive fibres were closely apposed to veins.

Immunoreactivity to GAL or VIP was observed in an intermediate number of nerve fibres supplying the salivary duct and acini, while solitary nerve endings found in a close apposition to these structures were SP- (Figure 10A) or CGRP-positive (Figure 10B). The majority of the NPY-positive nerve fibres were associated with intralobular and stromal blood vessels. Only solitary NPY-immunoreactive nerve terminals were closely apposed to salivary ducts and acini.

Double-labelling immunofluorescence revealed that VAChT-(cholinergic) and DβH-(adrenergic) positive intrinsic nerve fibres supplying the porcine MGl represented separate populations of the nerve terminals (no colocalisation of the markers was observed). Many VAChT-immunoreactive periacinar nerve fibres (Figures 8A and 9A) also stained positive for VIP (Figure 8B) or nNOS (Figure 9B).

RT-PCR

RT-PCR analysis revealed strong signals ChAT, VAChT, nNOS, VIP, NPY and GAL transcripts (Figure 11). For SP and DβH only very weak bands were observed in the gel. RT-PCR with primers for CGRP did not show any PCR product. The positive control for DβH with cDNA from the superior cervical ganglion and also for SP and CGRP with cDNA from the dorsal root ganglion showed strong bands of the expected sizes. There were no significant differences between males and females.

DISCUSSION

The present study has shown that the porcine MGl is richly supplied by nerve fibres exhibiting immunoreactivity to VAChT; therefore, they should be regarded as cholinergic nerve terminals. The VAChT-positive nerve fibres surrounded intra- and interlobular salivary ducts and were also observed around the acini. The general distribution of cholinergic nerve terminals in the porcine MGl is similar to that of AChE-positive axons found in the cebid monkey (Rossoni et al. 1992) as well as in the cow,

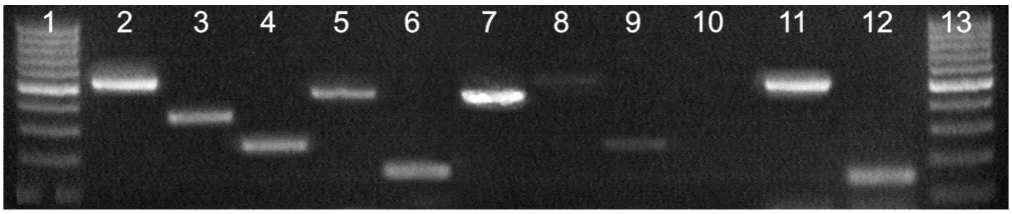

Figure 11. Gel electrophoresis of RT-PCR products corresponding to mRNA encoding the porcine neuropeptides and neurotransmitter-synthesising enzymes in the mandibular ganglion. 1, 13 = marker; 2 = ChAT; 3 = VAChT; 4 = nNOS; 5 = VIP; 6 = NPY; 7 = GAL; 8 = DβH; 9 = SP; 10 = CGRP; 11 = DβH, positive control; 12 = CGRP, positive control

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guinea pig and hamster (Bloom and Carlsoo 1973). In these species AChE-positive nerve fibres have been found to be distributed around blood vessels and secretory endpieces of all major and minor salivary glands. Cholinergic nerve terminals have also been observed to supply the intralobular ducts. A study on the presence of VAChT-positive nerve fibres in the MGl of rats provided no detailed information on their distribution (Schafer at al. 1998). The authors of this publication, which in general deals with the possibility of visualisation of cholinergic nerve structures in the peripheral nervous system using VAChT as a marker, only mentioned in the figure legend that such nerve terminals have "periglandular distribution".

Many nerve fibres supplying the porcine MGl exhibit immunoreactivity to DβH. These axons richly supply not only the arteries but also the glandular acini and ducts, and some of them contribute to the innervation of the veins. Double immunostainings have confirmed, for the first time, that cholinergic and adrenergic nerve terminals supplying the mammalian (porcine) salivary gland represent separate populations of nerve fibres. A similar distribution of adrenergic axons supplying the MGl has been demonstrated in the rat and cebid monkey (Soinila et al. 1991a; Soinila et al. 1991b; Rossoni et al. 1992). In the rat, nerve fibres exhibiting immunoreactivity to the adrenergic marker tyrosine hydroxylase were also observed in association with the acini and intra- and interlobular salivary ducts, and around blood vessels. Investigations dealing with the monkey MGl (Rossoni et al. 1992) utilised histochemical methods to visualise adrenergic nerve elements.

This study has revealed that the small number of intraglandular NOS-immunoreactive nerve fibres is mainly associated with the glandular acini, ducts and, in a smaller proportion, with the blood vessels. Immunoreactivity to nNOS has been found in a few nerve fibres supplying human, rat, cat and ferret salivary glands (Lohinai et al. 1995; Alm et al. 1997; Takai et al. 1999; Soinila et al. 2006). In the ferret and rat, NOS-positive varicose nerve terminals encircled the glandular acini and arteries of various sizes (Alm et al. 1997). Moreover, in the ferret, ducts of the sublingual, mandibular and parotid gland are also supplied with NOS-IR nerve fibres. In the rat, such fibres were observed only in a close association with the mandibular duct.

In humans, in contrast to the rat glands, which contained a dense plexus of NOS-positive nerve fibres, only solitary NOS-immunoreactive nerve terminals are encountered (Soinila et al. 2006). These authors have suggested that in humans NO plays a minor role as a neuronal regulator of saliva synthesis and excretion because this substance is expressed by only a small number of nerve fibres supplying the salivary gland tissue.

The porcine MGl is a mixed gland consisting of serous and mucous acini. The present study has shown that an intermediate number of GAL- and VIP-positive nerve fibres are proportionally associated with both kinds of acini.

In the human MGl (Soinila et al. 2006), nerve endings immunoreactive to VIP, GAL or NPY supplying the mucous acini have been found to be significantly more numerous than those apposing the serous acini. Furthermore, Del Fiacco et al. (2014) have revealed that besides VIP-positive axons, also PHM-immunoreactive nerve terminals found in close proximity to acini and ducts are relatively abundant in the human MGl. Therefore, in humans these peptides may play a more active part in the regulation of the secretion of saliva in the mucous acini than in the serous acini. In contrast, in the pig NPY-positive nerve fibres were mainly associated with blood vessels, and, to a lesser degree, with the mandibular intra- and interlobular ducts, and with the acini. Perivascular NPY-IR nerve fibres may be involved in controlling local blood flow through the gland. This assumption has been confirmed by Schultz et al. (1994) who have found that in the rat MGl, NPY-positive innervation of the acini was much less abundant than in the other salivary glands. In the rat MGl, NPY-positive nerve fibres were observed in close vicinity to blood vessels but not to the acini.

This study has shown that intraganglionic GAL-positive nerve endings are associated with ducts and acini. Our results are in accord with those of Konopka et al. (1992) who investigated GAL-like innervation of the rat mandibular and sublingual salivary glands.

The present investigation has revealed only solitary intraglandular nerve fibres immunoreactive to SP and CGRP. In humans, also only a relatively small number of SP- and CGRP-positive nerve endings were observed to be associated with the mucous and serous acini (Salo et al. 1993; Kusakabe et al. 1997; Soinila et al. 2006). In the rat MGl, varicose CGRP-positive nerve fibres were located around interlobular salivary ducts and small blood vessels. Only occasionally were CGRP-immunoreactive

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nerve fibres encountered between or around the acini (Soinila et al. 1989). CGRP is a well-known potent vasodilator (Brain et al. 1985); thus, CGRP-positive nerve fibres may play a role in the regulation of the glandular blood flow.

The MGn is a parasympathetic ganglion which contributes to the innervation of the sublingual and mandibular glands. It receives the preganglionic input from the anterior salivary nucleus.

The present study has shown that in the pig, the majority of neurons associated with the MGn form a larger ganglion located in the hilum of the gland, but some of them, as solitary nerve cells or small cell clusters, are also distributed within the gland, mainly along the inter- and intralobular ducts.

Double-immunostaining has revealed that nearly all neurons in the porcine MGn exhibit immunoreactivity to VAChT/ChAT (98.45 ± 0.59%) and nNOS (99.71 ± 0.18%). An intermediate number of the nerve cell bodies display immunoreactivity to NPY and VIP (18.67 ± 0.52% and 8.11 ± 0.36%, respectively), and single nerve cells are GAL-positive.

The data dealing with the chemical coding of the MGn neurons in other mammals are relatively scarce. In the rat, MGn neurons have been found to exhibit immunoreactivity to SP, VIP, NPY, MEAGL, TH (Soinila et al. 1991a; Soinila et al. 1991b; Ng et al. 1995; Jia et al. 1997) and [Met5]enkephalin-Arg6-Gly7-Leu8 (Ng et al. 1995). However, in contrast to the rat, in the monkey, MGn neurons stained only for NPY and SP (Ng et al. 1995). The presence of enkephalin (ENK) and TH in neurons of the rat MGn was also observed by Shida et al. (1991). Four types of neurons have been distinguished in the rat MGn: cells containing both TH and ENK (9% of all ganglion cells), cells containing only ENK (17%), cells containing only TH (4%) and cells lacking both ENK and TH. It should be emphasised that no TH- or DβH-positive neurons were found in the porcine MGn. Almost all perikarya in the porcine MGn are presumably cholinergic in nature (VAChT/ChAT-positive). ACHE-positive neurons were also occasionally observed in bovine, guinea pig and hamster salivary glands (Bloom and Carlsoo 1973).

Adrenergic nerves have been observed around the secretory acini of the bovine, guinea pig and hamster (Bloom and Carlsoo 1973) and rat (Norberg and Olson 1965) mandibular glands. DβH-IR nerve fibres which supply the MGn in the pig probably originate from the superior cervical ganglion. Arciszewski et al. (2004) found that the majority of sympathetic neurons projecting to the sheep MGl are found in the superior cervical ganglion, but some of them are also distributed in the middle cervical ganglion. Ng et al. (1995) observed a variable number of neurons in the superior salivary nucleus, superior cervical ganglion and trigeminal ganglion after fluorogold injections into the rat mandibular and sublingual glands.

In the present study, almost all MGn nerve cell bodies (99.71 ± 0.18%) exhibited immunoreactivity to nNOS. nNOS-positive neurons have also been found in the hilar region of the rat and ferret mandibular and sublingual glands (Alm et al. 1997; Takai et al. 1999). Lohinai et al. (1995) observed that all neurons in the cat mandibular ganglia were intensely positive for both NADPH-d and nNOS. These authors suggested that NO might play a physiological role in the MGl via the parasympathetic nervous system.

The present study has shown that single nerve cell bodies in the pocine MGn contain GAL. A subset of rat MGn has been found to exhibit immunoreactivity to this substance (Konopka et al. 1992). Experiments utilising retrograde neuronal tracing have demonstrated that GAL-immunoreactive sensory neurons in the trigeminal ganglion do not innervate the madibular ganglion. These results indicate that galanin-like innervation of the rat MGl derives from parasympathetic nerves to the glands (Konopka et al. 1992).

In the present study we have found small ganglia which are located intraglandulary. Similarly distributed ganglia were also observed in the stroma of the human MGl (Geerling et al. 2008; Tosios et al. 2010). In humans, the small ganglia were observed to be in close association with salivary parenchymal cells and blood vessels. The functional role of intrasalivary ganglia is not clear. In general, parasympathetic innervation of the MGl is responsible for fluid and electrolyte secretion, regulation of salivary secretion as well as regeneration of the MGl (Suzuki and Sakada 1972; Kawa and Roper 1984). The ganglion cells are considered to be responsible for the innervation of MGl structures.

In general the RT-PCR findings corroborated the results of the immunohistochemical analysis and confirmed the presence of ChAT, VAChT, nNOS, VIP, NPY and GAL at the level of mRNA and protein in the porcine MGn tissues. Immunohistochemical staining did not reveal any SP or DβH-positive peri-

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karya. However, the weak PCR product bands for DβH and SP suggest that these proteins are expressed at a low level in the studied tissues. This may be due to the presence of the mRNAs for DβH and SP in other, non-neuronal, cells found in the studied material. It is possible that the source of the detected mRNA for DβH and SP were leukocytes, because it is known that these cells show an expression of these substances (Lai et al. 1998; Kuci et al. 2006). However it cannot be excluded that a very low level of the expression of the genes may occur also in neurons of the porcine MGn.

Based on the results obtained it can be stated that the pattern of innervation (in terms of the chemical coding, distribution and frequency of intraglandular nerve fibres) of the MGl in the pig is, in general, similar to that found in other mammals investigated. However, it possesses also some species-characteristic features which distinguish it from the pattern determined, for instance, in the rat or primates (monkeys, humans). As in other species, in the pig, the glandular elements are richly supplied by both cholinergic and adrenergic axons (the latter also abundantly supply the blood vessels) which represent separate populations of nerve terminals. However, in contrast to humans, in the pig the frequency of the nerve fibres supplying the serous and mucous acini is comparable, respectively. In the context of the chemical coding of neurons in the porcine MGn, the expression of NOS by the nerve fibres appears to represent an interesting aspect. In the pig, an intermediate number of the cholinergic axons associated with the glandular elements express this enzyme; in the rat, the nitrergic nerve terminals seem to be more frequent while in humans, in contrast, they are very sparse. Due to the lack of proper data from tracing experiments, the origin of the cholinergic/NOS-positive nerve fibres in the pig can only be a matter of speculation. Nevertheless, it seems that the MGn should be considered to be their major, if not only source, because, interestingly, nearly all the neurons in this porcine ganglion simultaneously express VAChT and NOS. This finding, probably characteristic of the pig, also suggests that in this species, the NOS-negative cholinergic axons must derive from other neuronal centres. Finally, the lack of distinct differences in the innervation of the MGl and the chemical coding of neurons in the MGn between female and male pigs suggests that these particular nerve elements may not be specifically linked to endocrine circuits associated with sexual behaviour.

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